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CTC

CENTRO DE TERAPIA CELULAR

CENTER FOR CELL-BASED THERAPY

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CENTER FOR CELL-BASED THERAPY (CTC) - 2014 REPORT

The Center for Cell-based Therapy (CTC) is a scientific multidisciplinary project aiming to study the molecular, cellular, and biological characteristics of normal and abnormal stem cells and to critically test their potential for therapeutic use. Our research projects are focused on stem cells and and take advantage of two different approaches: basic research and clinical studies. Basic studies are being conducted on pluripotent stem cells: embryonic and induced pluripotent stem cells (iPS); and in somatic stem cells (hematopoietic, mesenchymal and endothelial).

Apart from the scientific findings obtained in basic and clinical investigation, this report also describes the advances of our Center in the field of Technology Transfer which includes the development of process for large-scale production of stem cells, manufacture of new recombinant proteins for clinical purposes, and the development of diagnostic tests of interest for blood transfusions, hematological diseases, and cellular therapy.

I) BASIC RESEARCH

The main areas of basic research are illustrated in Figure 1.

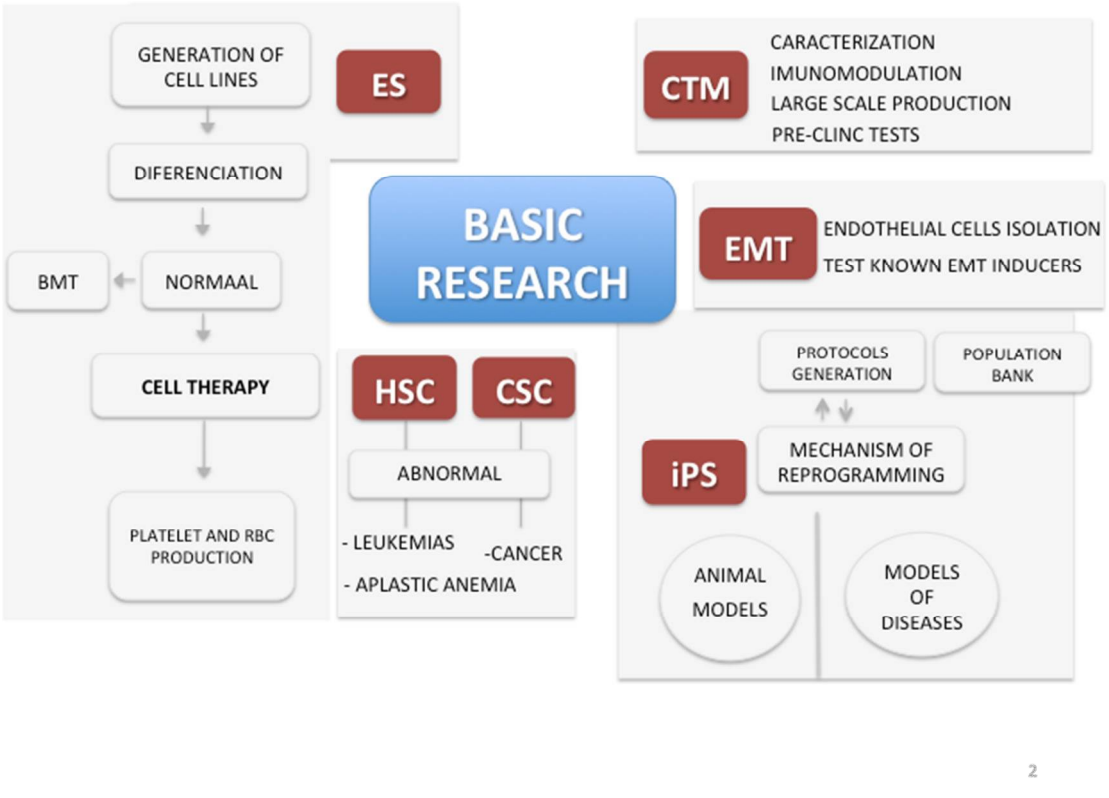


Figure 1. Illustrative scheme of the fields of BASIC research developed at CTC. Abbreviation: ESC: embryonic stem cell, MSC: mesenchymal stromal cells, HSC: hematopoietic stem cells, iPS: induced pluripotent stem cells and CSC: cancer stem cells.

This report is organized according to the proposed goals of the CTC for 2013. Results are reported in the same order described in Appendix

1) GOAL 1: ESTABLISHMENT OF HUMAN EMBRYONIC STEM CELLS (hESC) FOR THE USE IN PRE-CLINICAL STUDIES.

a) Culture and propagation of hESC in large scale and under GMP conditions.

At this step, two new human embryonic cell lines were generated: BR-4 and BR-5 under oxygen physiological conditions. Forty-three embryos (cell stage 4-8) were thawed and cultured. Among them, 16 reached the blastocyst stage and internal mass was isolated and cultured on mouse embryonic fibroblasts (MEF) at 5% O_2. Two months after culture, two independent lines were generated, BR-4 and BR-5, and were properly expanded and characterized.

Since 2012, two areas have been expanded and remodeled: i) the area of the Cell Therapy Laboratory (Hemocentro de Ribeirão Preto) which is dedicated to the stem cell facility for clinical research; and ii) the Center for Cell-based Therapy (CTC) – National Laboratory of Embryonic Stem Cells, funded by FINEP/BNDES which is being implemented in the Department of Genetics and Evolutionary Biology of the Biosciences Institute (USP). Those projects predict the renovation of areas where the procedures of manipulation, isolation, culture, expansion, and freezing of stem cells will be conducted in compliance with the Good Manufacturing Practices (GMP) for the use in clinical research.

2) GOAL 2: DIFFERENTIATION OF PLURIPOTENT CELLS INTO BLOOD CELLS.

a) Differentiation into hematopoietic progenitors from hESC

The transplantation of hematopoietic stem cells (HSC) represents the most successful type of cell therapy performed up to now. However, in spite of the importance of isolated HSCs from adults in clinical practice, there are some limitations inherent in their availability and immunological compatibility. Nowadays efforts are invested in the discovery of alternative sources for HSCs to meet the demand for transplant. HSCs are capable of differentiating into all blood cells. These cells can be produced *in vitro* from human embryonic stem cells (hESC). Although hESC have great potential for differentiation, cell therapies using differentiated cells from hESC are only possible if the cells generated are equivalent to adult cells. The *in vitro* differentiation systems currently used for hESC differentiation into hematopoietic cells (HC) are still ineffective. To overcome this obstacle, the identification of genes/proteins and molecular pathways involved in this process is needed. One of the methods widely used for obtaining HCs from hESC *in vitro* is the co-culture of pluripotent cells with murine stromal cells. The soluble factors secreted by these cells and the contact cell-cell show great impact on the differentiation process. Nevertheless, the molecular pathways activated in stromal cells, after the contact with hESC, which induce hematopoietic differentiation, are unknown. At the moment, we are conducting a comparative proteomic analysis of OP9 stromal cells before and after hematopoietic differentiation from hESC, with the purpose of identifying new targets and pathways involved in this differentiation. The analysis of cells obtained from hESC at the

differentiation peak (day 9) by flow cytometry revealed increase in the number of cells expressing markers of hematopoietic and endothelial cells. Approximately 40% of differentiated cells showed CD43 marker, 26% showed CD34 expression, 19% showed positivity for CD31, 5% showed positivity for CD45, 24% were positive for KDR marker, and 22% showed CD144 expression. A total of 759 proteins were identified in OP9 cells, 180 were upregulated at the hematopoietic differentiation peak (day 9), suggesting that these molecules take part in the generation of blood cells (Figure 2A). Among these proteins, 13 participate in the pathway of Nrf2, a regulatory gene of oxidative stress, described for playing a role in the development of hematopoietic cells and in the migration and retention of these cells in niche. The identification of these proteins offers new information about obtaining hematopoietic cells *in vitro* from pluriplotent cells. Thus, our next step is activating Nrf2 gene in stromal cells and evaluating its role in the generation of blood cells from hESC, in order to optimize the differentiation process and produce specific cell types of blood.

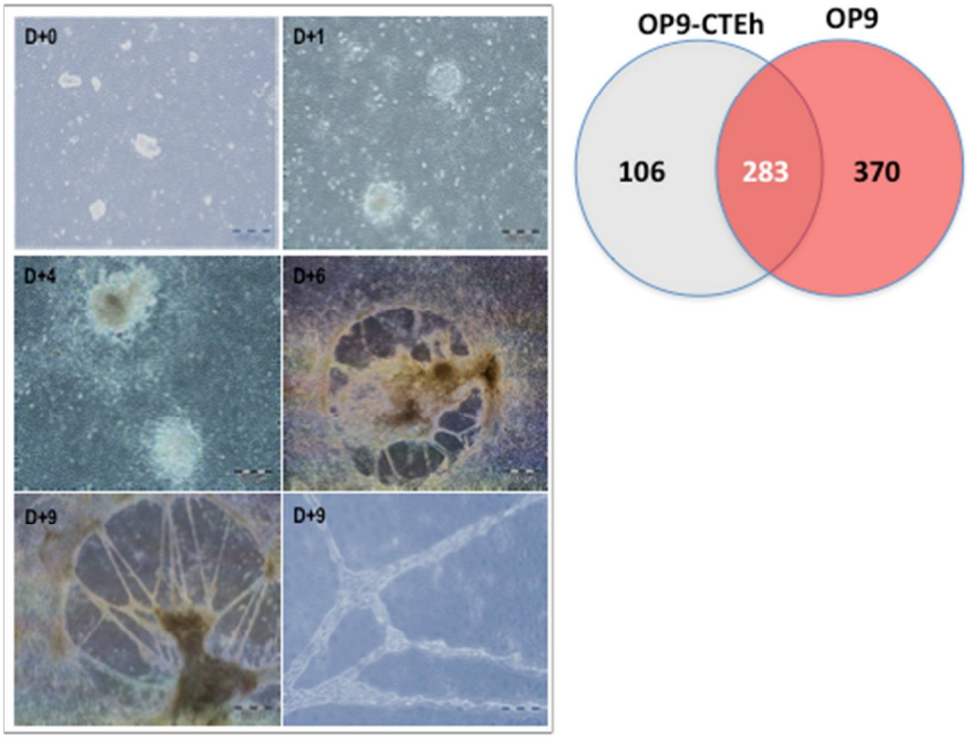


Figure 2: Hematopoietic differentiation in vitro from human embryonic stem cells by co-culture with OP9 stromal cells. $1.5x10^6$ hESC were cultured on monolayer of OP9 cells for 9 days. D+0: small fragments of hESC over OP9; D+1: hESC colonies adhered on OP9; D+4: presence of the first hematopoietic progenitors (D+4); D+6: presence of structures similar to capillaries and rounded cells (hematopoietic progenitors) at the border of the colonies; D+9: differentiation peak with large quantity of rounded cells at the border of the colonies and structures similar to capillaries on bigger increase. Scale indicated in every figure. B) A total of 759 proteins were identified. For OP9, 106 proteins are exclusive to OP9 during differentiation.

In addition to the studies conducted using standard differentiation protocols (as OP9), our research group developed a new differentiation protocol based on the co-culture system of

hESC with mouse embryonic fibroblasts (MEFs) in a differentiation medium containing fetal bovine serum, cytokines and hematopoietic growth factor (BMP-4, TPO, FLT3L, SCF, EPO, IL-3, IL-6, G-CSF and IGF) at low doses. It has been described that these cells are not able to promote hematopoietic differentiation of hESC. However, we demonstrated that after co-culture of sESC-H1 population with MEF in differentiation medium for 31 days, it is possible to obtain hematopoietic cells. Our differentiation protocol has already been performed in 5 independent experiments, suggesting that this protocol is reproducible and robust. The cells obtained are positive for CD45 94%(±4.3%), CD43 93%(±4.5%), CD31 92%(±1.6%), and $CD34^{low}$ 4%(±1%). The system also is capable of expressing lymphoid markers CD4 (20.7 ± 2.3%) e CD8 (18.5 ± 4.3%). 50% of $CD45^{+}CD4^{+}$ population also expresses CD8, suggesting that these cells would not be fully mature yet.

Differentiated cells express genes involved in definite and primitive hematopoiesis, as CD45, CD31, Runx1, Tal1, Lmo2, Prom1, CD34 and Notch1, and these cells have clonogenic potential of one colony per 574 plated cells. Therefore, our study demonstrated that hESC hematopoietic differentiation on MEF is possible. This system allows the development of cells with immunophenotypical and molecular characteristics of definite HSC, different from other research groups that have obtained cells with characteristics of primitive hematopoiesis. At the moment, this protocol is being tested in other embryonic lines (H9, BR1, HuES) and in iPS. *In vivo* assays of hematopoietic reconstitution will be performed to demonstrate the functional potential of these cells.

b) Differentiation of induced pluripotent cells (iPS) into hematopoietic line.

For differentiation experiments, three iPS cells generated in our group were used: one iPS cell line reprogrammed with Tcl-1, SOX2 and c-Myc (iPS TSM) and two iPS cell lines reprogrammed with c-MYC, Klf4, SOX-2, Oct-4. iPS cells were cultured on matrigel and submitted to hematopoietic differentiation. For this, 10^6 indifferentiated iPS cells were cultured on monolayer of OP9 stromal cells for nine days, in differentiation medium free from cytokines and growth factors. After the first four days of differentiation, only iPS 01 showed morphology of hematopoietic progenitor cells. However, it was not possible to perform the immunophenotypical analisys of differentiated cells, because they were in insufficient amount for analysis. The iPS cells (iPS 03_Cl8) and iPS TSM did not differentiate and died from the fifth and second day of co-culture, respectively (Tabel I). New differentiation experiments will be carried out to better evaluate the differentiated cells obtained from iPS cells.

Table 1. Hematopoietic diferentiation experiments using iPS cell lines.

Co-culture day	Pluripotent cell			
	iPS 01	**iPS 03_Cl8**	**iPS TSM**	**CTEh**
Day 0	Fragments of approximately 10^6 indifferentiated cells were transferred on MEF monolayer			
Day 4	First mesodermal colonies were observed	No presence of of mesodermal colonies. Many dead cells.	No presence of of mesodermal Colonies. Many dead cells.	Formation of the first mesodermal colonies
Day 9	Presence of hematopoietic Progenitor cells (rounded cells at the border of the colonies)	It was not possible to evaluate.	It was not possible to evaluate.	Presence of hematopoietic Progenitor cells (rounded cells at the border of the colonies)
Immunophenotype	It was not possible to evaluate due to the small quantity of cells for analysis.	It was not possible to evaluate.	It was not possible to evaluate.	Hematopoietic progenitor cells expressing CD34, CD43, CD45, CD31, KDR and CD144 markers

c) Induction of megakaryocytic differentiation from ESC by manipulation of culture conditions and gene induction.

The capacity of generating *in vitro* HLA-compatible platelets brings significant advantages over the current situation of availability of the product that depends on voluntary blood donations. Moreover, the production of platelets from a constant and safe source will offer a singular advance in transfusion medicine. The results of this study demonstrate that megakaryocytes can be generated *in vitro* from H1-hESC in three distinct systems of culture. Immunophenotypical analyses of the cells and platelets produced by the proposed systems demonstrate the expression of CD42b and CD71 markers. In addition, colonies of granulocytes, macrophages, and erythroid progenitor cells can be generated by these systems. Transmission electron microscopy analysis of the differentiated cells revealed several ultrastructural features of the platelets. The generated platelets were indistinguishable from normal platelets from pheripheral blood. hESC-derived platelets responded to thrombin stimulation, formed microaggregates, and facilitated clot formation/retraction *in vitro*.

The model established represents a promising tool for understanding the aspects not solved in the mechanisms of megakaryocytopoiesis and thrombocytopoiesis, apart from contributing to the screening of new therapeutic agents for the treatment of diseases related to platelet functions and production.

3) GOAL 3: GENERATION AND CHARACTERIZATION OF iPS CELLS OF PATIENTS AND HEALTHY INDIVIDUALS.

iPS cells can be used as study models for many diseases. CTC invested in the creation of a *Core Facility* for the production and distribution of vectors for the generation of iPS cells. The vectors produced are used by the laboratories of the Regional Blood Center of Ribeirão Preto, Medical School of Ribeirão Preto, School of Animal Husbrandry and Food Engineering (FZEA, USP- Pirassununga) and by the Bioscience Institute (USP-SP). The group developed two protocols for cell reprogramming:

Protocol 1: Lentiviral vectors.

We used polycistronic lentiviral vector containing factors Sox2, KLF4, Oct4 and c-Myc - Stemcca. Viral production is done by transitory transfection in 293FT cells. Viruses collected are ultracentrifuged at 48.960 x g for 1 hour and 40 minutes (rotor SW28) and viral titration is done by real time PCR. Viruses produced are used in the transduction of diverse cell types, such as: fibroblasts, mononuclear cells, lymphocytes and erythroblasts. Apart from viral vectors we also use pEB-C5 (OCT4, SOX2, KLF4, c-MYC and LIN28) plasmidal vectors and pEB-Tg for generating iPS with no integrations.

Protocol 2: Episomal vectors

Mononucleated cells were isolated from pheripheral blood by Ficoll gradient. A total of $4x10^6$ cells were cultured for 12 days on StemSpan medium (StemCell) containing dexamethasone IL-3, IGF-1, SCF and erythropoietin. At the 12^{th} day, 70% of erythroblasts (approximately 2.8 $x10^6$ cells) were obtained. Cells were transfected with two plasmids expressing a total of 6 reprogramming factors (pEB-C5: Oct4, Sox2, Klf4, cMyc and Lin28; and pEB-Tg: SV40-large T). A total of $2x10^6$ cells were transfected with 10 ug of both plasmids. After 24 hours, transfected cells were transferred to MEF plates, 14 days after in culture with hESC medium; colonies with the appropriate morphology were identified.

a) Hemophilia A

Hemophilia is a blood coagulation disorder, caused by the deficiency of coagulation factors VIII (hemophilia A) or IX (hemophilia B). Nowadays the treatment for hemophilia basically consists of the regular reposition of the deficient antihemophilic factor. However this therapy poses many difficulties that include the high cost of treatment, short half-life of the coagulation factor VIII and the development of inhibitor antibodies against the administered factors, unveiling the need for searching new therapies. In this context, gene therapy combined with cell therapy emerges like a promising alternative for the treatment of hemophilia. Our group intends to use this combination to treat hemophilia A by the genetic correction of factor VIII in iPS cells generated from fibroblasts of patients with hemophilia, and afterwards, to differentiate these cells into hepatocytes which produce functional factor VIII.

In this period, biopsies of the skin of patients with severe hemophilia A were performed. Physicians of the Regional Blood Center performed biopsy after patients signed a Free, Prior and

Informed consent (FPIC). Ethics Committee of the Hospital of the Medical School of Ribeirão Preto approved this project (n. 233.230). The material of the biopsy underwent enzyme treatment and fibroblasts were cultured until the establishment of the primary culture in DMEM medium with 20% of fetal bovine serum. Subsequently, samples of fibroblasts were transduced with lentiviral vectors as described in the item 3.1. iPS were generated from fibroblasts of patients with severe hemophilia A using the pluripotency factors Oct-4, Sox-2, Klf-4 and c-Myc (Figure 3).

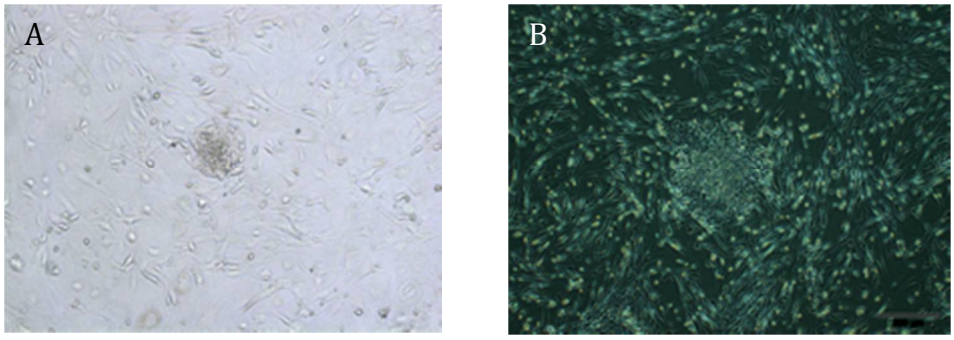


Figure 3. Reprogramming of fibroblasts from Haemophilia A patient. A) iPS-like colonies generated from reprogramming of hemophilic fibroblasts. B) Colonies after the passage of fibroblasts transduced to MEF (day 13).

b) Sickle Cell Disease

Sickle cell disease (SCD) is a monogenic disease which results of a recessive mutation in the gene β–globin, originating abnormal hemoglobin, S hemoglobin (HbS). The disease is caused by the homozygous point mutation, generating abnormal, rigid, sickle shape red cells, which survive less in circulation (10–20 days). The only potentially curative treatment for SCD is hematopoietic stem cell (HSC) transplantation, but it is a risk procedure, with elevated morbimortality, recommended only for the most severe cases that have compatible donor.

Gene therapy using HSC from the patient represented an alternative and potential cure. However the lack of methodology for efficient expansion and for genetic modifications of somatic HSC, in addition to the risk of insertional mutagenesis of the viral vectors, is a major limitation for the gene therapy based on HSC. Relying on the discovery of technology of induced pluripotent stem cells (iPSC) generation, it was possible to generate models of diverse types of *in vitro* diseases, allowing the increased understanding about them, enabling the development of new therapies.

For the selection of patients for sample donation, we chose patients who are not taking the medication hydroxyurea to prevent any interference in cell culture. Skin samples of a patient with sickle cell disease were obtained by biopsy for the isolation of fibroblasts, and collection of peripheral blood for the isolation of PBMC. PBMC were obtained by centrifugion in density gradient Ficoll-Paque PREMIUM, counted and frozen. Fibroblasts were obtained by processing tissue and culture under glass cover slip for approximately 30 days. After expansion and freezing, fibroblasts were used for reprogramming using polycistronic lentiviral vector.

At the first attempt of obtaining iPS from fibroblasts of healthy individuals, we used two viral concentrations: 10 and 20 viruses per cell. Cells infected with MOI of 20 did not resist and died. Fibroblasts infected with 10 viruses per cell were plated on MEF. The infected cells with 10 viruses per cell were plated on MEF and about 15 days after transduction, the first colonies were visible (Figure 4A). Nevertheless, after the first passage the colonies did not grow, maybe because they were not fully reprogrammed colonies. We made one more attempt with the concentration of 10**,** 5 and 3 viruses per cell to transduce fibroblasts from cell- sickle patients (Figure 4B). Seven days after transduction, cells were replated on MEF and the treatment with sodium butyrate (NaBu) 0.5mM for 7 days. This experiment is still ongoing.

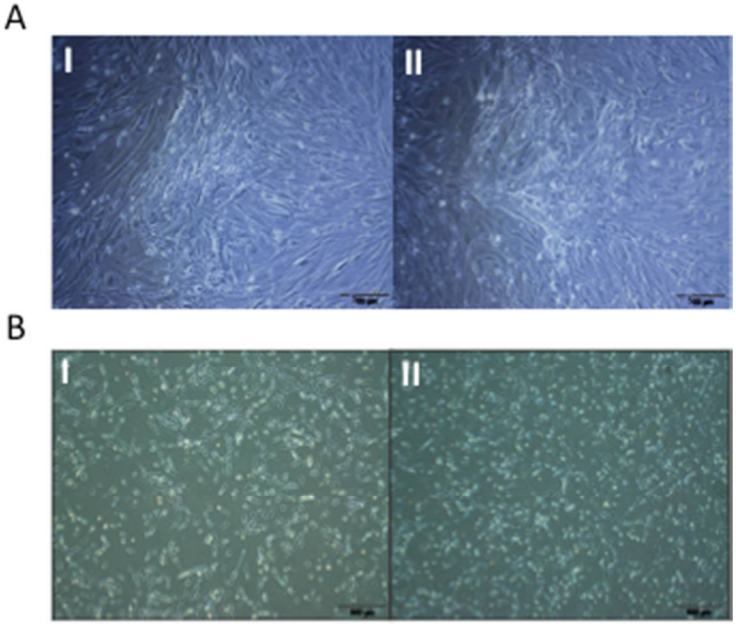


Figure 4. Reprogramming of fibroblasts from Sickle-cell disease patient. A) Images I and II showing the morphology of the first colonies of iPSC derived from healthy fibroblasts. B) I: Fibroblasts isolated from the skin of a patient with sickle cell disease. II: Sickle fibroblast transduced on MEF 7 days after transduction.

c) Dyskeratosis congenita

Dyseratosis congenita (DC) is an inherited bone marrow failure syndrome in which telomerase defects are etiologic. Telomere sequences shortens due to cellular mitotic division but are maintained in cells with high proliferative capacity by telomerase, which enzymatically adds telomere repeats to 3' ends of the chromosomes. Telomerase complex is composed of a reverse transcriptase (TERT), an RNA component (TERC), and proteins that provide stability, as dyskerin (encoded by *DKC1* gene). Mutations in telomerase genes may result in human diseases as aplastic anemia, pulmonary fibrosis, hepatic cirrhosis, and DC. In an attempt to elucidate telomere dynamics in DC, iPSCs were derived from dermal fibroblasts of a DC patient carrying a *DKC1* mutation (A353V substitution) by forced expression of *OCT4*, *SOX2*, *KLF4*, and *MYC* using an excisable polycistronic lentiviral vector (STEMCCA), which was removed by transient cre-recombinase expression. Telomere length of three iPSCs clones was assessed during 40 passages and a trend of telomere shortening was observed immediately after reprogramming; however,

after 20 passages, telomere elongation was noted over time, but telomeres remained shorter than parental fibroblasts. iPSCs differentiations were carried out into two tissues affected (blood and hepatocytes) and in one tissue not affected (intestinal tissue) in DC (Figure 5). Preliminary results in hematopoietic stem cell (HSC) differentiation of three iPSCs clones showed defective capacity of *DKC1* mutants in generate HSC lineages, though one clone presented elevated ability to generate hemogenic endothelial cells. Hepatic and intestinal tissue differentiations were successfully carried out into definitive endoderm, but iPSCs (both mutant and control) presented reduced or missing induction of terminal differentiation towards hepatic or intestinal lineages. Our finds suggest that *DKC1* mutant iPSCs present a tendency to telomere length stabilization and a reduced capacity in generating hematopoietic progenitors. Experiments to confirm these findings and to improve efficiency of hepatic and intestinal differentiation are ongoing.

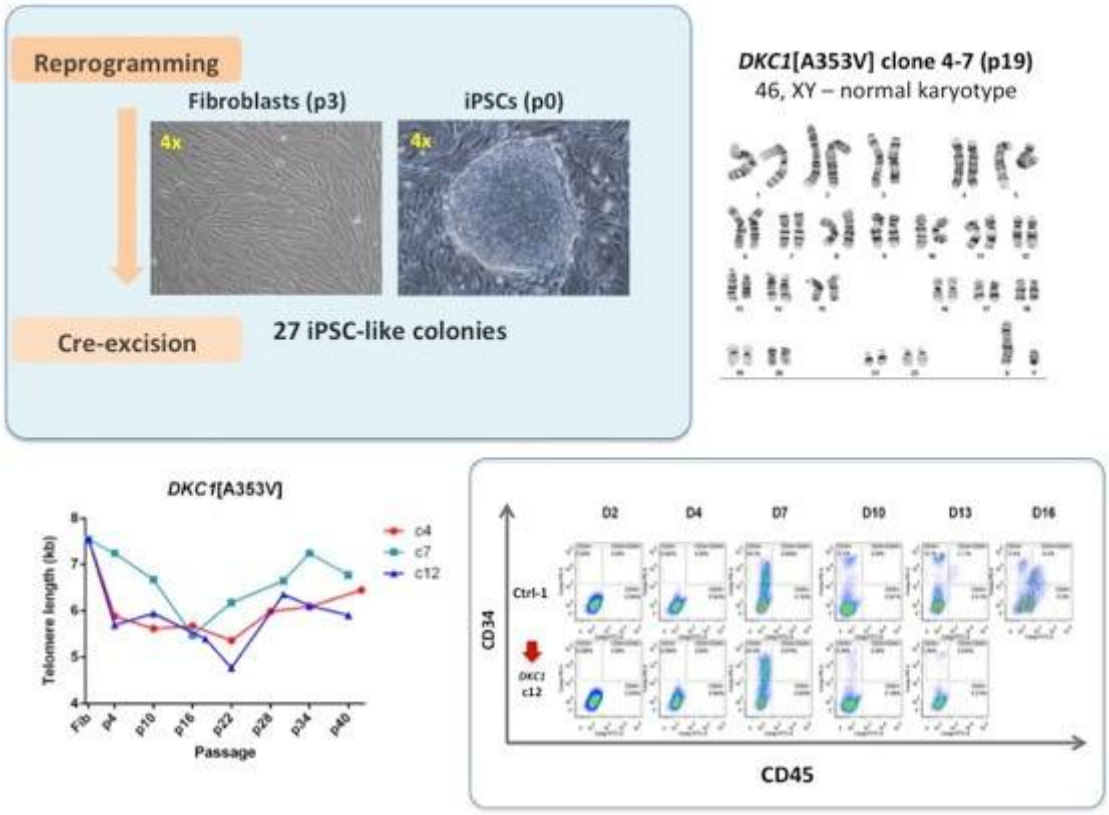


Figure 5. Reprogramming of skin fibroblasts of patients with dyskeratosis congenita. The 27 iPS-like colonies were selected and vector was excised using the Cre system (upper left panel). Patient-derived iPS colonies had normal karyotype (upper right panel) and showed defective telomere elongation over passages (lower left panel). Hematopoietic differentiation was defective in patient-derived iPS as compared to healthy controls.

d) Aplastic anemia

Aplastic anemia is a potentially fatal disease characterized by low peripheral blood cell counts and an empty bone marrow. It is the prototypical disease of hematopoietic stem cell (HSC) failure, as in aplastic anemia HSCs is not able to maintain the daily necessary production of blood cells. Aplastic anemia may be inherited or acquired; in inherited cases, gene defects in DNA or telomere repair may be etiologic; in acquired cases, HSCs are the target of a T-cell

mediated attack. Mutations in genes responsible for telomere maintenance are linked to a number of human diseases. We derived induced pluripotent stem cells (iPSCs) from 4 patients with aplastic anemia or hypocellular bone marrow carrying heterozygous mutations in the telomerase reverse transcriptase (*TERT*) or the telomerase RNA component (*TERC*) telomerase genes. Both mutant and control iPSCs upregulated *TERT* and *TERC* expression compared with parental fibroblasts, but mutant iPSCs elongated telomeres at a lower rate compared with healthy iPSCs, and the deficit correlated with the mutations' impact on telomerase activity. There was no evidence for alternative lengthening of telomere (ALT) pathway activation. Elongation varied among iPSC clones derived from the same patient and among clones from siblings harboring identical mutations. Clonal heterogeneity was linked to genetic and environmental factors, but was not influenced by residual expression of reprogramming transgenes. Hypoxia increased telomere extension in both mutant and normal iPSCs. Additionally, telomerase-mutant iPSCs showed defective hematopoietic differentiation in vitro, mirroring the clinical phenotype observed in patients and demonstrating that human telomere diseases can be modeled utilizing iPSCs. Our data support the necessity of studying multiple clones when using iPSCs to model disease (Figure 6).

