

## PRECLINICAL STEM CELL THERAPY IN CHAGAS DISEASE: PERSPECTIVES FOR FUTURE RESEARCH

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### Abstract

Although Chagas cardiomyopathy was first described more than hundred years ago, it still remains a challenging problem that is responsible for high morbidity and mortality in Central and Latin America. Myocardial Chagas disease disrupts blood microcirculation via various autoimmune mechanisms, causing loss of cardiomyocytes and severe impairment of heart function. The discovery of stem cells capable of contributing to cardiac regeneration has provided the hope that cell therapy could salvage the damaged myocardium; however, the role of cell therapy in improving myocardial loss in patients with Chagas disease is still unclear. Different cell types and delivery approaches have been studied in both preclinical models and clinical trials. The aim of these strategies is to cause myocardial regeneration and to improve heart function. Nevertheless, prior to the final clinical application of the concept of cell therapy, several aspects will have to be overcome on the experimental level to ensure the safety of this important technology. The main objective of the current research is to clarify the reasons as to why the benefits that have been seen with cell therapy in preclinical models fail to translate to the clinical setting. The failure of stem cell therapy in preclinical models of Chagas disease can be explained by crucial differences between the cellular types and pathophysiological mechanisms of the disease, as well as the differences between human patients and animal models. In this article, we discuss examples that demonstrate how the results from preclinical trials might have overestimated the efficacy of the myocardial regeneration therapies. Future research should focus not only on studying the best cell type to use but, very importantly, understanding the levels of safety and cellular interaction that can elicit efficient therapeutic effects in human tissue. Addressing the challenges associated with future research may ensure the success of stem cell therapy in improving preclinical models and the treatment of Chagas disease.

Keywords: Chagas disease, preclinical, stem cell, therapy, co-cultured

### Introduction

Chronic Chagas disease is the most common form of cardiomyopathy in Central and South America and is one of the leading causes of death from cardiovascular disease in endemic areas. To date, there is no effective treatment for this disease apart from pharmacological treatment. Patients as

described above may derive some benefits from beta-blockers, inhibitors of angiotensin conversion enzyme and diuretics<sup>[1]</sup>.

The only effective treatment available for individuals who develop a more severe disease, such as heart failure due to Chagas disease, is total organ transplantation, i.e. heart transplantation. This procedure is limited due to its high cost, and the scarcity of donated organs; the immune suppressor drugs used in this situation can also reactivate the disease.

On the other hand, regenerative medicine has emerged with new perspectives on cell-based therapy to add to the established drug therapy of Chagasic cardiomyopathy in order to prevent heart failure progressing or to prolong and improve the quality of life of patients.

However, these possibilities should be viewed with caution in light of the pathophysiological aspects of cardiomyopathy.

There are currently various cell types that can be used in cell therapy: isolated cells, or in combinations, and associated (or not) with the arrays. Another important variable is the manner in which cells are administered: catheterisation, epicardial or intramyocardial injection. In this context, preclinical research is fundamental for better identification of the type of cell therapy that is functionally effective for translation to humans as well as for identifying the therapeutic availability and risks involved. Cell therapy should be both feasible and safe.

The aim of cell therapy in relation to the heart, independently of ischemic or Chagas disease, is to obtain myocardial regeneration and to improve heart function by cell-replacement therapy, as well as to reverse the geometric remodeling of ventricular cavities.

Consensus on the most appropriate form of cell therapy should be based on the best functional outcomes in preclinical studies. Animal models are important tools in experimental medical science to better understand the pathogenesis of human disease and to test therapeutic approaches.

Many reasons have been proposed for the failures of clinical trials, including the choice of cellular type for therapy. In this article we discuss the selection of preclinical models because this is one of the main reasons why clinical translation has been unsuccessful thus far. This issue has received little attention, but it may have had dramatic implications for the expectations of clinical trials. We highlight crucial differences between cellular types and pathophysiological mechanisms of the disease, as well as the differences between human patients and animal models, with regards to a better understanding of the results obtained so far and to reflect on the perspectives for future research. We use examples to demonstrate why the results from preclinical trials might have overestimated the efficacy of the myocardial regeneration therapies that have been developed to date. We also suggest ways in which currently available animal models of Chagas could be translated for human use and also offer advice on how to work with existing models to avoid overestimating the efficacy of single bone marrow cell therapies.

All models have advantages and disadvantages and the choice of stem cell therapy model should be based on the specific pathophysiological mechanisms of the disease; nevertheless, for myocardial regeneration, cell therapy requires the development of myogenesis, for contraction as well as angiogenesis, independent of the disease pathophysiology, because the striated muscle, like the myocardial and its cardiomyocytes, needs nutrition.

## Pathophysiological mechanisms

For the development of therapeutics based on stem cells in Chagas disease, some authors have considered approaches or tests that are similar to those performed in ischemic cardiomyopathies, not taking into account the fundamental differences between the pathophysiologies. This explains why the results obtained in humans differ from preclinical results; the intracoronary injection of autologous mononuclear cells in humans has not improved left ventricular function or the quality of life of patients with chronic Chagas cardiomyopathy. These results were different from those obtained in a preclinical model<sup>[2]</sup>.

The death of CMCs may be due to many factors, such as myocardial infarction or other causes, like Chagas cardiomyopathy, which cause fibrosis in the remodeling of the left ventricle due to the fact that adult CMCs have only a limited capacity to regenerate and are insufficient to resolve heart tissue injury<sup>[3]</sup>.

