Nome do Centro:

CTC

CENTRO DE TERAPIA CELULAR

CENTER FOR CELL-BASED THERAPY

Instituição Sede: Fundação Hemocentro de Ribeirão Preto

Instituições Associadas:

Universidade de São Paulo

Faculdade de Medicina de Ribeirão Preto da USP

Faculdade de Zootecnia e Engenharia de Alimentos da USP

Instituto de Biologia da USP

Pesquisador Responsável: *Prof. Dr. Marco Antonio Zago*

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Abstract

We propose a program of basic and clinical research to isolate, culture, and characterize embryonic, somatic, and neoplastic stem cells in order to understand their biology and apply this knowledge to therapy, comprising four research lines: (1) pluripotent stem cells (SC) (embryonic SC and induced pluripotent SC); (2) somatic SC (hematopoietic SC, mesenchymal SC, endothelial SC, and cancer SC); (3) general mechanisms involved in maintaining "stemness"; and (4) clinical SC applications. This research plan is firmly grounded in the analysis of mechanisms and pathways of normal and abnormal stem cells to serve as targets or to help manipulate cells for therapy. The research team includes specialists in cell and molecular biology, embryology, genomics, bioinformatics, veterinary medicine, hematology, and oncology. The technology transfer plan involves cooperation with the private sector and with the government, the education of a new generation of scientists and technicians, and the direct transfer of high quality services to the public. The education plan is focused mainly in a long-term relationship of teachers and middle-school students with the center, researchers, and PhD students. ***(http://ctcusp.org/)***

Summary

The structure that we have built in the last decade serves as the basis for this new proposal, although it is now both expanded and redirected. The Center for Cell Therapy (CTC) (***http://ctcusp.org/)*** initially brought together a group of researchers in cell and molecular biology, genetics, protein chemistry, bioinformatics, and hematology who developed collaboration and synergism in research, and a strong capacity to develop a program in a field that was new at the time, linking it to the well-established cancer research, especially blood malignancies. This association had a strong positive effect on the scientific output. We defined many characteristics of the mesenchymal SC (gene expression profile, widespread occurrence, relationship with pericytes and fibroblasts, molecular mechanisms underlying their immunomodulatory role). We revealed the role of the NF kappa B pathway in primitive progenitors, and a wealth of molecular mechanisms in blood neoplastic cells (expression of CT-antigens, miRNA expression profile, and the role of TGF-beta, MDR1, and telomerase). We were the first Brazilian group to establish new human embryonic SC lines and to publish on iPS cells. On the clinical setting, we demonstrated that high-dose chemotherapy followed by autologous hematopoietic SC transplantation is a feasible way to treat type 1 diabetes mellitus.

The incorporation of experts in telomere biology, in large mammal ooplasmic transfer and cloning, and in embryonic and iPS cells allowed the expansion and redirection of our research. The present proposal is to develop basic and clinical research to isolate, expand, and characterize embryonic, pluripotent, somatic, and neoplastic stem cells (SC) in order to understand their biology and apply this knowledge to therapy, comprising four research lines: (1) pluripotent SC (embryonic SC and induced pluripotent SC); (2) somatic SC (hematopoietic SC, mesenchymal SC, endothelial SC, and cancer SC); (3) general mechanisms involved in maintaining "stemness" (epithelial-mesenchymal, mesenchymal-epithelial, and endothelial-mesenchymal transitions); and (4) clinical SC applications.

One of the reasons for the success of the Center is that it was entirely located in ample and well-equipped facilities on the University of São Paulo (USP) campus in Ribeirão Preto. It is not a "virtual" network, but a real research institute that allows permanent interaction between senior and younger researchers and interdisciplinary collaboration. Most laboratories and facilities are located at the Ribeirão Preto Blood Center, in addition to laboratories, wards, and outpatient clinics at the University Hospital at walking distance from the Blood Center, where experts work on hematology, immunology, protein chemistry, genetics, cell and molecular biology, virology, and bioinformatics. This is complemented by supporting facilities for animal care, teaching, the science education program, and management support (clerical, documentation, procurement, and accounting).

This new proposal includes two additional laboratories off campus. One is located at the School of Animal Sciences and Food Engineering, at the USP campus in Pirassununga (100 km away from Ribeirão Preto), where scientists working on ooplasmic transfer and mammal cloning are based on. In the other laboratory, scientists are experts on embryonic stem (ES) cell derivation and ES and iPS cell biology, and it is located at the Institute of Biology, at the USP campus in the city of São Paulo (300 km away from Ribeirão Preto).

These three groups have interacted in the last five years and constitute the National Institute for Stem Cell Research and Cell Therapy sponsored by the Ministry of Science and Technology since 2008.

The other strength of our Center is the capacity to ***train human resources***. Since the creation of the Center, we have qualified 60 MSc and 68 PhD students and 31 postdoctoral fellows. To strengthen this effort, we have organized a new graduate program (with two tracks, one academic and another professional) that will begin to enroll the first candidates in the fall of 2012.

We propose a three-fold approach for technology transfer: (a) cooperation with the private sector (new therapies, recombinant proteins, and diagnostic methods); (b) cooperation with the government; and (c) education of a new generation of scientists and high level technicians, fostering entrepreneurship, innovation, and generation of spin-off companies. An integral part of the technology transfer is the contribution of the Center for Cell Therapy to the Ribeirão Preto Blood Center by improving and diversifying services offered to the community. It is the only Blood Center accredited and certified by ISO9001 and the American Association of Blood Banks (AABB) in the State of São Paulo, with more than 160,000 registered donors, 8,000 donations/month, and 12,000 clinic visits of patients with blood disorders, and more than 150,000 blood components transfused yearly. The Center has developed a nucleic acid testing for HIV and HCV that is now applied routinely to 10,000 blood donations/month, and this technology has been transferred to the State Department of Health. The Center's experience led to the establishment of a Cryobiology Laboratory, Umbilical Cord Blood Bank, and a GMP Tissue Culture facility, which allows, for instance, the culture of mesenchymal SC for clinical trials. Within this model, our targets for the immediate future are: (1) **the generation of an iPS cell library based on the Brazilian ethnically diverse population, (2) to scale up processes for cell therapy, (3) use of stem cells as biofactories, and (4) the development of molecular diagnostics (telomere length in blood cells for telomere diseases and infections relevant to cell therapy: mycoplasma, CMV, human parvovirus B19, and HTLV**).

Thus far, more than 2,000 middle-school students and 150 teachers have participated in the science education program that will now be reinforced, keeping its basic feature of focusing on a long-term relationship between the center and middle-school students and teachers.

The two university institutions involved in the creation of the Center will provide significant ***counterpart to match FAPESP's support***. **USP** will provide two new full-time tenure-track faculty positions, three full-time technicians, and one administrative manager to the Center. Innovation and technology transfer is supported by USP Innovation Agency that comprises 35 dedicated personnel including innovation agents and lawyers. USP will continue to provide 1,500 m^2 of lab and ward space (paying for basic expenses). The **Blood Center** (and *Hemocentro Foundation*, the Blood Center supporting civil organization) will continue to provide 5,200 m^2 in laboratory space (paying also for basic expenses, such as utilities without overhead charges) for research and education, clerical, documentation, and management support, paying also for 21 technical personnel actively engaged in research, in addition to paying salaries for six full-time staff scientists, four of them with PhD degree.

For more information, go to http://ctcusp.org

Justification: Why to create a Center for Cell Therapy?

We propose a program for basic and clinical research to isolate, culture, and characterize embryonic, somatic, and neoplastic stem cells in order to understand their biology and apply this knowledge to therapy. We will focus on questions that derive from both our past experience and the present bottlenecks in the field, moving to a more "applied" research in normal and abnormal stem cells, without neglecting our strong basis in cell biology. A great deal of effort in stem cell research has been lost in trying to bring therapeutic tests prematurely to the bedside, based on untested, unproved, or unclear premises. Thus, our research program is firmly grounded in the analysis of mechanisms and pathways of normal and abnormal stem cells to serve as targets or to help manipulate cells for therapy.

The research project comprises four lines: *pluripotent stem cells*, which include both embryonic and induced pluripotent stem cells (SC); *somatic stem cells*, such as hematopoietic SC, mesenchymal SC, endothelial SC, and cancer SC; *general mechanisms* involved in maintaining "stemness", focusing on the epithelial-mesenchymal, mesenchymal-epithelial, and endothelial-mesenchymal transitions; and *clinical stem cell applications*. These are complex and inter-related issues that can only be approached by an inter-disciplinary team working together in long-term cooperation.

The first strong argument for the existence of this center is that it consolidates the close relationship and collaboration between basic and clinical researchers already established, fostering a research program that is truly "bench-to-bedside" and the reverse. There is a balanced proportion of clinicians, who attend in wards and outpatient clinics, working together in the same laboratories with cell and molecular biologists, biochemists, and geneticists; there are many examples of MSc and PhD theses of basic scientists who were supervised by clinicians, as well as papers authored jointly. The second relevant point of this project is that it brings together research in ***normal*** and ***abnormal*** stem cells. The research proposed for the bone marrow failure syndromes is an example of an approach largely used for normal stem cell research (*generation of iPS cells*) applied to the investigation of basic mechanisms of a group of diseases (*aplastic anemia, Fanconi anemia, dyskeratosis congenita*) in which the hematopoietic stem cell fails; at the same time, the question of the integrity and dynamics of telomeres, which are relevant in these diseases, as demonstrated recently by one of us,[1,2] is essentially involved in the maintenance of "stemness" and SC senescence and differentiation, as well as a key player in genomic instability and leukemogenesis, as one of us has recently demonstrated.[3]

Another example of the need to link the study of normal and cancer stem cells, and basic and clinical research is the project proposed for acute promyelocytic leukemia (APL) as a disease model for cancer studies. Based on the knowledge derived from the identification of murine hematopoietic stem cells, we have isolated and characterized APL stem cells in the transgenic mouse model for APL that contains the hCG-PML/RARA fusion gene. We now propose to apply this knowledge to isolate and characterize stem cells of human APL samples by xenotransplants into NSG mice of candidate APL cell populations. The

[1] Calado, R.T. and Young, N.S. N Eng J Med. 2009; 361(24):2353-65.
[2] Calado R.T. *et al*. Leukemia. 2011; doi: 10.1038/leu.2011.272.
[3] Int. Stem Cell Initiative. Nat Biotechnol. 2011; 29(12):1132-44.

intensive basic research on APL biology and creation of the International Consortium on APL (IC-APL) led by our Center resulted in obvious benefit to patients; one-year overall survival for patients with APL in Brazil dramatically increased from below 50% to above 75% after the IC-APL was implemented.[4] From the bedside back to the bench, the IC-APL enabled the collection of more than 400 APL samples and we now propose to use these samples to evaluate, for instance, changes in the p73 pathway as a modulator of disease severity and the molecular mechanisms responsible for the coagulopathy associated with APL.

Most of the research proposed by the Center can only be carried out in the long run. This can be illustrated by the use of mesenchymal SC for treatment or prophylaxis of immunologic diseases or reactions (*graft-versus-host disease, type 1 diabetes mellitus*); the results from bench work isolating and expanding mesenchymal SC for clinical use need to be carried out in a GMP Tissue Culture facility, then tested in a clinical setting, the material obtained from the patients need again to be reviewed in the laboratory, results guide both new experimental work and changes in the protocol to prepare the cells (in this case, for instance, mesenchymal SC priming with interferon to prevent or treat immunologic reactions), and finally going back to the clinical tests. This complete cycle takes years instead of months. Likewise, genome sequencing of neoplastic cells to identify relevant abnormalities, as for instance proposed here for myelodysplastic syndromes, requires selection of clinical samples, followed by sequencing and bioinformatics work, and again access to the patients and patient follow-up to extract meaningful results to correlate basic with clinical findings. This research cycle needs long lasting basic-clinical cooperation.

Also, the center is necessary to give long-term continuity to an in-depth approach to a particular issue, instead of selection of unrelated publications. Thus, the clinical applications of the present project reflect the ***past history*** of the Center, focused mainly on the basic biology of mesenchymal stem cells. On the other hand, the basic component of the ***present project*** has a much broader basis, and encompasses ES and iPS cells, tissue specific progenitors, and abnormal stem cells; only if this group of basic researchers and clinicians are kept together, with access to the facilities to separate and expand cells in GMP conditions and to clinical wards to test them in patients, the results of this basic research can be brought to the bedside.

One of the bottlenecks for the development of Brazil and the State of São Paulo is the education of human resources in quantity and quality. We have dedicated a lot of effort for these purposes in the past, and as a consequence we have organized a graduate program that begins to enroll its first students this fall. For the success of this graduate program, the core of this group will have to be kept together with access to laboratories and funding in order to reach our goal, which is to double the numbers of MSc, PhD, and postdoctoral fellows trained. The counterpart offered by USP includes two new tenure-track faculty positions and three high level technicians to the group, which will strengthen this educational component of project.

The technology transfer project also takes into account that the development of biotechnology and health-focused products and process usually demands long-term initiatives, which are compatible with the time

[4] Rego, E.M. *et al.* Blood. 2009: 114(suppl):6.

frame proposed for the Center. The educational project is firmly based on the success of the previous decade; it depends both on a long-term commitment of a dedicated team, which takes time to be put together, and infrastructure compatible with the proposed program.

Finally, this kind of long-term project involves international and national collaboration, including the exchange of researchers, training of young researchers, organization of workshops and meetings, but especially by carrying on complementary tasks as part of a research project (the table below lists active collaborations with research groups from Brazil or abroad; *see also http://ctcusp.org*). For instance, in the collaboration with the French group we are responsible for the DNA analysis of over 900 samples from patients collected in four European countries.