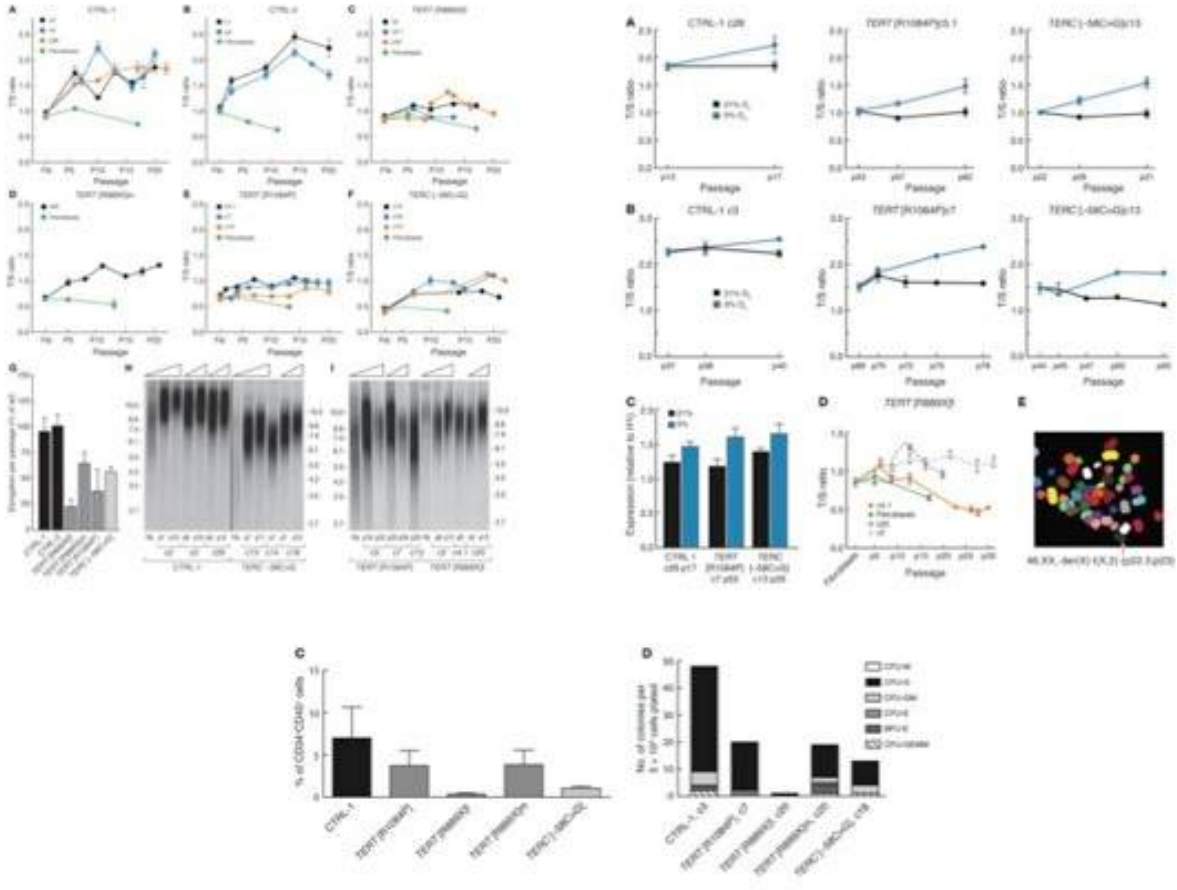


Figure 6. Telomere elongation, oxygen concentration and Hematopoietic differentiation in aplastic anemia-derived iPS cells. While Telomere elongation observed in iPS cells derived from healthy subjects, it is defective in aplastic anemia-derived iPS colonies (upper left). Environmental factors, such as oxygen concentration, are able to modulate telomere maintenance in iPS cells (upper right). Hematopoietic differentiation is defective in aplastic anemia-derived iPS cells (bottom).

e) Gaucher disease/Parkinson disease

The objective is to evaluate the association of Parkinson disease (PD) and mutations in the gene gene glucocerebrosidase (GBA) in hiPS. For this, we propose: i) the generation of hiPSC of patients with PD/with mutation in GBA; PD/without mutation in GBA(+/-); ii) differentiation of hiPSC of the different groups in dopaminergic neurons and iii) identification of the cell phenotypes associated with DP/GBA.

Up to now, hiPSC lines of three patients with PD/with mutation in GBA were established, in addition to the establishment of the protocol of generation of iPS from peripheral blood using episomal vectors.

f) Establishment of a hiPSC library of the Brazilian population

This project aims to establish a collection of hiPSC representing the diversity of the Brazilian genetics from the participants of the Estudo Longitudinal da Saúde do Adulto (ELSA). The samples of peripheral blood mononuclear cells (PBMC) from the participants of ELSA in the University Hospital – USP are collected and cryopreserved. Next the genotyping of a subgroup of samples for the genes involved in the metabolism of drugs was will be performed. Therefore, there is the need to develop methods in scale for the generation of hiPSC. This project has the purpose of generating a collection of 100 hiPSC of a Brazilian population selected among the participants of ELSA. PBMC of 2.000 participants of ELSA HU-USP were collected and cryopreserved as well as the methodology for hiPSC generation from peripheral blood was standardized without genome integration. Up to now 10 hiPSC lines were generated.

g) Mitochondrial diseases

Mitochondrial disfunctions caused by mutations in mitochondrial DNA (mtDNA) represent an important group of human pathologies. However, it is not possible to accurately predict the risk of a woman affected by a mutation in mtDNA transmitting the pathology to her descendants once we do not know the molecular mechanisms, which control mitochondrial heritage. With the development of methodologies, which enable induced pluripotent cells (iPSC) derivation from *in vitro* culture of somatic cells, iPSC became an interesting model for the study of mitochondrial heritage. Similarity to embryonic stem cells (ESC), induced nuclear reprogramming for the production of iPSC results in modifications in mitochondria which start to show underdeveloped cristae, low mitochondrial activity, and reduced amount of mitochondria and mtDNAs per cell.

Derivation of iPSC of patients with pathogenic mutation in mtDNAhas revealed that the percentage of mutant molecules decreased due to iPSC culture time, resulting, in some cases, on the complete elimination of mutant mtDNA. As it has been already reported in *in vivo* studies with humans and mice, these results suggest the existence of specific mechanisms of mtDNA selection that will populate the next generations. Thus the objective of this work is investigating in iPSC, derived from patients with mitochondrial disorders, the existence of a mechanism of negative selection of mtDNA mutant molecules. For this reason, we are using heteroplasmic

fibroblasts with a point mutation A3243G that causes the mitochondrial disorder MELAS; heteroplasmic fibroblasts with a 4.9 kb deletion in mtDNA that causes the Kearns-Sayre Syndrome (KSS); and homoplasmic fibroblasts with wild-type mtDNA (control). A minimal of three patients in each group was collected.

These cell lines were used to derive iPSC by the lentiviral mechanism (hSTEMCCA). Thus far we have produced four colony lines derived from KSS fibroblasts and three of control fibroblasts. The colonies are today between the 5^{th} and 10^{th} passage and we are performing the characterization of pluripotency. Additionally, the number of mtDNA copies and mitochondrial heteroplasmy are being analyzed in the culture of fibroblasts and iPSC at every three-cell passage and for a minimal period of 15 passages.

Relying on this work, we hope expand our knowledge regarding the mechanisms involved in mitochondrial heritage, especially in the cases where pathogenic mutations are present. The use of iPSC in this study seems to be a good alternative, since the access to human biological material (e.g., oocytes, embryos and ESC) from patients with mitochondrial diseases is rare and difficult because of ethical issues, and practically there are not animal models for mitochondrial diseases.

h) Derivation of iPS lines of animal models: bovine, equine and canine models in comparison with murine and human models.

ESC derivation of large mammalian animals would allow the development of diverse study models, and in consequence, it is greatly desired. However, these cells, although studied by many research groups have not showed yet consistency regarding maintenance of the pluripotent state *in vitro*, expression of pluripotency markers and have not produced chimeras either, one of their critical characteristics to prove their capacity of contributing to the development of a viable organism. The advent of pluripotency induction by the overexpression of known transcription factors showed that the production of induced pluripotent stem cells is possible even in domestic animals, and moreover, it seems to follow patterns similar to humans when compared to mice.

In this study human and murine iPS cell were generated by the overexpression of factors related to specific pluripotency (STEMCCA containing murine or human factors, apart from iPS bovine, equine and canine cells by interspecific mechanisms.

In total, we produced 8 human lines derived from two different cell types (5 from mesenchymal of adipose tissue and 3 from fibroblasts); 7 human lines derived from an experiment with patients with mitochondrial diseases; 21 bovine iPS derived from fibroblasts; 20 equine iPS lines, 10 derived from mesenchymal cells of adipose tissue and other 10 derived from umbilical cord cells; 3 murine iPS derived from fibroblasts; and 4 canine iPS derived from fibroblasts.

The characterization of these cells is in final process; at least 3 lines of every species were characterized in relation to alkaline phosphatase expression, production of embryoid bodies, *in*

vitro differentiation, gene expression, proteins related to pluripotency, and *in vivo* differentiation.

Moreover, the production of clone embryos by cells donating nuclei previously modified epigenetically was performed. In summary, bovine oocytes obtained from ovaries were collected from the slaughterhouse were maturated *in vitro* for 18h, enucleated and reconstituted with biPS cells (n=203) or bovine fetal fibroblasts (bFF, n=153), in five repetitions. After reconstruction, embryos were activated with ionomycin and 6-DMAP and cultured *in vitro* until the blastocyst stage. We evaluated the rates of fusion; cleavage (48h after activation) and blastocyst development (192h after activation) and the results were subjected to Chi-square test accepted significance level of 5%. We did not find difference among groups regarding cleavage rate (81.53 vs 88.96%) and blastocyst production (22.68 vs 29.63%, respectively); however the group reconstructed with iPS cells showed lower fusion rate (47.78 vs 70.59%). In conclusion, bovine iPS cells were produced and generated clone embryos when used in the nuclear transfer.

i) Epigenomic of Stem Cells

This project aims to map epigenomic signatures (transcriptome, methylome, and identification of active regulatory regions) of human pluripotent cell lines (ESC and iPS) generated from Brazilian individuals, using next-generation sequencing. Moreover, this study aims to map the epigenetic dynamic due to commitment of pluripotent stem cells in response to mesenchymal differentiation. We also aim to compare the efficiency of pluripotent differentiation among different cell lines and correlate with epigenetic changes resulting from the process. The prediction of an epigenetic signature that favors differentiation would be of great importance to assist in choosing the best cell type to be used for therapeutic purposes. So far, we expanded and characterized the following cell lines: BR1, iPS-03 Cl8, iPS-03 Cl11, iPS-2, and iPS-4. We are now expanding four cell lines: BR4, BR5, H1 e iPS-WiCell. The next steps are to start the mesenchymal differentiation protocols and to perform the sequencing and analysis.

4) GOAL 4: DETERMINATION OF *IMPRINTING* PATTERNS DURING CELL REPROGRAMMING.

a) Determination of the allele specific expression and allele-specific DNA methylation of genes that undergo imprinting in bovine cell lines induced to pluripotency under different conditions and in hybrid bovine embryos produced by nuclear transfer.

In the last years, autologous pluripotent cells were produced by our group by methods as nuclear transfer (TNCS) and induced pluripotency technology (iPS). Those methods are considered to be efficient strategies to the derivation of autologous pluripotent cells from somatic cells and consist in a promising therapeutic approach in regenerative medicine. Nevertheless both reprogramming methodologies show low efficiency. One explanation for this failure is the epigenetic reprogramming that results in incomplete repurchase the undifferentiated cell state. One of the most outstanding epigenetic characteristics affected by reprogramming technologies is genomic imprinting, once the correct reprogramming is strongly

correlated with the full development potential of embryonic stem cells. Thus, the use of animal models containing interspecific genetic variations, this model has been already validated by our group, enables screening of gene expression allele-specific methylation, making possible the analysis of the real impact of the reprogramming methodologies on the epigenetic control of imprinted genes using techniques for large-scale genomic evaluation.

Specifically, bovine iPS lines could be studied in relation to allele-specific methylation and imprinted gene expression known to be important in the reprogramming process: OCT4, H19 and SNURF/SNRPN. Such results were possible because of the model F1 *Bos taurus* x *Bos indicus* and were never seen in the international setting.

In summary, in gene gene H19 partial hypopmethylation of paternal DMR in three of the four iPS lines studied, none effect on H19 maternal DMR (demethylated), biallelic expression in hypomethylated lines and diminution of H19 global expression, accompanied by diminution of IGF2 global expression. These results are consistent with those obtained when cloned embryos were studied. Concerning to gene SNURF, we found partial hypomethylation of maternal DMR in two iPS lines, variation in the methylation leves of paternal DMR, exclusively monoallelic paternal expression and outstanding increase of global expression.

Very interesting results were observed when the gene related to OCT4 pluripotency was analyzed. A non-expected hypermethylation of the region rich in CG of exon 1 of OCT4 was observed, despite a remarkable increase of bovine specific OCT4 expression, mainly in two of the iPS lines studied. There are no SNP for allelic analysis of methylation in this region, and the next step is the study of hydroxymethylation in the promoter region of this gene. The results achieved might be discussed and possibly the regulation and maintenance of pluripotency in non-human species, particularly aiming at animal pre-clinical models, will be better understood.

b) Evaluation in large scale of gene expression (RNAseq) in hybrid bovine cells induced to pluripotency and produced under diferente conditions and evaluation of the impact of the undifferentiation system in the epigenetic mechanisms that control genes with imprinting by DNA analysis in large scale.

After the recent generation of bovine iPS, we will evaluate the expression of transcripts (RNA-seq) of hybrid cell lines produced under different conditions of undifferentiation (pluripotency induction and nuclear transfer), apart from the analysis regarding the epigenetic control mechanisms of imprinted genes.

5) GOAL 5: EVALUATION OF THE FUNCTIONAL ROLE OF MicroRNAs ASSOCIATED WITH PLURIPOTENCY AND DIFFERENTIATION OF PLURIPOTENT STEM CELLS.

a) Standardization of transfection protocols of pluripotent NTERA2 lines with pre- and anti-miRS for functional assays.

Pluripotency and the ability to self-renew are distinctive properties of embryonic stem cells (ESCs) that confer them an enormous potential in regenerative medicine. The understanding of the regulatory networks acting on ESCs, led to the identification of a core set

of transcription factors (TFs) able to control pluripotency and differentiation, leading to the discovery that somatic cells could be reprogramed into induced pluripotent stem cells (iPSCs), by introducing different combinations of these pluripotency-related TFs, such as OCT4, SOX2 and NANOG. In addition to the wide transcriptional control mediated by TFs, small (~22nt) RNA molecules, called microRNAs (miRNAs), have been shown to a large effect on gene expression by binding to several target mRNAs, leading to translation repression and mRNA degradation. Selected miRNAs are specifically expressed in pluripotent cells and are implicated in the maintenance of their characteristics and their ectopic expression can improve the reprograming induced by the classical TFs or, even, completely substitute them. Although a lot of work has been devoted to the characterization of miRNAs differentially expressed between pluripotent embryonic or iPSCs and their differentiated counterparts, there is a clear lack of systematic studies aiming at defining the functions played by each of the known miRNAs, in the biology of pluripotent stem cells. More specifically, only a handful of works evaluated the function of a few hundred miRNAs, on the proliferation of ESCs or in the reprograming process. Despite the reduced number, the aforementioned studies, helped to pinpoint important miRNA functions in the regulation of self-renewal, pluripotency and differentiation of mouse pluripotent stem cells. Importantly, it became evident that some of these roles are mediated by the regulation of a process called Mesenchymal-Epithelial Transition (MET) and its reverse process, the Epithelial-Mesenchymal Transition (EMT), which are also responsible for the remodeling involving cell migration and differentiation that occurs during embryonic development. EMT is characterized by the transition from epithelial morphology to a typical mesenchymal fibroblastic morphology with reduced levels of Epithelial-cadherin and increased levels of Neural-cadherin. Diverse signaling pathways cooperate in the initiation and progression of EMT and MET and, given their ability to post-transcriptionally target multiple components, miRNAs are considered potent regulators of these processes, largely impacting stem cell pluripotency. Functional studies, to elucidate the roles of miRNAs in the biological properties of pluripotent stem cells are, thus, fundamental to accelerate the progress in this important research field.

b) Standardization of High-Content Screening (HCS) assays to be used in functional assays.

b.1) Establishment of a high-content screening assay for the study of pluripotency and differentiation of stem cells

With that in mind, we established an experimental cellular model, for the functional assessment of factors that may influence distinct biological characteristics of pluripotent stem cells, to be used in conjunction with the technique called High-Content Screening (HCS), which allows the automated acquisition and analysis of fluorescence microscopy images, from 96 or 384-well plates. To this end, due its simple handling characteristics, the human pluripotent embryonal carcinoma cell line NTERA-2 was used. The process of differentiation was evaluated at 2, 4 and 8 days, in the presence or absence of all-trans retinoic acid - atRA (an inducer of cellular differentiation). The transcriptional levels of the pluripotency markers, Oct4 and Nanog, and of the EMT (epithelial-mesenchymal transition) marker N-cadherin, were assessed by real

time PCR. The proliferation of cells undergoing differentiation was assessed by XTT assay. Finally, the protein levels and cellular distribution of Oct4, Nanog and alpha-actin were assessed by fluorescence microscopy, using antibodies or labeled phalloidin and a HCS platform for image analysis. atRA inhibited proliferation and induced differentiation, as shown by the XTT assay results, and by the decay of Oct4 and Nanog, and concomitant increase of N-cadherin transcripts levels over time (Figure 8). Spontaneous differentiation in the absence of atRA, was also observed, although less markedly (Figure 6 and 8). Finally, HCS results showed that during the differentiation process, the nuclear expression of Oct4 and Nanog is lost (Figure 8 and 9) and is associated with altered cell morphology, with loss of epithelial organization, enlargement of cytoplasm, and redistribution of cortical actin into stress fibers (Figure 8), in addition to increased N-Cadherin expression (Figure 8), characterizing the epithelial-mesenchymal transition (EMT), an important mechanism involved in cell differentiation. These results demonstrate the feasibility of using the NTERA-2 cell line as a model for future HCS studies aiming at the identification of microRNAs that act in the modulation of fundamental properties of pluripotent stem cells.

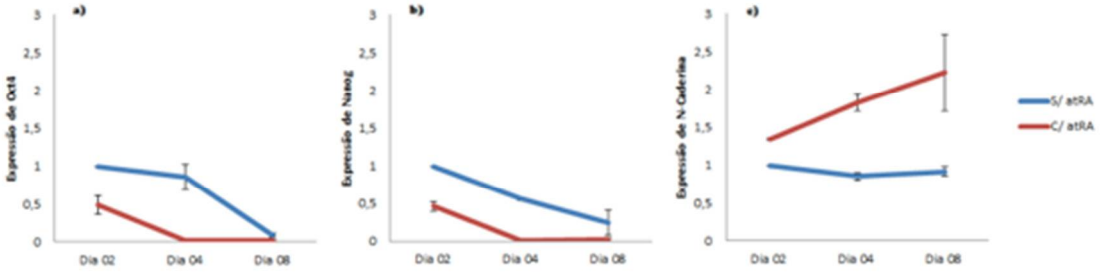


Figure 7. Relative expression levels of Oct4 (a), Nanog (b) and N-cadherin (c) transcripts. The groups were divided in treated or not with atRA, for 2, 4 and 8 days of cell culture cells, as compared to the control group on day 2.

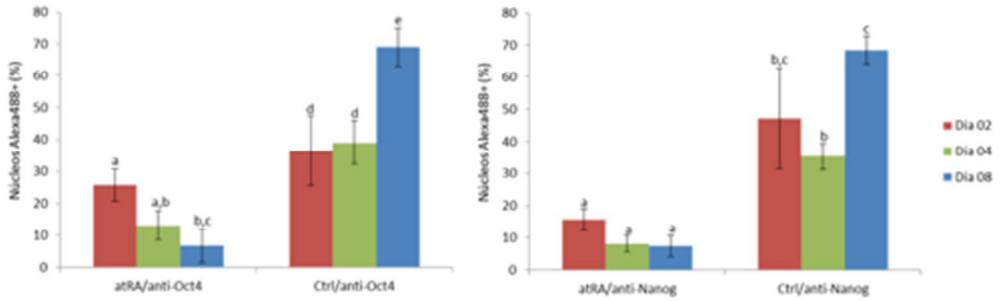


Figure 8. Percentage of cells positive for nuclear staining with anti-Oct4 (a) or anti-Nanog (b). The groups were divided in treated with atRA or non treated (Control) and maintained in cell culture for 2, 4 or 8 days. Different letters represent statistically significant differences ($p < 0.01$).

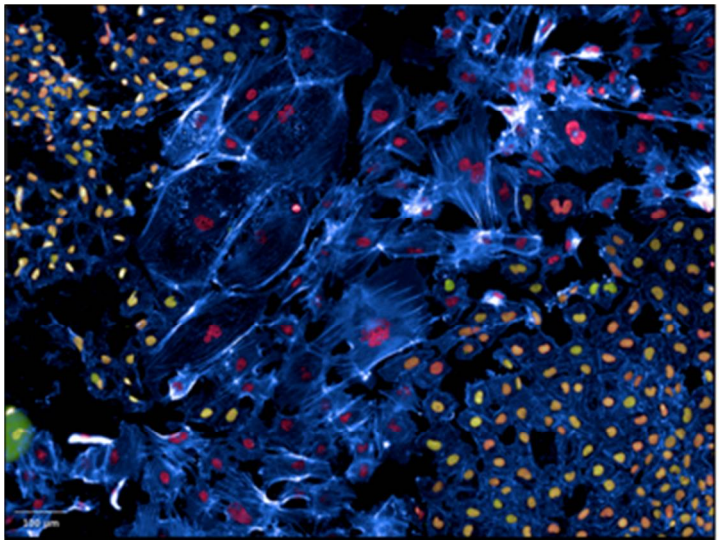


Figure 9. Image composition generated from four field micrographs. The image was obtained from cell after 8 days in cell culture, without treatment with all-trans retinoic acid. In blue, the structure of the actin cytoskeleton; in red nuclei labeled with DAPI and yellow/orange the double staining of nuclei with DAPI and Alexa488 (varying with the intensity of anti-Oct4 staining). In this figure differentiated (red nuclei) and undifferentiated cells (yellow/orange nuclei) can be seen, as well as, the structure of the actin cytoskeleton, which allows the visualization of stress fibers and also the cytoplasm of the larger differentiated cells (Magnification: 200X).

b.2) Focused functional high content screening of microRNAs regulating cell proliferation and viability in human fibroblasts

In order to gain know-how on the execution of functional microRNA screens, we carried a pilot High Content Screen study aimed to evaluate the effects of selected miRs on the proliferation, viability and cell cycle of a somatic cell line (BJ Foreskin Fibroblasts). For this, 2.000 cells were seeded in 96-well microplates and transfected with 50nM of 28 microRNAs mimics (pre-miR) and inhibitors (anti-miR) in quadruplicate. After 5 days, the proliferation was measured by XTT assay (used as a reference standard technique based os spectrophotometry). The nuclear stain Sytox Green was used to identify dead cells. A High-Content Screening platform (Operetta, Perkin Elmer, in demonstration in our lab, at the time) was used to acquire and quantify the images from the viability assays. We found 19 treatments that significantly altered the proliferation of cells. Importantly, the results from both approaches were concordant; while the transfection of pre-miR mimics of miR-20b, miR-101 and miR-181d (known to be expressed in BJ cells) decreased the proliferation of the fibroblasts; transfection of the corresponding anti-miRs had the opposite effect. Compared to controls, pre-miR-181d had the most cytotoxic effect, followed by miR-20b and miR-101, both, in the XTT (Figure 10) and in the Sytox Green assays (Figure 11).

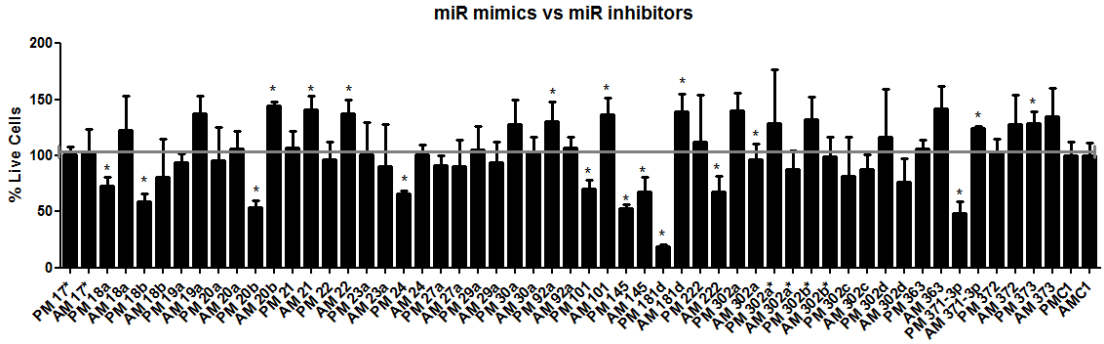


Figure 10. Percentage of viable cells after treatment with microRNAs. BJ fibroblasts were treated with 50 nM of synthetic miRNA (PM) or miRNA inhibitors (AM) for 5 days. After this period, cell proliferation was estimated by the XTT assay.

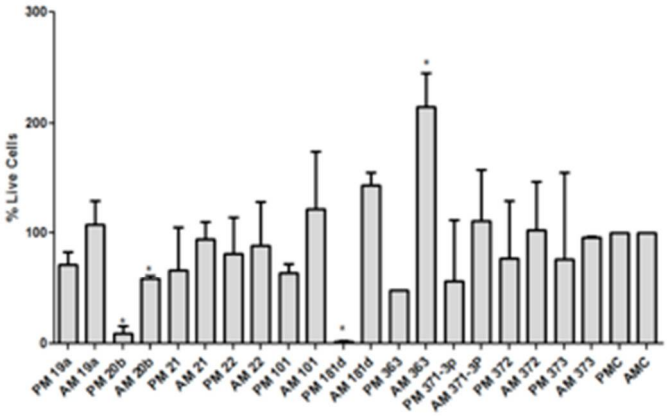


Figure 11. Percentage of living cells after treatment with microRNAs, as determined by the Sytox Green test using a HCS system. * p <0.05

c) Identification of microRNA targets and signalling pathways: miR-29a targets two central components of active DNA demethylation, TET1 and TDG, potential mechanism during reprogramming

As a complementary approach for the microRNA functional studies planed, we carried a study focused on miR-29a, a microRNA shown to modulate reprogramming of somatic cells into iPSC. Interestingly, while miR-29a (a miR highly expressed in Mouse Embryonic Fibroblasts – MEFs) inhibits reprograming; its depletion enhances reprogramming efficiency of MEFs by 2 to 3 fold. Moreover, the transduction of c-Myc, was capable to decrease miR29a levels, and subsequently regulate multiple signaling networks, boosting reprogramming.

In order to explore the molecular mechanisms by which miR29a influences iPSC generation we sought to investigate it targets, using a large-scale microarray-based strategy. Synthetic pre-miRs, inhibitory anti-miRs and corresponding mock transfectants were independently transfected into human fibroblasts and into pluripotent NTera2 lineage. After 72h, whole-genome microarrays transcriptomes were obtained. Confident miR-targets were identified by selecting experimentally modulated transcripts (downregulated by pre-miR and upregulated by corresponding anti-miR) showing evolutionary conserved predicted binding sites for miR-29a (microrna.org). Reprogramming-associated molecular changes were identified by comparing miR-induced changes to transcripts modulated upon iPSC reprogramming (as

determined by comparing two distinct iPSCs to the corresponding fibroblasts of origin) (general strategy on Figure 12).