In myocardial infarction there is a loss of cardiac vascular supply, accompanied by pro-inflammatory events with increased production of 6-interleukin and tumor necrosis factor, leading to cellular necrosis, loss of CMCs in the heart region and heart dysfunction<sup>[4,5]</sup>.

The pathophysiology of the chronic form of Chagas cardiomyopathy is still not very clear. Among the various mechanisms are: a parasite-mediated tissue destruction, denervation plexus infarction, platelet aggregation, and intravascular lesion tissue mediated autoimmune mechanisms. The disproportion of parasites suggests relationships with autoimmune mechanisms<sup>[4-9]</sup>.

In Chagas disease, infection by *Trypanosoma cruzi* causes a generalised inflammatory vascular disease, characterised by the presence of vasospasm, a reduction in blood flow, focal ischemia, thrombosis, increased platelet aggregation, and higher levels of thromboxane A2 and endothelin-1. Endothelial cell infection by the parasite increases with the synthesis of endothelin-1, which participates in the vasospasm of the coronary microcirculation<sup>[5]</sup>.

In summary, myocardial Chagas disease is a diffuse lesion due to the interrupted microcirculation of blood vessel supplies mediated by autoimmune mechanisms, causing the loss of CMCs and remodeling process with the impairment of heart function.

## Cellular types

To obtain myocardium regeneration, various cell types were evaluated; undifferentiated cells such as stem cells, and differentiated cells like CMCs or myoblasts. However, not all cellular types have been evaluated in Chagas disease.

As regards undifferentiated cells, there are embryonic stem cells (ESCs) or adult stem cells (ASCs). The ASCs can be of diverse origin and can include: bone marrow-derived stem cells; bone marrow mononuclear cells (BMMNC, known as hematopoietic stem cells) such as CD45+/CD34-; hematopoietic-derived mesenchymal stem cells (hMSC, known as bone marrow mesenchymal stem cells) such as CD45-/CD34-; adipose-derived stem cells (ADSC); mesenchymal fraction such as CD45-/CD34-/CD105+/CD90+/CD73+; umbilical cord blood-derived stem cells (UCBSC); mononuclear cells

such as CD45+/CD34- and mesenchymal cells CD45-/CD34- ; and induced pluripotent stem cells such as OKT4+ (IPSC)<sup>[10,11]</sup>.

There are only three preclinical models that have been tested for cell therapy in Chagas cardiomyopathy: (a) bone marrow mononuclear stem cells, (b) co-cultured cells; myoblasts such as CD56+ with bone marrow mesenchymal stem cells and (c) isolated bone marrow mesenchymal stem cells. (6-9) The other cellular types are still only a theoretical approach<sup>[12]</sup>.

### **Adult stem cells (ASCs)**

#### Bone marrow- derived stem cells

Bone marrow was the first source of stem cells for application in various preclinical models for many diseases, including heart disease. This followed extensive clinical experience with these cells in their use for the treatment of onco-hematological diseases. Cells obtained from bone marrow have many advantages; they are easy to obtain and they do not require cultivation (reduced risk of contamination and for transformation), which allows the possibility of autologous therapy without the need for immune suppressor drugs and their adverse effects.

These cells can be obtained by puncture of the iliac crest bone marrow, or from peripheral blood by apheresis with the aid of granulocyte stimulating factor, which mobilises the cells of the bone marrow into peripheral blood. In addition to these advantages, there is increased knowledge about the immune phenotypic characterisation and the quantification of these cells by flow cytometric analysis, ensuring standardisation of protocols.

To test the efficacy of cellular therapy with stem cells from bone marrow in the Chagasic cardiomyopathy, the experimental model of inbred mice chronically infected with the Colombian strain of *T. cruzi* has been used, which caused the development of Chagasic cardiomyopathy in these animals. Mononuclear cells from bone marrow were obtained by lavage of the femurs of the animals and they were injected intravenously into mice during the chronic infection. The degree of inflammation and fibrosis in the heart was assessed after euthanasia of the treated and control animals and the histological sections of the heart were compared<sup>[13]</sup>.

The results of the aforementioned research demonstrated that treated mice showed a significant improvement in myocarditis two months after transplantation when compared to untreated controls. This was explained by the authors as the result of an increase in apoptosis in the inflammatory cells, which caused the loss of CMCs. A decrease in the area of fibrosis was also demonstrated, suggesting that this is a reversible process<sup>[13,14]</sup>.

Another strategy to better understand the action of BMMSC cells in myocardium repair was recently carried out by Jasmin *et al.* (2012)<sup>[15]</sup>. This study demonstrated the beneficial effects of MSC therapy in mice model of Chagas disease, arising from an indirect action of the cells in the heart, rather than a direct action due to the incorporation of large numbers of transplanted BMMSC into working myocardium. The authors used cell tracking, following the labelling of MSCs with nanoparticles to investigate the migration of transplanted BMMSCs to the heart.