Project	***Foreign coordinator***	***Financial support***
Overexpression of EMT genes in endothelial cells during endothelial mesenchymal transition *(DT Covas)*	Robert Weinberg, MIT, USA	FAPESP (Brazil, 10/51962-9) & MIT
Telomere dysfunction as a mechanism of human stem cell disease; induced pluripotent stem cells to model telomere regulation and cell therapy *(RT Calado)*	Neal Young, NIH, Bethesda, USA	NIH Center for Regenerative Medicine Pilot Projects for Clinical Applications (2011-2012)
The role of *Dido* locus in the differentiation of stem cells and in myeloid neoplasias *(DT Covas and MA Zago)*	Carlos Martínez, National Center of Biotechnology, Madrid, Spain	CNPq (Brazil, 560884/2010-9) & MICINN (Spain)
Prognostic association of genetic polymorphisms of drug metabolism and innate immune response on umbilical cord blood transplantation outcomes *(MA Zago)*	Eliane Gluckman, Eurocord, Hôpital Saint Louis, Paris	
Generation of iPS cells from patients with Fanconi anemia belonging to different complementation groups, to study the DNA-repair mechanisms in the hematopoietic stem cells *(RT Calado and DT Covas)*	Ricardo Pasquini, UFPR, Curitiba, Brazil	
Impact of RNA processing and regulation in human normal and cancer stem cell differentiation *(WA Silva Jr)*	Gene Yeo, Cellular & Molecular Medicine, UCSD, USA	
Molecular Genetics of Prostate Cancer: Role of Stem Cells *(WA Silva Jr)*	Gerhard A Coetzee, Preventive Med, Norris Cancer Center, USC, USA	
Generation of a hiPSC library of the Brazilian population *(LV Pereira)*	Jeanne Loring, Scripps Institute, La Jolla, California, USA	BNDES (Brazil) and FINEP (Brazil)
Introducing embryonic and somatic mitochondria on parthenogenesis development in vitro *(FV Meirelles)*	Joanna Poulton, Univ. Oxford, UK Lawrence Smith, Univ. Montreal, Quebec, Canada	FAPESP (Brazil, 2010/13384-3) & INCT (Brazil)
iPS reprogramming prior to cloning *(FV Meirelles)*	Konrad Hochedingler, Harvard University, Boston, MA	FAPESP (Brazil, 2011/08376-4)

Research Project

In the last decade, our group has established a strong collaboration and synergism in research through the Center of Cell Therapy (CTC). As a result, there was significant improvement in the quality and impact of our scientific work, and more intensive international networking was established. Some important achievements during the previous period paved the way for this new proposal focused on embryonic, pluripotent, somatic, and neoplastic stem cells and include "applied" research in normal and abnormal stem cells, without neglecting our strong basis in cell biology. Some of the pivotal results of our previous proposition include one of the first characterizations of mesenchymal stem cells (gene expression profile,[5,6] its broad occurrence in human tissues,[2,7,8] relationship with pericytes and fibroblasts,[9] molecular mechanisms underlying immunomodulatory role[10,11]). In addition, we have demonstrated the role of the NFκB pathway in primitive progenitors[12] and TGF-beta signaling[13] and mitochondrial function[14] in acute myeloid leukemia cells. We have characterized crucial molecular mechanisms in hematological malignancies (expression of cancer-testis antigens,[15,16] miRNA expression profile,[17] multidrug resistance transporters,[18,19] and telomerase and telomere dysfunction[20,21,22,23]). We were the first Brazilian group establish new lines of human ES cells[24] and to to publish on iPS cells.[25] In the clinical areana, we demonstrated that high dose chemotherapy followed by autologous hematopoietic stem cell transplant is a feasible option to treat juvenile diabetes mellitus[26,27].

In the present proposal, the CTC will develop basic and clinical research to isolate, expand, and characterize embryonic, pluripotent, somatic, and neoplastic stem cells (SC) in order to understand their biology and apply this knowledge to therapy, comprising four research lines: 1. Pluripotent SC (embryonic SC and induced pluripotent SC); 2. Somatic SC (hematopoietic SC, mesenchymal SC, endothelial SC, and cancer SC); 3. General mechanisms involved in maintaining "stemness" (EMT, MET, EndMT); and 4. Clinical SC applications.

1. Pluripotent Stem Cells

[5] Silva Jr , W.A. *et al.* Stem Cells. 2003; 21(6):661-9.
[6] Panepucci, R.A. *et al.* Stem Cells. 2004; 22(7):1263-78.
[7] Covas, D.T. *et al.* Exp Cell Res. 2005; 309(2):340-4.
[8] Covas, D.T. *et al*. Exp Hematol. 2008; 36(5):642-54.
[9] Covas, D.T. *et al.* Exp Hematol. 2008; 36(5):642-54.
[10] Saldanha-Araújo, F. *et al.* J Cell Mol Med. 2011.
[11] Saldanha-Araújo, F. *et al.* Stem Cell Res. 2011; 7(1):66-74.
[12] Panepucci, R.A. *et al.* Stem Cells Dev. 2010; 19(3):321-32.
[13] de Figueiredo-Pontes, L.L. *et al.* PLoS One. 2011;6(10):e26713.
[14] dos Santos, G.A. *et al.* Leukemia. 2011; doi: 10.1038/leu.2011.216.
[15] Proto-Siqueira, R. *et al.* Leuk Res. 2006; 30(11):1333-9.
[16] Figueiredo, D.L. *et al.* Head Neck. 2006; 28(7):614-9.
[17] Zanette, D.L. *et al.* Braz J Med Biol Res. 2007; 40(11):1435-40
[18] Figueiredo-Pontes, L.L. *et al.* Cytometry B Clin Cytom. 2008; 74(3):163-8.
[19] Rocha, V. *et al.* Leukemia. 2009; 23(3):545-56.
[20] Scheinberg, P. *et al.* JAMA. 2010; 304(12):1358-64
[21] Calado, R.T. *et al.* Leukemia. 2011. doi: 10.1038/leu.2011.272.
[22] Calado, R.T. *et al.* Blood. 2009; 114(11):2236-43.
[23] Calado, R.T. *et al.* Proc Natl Acad Sci U S A. 2009; 106(4):1187-92.
[24] Fraga, A.M. *et al.* Cell Transplant. 2011;20(3):431-40.
[25] Picanço-Castro, V. *et al.* Stem Cells Dev. 2011;20(1):169-80.
[26] Couri, C.E. *et al.* JAMA. 2009; 301(15):1573-9.
[27] Voltarelli, J.C. *et al.* JAMA. 2007; 297(14):1568-76.

MicroRNAs in stem cell plutipotency, self-renewal, and differentiation

Embryonic stem cells (ESC) are able to self-renew while remaining pluripotent.[28] These properties make these cells potential tools in regenerative medicine, but its clinical use depends on the existence of immunogeneticaly compatible cells. Reprogramming somatic cells from a patient into induced pluripotent stem cells (iPS) may be a way to circumvent this issue, but still involves the genetic manipulation of cells for the introduction of pluripotency-related factors (OCT4, SOX2, NANOG, c-MYC, or LIN28).[29,30]

Similarly to the pluripotency-related transcription factors, the ectopic expression of microRNAs preferentially expressed in pluripotent SC may improve the reprograming efficiency of classical factors, or completely substitutes them.[31] Direct transfection of mature double-stranded synthetic microRNAs for mir-200c, mir-302 and mir-369 allows reprograming of mouse and human cells, eliminating the need for genetic manipulation.[32] Although not as efficient as the reprograming achieved by the ectopic expression of the miR302/367 cluster, this approach is simpler, what may turn it amenable for potential clinical applications.

These observations highlight the importance of further studies on microRNAs to induce and reprograming, self-renewal, and pluripotency. In fact, microRNAs studied so far are only part of the entire specrtrum in pluripotent embryonic or iPS cells and their differentiated counterparts.

In a murine microRNA library screening, several microRNAs capable of promoting reprograming were identified.[33] This approach takes advantage of High-Content Screening (HCS), bearing an enormous potential to improve the basic understanding of stem cell biology. HCS combines automated microscopy of multi-well plates and computational image processing methods, allowing the evaluation of diverse cellular and molecular processes simultaneously and in a limited number of cells submitted to distinct treatments. Importantly, HCS instruments, such as the one been requested in the present proposal (Image Xpress Micro, Molecular Devices) can deliver functional and morphometric information of individual cells within heterogeneous populations and across different time points, allowing stem cell subpopulations to be dynamically evaluated.

In the present proposal, we plan to investigate the role of different microRNAs in the regulation of pluripotency, differentiation, and in the modulation of several other biological parameters, such as proliferation, cell cycle, and apoptosis. For this, the pluripotent cell line NTera-2, derived from embryonic carcinoma will be used to establish (using lentivirus constructs) different cell lines containing, each one, a GFP reporter gene with the promoter regions under the control of different transcription factors, including Oct4 and Nanog as reporters associated with self-renewing and pluripotency; and the Retinoic acid receptor-RAR, associated with cell differentiation.

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[28] Thomsom, J.A. Science. 1998;282:1145-7.
[29] Takahashi, K. *et al.* Cell. 2007; 131, 861-72.
[30] Yu, J. *et al.* Science. 2007;318, 1917-1920.
[31] Anokye-Danso, F. *et al.* Cell Stem Cell. 2011; 8, 376-88.
[32] Miyoshi, N. *et al.* Cell Stem Cell. 2011;8, 633-8.
[33] Pfaff, N. *et al.* EMBO Rep. 2011;12:1153-9.

Additionally, the modulation of other pathways with known roles in stem cell self-renewal, pluripotency and differentiation will also be evaluated, through cell lines with a GFP reporter under the control of TCF/LEF, to identify modulators of Wnt/B-catenin signaling, RBP-jK, to identify modulators of Notch signaling, and NF-kB. These reporter cell lines will be used in HCS, based on the transfection of a human library of synthetic microRNAs (pre-MIRs) or the corresponding inhibitory anti-MIRs. For each cell line used in independent screens, the expression of the GFP reporter protein will be evaluated in parallel to other biological parameters, such as cell/colony morphology, proliferation, cell cycle and apoptosis. For this, specific assays will be developed, based on different fluorescent markers.

Once selected in the screening, the potential mRNA targets of these microRNAs will be identified through the combined use of microarrays and adequate bioinformatic tools and databases. The approach (currently used by us) will be based on the fact that microRNAs act by complementary binding to selected sites in several target mRNAs, not only blocking their translation, but predominantly leading to their degradation. Thus, by identifying mRNA transcripts down-regulated in the N-Tera2 cell line transfected with a specific pre-miR (as identified by comparison to cells transfected with nonspecific pre-miR controls) and that are simultaneously predicted as targets (in the microRNA-target database used), we expect to identify potential microRNA-mRNA signaling modules. The same but inverse approach will be carried out with the transcriptomes derived from cells transfected with the anti-miR molecules and the corresponding controls. The identified microRNA-mRNA modules will be further studied by independent experimental approaches in order to specifically demonstrate their roles.

With the results obtained from this microRNA HCS, we hope to significantly contribute to the understanding of the regulatory mechanisms involved in the molecular control of pluripotency, self-renewal and differentiation of pluripotrent stem cells. Finally, the gained knowledge may potentially lead to the establishment of new experimental protocols to induce pluripotency in somatic cells or to restrict or promote cell differentiation. *(PIs, Zago, Silva, and Covas)*

Imprinting patterns during cell reprogramming

In the last years, we have sucessfully produced autologous pluripotent cells by reprogramming methods such as stem cell nuclear transfer (SCNT)[34] and iPS[35] technologies. These methods are viewed as effective strategies to derive autologous pluripotent cells from somatic cells and are a promising therapeutic approach in regenerative medicine. However, both reprograming methods have low efficiency rates.[36] One explanation for this issue is the faulty epigenetic reprogramming that leads to an incomplete restoration of undifferenciated cell status. One of the most striking epigenetic features affected by reprogramming technologies is the genomic imprinting, since its correct reprogramming strogly correlates with full development potential of ES cells[37]. Hence, using animal models harboring interspecific genetic variations in order to track allele-specific methylation and expression and previously developed by our

[34] Miranda, MS. et al. Cloning and Stem Cells. 2009, 11:1-9.
[35] Picanço-Castro, V. et al. Stem Cells Dev. 2011, 20:169-180.
[36] Meirelles, FV. et al. Reprod. Fertility and Dev. 2010, 22:88-95.
[37] Stadtfeld M and Hochedlinger K., Genes Dev. 2010, 24(20):2239-63.

group,[38] we will develop studies to evaluate the impact of reprogramming methods on imprinting gene epigenetic control using wide genome evaluation technics. These studies will involve the production of pluripotent cells using SCNT, iPS, or the combination of both techniques in bovine and eventually equine or canine models, which, in association with chimeric experiments, will be used for the identification of epigenetic signatures of suitable reprogramming. *(PIs, Meirelles, Pereira, and Silva)*

Differentiation into blood

Transfusion medicine depends on volunteer donation of blood, but blood supply is frequenlty not sufficient to come up with patients' demand. Additionally, transfusion-related complications, such as transmission of blood-borne pathogens or alloimmunization, are unpredictable adverse events.

Vascular endothelial and hematopoietic cells arise *in vitro* from a common precursor cell, the hemangioblast, and that endothelial cells establish an instructive vascular niche that is necessary for normal hematopoietic regeneration. In this project, we aim to elucidate differentiation mechanisms of endothelial cells and HSCs from hESCs. Additionally, establishing a protocol to induce differentiation from either hESCs or iPSCs into hematopoietic progenitor and endothelial cells might provide an approach to recapitulate hematopoiesis during human ontogeny.

However, central questions about the molecular mechanisms that regulate hematopoietic and endothelial cell differentiation are unknown. We propose to generate erythrocyte, megakaryocyte and endothelial cells from hESCs and iPSCs based on our experience on co-culture with inactivated murine cells and embryoid bodies. According to "knockout" studies in mice that demonstrate the role of RUNX1 and FLI-1 factors during megakaryopoiesis, we propose to overexpress RUNX-1 and FLI-1 genes to establish an immortalized megakaryocyte cell line (MKCL). Our hypothesis is that the establishment of immortalized MKCL through gene manipulation could provide a platelet production system for clinical application.

Regarding the hematopoietic and endothelial differentiation from hESC, HOX genes are evolutionarily conserved genes and play important role in cell identity during embryonic development. Based on previous studies demonstrating that HOX expression is regulated by long noncoding RNAs, that overexpression of some specific HOX genes enables the detection of transplantable HSC, and that HOX genes regulate various vascular processes, we propose to study these gene expression programs during hematopoietic and endothelial differentiation. We aim to genetically modify hESC with GFP under hemangioblast and angioblast promoters. Then, GFP+ cells will be FACS-sorted and induced to differentiate into erythrocyte and endothelial cells.

The results of these studies will contribute to elucidate the role of long non coding RNAs in the regulation of conserved genes during hematopoietic and endothelial differentiation programs which have emerged as new paradigms in developmental biology and hematology. *(PIs, Covas, Pereira, and Zago)*

Hematopoietic stem cell failure syndromes

[38] Suzuki Jr, J. et al., Biol. of Reprod. 2011, 84:947-956.

Aplastic anemia (AA) is a rare hematological disorder in which the hematopoietic stem cell (HSC) fails to produce adequate numbers of mature peripheral blood cells, clinically translating in low peripheral blood cell counts and an empty bone marrow. AA can be acquired or congenital. Most acquired cases are the result of an immune process driven by oligoclonal cytotoxic T-cells toward HSCs, whereas inherited AA may be caused by defects in genes associated with DNA repair, as in Fanconi anemia, telomere maintenance, as in dyskeratosis congenita, or mitotic spindle stability, as in Shwachman-Diamond syndrome. Regardless whether acquired or constitutional, there is a profound reduction in the number of HSCs in the bone marrow. The main goal of treatment is to restore functional HSCs in order to recover hematopoiesis. In constitutional and acquired cases, blood counts may be restored by HSC transplantation from a matched sibling donor, but only a minority of patients have a compatible donor and alternative HSC sources are sought. Apart from transplant, we have shown that in some patients with congenital AA, androgens up-regulate telomerase expression in HSCs, alleviating marrow failure.[39]

The major hurdle in the laboratory investigation of these diseases is the paucity of hematopoietic and other affected primary cells at clinical presentation. The use of ectopic expression of SC-specific transcription factors enabled the reprogramming of murine post-natal somatic cells to induced pluripotent stem (iPS) cells, and human iPS cells also have been described. Human iPS cells have potential use in medicine and production of tissue-specific adult SCs; iPS cells potentially may work as a source of autologous HSCs in mutiple diseases, including AA, the prototypical disease of HSC failure. In the laboratory, iPS cells may serve to model disease and investigate cellular and molecular pathways. The difficulties today, though, is to induce safe and efficacious differentiation of iPS cells into tissue-specific SC, as well as understand and control cell phenotype during reprogramming.

