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,[1,2] its broad occurrence in human tissues,[2,3,4] relationship with pericytes and fibroblasts,[5] molecular mechanisms underlying immunomodulatory role[6,7]). In addition, we have demonstrated the role of the NFκB pathway in primitive progenitors[8] and TGF-beta signaling[9] and mitochondrial function[10] in acute myeloid leukemia cells. We have characterized crucial molecular mechanisms in hematological malignancies (expression of cancer-testis antigens,[11,12] miRNA expression profile,[13] multidrug resistance transporters,[14,15] and telomerase and telomere dysfunction[16,17,18,19]). We were the first Brazilian group establish new lines of human ES cells[20] and to to publish on iPS cells.[21] 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[22,23].

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.

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[9] de Figueiredo-Pontes, L.L. *et al.* PLoS One. 2011;6(10):e26713.
[10] dos Santos, G.A. *et al.* Leukemia. 2011; doi: 10.1038/leu.2011.216.
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[14] Figueiredo-Pontes, L.L. *et al.* Cytometry B Clin Cytom. 2008; 74(3):163-8.
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[17] Calado, R.T. *et al.* Leukemia. 2011. doi: 10.1038/leu.2011.272.
[18] Calado, R.T. *et al.* Blood. 2009; 114(11):2236-43.
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[21] Picanço-Castro, V. *et al.* Stem Cells Dev. 2011;20(1):169-80.
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1. Pluripotent Stem Cells

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

Embryonic stem cells (ESC) are able to self-renew while remaining pluripotent.[24] 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).[25,26]

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.[27] 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.[28] 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.[29] 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

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

Oct4 and Nanog as reporters associated with self-renewing and pluripotency; and the Retinoic acid receptor-RAR, associated with cell differentiation.

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)[30] and iPS[31] 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.[32] 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

[30] Miranda, MS. et al. Cloning and Stem Cells. 2009, 11:1-9.
[31] Picanço-Castro, V. et al. Stem Cells Dev. 2011, 20:169-180.
[32] Meirelles, FV. et al. Reprod. Fertility and Dev. 2010, 22:88-95.

with full development potential of ES cells[33]. Hence, using animal models harboring interspecific genetic variations in order to track allele-specific methylation and expression and previously developed by our group,[34] 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.

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[33] Stadtfeld M and Hochedlinger K., Genes Dev. 2010, 24(20):2239-63.
[34] Suzuki Jr, J. et al., Biol. of Reprod. 2011, 84:947-956.

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

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.[35]

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.[36] 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

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

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, 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.[37] 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

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

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 characterization of GD patients in the Brazilian population for 10 years, having analyzed mutations in the *GBA* gene in more than 1000 patients.[38][39] 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)*

<u>Diseases of mitochondrial DNA</u>

Mutations in the mitochondrial DNA (mtDNA) are etiologic in human diseasess that affects near 1/4,000 subjects.[40] 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.[41] Currently, only nuclear transfer (NT) has held promise to be used as a preventive strategy.[42] Our group had significantly contributed to the development of mammalian models in order to understand the mtDNA genetic bottleneck.[43] We have recently developed a method that enables the removal of as much as 90% of oocyte mtDNA[44]. By further

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

applying NT to this method, we showed that it is feasible to introduce foreign organelles of embryonic or somatic,[45] or of intra or interspecific origin.[46]

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 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,[47,48] 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.[4950] We will quantify p53, p21, MDM2 mRNA and protein levels in steady state and UV-stimulated *Dkc1^{m}* HSCs.

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[45] Ferreira, C.R. *et al*. Biol. of Reprod. 2010, 82:563-571.
[46] Chiaratti M.R. *et al*. Cellular Reprogramming. 2010, 12:231-236.
[47] Ruggero, D. *et al.* Science. 2003; 299(5604):259-62.
[48] Yoon, A. *et al.* Science. 2006; 312(5775):902-6.
[49] Bellodi, C. *et al.* EMBO J. 2010 Jun 2;29(11):1865-76.
[50] Westin, E.R. *et al.* Antioxid Redox Signal. 2011; 14(6):985-97

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.[51]

Hepatic cirrhosis is another clinical manifestation of telomere diseases, including dyskeratosis congenita.[52] 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, 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.[53] 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.[54] 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

[51] dos Santos, G.A. *et al.* Leukemia. 2011. doi: 10.1038/leu.2011.216.
[52] Calado *et al.* Hepatology. 2011; 53(5): 1600-7.
[53] Castilho-Fernandes et al. Exp Mol Pathol. 2011; 91(3): 664-72.
[54] Rego, E.M. *et al.* Proc Natl Acad Sci U S A. 2000;97(18):10173-8.

characterized the leukemia stem cells (LSCs) in APL.[55] 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 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,[56] 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

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[55] Guibal, F.C. *et al.* Blood. 2009; 114(27):5415-25.
[56] Rego, E.M. *et al.* Blood 2009; 114(22): Abstract 6

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.

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.[57] 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.[58] 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.[59] (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.[60] 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

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

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 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.[61] 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.[62] 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.[63] 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

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

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 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.[64,65,66] 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.[67]

The use of MSCs in the clinic has been mostly related to the treatment of graft-versus-host disease (GvHD).[68] 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.[69] In fact, we have demonstrated that MSCs up-regulate CD39 expression and adenosine production in response to activated

[64] Covas, D.T. *et al.* Braz J Med Biol Res. 2003;36:1179-83.
[65] Panepucci, R.A. *et al.* Stem Cells. 2004;22:1263-78.
[66] Silva, W.A. *et al.* Stem Cells. 2003;21:661-9.
[67] Covas, D.T. *et al.* Exp Hematol. 2008;36:642-54.
[68] Le Blanc, K. et al. Lancet. 2008;**371:**1579-86.
[69] Kamprera, M. Leukemia. 2011;25(9):1408-14.

T-cell-associated inflammatory stimuli, thus contributing to the suppression of proliferation of lymphocytes.[70,71]

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.[72] 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), 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.[73] The EMT is also activated in cancer progression and metastasis.[74] 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.[75] 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

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[70] Saldanha-Araújo, F. *et al.* Stem Cell Res. 2011;7:66-74.
[71] Saldanha-Araújo, F. *et al.* J Cell Mol Med. 2011;Epub ahead of print
[72] Polchert, D. *et al.* Eur J Immunol. 2008;38:1745-55.
[73] Thiery, J.P. *et al*. Cell. 2009; 139:871-90
[74] Wells, A. *et al.* Front Biosci. 2011; 16:815-37
[75] Nicolini, A. *et al.* Curr Pharm Biotechnol. 2011; 12:196-205

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) [76], 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 causes of fibrosis. Our Center has been studying MSC and endothelial cells (EC) for at least 10 years.[77,78] 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

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[76] Picanço-Castro, V. *et al.* Stem Cells Dev. 2011; 20:169-180.
[77] Covas, D.T., *et al.* Exp Hematol. 2008; 36(5): 42-54.
[78] Panepucci, R.A., *et al.* Stem Cells. 2004; 22(7): p. 1263-78.

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 published. Twenty-five recently diagnosed T1DM patients were included, 21 of which became insulin-free after transplantation.[79,80] 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.[81] 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.

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

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 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.[82,83,84,85] 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

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

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 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)*