### Co-cultured model of bone marrow mesenchymal stem cells and myoblasts

Carvalho *et al.* (2004)<sup>[6]</sup> proposed the autologous transplantation of the co-cultured bone marrow mesenchymal stem cells (BMMSC) and myoblasts for myocardial regeneration in Wistar rats. Their first report proposed the cultivation of both cellular types in a co-cultured model to obtain cells capable of promoting angiogenesis by BMMSC and myogenesis by myoblasts for ischemic myocardium, and at the same time to reduce costs and cultivation time. This co-cultured model had been tested previously in myocardial infarction and compared with myoblasts, co-cultured cells and control as SHAM (Dulbecco's Modified Eagle Medium-DMEM, without cells). The results demonstrated an improvement in left ventricular ejection fraction (LVEF) in both the groups that received cells, with additional results in histopathological analysis - the presence of angiogenesis and myogenesis in the group that received the co-cultured cells<sup>[6,16,17]</sup>.

This model was subsequently transferred for preclinical Chagas cardiomyopathy. In this particular study, 80 rats were inoculated with a single intraperitoneal injection of 150,000 trypomastigotes of *T. cruzi*. An ELISA test for Chagas disease was performed in a sample of the animals. After 8 months of inoculation, they underwent transthoracic echocardiography for baseline evaluation of heart function. Of the 15 animals that developed ventricular dysfunction with LVEF, less than 35% were randomly submitted to treatment. The incidence obtained of animals with Chagas cardiomyopathy was similar to that which has been described in humans<sup>[6,16-18]</sup>.

Seven animals underwent autologous co-cultured cell transplantation by direct injection ( $\times 10^6$  co-cultured product) in the epicardial in open surgery, *versus* eight animals in the control group, which was followed a natural evolution (not SHAM). At one month after treatment, all the animals were submitted to transthoracic echocardiography. The product of the co-cultured cells was identified by immunocytochemistry assay for identification; antibody anti-fast-myosin for skeletal muscle cells demonstrated by FITC immunofluorescence, and antibody anti-VIII factor for new vessels by demonstrated immunoperoxidase<sup>[16,17]</sup>.

One month after transplantation, in the echocardiographic functional analysis the group of Chagas disease that had received co-cultured cells demonstrated significantly improved LVEF,  $31.10 \pm 5.78$  to  $53.37 \pm 5.84$  *versus* natural evolution ( $p < 0.001$ ). There was also negative remodelling, which was demonstrated by left ventricular-end diastolic volume (LVEDV), co-cultured cells transplant group:  $0.83 \pm 0.08$  to  $0.64 \pm 0.16$  ( $p \leq 0.005$ ) *versus* natural evolution,  $0.68 \pm 0.12$  to  $0.72 \pm 0.16$  ( $p \geq 0.642$ ). Histopathological analysis demonstrated the presence of skeletal muscle cells, like myotube (immature skeletal muscle), and new vessels in hosted myocardial<sup>[16,17]</sup>.

This model demonstrates that negative LV remodelling, as well as reducing the progression of heart failure, may stabilise alterations in the biology of cardiomyocytes, (for example, hypertrophy) and maintain the contractile performance of myocardium<sup>[16,17]</sup>. On the other hand, Hagège *et al.* (2006)<sup>[19]</sup>, in relation to human ischemic cardiomyopathy, only transplanted myoblasts. In patients with severe heart failure, the clinical status and EF of patients improves in a stable manner over time, with a strikingly low incidence of hospitalisations for heart failure (0.13/patient-years) and arrhythmic risk can be

controlled by medical therapy and/or on-request automatic cardiac defibrillator implantation. In this preclinical model, arrhythmia was not observed<sup>[18]</sup>.

The co-cultured model seems to offer the promise of a treatment that adds to adjuvant therapy for Chagasic cardiomyopathy in patients and the bioprocess of this co-culture has been translated for use in humans; however, this model has not yet been evaluated in human Chagas Disease. Permission has been granted to test in I Phase Human by the Brazilian Human Research Ethics Committee, and testing should start soon.

## Perspectives

### Human Embryonic Stem Cells For Cell Therapy

In contrast to ASCs, ESCs have the potential to differentiate between the tissue derivatives of all three embryonic germ layers and therefore they are termed pluripotent. CMCs have been obtained from all three types of murine embryo-derived stem cells: embryonic carcinoma (EC), embryonic stem (ES), and embryonic germ (EG) cells. We focus our attention on ESCs due to their potential clinical application. hESC lines, isolated from the inner cell mass (ICM) of embryos, can be propagated continuously in the undifferentiated state when grown on top of a mouse embryonic fibroblast (MEF) feeder layer. When removed from these conditions and grown in suspension, they begin to generate three-dimensional differentiating cell aggregates, termed embryoid bodies (EBs)<sup>[19]</sup>.

Given the versatility of hESCs, and the possibility of obtaining beating CMCs from them, they appear to be the main candidate for cell-based applications for cardiac repair. In fact, hESCs apparently fulfill most, if not all, of the properties of an ideal donor cell line<sup>[20]</sup>.

A possible strategy for cell-replacement therapy could be to initially allow the spontaneous differentiation of ESCs into multiple lineages *in vitro*, followed by selective purification of the cardiomyogenic lineage isolated from embryoid bodies. On this issue, Kehat *et al.* (2001, 2004) showed that transplanted hESC-derived CMCs substituted damaged pacemaker cells in a swine model of atrioventricular block, and were responsible for eliciting an ectopic rhythm compatible with the animal's survival. Their results provide compelling evidence that this type of graft integrates electromechanically within the recipient tissue, as discussed by Menasché (2004)<sup>[20-23]</sup>.