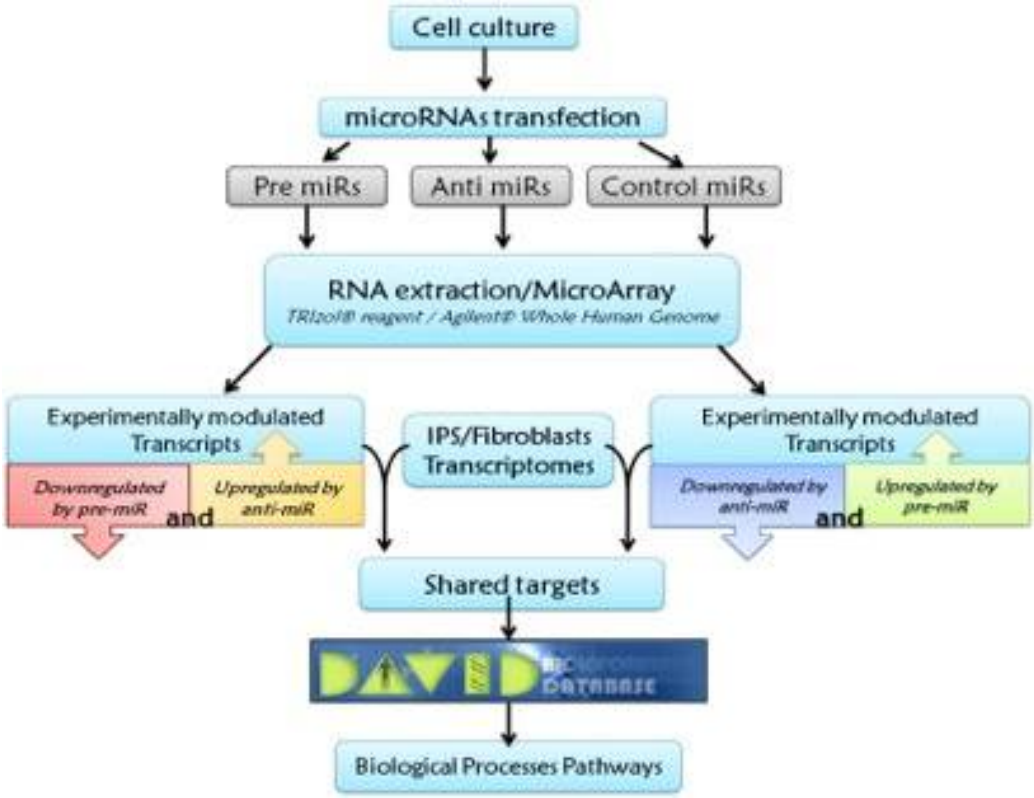


Figure 12. Workflow of experimental procedures for mRNA MicroArray analysis.

While evaluating the targets, we identified transcripts encoding for members of the TET family and TDG proteins whose roles in epigenetic regulation, have only begun to be envisioned. In addition to the microarray data, we have carried transfection experiments in triplicates, and evaluated Tet1 and TDG transcript levels, by real time PCR. These results confirm the down-regulation of Tet1 and TDG transcript levels by miR-29a, and indicate that their levels at least start to accumulate 72h following transfection of the anti-miR-29a (Figure 13). Importantly, Tet1 transcription is directly controlled by Oct4 and Sox2, and during reprogramming, CpG dinucleotides in the promoters of pluripotency factors bound by Tet1 (such as OCT4), are hydroxylated, becoming enriched for 5hmC, finally becoming demethylated and reactivated. Furthermore, it has been shown that Tet1 and NANOG synergistically act enhancing reprogramming efficiency.

Taken together with the data in the literature, our novel finding of Tet1 and TDG as miR-29a targets, may indicate that endogenous fibroblast miR-29a may impair iPSC generation by targeting Tet1 and TDG, and that its inhibition (through the use of anti-miR29a), may favor reprogramming by allowing Tet1 and TDG to accumulate. The confirmation of this hypothesis may help to elucidate how miR-29a modulates the epigenetic machinery during reprogramming.

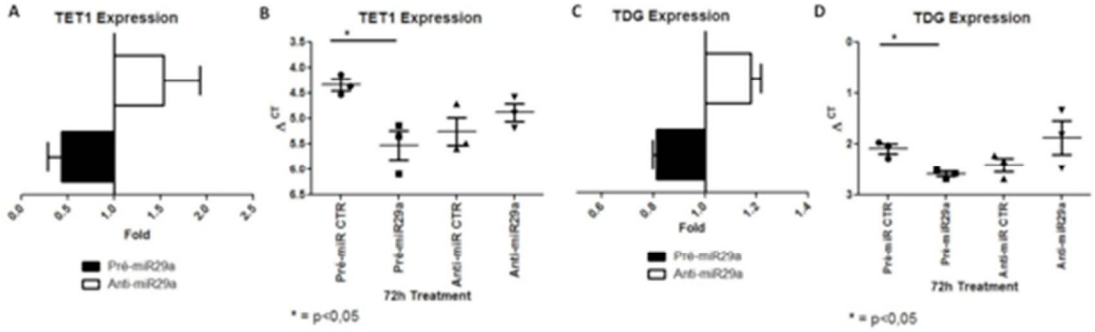


Figure 13. Microarray and Quantitative Real-Time PCR of TET1 and TDG Transcripts. Transcript levels were determined by qPCR. Normalized ΔCt values in the y axis correspond to the difference between the Ct of the evaluated transcript and the Ct obtained for GAPDH. The y axis was inverted for clarity, as smaller ΔCt values correspond to higher expression levels (B and D). *One-tailed non-parametric T-test. Microarray results are shown in column graphs (A and C).

6) GOAL 6: ENDOTHELIAL CELLS FROM DISTINCT TISSUE ORIGINS AND ENDOTHELIAL MESENCHYMAL TRANSITION (EndMT) INDUCTION *IN VITRO*

a) Isolation and expansion of endotelial cells of umbilical vein and evaluation of known inducers of EMT, as TGF-b and evaluating the molecular mechanisms involved in EndMT. Overexpression of transcription factors SNAIL, SLUG, TWIST and ZEB1.

Endothelial-mesenchymal transition (EndMT) is an important and specialized category of epithelial-mesenchymal transition (EMT) and is involved in physiological and pathological processes. In EndMT, endothelial cells (EC) lose intercellular junctions and the endothelial markers and acquire invasive and migratory properties, with mesenchymal phenotype. In physiological conditions, EndMT is involved in embryogenesis and recent studies demonstrate its role in origin and maintenance of mesenchymal stem cells (MSC) in the adult organism. In pathological conditions, EndMT is responsible for the formation process of fibrosis and also participates in tumor progression.

We postulate that EMT and EndMT share the same molecular mechanisms concerning to transition induction, however, further studies are need to clarify similarities and differences between both processes. In this context, the objectives of this project were widely approaching EC biology during EndMT induction and evaluating the role of this process in tumor progression.

In this period, immunophenotypic characterization of endothelial cells was performed. The following assays were also conducted: i) EMT in MCF-10^{A} cells; ii) EndMT with induction by TGF-β2; iii) EndMT with induction by ectopic expression of Snail transcription factor; and iv) evaluation of Snail expression during EMT process in mammalian cells. This last topic of the work was developed in collaboration with Prof. Robert A. Weinberg, from *Massachussets Institute of Technology* – MIT (Cambridge, EUA), who was a scientific adviser and offered laboratory training to the doctorate students Tathiane Maistro Malta Pereira and Lucas Eduardo Botelho, as well as the infrastructure for conducting the study. This project also involves a master and a doctorate thesis that are ongoing with conclusion in 2014 and 2015, respectively.

Among the most relevant results obtained in the period, we can highlight: a) TGF-β2 induced EMT *in vitro* by signaling pathway dependent on Smad, as well as by the activation of pathway Erk1/2 that is independent of Smad; b) Endothelial cells from different anatomic sources were analyzed: EC from aorta (PAEC), primary EC from umbilical cord vein (HUVEC), EC from coronary artery (CAEC) and pulmonary artery (HPAEC). PAEC were the only EC that responded to treatment with TGF-β2 obtaining results consistent with EndMT process *in vitro*; c) The studies of gene and protein expression suggest that the alterations observed in PAEC were started by the activation of Erk1/2 pathway, which is a pathway related to TGF-β signalling, but independente of Smad. Once these EMT/EndMT processes may cause ou aggravate some pathologic conditions like fibrosis, the selective inhibition of Erk pathway could suppress these pathologies induced by TGF-β2; d) Transduction of MCF-10A epithelial cells with Snail transcription factor, associated with treatment with TGF-β2, was the most favorable condition to induce EMT; e) The most appropriate condition to induce EndMT was by culturing EC (HPAEC, CAEC e PAEC) transduced with Snail in EGM2 without serum for five days with the addition of TGF-β2, once this condition was the one that showed higher expression of mesenchymal markers, as well as a slight reduction in the expression of endothelial marker CD31; f) The mammary epithelial cells that spontaneously activated EMT (NAMECs) may show capacity to differentiate into adipocytes and oosteocytes *in vivo*, suggesting that EMT process may reprogramme cells to a phenotype of progenitor cells; g) Snail expression is able to identify multipotent cells derived from mammary epithelium that assumed a mesenchymal phenotype after EMT activation. Therefore, Snail can be a determinant transcription factor to activate the molecular circuit that leads to the acquisition of capacity of differentiating into multiple mesodermic lines after EMT.

The next steps involve conducting the global analysis of gene expression for CAEC cells transduced with Snail and then identifying possible regulation mechanisms of this process.

7) GOAL 7: STUDIES OF THE MOLECULAR MECHANISMS INVOLVED IN THE INCREASE OF IMMUNOMODULATORY PROPERTIES OF MESENSHYMAL CELLS BY INFLAMMATORY STIMULI

a) Evaluating the effect of different Toll-like (TLRs) receptors and their ligands in the proliferation of mesenchymal, after additional inflammatory stimuli. And also evaluating the capacity of mesenchymal cells to inhibit the proliferation of activated T lymphocytes.

b) Evaluating the potential action mechanisms of TLRs ligands.

Mesenchymal stem cells (MSC) represent a promising cell-based therapy for Graft Versus Host Disease (GVHD), for they can suppress the proliferation of activated lymphocytes in vitro, through either direct contact or secretion of anti-inflammatory cytokines. However, clinical trials performed in the past decade evaluating the effectiveness of infused MSC to improve the outcome of GVHD patients had variable results so far. Since the presence of associated infections are frequent in patients with GVHD, and given that priming to different Toll-like receptors (TLR) may elicit opposite immunosuppressory proprieties in MSC, in this study we investigated the modulation of the immune suppressive potential of bone marrow MSC (BM-MSC) by different TLR ligands.

PBMC obtained from healthy donors (n= 3) were utilized to evaluate the protein expression levels (using flow cytometry) of TLR2, TLR3, TLR4 and TLR9 in gated monocytes, cells from the innate immune system known to express those TLRs. As expected, monocytes expressed high levels of all TLR evaluated. Importantly, BM-MSC had the protein expression of TLR3, TLR4 and TLR9 similar to those observed in monocytes, with significant lower levels of TLR2 when compared to monocytes (Figure 14).

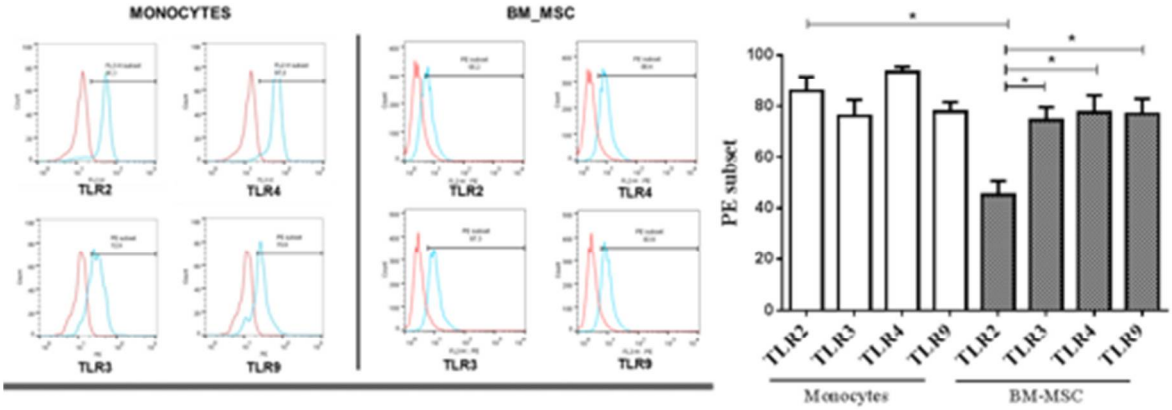


Figure 14. Protein expression levels (PE subset) of Toll-like receptors on monocytes isolated from PBMC and BM-MSC, after incubation with antibodies anti TLR2, TLR3, TLR4 and TLR9, followed by acquisition on flow cytometry. BM-MSC protein levels of TLR3, TLR4 and TLR9 were similar in comparison with monocytes protein levels. Otherwise, TLR2 levels were significant lower in BM-MSC in comparison to monocytes levels. * = significant difference at $p < 0.05$.

In order to perform an initial screening, BM-MSC derived from a single patient were stimulated (in experimental triplicates) with LPS, POLY IC, CpG-A, CpG-B or DSP30, and then subjected to an immunosuppression assay (Figure 13A). For this, peripheral blood CD3+ lymphocytes were labeled with the fluorescent dye CFSE, activated with antibody-loaded beads anti CD2, CD3 and CD28, and cocultured for 5 days with treated or untreated BM-MSC. Suppression of lymphocyte proliferation, by BM-MSC in different conditions, was evaluated by flow cytometry. As expected, compared to activated lymphocytes alone, non-stimulated BM-MSC significantly suppressed the proliferation of lymphocytes by about 50%, however, stimulation of TLR4 with LPS impaired the immunosuppressive capacity of MSC. In contrast, stimulation with DSP30, but not other TLR9 ligands, leads to a significant enhancement of MSC suppressive capacity, when compared to non-stimulated BM-MSC.

Because of these interesting results, we perform additional immunosuppression assays with BM-MSC derived from five distinct donors, stimulated or not with LPS, DSP30 and, additionally, with both ligands simultaneously (Figure 15). In accordance to the previous results, as compared to untreated control MSC, the immunosuppressive capacity of TLR4 primed BM-MSC was significantly hampered, while that of TLR9 primed BM-MSC was enhanced. Importantly, simultaneous priming of MSC with both ligands was able to keep the immunosuppression at levels comparable to that of untreated BM-MSC.

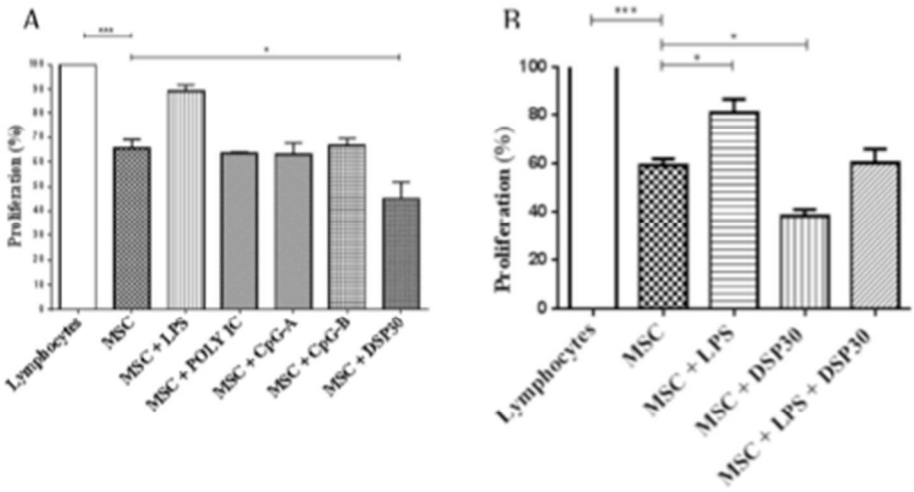


Figure 15. The modulatory activity of TLR stimulation on the capacity of BM-MSC to suppress the proliferation of CFSE-labeled and activated lymphocytes.(A) Experimental conditions at experimental replicates: significant immunosuppression of non-stimulated BM-MSC (MSC), maintenance of immunosuppression at non-stimulation levels after stimulation with POLY IC (MSC + POLY IC), CpG-A(MSC + CpG-A) or CpG-B(MSC + CpG-B), hampering and enhancement of immunosuppression after stimulation with LPS (MSC + LPS) and DSP30 (MSC + DSP30), respectively. (B) Experimental conditions at biological replicates: hampering and enhancement of immunosuppression of BM-MSC after stimulation with LPS (MSC + LPS) and DSP30 (MSC + DSP30), respectively, maintenance of the immunosuppressive capacity at non-stimulated levels after stimulation with LPS and DSP30 (MSC + LPS + DSP30) . * = significant difference at $p < 0.05$.

In order to identify the possible mechanisms involved in this modulatory activity, BM-MSC (n=3) in same conditions were evaluated for changes in proliferation, using an assay of EdU incorporation into DNA and by direct cell count of fluorescently labeled cells, using an automated High Content Screening (HCS) imaging device (Image Xpress Micro, Molecular Devices). TLR9 priming with DSP30 significantly promoted the proliferation of BM-MSC, as demonstrated by higher EdU incorporation and cell count ($p<0.05$); while TLR4 priming by LPS had no effect in these parameters.

To investigate if the observed LPS and DSP30 effects on MSC immunosuppression potential could result, at list in part, from altered profile of secreted cytokines, transcript levels of the pro-inflamatory cytokine IL-1β and the immmuneregulatory factor TGF-β1 were evaluated by RT-qPCR, on BM-MSC stimulated with LPS, DSP30 or both ligands. Stimulation with LPS, but not DSP30, induced IL1B expression. In contrast, stimulation with DSP30 led to a significant increase on TGFB1 expression. Overall, the simultaneous stimulation with LPS and DSP30, led to an intermediate expression level of both transcripts (Figure 14).

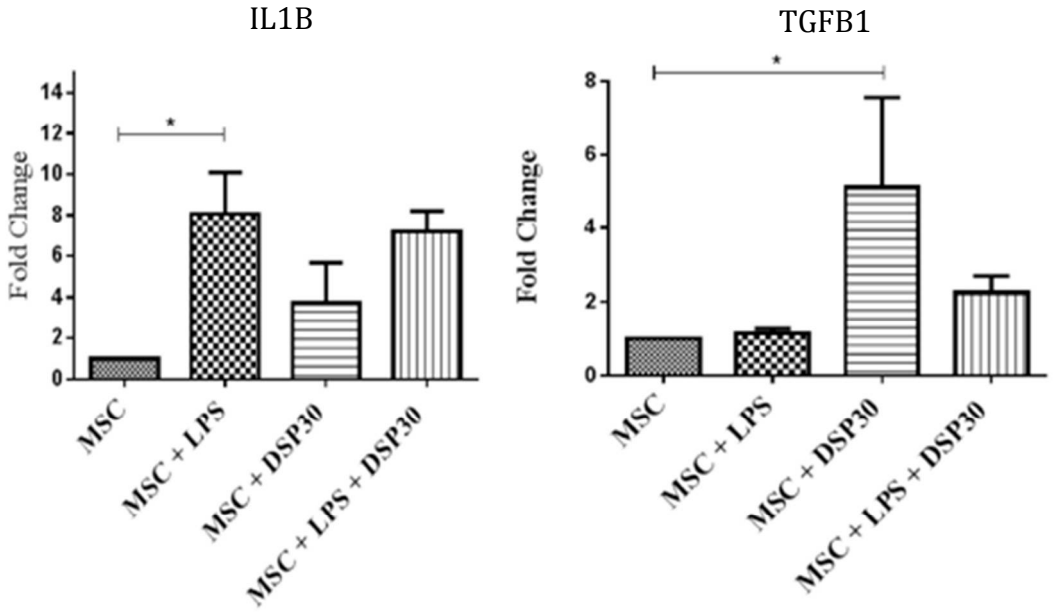


Figure 16. Modulatory effects on gene expression in virtue of stimulation with Toll-like receptors agonists. Cells were stimulated or not (MSC) with LPS (MSC + LPS), DSP30 (MSC + DSP30) or both agonists simultaneously (MSC + LPS + DSP30), being then quantified the expression of genes coding cytokines by qPCR. Significant increase on gene expression of IL-1β1 due to LPS stimulation (MSC + LPS), but not DSP30 or LPS + DSP30. Significant increase on gene expression of TGFβ1 after DSP30 stimulation and maintenance of non-stimulated levels after stimulation with LPS or LPS + DSP30.* = significant difference at $p < 0.05$.

Since priming by TLR4 and TLR9 may lead to the respective activation of canonical and non-canonical NF-kB pathways (which are mediated by the transcription factors RelA and RelB, respectively); we used chromatin immunoprecipitation (ChIP) to evaluate the binding of RelA and RelB to the gene promoter of the vascular cell adhesion molecule-1 (VCAM-1, a known NF-kB target with roles in inflammation, as well as in immunomodulation) in BM-MSC (n=3) primed with TLR4 and/or TLR9. Priming of TLR4 led, exclusively, to the binding of RelA (three fold enrichment, compared to unprimed BM-MSC). Moreover, simultaneous priming of TLR4 and TLR9 resulted in an increased binding of both RelA and RelB.

Altogether, our results indicates that BM-MSC had its immunosuppressory capacity hampered by priming of TLR4 by LPS (possibly due to the activation of canonical NF-Kb pathway), while TLR9 priming by CpG oligonucleotide enhances the immune suppression, which may be related to an increase in proliferation and, possibly, due to the activation of non-canonical NF-Kb pathway in MSC simultaneously primed for TLR4 and TLR9. These observations may help to elucidate the cellular and molecular mechanisms underlying the heterogeneous results in clinical studies of GVHD patients receiving BM-MSC infusions.

As patients subjected to bone marrow transplantation are more prompt to infections by different pathogens (potentially exposing BM-MSC to different TLR ligands), the identification of the CpG oligonucleotide as a TLR9 ligand capable of enhancing and protecting the immunosuppressive activity of BM-MSC, may lead to the establishment of a laboratory protocol aiming the production of MSC for the treatment of GVHD patients. In this sense, priming of TLR9 or the specific activation of non-canonical NF-Kb pathway, could be used in order to boost BM-MSC efficiency, or to protect it against unwanted effects other TLR4 ligands (such as LPS).

08) GOAL 8: TO PEFORM A COMPREHENSIVE EVALUATION OF THE BIOLOGICAL DIFFERENCES IN CLONAL CELLS BETWEEN MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL) AND CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

Monoclonal B-cell lymphocytosis (MBL) is an asymptomatic hematologic condition defined by the presence of a small (<5 x 10(9)/L) clonal B-cell population in the peripheral blood in the absence of lymph-node enlargement, cytopenias or autoimmune diseases. It is found in approximately 3-12% of normal persons depending on the accuracy of analytical techniques applied. According to the immunophenotypic profile of clonal B-cells, the majority of MBL cases (75%) are classified as chronic lymphocytic leukemia (CLL)-like. This form may progress into CLL at a rate of 1-2% per year. It is thought that CLL is always preceded by MBL**.** The aim of the present study is to compare the microRNA profile and telomere length of B-cells of patients with MBL and CLL. In order to compare the same cell subsets in the two disorders, CD19+CD5+ cells will be sorted and molecular analyses will be performed only in this putatively preleukemic counterpart of the CLL cells. So far we have included 18 CLL and 16 MBL patients, among the latter 6 presented a rare familial form that will provide additional information. The methodology for the isolation of CD19+CD5+ have been established and yield cell suspensions with purities > 80%. To check the quality of samples to be used in the microRNA analysis, we performed RQ-PCR reactions for the RNU44 e RNU48 endogenous microRNAs. In all cases, the samples had very good quality and are adequate to proceed with the large scale analysis. For the telomere length analysis, the methodology has already been established in the lab by Prof. Dr. Calado and we have samples from another 5 CLL and 4 MBL patients ready for processing.

09) GOAL 9: GENOME WIDE ANALYSIS OF CANCER CELLS AND CANCER STEM CELLS FROM DIFFERENT SOLID TUMORS

a) **To establish genetic and epigenetic signatures (DNA methylation patterns and expression of coding and non-coding genes), and structural variations (SNVs, CNVs, and rearrangements) specific of tumoral cells and cancer stem cells isolated from astrocytomas, and from head and neck and colon tumors.**

We present here two studies that investigated 1) the role of a long non-coding gene in EMT and stemness acquisition, and 2) the expression profile of HOX gene family in the progression of head and neck tumors.

1. Hotair is a member of the recently described class of noncoding RNAs called lincRNA (large intergenic noncoding RNA). Various studies suggest that Hotair acts regulating epigenetic states by recruiting chromatin-modifying complexes to specific target sequences that ultimately leads to suppression of several genes. Although Hotair has been associated with metastasis and poor prognosis in different tumor types, a deep characterization of its functions in cancer is still needed. Here, we investigated the role of Hotair in the scenario of epithelial-to-mesenchymal transition (EMT) and in the arising and maintenance of cancer stem cells (CSCs). We found that treatment with TGF-β1 resulted in increased Hotair expression and triggered the EMT program.

Interestingly, ablation of Hotair expression by siRNA prevented the EMT program stimulated by TGF-β1, and also the colony-forming capacity of colon and breast cancer cells. Furthermore, we observed that the colon CSC subpopulation (CD133(+)/CD44(+)) presents much higher levels of Hotair when compared with the non-stem cell subpopulation. These results indicate that Hotair acts as a key regulator that controls the multiple signaling mechanisms involved in EMT. Altogether, our data suggest that the role of Hotair in tumorigenesis occurs through EMT triggering and stemness acquisition (Figure 17).

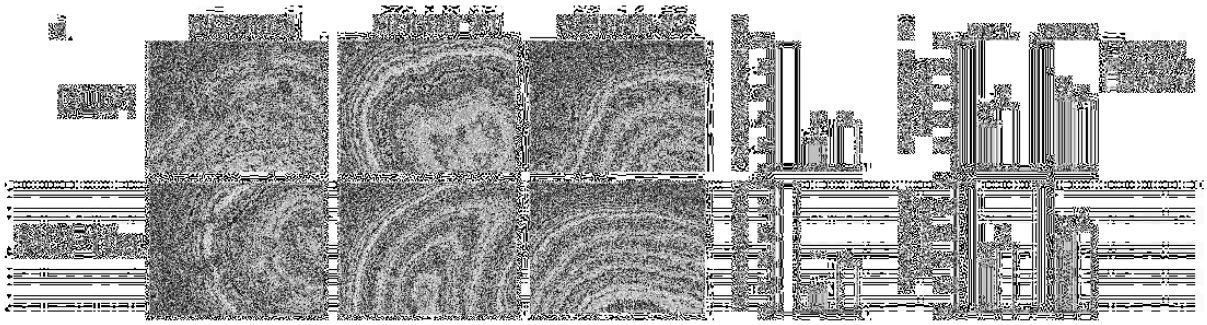


Figure 17. Colonosphere and mammosphere formation are inhibited by Hotair knockdown. Representative images (A) and quantification (B) of colonosphere and mammosphere formed from DLD1 and MCF10a cells, previously transfected with siHotair or siControl. Spheres were counted by visual inspection in light microscopy. Data are shown as mean ± SD from 3 independent experiments. (C) qPCR was performed to evaluate the expression of Hotair in day 1 and 14 of the sphere assays. The HPRT gene was used to normalize RNA inputs. Data are shown as mean ± SD from 3 independent experiments. (*p<0,05).

2. Larynx squamous cell carcinoma (LSCC) is the second most aggressive cancer from head and neck (HSCC). Survival of LSCC patients has been found worsening, albeit significant advances in technology and therapeutics were made. We sought to clarify new genetic targets driven the development of LSCC. Thirty-two samples were collected from patients undergoing surgical ablation of LSCC according to Ethic Committee guideline and patient pre-informed signed consents. Whole human genome microarray analysis was applied to investigate new genetic targets in LSCC. Validation was carried out with qPCR. To clarify transcriptomic dataset, human FADU cell line was used together with siRNA, flow cytometry, and colony formation analyses. Analyzing tumors revealed that eight members of the homeobox gene family (HOX) were expressed 200 times more in LSCC samples than in normal larynx tissue. RT-qPCR analysis validated all transcriptomic findings for the HOX gene family. Receiver Operating Characteristic statistical method (ROC curve) predicted that 8 members of the HOX gene family differentiate tumors from their normal surrounding tissue. Enabling the comparison of patient clinics with gene expression data, ROC curve analysis yet revealed that HOXC8 and HOXD11 genes are related to the tumor differentiation degree and regional lymph node metastasis, respectively. From siRNA assay, we found that *HOXC8* and *HOXD11* genes are essential for the expansion of FADU cell colonies. Our findings strongly suggest that members of the HOX family might be associated with the development of LSCC (Figure 18).

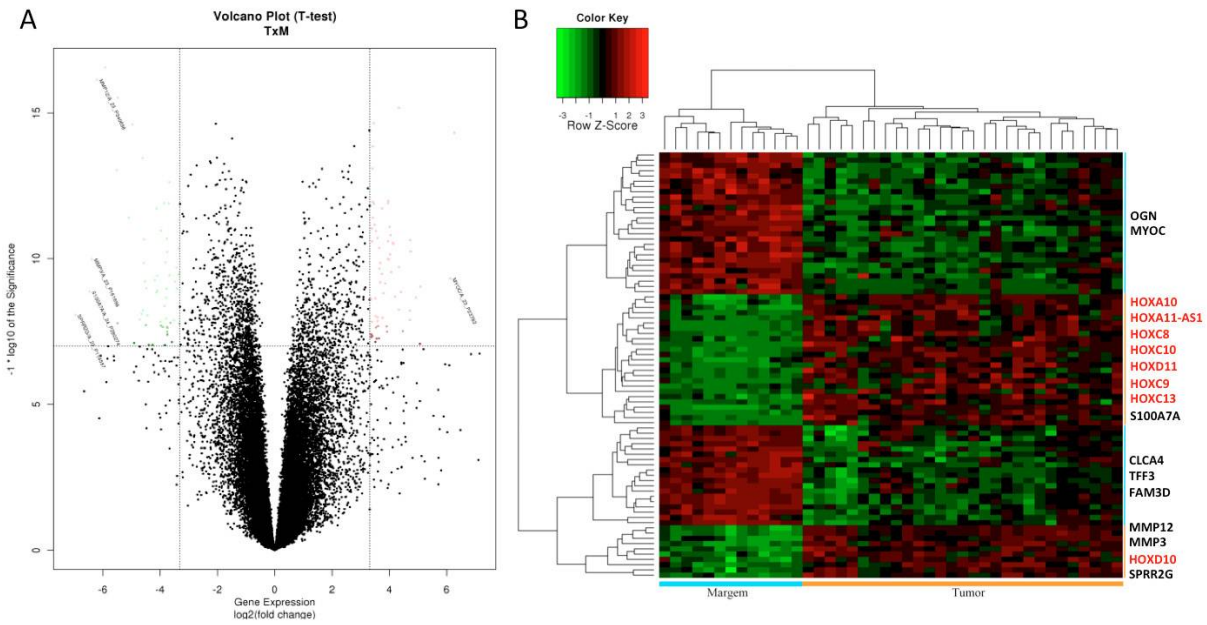


Figure 18. Microarray analysis of differential gene expression in normal (blue dash) versus LSCC (orange dash) samples. **A)** *Volcano* Plot **B)** Hierarchical cluster diagram. Data are visualized as heat plots, 'red' representing elevated gene expression and 'green' decreased gene expression.

b) To identify and characterize the gene pathways (DNA repair genes, detoxifying enzymes, efflux pumps and apoptosis genes) associated with the resistance of cancer stem cell to radio/chemotherapy.

