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ARTICLE

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# Human cardiomyocytes for drug discovery

## Cardiomiócitos humanos para descoberta de drogas

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### ABSTRACT

**Introduction:** Cardiac drug discovery are based in old methods that use animals, animal cells or modified cells that do not faithfully represent human cardiac phenotypes. **Objective:** Here, we aimed to show that cardiomyocytes derived from human iPS cells represent a new tool for cardiac drug discovery and could contribute to reduce animal use in research. **Method:** Generation of human iPS cells derived cardiomyocytes and its use for cardiotoxicity evaluation and infection with *T. cruzi* for drug discovery. **Results:** Definition of robust protocol for human iPS cells reprogramming, maintenance and cardiac differentiation. Derivation of high purity cardiomyocytes from hiPSCs that presented toxicity to different doses of doxorubicin and were amenable to infection of *T. cruzi*. **Conclusions:** Human cardiomyocytes derived from human iPS cells can be a great tool for drug discovery and can replace several assays done in animals helping to reduce animal use in research.

**KEYWORDS:** Human Cardiomyocytes; Drug Discovery; Doxorubicin; Animal Replacement

### RESUMO

**Introdução:** A descoberta de novas drogas para doenças cardíacas é baseada em métodos antigos que usam animais, células de animais ou células modificadas que não representam fielmente fenótipos cardíacos humanos. **Objetivo:** Neste trabalho, temos o objetivo de mostrar que cardiomiócitos derivados de células iPS humanas representam uma nova ferramenta para a descoberta de drogas cardíacas e poderiam ajudar na diminuição do uso de animais na pesquisa. **Método:** Geração de cardiomiócitos derivados de células iPS e seus usos para avaliação de toxicidade cardíaca e infecção por *Trypanosoma cruzi* para descoberta de drogas. **Resultados:** Definição de um protocolo robusto de reprogramação, manutenção e diferenciação de células iPS. Diferenciação de células iPS em cardiomiócitos com alta pureza que apresentam toxicidade a diferentes doses de doxorubicina foram suscetíveis a infecção com *T. cruzi*. **Conclusões:** Cardiomiócitos humanos derivados de células iPS podem ser uma potente ferramenta para descoberta de novas drogas e podem substituir diversos ensaios feitos em animais ajudando a diminuir o uso de animais em pesquisa.

**PALAVRAS-CHAVE:** Cardiomiócitos Humanos; Descoberta de Drogas; Doxorubicina; Substituição de Animais

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## INTRODUCTION

Since early 1920, animals have been used as models to evaluate toxicity of compounds before human use<sup>1</sup>. This is important to prevent harsh and dangerous side effects due to the administration of unknown drugs to humans. For more than 40 years, the toxicological field has evolved little in respect to the traditional way of measuring the toxicity of new compounds in animals, relying basically on the administration of different dosage (chronic or acute) to a large number of animals to observe tissue distribution and pathological implications of the drug use<sup>1</sup>.

Although very established and with unparalleled characteristics, *in vivo* pre-clinical safety and toxicological studies have remained costly, time consuming and, for some tissues (like heart, liver and brain), very superficial. Not surprisingly, problems in heart and liver account for almost 50% of the causes of drug attrition rate<sup>2</sup>, that is, when drugs fail in human tests (clinical trials), despite the fact that were previously thought to be safe in pre-clinical testing. This serves to show that we are in need of new tools that are able to evaluate more precisely toxicity and safety aspects of new drugs preferably in an efficient, high throughput and cheaper way than *in vivo* animal testing.

Since the advent of the human induced pluripotent stem cells (so called iPSCs) by Takahashi et al. in 2007<sup>3</sup>, the scientific community has heralded iPSCs as disruptive. Being able to induce pluripotency from adult cells from different donors open the avenue for a much-needed evolution in cellular models for normal and diseased tissues. Scientific community has always had problems in accessing various tissues types from donors. Tissues such as heart and brain are very scarce and hampered the advance of scientific knowledge about them. However, with iPSCs (and differentiation protocols)<sup>4</sup>, scientists are now able to obtain large amount of cells, from different tissues and genetic backgrounds (donors), including diseased ones<sup>5</sup>. Looking at this perspective, many institutions around the world have financed induced pluripotent stem cell banks that offer access to a great variety of material<sup>5</sup>.