Nevertheless, the following obstacles still remain unsolved:

(a) the yield of CMC production has to be dramatically improved. It is fundamental to work on the 'ideal' culture conditions for CMC differentiation. Unfortunately, the definition of strategies useful for this aim is not easy. The inherent differences between hESCs and their murine counterpart necessitate the obligatory use of hESCs as a model; laws and ethical considerations place strong limitations on what can be done. A further complication is represented by differences between the various protocols<sup>[23,24]</sup>.

(b) hESC lines and their characterisation which, to date, has been unsystematic<sup>[25-32]</sup>. It appears that each hESC line possesses a unique expression signature and a distinct cardiomyogenic inclination<sup>[33]</sup>. Stimuli useful for directing hESCs through the cardiac lineage are still only being investigated<sup>[32-34]</sup>. A methodic, combinatorial approach, using various stimuli (trans-stimuli, extra-cellular matrices, co-culture,

physical stimuli) could be the best way of directing the differentiation of stem cells *in vitro* in a cardiac stringent-specific way. This speculation is supported by the fact that, when in their natural milieu, cardiomyogenic differentiation of stem cells probably involves multiple signalling pathways. This may be mimicked *in vitro* with a combination of various methods that achieve a synergistic effect. In fact, *in vitro*-derived, prevascularised scaffold-free cardiac tissue patches from co-culture of CMCs, endothelial cells and fibroblasts were found to greatly improve cell viability, post-transplantation<sup>[34]</sup>.

(c) Culture media. For clinical applications, it is imperative to develop well-defined and efficient *in vitro* protocols for the cardiomyogenic differentiation of stem cells, which use chemically defined culture media supplemented with recombinant cytokines and growth factors. The main drawback of the current xenosupport system is the risk of cross-transfer of animal pathogens that might hamper future clinical applications. It was recently shown that non-human sialic acid Neu5Gc (against which many humans have circulating antibodies) was incorporated into hES cells grown on mouse feeder layers<sup>[35]</sup>. The use of human plasma-derived serum, and the development of a serum-free support system and animal-free feeder layer consisting of human fetal fibroblasts and adult epithelial cells or foreskin cells, may provide an appropriate solution to these risks. Nevertheless, *in vitro* up-scaling of clinical grade cell products that are essentially free of xenogenic products, in compliance with good manufacturing practice (GMP), remains a significant hurdle<sup>[36-40]</sup>.

(d) Competency of derived CMCs in terms of excitation-contraction coupling. Another important issue is to what extent these cells can be considered mature CMCs as regards excitation-contraction coupling. Indeed, heterogenous electrophysiological properties have been demonstrated in CMCs derived from separate differentiation methods within the same group<sup>[40]</sup>. This question cannot be accurately answered at the moment since the differentiation procedure has not been efficiently or even minimally standardised. However, some data provide fairly convincing evidence that hESCs can integrate electrically with the recipient myocardium, suggesting that they are capable of contributing to the augmentation of pump function following injury<sup>[20]</sup>.

(e) Immune rejection has to be blocked. Upon differentiation, ES cells express molecules of the major histocompatibility complex (MHC), in particular MHC I, while MHC II expression levels are low or absent<sup>[41]</sup>. Thus, decreasing the expression of MHC I by genetic modification could improve immunologic tolerance. Alternatively, minimal but targetted conditioning of CD4 and CD8 T-cells may be an option to promote tolerance of embryonic stem cell-derived tissues<sup>[42]</sup>.

(f) Tumorigenicity may be a problem, even when terminally differentiated CMCs are used for cell replacement. The implantation of undifferentiated ES cells leads to the formation of benign teratomas in the recipients <sup>[43-46]</sup>. Those risks are also present in all cultured cells, as demonstrated by Irioda *et al.* (2011)<sup>[47]</sup>.

As discussed by the aforementioned authors, an ES-derived teratoma is not essentially malignant, but its natural propensity to grow makes it potentially dangerous when implanted into an individual and, as such, a crippling obstacle on the path to ES cell therapeutics<sup>[48,49]</sup>. Recent experiments suggest that the formation of a teratoma may be dependent upon experimental conditions. For instance, Bjorklund *et al.* (2002)<sup>[50]</sup> have shown that teratoma formation could be prevented in a majority of cases, when pre-differentiated mouse ES cells were implanted into the brains of rats at a very low density.

Asano *et al.* (2003)<sup>[51]</sup> showed that ES cells implanted allogeneically into a non-human primate fetus in utero formed a teratoma when developed in a natural cavity, but conversely integrated normally in tissues when implanted within various organs. Therefore, teratoma formation does not appear to be an unavoidable consequence of ES cell implantation but rather as a phenomenon, the mechanisms of which require further investigation in order to identify the safest procedures for clinical application. Tumorigenicity demands the use of an extensively characterised, pure, differentiated cell population as well as rigorous cell screening.

The negative selection of Oct4 (undifferentiated cell marker) expressing cells might be a solution. New strategies and methodologies need to be developed to isolate the terminally differentiated cells. ES cell implants can be tagged with some kind of death signal in such a way that when they start to form tumors, or cause severe complications, they can be cleared from the body, leaving the host unaffected. Other safeguards proposed to purify CMCs, such as flow cytometry, cell sorting using cardiomyocyte-specific fluorescent dye or cardiac plasma membrane surface marker, and other strategies reviewed elsewhere, would further enhance the safety profile of these exogenously derived CMCs. As yet, there is no validated solution to this problem<sup>[51-54]</sup>.