In this section, we initiated the functional analysis of a novel DNA repair gene in the maintenance of tumor cells isolated from glioblastomas, which are the most common type of primary brain cancer in adults. Astrocytomas present a wide variation in differentiation and aggressiveness, being classified into three different grades: low-grade diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma multiforme (grade IV), the most frequent and the major lethal type. Recent studies have highlighted the molecular heterogeneity of astrocytomas and demonstrated that large-scale analysis of gene expression could help in their classification and treatment. We showed that HJURP, a novel protein essential for the repair of double strand breaks in DNA, is remarkably overexpressed in a cohort composed of 40 patients with different grade astrocytomas. We also observed that tumors presenting the higher expression levels of HJURP are associated with poor survival prognosis, highlighting HJURP overexpression as an independent prognostic factor of death risk for astrocytoma patients. More importantly, we found that HJURP knockdown strongly affects the maintenance of glioblastoma cells in a selective manner. Glioblastoma cells showed remarkable cell cycle arrest and premature senescence that culminated in elevated levels of cell death, differently from non-tumoral cells that were minimally affected. These data suggest that HJURP has an important role in the maintenance of extremely proliferative cells of high-grade gliomas and point to HJURP as a potential therapeutic target for the development of novel treatments for glioma patients (Figure 19).

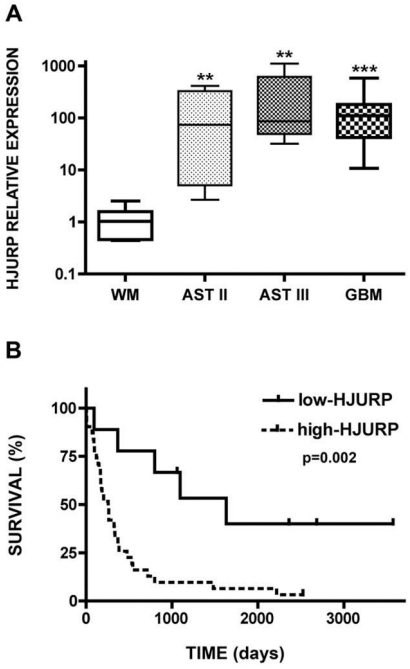


Figure 19. Levels of HJURP mRNA are increased in different grade astrocytomas and correlated with survival prognosis. 9A) Expression levels of HJURP mRNA in samples of normal whi matter (WM, n = 7), diffuse atrocytoma (AST II, n = 5), anaplast astrocytoma (AST III, n = 5) and glioblastoma multiforme (GBM, n 30) were evaluated by quantitative RT-PCR. Boxes represent low and upper quartiles of HJURP relative expression ranges, wi medians indicated. Whiskers represent the 10th and 90th percentile **P-value = 0.0025 and ***P-value <0.0001 in comparison wi normal white matter, Mann-Whitney test. (B) Kaplan Meier surviv curves for glioma patients according to the HJURP expression levels the tumors. Patients were divided in two groups: i) HJURP relati quantities below 39.7 (threshold value determined by ROC curv (solid line, n = 9), and ii) HJURP relative quantities above 39.7 (dash line, n = 31).

c) To identify genetic and epigenetic signatures that can discriminate different stages of tumor development

The general objective of this goal is to identify proteins differentially expressed during the tumor progression of neoplasias of the central nervous system (CNS) by proteomic approach, analizyng protein profile of cancer tissues and in relation to normal brain tissue, as well as the functional proteomic study in cell lines derived from neoplasias of the CNS.

Proteomic analyses of medulloblastoma

Label-free LC-MS/MS quantitative proteomic analysis was conducted in a mass spectrometer electrospray Q-Exactive to identify and quantify proteins among the samples of classical infantile (Group 1, 2-10 years old, n=4) and adult MB (Group 2, 17-29 years old, n=3). Three independent programs (Sieve, Scaffold and Mascot Distiller) were used to quantify the proteins of the samples analyzed. The present work was pioneer in the investigation of tumor samples of medulloblastoma by label-free quantitative proteomics. Proteomic quantitative analyses were rigorous and from them we were able to select proteins differentially expressed, which are interesting for medulloblastoma. The protein Ub2l3 identified only in the group of infantile tumors and in DAOY regulates the estabilization of proteins involved in the biology of medulloblastoma such as p53. Proteins increased in infantile tumor samples are related to proteins folding, response to non-folded proteins and biosynthesis of N-glycans: GRP78, endoplasmin, calreticulin, PDIs, glucosidade II, cyclophilin B and RPN1. In relation to the proteins increased in tumors of adults, we highlight MAPs and the proteins involved in the processes of differentiation and neuronal development: TAU, MAP2, NCAM1 and CRMP-2. Moreover, we point the protein NEDD8 increased in adults as a potential therapeutic target.

This proteomic study on medulloblastomas of adult and infantile tumors was important to improve our understanding of the biology of medulloblastoma type classical, and it was able to contribute to define new targets for diagnoses, prognoses and therapeutic targets that might enable a differentiated and effective treatment to patients.

Functional proteomics of glioblastoma, using U87MG (tumorigenic) and T98G (non-tumorigenic) cell lines as study models.

In this experiment, U87MG and T98G cell lines were treated with 20ug/mL of epidermal growth factor (EGF) for 18 hours in culture with amino acids not labeled with SILAC (K and R). Samples were collected and proteins were extracted according to the protocol established in the laboratory. Proteins were quantified by Bradford's method and subsequently mixed at ratio of 1:1 with the protein extract from the corresponding line enriched with SILAC control (<90% of metabolic incorporation). Samples mixed were subjected to sorting by SDS-PAGE electrophoresis and gels fractioned in up to 14 fractions with different faixas/ranges of molecular mass. Every piece of gel was treated with trypsin ("in situ") for 24 hours at 37°C and peptides were extracted in 0.1% formic acid solution containing 5% of acetonitrile. Gel fractions were then sorted in UPLC (Waters, Beverly, MA) modified for sorting in two phases, ionic exchange and reverse phase (RP), the first phase was a column of strong cation exchange (SCX) followed by reverse phase (RP) with 3 to 5 plugs of ammonium formate solution at growing concentration of NH4+ (25, 50 and 150 mM). Peptide samples were analyzed online on an electrospray source linked to a Q-TOF mass spectrometer (ESI-Q-TOF-MS, Ultima, Waters). The data of spectra of induced collision (CID-MS/MS) were automatically collected by data dependent acquisition (DDA), selecting the four most intense ions/chromatography time.

Mass spectra were treated in the program Mascot Distiller, selecting trypsin, SILAC, pSer, pThr and pTyr as peptide modifications, 1.2Da for precursor ion and 0.8Da for product ions (fragments), 1 trypsin cleavage lost. Files were exported in the format .xml and analyzed in the program Scaffold 4.3.1.

In U87MG, 24 phosphopeptides corresponding to 17 phosphoproteins were detected, whereas for T98G, 10 phosphopeptides corresponding to 8 phosphoproteins were detected. In both analyses, some phosphoproteins showed more than one phosphorylation site detected. The biological importance in EGF stimulus setting is being analyzed by a net/network of interactions like String (http://string-db.org). At this level of investigation, validated by Ascore algorithm, only the protein Proteasome 26S subunit ATPase 1 (PRS4) was detected in both cell lines.

U87MG proteomic analysis treated with EGF for 18 hours showed the identification of 237 proteins, 13 proteins quantified as >1.5 times in relation to the one not treated (Table 4). Only the proteins K2C1, ACTN4, PLEC, IF4A1 and NAMPT were significant (<0.05 Mann Whitney test). Forty phosphoproteins were also identified; however none of them was quantified by the ration/razão between Control/EGF. The analysis by Scaffold software demonstrated that 143 proteins were modified by SILAC and 234 were not modified, this higher number indicates a

flaw in the detection by DDA in ESI- Q-TOF-MS, because the samples containing SILAC were mixed 1:1 and quantified in the same lot of protein determination. Manual evaluation will be conducted in the spectra of these phosphoproteins to find and quantify phosphopeptide pairs.

Proteomic analysis of T98G line showed 139 proteins identified, 137 from the sample treated by EGF, 83 proteins of control samples labeled with SILAC. In the total, 25 phosphoproteins were identified and only one PRS4 had quantification 1.5 times less expressed in the sample treated with EGF in relation to control, but this was not a statically significant difference ($p=0.32$ Mann Whitney test). Differentially expressed proteins are demonstrated in Table 5. Among them, ACTB, H2A1D and AK1C2 were significantly different ($p<0.05$ Mann Whitney test).

By inter-relating all the proteins identified (including the not phosphorylated) between U87MG and T98G, we observed that there were 127 proteins in common, 11 exclusive to T98G and 36 proteins exclusive to U87MG. There was no discrimination between proteins and cell lines. Other activities are ongoing.

d) To identify genetic mechanisms associated with the triggering and control of epithelial-mesenchymal transition (EMT) in tumor cell lineages

The general objective of this goal is to identify proteins differentially expressed during the tumor progression of neoplasias of the central nervous system (CNS) by proteomic approach, analizyng protein profile of cancer tissues and in relation to normal brain tissue, as well as the functional proteomic study in cell lines derived from neoplasias of the CNS.

10) GOAL 10: ANALYSIS OF GENETIC AND EPIGENETIC CHANGES IN CANCER/LEUKEMIA STEM CELLS

a) Analysis of the global methylation pattern in leukemic stem cells of APL

In this goal, we will determine if the increase of methylation in CpG islands located in the promoter region of genes EGF and EGFR of blasts from patients with acute promyelocytic leukemia (APL) (previous results of our laboratory) correlates with clinical response. Determining the molecular mechanisms by which hypoexpression of EGF and EGFR affects proliferation, resistence to apoptosis and tumor growth in APL.

Our hypothesis is that the lowest intracellular concentrations of EGF and EGFR favor leukemogenesis causing blockage of cell differentiation and deregulation of cell cycle with consequent greater tumor proliferation.

The value of EGF and EGFR expression as prognostic marker was evaluated in a cohort of patients with acute promyelocytic leukemia (APL) treated in accordance with a protocol of the International Consortium on Acute Promyelocytic Leukaemia. We detected significant correlation between the lowest EGF expression and the worst response to treatment, associated with the greatest risk of relapse and the lowest global survival. Patients classified in the group of EGF low expression showed higher count of leukocytes, lower serum concetration of albumin and higher plasma concentrations of fibrinogen. For functional studies, cDNA of the

genes EGF and EGFR were cloned and pMEG lentiviral vectors were constructed. NB4 and NB4-R2 transfected cells for induction of EGF and EGFR expression showed lower cell proliferation rate and higher expression of the CD11b differentiation marker, independent of the stimulus with all-trans retinoic acid (ATRA). c-Balb/Nude animals inoculated sc with NB4 cells transduced to EGF or EGFR developed lower tumor mass in comparison with the animals inoculated with control cells.

b) Analysis of the change in gene expression and interleukin production induced by cafeic acid.

This step will still be initiated.

c) Analysis in vitro and in vivo of induced deltaN p73 expression in leukemic stem cells of the transgenic model of APL.

This step will still be initiated.

11) GOAL 11: PHENOTYPIC, MOLECULAR AND FUNCTIONAL ANALYSIS OF MESENCHYNAL STROMAL CELLS FROM HEALTHY INDIVIDUALS AND PATIENTS WITH VIRAL INFECTIONS AND HEMATOLOLOGICAL DISEASES.

a) *New* Evaluation of the phenotypic, molecular and functional analysis of mesenchynal stromal cells of patients with chronic myeloproliferative diseases.

This project will be iniciated in the second semester of this year.

b) *New* Evaluation of the phenotypic, molecular and functional analysis of mesenchynal stromal cells of patients with HTVL-1 infection.

The typical characteristics of mesenchymal stem cells (MSCs) can be affected by inflammatory microenvironment; however, the exact contribution of HTLV-1 to MSC dysfunction remains to be elucidated. In this study, we demonstrated that MSC cell surface molecules VCAM-1 and ICAM-1 are upregulated by contact with HTLV-1, and HLA-DR was most highly expressed in MSCs co-cultured with MT2 cells. The expression levels of VCAM-1 and HLA-DR were increased in MSCs cultured in the presence of PBMCs isolated from HTLV-1-infected symptomatic individuals compared with those cultured with cells from asymptomatic infected individuals or healthy subjects. HTLV-1 does not impair the MSC differentiation process into osteocytes and adipocytes. In addition, MSCs were efficiently infected with HTLV-1 in vitro through direct contact with HTLV-1-infected cells; however, cell-free virus particles were not capable of causing infection. In summary, HTLV-1 can alter MSC function, and this mechanism may contribute to the pathogenesis of this viral infection.

c) *New* Characterization of cell subpopulations of stromal vascular fraction of adipose tissue.

Multipotent stromal mesenchymal cells of adipose tissue (CEMM-TAs) belong to a population of heterogeneous cells named stromal vascular fraction (SVF). Different populations

can be found in SVF and up to now CD34 expression and the absence of CD146 marker are the main phenotypes observed in CEMMs-TA. The identification of a marker for CEMM-TAs meets another important issue related to the true developmental origin of CEMMs-TA, where stands the hypothesis that CEMMs can also be originated from neural crest by epithelial-mesenchymal transition. CD271 is a typical cell marker originated from neural crista, and recently it has been associated with human CEMMs-MO, identifying a group of mesenchymal progenitor cells. Considering then the hypothesis that CEMM may also have a neuroectodermal origin, and that cells isolated by a typical marker of cells originated from neural crest like CD271 can identify mesenchymal progenitors. This study will evaluate the capacity of CD271 marker to isolate CEMM-TA by population sorting from SVF of adipose tissue ($CD31^{neg}CD45^{neg}CD271^{pos}CD34^{pos}$ and $CD31^{neg}CD45^{neg}CD271^{neg}CD34^{pos}$). Populations obtained will be characterized regarding morphology, proliferative potential, their immunophenotypic profile in culture, differentiation capacity *in vitro* in osteoblasts and adipocytes, the capacity to generate fibroblastoid colonies (CFU-F) and their profile of gene expression by real time PCR. The results obtained so far are based on analyses involving two fragments of adipose tissue from the abdominoplasty of patients between 30 – 40 years old.

The results obtained from immunophenotyping of isolated cells without culture have already shown typical characteristics of an immunophenotype compatible with CEMMs, once the expression of markers related to CEMMs, such as CD90 and CD73, showed high percentages. In addition, we also observed high expression of CD34, a characteristic marker of CEMMs-TA in recent cultures.

For cell sorting process, cells were subjected to a negative selection of CD31 (endothelial) and CD45 (hematopoietic) markers and from this double negative population, then the population of interest $CD271^{pos}CD34^{pos}$ and $CD271^{neg}CD34^{pos}$ was selected.

The results of the assay of formation of fibroblastoid colonies (CFU-Fs) demonstrated that cell population $CD271^{neg}CD34^{pos}$ has greater potential to generate CFU-Fs in relation to other populations studied, although all cells were able to generate fibroblastoid colonies.

Immunophenotypic analyses of the cells in culture after the 1^{st} passage revealed a similar increase in all populations studies regarding markers CD90, CD73 and CD105, demonstrating that by the end of primary culture a great percentage of cells already exhibits typical characteristic of CEMMs. As expected, we observed an expressive decrease in the percentage of positive CD34 cells. Nevertheless, we verified an increase in all populations, in the expression of CD271 marker. This increase has recently been reported by other group of researchers, where they observed that CEMMs-DA, even when subjected to a negative selection for CD271, showed gradual increase in CD271 expression throughout culture of CEMMs-DA (Braun et al., 2013).

Although different cell populations show the same immunophenotypic profile in culture, suggesting that there are no significant differences among the populations subjected to sorting and SVF, we observed after culture in specific media inducing differentiation for adipocytes that $CD271^{neg}CD34^{pos}$ population had greater capacity to accumulate cytoplasmic lipid vesicles in relation to other cell populations (n=1)(Fig. 5).

The results obtained up to now suggest that CD271 cell surface marker does not identify mesenchymal cells of adipose tissue with characteristics of progenitor cells, once $CD271^{neg}CD34^{pos}$ population obtained greater capacity to generate CFU-Fs in relation to SVF and $CD271^{pos}CD34^{pos}$ population. Moreover, $CD271^{neg}CD34^{pos}$ cells showed higher differentiation potential in adipocytes mainly in relation to SVF.

d) *New* Characterization of mesenchymal stromal cells derived from umbilical cord.

Mesenchymal stromal cells (MSC) need to go through a culture and expansion process, therefore it is essential that our knowledge is well elucidated concerning to their functional biology in culture. This project proposes a wide characterization of MSC-UC regarding the identification of subpopulations which we assume to be responsible for the beneficial clinical effect, as well as for the immunomodulatory abilities of heterogeneous population in culture.
Our first results of the non-cultured population, by flow citometry analyses, show the frequencies of every subpopulation (Table II). The result indicates similarity in the frequency of MSC present in bone marrow.

Table 2. Characterization of cell subpopulations derived from umbilical cord.

Subpopulation	Frequency (% of total population)
CD45- CD44+ CD271+ CD146+	0.15%
CD45- CD44+ CD271+ CD146-	0.42%
CD45- CD44- CD271+ CD146+	0.82%
CD45- CD44- CD271+ CD146-	3.91%

MSC were characterized regarding the expression of surface receptors, by flow cytometry, from passage 1 to passage 3. The results show that only chemokine receptors were kept in culture, as it can be seen in the graphs below.

We performed CFU-F assays with MSC from the first until the fourth passage, and the results demonstrated that the efficiency of colonies formation (CFE) is kept throughout the passages.

MSC were subjected to protocols of osteogenic and adipogenic differentiation in a period of 21 days, and demonstrated that they were able to differentiate into osteocytes and adipocytes.

e) *New* Human adipose tissue as ectopic vehicle to produce coagulation factor VIII in a murine model of hemophilia A.

Hemophilia A (HA) is coagulopathy caused by mutations in the gene of factor VIII of human blood coagulation (FVIII). Carriers of these mutations do not produce functional FVIII, therefore, they are not able to generate sufficient amounts of thrombin during coagulation, what may result in hemorrhages in various body sites that affect quality of life and can be fatal.

The current strategy for Hemophilia treatment is the replacement therapy by the infusion of FVIII protein. In spite of the benefits of this approach, the development of a lasting prophylactic therapy, less expensive and that requires less infusions still represents one of the major challenges for HA treatment. The transplantation of mesenchymal stromal cells (MSC) producing FVIII is a strategy potentially able to accomplish these objectives. However the attempts reported so far bump in the limited survival after transplantation of MSC. It is known that stem cells are protected from cell death by the interactions that occur with extracellular matrix and other cells, which compose the niche. Moreover, the interaction among all those elements is responsible for homeostasis of organs and tissues. Therefore, our hypothesis is that the creation of a MSC ectopic niche will serve as a vehicle of longer production of therapeutic proteins than cells systemically infused. Considering that MSC perivascular niche is well characterized, the objective of this work is modifying vascular endothelial cells and MSC obtained from adipose tissue stromal cells (referred here as ATSC) to express FVIII and use them in the generation of ectopic adipose tissue producing the mentioned factor in hemophilic A mice. The fundamental assumption of this project is that SVF obtained after enzymatic digestion of adipose tissue contain cells capable of recapitulating the formation of their origin tissue *in vivo*. To test this assumption, non-cultured SVF cells were mixed in a Matrigel+PBS (1:1) solution and subcutaneously injected in 2 Nude immunodeficient mice (n = 4 infusion focus). Forty days after transplantation, we detected the formation of ectopic adipose tissue.

Next, we established the isolation protocol of endothelial cells from adipose tissue. The procedure consisted of enzymatic dissociation of adipose tissue with collagenase IA and subsequent filtering of the capillaries not digested with trypsin. Endothelial cells were selected with anti-CD31 immunomagnetic columns and cultured. Both the polygonal morphology and CD31 expression, typical of endothelial cells, were present in the cell population obtained, showing the success in the isolation.

Adipose tissue endothelial cells (ATEC) were obtained by the culture of $CD31^-$ fraction after immunomagnetic selection of endothelial cells. ATSC showed fibroblast morphology and immunophenotypic profile compatible with stromal cells.

Next we demonstrated that ATSC cultured in ECM medium, containing pro-angiogenic cytokines (VEGF, IGF-I, bFGF and EGF), keep their adipogenic differentiation capacity after *in vitro* expansion. On the contrary, cells kept in aMEM medium lose differentiation potential throughout culture (Figure 20). Thus, we intend to generate ectopic adipose tissue by the infusion of ATSC previously cultured in ECM medium.

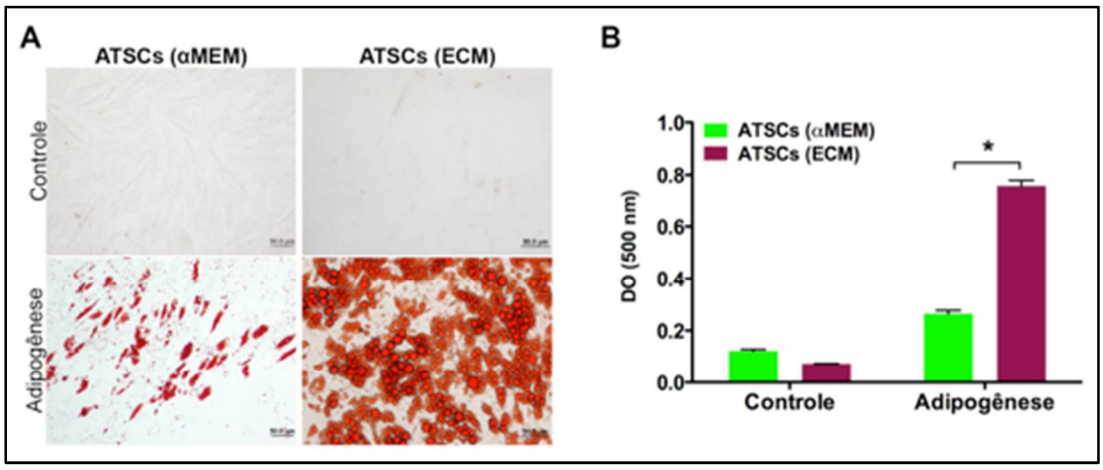


Figure 20. ATSCs cultured in ECM medium show greater differentiation potential. (A) Photomicrographs of ATSC colored with Oil Red O after adipogenesis induction. The majority ATSC cultured in ECM accumulated lipid vesicles during adipogenesis induction (low panel in the right). On the other hand, only some adipogenic differentiation focuses were observed in ATSC population cultured in αMEM medium (low panel in the left). **(B)** Quantification of red Oil Red O staining by optical densitometry. Quantitative analysis confirmed that ATSC cultured in ECM medium show higher differentiation potential in relation to the ones cultured in αMEM medium (*$p < 0.001$; t Student test; $n = 5$).

Finally, we were successful in generating immunodeficient hemophilic mice. Females with hemophilia A were mated with males of NSG **(NOD scid gamma)** line, which are carriers of the mutation in the gene Prkdc causing severe combined immunodeficiency syndrome (absence of T and B lymphocytes). The offspring, generation F1, were backcrossed to obtain generation F2. Animals of generation F2 were genotypically characterized regarding the presence of mutation in genes F8 and Prkdc. Animals with two mutations were identified and the colony is in phase of expansion. Genotypings assays were performed and the results are summarized in Figure 21.

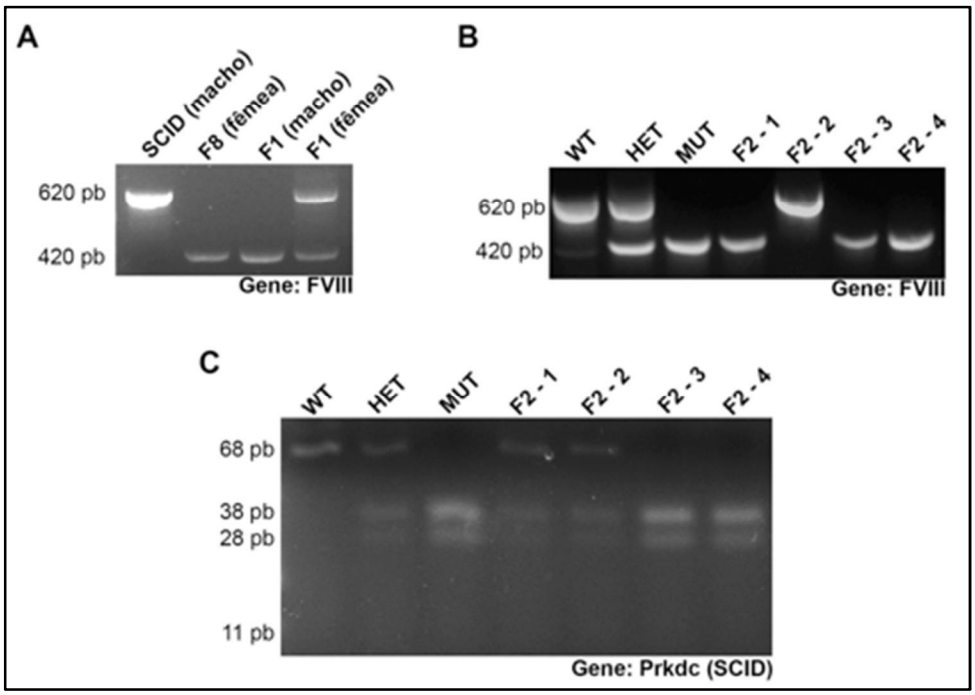


Figure 21. Genotyping of mice during the generation of F8/SCID animals. (A) Genotyping representing parents and two pups of generation F1. Animals carrying wild-type alleles show only fragments of 620 pb after PCR. Heterozygous animals have fragments of 620 pb and 420 pb. Animals carrying mutant alleles only generate fragments of 420 pb. (B) Representive genotyping for FVIII alleles of 4 animals of the generation F2, one animal carrying wild-type alleles (WT), one heterozygous animal (HET) and one animal carrying mutant alleles (MUT). We conclude that individuals F2-1, F2-3 and F2-4 are hemophilic. (C) Representive genotyping for Prkdc alleles of 4 animals of generation F2, one animal carrying wild-type alleles (WT), one heterozygous animal (HET) and one animal carrying mutant alleles (MUT). Animals carrying wild-type alleles of Prkdc (first gel lane) showed the expected pattern of bands after sorting of the fragments by electrophoresis: fragments of 68 pb and 11 pb, fragments of 11 pb are very hard to visualize in this image. Heterozygous animals (second gel lane) showed 4 sizes of expected fragments (68 pb, 38 pb, 28 pb and 11 pb) and the animals carrying SCID (third gel lane) showed 3 expected fragments (38 pb, 28 pb and 11 pb).

II. CLINICAL RESEARCH

The main topics enrolled in the clinical research are illustrated in Figure 22.

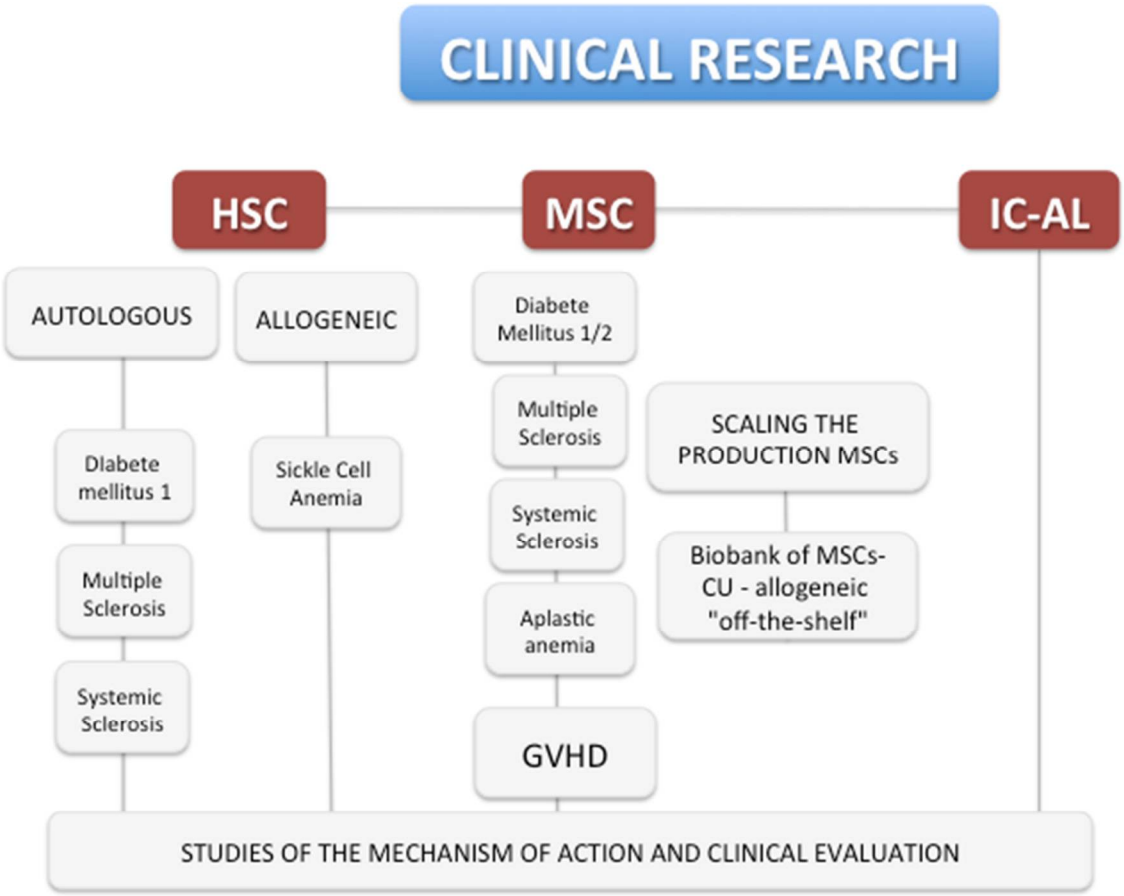


Figure 22. Illustrative scheme of the field of CLINICAL research developed at CTC. Abbreviations: HSC: hematopoietic stem cells, MSC: stromal mesenchymal cells, IC-AL: International Consortium of Acute Leukemia.