Looking at this perspective, establishing a robust and powerful protocol for heart cell differentiation from iPSCs will provide a new tool to access cardiotoxic profiles of new compounds being developed for therapeutic applications. Our goal was to efficiently differentiate human induced Pluripotent Stem Cells (hiPSCs) into cardiomyocytes with high purity and robustness and evaluate their use for drug cardiotoxicity.

## METHOD

### iPSCs reprogramming and maintenance

Erythroblasts were cultured in a serum-free mononuclear cell (MNC) medium containing the following cytokines diluted in Stem Span Serum Free Expansion Medium (Stem Cell Technologies, USA): insulin-like growth factor 1 (IGF-1): 40 ng/ml; Stem Cell Factor (SCF): 100 ng/ml; Interleukin 3 (IL-3): 10 ng/ml; erythropoietin (EPO): 2 U/ml (all cytokines are from R&D Systems, EUA). Two million cells

were transfected with plasmids pEB-C5 and pEB-Tg (Addgene, USA), containing reprogramming factors Oct4, Sox2, Klf4, cMyc, Lin28 and SV40-T, using the Human CD34+ nucleofector kit and the Nucleofector II device, both by Lonza (Basel, Switzerland) following manufacturer's instructions. Reprogrammed erythroblasts were incubated in MEF-coated plates in MEF medium and FBS ES-Cell Qualified (Thermo Fisher, USA) with basic fibroblast growth factor (bFGF; 20 ng/ml) overnight. Then, they were transferred into embryonic stem cell (ESC) medium containing Knockout DMEM, Knockout Serum replacement, Antibiotic-antimycotic, Glutamax 200 mM, MEM non-essential amino acid solution, 2-mercaptoethanol supplemented with bFGF (20 ng/ml) (all from Thermo Fisher, USA) and Sodium butyrate (0.25 mM) (Cayman, USA). hiPSC colonies were passaged from a 6-well MEF-coated plate into Matrigel (BD)-coated plates with E8 medium (Thermo Fisher, USA), using Gentle Cell Dissociation Reagent (Stem Cell Technologies, USA) and 10  $\mu$ M ROCK inhibitor Y-27632 (Stemgent, USA).

For maintenance, colonies were passaged twice per week with Versene (Thermo Fisher, USA), following manufacturer's instruction, and plated as single-cells in a concentration of  $2,5 \times 10^5$  cells/cm<sup>2</sup>. E8 media (Thermo Fisher, USA) was used during the week and E8 flex (Thermo Fisher, USA) during the weekend. hiPSCs were routinely tested for mycoplasma and none contamination was detected.

### Cardiac differentiation of hiPSCs

iPSCs were differentiated using a monolayer differentiation method modified from previous reports<sup>6,7</sup>. iPSCs were grown in feeder-free conditions until they reached 60%-70% confluence. Cells were singularized, counted and plated ( $2,5 \times 10^5$  cells/cm<sup>2</sup>) E8 with 5  $\mu$ M of Ri (Cayman, USA). E8 medium was changed daily until cells reached 100% confluence. This day was considered day 0 and medium was changed to RPMI supplemented with 1X B27 supplement (Thermo Fisher, USA) without insulin (RB-) and 4  $\mu$ M of CHIR99021 (Merck, USA). 24 hours later, medium was changed to RB- supplemented with 10 ng/mL BMP4 (R&D Systems, USA). In day 2, medium was changed to fresh RB- supplemented with 2,5  $\mu$ M of KY2111 and XAV939 (both from Cayman). At day 4 and every two days, medium was changed to fresh CDM3<sup>7</sup>. Cells were grown for 30 days when passaged as single-cells to specific experiments.

### Embryoid bodies formation

Embryoid Bodies (EB) were generated as described by Lin and Chen<sup>8</sup> with minor modifications. Briefly, cells were cultured until reach 90% confluence and passage with Dispase (StemCell Technologies, USA), after complete dissociation, PBS (LGC Technologies, Brazil) were added and a gently up and down pipetting was performed to break big colonies. Cells were transferred to 15 mL tubes washed twice with PBS after centrifugation 100 x g for 2 minutes. Pellets were gently resuspended in E8 media supplemented with 4  $\mu$ g/mL of PVA (Sigma, USA) and cultivated in non-adherent plates (Sarstedt, German) for 1 day. Medium was changed to E6 (Thermo Fisher, USA) carefully to not remove EB



in suspension. Half of the medium was changed every 3 days until day 13, when RNA was collected using Trizol (Thermo Fisher, USA).