Hence, it is probably unrealistic to assume that an approach designed to improve cardiac differentiation would be applicable to all hESC lines. Clearly, systematic characterisation is necessary in order to identify sub-categories of hESC lines. According to Stojkovic *et al.* (2004)<sup>[55]</sup>, one possible solution to this problem is the establishment of national or international hESC banks, which would allow comparable and detailed characterisation of deposited cells and provide scientists with all the necessary information to choose the most suitable hESC line for their own research<sup>[56]</sup>.

### **Somatic cell nuclear transfer (SCNT)**

Recently, high-profile reports of the derivation of human embryonic stem cells from human blastocysts produced by SCNT have highlighted the possibility of making autologous cell lines specific to individual patients<sup>[55]</sup>. Given the range of immunophenotypes of hESC lines currently available, rejection of the differentiated cells by the host is a potentially serious problem. SCNT offers a means of circumventing this by producing embryonic stem cells of the same genotype as the donor. However, this technique is not without problems since it requires the resetting of the gene expression programme of a somatic cell to a state consistent with embryonic development<sup>[43,56,57]</sup>.

The use of SCNT is currently under investigation from several points of view (ethical, scientific, technical/technological) and it has promising potential for the treatment of a variety of degenerative diseases. Furthermore, with the advent of other techniques such as xenofree, and direct differentiation of resident cells to CMCs, this may offer additional and exciting avenues for autologous cell therapy in the future<sup>[58-60]</sup>.

ESC and SCNT have excellent perspectives for future study in preclinical models of cardiomyopathy, such as Chagas or ischemic, but there are still many questions to be answered and those cells have not yet been evaluated in this preclinical model.



## Conclusion

The success of stem cell therapy in a preclinical model for treating Chagas disease is unsuccessful in human translation. Solutions are needed to provide acceptable levels of safety and strict quality control that would make possible the clinical applications of conducting therapy with stem cells in Chagas cardiomyopathy. Addressing the challenges associated with future research may ensure the success of stem cell therapy in the improvement of preclinical models and the treatment of Chagas disease.

## REFERENCES

- 1 **Perin EC**, Silva GV. Stem cell therapy for cardiac diseases. *Curr Opin Hematol* 2004; **11**: 399-403 [PMID: 15548994 DOI: 10.1097/01.moh.0000143359.77689.aa]
- 2 **Ribeiro SR**, Rassi S, Feitosa G, Grecco OT, Rassi AJr, da Cunha AB, de Carvalho VB, Guarita-Souza LC, de Oliveira WJr, Tura BR, Soares MB, Campos de Carvalho AC. Cell therapy in Chagas cardiomyopathy (Chagas arm of the multicenter randomized trial of cell therapy in cardiopathies study): a multicenter randomized trial. *Circulation* 2012; **125**: 2454-2461 [PMID: 22523306 DOI: 10.1161/CIRCULATIONAHA.111.067785]
- 3 **Mann DL**. Mechanisms and models in heart failure: a combinatorial approach. *Circulation* 1999; **100**: 999-1008 [PMID: 15927992 DOI: 10.1161/CIRCULATIONAHA.104.500546]
- 4 **Givertz MM**, Colucci WS. New targets for heart-failure therapy: endothelin, inflammatory cytokines, and oxidative stress. *Lancet* 1998; **352** Suppl 1: S134-S138 [PMID: 9736478 DOI: 10.1016/S0140-6736(98)90017-4]
- 5 **Nabors GS**, Tarleton RL. Differential control of IFN-gamma and IL-2 production during *Trypanosoma cruzi* infection. *J Immunol* 1991; **146**: 3591-3598 [PMID: 1902857]
- 6 **Carvalho KA**, Guarita-Souza LC, Rebelatto CL, Senegaglia AC, Hansen P, Mendonça JG, Cury CC, Francisco JC, Brofman PR. Could the coculture of skeletal myoblasts and mesenchymal stem cells be a solution for postinfarction myocardial scar? *Transplant Proc* 2004; **36**: 991-992 [PMID: 15194344 DOI: 10.1016/j.transproceed.2004.03.056]
- 7 **Soares MBP**, Pontes-de-Carvalho L, Ribeiro SR. The pathogenesis of Chagas' disease: when autoimmune and parasite-specific immune responses meet. *An Acad Bras Cienc* 2001; **73**: 547-559 [PMID: 11743602 DOI: 10.1590/S0001-37652001000400008]
- 8 **Soares MBP**, Lima RS, Rocha LL, Takya CM, Pontes-de-Carvalho L, Carvalho AC, Ribeiro-dos-Santos R. Transplanted bone marrow cells repair heart tissue and reduce myocarditis in chronic Chagasic mice. *Am J Pathol* 2004; **164**: 441-447 [PMID: 14742250 DOI: 10.1016/S0002-9440(10)63134-3]
- 9 **Pontes-de-Carvalho L**, Santana CC, Soares MB, Oliveira GG, Cunha-Neto E, Ribeiro SR. Experimental chronic Chagas' disease myocarditis is an autoimmune disease preventable by induction of immunological tolerance to myocardial antigens. *J Autoimmun* 2002; **18**: 131-138 [PMID: 11908945 DOI: 10.1006/jaut.2001.0574]
- 10 **Weissman IL**, Shizuru JA. The origins of the identification and isolation of hematopoietic stem cells and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood* 2008; **112**: 3543-3453 [PMID: 18948588 DOI: 10.1182/blood-2008-08-078220]
- 11 **Ma T**, Xie M, Laurent T, Ding S. Progress in the reprogramming of somatic cells. *Circ Res* 2013; **112**: 562-574 [PMID: 23371904 DOI: 10.1161/CIRCRESAHA.111.249235]
- 12 **Abdelwahid E**, Siminiak T, Guarita-Souza LC, Carvalho KAT, Gallo P, Shim W, Condorelli G. Stem cell therapy in heart diseases: a review of selected new perspectives, practical considerations and clinical applications. *Curr Cardiol Rev* 2011; **7**: 201-212 [PMID: 22758618 DOI: 10.2174/157340311798220502]
- 13 **Rossi MA**, Gonçalves S, Ribeiro SR. Experimental *Trypanosoma cruzi* cardiomyopathy in balb/c mice: The potential role of intravascular platelet aggregation in its genesis. *Am J Pathol* 1984; **114**: 209-215 [PMID: 6230012]
- 14 **Rocha A**, de Meneses AC, da Silva AM, Ferreira MS, Nishioka SA, Burgarelli MK, Almeida E, Turcato Júnior G, Metze K, Lopes ER. Pathology of patients with Chagas' disease and acquired immunodeficiency syndrome. *Am J Trop Med Hyg* 1994; **50**: 261-268 [PMID: 8147485]
- 15 **Jasmin JLA**, Koba W, Tanowitz HB, Mendez-Otero R, Campos de Carvalho AC, Spray DC. Mesenchymal bone marrow cell therapy in a mouse model of Chagas disease. Where do the cells go? *PLoS Negl Trop Dis* 2012; **6**: e1971 [PMID: 23272265 DOI: 10.1371/journal.pntd.0001971]
- 16 **Carvalho KA**, Guarita-Souza LC, Hansen P, Rebelatto CL, Senegaglia AC, Miyague N, Olandoski M, Francisco JC, Furuta M, Gremski W. Cell transplantation after the coculture of skeletal myoblasts and mesenchymal stem cells in the regeneration of the myocardium scar: an