Dyskeratosis congenita. We and others have demonstrated that mutations in telomerase-related genes cause telomere shortening, clinically translating into marrow failure, idiopathic pulmonary fibrosis, and liver disease.[40] Our aims are to understand the mechanisms of telomere maintanence during human iPS derivation and identify the pathways responsible for telomerase activation. In collaboration with Dr Neal Young from the U.S. National Institutes of Health (NIH), in Bethesda, Maryland and using a set of four transcription factors (Oct-4, Sox2, Klf4 and c-Myc) in a bicistronic lentiviral vector, we will generate iPS from dermal fibroblasts of patients with mutations in several telomerase genes (*TERT*, *TERC*, *DKC1*, *TINF2*). These cells will serve as a powerful tool to understand the mechanisms of telomere diseases as well as telomere and telomerase bioogy during reprogramming to a pluripotency state. First, we will determine the specific contributions of telomerase and telomerase-independent mechanisms to telomere elongation during reprogramming of mutant and healthy human cells. We also will investigate the telomere dynamics during reprogramming of telomerase-mutant cells. Preliminary results indicate that telomeres are elongated but at a reduced rate as compared to control cells. If telomeres are effectively elongated during reprogramming of telomerase-haploinsufficient cells, using gene expression analysis, iPS cells will serve to identify the specific cellular pathways responsible for net telomere gain. In addition,

[39] Calado, R.T. *et al.* Blood. 2009; 114(11):2236-43.
[40] Calado, R.T. & Young, N.S. NEJM. 2009; 261(24): 2353-65.

these cells may help us identify cellular pathways that are modulated by telomerase deficiency or telomere erosion. In addition, our prelimimary results suggest that, apparently, chromosomal aberrations acquired during reprogramming are associated with abnormal telomere elongation; these findings will enable us to further identify genetic, epigenetic, and environmental factors modulating telomere elongation as well as clinical phenotype. The identification of these pathways may allow us to use them as therapeutic targets in HSCs.

We also will address the HSC differentiation capacity of telomerase-mutant iPS cells using the embryoid body-based hematopoietic differentiation with cytokines and BMP-4. In addition, the function properties of derived HSCs will be investigated both *in vitro* in colony formation assays and flow cytometry and *in vivo* by transplant into NSG mice. These results will help us to determine the potential pre-clinical and clinical utility of these cells for telomere diseases.

Direct conversion of dermal fibroblasts into HSCs also has been recently reported. However, telomere dynamics during this reprogramming is unknown. Using the Oct-4 transcription factor in a lentiviral vector, we will induce the conversion of fibroblasts of healthy individuals into HSCs and observe the dynamics in telomere length and telomerase expression. These findings will contribute to the identification of molecular differences between iPS cells and direct conversion. If telomerase also is expressed during this process, we will directly convert fibroblasts of patients with telomerase mutations to observe the fate of their telomeres and the functional characteristics of the HSCs.

Fanconi anemia (FA). Although rare, FA is the most common type of inherited marrow failure syndrome and is caused by mutations in genes associated with DNA repair. Mutations in at least 13 genes were involved in FA. Mutations in genes of the FA complex cause a reduced DNA-damage response, leading to marrow failure and cancer development. However, it is still unclear how defects in DNA repair preferentially affects HSCs leading to exhaustion of the HSC pool. iPS generation will enable the production of cells to study pathways affected by FA gene lesions. Belmonte's group has sucessfully generated iPS cells from genetically corrected FA dermal fibroblasts. In collaboration with Professor Ricardo Pasquini at the Federal University of Paraná (UFPR), in Curitiba, Brazil, our aim is to derive iPS cells using the same approach as above from patients with FA from a variety of complementation groups without previous genetic correction. Although Belmonte's group reported that iPS-like cells derived from non-corrected fibroblasts failed to survive over three passages, this has not been systematically investigated. If non-corrected FA iPS cells are unable to survive, it is important to address what cellular and molecular mechanisms are activating cell senescence or death. On the other hand, it is possible that only a few complementation groups are unable to maintain "stemness" over passages.

Acquired AA. As acquired AA is the prototypical disease of of HSC failure, we also will generate iPS cells from dermal fibroblasts of patients with acquired AA and determine its ability to differentiate into HSC using the embryoid body-based method.

Together, we will produce significant knowledge on the molecular biology of HSC failure syndromes and generate iPS cells that can potentially be used in the clinic. As mentioned above, the therapeutic goal in AA is to restore the HSC function to produce blood cells. Derivation of autologous iPS cells to produce

HSCs or direct conversion of fibroblasts into HSCs may be an attractive threapeutic tool. *(PIs, Calado, Falcão, Pereira, and Zago)*

Hemophilia A

Hemophilia A is a blood clotting disorder caused by deficiency of the factor VIII. The treatment for hemophilia consists in the replacement of the deficient antihemophilic factor. However, this therapy raises several concerns, such as high cost, short half-life of coagulation factors, and the development of inhibitors against transfused factors, opening the possibility of new therapies. In this context, gene therapy combined with cell therapy is emerging as a promising alternative for the treatment of hemophilias.

Our group has extensive experience on HA research, developing FVIII-expressing vectors for replacement therapy. In this proposal, our goal is to combine gene and cell therapies for the development of new treaments for HA in animal models. We plan to genetically correct the deficiency of factor VIII by the insertion and integration of the wild factor VIII into the genome of induced pluripotent stem cells (iPSCs) generated from fibroblasts and/or keratinocytes of HA patients. iPSCs will be differentiated in hepatocytes and endothelial cells producing a functional factor VIII. Then, hemophilia-corrected cells will be applied in pre-clinical models using imunodeficient HA mice and its therapeutic effect will be evaluated. *(PIs, Covas and Meirelles)*

Gaucher disease and Parkinsonism

Parkinson's disease (PD) is one of the most common neurodegenerative disorders affecting ~1% of the population over 50 years and is characterized by tremor, rigidity, bradykinesia, and postural instability, associated with progressive neuronal loss in the substantia nigra and other brain structures. A strong association between PD and mutations in the *GBA* gene—which encondes the β-glucocerebrosidase enzyme—has been recently demonstrated by a multicenter study including our group, making the *GBA* locus the most common genetic risk factor for PD.[41] However, hmozygous GBA mutations are etiologic in Gaucher disease (GD), the most prevalent lisossomal storage disorder in humans and characterized by the accumulation of glucosylceramide primarily within cells of mononuclear phagocyte origin. Interestingly, in some forms of GD (types II and III) there is involvement of the central nervous system leading to neurologic manifestations.

Some models suggest that glucosylceramide and glucosylsphingosine accumulation in lysosomes may interfere with the normal autophagic process leading to axonal dystrophy and degeneration. According to these models, abnormal autophagy triggered by glucosylceramide and glucosylsphingosine storage leads to degenerative axonal process, the most important pathogenesis of neuronopathic GD.

The association between *GBA* mutations and PD revealed potential novel mechanisms of disease involving the involving the β-glucocerebrosidase enzyme, including gain-of-function in protein aggregation, lysosomal dysfunction, or loss-of-function related to fluctuations in ceramide.

In the present prososal, we aim to use the technology of cell reprogramming to create an experimental system to model and investigate the contribution of *GBA* mutations to PD. We worked on the genetic

[41] Sidransky E. NEJM. 2009;361(17):1651-61.

characterization of GD patients in the Brazilian population for 10 years, having analyzed mutations in the *GBA* gene in more than 1000 patients.[4243] In collaboration with Dr. Dr. Egberto Reis Barbosa, Department of Neurology, University of São Paulo, we will establish iPS cell lines from patients with (1) GD types 1, 2, and 3; (2) PD with heterozygous *GBA* mutations; (3) PD with GD; (4) PD without *GBA* mutatoins; (5) heterozygous *GBA* mutations without PD, and (6) healthy controls. Dopaminergic neuronal cell will be derived from these cell lines, and subjected to comparative analysis by gene expression profiling in order to identify molecules and pathways involved in PD associated with *GBA* mutations. In addition, the iPS cells generated from patients with GD will be used to understand the mechanisms involved in the development of neuropathy in types 2 and 3 GD. In collaboration with Dr. Stevens Rehen from the Federal University of Rio de Janeiro (UFRJ), we will induce neuron differentiation to evaluate anc compare the neurogenesis capacities and dopaminergic neuron functions among the iPS cell types with specific genetic lesions.

The findings from these studies and provide practical information on cellular pathways involved in the pathogenesis of PD, more specifically on how β-glucocerebrosidase deficiency may lead to axonal degeneration. If these pathways are successfully identified, the will be potential therapeutic targets for testing also in iPS-derived neurons from patients with specific mutations. *(PIs, Pereira and Covas)*

Diseases of mitochondrial DNA

Mutations in the mitochondrial DNA (mtDNA) are etiologic in human diseasess that affects near 1/4,000 subjects.[44] Nonetheless, due to the unique inheritance pattern of pathogenic molecules, their transmission is not preventable. The number of relevant therapeutic approaches aiming the mitochondira are limited by our lack of basic knowledge on mitochondrial biology.[45] Currently, only nuclear transfer (NT) has held promise to be used as a preventive strategy.[46] Our group had significantly contributed to the development of mammalian models in order to understand the mtDNA genetic bottleneck.[47] We have recently developed a method that enables the removal of as much as 90% of oocyte mtDNA[48]. By further applying NT to this method, we showed that it is feasible to introduce foreign organelles of embryonic or somatic,[49] or of intra or interspecific origin.[50]

Using the knowledge accumulated in the last years, we will use murine and bovine models for mtDNA silent mutations. NT will be applied to produce embryonic SCs of homologous or heterologous origin. Analog studies will be conducted to produce iPS cells also harboring mutated mitotypes. Cells harboring different sources of mtDNA will be re-introduced into animals by chimera production or by adult administration throughout different methods. Comparisons of the different methods will allow us to

[42] Rozenberg, R. *et al.* Braz J Med Biol Res. 2006;39(9):1171-9.
[43] Rozenberg, R. *et al.* Blood Cells Mol Dis. 2006;37(3):204-9, 2006.
[44] Chiaratti, M.R. *et al.* Mitochondrion. 2011,11:820-828.
[45] Wallace, D.C. Genetics 2008. 179(2):727-735.
[46] Poulton, J. *et al.* PLoS Genetics. 2010, 6:e1001066-08.
[47] Meirelles, F.V. & Smith, L.C. Genetics. 1997, 145:445-451.
[48] Chiaratti, M.R. & Meirelles, F.V. Biol. of Reprod. 2009, 82: 76-85.
[49] Ferreira, C.R. *et al*. Biol. of Reprod. 2010, 82:563-571.
[50] Chiaratti M.R. *et al*. Cellular Reprogramming. 2010, 12:231-236.

evaluate the feasibility of the adult cell therapy and the extent of contribution achieved with different approaches.

Moreover, based on recent studies with mtDNA depleted cells, we showed that TFAM (mitochondrial transcription factor A) plays a key role on organelle DNA replenish with possible species-specific characteristics (unpublished results). In order to control the mtDNA population we will produce TFAM knockout bovine cells and introduce a buffalo mtDNA together with a buffalo TFAM. In this longer-term study we expect that cells harboring the transgenic TFAM will preferentially replicate the specific mtDNA allowing the selection of the desired interspecific mitotype. *(PIs, Meirelles and Silva)*

2. Somatic Stem Cells

Telomeres and somatic stem cell failure

In the present proposal, we will further investigate the cellular and molecular events taking place in somatic stem cells in telomeropathies and dyskeratosis congenita by taking advantage of murine models. We have previously collaborated with Professor Pier Paolo Pandolfi at the Sloan-Kettering Memorial Hospital in New York in the generation of a murine model for X-linked dyskeratosis congenita,[51,52] the hypomorphic *Dkc1*^{m} mouse, which faithfully recapitulates the AA features. Here, we propose experiments to determine how dyskerin regulates the generation and differentiation of hematopoietic cells. We will isolate hematopoietic stem cells from *Dkc1*^{m} and from wild-type controls based on the expression of lineage markers (Lin), Sca-1, c-Kit, as well as SLAM markers and quantify the number of quiescent cells. Our hypothesis is that *Dkc1*^{m} present decreased number of quiescent HSCs, which will be less efficient to repopulate lethally irradiated recipients. To test our hypothesis, *Dkc1*^{m} mice will be backcrossed with Ly5.1 mice and after several generations, we will perform competitive serial transplantations (*Dkc1*^{m} are Ly5.2 positive) and determine the percentage of HSCs, myeloid and erythroid progenitors, and mature hematopoietic cells originated from *Dkc-1*^{m} *versus* wild-type HSCs in the recipients. In addition, we will analyze the p53 pathway in *Dkc1*^{m} HSCs, as *DKC1*^{m} cells signal the DNA damage response via p53 and its downstream mediator, p21 (WAF/CIP), accompanied by an elevation in steady-state levels of superoxide and glutathione disulfide.[5354] We will quantify p53, p21, MDM2 mRNA and protein levels in steady state and UV-stimulated *Dkc1*^{m} HSCs. Finally, the role of mitochondria function in oxidative stress will be assessed by respiratory chain complexes I and II activity as well as oxygen consumption in digitonin-permeabilized cells.[55]

Hepatic cirrhosis is another clinical manifestation of telomere diseases, including dyskeratosis congenita.[56] However, how short and dysfunctional telomeres lead to a hepatic process is not well understood. It is possible that short telomeres hamper hepatocyte response to chronic injuries, causing hepatocyte loss, which is replaced by fibrosis. On the other hand, short telomeres of inflammatory cells (derived from the bone marrow) also may predispose to an abnormal pro-fibrotic response. Ultimately,

[51] Ruggero, D. *et al.* Science. 2003; 299(5604):259-62.
[52] Yoon, A. *et al.* Science. 2006; 312(5775):902-6.
[53] Bellodi, C. *et al.* EMBO J. 2010 Jun 2;29(11):1865-76.
[54] Westin, E.R. *et al.* Antioxid Redox Signal. 2011; 14(6):985-97
[55] dos Santos, G.A. *et al.* Leukemia. 2011. doi: 10.1038/leu.2011.216.
[56] Calado *et al.* Hepatology. 2011; 53(5): 1600-7.

stellate cells, thought to mediate fibrosis in cirrhosis, also may be abnormally affected by short telomeres. We have recently shown that human stellate cell lines are phenotypically similar to bone marrow-derived mesenchymal cells.[57] Ron DePinho's group has shown that telomerase "knockout" (*Tert*$^{-/-}$) mice are more susceptible to chemically induced cirrhosis and liver injury may be alleviated by *Tert* ectopic expression.