#### rt-PCR and qPCR

All procedures followed manufactures' instructions. For cDNA synthesis were used Promega's GoScript Reverse Transcription System # A5001, samples were diluted for 10 ng/mL. For the endpoint PCR were used Promega's GoTaq DNA Polymerase # m3008 and for quantitative PCR was used Power Syber Green Master Mix PCR #4367659. Primers sequences are available upon request.

#### Karyotype

iPS cell were grown in a 60 mm plate to reach 90% confluence. At this moment, cells were detached using Versene and collected in a 15 mL tube. Cells were treated with E8 supplemented with Colcemid (20 ng/mL) (Thermo Fisher, USA) for 1 hour in 37°C, washed with PBS and treated for 20 minutes 37°C with hypotonic solution (PBS supplemented with KCl 0,075 M). Cells were wash with PBS and a fixation step was performed. Fixation solution was Methanol: Acetic acid (3:1). All centrifugation steps were 200 x g for 4 minutes. Conventional chromosome analysis was performed on iPSCs cultures, using GTG banding at 400-band resolution according to standard protocols. A total of 10 metaphase cells were analyzed. Cell images were captured using the CytoVysion system (Applied Imaging Corporation, USA).

#### Immunofluorescence

iPS cells were plated in 96-well plates coated with Geltrex and cultivated for 2 days until 50% confluence. They were fixed with 4% PFA and stained with OCT4 (Santa Cruz # sc5279; 1:100 dilution), NANOG (R&D # af1997; 1:25 dilution) and TRA1-81 (Stemgent # 09-0006; dilution 1:100) antibodies and DAPI (Sigma, USA) to show nuclei. Image was generated in EVOS FL (Thermo Fisher, USA).

#### Flow Cytometry

For iPS cytometry, cells were grown until they reach 70-80% confluence and passaged with Versene. BD Stemflow #560589 was used following manufacturer's instructions.

Cardiomyocytes were plated in 6-well plates coated with Geltrex and cultivated for 7 days. After cell dissociation, they were fixed with 4% PFA and stained with anti-cardiac Troponin T (Fitzgerald # 20R-3024, dilution 1:10000), Myosin (AbCam # ab207926, dilution 1:4000), alfa-actinin (AbCam # AB9465, dilution 1:4000) and cardiac troponin I (BD # 564409, dilution 1:400) antibodies. Titration of antibody was done until no staining was found in iPS cells. For iPSCs and cardiomyocytes, data was acquired using Canto BD equipment and analyzed by FlowJo Software considering 2% of false positive events.

#### Cardiotoxicity assays

In Biosintesis toxicity test, three different batches of cardiomyocytes (05C146, 05C138, 05C146) were exposed to DMSO (20%) and Doxorubicin (1 and 10 µM) in 96-well plates. Toxicities of the substances were evaluated after 48 h of exposure using Neutral Red dye.

In CIEnP (Centro de Inovação e Ensaios pré-clínicos) toxicity test, three different batches of cardiomyocytes (05C022, 05C014, 05C065) were exposed to Doxorubicin (1 and 10 µM) in 96-well plates. Toxicities of the substances were evaluated after 48h of exposure using MTT<sup>9</sup> and Neutral Red<sup>10</sup> dye, for both the absorbance was measured using SpectraMax i3X (Molecular Devices). Results were shown using percentage of live cells related to control group treated with the vehicle.

#### Statistic

CIEnP data: data were expressed as mean ± standard error. To evaluate the significant differences between groups, an on way ANOVA followed by Newman-Keuls test was performed. Values below 0,05 were considered as an indication of significance. Analysis were carried out by GraphPad Prism Software (GraphPad Software Inc, USA).

Biosintesis data: The calculation of cellular viability in percentage (CV%) was done according to the formula:

$$CV\% = \frac{(\text{Average of Optical Density readings of the test substance / references substance})}{\text{Average of Optical Density readings of the cells control}} \cdot 100$$

Results of cardiotoxicity were subjected to ANOVA One-way variance analysis. Statistical difference among the groups was checked in the Bonferroni test with a reliability interval of 95%.