- experimental study in rats. *Transplant Proc* 2006; **38**: 1596-1602 [PMID: 16797364 DOI: 10.1016/j.transproceed.2006.03.023]
- 17 **Guarita-Souza LC**, Carvalho KA, Woitowicz V, Rebelatto C, Senegaglia A, Hansen P, Francisco JC, Olandowski M, Brofman PR. Simultaneous autologous transplantation of cocultured mesenchymal stem cells and skeletal myoblasts improves ventricular function in a murine model of Chagas disease. *Circulation* 2006; **114** Suppl 1:I120-124 [PMID: 16820560 DOI: 10.1161/CIRCULATIONAHA.105.000646]
  - 18 **Sabino EC**, Ribeiro AL, VMC Salemi, Oliveira DIL, Antunes AP, Menezes MM, Ianni BM, Nastari L, Fernandes F, Patavino GM, Sachdev V, Capuani L, de Almeida-Neto C, Carrick DM, Wright D, Kavounis K, Goncalvez TT, Carneiro-Proietti AB, Custer B, Busch MP, Murphy EL. Ten-year incidence of Chagas cardiomyopathy among asymptomatic, *T. cruzi* seropositive former blood donors. *Circulation* 2013; **127**: 1105-1115 [PMID: 23393012 DOI: 10.1161/CIRCULATIONAHA.112.123612]
  - 19 **Hagège AA**, Marolleau JP, Vilquin JT, Alhéritière A, Peyrard S, Duboc D, Abergel E, Messas E, Mousseaux E, Schwartz K, Desnos M, Menasché P. Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients. *Circulation* 2006; **114** Suppl 1: I108-i113 [PMID: 16820558 DOI: 10.1161/CIRCULATIONAHA.105.000521]
  - 20 **Mummery C**, Ward D, Van den Brink CE, Bird SD, Doevendans PA, Opthof T, Brutel de la Riviere A, Tertoolen L, van der Heyden M, Pera M. Cardiomyocyte differentiation of mouse and human embryonic stem cells. *J Anat* 2002; **200**: 233-242 [PMID: 12033727 DOI: 10.1046/j.1469-7580.2002.00031.x]
  - 21 **Kehat I**, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001; **108**: 407-414 [PMID: 11489934 DOI: 10.1172/JCI12131]
  - 22 **Kehat I**, Khimovich L, Caspi O, Gepstein A, Shofti R, Arbel G, Huber I, Satin J, Itskovitz-Eldor J, Gepstein L. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat Biotechnol* 2004; **22**: 1282-1289 [PMID: 15448703 DOI: 10.1038/nbt1014]
  - 23 **Menasché P**. Embryonic stem cells pace the heart. *Nat Biotechnol* 2004; **22**: 1237-1238 [PMID: 15470458 DOI: 10.1038/nbt1004-1237]
  - 24 **Odorico JS**, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* 2001; **19**: 193-204 [PMID: 11359944 DOI: 10.1634/stemcells.19-3-1]
  - 25 **Carpenter MK**, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T, Lucero M, Rao MS. Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. *Dev Dyn* 2004; **229**: 243-258 [PMID: 14745950 DOI: 10.1002/dvdy.10431]
  - 26 **Rosler ES**, Fisk GJ, Ares X, Irving J, Miura T, Rao MS, Carpenter MK. Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn* 2004; **229**: 259-274 [PMID: 14745951 DOI: 10.1002/dvdy.10430]
  - 27 **Abeyta MJ**, Clark AT, Rodriguez RT, Bodnar MS, Pera RA, Firpo MT. Unique gene expression signatures of independently-derived human embryonic stem cell lines. *Hum Mol Genet* 2004; **13**: 601-608 [PMID: 14749348 DOI: 10.1093/hmg/ddh0]
  - 28 **Richards M**, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol* 2002; **20**: 933-936 [PMID: 12161760 DOI: 10.1038/nbt726]
  - 29 **Pekkanen-Mattila M**, Kerkelä E, Tanskanen JM, Pietilä M, Peltö-Huikko M, Hyttinen J, Skottman H, Suuronen R, Aalto-Setälä K. Substantial variation in the cardiac differentiation of human embryonic stem cell lines derived and propagated under the same conditions – a comparison of multiple cell lines. *Ann Med* 2009; **41**: 360-370 [PMID: 19165643 DOI:10.1080/07853890802609542]
  - 30 **Heng BC**, Haider HK, Sim EK, Cao T, Tong GQ, Ng SC. Comments about possible use of human embryonic stem cell-derived cardiomyocytes to direct autologous adult stem cells into the cardiomyogenic lineage. *Acta Cardiol* 2005; **60**: 7-12 [PMID: 15779844 DOI: 10.2143/AC.60.1.2005041]
  - 31 **Heng BC**, Cao T, Haider HK, Wang DZ, Sim EK, Ng SC. An overview and synopsis of techniques for directing stem cell differentiation *in vitro*. *Cell Tissue Res* 2004; **315**: 291-303 [PMID: 14758536 DOI: 10.1007/s00441-003-0847-5]
  - 32 **Heng BC**, Cao T, Tong GQ, Ng SC. Potential utility of cell-permeable transcription factors to direct stem cell differentiation. *Stem Cells Dev* 2004; **13**: 460-462 [PMID: 15588503 DOI: 10.1089/scd.2004.13.460]
  - 33 **Heng BC**, Haider HK, Sim EK, Cao T, Ng SC. Strategies for directing the differentiation of stem cells into the cardiomyogenic lineage *in vitro*. *Cardiovasc Res* 2004; **62**: 34-42 [PMID: 15023550 DOI: 10.1016/j.cardiores.2003.12.022]
  - 34 **Stevens KR**, Kreutziger KL, Dupras SK, Korte FS, Regnier M, Muskheli V, Nourse MB, Bendixen K, Reinecke H, Murry CE. Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. *Proc Natl Acad Sci USA* 2009; **106**: 16568-16573 [PMID: 19805339 DOI:10.1073/pnas.0908381106]