However, it is still unclear what telomerase-deficient cells are responsible for fibrosis. In the present project, we propose a different approach. Using the *Dkc1*^{m} hypomorphic, the *Terc*$^{-/-}$ "knockout" and wild-type mice, we will do bone marrow transplant experiments to produce animals with either hepatocyte stem or bone marrow stem (inflammatory) cells that are deficient for telomerase (using two different telomerase-deficient murine models). In these chimeric mice, we will induce cirrhosis by experimental schistosomiasis (*S. mansoni*), which induces much more predictive and chronic disease in comparison to CCl_4-induced cirrhosis. These experiments will allow us to determine what animals are more susceptible to cirrhosis development (whether hepatocyte or marrow telomerase deficient or a combination of both), which has immediate clinical implications, as bone marrow transplant is one of the therapeutic options for dyskeratosis congenita. Additionally, we will evaluate the inflammatory pathways involved in fibrosis development in telomerase-deficient setting both by cytokine profile and gene expression analyses. These findings may shed some light on the elusive proclivity to fibrosis in telomere diseases. *(PIs, Rego and Calado)*

Cancer stem cell biology

Leukemia stem cells. In the last 10 years, we have been interested in the molecular basis of leukemogenesis and have chosen the acute promyelocytic leukemia (APL) as disease model.[58] This subtype of acute myeloid leukemia (AML) is characterized by its invariable association with chromosomal translocations involving the *Retinoic Acid Receptor alpha* (*RARA*) locus on chromosome 17. In about 98% of patients with APL, the t(15;17) is detected, leading to the generation of the PML/RARA fusion gene. Transgenic mice (TM) expressing the PML/RARA fusion gene under the control of the human Cathepsin G promoter (hCG-PML/RARA TM) develop a form of myeloid leukemia that closely resembles human APL. The disease occurs after a long latency and only in 10-15% of the animals, demonstrating that PML/RARA is necessary but not sufficient to full blown leukemogenesis. We have recently isolated and characterized the leukemia stem cells (LSCs) in APL.[59] LSCs as well as Cancer Stem Cells (CSCs) were defined, in analogy to normal stem cells (NSC), as cells presenting the capacities of self-renewal, unlimited proliferative potential, and differentiation competence. APL LSCs expressed CD34^{+}, c-kit^{+}, FcgammaRIII/II^{+}, Gr1^{int}, and were capable of causing leukemia in secondary transplant experiments. We now propose to determine whether the LSCs, similarly to what has been described in other Cancer Stem Cells, are quiescent, and to determine the contribution of PML/RARA to cell quiescence. We will study bone marrow samples from patients with APL, isolate different cell subpopulations and perform xenotransplants into NSG mice in order to identify the LSCs in humans. Our preliminary data indicate that

[57] Castilho-Fernandes et al. Exp Mol Pathol. 2011; 91(3): 664-72.
[58] Rego, E.M. *et al.* Proc Natl Acad Sci U S A. 2000;97(18):10173-8.
[59] Guibal, F.C. *et al.* Blood. 2009; 114(27):5415-25.

microRNAs act as key regulators of differentiation in APL blasts, and we intend to characterize the LSC microRNA profile in the transgenic model in order to compare to their normal counterparts and leukemic cells devoid of stem-cell properties. Based on this comparative analysis, we will perform *in vivo* assays to determine whether the down-regulation or up-regulation of selected microRNAs modulates the leukemogenic potential of murine APL LSCs.

Since 2006, our group is coordinating in Brazil the International Consortium on Acute Promyelocytic Leukemia study (IC-APL), which is a unique international trial aiming to improve the treatment outcome of patients with APL in emerging countries, sponsored by both the American Society of Hematology and the European Hematology Association. Our interim analysis was presented at the Plenary Session of the American Society of Hematology in 2009,[60] and demonstrated a significant improvement in early mortality rates, disease-free survival, and overall survival compared to historic controls. As result of this network, we have gathered more than 400 bone marrow samples collected at diagnosis and at different time points during treatment. Detailed clinical and laboratory data have been recorded in a web-based database. Now we intend to analyze (1) if the breakpoints in the intronic regions of the *PML* and *RARA* genes are different in patients with APL in developing countries compared with those in Europe (colaboration with Dr David Grimwade, UK); (2) if the differential expression of isoforms of the *p73* gene, which differ from being transcriptionally active or not, is associated with the treatment outcome and, if so, through which mechanism; (3) the role of microparticles and annexin II expression in APL-associated coagulopathy; (4) if the monitoring of the PML/RARA fusion gene using RQ-PCR technology can predict relapse better than the routinely used RT-PCR method; (5) to determine if leukemia relapse is associated with additional mutagenic events, we will apply next-generation sequencing and compare samples of the same patient from diagnosis and relapse.

Clonal lymphoproliferative disorders. Another group of neoplastic diseases we focus our interest on are the lymphoproliferative disorders, in particular chronic lymphocytic leukemia (CLL), a condition characterized by a clonal proliferation of small monomorphic $CD19^{+}CD5^{+}$ B lymphocytes in the bone marrow, peripheral blood, lymph nodes, and spleen, necessarily with greater than $5x10^{9}$ cells/L in peripheral blood. A benign condition, monoclonal B-cell lymphocytosis (MBL), in which affected cells have a phenotype similar to CLL, may be in some cases a pre-CLL disorder. The biological proximity between CLL and MBL occurs at various levels: immunophenotypically, MBL cells also express CD5, CD23 and $CD20^{dim}$, which is a typical pattern in CLL cells. Protein expression profile shows that CLL and MBL are identical even when those diagnostic markers are excluded. Finally, the proportion of $CD5^{+}CD23^{+}$ MBL cases that have 13q14 deletion or trisomy 12 is similar to CLL. In contrast, there also are significant biological differences; approximately half of CLL patients have mutated IgVH gene in contrast to 75-90% in MBL. Ig repertoire also differs.

[60] Rego, E.M. *et al.* Blood 2009; 114(22): Abstract 6

We have recently determined the prevalence of MBL in 167 first-degree relatives deriving from 42 families of patients with sporadic CLL, indicating a common genetic basis for both disorders.[61] MBL was found in 7 of these 167 subjects (4%); the prevalence increased with age reaching 16% in individuals over 60 years. However, the clinical relationship between MBL and CLL is not fully understood.

In the present project, we propose to peform a comprehensive evaluation of the biological differences in clonal cells between MBL and CLL. Two biological factors, the telomere length and the microRNA, which is known to to be abnormal in CLL, will be studied in MBL. Telomeres of CLL cells are shorter in comparison to their normal B cell counterparts, suggesting that CLL cells have increased proliferative history and that CLL is not only an cumulative disease. Futhermore, telomere length and telomerase activity appear to be important prognostic factors in CLL.[62] The significance of telomere length and telomerase activity in MBL are unknown and this information might help to understand the relationship with CLL. Using high throughput qPCR-based techniques and flow-FISH, we will determine the telomere length of MBL, CLL, and normal B cells in affected family members and observe its dynamics as diseases progress. We also will use telomere repeat amplification protocol (TRAP) assays to address telomerase function.

CLL has distinct microRNA (miR) signatures.[63] (Calin et al,2004) which are associated with prognosis and progression (Calin et al,2005). We also have shown that miR332, miR29a, miR195, miR34a, and miR29c are highly expressed in CLL.[64] However, there is no information on miR expression in MBL is available. In this proposal, we will analyze the microRNA signature profile in MBL cells and compare it to the normal B cell counterparts and CLL cells.

In sum, the results from our research will contribute to the understanding of clonal evolution from B cells to MBL to CLL, to identify specific biological behaviors in disease progression operating the leukemic stem cells, more specifically the role of miRs to disease pathogenesis and dynamics and clinical effects of telomere shortening to evolution.

Genetic analysis of solid cancer stem cells. We also are interested in the characterization of CSC biological properties in solid tumors, since these cells are extremely resistant to radio and chemotherapies and, as a consequence, are more prone to relapse. The purpose of this initiative is to globally characterize CSCs derived of different tumor types to identify new biomarkers for diagnosis and prognosis, and targets for the development of more efficient therapies. For this, we will study several solid tumors including head and neck carcinoma, colorectal carcinoma, glioblastoma multiforme, and osteosarcoma. To achieve this aim, we will apply next-generation sequencing in order to profile gene expression, characterize epigenetic alterations, detect chromosomal rearrangements, and single nucleotide variations in coding and regulatory gene regions (RNAseq, ChIP-Seq, DNA methylation, Exome, and Paired-End protocols). In

[61] Matos, D.M. *et al.* Brit J Haemtol. 2009;147(3):339-46.
[62] Rampazzo, E. *et al.* Haematologica. 2012; 97(1):56-63.
[63] Calin, G.A. *et al.* PNAS. 2004;101(32):11755-60.
[64] Zanette, D.L. *et al.* Braz J Med Biol Res. 2007;40(11):1435-40.

addition, proteomic high throughput tools will be applied to obtain the complementary set of proteins expressed in the same samples.

The data generated will be integrated by robust bioinformatic processing to estabilish transcriptome signatures, epigenome, and genomic instability of CSCs, as well as the concordances and discordances with the respectives proteomes. Previous analysis performed by our bioinformatic group using next-generation sequencing data revealed that breast cancer cells present, besides the higher levels of genomic instability, mutations preferentially located in functionally related genes, which appears to be essential for their tumorigenic potential.[65] We expect that the exploration of CSC signatures will result in the identification of new genetic markers associated with CSC self-renewal, proliferation, and resistance to radio and chemotherapeutic agents.

Whole genome sequencing in myelodysplastic syndromes. Myelodysplastic syndromes (MDS) are a very heterogeneous group of clonal diseases of the hematopoietic stem cell characterized by ineffective hematopoiesis, dysplasia, and marrow failure.[66] MDS may progress and eventually evolve to acute myeloid leukemia. A few MDS cases have distinct etiology and pathophysiology, such as $5q^-$ syndrome, in which a spe- cific chromosomal lesion leads to abnormal gene expression (RPS14) and disease phenotype, or monosomy 7, which usually evolves from aplastic anemia and associated with very poor prognosis. However, a significant proportion of MDS is cytogenetically normal and the pathogenesis is not well understood. For instance, although *RPS14* haploinsufficiency in $5q^-$ syndrome ultimately causes p53 activation, p21 accumulation, and cell cycle arrest, how an acquired haploinsufficient deletion results in proliferation advantage of the abnormal clone over normal hematopoietic stem cells is elusive. Likewise, monosomy 7 MDS have distinct gene expression profile, but it is not clear what haploinsufficient genes in chromosome 7 are responsible for disease phenotype and growth advantage. Finally, in normal karyotype MDS, the mechanisms of disease are even less poorly understood. Based on our current knowledge on acute myeloid leukemia genomics, several genes have been screened in MDS for mutations and a few of them (TP53, EZH2, ETV6, RUNX1, and ASXL1) appear to harbor acquired mutations with prognostic significance.[67] However, the biological pathways modulating disease behavior are not well characterized. This is also true for acquired mutations in *SF3B1*, a splicing factor. Abnormal *SF3B1* is associated with ringed sideroblasts, but does not necessarily cause MDS. Here we are proposing to select few well characterized cases of $5q^-$ syndrome, monosomy 7, and normal karyotype MDS for whole genome sequencing of sorted marrow $CD34^+$ cells in order to seek genes that may be mutated or deleted in these syndromes and may contribute to leukemogenesis. This may lead to the discovery of new pathways involved in disease pathophysiology. In addition, we plan to investigate the mechanisms of haploinsufficiency in $5q^-$ syndrome and monosomy 7, by addressing the pattern of inheritance and gene imprinting in the remaining chromosomes as well as potential uniparental dysomies that may exist in these illnesses. This may help to elucidate how haploinsufficient chromosomes may eventually outnumber

[65] Galante, P.A. *et al.* Nucleic Acids Res. 2011;39:6056-68.
[66] Calado, R.T. Semin Oncol. 2011;38(5):667-672.
[67] Bejar, R. *et al* NEJM. 2011;364(26):2496-506.

normal hematopoiesis. In collaboration with Dr. Neal Young at NIH, we propose to further investigate the genetic events associated with disease progression in monosomy 7 MDS. We currently have bone marrow samples of patients with aplastic anemia (and normal karyotype) who eventually evolved to monosomy 7 and leukemia, which were uniquely prospectively collected at various time points, from aplastic anemia to leukemia. Whole genome sequencing of these samples may help to elucidate the chronological pattern of genetic events involved in leukemogenesis. In addition, this analysis also may contribute to understand the issue of somatic mosaicism and its confounding effects on the interpretation of whole genome sequencing in cancer. *(PIs: Zago, Rego, Falcão, Calado, Greene, and Silva)*

Mesenchymal stromal cells

Among somatic stem cells, multipotent mesenchymal stromal cells—or mesenchymal stem cells—(MSCs) attract clinical interest due to its immunomodulatory and niche-forming abilities. We were the first ones to demonstrate that MSCs can be obtained from the umbilical cord vein subendothelial layer and that these cells are transcriptionally similar to bone marrow-derived MSCs.[68,69,70] These findings compelled us to explore the MSC distribution throughout the body, leading to the demonstration that these cells can be obtained from virtually any adult and fetal human tissue (including the wall of veins and arteries) and that they closely resemble pericytes and, to a lesser extent, to smooth muscle cells and dermal fibroblasts.[71]

The use of MSCs in the clinic has been mostly related to the treatment of graft-versus-host disease (GvHD).[72] However, its clinical use is hampered by the high cost and time-consuming processes necessary to obtain a sufficient numbers of cells for each patient. Moreover, the MSC immunomodulatory properties are heterogeneous both *in vitro* and *in vivo*, and it appears that they need first to be activated and conditioned (or "licensed") in order to achieve full immunomodulatory potential.[73] In fact, we have demonstrated that MSCs up-regulate CD39 expression and adenosine production in response to activated T-cell-associated inflammatory stimuli, thus contributing to the suppression of proliferation of lymphocytes.[74,75]

The importance of MSC "licensing" in the clinical setting is supported murine models for GvHD; MSCs that are pre-treated with interferon-γ suppress GvHD more efficiently than non-treated MSCs.[76] However, the mechanism(s) responsible for MSC licensing remains largely unknown. Signaling through the NF-kB pathway may have a role in controlling the net outcome of signals derived from the pro-inflammatory stimuli. In the present project, we propose to investigate the molecular basis responsible for MSC licensing by different inflammatory stimuli (including IFN-y, TNF-a, IL1B; and LPS, poly(I:C),

[68] Covas, D.T. *et al.* Braz J Med Biol Res. 2003;36:1179-83.
[69] Panepucci, R.A. *et al.* Stem Cells. 2004;22:1263-78.
[70] Silva, W.A. *et al.* Stem Cells. 2003;21:661-9.
[71] Covas, D.T. *et al.* Exp Hematol. 2008;36:642-54.
[72] Le Blanc, K. et al. Lancet. 2008;**371:**1579-86.
[73] Kamprera, M. Leukemia. 2011;25(9):1408-14.
[74] Saldanha-Araújo, F. *et al.* Stem Cell Res. 2011;7:66-74.
[75] Saldanha-Araújo, F. *et al.* J Cell Mol Med. 2011;Epub ahead of print
[76] Polchert, D. *et al.* Eur J Immunol. 2008;38:1745-55.

dsRNA). The immunosuppressive properties of MSCs treated with various factor combinations will be evaluated by its effects on T-cell proliferation by CFSE. NF-kB subunit activation will be measured by gel shift assays and high content screening (HCS). In addition, MSC treated with selected factor combinations will be further analyzed using oligonucleotide gene expression microarrays and the ChIP-chip technique, in order to identify the promoter targeted by NF-kB subunits.