#### Trypanosoma cruzi culture

Trypomastigote infection: *T. cruzi* trypomastigotes (Y strain labelled with GFP)<sup>11</sup> were derived from the supernatants of infected LLC-MK2 culture cells (ATCC CCL-7; American Type Culture Collection, Rockville, MD). Cells were cultivated with RPMI 1640 medium plus 10% fetal bovine serum (FBS), at 37°C in 5% CO<sub>2</sub>. Free trypomastigote forms are found in the cell supernatants daily. Supernatant was harvest, centrifuged at 3000 rpm for 15 minutes and pellet was resuspended in RPMI media supplemented with B27 for cardiomyocyte infection. Cardiomyocytes were incubated during 24 hours with RPMI media supplemented with B27 containing 2.5 x 10<sup>5</sup> trypomastigotes (MOI 5:1). After 24 hours of infection, the parasites were discarded and the cells were washed 3 times with PBS, followed by addition of RPMI media supplemented with B27 free of parasites. Cells were cultivated for another 24 hours, up to 48 hours of infection.

#### Staining

After 48 hours of infection, cells were washed 3 times with PBS and fixed with 4% PFA for 30 minutes. PFA was discarded and cells were washed 3 times with PBS. Cells were incubated for one hour with DAPI (NucBlue - Molecular Probes) to stain nuclei and Alexa Fluor 555 phalloidin (Invitrogen) for actin staining. After staining, cells were washed with PBS and kept at 4°C until image acquisition in the Cellomics® ArrayScan® VTI High Content Analysis Reader with automatic autofocus acquisition of 50 pictures per well under 40X magnification.



**Amastigotes infection:** Cardiomyocytes attached to laminin-coated glass coverslips were infected with G strain extracellular amastigotes from *T. cruzi* as previously described<sup>12</sup>. After 72 hours, cells were fixed with 4% paraformaldehyde in PBS and parasites labeled with a human chagasic serum (with Alexa-488 anti-human Ig (Thermo Fisher, USA), in green), actin filaments stained with phalloidin-TRITC (Thermo Fisher, USA) and DAPI (Thermo Fisher, USA) was used to label nuclei and kinetoplasts.