12) GOAL 12: CLINICAL APPLICATION OF STEM CELL

a) Recruit and follow 10 patients with refractory/relapsed aplastic anemia for treatment with immunosuppression and mesenchymal stromal cells and observe safety, hematologic response and MSC homing to the bone marrow niche

Acquired aplastic anemia (AA) is a bone marrow failure characterized by pancytopenia and empty bone marrow. For patients with AA who are refractory or relapse after first line immunosuppressive therapy lacking a suitable donor for transplant, therapeutic options may be challenging due to insufficient immunosuppression or profound depletion of the hematopoietic stem cell compartment. Given the immunomodulatory effects of mesenchymal stromal cells (MSC), we conducted a phase I trial adding allogeneic MSC infusion to standard rabbit ATG plus cyclosporine as second line treatment. Nine refractory or relapsed AA patients were recruited

and treated with 2-5 weekly intravenous infusions of allogeneic unrelated non-HLA-matched bone marrow MSC (mean of 2.51 x10^6 cell/kg/infusion ± SD: 0.83). The primary endpoint was safety and secondary endpoints were response at 6 months, MSC engraftment measured by FISH X/Y (MSCs were sex-mismatched) and by PCR for VNTR genes; and lymphocyte profile, measured by flow cytometry. No acute or chronic adverse effects related to infusions were observed after a 14-month median follow-up (range, 2-36.8). Seven patients were still alive and two patients had partial response; no complete response was achieved. Two deaths occurred, one due to acute heart failure related to severe hemochromatosis one week after the third MSC infusion, and one patient succumbed to bacterial lung infection 14 months after treatment. Patients' marrows were sampled at days 0, 30, 90, and 180 after treatment and MSCs were separated. We failed to observe any engraftment of infused MSCs by FISH or VNTR. The numbers of CD4, CD8, and Treg cells in bone marrow remained unchanged before and after infusions. We concluded that the use of intravenous MSC infusion in patients with AA was clinically safe, but its addition to standard IST appeared not to translate into clinical benefit. We demonstrated that intravenously infused MSCs did not engraft in bone marrow of AA patients, which should be taken into account in future clinical trials for MSC use (clinicaltrials.gov number, NCT01297972).

b) To investigate novel biomarkers in Acute Promyelocytic Leukemia and to determine the impact of networking in the outcome of patients with acute promyelocytic leukemia in developing countries

b.1) Analysis of* MLL5 *transcript levels as a biomarker in Acute Promyelocytic Leukemia

To determine if the MLL5 (currently named KMT2E) transcript levels in samples from APL patients enrolled in the IC-APL trial are associated with treatment outcome. We assessed the impact of KMT2E expression on the prognosis of 121 APL patients treated with ATRA and anthracycline-based chemotherapy. Univariate analysis showed that complete remission (P=0.006), 2-year overall survival (OS) (P=0.005) and 2-year disease-free survival (DFS) rates (P= 0.037) were significantly lower in patients with low KMT2E expression; additionally, the 2-year cumulative incidence of relapse was higher in patients with low KMT2E expression (P= 0.04). Multivariate analysis revealed that low KMT2E expression was independently associated with lower remission rate (odds ratio [OR]: 7. 18, 95% confidence interval [CI]: 1.71– 30.1; =0.007) and shorter OS (hazard ratio [HR]: 0.27, 95% CI: 0.08– 0.87; P 0.029). Evaluated as a continuous variable, KMT2E expression retained association with poor remission rate (OR: 10.3, 95% CI: 2.49– 43. 2; P= 0.001) and shorter survival (HR: 0. 17, 95% IC: 0. 05– 0. 53; P=0. 002), while the association with DFS was of marginal significance (HR: 1. 01; 95% CI: 0. 99– 1. 02; P=0.06). In summary, low KMT2E expression may predict poor outcome in APL patients.

Manuscript accepted by the British Journal of Haematology (under revision): "Prognostic impact of KMT2E transcript levels on outcome of patients with acute promyelocytic leukaemia treated with all-trans retinoic acid and anthracycline-based chemotherapy: an International Consortium on Acute Promyelocytic Leukaemia study".

b.2) To investigate the role of EGFR signaling in Acute Promyelocytic Leukemia

The aims of this subproject are: a, to determine if the increased CPG-methylated islands situated in the promotor region of the EGF and EGFR genes (previous results of our laboratory) in blasts of patients with Acute Promyelocytic Leukemia (APL) correlates with patient clinical response; b, to determine how the molecular mechanisms involved in the hypoexpression of EGF and EGFR affects the proliferation, apoptosis resistance and tumor growth in APL patients. The expression value of EGF and EGFR as prognostic markers were evaluated in the cohort of ALP patients treated by the protocol of the International Consortium in ALP. There was significant correlation between the low expression of EGF and the worse response to treatment. Patients with low expression value of EGF presented higher leucocyte counts, lower albumin concentrations and higher plasmatic fibrinogen concentration. For the functional studies, the cDNA of EGF and EGFR were cloned and lentivirals vectors were constructed. NB4 e NB4-R2 cells were transduced with these vectors (containing EGF or EGFR gene) showed lower cell proliferation ratio and higher expression of the differentiation marker CD11b. Balb/Nude mice were inoculated (sc) with EGF or EGFR- transduced NB4 cells developed lower tumor mass compared with controls animals.

c) To determine the relevance of p73 isoforms in the response to treatment of APL

The aim of this study was to determine whether the ΔN/TA expression ratio was associated with treatment outcome of APL patients and to investigate the mechanisms by which ΔNp73 may contribute to PML-RARa+ cell survival. Using isoform-specific probes for ΔN*p73* and TA*p73*, their expression was analyzed in 166 APL patients by Real-time quantitative polymerase chain reaction (RQ-PCR). Patients were divided into tertiles for ΔN/TA expression ratio (median = 1.12; percentile $33^{rd}/66^{th}$ = 0.68/2.54) and their clinical and laboratory characteristics were compared. Patients in the highest tertile presented higher white blood cells (WBC) counts than those in intermediate/lower tertiles ($p < 0.001$), but no significant differences were observed for age, gender, PML breakpoint, or platelets count. Higher ΔN/TA expression ratio values were significantly associated with the presence of *FLT3-ITD* ($p = 0.001$). Treatment outcome was obtained for 131 APL patients enrolled in the APL99 (n = 41) and IC-APL (n = 90) trials. The mean follow-up was 29.1 months, ranging from 1 to 85.5 months. The mean overall survival (OS) of all patients was of 66.8 months [95%CI; 60.8 to 72.8], whereas it was of 67.1 months [56.5 to 77.7] for patients in the lower and 41.7 months [32 to 51.4] for those in the higher tertile for ΔN/TA expression ratio (p = 0.014). Univariate analysis identified WBC counts above 10,000/µl ($p = 0.003$), *FLT3-ITD* mutation ($p = 0.011$) and ΔN/TA expression ratio ($p=0.014$) as predictive factors for OS. However, in multivariate Cox analysis, these three prognostic factors were not independent. Until April 2011, a total of eight relapses (6.1%) were recorded. The disease free survival (DFS) rate at five-years for all patients was of 88.3% ± 4.2% and the mean DFS was of 76.1 months [71.2 to 80.9]. DFS was significantly shorter in patients at the higher tertile ΔN/TA expression ratio compared with patients at the lower tertile (72.1 ± 11.2% *vs* 97.1 ± 2.8%, respectively; $p < 0.001$) and was the only variable found to be significant in the univariate

analysis. To test the functional significance of the association of PML-RARa with high ΔN*p73* gene expression, primary murine bone marrow cells from hCG-PML-RARa transgenic mice were transfected with MSCV-based retroviral vector carrying the ΔN*p73* cDNA upstream of IRES-GFP cassette (PML-pMIG-ΔN). Expression of ΔN*p73* in PML-RARa+ cells increased cell proliferation rate by 2.5-fold compared to PML-RARa+ transfected with the empty vector (p=0.03). This increase resulted from accumulation of cells at the G2/M phase (5.79 ± 0.08% for PML-pMIG *vs* 9.8 ± 0.35% for PML-pMIG-ΔN, p<0.001), as well as at S phase of the cell cycle (27.74 ± 0.89% for PML-pMIG vs. 36.78 ± 0.81% for PML-pMIG-ΔN, p = 0.001).

d) Treatments of Acute Myeloid Leukemia

d.1 Establishment of an international multicentric colaborative group to study the role of autologous stem cell transplantation in the treatment of acute myeloid leukemia of favorable and intermediate prognosis

The protocol entitled 'Feasibility Study of the Use of Intermediate Doses of Cytarabine Associated with Autologous Hematopoietic Stem Cells as Consolidation Treatment of Adults with Low- or Intermediate-risk de Novo Acute Myeloid Leukemia' was just approved by the National IRB (CONEP) and is going to start to recruit patients soon.

d.2 Use of Genome-wide DNA methylation mapping of Leukemic Stem Cells (LSCs) to identify novel biomarkers of daunorubicin e cytarabine response in Acute Myeloid Leukemia (AML)

The aim of this subproject is to map the DNA methylation profile of a highly purified cell population enriched for Leukemia Stem Cells (CD34+CD38- ALDH1+int) in well-annotated clinical samples. This will allow us to identify novel biomarkers of daunorubicin e cytarabine response in AML. We have successfully established the methodology to isolate cell $CD34^{+}CD38^{-}$ $ALDH1^{+int}$ leukemic stem cells (LSCs), $CD34^{+}CD38^{-}$ $ALDH1^{+high}$ hematopoietic stem cells (HSCs) and $CD34^{+}CD38^{-}$ $ALDH1^{-}$. So far, these cells subpopulations were obtained from bone marrow samples of 12 patients with AML and normal counterparts were isolated from 4 healthy subjects. We have successfully established the phosflow methodology to analyze the phosphorilation of STAT5, STAT3, MAPK but not of AKT. The cell subsets of interest were incubated with GM-CSF and the phosphorylation status of STAT and MAPK pathways were evaluated by flow cytometry. The preliminary analyses suggest a differential activation between (LSCs) $CD34^{+}CD38^{-}$ $ALDH1^{+high}$ hematopoietic stem cells (HSCs) from the same patient. We have performed xenotransplants using $CD34^{+}CD38^{-}$ $ALDH1^{+int}$ LSCs and $CD34^{+}CD38^{-}$ $ALDH1^{+high}$ HSCs obtained from 7 patients with AML. After 8 weeks the engraftment was evaluated based on the alternative staining for human versus murine CD45. In 5 experiments, human cells were detected indicating the successful engraftment.

e) Treatment of patients with inflammatory multiple sclerosis with high dose immunosuppression followed by autologous hematopoietic stem cell transplantation (HDI/AHSCT).

Eighty-four multiple sclerosis patients were transplanted at the Ribeirão Preto Medical School – University of São Paulo, under the cyclophosphamide plus ATG regimen followed by infusion of autologous hematopoietic stem cells. Results demonstrated that the procedure was beneficial for the general population of patients, although more specifically in the inflammatory subset of patients. Therefore, since 2009, we have been enrolling relapsing-remitting multiple sclerosis patients under the new multicenter, randomized MIST protocol, coordinated by Dr. Richard Burt at the Northwestern University, Chicago, USA. Since 2013, one additional patient was included and transplanted at the Ribeirão Preto Medical School under the MIST protocol. Transplanted patients present less clinical and magnetic resonance-detected relapses than before AHSCT. The real efficacy of the procedure will be compared with patients treated with standard treatment (control group) after end of enrolment and follow-up, and unblinding of results.

f) Treatment of patients with early-diagnosed type 1 diabetes with HDI/AHSTC

One recent-onset type 1 patient was included and transplanted at the Ribeirão Preto Medical School under the multicenter randomized, open label, protocol coordinated by our center. Two additional control patients have been recently included by the Northwestern University, Chicago (USA) center. A total of four patients have been already transplanted, leading to insulin suspension in three and to decrease of exogenous insulin doses in one patient, in mean follow-up of 25 months. Serum glycated hemoglobin levels remained stable, indicating adequate metabolic control after the procedure. Serum samples for C-peptide measurements have been stored and will be analyzed at the end of the study.

g) Treatment of patients with systemic sclerosis with HDI/AHSTC

Eight patients with diffuse systemic sclerosis were included and underwent autologous hematopoietic transplantation (AHSCT) from May 2013 to May 2014. Therefore, 51 patients have already been transplanted for this disorder at the Ribeirão Preto Medical School, University of São Paulo, since the beginning of the program. Mean follow-up is 44 months. Skin improvement has been detected through modified Rodnan's Skin Score (mRSS) evaluations. Mean baseline mRSS of 27.6 decreased to 22.1 ($p<0.05$) and 16.7 ($p<0.01$), 6 and 12 months after AHSCT, respectively, and stabilized thereafter, until end of follow-up (Figure 23A). Lung function was evaluated through pulmonary function tests, before and periodically after transplantation, evidenced stabilization of forced vital capacity (FVC) and of carbon monoxide lung diffusion (DLCO) (Figure 23B). A retrospective combined analysis of 90 systemic sclerosis patients transplanted at the Ribeirão Preto Medical School and at the Chicago, Northwestern University (USA) centers showed that cardiac involvement before AHSCT was associated with higher transplant-related mortality rates and with worse pulmonary outcome after transplantation (Burt et al, Lancet 2013).

In this setting, we decided to start a new protocol, which includes a thorough heart evaluation before enrolment. The aim is to exclude patients with subclinical heart involvement and therefore decrease transplant-related mortality and improve outcomes. This new prospective protocol will also be combined with Chicago Northwestern University, under Dr. Burt's coordination.

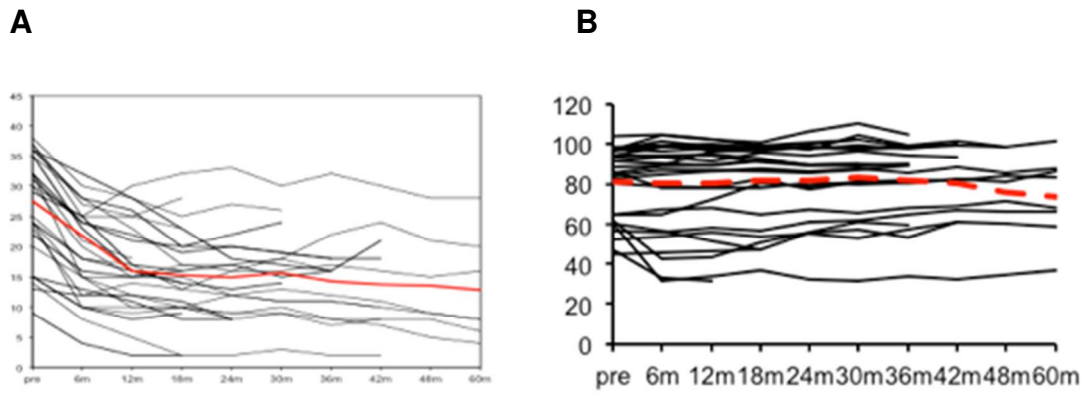


Figure 23. **Evaluations of Rodnan's skin score** (A) and lung function (B) before and after AHSC for systemic sclerosis in patients enrolled at the Ribeirão Preto Medical School – University of São Paulo.

h) Evaluation of the immune mechanisms of action of the HDI/AHSCT in type 1 diabetes, multiple sclerosis and systemic sclerosis patients

Type 1 diabetes

In autoimmune diseases stem cell transplantation has attempted to reset the immune system and induce self-tolerance towards autoantigens. We have previously shown that autologous hematopoietic stem cell transplantation increased C-peptide levels and induced transient or continuous suspension of insulin use in most patients with type 1 diabetes. However, the immune mechanisms associated with clinical outcomes were not elucidated. In the present study, we monitored the immune system in long-term follow-up of 25 type 1 diabetes patients treated with hematopoietic stem cell transplantation, and investigated how these markers were associated with duration of insulin independence and C-peptide levels. Frequencies of islet-specific autoreactive T-cells, distribution of regulatory lymphocyte subsets, as well as T-cell receptor excision circle and T-cell repertoire diversity were evaluated. In mean follow-up of 67.5 months, 21 of 25 patients became insulin-independent, and C-peptide levels remained higher than baseline until 4 years post-transplantation. However, most patients resumed insulin after median of 43 months. Patients with lower frequencies of autoreactivity presented longer duration of insulin independency, with higher C-peptide levels. Baseline islet-specific autoreactivity did not change, but immunoregulatory T-cell counts increased after transplantation. Although recent thymic emigrants increased after transplantation, broad T-cell repertoire diversity was not sustained beyond 30 months. In conclusion, although the highly immunosuppressive conditioning regimen was not sufficient to eliminate islet-specific

autoreactivity in type 1 diabetes patients, the procedure successfully promoted long-term insulin-independency in most patients. Beneficial effects of regulatory mechanisms may have counterbalanced autoreactivity thus explaining better metabolic outcomes in patients with lower frequencies of autoreactive islet-specific T-cells (Figure 24, 25 and 26).

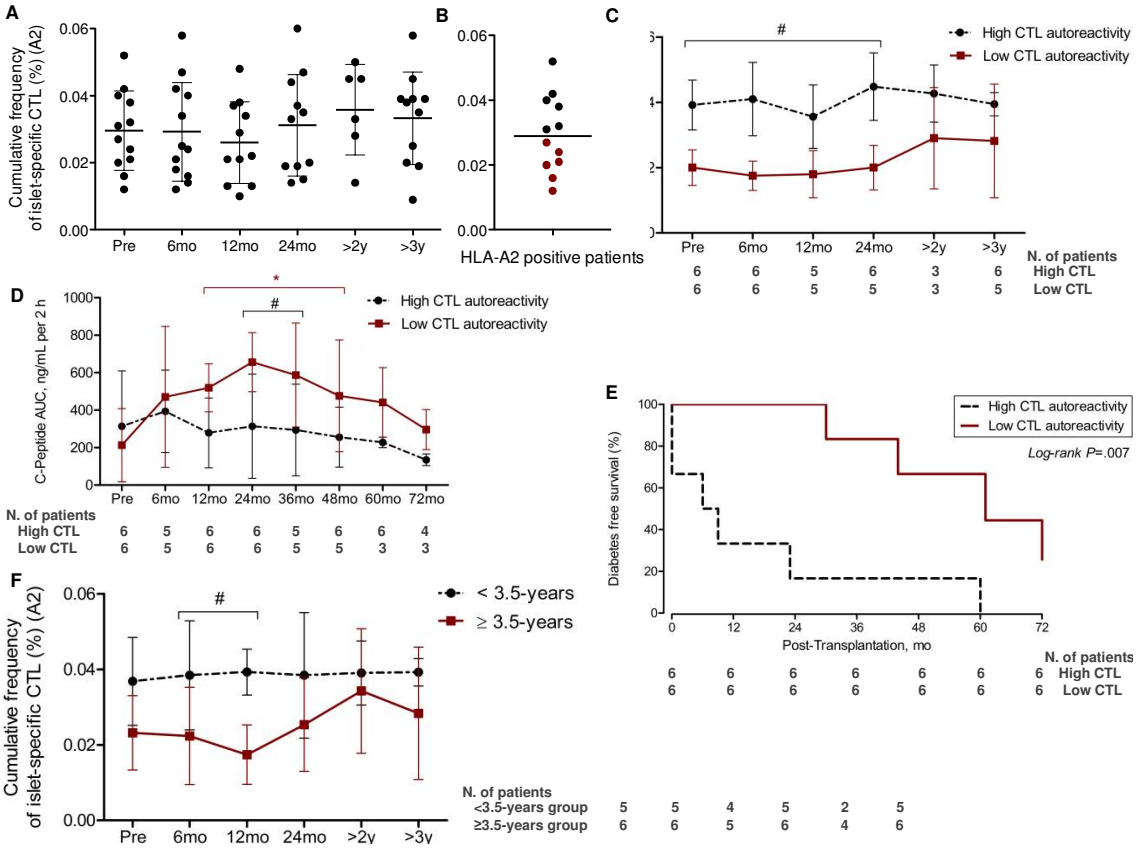


Figure 24. Cumulative frequency of autoreactive islet-specific CD8 T-cells at baseline predicts clinical outcome of AHSCT in T1D patients. (**A**) Cumulative frequency (%) and dynamics of autoreactive islet-specific CD8 T-cells (CTL) in 12 HLA-A2 positive patients. (**B**) Patients were divided in two groups with high or low CTL autoreactivity. (**C**) Dynamics of autoreactive CD8 T-cell frequencies after AHSCT in 6 patients with high CTL autoreactivity and 6 patients with low CTL autoreactivity. (**D**) Time course of total area under the curve (AUC) of C-peptide levels during mixed-meal tolerance test in 6 patients with high CTL autoreactivity and 6 patients with low CTL autoreactivity. (**E**) Diabetes-free survival. (**F**) Dynamics of autoreactive CTL frequencies after AHSCT in 6 patients insulin-free ≥3.5-years and 5 patients insulin-free <3.5-years groups. Statistical analysis was performed using a model of multiple regression of mixed effects. ■, patients insulin-free for at least 3.5 years (≥3.5-years-group). ●, patients insulin-free less than 3.5 years (<3.5-years-group). *, p<0.05: ≥3.5-years-group at cited period vs pre-transplantation period. *, p<0.05: <3.5-years-group at cited period vs pre-transplantation period. #, p<0.05 between the groups at cited period. Pre, pre-transplantation period; mo, months; y, years.

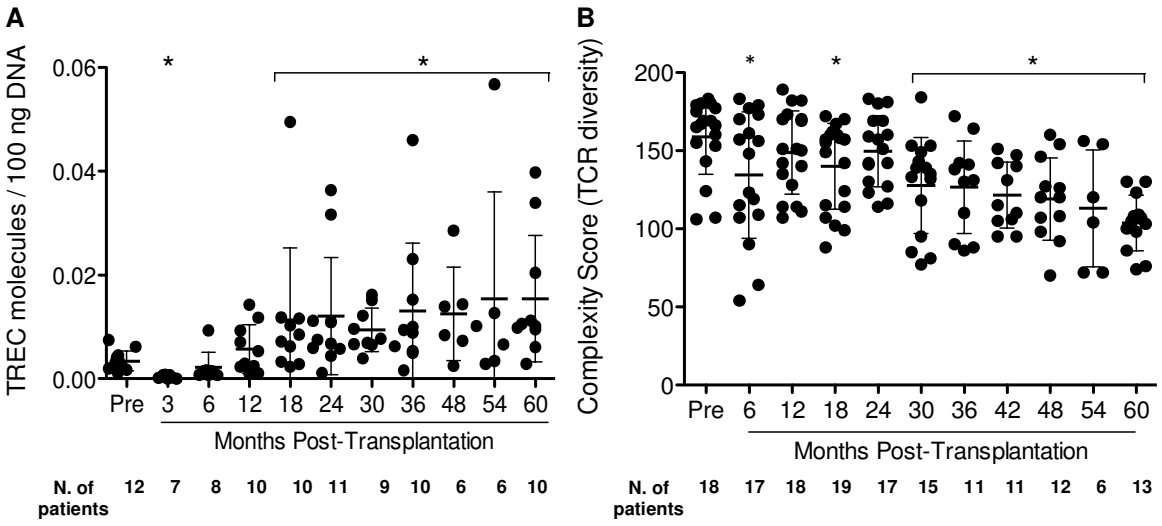


Figure 25. Dynamics of thymic output and of overall T-cell repertoire diversity in type 1 diabetes patients following AHSCT. (**A**) T-cell receptor excision circle (TREC) levels over time. TREC levels were determined by real-time PCR. TREC levels are expressed as TREC molecules/100 ng DNA of PBMCs. (**B**) T-cell receptor repertoire diversity represented by complexity scores. T-cell receptor repertoire diversity was analyzed by TCRBV CDR3 Length Spectratyping, as detailed in Materials and Methods. The complexity score was determined by counting the number of complexity scores of each Vβ family. Data are shown as mean±SD. *p<0.05 between cited period vs pre-transplantation period. Pre: pre-transplantation.

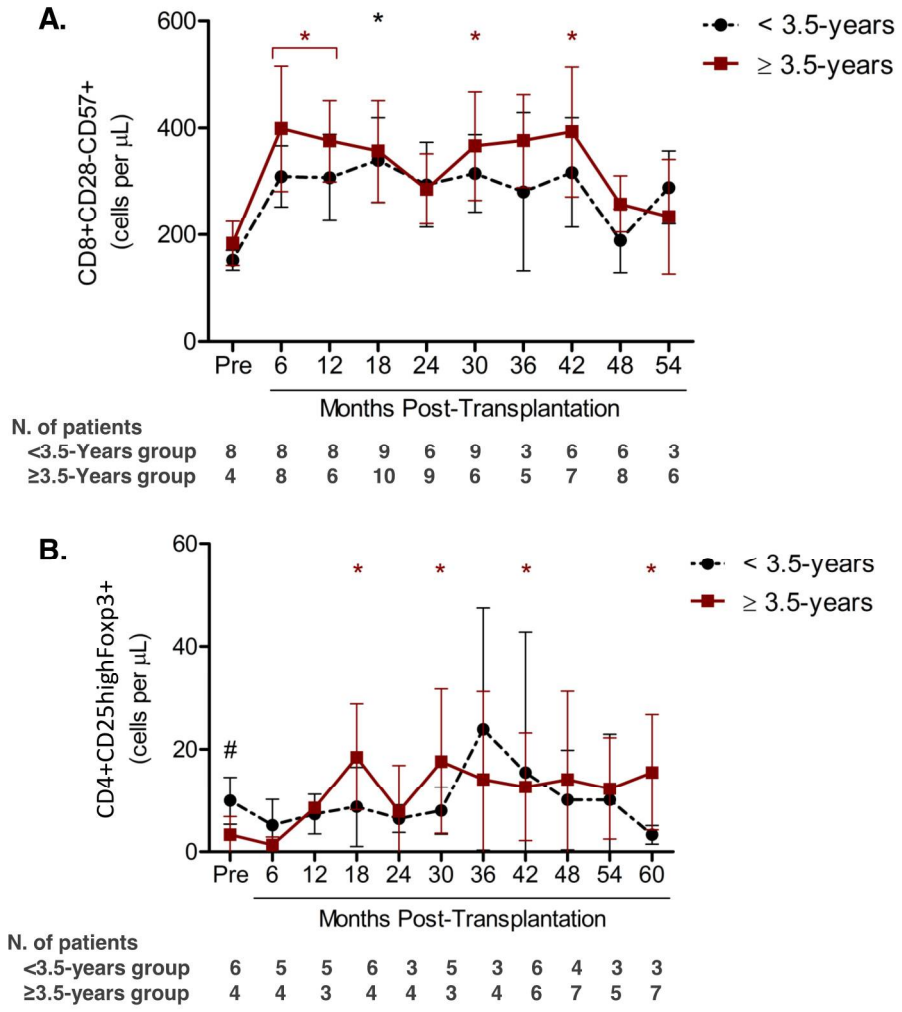


Figure 26. Reconstitution kinetics of immunoregulatory T-cell subsets in T1D patients after AHSCT. Reconstitution of absolute numbers of $CD8^{+}CD28^{-}CD57^{+}$ suppressor T-cells (**A**) and regulatory $CD4^{+}CD25^{high}FoxP3^{+}$ T-cells (**B**). The immmunophenotypic analysis was assessed by flow cytometry of samples of whole peripheral blood. T1D patients were divided in groups according to duration of insulin dependence after treatment with AHSCT. Statistical analysis was performed using a model of multiple regression of mixed effects. ■, patients insulin-free for at least 3.5 years (≥3.5-years-group). ●, patients insulin-free less than 3.5 years (<3.5-years-group). *, $p<0.05$: ≥3.5-years-group at cited period vs pre-transplantation period. *, $p<0.05$: <3.5-years-group at cited period vs pre-transplantation period. #, $p<0.05$ between the groups at cited period. Pre, pre-transplantation period.