- 35 **Martin MJ**, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 2005; **11**: 228-232 [PMID: 15685172 DOI: 10.1038/nm1181]
- 36 **Ellerström C**, Strehl R, Moya K, Andersson K, Bergh C, Lundin K, Hyllner J, Semb H. Derivation of a xeno-free human embryonic stem cell line. *Stem Cells* 2006; **24**: 2170-2176 [PMID: 16741223 DOI: 10.1634/stemcells.2006-0130]
- 37 **Rajala K**, Hakala H, Panula S, Aivio S, Pihlajamäki H, Suuronen R, Hovatta O, Skottman H. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. *Hum Reprod* 2007; **22**: 1231-1238 [PMID: 17251355 DOI: 10.1093/humrep/del523]
- 38 **Amit M**, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, Itskovitz-Eldor J. Human feeder layers for human embryonic stem cells. *Biol Reprod* 2003; **68**: 2150-2156 [PMID: 12606388 DOI: 10.1095/biolreprod.102.012583]
- 39 **Unger C**, Skottman H, Blomberg P, Dilber MS, Hovatta O. Good manufacturing practice and clinical-grade human embryonic stem cell lines. *Hum Mol Genet* 2008; **17**: 48-53 [PMID: 18632697 DOI: 10.1093/hmg/ddn079]
- 40 **Pekkanen-Mattila M**, Chapman H, Kerkelä E, Suuronen R, Skottman H, Koivisto AP, Aalto-Setälä K. Human embryonic stem cell-derived cardiomyocytes: demonstration of a portion of cardiac cells with fairly mature electrical phenotype. *Exp Biol Med (Maywood)* 2010; **235**: 522-530 [PMID: 20407085 DOI:10.1258/ebm.2010.009345]
- 41 **Drukker M**, Katz G, Urbach A, Schuldiner M, Markel G, Itskovitz-Eldor J, Reubinoff B, Mandelboim O, Benvenisty N. Characterization of the expression of MHC proteins in human embryonic stem cells. *Proc Natl Acad Sci USA* 2002; **99**: 9864-9869 [PMID: 12114532 DOI: 10.1073/pnas.142298299]
- 42 **Robertson NJ**, Brook FA, Gardner RL, Cobbold SP, Waldmann H, Fairchild PJ. Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance. *Proc Natl Acad Sci USA* 2007; **104**: 20920-20925 [PMID: 18093946 DOI: 10.1073/pnas.0710265105]
- 43 **Hwang WS**, Ryu YJ, Park JH, Park ES, Lee EG, Koo JM, Jeon HY, Lee BC, Kang SK, Kim SH, Ahn C, Hwang JH, Park KY, Cibelli JB, Moon SY. Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. *Science* 2004; **303**: 1669-1674 [PMID: 14963337 DOI: 10.1126/science.1094515]
- 44 **Armstrong L**, Lako M, Dean W, Stojkovic M. Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem Cells* 2006; **24**: 805-814 [PMID: 16282443 DOI: 10.1634/stemcells.2005-0350]
- 45 **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556 DOI: 10.1126/science.282.5391.1145]
- 46 **Thomson JA**, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP. Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA* 1995; **92**: 7844-7848 [PMID: 7544005]
- 47 **Irioda AC**, Zocche L, Souza CMCO, Ferreira RJ, Aliprandini E, Cunha RC, Francisco JC, Guarita-Souza LC, Malvezzi M, Beltrame MP, Mesquita LAF, Kuczerka D, Chachques JC, Carvalho KAT. Pap test as the first step in screening genetic stability in cell-based therapy. *J Stem Cell Res Ther* 2011; **1**: 101-106 [DOI:10.4172/2157-7633.1000106]
- 48 **Doss MX**, Koehler CI, Gissel C, Hescheler J, Sachinidis A. Embryonic stem cells: a promising tool for cell replacement therapy. *J Cell Mol Med* 2004; **8**: 465-473 [PMID: 15601575 DOI: 10.1111/j.1582-4934.2004.tb00471.x]
- 49 **Mitjavila-Garcia MT**, Simonin C, Peschanski M. Embryonic stem cells: meeting the needs for cell therapy. *Adv Drug Deliv Rev* 2005; **57**: 1935-1943 [PMID: 16257083 DOI: 10.1016/j.addr.2005.06.001]
- 50 **Bjorklund LM**, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci USA* 2002; **99**: 2344-2349 [PMID: 11782534 DOI: 10.1073/pnas.022438099]
- 51 **Asano T**, Ageyama N, Takeuchi K, Momoeda M, Kitano Y, Sasaki K, Ueda Y, Suzuki Y, Torii R, Hasegawa M, Ookawara S, Harii K, Terao K, Ozawa K, Hanazono Y. Engraftment and tumor formation after allogeneic in utero transplantation of primate embryonic stem cells. *Transplantation* 2003; **76**: 1061-1067 [PMID: 14557753 DOI: 10.1097/01.TP.0000090342.85649.81]
- 52 **Hattori F**, Chen H, Yamashita H, Tohyama S, Satoh YS, Yuasa S, Li W, Yamakawa H, Tanaka T, Onitsuka T, Shimoji K, Ohno Y, Egashira T, Kaneda R, Murata M, Hidaka K, Morisaki T, Sasaki E, Suzuki T, Sano M, Makino S, Oikawa S, Fukuda K. Nongenetic method for purifying stem cell-derived cardiomyocytes. *Nat Methods* 2010; **7**: 61-66 [PMID: 19946277 DOI:10.1038/nmeth.1403]
- 53 **Van Hoof D**, Dormeyer W, Braam SR, Passier R, Monshouwer-Kloots J, Ward-van Oostwaard D, Heck AJ, Krijgsveld J, Mummery CL. Identification of cell surface proteins for antibody-based selection of human embryonic stem cell-derived cardiomyocytes. *J Proteome Res* 2010; **9**: 1610-1618 [PMID: 20088484 DOI: 10.1021/pr901138a]
- 54 **Hattori F**, Fukuda K. Strategies for ensuring that regenerative cardiomyocytes function properly and in cooperation with the host myocardium. *Exp Mol Med* 2010; **42**: 155-165 [PMID: 22172453 DOI:

- 10.1016/j.ttre.2011.09.003]
- 55 **Stojkovic M**, Lako M, Strachan T, Murdoch A. Derivation, growth and applications of human embryonic stem cells. *Reproduction* 2004; **128**: 259-267 [PMID: 15333777 DOI: 10.1530/rep.1.00243]
  - 56 **Reubinoff BE**, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T. Neural progenitors from human embryonic stem cells. *Nat Biotechnol* 2001; **19**: 1134-1140 [PMID: 11731782 DOI: 10.1038/nbt1201-1134]
  - 57 **Lanzendorf SE**, Boyd CA, Wright DL, Muasher S, Oehninger S, Hodgen GD. Use of human gametes obtained from anonymous donors for the production of human embryonic stem cell lines. *Fertil Steril* 2001; **76**: 132-137 [PMID: 11438331 DOI: 10.1016/S0015-0282(01)01825-8]
  - 58 **Rodriguez-Piza I**, Richaud-Patin Y, Vassena R, González F, Barrero MJ, Veiga A, Raya A, Izpisua Belmonte JC. Reprogramming of human fibroblasts to induced pluripotent stem cells under xeno-free conditions. *Stem Cells* 2010; **28**: 36-44 [PMID: 19890879 DOI: 10.1002/stem.248]
  - 59 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]
  - 60 **Ieda M**, Fu JD, Delgado-Olguin P, Hayashi Y, Bruneau BG, Srivastava D. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 2010; **142**: 375-386 [PMID: 20691899 DOI: 10.1016/j.cell.2010.07.002]