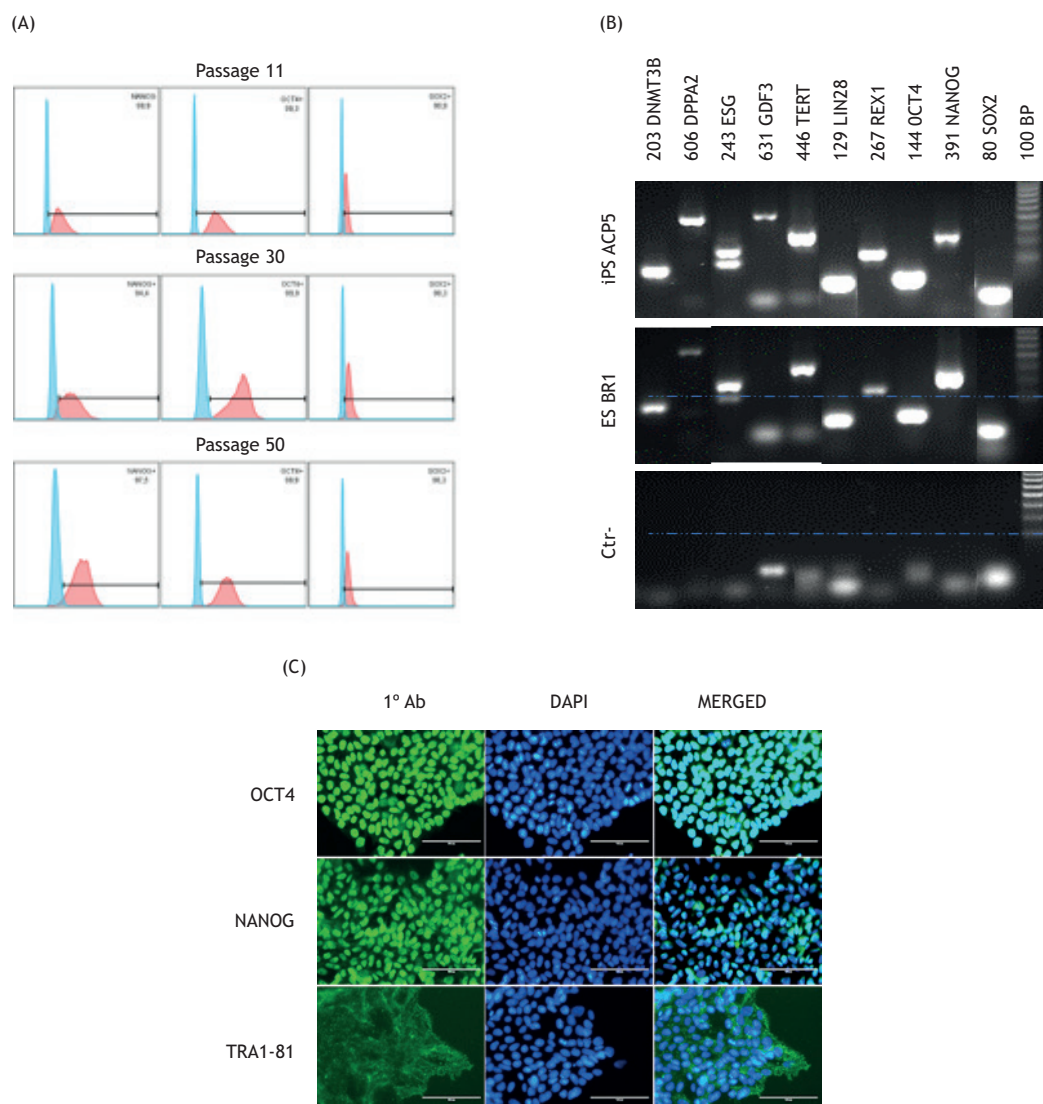
## RESULTS

### Generation of human iPS Cells

Erythroblast from a healthy donor (ACP) were reprogrammed using episomal vectors. iPS ACP5 clone was collected after colonies formation during reprogramming and expanded. Figure 1A shows flow cytometry analysis of hiPSCs transcription factors

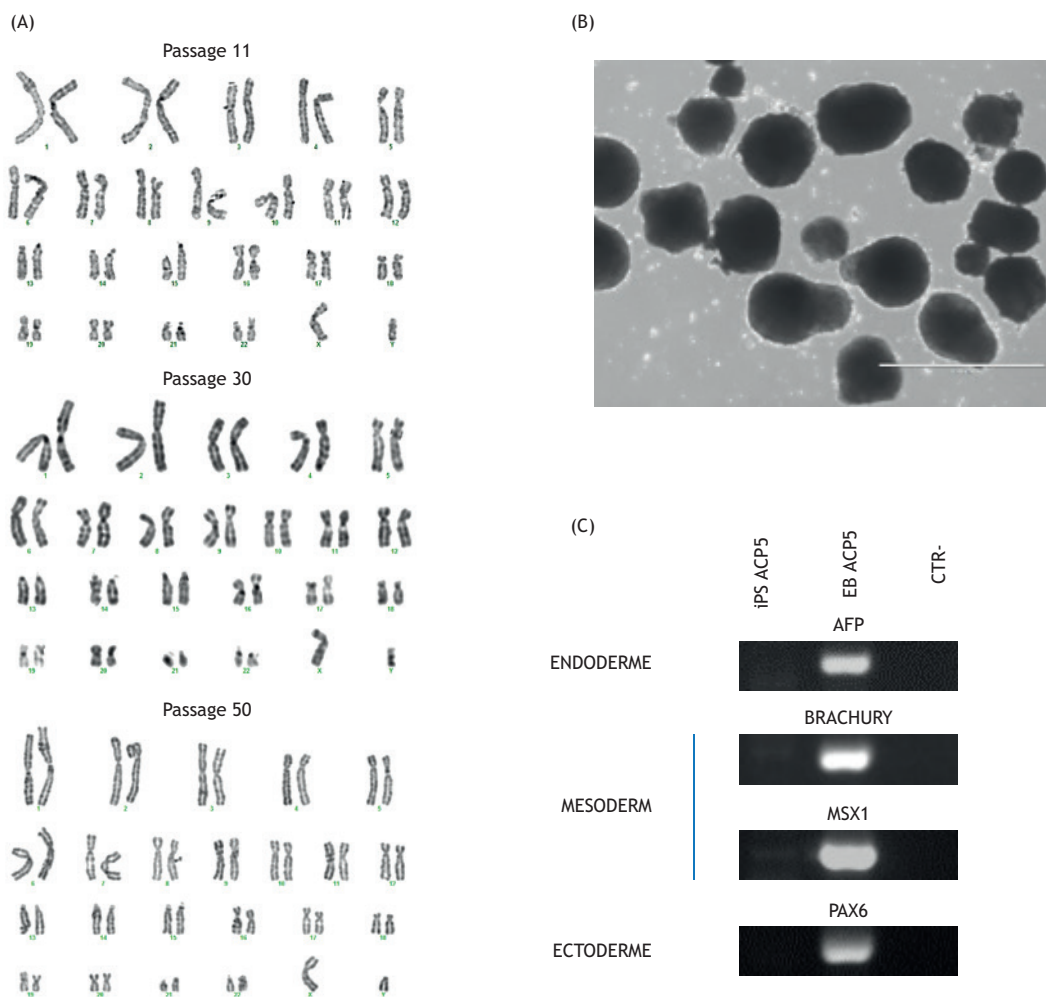
markers, cells show high positive staining for all markers even after long-term cultivation (50 passages). Transcription factors and membrane markers are also showed by immunofluorescence in Figure 1B. ACP5 shows expression of pluripotency markers genes for hiPSC (Figure 1C). To validate our method of passing as single-cells, we run karyotype analysis during long term cultivation, ACP5 clone presented normal karyotype until passage 50 (Figure 2A).