Systemic Sclerosis

High dose immunossuppression (HDI) followed by autologous hematopoietic stem cell transplantation (AHSCT) emerged in last past years as a therapeutic alternative for systemic sclerosis (SSc) patients. The evaluation of immune reconstitution is important to elucidate the therapeutic mechanisms and improve treatment efficacy. Objectives: To evaluate the reconstitution of regulatory T and B cells, suppressor T cells, and naive and memory B cells after HDI/AHSCT. Methods: Peripheral blood was collected from 15 diffuse SSc patients at baseline, 6 and 12 months after transplantation. Peripheral blood mononuclear cells were

immunophenotyped by flow cytometry and absolute numbers of T or B cell subsets were quantified. Results: Numbers of regulatory B cells ($CD19^{+}CD24^{hi}CD38^{hi}$) were significantly increased at 6 and 12 months post-AHSCT, compared with baseline, despite no alterations in $CD4^{+}CD25^{hi}FoxP3^{+}$ regulatory T cell numbers. Absolute counts of $CD8^{+}CD28^{-}CD57^{+}$ suppressor T cells were also significantly increased at 6 and 12 months post-therapy, when compared with the pre-treatment period. Simultaneously, numbers of $CD19^{+}CD27^{+}IgD^{-}$ memory B cells were significantly decreased at 6 and 12 months, while $CD19^{+}CD27^{+}IgD^{+}$ naive B cells transiently increased at 6 months, when compared with baseline. Conclusions: Our results suggest an early improvement of peripheral immune regulatory mechanisms and ablation of memory B cells after HDI/AHSCT, which may contribute to reestablishment of self-tolerance and control of autoimmunity in SSc patients.

I) Treatment of patients with graft-versus-host disease, in the setting of haploidentical allogeneic HSCT, with allogeneic bone marrow-mesenchymal stromal cells infusions

From June 2013-May 2014, four patients with graft-versus-host disease in the setting of haploidentical allogeneic HSCT were treated with infusions of bone marrow derived-MSCs. The MSCs were isolated from bone marrow of non-related donors and expanded in flasks in GMP conditions until the third passage. Quality controls tests, potency evaluation and lot validation were performed, and the cells were infused in the patients by the physicians specialized in hemotherapy. Each patient received 2-4 infusions of MCSs (dose containing 2 $x10^{6}$/Kg). The intravenous infusion of MSCs were safe Initial results were clinically promising, especially of the GVHD affecting the intestine and liver.

j e k) Development of an allogeneic mesenchymal stromal cells from umbilical cords (MSCs-UC) bank for "off-the-shelf" clinical use. Treatment of patients with acute graft-versus-host disease, refractory to corticosteroids, with allogeneic umbilical cord-mesenchymal stromal cells infusions

In order to attend the new Brazilian regulation for production of cells for therapy purpose, the "Cell Therapy laboratory" of our center was reformed and adapted during last year. The new Lab will start operating next June 2014 and the clinical trial of infusions allogeneic umbilical cord-mesenchymal stromal cells (UC-MSCs) in acute graft-versus-host disease refractory to corticosteroids will start. From June 2013-May 2014, the production of mesenchymal stromal cells of umbilical cord cultivated in xenoantigen-free and GMP conditions for clinical application is currently being optimized. MSCs were isolated from 10 umbilical cords, were expanded in 175 cm2 until the third passage under in GMP conditions. MSCs lots were cryopreserved in cryogenic vials for future clinical use. The quality controls tests, potency evaluation and lot validation were currently being validated. In addition, the cryopreservation of MSCs in transfusion cryogenic bags area also being optimized and validated. To date, 10 bags containing UC-MSCs (1,5 x 10^{8} cells) were cryopreserved in 25,0 mL using the "BioArchive" cryopreservation system. The cryopreservation solution is composed of 90% human serum-converted plasma and 10% of hydroxietilamide-6% and dimethysulfoxide (DMSO). These bags

will be thawed and cells will be evaluated regarding viability, differentiation potential, immunophenotyping for 24validation of the cryopreservation method of UC-MSCs for clinical application.

Bone marrow mesenchymal stromal cells in the treatment of refractory or relapsed acquired aplastic anemia

Acquired aplastic anemia (AA) is a bone marrow failure syndrome characterized by pancytopenia and an empty marrow. For patients with AA who are refractory or relapse after first line immunosuppressive therapy lacking a suitable donor for transplant, therapeutic options may be challenging due to insufficient immunosuppression or profound depletion of the hematopoietic stem cell compartment. Given the immunomodulatory effects of mesenchymal stromal cells (MSC), we conducted a phase I study adding allogeneic MSC infusion to standard rabbit antithymocyte globulin (ATG) plus cyclosporine as second line treatment. Nine refractory or relapsed AA patients were recruited and treated with 2-5 weekly intravenous infusions of allogeneic unrelated non-HLA-matched bone marrow MSC (mean of 2.51 $x10^6$ cell/kg/infusion ± SD: 0.83). The primary endpoint was safety and secondary endpoints were response at 6 months, MSC engraftment measured by FISH X/Y (MSCs were sex-mismatched) and by PCR for VNTR genes; and lymphocyte profile, measured by flow cytometry. No acute or chronic adverse effects related to infusions were observed after a 14-month median follow-up (range, 2-36.8). Seven patients were still alive and two patients had partial response; no complete response was achieved. Two deaths occurred, one due to acute heart failure related to severe hemochromatosis one week after the third MSC infusion, and one patient succumbed to bacterial lung infection 14 months after treatment. Patients' marrows were sampled at days 0, 30, 90, and 180 after treatment and MSCs were separated. We failed to observe any engraftment of infused MSCs by FISH or VNTR. The numbers of CD4, CD8, and Treg cells in bone marrow remained unchanged before and after infusions. We concluded that the use of intravenous MSC infusion in patients with AA was clinically safe, but its addition to standard IST appeared not to translate into clinical benefit. We demonstrated that intravenously infused MSCs did not engraft in bone marrow of AA patients, which should be taken into account in future clinical trials for MSC use. (clinicaltrials.gov number, NCT01297972), Figure 27.

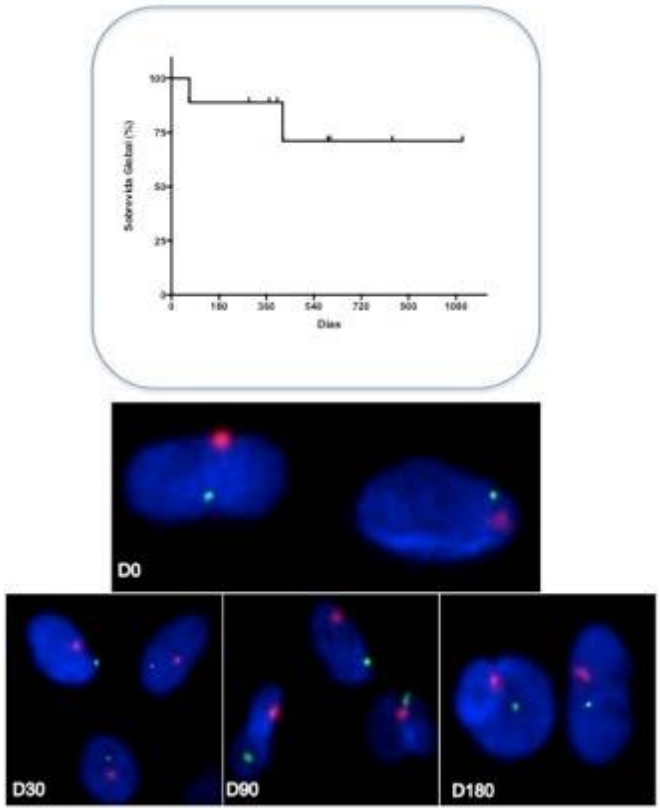


Figure 27. Overall survival for patients with aplastic anemia treated with MSC infusion was 71%. By FISH analysis of recipient's marrow MSCs for X/Y chromosome, we determined that infused MSCs did not engraft in patients' marrows.

III. TRANSFER TECNOLOGY

1) Production of Coagulation Factors

Hemophilia A and B are genetic diseases linked to the X chromosome that results in protein deficiency or abnormality of factor VIII and FIX. Replacement therapy using proteins derived from human plasma or recombinant produced in murine cell lines are the only options of treatment available at the moment. However, a major problem of augmentation therapy is the formation of inhibitory antibodies to FVIII, which currently is the most significant complication related to the treatment of hemophilia patients. All recombinant clotting factors available in the market are produced in murine cell lines. However, the use of murine cell lines can generate a differente glycosilation profile in the recombinant proteins and this can affect the half-life and the immunogenicity of the protein.

The use of human cell lines provides increased security, lower cost product and less immunogenicity of protein therapeutics. During ten years of research, we developed at the Blood Center of Ribeirão Preto human cell lines producing recombinant clotting factors (FVII , FVIII and IX). In the past year, we increased our production scale of FVIII from 10 mL per plate cultivation to 40 liters in a bioreactor operated in perfusion mode using microcarriers. Currently, in 10 days of production we can produce about 50.940,55 IU of FVIII. In the last six months, we developed a purification method that permits a recombinant product with 90% purity. The technology of production of recombinant proteins using human cells have been developed by our research group and this technology has been gaining attention in the international scientific literature. Besides the technology generated, this project contributed to the training of human resources with multidisciplinary characteristics capable of dealing with this type of technology and market development.

a) Cloning and expression of blood clotting factor VII in human cell lines.

The recombinant Factor VII (rFVII) has been the primary therapeutic choice for patients with hemophilia who develop inhibitors against FVIII. Human FVII is a vitamin K dependent glycoprotein which serves to initiate the blood coagulation cascade to form a complex with tissue factor. Currently, the comercial product used is produced in murine cells (BHK). Factor VII requires speficic post- translational modifications of essential for the activity of the protein. Recombinant proteins produced in mouse cells may have the same pattern of post- translational modifications of human cells and can lead to generate inhibitory antibodies in patients. An alternative would be the production in human cell lines. The main objective of this work was to clone and express the FVII clotting of blood in 3 human cell lines (HepG2 , Sk - Hep , HKB - 11) and select the best production of the recombinant protein. The BHK-21 murine strain were used as controls. Initially, the FVII gene (ATCC) was cloned into the bicistronic lentiviral vector p1054-CIGWS, which contains the GFP gene , encoding a fluorescent protein that allows observing the efficiency of modifying cell lines. After the modification of the cells, the expression of the marker gene GFP was observed by fluorescence microscopy and flow

cytometry. BHK-FVIII cells showed 80% expression of GFP. HepG2-FVII cells showed expression of 73 % while the HKB-11-FVII cells showed 32 % of GFP. Since the cell line Sk-Hep-rFVII showed the best expression of the marker gene, the next step was to characterize the rFVII produced by the modified cell lines. The analysis showed that the cells produce: HepG2-rFVII: 847 ng/ml, SKHep-rFVII 744 ng/ml, HKB-11-rFVII: 676 ng/ml and BHK-21-rFVII: 140 ng/ml. In order to analyze the mRNA expression related to rFVII well as the one related to γ-carboxylation enzymes, we performed real-time PCR. Regarding enzymes γ-carboxylation, it was observed that γ -carboxylase , VKORC1 and the calumenina inhibitor showed increased levels of mRNA expression when treated with vitamin K. To date, our data suggest that the best production is done by the HepG2 cell line, however other analyzes are being conducted.

2) Production of new molecules FIX

The CTC has already a patent related to FIX production. Our current objective is to improve the biochemical and biophysical properties of FIX. In this work, we use the tool of site-directed mutagenesis to modify FIX to improve its biological activity. This combined with the selection of human hepatic cell lines with an effective cell expression system for recombinant FIX biologically active can contribute to a more effective and efficient replacement therapy. These modified molecules may have improved its biological activity, the reduction or elimination of the generation of FIX inhibitor which reduces side effects and treatment costs. Thus, the production of new molecules with increased activity of recombinant FIX expression system using a human cell is an important innovation and may alternatively be a great improvement in the treatment of hemophilia B.

3) Evaluation of transient expression of FVIII with mutations in domain A1 and A2 .

Mutations in some domains of FVIII, especially in A1 may lead to increased secretion of factor FVIII in cell cultures. A vector containing the mutations F309S, R484A, R489A and P492A was developed to evaluate whether these mutations can increase the production of FVIII *in vitro*. To assess the functionality of the protein produced from the vector with mutations transient expression was analyzed and cell supernatant was collected in 24, 48 and 72 hours. The results showed no differences between biological activities obtained from the vectors and FVIIIΔB FVIIIMmutΔB (Figure 28).

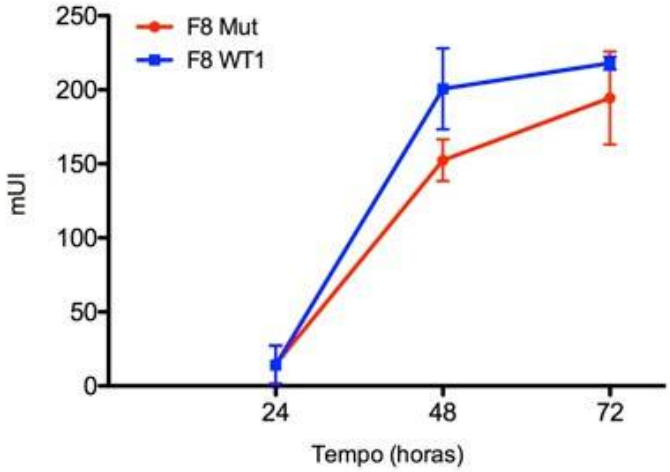


Figure 28. **Transient production of FVIII wild-type and FVIIIMUTΔB.**

Further tests are being conducted to determine whether these mutations actually are able to increase the secretion of FVIII in vitro.

4) Large-scale production of MSC in bioreactors.

- **MSC expansion on microcarriers in spinner flasks**

Human mesenchymal stromal cells (hMSCs) cells are attractive for applications in tissue engineering and cell therapy. Because of the low availability of hMSCs in tissues and the high doses of hMSCs necessary for infusion, scalable and cost-effective technologies for *in vitro* cell expansion are needed to produce MSCs, while maintaining their functional, immunophenotypic and cytogenetic characteristics. Microcarrier-based culture systems are a good alternative to traditional systems for hMSC expansion based on static flasks. The aim of this project was to develop a scalable bioprocess for the expansion of human bone marrow mesenchymal stromal cells (hBM-MSCs) on microcarriers to optimize growth and functional harvesting. In general, the results obtained demonstrated the feasibility of expanding hBM-MSCs using microcarrier technology in spinner flasks. The maximum cell concentration (n=5) was $4.82 \pm 1.18 \times 10^5$ cell/mL at day 7, representing a 3.9-fold increase relative to the amount of inoculated cells. At the end of culture, 87.2% of the cells could be harvested (viability=95%). Cell metabolism analysis revealed that there was no depletion of important nutrients such as glucose and glutamine during culture, and neither lactate nor ammonia by-products were formed at inhibitory concentrations (Figure 29). The cells that were recovered after the expansion retained their immunophenotypic and functional characteristics (Figure 30). These results represent an important step towards the implementation of a GMP-compliant large-scale production system for hMSCs for cellular therapy. As the system used allows the scale-up, if we had used a bioreactor of 1L it would have been possible to produce approximately 5×10^8 cells what would be enough to treat more than three patients of up to 70kg with a dose of 2×10^6 cells/kg. For the expansion of the same amount of cells in the traditional way, it would be necessary 135 static flasks of 175

cm^2 with total cost twice higher than the estimated cost of expansion using microcarriers.

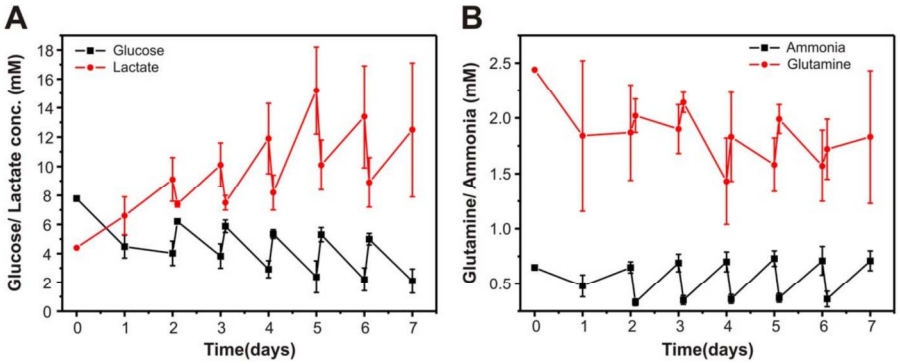


Figure 29. Metabolic profiles of the hBM-MSCs cultured on Cytodex 3 microcarrier in spinner flasks for 7 days. The results are expressed as the mean ± SD of five independent experiments (n=5).

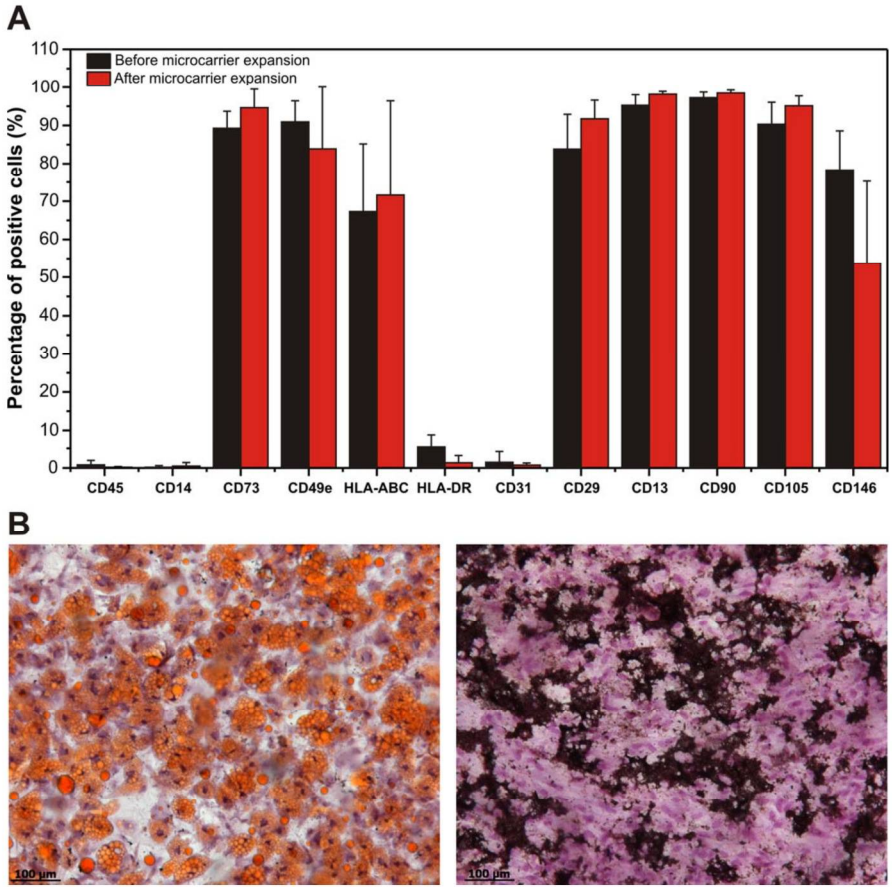


Figure 30. (A) Immunophenotypic analysis and (B) differentiation potential (adipogenic and osteogenic) of hBM-MSC after expansion on the Cytodex 3 microcarrier.

- **MSC expansion on microcarriers in stirred tank bioreactors**

Once the feasibility of hMSC cell expansion on microcarriers in spinner flasks has been demonstrated, the scale-up of this process was performed in stirred tank

bioreactor. It was observed an intense cell growth (μ_{max} 0,30 d^{-1}, r^2 = 0,999) with production of 5,31(±0,09)x10^8 cells at day 7, representing a 8,84 (±0,397)-fold increase (n=3). At the end of culture, 60,6 % (±15,72) of the cells could be harvested, retaining their immunophenotypic and functional characteristics.

Despite the large amount of cells produced, there is still the need to optimize the method for cell recovery. Such studies are in progress.

- **MSC expansion in disposable fixed-bed bioreactors**

The need for efficient and reliable technologies for clinical-scale expansion of mesenchymal stromal cells (MSC) has led to the use of disposable bioreactors and culture systems. Here, we evaluate the expansion of cord blood-derived MSC in a disposable fixed bed culture system. Starting from an initial cell density of 6.0x10^7 cells, after 7 days of culture, it was possible to produce 4.2 (±0.8)x10^8 cells, which represents a fold increase of 7.0 (Figure 31)(n=3). After enzymatic retrieval from Fibra-Cell disks, the cells were able to maintain their potential for differentiation into adipocytes and osteocytes and were positive for many markers common to MSC (CD73, CD90, and CD105)(Figure 32). The results obtained in this study demonstrate that MSC can be efficiently expanded in the culture system. This novel approach presents several advantages over the current expansion systems, based on culture flasks or microcarrier-based spinner flasks and represents a key element for MSC cellular therapy according to GMP compliant clinical-scale production system.

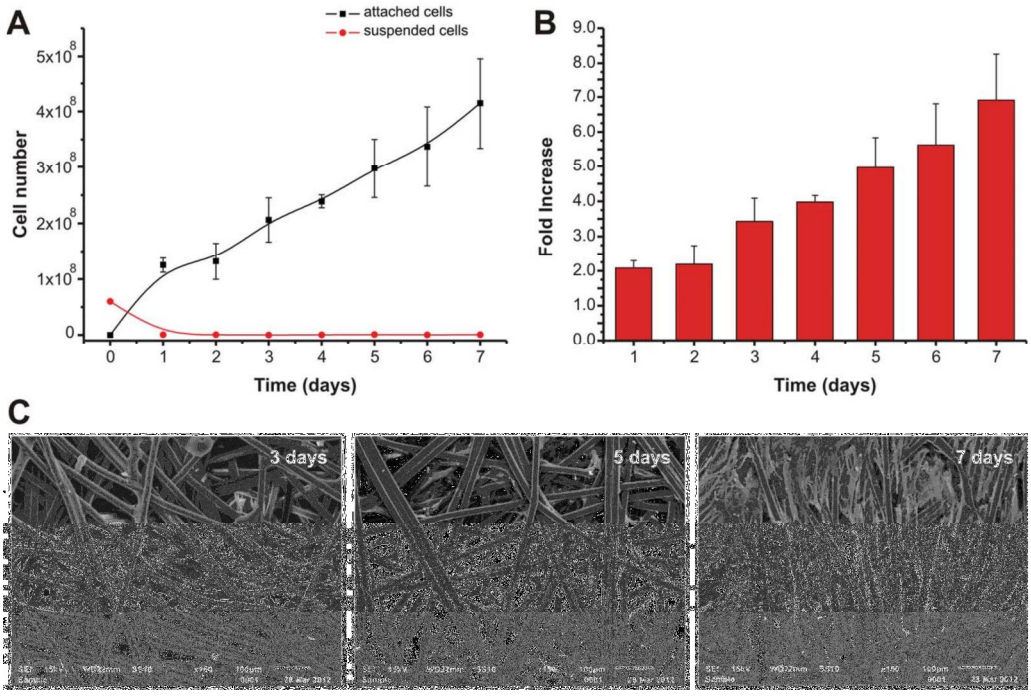


Figure 31. **Cell proliferation of hCBMSC** (n 5 3, mean 6 SD).(A) Growth profile; (B) cell expansion in terms of fold increase in total cell number; and (C) scanning electron microscopic (SEM) photographs (150x) of hCBMSC on Fibra-Cell disks at 3, 5, and 7 days after inoculation.

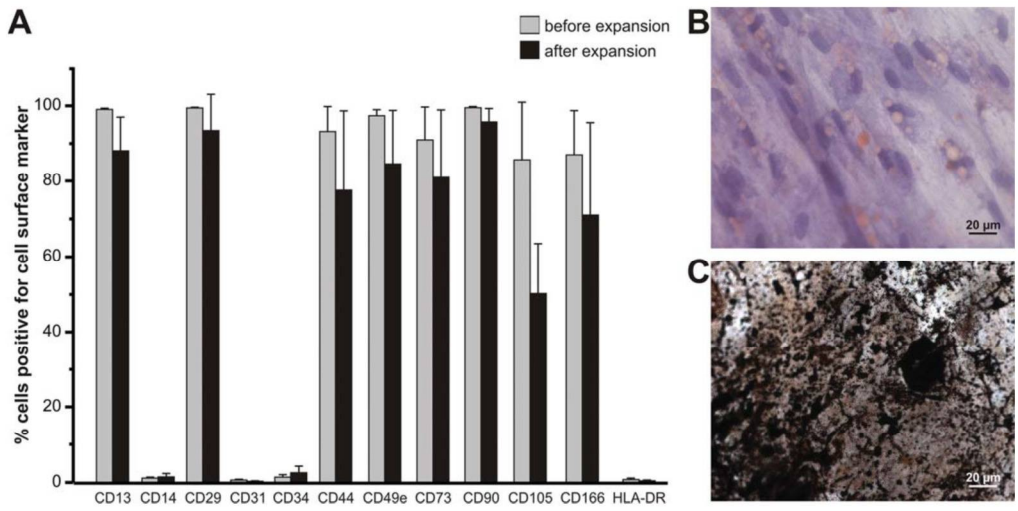


Figure 32. (A) Immunophenotype analysis and (B, C) differentiated potential of hCBMSC after expansion in Fibrastage culture system. B: Adipocytes at day 21 and (C) osteocytes at day 28 post-differentiation induction.

5) Development of a bioprocess for T-cells expansion

This project is the beginning and we are still evaluating the best strategy to start the experiments.

6) Profiling of MSC secretome grown in bioreactor AND 5) Initial evaluation of large scale production of therapeutically relevant proteins by MSC cultured in bioreactors.

MSCs are in the final phase of expansion in bioreactors and after finding the best bioreactor for expansion of these cells these next steps will be initiated.

7) Development of molecular diagnosis platform for infectious diseases (mycoplasma, citomegalovirus, human parvovirus B19 and HTLV) which are relevant to cell therapy in accordance with GMP and hemotherapy needs.

Nucleic acid test (NAT) for detection of infectious diseases agents associated to transfusion transmission.

a) Human T Lymphotropic Virus (type 1 and 2)

Due to the high prevalence of the Human T Lymphotropic virus type 1 and 2 (HTLV-1/2) infection in our country, the serologic screening in blood banks has become mandatory since 1993. In comparison with other human retroviruses like HIV, where the infection confirmation is mandatory, there is no obligatory test for HTLV confirmation. Nowadays the confirmatory diagnosis of HTLV infection using Western Blot shows some drawbacks such as high inefficiency (high proportion of indeterminate results) and elevated costs. Additionally, despite of the wide range of commercial molecular tests for a variety of viral infections, there is no commercial test available for HTLV infection. In

this relation, since 2011 our group has been developing a molecular platform for confirmatory HTLV-1/2 diagnosis. Our platform is based on TaqMan® real-time PCR technology using noncompetitive multiplex format (triplex for HTLV-1, HTLV-2, and endogenous gene) that enables simultaneous detection and differentiation of HTLV-1/2 in the same reaction. We have started the validation process, following the Verification and Validation of Nucleic Acid Assays guidelines from the Clinical and Laboratory Standards Institute (CLSI). We observed a limit of detection (LOD) of 2.61 and 6.38 copies/reaction for HTLV-1 and HTLV-2, respectively. The test showed 92% of diagnostic sensibility and 100% of diagnostic specificity. This test did not cross-react with HIV, HBV, HCV and B19V viruses. The inter- and intra-assay variation coefficients were below 3%. Currently, we are in process of routine implementation of this test as a confirmatory and discriminatory diagnostic test. We also started writing the patent process. The application of a molecular-based real-time multiplex technology optimized in Brazil will have lower cost compared to the serological diagnostic kits. This test will be also important to the Brazilian Public Health – as a screening and confirmatory test for blood donors, pregnant women, indigenous groups, intravenous drug users, and in the economy aspect (low cost) as well as it will contribute for the technologic fortification of Brazil.

b) Human Parvovirus B19 (B19V)

In 2013, the optimization of TaqMan® real-time PCR-based method for detection of the all three genotypes (1,2 and 3) of human parvovirus B19 (B19V) was finalized. It used specific in-house designed primers/probe amplifying 110 bp fragment from the VP1 genomic region. This detection platform was applied in different studies and a recent one determined the prevalence of B19V viremia in hemophilic patients (hemophilia A and B). Among them, 28 (35.7%) showed low viral load viremia with high seroprevalence for B19V-IgG (78.6%) compared to healthy blood donors. This initial analysis reveals the significance of B19V molecular screening in hemophilic patients since this infection is responsible for severe clinical conditions, including lethal infections. Since 2010 genotyping of the circulating B19V strains among patients with hemoglobinopathies is also performed with frequent detection of B19V genotype 1A.

c) Human Cytomegalovirus (HCMV)

In 2013, the optimization of TaqMan® real-time PCR for the detection of human cytomegalovirus (HCMV) was also concluded. The in-house reaction showed increased sensitivity (0.5 IU/reaction, WHO-based standard) and detected a 67 bp fragment from the UL97 region. Molecular detection of HCMV in patients with hemoglobinopathies (sickle cell anemia and beta-thalassemia major) demonstrated positive results in 13.8%, and 7.6 % of the patients with sickle cell anemia and beta thalassemia major, respectively. Some infections were characterized by a high viral load and clinical symptoms like retinopathy. In these patients, a partial UL 55 gene region (encoding the gB protein) was sequenced and genotyped. Ninety percent of the detected genotypes

belonged to gB2 genotype and only 9% to gB1. These genotypes could not be associated with the clinical status of these patients, since gB2 was detected both in patients with severe clinical conditions and asymptomatic ones. It was also observed that the frequency of viral reactivation is positively correlated to the multiple transfusions which can induce a mild immunosuppression of the patient. This is the first study even in world aspect for the clinical impact of HCMV in patients with hemoglobinopathies and could have important implications during their therapy and management.

We extended the studies of HCMV infection to the emergence of ganciclovir resistance in patients with HIV-infection undergoing treatment for HCMV. In-house method for genotyping of the UL97 gene responsible for HCMV resistance was developed. We determined that HCMV resistance appears approximately 240 after the start of the ganciclovir treatment in HIV infected patients undergoing continuous treatment with ganciclovir. A594V and L595W were the mutations most frequently observed, but they do not interfere the treatment as confer low-dose ganciclovir resistance.