The results derived from this project may contribute to the development and optimization of clinical protocols using MSCs for the treatment of GvHD and other immune-mediated diseases, such as diabetes mellitus type 1 or multiple sclerosis (which are currently been on clinical trials in our institution). *(PIs, Zago, Greene and Covas)*

3. Maintenance of stemness

The epithelial-mesenchymal transition (EMT) is a key process in embryonic development and tissue injury repair in which epithelial cells undergo a transition to a mesenchymal migratory phenotype.[77] The EMT is also activated in cancer progression and metastasis.[78] The EMT program has emerged as an important regulator of phenotypic plasticity in cancer cells, including their entrance into stem-cell states. These cancer stem cells are defined by their capacity to form new tumors and are thought to be chemoresistant.[79] Microenvironmental signaling plays an important role in EMT induction. Therefore, we are currently investigating the interaction between MSCs and melanoma cells during EMT induction and metastasis, in a mouse model. Preliminary results suggest that MSCs' secretome triggers EMT in melanoma cells, thereby endowing them with metastatic traits *in vitro* and promoting metastasis *in vivo*. Also, this approach provides a model to understand how heterotypic interactions during EMT activation affect CSC's biology. In this way, our group also propose to explore the genetic and epigenetic mechanisms that underlie the emergence and plasticity of CSC in different tumors during EMT, and to identify molecular signatures of EMT with clinical applications for diagnostics and therapy.

The reverse process, mesenchymal-epithelial transition (MET), is also observed in normal development and pathologies. Morever, colonization of a secondary site by metastatic cells and reprogramming of fibroblasts into iPS require the loss of their mesenchymal features and acquisition of an epithelial-like phenotype. By studying the gene expression profile of an iPS generated with a distinct combination of transcription factors (TCL1A, SOX2 and C-MYC) [80], we detected modulation of MET machinery, showing that combination of these factors were able to promote the initial steps of reprogramming. Our aim now is to explore the contribution of these genes, specially TLC1A, in the reprogramming process and in the EMT/MET pathway in order to enhance the efficiency and speed of iPS generation.

A specialized form of EMT, the Endothelial-Mesenchymal Transition (EndMT), occurs in the embryonic development during the formation of the heart valves and is considered one of the possible

[77] Thiery, J.P. *et al*. Cell. 2009; 139:871-90
[78] Wells, A. *et al.* Front Biosci. 2011; 16:815-37
[79] Nicolini, A. *et al.* Curr Pharm Biotechnol. 2011; 12:196-205
[80] Picanço-Castro, V. *et al.* Stem Cells Dev. 2011; 20:169-180.

causes of fibrosis. Our Center has been studying MSC and endothelial cells (EC) for at least 10 years.[81,82] Our data demonstrated that MSC present a high-level expression of genes involved in EMT, such as Twist, Snail and TGF-β and these genes were repressed in EC. We also observed that a transition of adult EC to mesenchymal cell-like occurs *in vitro*. In this propoal, we asked if EC of several origins are able to transit to mesenchymal phenotype *in vitro* through EndMT, and which inductors and molecular mechanisms could be involved. We will test different known EMT inducers, such as TGFB and the overexpression of transcription factors (SNAIL, SLUG, TWIST, and ZEB1) testing the hypothesis that EMT and EndMT share the same mechanisms of action.

These projects involving will be developed in collaboration with Professor Robert A. Weinberg from the Whitehead Institute/MIT (Proposal FAPESP-MIT 10/51962-9). *(PIs, Covas and Silva)*

4. Clinical Stem Cell Application

The clinical studies in this proposal involve the therapeutic use of hematopoietic stem cells (HSC) and multipotent mesenchymal stromal cells (MSCs) for autoimmune and inflammatory diseases.

High dose immunosuppression followed by autologous hematopoietic stem cell transplantation (AHSCT) has been evaluated worldwide as a therapeutic strategy to restore immune tolerance and halt immune-mediated tissue destruction. Our center started to perform this therapeutic strategy for severe neurological and rheumatic autoimmune diseases patients in 2001, within the Center for Cell Therapy. The rationale for using AHSCT in autoimmune diseases is the purging of pathologic immune cells and regeneration with a healthy repertoire of immune cells. Thus, it is proposed that AHSCT, by ‘‘resetting’’ the immunologic memory, may bring the individual’s immune system back to a premorbid state, resulting in a prolonged clinical remission.

Today, the most frequently transplanted autoimmune disease worldwide is multiple sclerosis (MS), due to its high prevalence and lack of effective therapies. In our center, 78 patients have been transplanted in a phase I/II clinical trial. Approximately 70% of the MS patients showed stabilization of neurological disability, indicating successful control of autoimmune neural aggression. Facing these encouraging results, and associated with observations that patients with the inflammatory, relapsing-remitting subtype of the disease present better outcomes, a new multicenter, randomized, we now propose a phase III trial in collaboration with Dr. Richard Burt, from the Northwestern University Immunotherapy Division, in Chicago, USA. Patients with the inflammatory form of MS will be included in this trial, which aims to evaluate, through clinical and magnetic resonance imaging criteria, the effectiveness of the procedure to control disease progression. We aim to recruit 55 patients in each arm (transplant and non-transplant arms).

Our center pioneered the treatment of type-1 diabetes mellitus (T1DM) with high dose cyclophosphamide plus anti-thymocyte globulin (ATG) followed by AHSCT with the objective of decreasing pancreatic β-cell immune-mediated destruction and preserving remaining pancreatic function. The unprecedented results were better than any other immunosuppressive trial for the disease already

[81] Covas, D.T., *et al.* Exp Hematol. 2008; 36(5): 42-54.
[82] Panepucci, R.A., *et al.* Stem Cells. 2004; 22(7): p. 1263-78.

published. Twenty-five recently diagnosed T1DM patients were included, 21 of which became insulin-free after transplantation.[83,84] Significant increase in C-peptide levels was detected, indicating that the procedure was effective in enhancing pancreatic insulin production. However, despite successful initial glycemic control, most patients relapsed due to loss of immune tolerance. To circumvent such outcome, we now propose a new multicenter, randomized, phase III trial involving a more intensive immunosuppressive regimen, aiming to reduce or eliminate relapse after transplantation. This clinical trial will involve collaboration with the Northwestern University Immunotherapy Division (Chicago, USA), Saint Louis Hospital (Paris, France), and Leiden University Medical Center (Leiden, The Netherlands) and aims to recruit 30 patients in each arm (controls and transplanted subjects).

During the last years, another disease that has become an indication for AHSCT worldwide is systemic sclerosis (SS). Cases with diffuse cutaneous or visceral involvements are considered severe and associated with mortality rates of up to 50% in 5 years. AHSCT is very effective in improving skin fibrosis, restoring elasticity, range of motion and facial expressions. Moreover, AHSCT stabilizes lung fibrosis, preventing progression to respiratory failure. In our center, 31 patients have been included in an AHSCT clinical trial. These patients showed significant skin improvement and stabilization of lung function. We have recently started skin biopsy evaluations, comparing pre and post-HSCT cutaneous fibrosis and inflammation. A recently published randomized trial demonstrated that AHSCT was more effective as first choice treatment for systemic sclerosis than conventional treatment with monthly cyclophosphamide pulses.[85] Since our observations agree with the published data, indicating that AHSCT is more effective in patients with shorter disease duration, we now plan to perform earlier transplant for recently diagnosed patients with severe systemic sclerosis. We hypothesize that transplant at an earlier time point will result higher disease reversibility, and perhaps may provide better functional outcomes and quality of life.

Concomitantly to the clinical trials, our center has been studying the mechanisms of action of the AHSCT in autoimmune diseases (MS and T1DM), which remains largely unknown. Scarce data from animal studies and human clinical trials indicate that, that following autologous transplantation, the immunological resetting occurs via immune repertoire replacement and via restoration of immune regulation. Our results on the immunological mechanisms of action of AHSCT in T1DM patients showed, after transplantation, increased frequency of $CD8^{+}CD28^{-}$ and $CD4^{+}CD25^{high}Foxp3^{+}$ regulatory T cells, increased expression of the immunoregulatory genes, quantitative and qualitative changes in the composition of the T cell repertoire, modulation of apoptosis-related genes. Besides, we observed that the frequencies of auto-reactive CD8 T cells before HSCT predicted clinical outcome. The rate of cellular autoimmunity before HSCT proves to be an immune correlate predicting clinical efficacy and pointing to disease and patient heterogeneity, requiring additional therapeutic immune intervention strategies that cope with existing loss of immune tolerance to islets. Besides studying the mechanisms of action of

[83] Voltarelli, J.C. *et al.* JAMA. 2007;297:1568-76.
[84] Couri, C.E. *et al.* JAMA. 2009: 301:1573-9.
[85] Burt, R.K. *et al.* Lancet. 2001;378:498-506.

AHSCT on MS and T1DM patients more deeply, future studies will be performed on the mechanisms of action of this intervention on SS patients.

Multipotent mesenchymal stromal cells (MSCs) have also been investigated as a potential cell source for the treatment of autoimmune and inflammatory diseases, based on their immunosuppressive and immunomodulatory properties, and possible regenerative potential. However, in spite of many currently active clinical trials around the world, the therapeutic role of MSCs is still undefined.

Our center started studying MSCs in 2001.[86,87,88,89] We have gained expertise to launch in 2007 the Cell Therapy Laboratory, in which we isolate and expand bone marrow MSCs (BM-MSCs) for clinical purposes under GMP conditions. We have already provided more then 160 BM-MSCs units for 46 patients enrolled in clinical trials or for compassionate use in or center or in others hospitals around the country. Our center is the only one in Brazil able to provide on a regular basis BM-MSCs for clinical purposes.

Our center started in 2008 a phase I/II clinical trial of infusion of BM-MSCs for the treatment of recently diagnosed T1DM patients. The aim is to investigate the clinical, immunological, and metabolic effects of BM-MSCs in these patients, as well as their homing in recipients; it is not clear whether MSCs exert their effect via humoral mechanisms or directly homing to affected tissues. We hypothesize that cells may reverse the immune-mediated destruction of pancreatic β-cells and restore insulin production. Unlike the AHSCT trials, the low toxicity of MSCs allows us to treat younger subjects; our current protocol includes patients beginning at age of 5 years. Allogeneic BM-MSCs (from relative donors) are injected intravenously, in multiple infusions of approximately 2 million cells/kg each. So far, the trial has included eight patients, four adults and four children. The initial clinical results are encouraging, showing reduction of exogenous insulin requirements, although none of the patients has yet become insulin-free. C-peptide dosing and immunological evaluations are underway. Besides, the initial outcomes indicated that this therapy is safe with few early adverse effects. The patients' follow-up will allow evaluating long-term adverse effects. Cell homing will be tracked by PET/CT, in order to better understand the fate of MSCs in recipients. In the context of this research project we intend to increase the number of patients treated, to extend treatment for chronic T1DM patients and to evaluate profoundly the immunological mechanisms of MSCs infusion.

The ability of BM-MSCs to prevent and/or alleviate graft-versus-host disease (GVHD) will be investigated in the setting of haploidentical allogeneic HSCT in a different clinical trial. In haploidentical transplants, the HLA mismatch between donor and recipient increases the risk of GVHD and graft failure following transplantation, warranting the use of immune interventions. In our trial, patients will be prophylactically treated with intravenous injections of BM-MSCs to prevent acute GVHD. In the event of high grade acute GVHD occurs, additional BM-MSCs will be injected, associated with conventional

[86] D. T. Covas, D.T. *et al.* Braz J Med Biol Res. 2003;36:1179-83.
[87] Silva, W.A. Jr. *et al.* Stem Cells. 2003;21, 661-9.
[88] Panepucci, R.A. *et al.* Stem Cells 2004:22:1263-78.
[89] Lima Prata, K. *et al.* Exp Hematol. 2010;**38**:292-300.

GVHD immunosuppressive treatment. This study has a strong relevance in the transplant field, since it allows transplantation for those who have no HLA-matched donors available.

As mentioned above, most cases of acquired aplastic anemia (AA) are immune-mediated; cytotoxic T-cells target hematopoietic stem and progenitor cells, causing marrow failure. Patients who lack a matched related bone marrow donor for transplant are treated with intensive immunosuppressive therapy based on antithymocyte globulin (ATG) and cyclosporine in order to cease the immune process. However only two-thirds of patients respond to current immunosuppressive regimens, and one third of responders eventually relapse. Several clinical trials aiming to increase the intensity of immunosuppression by addition of drugs failed to produce results better then standard treatment. The lack of better results may be explaining by the mechanism of action of added drugs, which may not act on the immune processes taking place in AA. Alternatively, the HSC niche may be severely destroyed and incapable of restoring hematopoiesis even if the immune process is ceased. In addition, HSCs may be exhausted by the time of treatment. In the present proposal, we will take advantage of the immunomodulatory and niche-forming properties of BM-MSCs to treat refractory and relapsed patients with acquired AA. BM-MSCs have produced promising results in the treatment of GVHD after other immunosuppressive drugs were tried. In our clinical study, refractory or relapsed AA patients will be treated with standard immunosuppressive regimen (ATG plus cyclosporine) complemented by the infusion of allogeneic (sex-mismatched) BM-MSCs. The main endpoint of this study will be the safety of BM-MSC use in AA. As secondary endpoints, we will evaluate the hematological response to the regimen and its immunological effects by cytokine and flow cytometry analyses of peripheral blood and bone marrow samples up to six months after treatment. That bone marrow biopsy is part of the standard analysis of response gives us a unique opportunity to verify MSC homing to the marrow by fluorescence in situ hybridization (FISH) and flow-FISH of sex chromosomes (as BM-MSCs will be sex-mismatched) and PCR-based VNTR. If the use of BM-MSCs proves to be safe in patients with AA and appears to result in hematological responses, we will consider its use in combination with ATG and cyclosporine as first line therapy for acquired AA.

Our center is currently investigating alternative sources of MSCs, such as the umbilical cord (UC). The advantages of UC as a source are the abundant supply lacking ethical concerns, painless collection, lower risk of viral contamination, easier and faster expansion *in vitro*, and capacities to differentiate into a variety of cells and to synthesize and secrete a set of cytokines and trophic factors. We have already established the procedure of UC-MSC isolation and expansion in xeno-free conditions. In this proposal, we will create and allogeneic MSC-UCs bank, where the cryopreserved banked cells will be previously tested, thus ready for “off-the-shelf” clinical use. The UC-MSC bank will be the first one in Brazil and will enable the execution of future clinical trials.