Finally, initial *in vitro* differentiation capability was evaluated by spontaneous differentiation of EB, after 13 days in suspension culture with no compound to induce specific differentiation. RT-PCR shows that differentiated cells expressed endoderm (AFP), mesoderm (BRACHURY and MSX1) and ectoderm (PAX6) markers (Figure 2B) demonstrating ACP5 clone can differentiate into the three germ layers *in vitro*. Taking together, these data confirm that our reprogramming and cultivation process of



**Figure 1.** iPS cells characterization; (A) Flow Cytometry for pluripotency nuclear markers at passages 11, 30 and 50; (B) endpoint PCR for pluripotency markers (DNMT3B = 203 bp); (DPPA2 = 606 bp); (ESG = 243 bp); (GDF3 = 631 bp); (TERT = 446 bp); (LIN28 = 129 bp); (REX1 = 267 bp); (OCT4 = 144 bp); (NANOG = 391 bp) ( Control 100 bp); (C) Immunofluorescence of pluripotency markers (OCT4, NANOG and TRA1-81). DAPI stain for the nucleus and MERGED stands for markers and nucleus staining together, scale bar: 100 µm.





**Figure 2.** iPS characterization. (A) Normal karyotypes during long term cultivation at passages 11, 30 and 50. (B) Embryoid body formation, scale bar 1000 µm. (C) endpoint PCR for the three lineages derivatives markers: Endoderm - marker AFP; Mesoderm - markers BRACHURY and MSX1 and Ectoderm - marker PAX6..

hiPSCs have high reproducibility and generated cells with good quality capable to differentiate in any cell type.

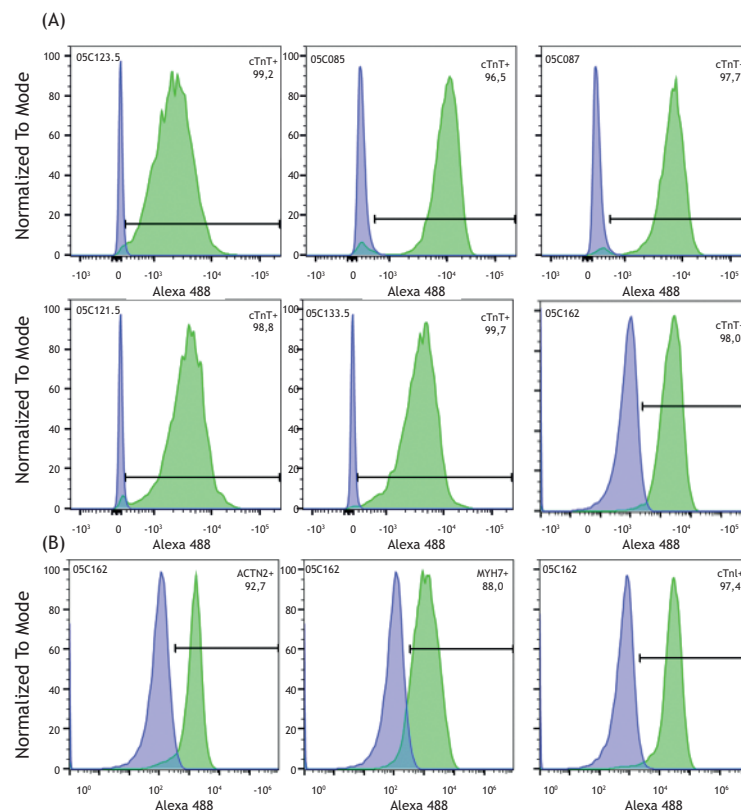
#### Cardiac differentiation of hiPSCs and its use for drug discovery