Publications:

Slavov SN, Kashima S, Wagatsuma WMD, Silva-Pinto AC, Favarin MC, Covas DT. Glycoprotein B (gB) genotyping of Human Cytomegalovirus (HCMV) strains isolated from Brazilian patients with sickle cell disease and beta-thalassemia major. Viral Immunology (submission phase).

Slavov SN, Vilar FC, Wagatsuma VMD, Santana R, Machado AA, Fonseca BAL, Kashima S, Covas D. Ganciclovir treatment failure of cytomegalovirus infection in a long-term HIV progressor associated with late emergence of A594V and L595W resistance mutations. Journal of Clinical Virology (submission phase).

Use in practice: Wide application for HCMV diagnosis and suspected cases in different cohorts of patients. Especially in the area of hemotherapy for the diagnosis and management of HCMV infection in patients with hematopoietic stem cell transplantation. The application of the test for HCMV ganciclovir resistance is also recommended in the management of patients with hematopoietic transplantation.

d) Emerging Viruses: Human Parvovirus 4 (PARV4) and Xenotropic Murine Leukemia Virus-associated virus (XMRV)

Studies regarding emerging viruses like PARV4 and XMRV were performed. PARV4 infection was detected in 2.9% of the patients with hemophilia, 3.6% of the patients with beta-thalassemia and 1.5% of the healthy blood donors. This is the first study in Brazil evaluating the presence of this emerging virus among patients with hematological disorders and its clinical impact is unknown. However, as it is detected in coagulation factor concentrates it presents threat to blood transfusion. All of the positive patients showed very low viral load and were clinically asymptomatic. Our studies involving the other emerging virus XMRV involved multiply transfused patients and healthy blood

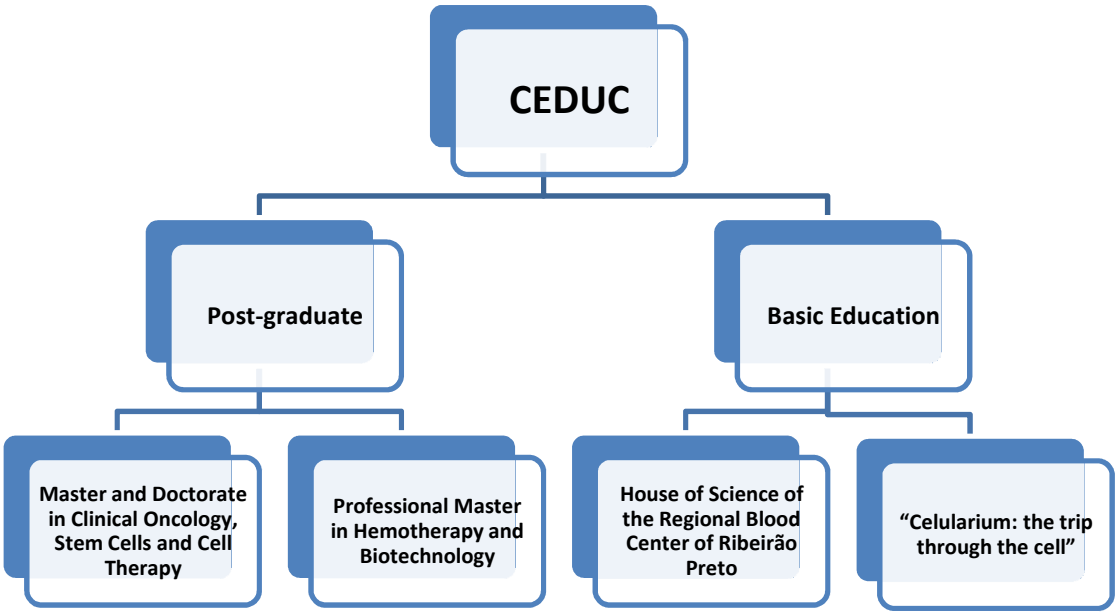
donors. Although, positive results were obtained from three patients with sickle cell disease, they were concordant with laboratory contamination due to their complete genetic similarity to the positive control (obtained from a murine leukemia retrovirus). Therefore our results confirm other studies describing the contaminative nature of XMRV infection and the low risk of transfusion transmission.

e) Mycoplasma

With the introduction of antibiotic treatment of the culture mediums of cultivated cells the problem with the bacterial contamination was minimized. However, the detection of mycoplasma species in cultivated cells has become more frequent. Due to the importance of the mycoplasma detection for our institution (mainly in products for cellular therapy) a real-time PCR reaction was optimized. This reaction used a type of intercalating dye (similar to SYBR® Green). The test is currently in its validation process (analytical and diagnostic sensitivity and specificity, precision, and robustness). Additionally scanning electron microscopy and phylogenetic analysis of the detected strains were performed. Our validation results will be compared to the currently used commercial test for mycoplasma detection MycoAlertTM mycoplasma detection kit. In addition our test will be evaluated in terms of costs per sample. After the complete optimization of the test it will be applied as a quality marker for the cells used for cellular therapy.

IV. EDUCATIONAL AREA

The educational activities of the CTC-CEPID are coordinated by CEDUC – Center of Education of the Regional Blood Center of Ribeirão Preto. In addition to the permanent educational program, which includes training and development of professionals such as physicians, nurses, biologists, physical therapists, and laboratory technicians, the activities are extended to specialists and researchers of the area, among them post-graduate students, residents in hematology and hemotherapy, and undergraduate students, promoting articulation between the knowledge produced in the research areas and teachers and students.



CEDUC relies on a team formed by a translator, a biologist and two secretaries, serving as interlocutor among the diverse educational projects, as support to continuous projects and seasonal event, revision of educational material and support in practical activities. It has a nucleus in the Regional Blood Center and an office in the campus of USP. The students of the post-graduate course can even rely on a follow-up of their courses by using a platform in ***Moodle*** system (http://ead.hemocentro.fmrp.usp.br/moodle/), apart from the follow-up by the system of USP.

As part of its physical structure, CEDUC has four classrooms with total seating capacity for 200 people and two amphitheaters, one with seating capacity for 170 people and another for 40 people, all with infrastructure for classes, for instance, interactive multimedia projectors, internet access to professors and students by Wi-fi network and support for the use of personal computers.

It has also a **Laboratory of Research and Teaching,** with a total area of 34 m^2, equipped with infrastructure of research on cellular and molecular biology, available to

the development of scientific initiation projects by teachers and students of Basic Education.

House of Science of the Regional Blood Center of Ribeirão Preto

The House of Science was started in 2000 as part of the educational program of the Center for Cell-based Therapy (CTC), one of the Centers of Research, Innovation and Diffusion (CEPID). Since its establishment, it is coordinated by Professor Marisa Ramos Barbieri.

At the moment, the team relies upon the support of four students under scholarship and one assistant that develop a serie of programs which search for the interaction between teachers and students of Basic Education and prominent researchers in Brazilian Science.

In the permanent space for exhibitions, , MuLEC (Museum and Laboratory for Science Teaching), the activities conducted have been reaching public and private schools. The proposal of working with after school programs of teaching support, specially in scientific initiation (less frequent nowadays at elementary and high schools), has the purpose of supporting the exhibitions.

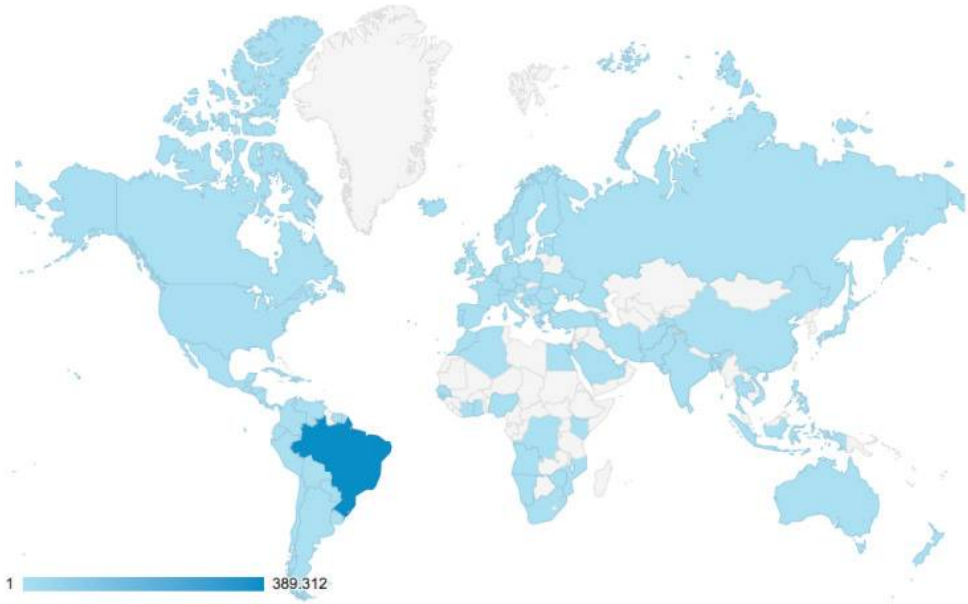
Produced Material

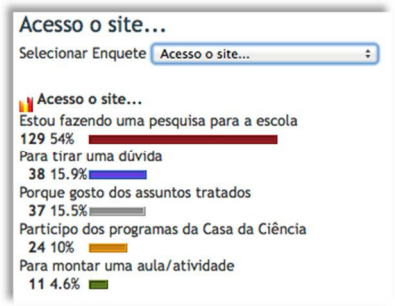
House of Science website

The website of the House of Science is one of the foundations for its plan of actions (http://ead.hemocentro.fmrp.usp.br/joomla/). It is an interactive access portal where the visitor can be updated on what is new on Cell and Molecular Biology and they can also learn more about the programs offered.

In 2013, the website had 166,510 visits. This year it has registered more than 70,000 visits up to now.

Since 2005 until the present time, it has received the vistit of 421,324 people from over 100 countries, Portugal is the first in the ranking with 11,411 visits, followed by United States, Angola, India, and Mozambique





The differential point of this portal is the participation of students from diverse programs in its development, thus it is the fruit of the interaction among reserchers, students, teachers and the House of Science, what makes its language more understandable and often desmystifying what is divulged by blogs and news agencies. In 2013, a poll designed to the public of the website was conducted in order to know the reasons for access and it was shown that the majority (54%) uses the website as a reference source for school assignments.

Science Journal

(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/publicacoes/jornal-das-ciencias)

With its first edition in August 2001, the ***Science Journal*** was launched with the purpose of divulging and popularizing science teaching, approaching scientific subjects and the research that was produced in the university with the collaboration of researchers and professors at CTC in a very simple and instructive way.

The Science Journal aims to support the teacher in the classroom, particularly in current themes, which the textbook does not cover or superficially covers. All the editions are printed and freely distributed to schools and available on the House of Science website.

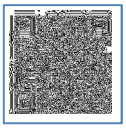
It has already an editorial line designed to meet the demand of both students and teachers, contributing to their education and bringing what is new, using a more understandable language. The Journal also possesses a complement (Science Track), written to students, where part of their activities in many programs of the House of Science is shown, always with the attempt to attract new students.

Folhetins

(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/publicacoes/folhetins/)

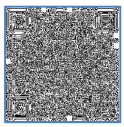
Folhetins are scientific texts written by researchers and specialists linked to the CTC group, they approach themes which have the aim of updating and increasing the knowledge of teachers and students. In addition to the publication that have already been printed, distributed, and tested, one can find there new material produced by post-graduate students of the Medical School of Ribeirão Preto (FMRP) and post-graduate students collaborating in the activities of the House of Science.

In a small format, with short and illustrative text, it invites to a fast reading and can be characterized by the diversity of theme concepts. Folhetins approach current scientific themes and promote a review of subjects and the understanding of basic concepts – generally poorly formulated in school books. The Folhetim "***DNA, o sentido da vida?***" (DNA, the sense of life?), produced in 2013, was printed more 1,500 times from its digital version available on the website.



Single Sheet
(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/publicacoes/folha-avulsa)

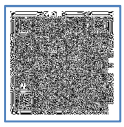
"Sheet" because it is the record of moment considered to be significant when learning takes place. "Single" to facilitate the record throughout the learning process. The proposal is mischaracterizing the trend of Science Teaching marked by the content imposed by the Sciences, without considering the educational aspect. Because it is "single", it encourages the investigation of its sequence in accordance with the same methodology proposal that organizes all programs.



Programs of the House of Science
Adote um cientista
(***http://ead.hemocentro.fmrp.usp.br/joomla/index.php/programa/adote-um-cientista/)***

The Adopt a Scientist program proposes that students from elementary school attend meetings with undergraduate and postgraduate students. In these meetings, they bring specific subjects and discuss with students.

The goal of the program is to bring together academic science makers and young students of elementary and high school in order to make them think scientifically and to learn to develop concepts, in a pleasant and difficult way.



Little Scientist
(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/programa/)

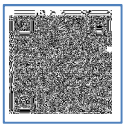
The programs predicts the development of scientific initiation by the students of public schools, as previously seen in programs like Caça-Talentos (Talent Hunt) (2001-2003), FAPESP-Júnior (2004-2005) and Pre-scientific Initiation (from 2009 up to now).

In this program, participating students develop a small scientific project throughout ten meetings, the projected is presented in a event by the end of the semester (***Mural***) to other students, employees, and researchers of the Center for Cell-based Therapy, Blood Center and USP community.

Science Saltimbancos
(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/noticias/ultimasnoticias/530-saltimbancos-casa-da-ciencia-inicia-novo-programa-educacional)

This program encourages research projects in schools, with the intense participation of the teacher and House of Science team. Encounters take place while students participate in other activities offered. It is the teacher's chance, supported by the House of Science team, to bring the research environment to school, making science diffusion and dissemination stronger and showing that the teacher, when supported, is capable of developing a serie of supplementary projects. The results achieved in this first year of Saltimbancos were presented in the Murals of 2013 (in the first and second semester).

Educational Events

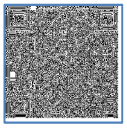


Mural

(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/eventos/mural/)

The Mural is an event organized by the House of Science that allows information exchange among students, it is the moment when they fully grasp the concepts learned during the semester. The students experience concept constructtion when they are presenting the outcomes of their activities with the projects of the House of Science.

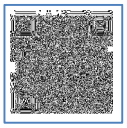
In 2013 were held, the 17th (June 27th) and the 18th (November 28th) where the students shared their discoveries with classmates and teachers. In the 18th edition, some outcomes of Science Saltimbancos were also shown.



Adopt an experiment

(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/laboratorio/adote-uma-experiencia/)

It is about na activity of the program ***Adopt a Scientist*** which brings classical experiments of science proposed to students on the internet. This challenge is followed by a video that arouses curiosity and leads the student to learn more about the themes adressed. The activities are planned to be conducted at home.



Vacation with Science

(http://ead.hemocentro.fmrp.usp.br/joomla/index.php/eventos/ferias-com-ciencia/)

Learning and fun are the foundation of this program, in which science aspects normally are left out in the classroom. This project playfully inctroduces science to students during their vacation time and shows a new way of learning without missing all the fun.

In 2013 three editions of the project were held:

The 5th Edition (January/2013) with the subject "*Sistemas dinâmicos naturais: entre a organização e a desorganização*" (Natural dynamic systems: between organization and disorganization), the team of the House of Science prepared practical activities like modelling workshops, games and simulations that led young students to investigate time factor in the formation of complex systems, going through evolution issues such as extinction, speciation, mutation, homeostasis and co-evolution.

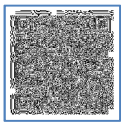
- 01/22 – Dynamic systems and their organization.
- 01/ 23- Nature that surrounds us and time factor.
- 24/01 - Disorganization: is it the end or the beginning?

The 6th Edition was held in July 23, 24 and 25. In this edition, entitled "Science et al… in the backyard", it encouraged the development of scientific initiation projects, beginning from "simple" daily resources, going through the common steps in the production of scientific knowledge. Organisms like scorpion and pseudoscorpion, terrestrial and aquatic ecosystems were analyzed by students and researchers.

The 7th Edition was held in January/2014 with an activity entitled ***"Dando linha (para a Matemática)"***, the event received young students interested in learning more about the relations between life science and exact science. The students participated in theoretical and field activities which even involved the construction of kites and flight tests.

National Week of Science and Technology (http://ead.hemocentro.fmrp.usp.br/joomla/index.php/eventos/snct)

The House of Science has taken part in the National Week of Science and Technology (SNCT) since its first edition in 2004, always with activities that playfully lead population to science, where the learning process is facilitated to arouse students' curiosity about diverse science paths, finding enjoyment in discovering. In 2013 (from October 21 to 27), the theme proposed by the Ministry of Science, Technology and Innovation of the Federal Government was "Science, Health and Sports".



The team of the House of Science got in the mood and developed many activities to the exposition "Mitochondrion: the number 10 in any team" which is focused on the energy used during physical activity and the most essential organelle for energy synthesis en the cell.

This exhibition has also gained an **online** version that offers the opportunity for learning more about this activity planned for the SNCT and now can be accessed by people all over the world. The virtual exhibition, apart from showing what happened in the exhibition held by the House, leads the visitor to access every space of the exhibition, check all the experiments done and even reproduce them at home.

Celularium

The *Celularium* is an inflatable dome of 22m^2, with a fulldome digital technology projector, that is used to exhibit a movie which stimulates the perspective of the public for the cellular universe. The movie, exhibited in 360º, simulates a trip inside the components of an animal cell. Conceived by the researchers of the Center for Cell-based Therapy, the exhibition invites the visitor to dive into one of the selective channels of the membrane until the cytoplasm, going through organelles, nucleus, participating in the arresting of extracellular particles by the lysosome.

The theme Cell, accentuated by the molecular area, is a current highlight in research and media. The understanding of basic concepts to grasp more complex subjects, such as gene therapy, stem cells and cancer, is fundamental to knowledge construction. Thus a tool like the *Celularium* becomes indispensable. This movie is the first produced in fulldome technology for the biological area in Brazil, and the approval of CTC researchers granted the movie scientific rigor and excellence in graphic resources.

In 2013, the *Celularium* was exhibited in the 59th Congresso Brasileiro de Genética, held in Águas de Lindóia. The congress took place from September 16 to 19. In the 18th, the Celularium was opened to attendees and students from schools for the exhibition of

the movie “A trip through the cell”. Approximately 550 attendees and students saw the sessions which started at 9 am and were extended until 11 pm.

During the National Week of Science and Technology, in partnership with SESI, the Celularium was exhibited in three units, with the participation of approximately 1000 students and teachers. The exhibitions were held at the Centro Educacional 346 (Ipiranga), Centro Educacional 298 (Castelo Branco), Centro Educacional 345 (Vila Virgínia).

Semana Nacional de Ciência e Tecnologia de 2013 *(21 a 27 de outubro)* apresentação em 3 unidades do **SESI**
989 alunos e professores.



59° Congresso Brasileiro em Águas de Lindóia - 16 e 19 de setembro
550 congressistas e estudantes


Hemocentro TV

The use of videos as supplementary resources is of vital importance for science democratization. Hemocentro TV, jointly with CEDUC, has an important role in this cause, producing high quality interviews, documentaries and video classes, allowing that spectators establish a relation between the concepts shown and the ones which already exist in their cognitive structure.

The newly created Hemocentro TV demonstrated a great potential for expansion, either to meet the need for professional training in the field of biotechnology and production of blood derivatives, giving support to the elaboration of video classes, or to the production of scientific diffusion and dissemination material, enhancing the diffusion of the scientific knowledge produced in the Center for Cell-based Therapy.

Post-graduate Programs

1) Clinical Oncology, Stem Cells and Cell Therapy

The program aims to train high level professionals to work both in the academic area and in the private or governmental sector. The program covers a set of disciplines and activities designed to the development of skills in clinical oncology and in cell therapy and stem cells, apart from a set of disciplines designed to the development of the post-graduate student’s critical sense, involving scientific writing, argumentative

training, communication techniques and teaching. At the moment, 21 students are enrolled, 12 in the Master program and 9 in the Doctorate program.

2) Professional Master in Hemotherapy and Biotechnology

The program aims to train specialized professionals capable of taking part in the development and promotion of health in Brazil, improving their technical and scientific knowledge, being able to conduct activities that enable the continuity of their training to contribute to the improvement of the services provided. In this program, applied and basic disciplines are offered, in a multidisciplinary way, which aim to train a professional with integrated vision of hemotherapy and biotechnology applications and who is able to deal with market evolution in the field. At the moment, 42 students are enrolled, 36 in hemotherapy and 5 in biotechnology. The map below shows the State in which the student lives and works.

Scientific Events

São Paulo School of Advanced Sciences on Oncogenesis and Translational Medicine

The event, held from February 17 to 22, received de 65 invited researchers and 96 students from 16 countries. The program included conferences, discussion panels, mini-courses and a theoretic-practical course on Bioinformatics. Subjects like cancer biology, leukemia and cancer immunotherapy were covered, among others. http://bioinformatics.fmrp.usp.br/ESPCAoncogenesis/

1º First Brazilian Meeting of High Content Screening

This pioneer event was held from March 30 to April 04, with the purpose of establishing the diffusion of HCS approaches among Brazilian researchers. The event was composed of four lectures of international specialists (scientific administrators of HCS facilities in renowned institutions), two lectures of national researchers, five technical lectures of the major manufacturers of equipment and consumable in the field of HCS, two round-tables gathered representatives from all Brazilian groups and facilities working with HCS and three theoretic-practical mini-courses.

Three workshops were also organized:

1) II Workshop on Mesenchymal Stromal Cells, course load of 8 hours, held in 07/03/2013, where 35 researchers met to discuss subjects related to basic research with Mesenchymal Cells.

Title	**Speaker**
Opening	Prof. Dr. Dimas Covas
Basic Research with MSC	
Cytoplasmic heritage and production of RHO mesenchymal cells	Prof. Dr. Flávio Vieira Meirelles
Animal mesenchymal cells bank and use of stem cells from fetal membranes	Prof. Dr. Carlos Eduardo Ambrósio
Break	
Human adipose tissue as ectopic vehicle to produce coagulation factor VIII in a murine model of hemophilia A Isolation and characterization of subpopulations of cells of stromal vascular fraction from adipose tissue	Msc. Lucas Eduardo Botelho de Souza Liziane Raquel Beckenkamp
Characterization of subpopulations of mesenchymal stromal cells derived from umbilical cord	Thaís Pimentel
Lunch	
Expansion *in vitro* of stromal mesenchymal cells and secretome characterization: biotechnological and therapeutic applications	Msc. Amanda Mizukami
The use of mesenchymal stem cells in ventilated fetus of mice with congenital diaphragmatic hernia	Dr. Frances Lilian Lanhellas Gonçalves Fernanda Ursoli Ferreira Melo
MSC use in Diabetes Type 1	Msc. Juliana Ueda
Break	
Cell therapy for cutaneous regeneration	Msc. Carolina Caliari
Production of multipotent mesenchymal cells in large scale for therapeutic purposes	Msc. Maristela Delgado Orellana
Manuscript: Regulation of stem cell therapies under attack in Europe: for whom the bell tolls	Msc. Lucas Eduardo Botelho de Souza
Closure	

2) I Workshop on Pluripotent Stem Cells, course load of 8 hours, held in 11/29/2013, with the participation of 56 researchers and post-graduate students.

CTC
CENTRO DE TERAPIA CELULAR
CENTER FOR CELL-BASED THERAPY

I Workshop sobre Células-tronco Pluripotentes

29/11/2013 - 8 às 17h
Anfiteatro Vermelho - Hemocentro RP

Programação

Horário	TEMA	Palestrante
8h30-9h30	Perspectivas em Células-Tronco Pluripotentes	*Prof. Dr. Dimas Tadeu Covas*
9h30-10h30	iPS como modelo de Doença Hematológica	*Prof. Dr. Rodrigo Calado*
10h30-10h45	**COFFEE BREAK**	
10h45-11h30	Biblioteca Celular da Diversidade Genética Brasileira	*Maximiliano Dasso*
11h30-12h15	Mecanismos Moleculares Envolvidos na Manutenção e na Diferenciação de Células-Tronco Pluripotentes	*Rodrigo Pannepuci*
12h15-13h15	**ALMOÇO**	
13h15-14h	iPS e Hemofilia A	*Aline Fernanda Ferreira*
14h -15h15	**MESA REDONDA- Pluripotência e modelos animais**	
14h-14h20	Reprogramação celular por trasferência de núcleo	*Prof. Dr. Flavio Meireles*
14h20-14h40	Reprogramação celular através da indução gênica e transferência de núcleo no modelo animal	*Fabiana Bressan*
14h40-15h	Geração de células de pluripotência induzida: avanços e perpectivas em cães e coelhos	*Natalia Juliana Nardelli Gonçalves*
15h-15h15	Discussão	
15h15-15h45	**COFFEE BREAK**	
15h45-16h15	Diferenciação de células hematopoieticas a partir de células embrionárias	*Maristela Delgado Orellana*
16h15-17h15	**Desenvolvimento de Novas Pesquisas**	
16h15-16h45	iPS e Anemia falciforme	*Luiza Junqueira*
16h45-17h15	Epigenética das Células-Tronco Pluripotentes	*Tathiane Maistro Malta Pereira*

Informações: tathimalta@hemocentro.fmrp.usp.br

USP FAPESP CEPID Hemocentro RP

3) I Workshop on Coagulation Factors, course load of 8 hours, held in 09/18/2013, participation of 48 researchers and post-graduate students. Subjects like Recombinant FVIII production in large scale, FVIII Purification, Recombinant FVII production, and Recombinant FIX production were covered.

Summer Courses

The Summer Course “Genome, Proteome and Cell Universe” started in 2001. It is a practical course that takes the students to research laboratories, where the access to different technology approaches have allowed great advance on medicine and biology in general. The XIV edition of the Course received 42 students from Brazil and Peru.

The Summer Course on Bioinformatics is annually offered and has the objective of presenting the real scenery of elaboration and development of a bioinformatics project

to undergraduate and/or post-graduate students. The X edition of the Course received 100 participants, 12 of them were foreign students, in addition to 2 American researchers and 1 Canadian researcher.

Winter Course of Biochemistry and Molecular Biology

The Winter Course of Biochemical and Molecular Biology is in its third year, in all editions we received students from almost all Brazilian states, creating opportunity of entering in post-graduate program in Biochemistry, offered by FMRP, and opening a communication channel among the participants. This course is an activity designed to undergraduate or recently graduate students who have taken Biochemistry classes or are developing research in the field and are interested in learning more about the different fields of activity. The objective of this event is divulging the research lines conducted by the Department of Biochemistry and Immunology FMRP/USP and keeping the communication channel among people who are interested in the field.

EVENT ORGANIZATION AND COORDINATION

FAÇA, V; KOIDE, T. ; LUCENA, M. N. . XII Curso de Inverno em Bioquímica e Biologia Molecular. 15-26/jul/2013.

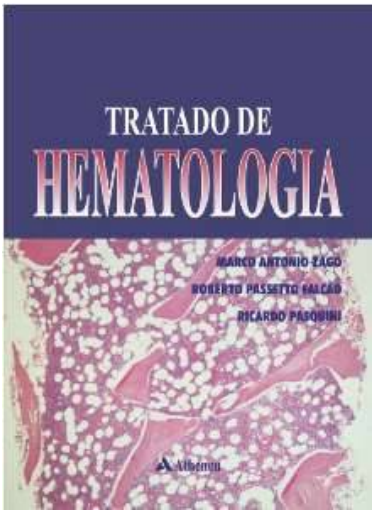
HUMAN RESOURCES TRAINING

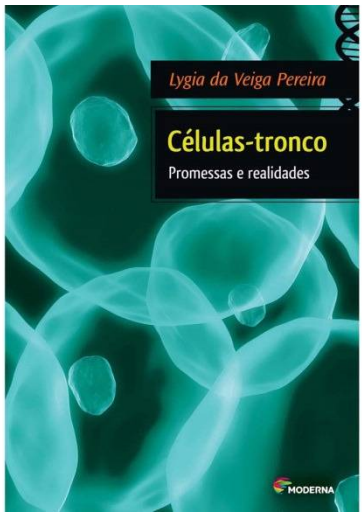
Type	Concluded in 2014	Ongoing in 2014
Doctorate	15	32
Post-doctorate	9	12
Master	13	19
Scientific Initiation	2	5
Young Researcher	1	

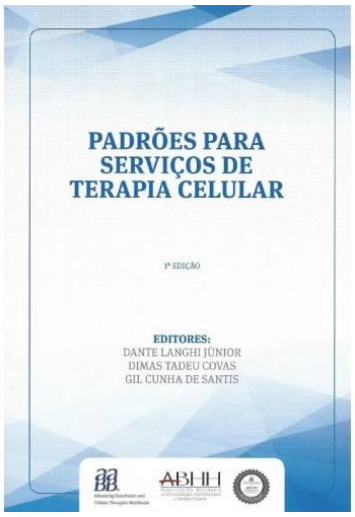
Scientific Production

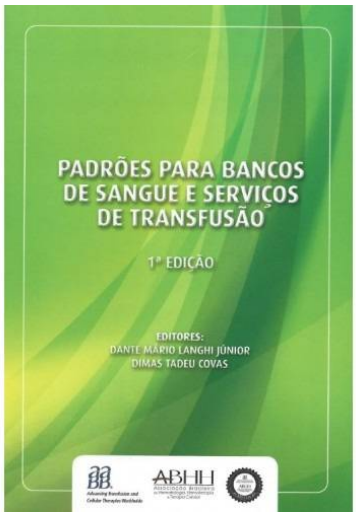
50 Scientific manuscripts

2 Books









V. HUMAN RESOURCES

1) Grants Concluded

Master

- Erika da Silva Czemisz. Análise Proteômica de Meduloblastomas. Supervisor: José César Rosa.
- Fernanda Gutierrez Rodrigues. Utilização do método flow-FISH no diagnóstico de doenças dos telômeros: síndromes de falência medular e fibrose pulmonar idiopática. Supervisor: Rodrigo do Tocantins Calado Saloma Rodrigues.
- Helena Debiazi Zomer. Estabelecimento de cultura de células de Pluripotência Induzida a partir de células-tronco derivadas do tecido adiposo de coelhos. Supervisor: Carlos Eduardo Ambrósio.
- Julia Teixeira Cottas de Azevedo. Avaliação do repertório de células T em pacientes com diabetes mellitus tipo 1 submetidos ao transplante autólogo de células-tronco hematopoéticas. Supervisor: Kellen Cristina Ribeiro Malmegrin de Farias.
- Lucas Coelho Marliére Arruda. Avaliação de Mecanismos Imunológicos envolvidos na Resposta Terapêutica de pacientes com Esclerose Sistêmica ao Transplante Autólogo de Células-Tronco. Supervisor: Kellen Cristina Ribeiro Malmegrin de Farias.
- Luiza Ferreira de Araújo. Instabilidade do genoma mitocondrial em adenoma e adenocarcinoma colorretal. Supervisor: Wilson Araújo da Silva Júnior.
- Renato Zonzini Bocabello. O uso da Condroitinase ABC combinada com células Tronco do Epitélio Olfatório de coelhos em modelo de lesão medular por Hemissecção dorsal em coelhosS. Supervisor: Carlos Eduardo Ambrósio.