Planned clinical applications include type 2 diabetes mellitus (T2DM), which shares some of the inflammatory mechanisms involved in the pathogenesis of T1DM, leading to less insulin production than required for glycemic control. Thus, we aim to decrease pancreatic inflammation through intravenous infusions of UC-MSCs, expecting to increase endogenous insulin production. This is a novel therapeutic

approach to treat this highly prevalent metabolic disease. The investigation of the mechanisms of action of UC-MSCs on pancreatic inflammation may lead to future therapeutic pathways and possibly, development of new drugs. In the future, we aim to expand the study, evaluating not only systemic intravenous infusions, but also local intra-pancreatic injections in T2DM patients.

Second planned therapeutic targets for UC-MSC, in the context of this research proposal, are the complications of allogeneic BMT, which include graft-versus-host-disease (GVHD), graft failure, and hemorrhagic cystitis. Despite HLA-match, these complications are often observed in BMT and vary from patient to patient, with incidence and severity increasing as the number of mismatches increases. Unlike haploidentical HSCT, previous donor BM-MSCs expansion and prophylactic injections are not justified, since most patients do not develop severe, life-threatening events. Therefore, a readily available source of cells, previously tested and expanded, such as the allogeneic UC-MSCs bank, is very useful when these non-anticipated complications emerge. So far, our center has used BM-MSCs in compassionate cases of severe and refractory GVHD complications, with variable, but in most cases favorable clinical results. Future studies intend to perform immunological evaluations, aiming to establish specific effects of UC-MSCs on these disease courses. *(PIs, Voltarelli, Calado, and Covas)*

Education and Dissemination of Knowledge

The educational program was one of the most successful activities carried out in the context of the CEPID program 2000-2011. Thus, we do not foresee significant changes in its design, and the present program is a continuation and a scale-up of the educational activities developed in the last decade.

Strategy. The core of this program is a long-term large-scale interaction of the researchers (senior and young) with the middle schools, their students and the teachers, especially in the field of science teaching and learning. It is necessary to emphasize that Brazil still endures a very inadequate and inefficient basic and middle education, as demonstrated, for instance, by the poor results obtained by Brazilian students at the international PISA evaluation, in which the country ranks 53th in sciences (of the total of 65 evaluated)[90]; this contrasts sharply with the fact that the country ranks 13th in the world in number of scientific articles published. Two of the reasons for this situation are the defective education of many school-teachers who do not have an opportunity to expand or update (and who are paid low salaries), and the other is the structure of the medium school curriculum, usually inflexible and formal, putting a strong emphasis in memorized contents with little practical approximation of real-life problems. This situation is aggravated at the lower social stratus of the society, which concentrates at the periphery of big cities and small townships located near big towns such as Ribeirão Preto, and do not have the opportunity to attend high quality private colleges.

Thus, we organized our education program around the relationship of the triad school-teacher-student from public middle schools with the Center and its researchers. This relation unfolds in a series of activities, some shorter, other more extended, that have in common the challenge for the student-teacher to produce a result (an assay, a model, an experiment, a theater play, for instance) originated by a question, a doubt or the explanation of a previous problem. The emphasis is thus a change from a formal-bookish to an every-day hands-on education centered in scientific subjects. Around this central axis there is a whole infrastructure, a group of dedicated researchers and supporting personnel, and practical activities that have been selected along ten years. We will summarize the activities accomplished and indicate the changes for this new period.

Science Education at the Center for Cell Therapy 2000-2011. Since 2000, more than 66 educational activities were developed as part of our program (see **Education** at the site http:/ctcusp.org); we summarize some relevant examples:

Course of Specialization in Science Education called "*The Cells, the Genome, and You, Teacher*", administered together with the University of Sao Paulo (USP) and the Secretary of Education of the State of Sao Paulo, with duration of 2 years, enrolled 200 teachers of basic schools and was offered in 2001 and 2003;

University extension courses with duration of 30 hours, for school-teachers;

Projects of scientific initiation supported by FAPESP;

[90] OECD (2010), PISA 2009 Results: What Students Know and Can Do – Student Performance in Reading, Mathematics and Science (Volume I). http://dx.doi.org/10.1787/9789264091450-en

Talented Students Program in which senior investigators from CTC held weekly meetings with teachers and students of basic schools;

Adopt a Scientist in which graduate students from USP oriented a small group of middle school students to carry out a simple research project, and to generate educational material for science diffusion;

Journal of Science, a newsletter that is at its 21st edition with a circulation of 4,000 (all the editions are on-line at the site www.ctcusp.org/link1.php, **Journal of Science**); the educational material in format of news is produced by teachers and students, and is distributed among students and school-teachers;

Educational games and theatrical plays. For instance, the students and teachers, under the supervision of the Center's researchers, wrote and produced the play ***"A Cell's Agony"*** that describes the phenomenon of apoptosis; this play was extensively presented as for instance during the Science and Technology Week in Brasilia, the performance was repeated 4 times by request of schools in the satellite cities around Brasilia. Another example is the play ***"Por uma gota" ("For a drop")*** that stimulates blood donation (see YouTube Channel http://www.youtube.com/user/CasadaCienciaHRP)

Educational brochures, such as "Talking about HIV and AIDS" that was funded by Ministry of Health and distributed to all Brazilian schools. This booklet was planned, developed and written in close collaboration of students and researchers, and has the virtue of using simple and direct language of adolescents talking among themselves; even the illustrations were produced by a medium school student.

Annual Summer Courses. In addition to the activities focused on the middle-school students, we developed initiatives aimed at other targets. Thus, we have offered a two-week ***Annual Summer Course*** for undergraduate students in the life science fields of Brazil. Thus far eleven have been made available, with the enrollment of more than 350 students. The subjects of the courses have been: genomics, proteomics and stem cells.

Specialization Courses for physicians and other professionals in the fields of transfusion medicine, nursing, social assistance and management of health services, that incorporate expertise and scientific knowledge derived from the Center for Cell Therapy.

General Population. The general public also benefits from Center's outreach program, since a part of our activities involves information to the general public, both on specific subjects related to the medical activity carried out (potential use of stem cells for therapy, transplants, HIV/AIDS, leukemias, anemias) and on general aspects that help in building the public perception of science (ethics, innovation, scientific knowledge for development). The table summarizes the subjects of 89 reports appearing in the news in 2011 that originated in the Center.

In 2011, 89 reports appearing in the media originated in the Center

Subject	*Radio*	*TV*	*Print*
Health care	2	19	14
Education	1	9	8
Research	4	9	20

A Trip through the Cell. A representative example of the educational activities carried outside of the Center, looking to reach the general population, is the week-long annual exhibition during the National Week of Science and Technology. The Center's last four exhibitions were held in one of the largest shopping malls of the city and were visited by more than 10,000 people (http://www.ctcusp.org/link2.php). One component of this exhibition is the **"Cellularium"**, an adapted inflatable planetarium where astronomy movies are replaced with biology and cell related movies. The movies were produced by a ***team of experts hired by the Center*** especially to develop and produce educational movies intended to be released in the cellularium or in regular theaters. The team of animators also has produced a 3D movie called "***A Trip through the Cell***" (http://ctcusp.org/?page_id=2096). Thus far more than 7,000 people have attended the Cellularium's sessions, in its itinerant exhibitions in different places in Ribeirao Preto and other towns.

Scientific Journalism. An integral part of science diffusion is scientific journalism, which is still very incipient in Brazil. Along 11 years we have promoted many initiatives to bring journalists for short stays, as regular members of the team or as partners. Along these years at least 15 journalists had medium or long-term association with the Center. At present the Center has two journalists hired specifically for diffusion of science and health topics. We also promoted a training course named **Partnership in Science Dissemination.** We plan to expand both the participation of professional journalists and to bring undergraduate and graduate students from Courses in Journalism as part of this project.

Infrastructure, Support and Personnel. The educational activities are offered within and outside the Center. Within the Center there are spaces created specifically to host the educational dissemination project of the Center: the **House of Science**, the **Museum and Laboratory for Science Teaching (MuLEC)**, the new **Laboratory for Research Teaching (LRT)**, and the new **TV Studio (TV).**

The **House of Science** is an area of 50 m^2 which contains spaces for practical activities of biology, chemistry, physics and mathematics, a small library, and a very interesting collection of class plans and other didactic materials.

The **MuLEC Lab and Museum** is an old house of 242 m^2 located at the University Campus, belonging to USP and transferred to the Center specifically for the educational program. The house was renovated and now is integrally used to host public exhibitions of educational and scientific material produced by the participants of the various activities**. MuLEC** has received visitors from the general public and from basic schools since 2004.

The **LRT** is a new lab of 36,8 m^2 equipped with research infrastructure in molecular and cellular biology, dedicated to experiments that are part of the introductory research projects carried out by basic schools teachers and students.

The **TV-studio** equipped with modern TV resources for generation and edition of movies was recently opened. The **TV** produces educational and scientific material to be released for basic schools and to the public in general (***see examples of this material at the site*** http://ctcusp.org/?page_id=414 ***under the heading Educational Clips***). More than twenty video classes are available in the HS website, or in the

related YOUTUBE channel http://www.youtube.com/user/CasadaCienciaHRP. ***The House of Science website has received more than 35.000 visitors in the last four months.***

Management. All the educational activities carried out are coordinated jointly with the Educational Center of the Regional Blood Center, an administrative and technical division of the Center linked directly to the Presidency. Physically it is located in an area of 1,600 m^2 divided into three offices, four classrooms, the TV studio, the House of Science, and the MuLEC Museum and Lab. The activities are conducted by a team of ten employees, including administrators, teachers, journalists, video makers, and technicians ***paid by the Hemocentro Foundation*** (the Blood Center supporting civil organization).

Plans for the new Term. All previously described educational activities will be maintained and extended in the new phase of Center for Cell Therapy. Specifically, the following programs that are being developed: Headhunter, Adopt a Scientist, FAPESP Jr, Scientific Initiation USP, Courses of Specialization in Science Education, Postgraduate Courses, Journal of Sciences, MuLEC Exhibits, Cellularium Exhibits, Summer Courses, Video Classes, Vacation with Sciences, Science in Focus, House of Science Activities.
The maintenance and the expansion of these activities will occur in physical facilities that already exist and in the new facilities designed to be built in the next two years, including a new building for the House of Science and new spaces for academic coexistence.

Online Scientific Courses. In the past we offered courses online of scientific formation using the platform MOODLE and the system of e-learning developed by USP (COL). We intend to continue to offer this type of resource since it can be accessed from anywhere in the country with connection to the Internet. Recently, we have installed 15 new computers in a classroom especially reserved for online activities of the students of the House of Science and for computer-based education and learning.

The New House of Science. Thus, the Center has already available a considerable infrastructure and support personnel to develop an advanced outreach program, and ***USP will provide a new full-time technician dedicated to educational activities***. Meanwhile, a new physical space is planned in view of the expansion and the important place that these activities have acquired in the last years. Thus we have planned a New House of Science in a modern 2,240 m^2 containing laboratories, conference room, space for the Cellularium, library, astronomic observatory, classrooms, belvedere, production center, room for the collections. The estimated cost of this building is of R$ 6,4 million (US$ 3.6 million); part of the costs will come from the Hemocentro Foundation as counterpart to match the FAPESP financing, and we are looking for additional funds from other sources, among them the USP. The **laboratories of education** are designed to accommodate the practical activities of biology, physics, chemistry and mathematics, to develop the projects of the program "Adopt a Scientist" and the experimental activities of scientific initiation sponsored by FAPESP and USP. We are confident that teachers and students will continue to transfer to the classroom the patterns and applications of the projects and experiments, as they do at the present. To extend the present activities that emphasize mostly biology and physics to chemistry and

mathematics we will hire teachers who are specialist in these areas for guiding the participants**.** The **conference room** is intended to shelter scientific presentations such as symposia, conferences, the artistic activities such as theater plays and musical shows, and other activities carried out by the various participants in educational activities. This room will be equipped with technical resources that will permit a multidisciplinary and appropriate use for the various activities that will be undertaken. The space of **Cellularium** is intended to allow the mounting of the equipment, previously described, for exhibits or programed visits to the Center**.** The l**ibrary** will house books, leaflets, newspapers, magazines, and other educational materials collected by the House of Science. In the current HS there is already a small library with 2,100 books mainly in the area of scientific education and biology; this collection will be expanded. The new library will also provide a space for study and research with easier access to the Internet. In the new HS was also designed to house a ***small astronomical observatory*** equipped with telescopes suitable for the observation and photography of planets of the solar system and distant galaxies. An astronomer collaborator will supervise the operation of the observatory. The new HS will also include classrooms, social spaces and administrative area. This new space was designed to be ***environmentally sustainable*** and should be completed in two years.

Partnership. The educational project of the CTC is centered on the exchange and constant partnership with schools, students, and teachers of basic education. In this sense, the participation of the municipal and state education departments is essential. Thus far, the cities of Dumont, Sertãozinho, Batatais and Ribeirão Preto have formally communicated the interest in renewing their participation in the project.

Methodological Issues. In our experience the joint participation of scientists and graduate students in science education for students and teachers of the basic and middle school played a central role in the success of our program of science education. In our view, the elements that lead to this improvement involve structural and pedagogical aspects of which we emphasize: 1. The educational work in the Center for Cell Therapy can be characterized as "active learning" that involves thinking, hands-on experience and learning to communicate; 2. The issues addressed are practical and related to the real life; 3. Researchers are highly qualified specialists who guide the discussion of themes, often eliminating the need for secondary sources of information; 4. Teams of researchers-and-students are generally highly motivated and committed to the teaching/learning process; 5. High-order cognitive aspects, according to Bloom's taxonomy, such as the analysis, synthesis and critical and creative thinking are treated continuously; 6. Laboratory infrastructure is available to carry out practical activities.

These positive aspects contrast with the situation of public basic and middle schools in Brazil. Therefore, the model deployed in educational activities of the Center for Cell Therapy may ***serve as a guide for a reform and improvement of science education in the country***. As a consequence, the teachers, the graduate students and the technical personnel are encouraged to keep records of the experience, as well as to write reports that can be used to document the progress observed. We hope that with the approval of the present project this whole educational experience can serve as a basis for ***scientific research in education*** for researchers and graduate students.

Technology Transfer Project

Our group had a long experience in transferring technology to the community, including many examples ranging from methods for handling blood samples to very specific clinical trials in which *in vitro* expanded cells were successfully transplanted into patients with diabetes mellitus or GVHD. Most of this technology was extended to the population in partnerships with the State of São Paulo Health Department or directly to patients, via the Brazilian Unified Health System (SUS) in leading hospitals (the University Hospital in Ribeirão Preto, Amaral Carvalho in Jaú, Albert Einstein, Sírio-Libanês, and the Children's Hospital GRAACC/UNIFESP in São Paulo). More recently, the Center also initiated efforts to make in-house generated technology available to the private sector. Many patent deposits requested by the Center's PIs are underway (Table), and a greater number of technological processes are in development, some of them with Confidential Disclosure Agreements already signed or under implementation.