iPS ACP5 clone was then used to generate human iPSCs derived cardiomyocytes (iPSC-CM). Cells from passage 20 to 60 were used resulting in equally efficient cardiomyocyte differentiation (<https://youtu.be/07Thu63V-5o>). We show by flow cytometry the percentage of cardiac specific Troponin T expression (TNNT2 gene) in different passages (Figure 3A). Highly pure iPSC-CM population (above of 90%) was achieved regardless hiPSCs passage. Using hiPSC-CMs derived from hiPSCs at passage 60, we also show the presence of other specific cardiac proteins as  $\alpha$ -actinin (ACTN2); heavy-chain myosin (MYH7) and the mature form of Troponin I (TNNI3) (Figure 3B).

We next used cardiomyocytes with purity above 90% for the cardiac troponin T protein (Figure 4A and C) to see if these cardiomyocytes could be able to recapitulate doxorubicin cardiotoxicity.

We ran different analysis in two different laboratories that performed the assays under Good Laboratory Practice (GLP) conditions. Figure 4B (upper panel) shows doxorubicin toxicity assay performed by Biosintesis. Figure 4D (lower panel) shows doxorubicin toxicity assay performed by CIEnP. In both analysis, different concentrations of doxorubicin resulted in different levels of cardiotoxicity. Figures 4E-4H show the effect of different in-development possible drugs (the names are confidential) not related to heart diseases in human cardiomyocytes, for some drugs, toxicity was observed in low and high doses; and for other drugs, toxicity was only observed in higher doses. Taken together, these data suggest that cardiomyocyte generated from clone ACP5 can offer a great and reliable cell assay to test human cardiotoxicity.

Finally, we evaluated if human cardiomyocytes derived from iPSCs could be infected with *T. cruzi*. Figure 5 shows efficient infection despite the *T. cruzi* form used for the infection, both trypomastigotes (A) and amastigotes (B) forms successfully infected the cells. Cardiomyocytes continue beating even after nine days of infection (<https://youtu.be/SjRsB0sjd-M>).



**Figure 3.** (A) Flow Cytometry of cardiac Troponin T (cTnT) for different batches of differentiation. (B) Flow cytometry of  $\alpha$ -actinin (ACTN2), myosin heavy-chain (MYH7) and adult form of cardiac troponin I (cTnI). Numbers inside the chart in upper left position indicate lot, in upper right position indicate percentage of positive cells.