Doctorate

- Gustavo Ribeiro Fernandes. Variabilidade fenotípica de um modelo murino para a Síndrome de Marfan - Triagem de genes modificadores do fenótipo. Supervisor: Lygia da Veiga Pereira.
- Germano Aguiar Ferreira. Proteoma de Microparticulas Oriundas de Células da Linhagem NB4 de Leucemia Promielocítica Aguda. Supervisor: Lewis Joel Greene.
- Julio Cesar Cetrulo Lorenzi. Assinatura de expressão de miRNAS de subpopulações de linfócitos T em indivíduos normais e pacientes com esclerose múltipla. Supervisor: Wilson Araújo da Silva Júnior.
- Lucas Oliveira Sousa. A proteína SET modula os níveis de miRNAs e proteínas envolvidas na manutenção e progressão do câncer oral. Supervisor: Lewis Joel Greene.
- Lucas Sacchini Del Lama. Caracterização e Adaptação do Dosímetro Friche para Dosimetria em Irradiação de Sangue. Supervisor: Adelaide de Almeida.
- Lucila Habib Bourguignon Oliveira. Bases moleculares da diferenciação "in vitro" de células T a partir de células CD34+ isoladas de sangue de cordão umbilical. Supervisor: Marco Antonio Zago.
- Naja Vergani. Triagem Funcional de Genes Envolvidos no Processo de Manutenção da Inativação do Cromossomo X em Humanos. Supervisor: Lygia da Veiga Pereira.
- Tathiane Maistro Malta Pereira. Estudo dos mecanismos reguladores da pluripotência em Células-Tronco Pluripotentes Induzidas (iPS) Humanas. Supervisor: Simone Kashima Haddad.
- Thiago Yukio Kikuchi Oliveira. Análise in-silico de rearranjos cromossômicos em células B selvagens e 53BP-/- comparadas com linfomas de células B 53BP1-/- e P53-/- utilizando sequenciamento em larga escala. Supervisor: Wilson Araújo da Silva Júnior.

Pos-Doctorate

- Aline Poersch. Estudo proteômico de fluidos intra-tumorais na identificação de novos biomarcadores para o diagnóstico precoce de câncer de ovário. Supervisor: Lewis Joel Greene.
- Celina Almeida Furlanetto Mançanares. Multipotencialidade das células-tronco do saco vitelino em embriões bovino. Supervisor: Carlos Eduardo Ambrósio.
- Paula Fratini. 2013. Faculdade de Medicina Veterinaria e Zootcnia, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Supervisor: Maria Angélica Miglino.
- Phelipe de Oliveira Favaron. Faculdade de Medicina Veterinaria e Zootcnia, Fundação de Amparo a Pesquisa do Estado de São Paulo Bolsa TT. Supervisor: Maria Angélica Miglino.
- Raquel Tognon Ribeiro. Alterações moleculares e epigenéticas da apoptose, ciclo celular e via de sinalização Jak/Stat em neoplasias mieloproliferativas crônicas JAK2 V617F P. Supervisor: Fabiola Attié de Castro.
- Silvio Henrique de Freitas. Avaliação Morfológica e imuno-histoquímica dos tecidos hepático, pulmonar, renal, esplênico, pancreático, músculos esqueléticos e cardíaco após clampeamento total do pedículo hepático e reperfusão em ratos, em diferentes tempos (manobra de Pringle modificada). Supervisor: Carlos Eduardo Ambrósio.
- Svetoslav Nanev Slavov. Desenvolvimento de plataforma molecular para o diagnóstico confirmatório e discriminatório da infecção pelo HTLV-1/2. Supervisor: Dimas Tadeu Covas.
- Valeria Ferreira da Silva. Avaliação do potencial de diferenciação neural das células mesenquimais estromais multipotentes da medula óssea humana após fototerapia a laser. Supervisor: Dimas Tadeu Covas.

Scientific Initiation

- Luiz Henrique Santana Conceição. Mensuração Quantitativa e Objetiva do Desempenho Motor e de Aspectos Mnemônicos e Emocionais de Macacos-Prego induzidos a Doença de Parkinson:Contribuições da análise. Supervisor: Klena Sarges Marruaz da Silva.
- Lygia Elyse Chaves da Silva. Estudo da ação terapêutica das células-tronco mesenquimais na neurogênese endógena em modelo experimental da doença de Parkinson em Cebus apella. Supervisor: Klena Sarges Marruaz da Silva.

2) Current Grants

Master

- Alessandra de Oliveira Pinheiro. Tratamento de cães infectados naturalmente pelo vírus da cinomose canina na fase aguda com o uso de células-tronco de epitélio olfatório. Supervisor: Carlos Eduardo Ambrósio.
- Bruna Ferreira de Souza. Caracterização estrutural do gene CR596471. Supervisor: Wilson Araújo da Silva Júnior.
- Daianne Maciely Alves de Carvalho. Geração de uma linhagem celular humana portadora do FVIII sintético com mutações nos domínios A1 e A2 utilizando o sistema lentiviral. Supervisor: Dimas Tadeu Covas.
- Daniel Fantozzi Garcia. Análise do perfil genotípico de pacientes com galactosemia clássica e estudo da relação do genótipo-fenótipo. Supervisor: Wilson Araújo da Silva Júnior.
- Diego Villa Clé. Células mesenquemais estromais multipotentes da medula óssea no tratamento da anemia aplastica refreatária. Supervisor: Roberto Passetto Falcão.
- Eduarda Morgana da Silva. Determinação da Base Molecular da Síndrome Ablefaria-Macrostomia. Supervisor: Wilson Araujo da Silva Júnior.
- Fabianna Pansani. Biomarcadores preditores de resposta a Quimiorradioterapia Neoadjuvante no Adenocarcinoma de Reto. Supervisor: Eduardo Magalhães Rego.
- Fernanda Ursoli Ferreira Melo. Estudo da transição endotélio-mesenquimal (ENDMT) na biologia de células endoteliais. Supervisor: Dimas Tadeu Covas.
- Fernando Sábio. Identificação de um gene do cromossomo X imprintado na pacenta humana. Supervisor: Lygia da Veiga Pereira.
- Gabriella Mamede Andrade. Estudo retrospectivo do constituinte lipídico do liquido folicular que banhava oocitos competentes ou incompetentes. Supervisor: Flávio Vieira Meirelles.
- Helder Teixeira Melo. Tendência evolutiva da legislação referente aos critérios para seleção e triagem de doadores, comparada à evolução tecnoológica da hemoterapia. Supervisor: Dimas Tadeu Covas.
- Juliana Bernardes Elias Dias. Comprimento telomérico no transplante de medula óssea para anemia aplástica adquirida. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Katarina Holanda. Efeito in vitro e in vivo da Mitoquinona-Q associada ou não ao As2O3 em células de melanoma. Supervisor: Eduardo Magalhães Rego.
- Larissa Ananias Candido. Estudo do efeito da administração de células tronco mesenquimais na lesão aguda pulmonar induzida por transfusão sanguínea. Supervisor: Eduardo Magalhães Rego.
- Liziane Raquel Beckenkamp. Geração de uma linhagem de amniócitos humanos com produção de altos níveis de proteína Fator VIIAB selvagem e sintético da coagulação sanguínea. Supervisor: Dimas Tadeu Covas.
- Maria do Carmo Favarin. Análise da granularidade da série mielóide em pacientes com síndrome mielodisplásticas. Supervisor: Roberto Passetto Falcão.
- Maria Florencia Tellechea. Geração de células pluripotentes induzidas de pacientes com anemia aplástica adquirida. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Mariana Três Cardoso. Estudo molecular comparativo de células-tronco mesenquimias de amnion canino e felino. Supervisor: Carlos Eduardo Ambrósio.
- Natália Ferreira Scatena. Correlação entre o comprimento telomérico e o risco de hepatocarcinoma na infecção crônica pelo virus da hepatite C. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Renan Grigoletto. Avaliação da concentração intra-articular de gentamicina, associada ou não ao DMSO, administrada por perfusão regional intravenosa em membros de equinos sadios. Supervisor: Daniele dos Santos Martins.

- Thais Valeria Costa de Andrade Pimentel. Avaliação do potencial imunomodulatório de células estromais mesenquimais. Supervisor: Dimas Tadeu Covas.

Doctorate

- Aline Fernanda de Souza. Derevição de células Germinativas através da indução de cultura de células de pluripotência induzida caninas. Supervisor: Daniele dos Santos Martins.
- Aline Simoneti Fonseca. Assinatura gênica e microRNAS (miRNAS) entre adenoma e adenocarcinoma colorretal. Supervisor: Wilson Araujo da Silva Júnior.
- Amanda Mizukami. Expansão in vitro de células estromais mesenquimais e caracterização do secretoma: Aplicações Terapêuticas e Biotecnológicas. Supervisor: Dimas Tadeu Covas.
- Ana Carolina Furlanetto Mançanares. O estudo das células T gama delta na manutenção da tolerância materna em vacas gestantes. Supervisor: Daniele dos Santos Martins.
- Ana Cristina Silva Pinto. A hidroxicarbamida atua sobre componentes do metabolismo da adenosina em células sangüíneas de pacientes com anemia falciforme. Supervisor: Marco Antonio Zago.
- Ana Paula Alencar de Lima Lange. Estudo do papel do C/EBPalfa na leucemogênese induzida pela proteína de fusão CALM/AF10. Supervisor: Eduardo Magalhães Rego.
- Anna Aalbers. Telomerase mutations in pediatric acute myeloid leukemia. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Antônio Roberto Lucena de Araújo. Análise *in vitro* e *in vivo* da forma truncada deltaN-p73 na leucemia promielocítica aguda. Supervisor: Eduardo Magalhães Rego.
- Atanasio Serafim Vidane. Modelo pré-clinico do uso de células tronco mesenquimais da membrana amniótica para o tratamento de insuficiência renal crônica em gatos. Supervisor: Carlos Eduardo Ambrósio.
- Bruna Rodrigues Muys. Estudo funcional dos microRNAs miR-450a e miR-450b-5p na tumorigênese. Supervisor: Wilson Araújo da Silva Júnior.
- Carolina Arruda de Faria. Emprego de Terapia Celular em pacientes com doença obstrutiva crônica/Enfisema Pulmonar. Supervisor: Wilson Araújo da Silva Júnior.
- Cynthia Quinderé Cardoso. Análise Histomorfológica óssea de Animais Modelo (MUS MUSCULUS) para a Síndrome de Marfan. Supervisor: Lygia da Veiga Pereira.
- Cláudia Regina Gasque Schoof. Validação de Micrornas Candidatos à Regulação da Expressão da DNA METILTRANSFERASE DNMT3B. Supervisor: Lygia da Veiga Pereira.
- Daiani Cristina Ciliao Alves. Perfil de Expressão de mRNAS, miRNAS e de Moléculas Regulatórias da resposta Imune em Aloenxertos Renais. Supervisor: Eduardo Antonio Donadi.
- Diego Villa Clé. Células mesenquimais estromais multipotentes da medula óssea no tratamento da anemia aplástica refratária.. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Everton de Brito Oliveira Costa. Estudo do nicho hematopoético e das propriedades funcionais das células tronco hematopoéticas obtidas de células tronco embrionárias humanas. Supervisor: Dimas Tadeu Covas.
- Fabiana Fernandes Bressan. Efeitos da indução Gênica e reprogramação através da transferência de núcleo na produção in vitro de embriões bovinos. Supervisor: Flávio Vieira Meirelles.
- Fabio Sergio Cury. Desenvolvimento placentário e descrição morfológica do órgão reprodutor feminino em Coendou prehensilis (Porco-espinho Caixeiro). Supervisor: Carlos Eduardo Ambrósio.
- Felipe Magalhães Furtado. Telomere lenght and miRNAs expression in individuals with monoclonal B lymphocytosis. A comparison with Binet A CLL. Supervisor: Roberto Passetto Falcão.
- Fernanda De Luca Silvestro. Avaliação das Células Pluripotentes Humanas como um Sistema para Screening de Toxidade in Vitro. Supervisor: Lygia da Veiga Pereira.

- Flávia Sacilotto Donaires. Diferenciação de células iPS derivadas de pacientes com telomeropatias. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Francisco Paula Caretta. Inibição de aurora cinase A e B reduz a proliferação celular e promove apoptose em leucemia linfocítica crônica. Supervisor: Marco Antonio Zago.
- Giovana Pirolla Cardozo. Biologia Genética. Supervisor: Lygia da Veiga Pereira.
- Helder Henrique Paiva. Identificação e caracterização funcional das células tronco leucêmicas na leucemia promielocítica aguda humana. Supervisor: Eduardo Magalhães Rego.
- Helder Teixeira de Freitas. Estudo dos mecanismos de geração de células T regulatórias a partir de células T naive: papel da sinalização da Adenosina. Supervisor: Rodrigo Alexandre Panepucci.
- Ildercilio Mota de Souza Lima. O papel dos miRNAs na proliferação e diferenciação celular da linhagem pluripotente de carcinoma embrionário humano NTera-2". Supervisor: Rodrigo Alexandre Panepucci.
- Joana Carvalho Moreira de Mello. Estado Epigenético do Cromossomo X em Tecidos Extra Embrionários Humanos e Bovinos. Supervisor: Lygia da Veiga Pereira.
- Julia Teixeira Cottas de Azevedo. Reconstituição imuno-hematológica em pacientes com anemia falciforme tratados com transplante alogênico de células-tronco hematopoéticas. Supervisor: Kelen Cristina Ribeiro Malmegrim de Farias.
- Juliana Barbosa Casals. Desvendando as células germinativas da placenta de carnívoros (Canis familiaris e Felis domesticus). Supervisor: Carlos Eduardo Ambrósio.
- Juliano Rodrigues Sangalli. Reprogramação núclear em bovinos, um modelo usando agentes demetilantes. Supervisor: Flávio Vieira Meirelles.
- Julio César Cetrulo Lorenzi. Assinatura gênica de miRNA em células T CD4+ e CD8+ periféricas em indivíduos normais e pacientes com esclerose múltipla. Supervisor: Wilson Araújo da Silva Júnior.
- Katia Kaori Otaguiri. Avaliação do efeito dos exossomos na infecção pelo vírus linfotrópico humano de células T do tipo 1 (HTLV-1). Supervisor: Simone Kashima Haddad.
- Laís Vicari de Figueiredo Pessôa. Produção de células tronco equinas para modelos de estudo de diferenciação Supervisor: Flávio Vieira Meirelles.
- Leonardo Rippel Salgado. Anotação funcional probabilística do transcriptoma da Seringueira (Hevea brasiliensis). Supervisor: Wilson Araújo da Silva Júnior.
- Lilian Figueiredo Moreira. Indução da Diferenciação de Células-Tronco Embrionárias Humanas (hCTE) em Células de Linhagem Endotelial através da Via Wnt. Supervisor: Dimas Tadeu Covas.
- Lucas Coelho Marliére Arruda. Estudo dos mecanismos imunológicos envolvidos na resposta terapêutica de pacientes com esclerose sistêmica ao transplante autólogo de células-tronco. Supervisor: Eduardo Antonio Donadi.
- Lucas Eduardo Botelho de Souza. Avaliação do nicho de células estromais mesenquimais do tecido adiposo como veículo produtor do Fator VIII da coagulação sanguínea humana. Supervisor: Dimas Tadeu Covas.
- Luciana Ribeiro Jarduli. Avaliação da Função Tímica em pacientes com Anemia Falciforme. Supervisor: Kelen Cristina Ribeiro Malmegrim de Farias.
- Luiza Ferreira de Araujo. Estudo do metabolismo energético na progressão do melanoma com base na instabilidade do genoma mitocondrial. Supervisor: Wilson Araújo da Silva Júnior.
- Marcela Cristina de Freitas. Clonagem e expressão do fator VII de coagulação sanguínea em linhagens celulares humanas. Supervisor: Dimas Tadeu Covas.
- Mariana Tereza de Lira Benicio. Análise das vias de sinalização nas células tronco da leucemia mielóide aguda. Supervisor: Eduardo Magalhães Rego.
- Mariana Tomazini Pinto. Avaliação dos fatores de transcrição indutores da transição epitélio-mesenquimal (EMT) na Biologia das Células Endoteliais. Supervisor: Simone Kashima Haddad.

- Mariane Serra Fraguas. Manipulação de vias inibitórias da indução de pluripotência visando o aumento de eficiência no processo de geração de iPSs. Supervisor: Marco Antonio Zago.
- Maristela Delgado Orellana. Caracterização das MSCS derivadas de células-tronco embrionárias HI CD-133+. Supervisor: Dimas Tadeu Covas.
- Mauricio Cristiano Rocha Júnior. Desenvolvimento de uma plataforma molecular in house para o Diagnóstico Confirmatório da infecção pelo HTLV. Supervisor: Simone Kashima Haddad.
- Naira Caroline Godoy Pieri. Espermatogênese xenogênica pós-transplante de células tronco caninas no testículo de camundongos. Supervisor: Daniele dos Santos Martins.
- Natalia Juliana Nardelli Gonçalves. Geração de células tronco pluripotentes caninas através de mecanismos in vivo e in vitro.. Supervisor: Carlos Eduardo Ambrósio.
- Pedro Henrique Padilha. Mutações no gene THPO em pacientes com anemia aplástica. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Pedro Ratto Lisboa Pires. Lipidômica da reprogramação celular. Supervisor: Flávio Vieira Meirelles.
- Rafael Vilar Sampaio. Hidroximetilação na reprogramação nuclear, um modelo bovino. Supervisor: Flávio Vieira Meirelles.
- Rafaela de Barros e Lima Bueno. Estudo farmacogenético das enzimas metabolizadoras de quimioterápicos usados no carcinoma espinocelular de cabeça e pescoço. Supervisor: Wilson Araújo da Silva Júnior.
- Reno Roli de Araujo. Tranferência de ooplasto em equinos Supervisor: Flávio Vieira Meirelles.
- Rodrigo Cesar dos Santos Vida. Shotgun Proteomics em estudos de glioma: obtenção do proteoma e fosfoproteoma de linhagens celulares derivadas de glioblastoma multiforme (GBM) estimuladas por EGF e tratadas com Temozolamida. Supervisor: José César Rosa.
- Rodrigo da Silva Nunes Barreto. Modificações epigenéticas na placenta. Supervisor: Flávio Vieira Meirelles.
- Sarah Cristina Bassi. Análise do inibidor do fator tecidual na coagulopatia da leucemia promielocítica aguda por meio de um modelo animal. Supervisor: Eduardo Magalhães Rego
- Thiago Yukio Kikuchi Oliveira. Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. Supervisor: Wilson Araújo da Silva Júnior.
- Vanessa Cristina de Oliveira. TALENs (Transcription activator- like nucleases) engenharia de sistema de edição para o gene TFAM fator de transcrição A, mitocondrial em modelo bovino. Supervisor: Carlos Eduardo Ambrósio

Post-doctorate

- Alana Maria Cerqueira de Oliveira. Efeitos de estímulos de maturação na expressão gênica de células dendríticas maturadas com IL-18, PGE2 ou IL-18+PGE2. Supervisor: Lewis Joel Greene
- Angel Mauricio Castro Gamero. Efeitos do ácido tetra-O-metil nordihidroguaiarético em células de glioblastoma: um estudo transcriptômico e proteômico. Supervisor: José César Rosa.
- Antonio Roberto Lucena de Araujo. Análise funcional e proteômica das células progenitoras hematopoiéticas do modelo animal de disceratose congênita. Supervisor: Eduardo Magalhães Rego.
- Carolina Hassibe Thome. Estudo da via de sinalização PI3K/AKT em Leucemia Mielóide Aguda utilizando o Monitoramento Seletivo de IONS (SRM). Supervisor: Vitor Marcel Faça.
- Cleidson de Pádua Alves. Fundação Hemocentro de Ribeirão Preto Faculdade de Medicina de Ribeirão Preto. Supervisor: Wilson Araújo da Silva Júnior.
- Fabio Morato Oliveira. Faculdade de Medicina de Ribeirão Preto-USP, Fundação de Amparo à Pesquisa do Estado de São Paulo. Supervisor: Roberto Passetto Falcão.

- Dalila Luciola Zanette. Fundação Hemocentro de Ribeirão Preto Faculdade de Medicina de Ribeirão Preto. Supervisor: Wilson Araújo da Silva Júnior.
- Germano Aguiar Ferreira. Estudo do efeito do alquilfosfolipídio perifosine sobre a composição e a quantidade relativa de proteínas palmitoiladas em frações rafts de células da linhagem Granta-519 de linfoma de células do manto. Supervisor: Lewis Joel Greene.
- Guilherme Augusto Silva dos Santos. Faculdade de Medicina de Ribeirão Preto, Fundação de Amparo à Pesquisa do Estado de São Paulo. Supervisor: Eduardo Magalhães Rego.
- Lilian de Jesus Oliveira. Universidade de São Paulo, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Supervisor: Flávio Vieira Meirelles.
- Luis Ernesto Farinha Arcieri. Estudo das vias de sinalização envolvidas na diferenciação óssea de células tronco embrionárias em um modelo murino da Síndrome de Marfan. Supervisor: Lygia da Veiga Pereira.
- Maximiliano Carlos Dasso. Criação de uma biblioteca Celular da População Brasileira como Ferramenta para Triagem de Drogas de Alto Rendimento. Reprogramação de células sanguíneas humanas Periféricas em células-tronco pluripotentes induzidas. Supervisor: Lygia da Veiga Pereira.
- Priscila Santos Scheucher. Faculdade de Medicina de Ribeirão Preto, Fundação de Amparo à Pesquisa do Estado de São Paulo. Supervisor: Eduardo Magalhães Rego.
- Raquel de Melo Alves Paiva. Faculdade de Medicina de Ribeirao Preto da Universidade de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.
- Roberta Ferreira. Universidade de São Paulo, Fundação de Amparo à Pesquisa do Estado de São Paulo.Supervisor: Flávio Vieira Meirelles.
- Simone Aparecida Siqueira da Fonseca. Instituto de Biociências, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Supervisor: Lygia da Veiga Pereira

Post-Doctorate Junior

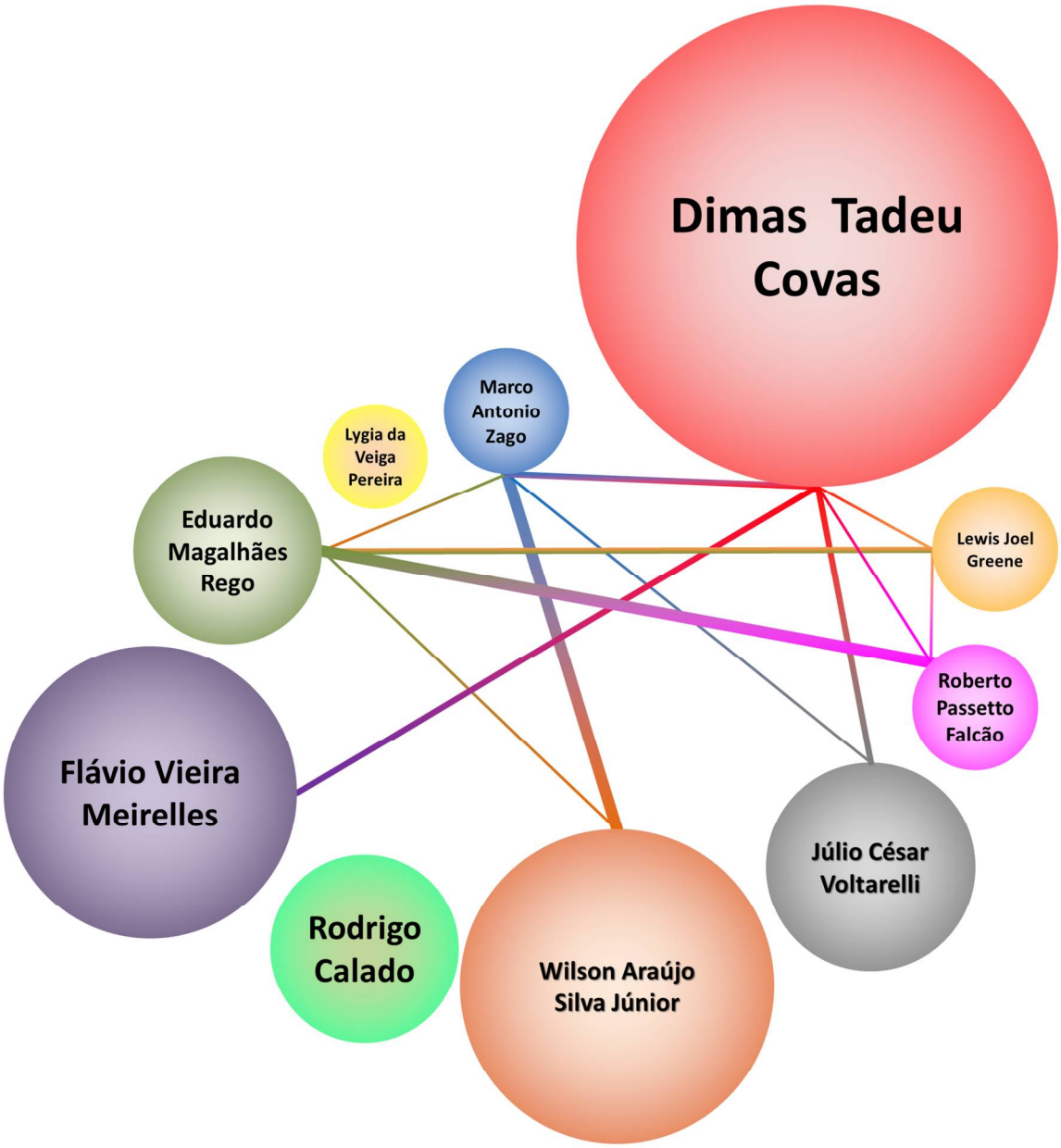
- Aline Fernanda Ferreira. Correção fenotípica da hemofilia A humana utilizando terapia celular baseada em células-tronco pluripotentes induzidas (iPS). Supervisor: Dimas Tadeu Covas.
- Germano Aguiar Ferreira. Efeito do alquilfosfolipídio, perifosine sobre composição e quantidade relativa de proteínas palmitoiladas em frações rafts de células da linhagem Granta-519 de linfoma de células do manto. Supervisor: Lewis Joel Greene.
- Tathiane Maistro Malta Pereira. Análise integrada do transcriptoma e metiloma de linhagens celulares pluripotentes brasileiras. Supervisor: Houtan Noushmehr.

Scientific Initiation

- Allan Gomes da Silva. Papel da Endotelina-1 no modelo experimental de TRALI em camundongos. Supervisor: Eduardo Magalhães Rego.
- André Luiz Silva. Aplicação de ferramentas computacionais de Bioinformática na análise de dados biológicos. Supervisor: Wilson Araújo da Silva Júnior.
- Isabela Gerdes Gyuricza. Ensaio de formação de teratoma em camundongos para avaliação da pluripotência de células-tronco pluripotentes induzidas (iPS). Supervisor: Simone Kashima Haddad.
- Lucas Assis Pereira. Biologia Genética. Instituto de Biociências, Fundação de Amparo à Pesquisa do Estado de São Paulo. Supervisor: Lygia da Veiga Pereira.
- Lucas Clementino. Mutações no gene MPL na anemia aplástica. Faculdade de Medicina de Ribeirao Preto da Universidade de São Paulo. Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.

- Robson Luis Ferraz do Amaral. Adaptação para cultivo em suspensão e meio de cultura livre de soro fetal bovino da linhagem celular 293-FIX-GFP para produção de FIX recombinante. Supervisor: Kamilla Swietch.
- Rodrigo Silva de Sousa. Avaliação do uso do levamisol como imunoestimulante na produção de celulares mononucleares em medula óssea de Chlorocebus aethiops. Supervisor: Klena Sarges Marruaz da Silva.
- Thiago José Ferro. Biologia Genética. Instituto de Biociências. Supervisor: Lygia da Veiga Pereira.
- Thaiene Aizza. Estudos de incorporação metabólica de azido-açucares em glicoconjugados durante o processo de diferenciação de células tronco mesenquimais de rato. Supervisor: José César Rosa.
- Talita Nishikawa. Pesquisa da doença residual mínima em pacientes com leucemia mielóide aguda por meio de RQ-PCR para o gene WT-1. Supervisor: Eduardo Magalhães Rego.
- Vinicius Tomazini. Efeito da quimioterapia sobre o comprimento telomérico do sangue periférico . Supervisor: Rodrigo do Tocantins Calado de Saloma Rodrigues.

VI. SCIENTIFIC PRODUCTION



The diameter of the circumference illustrates the number of publications by PI and the width of lines illustrates the number of joint publications.

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