TABLE 1. INTELLECTUAL PROPERTY APPLICATION PUBLISHED IN THE NAME OF THE CTC PI's.

TITLE	PUBL YEAR	AUTORS	Derwent Number
Produção estável e em larga escala de FVIII humano em linhagem celular humana sk-hep-1.	2012*	COVAS TC, CASTRO VP, et al.	000022110 0379104**
New human blood coagulation factor IX recombinant protein comprising specific substitution of the aspartic acid amino acid in specific position by valine for treating hemophilia B	2011	FONTES A M, COVAS D T	2011 - B36595
New recombinant human blood coagulation protein of factor VIII useful in preparation of medicine for treating hemophilia A, comprises reduced domain B.	2010	FONTES A M, TADEU COVAS D	2010 - D97518
Recombinant protein factor for use in preparing drug for treating hemophilia B and for coagulating human blood comprises amino acid sequence, where aspartic acid is substituted by valine and is produced by human kidney or liver cell lines.	2010	COVAS D T, FONTES A M	2010 - D18561
Animal cloning method, involves transferring somatic cell and/or embryonic cell during induced and/or natural programmed cell death i.e. apoptosis.	2009	BRESSAN F, MEIRELLES F V, , et. al	2010 - A51391
Use of crotamine for transporting molecules such as nucleic acids to the surface, cytoplasm, or nucleus of the cells, or for preparing a composition for treating diseases or dysfunctions linked to cell proliferation	2006	YAMANE T, KERKIS I, PEREIRA L D V, et. al	2006 - 708599
Serological diagnosis of chagas' disease.	1999	RAJA G.T. L R, CORREIA A.I, COVAS D T, et. al	1999 - 518469

* PROBABLE YEAR OF PUBLICATION IN INPI-BRAZIL.
** INPI ASSESSION NUMBER.

The present proposal aims to steadily expand and generate science and technology transferable to the community with special focus on public health. In this section we describe our present and planned initiatives as well as some of the specific products expected to be developed in the Center's Project. We also present the currently available infrastructure and the strategies that we rely upon in order to promote an efficient process of technology transfer. Our technology transfer proposal incorporates a three-fold approach to close the cycle of translational medicine, benefiting society and patients:

a) To cooperate and continuously communicate with the private sector interested in new therapeutic approaches, new recombinant proteins, and new diagnostic methods;
b) To cooperate with the government, responsible for public policies to improve the Unified Health System;
c) To educate a new generation of scientists and leaders; to stimulate the entrepreneurship, innovation and generation of spin-off companies in order to deliver qualified human resources to the market, as well as new products and services derived from the knowledge generated at the Center.

All the three goals take into account that biotechnology and health-focused products usually required long-term initiatives, which are compatible with the time frame proposed for the Center. It must be emphasized that ***we see the technology transfer*** as encompassing not only new products and process that can be transferred to private and public companies or the government, but also the direct delivery of high quality technological services to the population (that may serve as models or pilots), and the social and legal issues involved, such as standards, regulation and laws.

The Innovation Environment. Technology transfer from the Center will be facilitated by several local factors: (1) patent applications for processes or biotechnology products have the permanent support of

USP Innovation Agency, an institutional division within the USP Research Provost Office, established in 2005, which functions both as the university office of technology transfer and the industry liaison office; (2) The Center and the Blood Center are already associated with a business incubator (***Supera***), located within the Center facility, which incorporates the Inbios initiative (Business Incubator in Health Biotechnology) implemented in 2002. At this time, Supera supports 9 resident companies, 3 pre-resident companies, 1 associated company and 20 projects; a branch called ***Supera-Hemocentro*** is installed in a 128-m^2 area at the Blood Center. (3) Some companies have already demonstrated interest in licensing future IPs or to act as private partners in the development of specific products, such as DNApta (Ribeirão Preto), focused on the applications of nucleic-acids-based affinity reagents (aptamers) in diagnostics and therapeutics, and Qiagen (Germantown, Maryland, USA) interested in the development of new qPCR-based kits for fast and high-throughput methods for telomere length measurement for clinical purposes (http://www.qiagen.com/literature/qiagennews/weeklyarticle/11_04/telomere/default.aspx). (4) A partnership between USP and the State Administration has created a ***Technology Park*** in the vicinities of our Center. (5) The technology transfer merges with the educational component, especially with the Professional Biotechnology Master Program, planned to foster professional competence in the biotechnology industry, as well as the Professional Transfusion Therapy Master Degree, planned to fulfill the country's critical need in this area for professionals able to create, absorb, and apply new technologies.

The History of the Team Accomplishments. The Blood Center already incorporates technology generated by the Center in its services to the community in pursuit of excellence. Advanced practices and standards for transfusion and cellular products place the Blood Center as a major reference leader in the country. The institution is the only public blood bank granted with ISO 9001 certification and the American Association of Blood Banks (AABB) accreditation. In this regard, it is worth mentioning that the ***ISO certification standards for Blood Banks were developed on the basis of a project carried out by our Center***. The Center has more than 160,000 registered blood donors, receives 10,000 donations/month, and performs 12,000 outpatient clinic visits of patients with blood disorders. It is responsible for providing care to many of the patients and in 2011 it transfused more than 150,000 blood components bags.

The Center also developed an in-house nucleic acid testing (NAT) protocol for HIV and HCV for blood donor screening in order to contribute to blood safety and provided new insights into the incidence of acute infections. Since 2009, 10,000 blood donations have been screened per month, with an effective cost three times lower as compared to commercial methods. ***This method was made available to the State of São Paulo Department of Health***. As a consequence, the Blood Center is the first public blood bank in Brazil that applies NAT to 100% of blood donated.

The Center's innovation experience resulted in the establishment of a Cryobiology Laboratory, an Umbilical Cord Blood Bank, an Apheresis Laboratory, and a GMP Tissue Culture Facility. These facilities work in a routine basis and give essential support to stem cell transplantation ongoing ***clinical trials*** in the institution, by allowing the culture of mesenchymal stem cells and the isolation and cryopreservation of autologous mobilized hematopoietic stem cells.

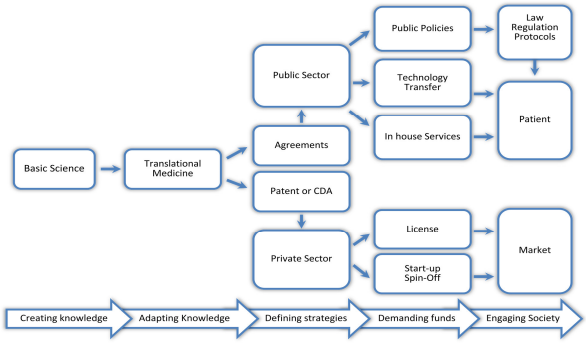


Figure: Technology transfer strategy. The CTC will within this proposal develop high standard Basic Research and also technological and translational medicine studies, which benefits may potentially be transferred to the society. In general we divided our contribution to the society on products, services and human resources, with the latter being essential to a successful technology transfer. In the CTC organization the definition on what strategy will be elected will be held by the inventors along with the Technology Transfer Committee, when required, with the assistance of innovation agency (agência USP de Inovação). Due to the characteristics of the project the results may be offered to the public sector through agreements for training people, defining protocols regulations, or integrating products or services to the public heath system. In other hand, products and services may be offered to private companies throughout a direct licensing or CDA (confidential disclosing agreement). If needed the technology may be further evaluated in the form of in house services within the Hemocentro and the local University Hospital (H. Clinicas de Ribeirão Preto) or as an incubated company in the Supera Hemocentro or Technological Park before delivery in lager scale to the Patients or Market.

In the last decade, 392 autologous hematopoietic stem cell transplants were performed; from 2006 to date, 142 mesenchymal stem cell (MSC) infusions were performed in patients with GVHD or type 1 diabetes mellitus (median dose of $2x10^6$ cell/kg body weight). MSC are currently produced in the GMP Tissue Culture Facility in static flasks and we are optimizing ***large-scale production of stem cells*** in bioreactors (a project supported by the Brazilian Innovation Agency Finep, Project 0108059100 and BNDES Project 09.2.0706.1). Within the timespan of the present proposal, this bioprocess will be used to expand cells for clinical trials proposed herein, and the technology developed will be made available to other research institutions, as well as to offer to private and public sectors.

Recombinant proteins have also been the focus of the Center in the latest years, resulting in cell lines producing coagulation Factors VIII and IX, for which PIs of the Center have submitted three patent requests (table 1). The development of a bioprocess to produce these factors in large scale was made in agreement with the public initiative “Instituto de Pesquisa Tecnológica” (IPT) and Butantan Institute (Finep Project 01.07.0652.00). The Center is now developing a suspension culture system (bioreactor) of human cell lines that results in a glycosylation pattern identical to that found in the plasma-derived coagulation factors.

Due to a demand for preclinical trials and the possibility of working on a variety of animal models, we started collaborating with the Veterinary Medicine facility of the University São Paulo in Pirassununga (100 km from Ribeirão Preto) with extensive expertise on embryonic development, cytoplasmic inheritance, and nuclear reprogramming. This relationship made available to our group a whole Experimental Veterinary Hospital facility dedicated to innovative therapy financed by the Brazilian Innovation Agency (Finep Project 01.10.0023.00), which will extend the technology transfer initiative to a broad diversity of domestic and wild animals.

This relationship started with fruitful results within previous collaborative projects, such as the National Institute of Science and Technology, to deliver cloned calves harboring the coagulation factor VIII expression vectors controlled by the *Bos indicus* beta-lactoglobulin promoter using an in-house patent-protected process of nuclear transfer (Table 1). Hence, the same process was offered to private laboratories in Brazil that together produced more than 200 cloned animals over the last 8 years, including bovine, ovine, and more recently pregnancies in equines. This large number of animals resulted in a demand for ***regulatory processes***, which were the subject of a ***bill*** proposed in the Federal Senate by Sen. Katia Abreu and member of our group served as consultants to the Senator.

The education of highly trained human resources is the present bottleneck for technological development in Brazil and in the State of São Paulo. Thus, we have organized two professional Master Programs in

Biotechnology and in Hemotherapy (TT), which received an initial grade of 5 from CAPES (the higher grade granted to new proposals). This year we have started to enroll students interested those courses. This is the first professional master program implemented by the University of São Paulo in the biomedical area. The program naturally includes motivation for entrepreneurship and business administration as we expect that our students will be leaders in biotechnology R&D and innovation in the future. The master program in TT is sponsored by the Ministry of Health and is a direct transfer of the technology developed at the Center to all the public transfusion centers in the country.

Strategies. The Center will hold annually an "Innovation Day", to which potential industrial and business partners will be invited to exchange information with the researchers. The technology transfer committee will be responsible for monitoring all research activities within the Center (research projects, article submissions, theses in development, reports) and taking action in selecting the appropriate strategy to turn science into applications. The results of these projects will be made available to the scientific community, but especial attention will be paid to protect inventions generated within the projects. PIs will fill an annual report indicating the potential innovations in process of development and the technology transfer committee will follow the areas closely in order to stimulate the researchers to think of possible commercial applications of their inventions, and to apply for intellectual protection. With support from our local technology transfer office (USP Innovation Agency), when appropriate, confidentiality disclosing agreement (CDA) will be implemented. In general, the foreseen routes to accomplish the technological transfer process are shown in the figure.

Prospection of potential partners will be achieved through regular contacts, facilitated by initiatives from the USP Innovation Agency, the Supera Incubator and the Technology Park, as well as the participation of the Coordinator Committee for Technology Transfer in partnering events, such as the Biotechnology Industry Organization Convention.

Opportunities and Perspectives. The wide spectrum of stem cell research has great potential for technology transfer in many aspects: cell therapy, drug screening, disease target identification for therapy and diagnostics, and production of recombinant proteins. As outlined in the research project, we will develop a program of basic, translational, and clinical research, centered on the study of normal and abnormal stem cells, in order to stimulate the development of products, protocols, and tools that could impact in the clinical setting. Therefore, special and continuous attention will be dedicated to the transfer of technology derived from each one of the proposed studies. Below we present examples of opportunities in the short-term identified in the proposal. A complete list can be found in the ***http://ctcusp.org***

<u>hiPSC library from the Brazilian population</u>: A project to establish a library of hiPSCs from a subset of the Brazilian population participating in the Longitudinal Study of the Adult Health (ELSA), sponsored by the Ministry of Health. This library will enable different basic and applied research projects, including (1) *in vitro* pharmacogenomics studies of known drugs; (2) *in vitro* "clinical" trials of new drugs, i.e, to attend the ever-increasing need of the pharmaceutical industry for assays to predict early the adverse effects of new compounds, and there is a great effort to develop cell-based assays to address these issues *in vitro.* In addition, drugs tested in a given population may have different effects on populations with diverse genetic

background. Thus, we identify the opportunity for licensing cell libraries for use by different companies interested in testing their new products in the Brazilian population *in vitro* before starting clinical trials.

Scale-up processes for therapeutic cells: As more cell-based therapeutics progress toward clinical testing, the development of reproducible, robust, and efficient processes for large-scale cell production becomes an imperative. Bioreactors allow for translating research-based experimental processes to be transformed into scalable cell-production processes in a safe and cost-effective manner. Thus, one of our main purposes is to develop bioreactor-based processes for cells already in clinical use such as MSC, and other potentially therapeutically cells in the future, such as regulatory T-cells, iPS cells, and platelets and erythrocytes derived from embryonic SC. Concomitant development of media may also provide opportunities for outlicensing.

The ***National Laboratory for Embryonic Stem Cell Research*** (LaNCE), a collaborator in this proposal, has a GMP facility for the ***production of hESCs and hESC-derived cells*** for therapy in small scale. Once the production is established and these cells prove to be safe in phase-I clinical trials, there will be also a need to expand production.

Stem cells as biofactories: MSCs can serve as cellular factories that secrete mediators (growth factor, cytokines, and chemokines) to stimulate *in situ* regeneration of injured tissues. Combined with the approach of improving the large-scale cell production, the characterization of their secretome will likely identify proteins with biotechnology potential and therapeutic applications, turning them into immediate products.

Molecular Diagnostics: In this context we will develop molecular diagnosis platforms for infectious diseases (mycoplasma, citomegalovirus, human parvovirus B19 and HTLV human T lymphotropic cell virus) which are relevant to cell therapy in accordance with GMP and hemotherapy needs.

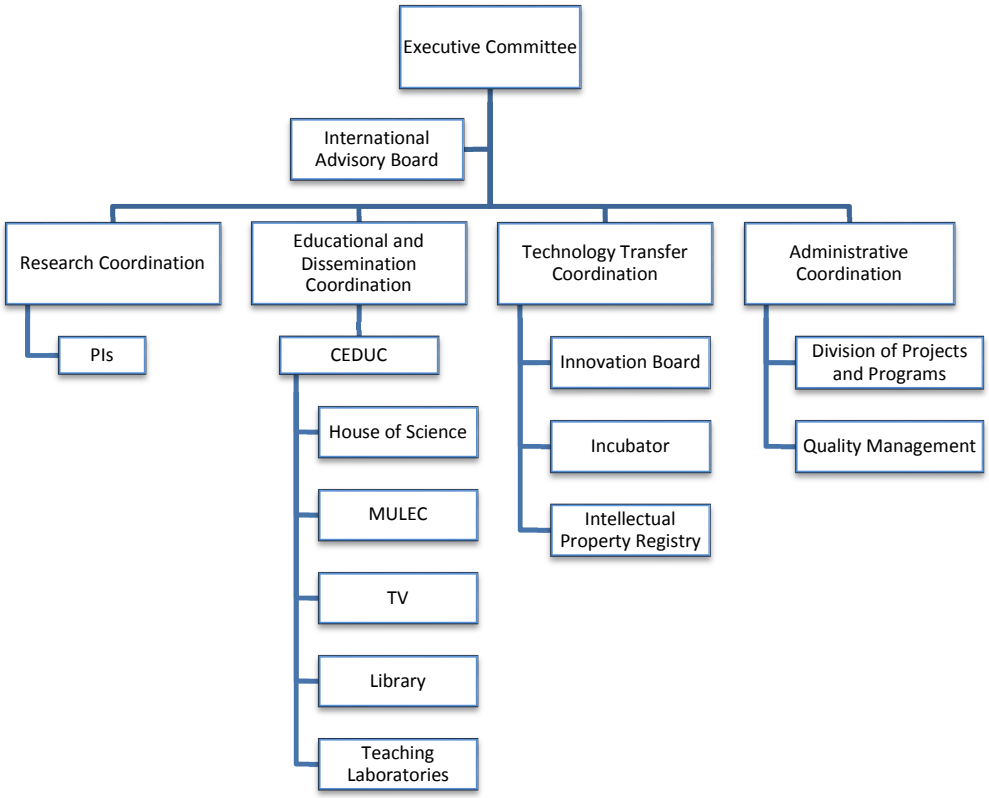
The major interest in telomere dysfunction causing disease prompted one of the PIs to collaborate with Qiagen (Germantown, Maryland, USA) to develop ***semi-automated high-throughput methods for measuring telomere length*** in large numbers of samples. This has already resulted in the development of a novel method for telomere length measurement using quantitative PCR (qPCR). Our Center is now working with Qiagen to improve this technology for the development of a multiplex monochromome method to measure telomere length. The potential goal is to collaborate with Qiagen to develop a standard kit for qPCR-based telomere length measumement, which would reduce the turn-around as well as standardize results in different laboratories.

Biotherapeutics: The center is also focused on the development of new recombinant coagulation factors (such as FVII, FVIII and FIX) with improved properties. The platform to produce these new recombinant proteins is based on human cell lines that are able to secrete recombinant proteins with the same glycosylation pattern found in the plasma-derived coagulation factors.

Molecular markers and new therapeutic targets in cancer: The Center will work to identify and characterize potential molecular prognosis markers that can help to identify patients with poor prognosis or tumors with refractory profile to therapeutic. The knowledge can provide important information to the discovery of new potential therapeutic targets in cancer.

Management Plan

The management of the CTC will follow the chart below.



Executive Committee (EC): composed by the Coordinator of the Center, Deputy Coordinator, Coordinator of Education and Knowledge Dissemination, Coordinator of Transfer of Technology and the Administrative Coordinator will be responsible for the overall coordination of the CTC. The EC will act in line with the recommendations of the ***International Advisory Board***.

***Coordination of Research(CR)*:** Composed by all the principal investigators (PIs) and represented in the EC by the Deputy Coordinator of the Center. This coordination will meet at least once every two months to suggest priorities in relation to acquisition of equipment, small items and quotas for consumables, and in relation to space usage.

Educational and Dissemination Coordination (EDC): Responsible to coordinate the outreach activities (education and dissemination of knowledge), carried out by the Educational Center of Blood Center of Ribeirão (CEDUC). The CEDUC, that includes the House of Science, the Museum and Laboratory for Science Teaching (MULEC), the TV studio, the library and the laboratories of education.

Technology Transfer Coordination (TTC): TTC will be responsible for innovation and issues related to intellectual property (generation, registration and licensing of new technology, products, processes and services generated by the Center). A permanent group of technicians and researchers will dedicate to the evaluation of the degree of innovation contained in the various projects in development, as well as the opportunities for the development of products, processes and services likely to be protected by patents or

registry of process. The transfer of these products, processes and services for the public or private sector should be evaluated preliminarily in this coordination and after submitted to the assessment of the EC. The TTC also must represent the Center in the ***Business Incubator of the Blood Center*** (SUPERA Hemocentro) and in the future ***Technological Park of Ribeirão Preto***. This coordination will be the responsible or the link with the ***USP Innovation Agency***, an administrative branch of the University of S. Paulo organized in 2005, which deals with all issues of technology transfer and intellectual property of that university.

Administrative Coordination (AC): AC will be formed by the Division of Programs and Projects of the Blood Center (business, purchases, contracts, services) and the Quality Management Department in charge of the systematic evaluation of the quality of administrative and technical processes developed by the Center.

The purchasing processes of equipment and consumables, the demands for the provision of services and the payments, once approved by the Coordinator of the Center, will be sent by Administrative Coordination to the competent sectors of FUNDHERP that will implement them in accordance with the current standards. The FUNDHERP is a foundation with an annual budget of about R$ 47 million, and operates under the control rules of State of São Paulo Audit Office.

Annual Meeting. The Member of the International Advisory Board, a FAPESP observer, the national and foreigner collaborators and all the participants of the Center will be invited to the Annual Meeting for performance assessment. Additionally the Center will hold quarterly meetings with the goals of team integration, exchange of scientific information, and critical evaluation of ongoing projects.

Executive Committee (EC): composed by the Coordinator of the Center, Deputy Coordinator, Coordinator of Education and Knowledge Dissemination, Coordinator of Transfer of Technology and the Administrative Coordinator will be responsible for the overall coordination of the CTC. The EC will act in line with the recommendations of the ***International Advisory Board***.

Executive Committee:

- Prof. Dr. Marco Antonio Zago
- Prof. Dr. Dimas Tadeu Covas
- Profa. Dra. Marisa Ramos Barbieri
- Prof. Dr. Flávio Vieira Meirelles

International Advisory Committee

The five scientists we have invited to become members of the international committee have large experience as researchers, as well as in science management and in the evaluation of research proposals and scientific papers; they also know all or some of the PIs personally, have followed our work for many years (in three cases more than 10 years) and three of them have visited the center more than once.

Peter Lansdorp, MD, PhD is a Distinguished Scientist at Terry Fox Laboratory, Professor of Medicine at the University of British Columbia, both in Vancouver, Canada, and Scientific Director of the European Institute on the Biology of Aging, University of Groningen, in Groningen, The Netherlands. Professor Lansdorp is a renowned physician-scientist on stem cells with ~250 publications and ~21,000 citations (h-index, 76), who worked on the purification and culture of human and murine hematopoietic stem cells in the 1980s, which led him to studies of telomere biology and the role of telomeres in stem cells.

Bob Löwenberg, MD, PhD is a Professor of Hematology at Erasmus University in Rotterdam, The Netherlands, and is known for his contributions to the pathobiology, diagnosis, and treatment of acute myeloid leukemia. Between 1982-1990, Professor Löwenberg was the scientific director of the Daniel den Hoed Cancer Center, Rotterdam, and between 1990-2011 he was Chairman of the Department of Hematology at Erasmus University Medical Center. He is member of the editorial board of the New England Journal of Medicine and elected Editor-in-chief of the journal Blood. Professor Löwenberg has published extensively (~600 publications; ~19,000 citation; h-index, 76) in leading scientific journals and has significantly contributed to the area of stem cell transplantation and molecular diagnostics and developmental therapeutics.

Gillermo Dighiero, MD, PhD is a Professor at Pasteur Institute, Associate Professor at the School of Medicine Paris VI, both in Paris, France, Executive Director of the Pasteur Institute in Montevideo, Uruguay, and member of the French National Academy of Medicine. Professor Dighiero is known worldwide for his extensive contribution to the investigation of the biology of chronic lymphocytic leukemia, the establishment of a prognostic classification, and the development of novel therapeutic strategies for this disease. Professor Dighiero has published ~200 articles in leading journals and has ~9,000 citations (h-index, 46).

Paolo Bianco, MD is a Professor at the Sapienza University of Rome, and Director of Stem Cell Laboratory of the Department of Molecular Medicine at the same University. He is known worldwide for his pioneering contributions to the field of somatic stem cells, especially Mesenchymal or Skeletal Stem Cell. He has published ~140 articles and has ~8,550 citations (h-index, 46).

Manoel Barral Netto, MD, PhD is a Professor at the Federal University of Bahia and Oswaldo Cruz Institute at Salvador, Bahia. At present he is one of the four directors of the Brazilian Research Council (CNPq), and has been the Dean of the Medical School and the Research Provost of the Federal University of Bahia. He is one of the most recognized Brazilian researchers in human immunology and parasitology. On the basis of Web of Science he has published 154 articles and received 3,000 citations (h=28).

The Research Team. The joint effort of the PIs for more than 10 years resulted in significant accomplishments in the scientific quality, infrastructure development, and education. The group was the first to propose and conduct clinical trials on autologous hematopoietic stem cell transplantation for the treatment of type 1 diabetes mellitus (Voltarelli et al. *JAMA 2007*; Couri et al. *JAMA 2009*). This study was made possible by the establishment of GMP-grade tissue culture and cryopreservation laboratories coordinated by Covas; analyses of cell transcriptome and proteome by Silva, Zago, and Greene; and clinical trials in the bone marrow transplant unit led by Voltarelli. The group also extensively collaborated in the study of mesenchymal stromal cells (Panepucci et al. *Stem Cells 2007*; Covas et al. *Exp Hematol 2008*; Saldanha-Araujo et al. *Stem Cell Res 2011*). Zago, Calado, Silva, and Covas worked jointly to identify the origins of these cells, as well as gene expression signatures and immunomodulatory properties. The Center is now the only one in the country able to provide mesenchymal stromal cells for clinical purposes and several clinical trials in the present proposal are based on this infrastructure. The investigation of acute promyelocytic leukemia as a model for cancer biology is an example of "bench-to-bedside" research established by the group (Figueiredo-Pontes et al. *PLOS One 2011*, Rego et al. *Blood 2009*, dos Santos et al. *Leukemia 2011*). We have developed new genetically-modified animal models for leukemia at a new animal care facility built specifically for this group of PIs for pre-clinical studies and involving the collaboration among Rego (animal models), Falcão (flow cytometry), and Greene (proteomics). Our group coordinates in Brazil *The International Consortium on Acute Promyelocytic Leukemia* (IC-APL), an initiative of the American Society of Hematology aiming to reduce the gap between developing and developed countries on the treatment outcome of patients with acute promyelocytic leukemia. This study involves researchers of our group (Rego, Silva, and Falcão) and other seven hematology centers across the country. The group worked together in the identification of molecular characteristics of other leukemia cells, such as the cancer-testis antigen expression in lymphoproliferative disorders (Proto-Siqueira et al. *Leuk Res 2006*; Figueiredo DL), which served as the basis for testing monoclonal antibodies for therapy. The interaction among Calado, Falcão, and Zago originated a pan-American effort (Brazil, US, and Canada) that established telomerase lesions as a risk factor for leukemia (Calado et al. *PNAS 2009*). The collaboration between Covas and Pereira resulted in the first Brazilian publication on induced pluripotent stem cells (Picanço-Castro et al. *Stem Cell Dev 2011*). In biotechnology, the group developed vectors and cellular systems for the production of the clotting factor IX, and the establishment of infrastructure for large-scale production for pre-clinical purposes. The collaboration between Covas and Meirelles has generated genetically modified calves harboring the human factor IX gene under the control of beta-lactoglobulin promoter (Monzani et al. submitted). On education, two major accomplishments should be highlighted. The creation of "Casa da Ciência" (House of Science) allowed the interaction of our scientists with more than 2,000 middle-school students and 150 teachers to learn about science and foster their future careers in science. The group jointly created the first *professional* Master program in biological sciences at the University of São Paulo as well as a new graduate program (MSc and PhD) in stem cells and oncology that will begin in 2012.

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Júlio César Voltarelli	Professor of Medicine, FMRP/USP
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Bruna Rodrigues Muys.	Master
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Júlia Teixeira Cottas de Azevedo.	Master
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Kalil William Alves de Lima	Master
Lucas Coelho Marliére Arruda.	Master
Katarina Holanda	Master
Katia Kaori Otaguiri.	Master
Lais Vicari de Figueiredo Pessoa.	Master
Livia Gonzaga Moura.	Master
Lucas Eduardo Botelho de Souza.	Master
Luiza Cunha Junqueira Reis.	Master
Luiza Ferreira de Araújo.	Master
Mariana Rodrigues Davanso.	Master
Mariana T. Benício	Master
Mariane Ferracin Martucce	Master
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Priscilla Carnavale Gomes Ferreira.	Master
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Tiago Henrique Camara de Bem	Master
Willys Tristão.	Master
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Daianne Maciely Alves de Carvalho	Scientific Initiation
Danielle Aparecida Rosa Magalhães	Laboratory Technician
Everton de Brito Oliveira Costa	Technical and Industrial Development Scholarship
George Maurício Navarro Barros	Medical Doctor
Heidge Fukumasu	Professor
Hudson Bezerra	Technical and Industrial Development Scholarship
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Cristiane Ayres Ferreira	Technical Administrative Support
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Elisete Luzia Gaspar de Carvalho	Technical Administrative Support

Fabiola Thais de Franca	Technical Administrative Support
Fernanda Teresinha Udinal	Technical Administrative Support
Greice Andreotti de Molfetta	Technical Administrative Support
Gustavo de Souza Arcodepani	Technical Administrative Support
Harrison Matheus Trevisan	Technical Administrative Support
João Pedro Pignata Júnior	Technical Administrative Support
Luis Henrique Rimel	Technical Administrative Support
Marcelo Gomes de Paula	Technical Administrative Support
Marcia Aparecida de Oliveira Brito	Technical Administrative Support
Maria Aparecida de Carvalho Grepe	Technical Administrative Support
Maria Cristina Morales da Silva	Technical Administrative Support
Maria Helena Silva Martins	Technical Administrative Support
Marlene Rossato	Technical Administrative Support
Monica Facincani Camacho	Technical Administrative Support
Patrícia Ferreira Pinho	Technical Administrative Support
Raquel Aparecida Botelho	Technical Administrative Support
Renata Aparecida Kurukawa	Technical Administrative Support
Rodrigo Gomes Teixeira	Technical Administrative Support
Sheila Aparecida Nakamura Prates	Technical Administrative Support
Silvia Elena Bonfarini	Technical Administrative Support
Sonia Aparecida Tomazini Pinto	Technical Administrative Support
Vanderlei de Paula Mendes	Technical Administrative Support
Vinicius Moreno Godoi	Technical Administrative Support