## DISCUSSION

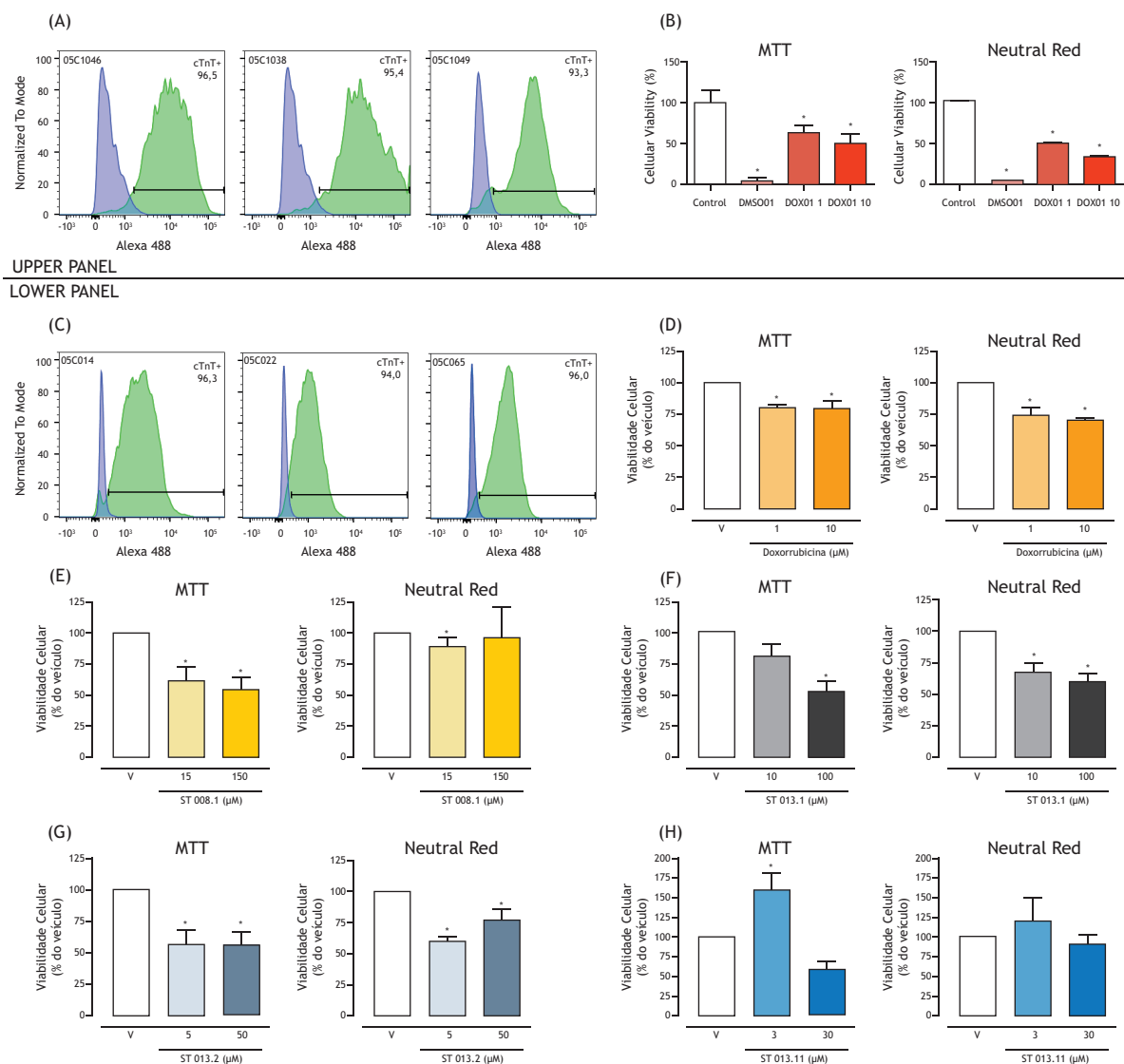
In this study, we have shown reliable reprogramming of blood from a single patient using non-viral and non-integrative approaches (Figure 1). Non-integrative approaches are desirable to prevent genetic modification of the cell line that could potentially damage an important component of the cell. Our results show that the lineage ACP5 has been faithfully reprogrammed showing all the basic characteristics of a pluripotent cell line reported in the literature such as expression of the key markers, morphology and genomic stability (Figure 2 and Heng and Fussenegger<sup>13</sup>). Karyotype analysis was carried out in different cell passages and cultivation up to passage 50 confirming genomic stability, which is in accordance to the literature<sup>14</sup>.

As for the cardiomyocyte differentiation protocol, we have been able to show that the cells obtained under our proprietary process are reliable and show the basic characteristics necessary for identification of cardiotoxic compounds (Figure 4). Doxorubicin is an anthracyclin used as an antineoplastic drug that has been demonstrated to clinically induce cardiotoxicity in a fraction of the treated patients<sup>15</sup>. In this study, we have shown that our cells respond to biological relevant doses of doxorubicin by undergoing cell death. It is important to note that the tests were done with 2 different partners (CiEnP e

Biosintesis), both of which work under GLP conditions and performed the test according to reference guidelines.

We have also been able to show that our cells are amenable to infection by *T. cruzi*, the infectious agent of Chagas' disease (Figure 5 and <https://youtu.be/SjRsB0sjd-M>), a major research interest among Brazilian scientific community for its public health impact. By developing this new model, we are able to bring many advantages to build upon the work of the Chagas disease scientific community, such as: 1) a more relevant model, by using human heart cells instead of dealing with the differences between animal and human heart cells; 2) replacing animal use by offering an alternative method that relies solely on human cells; 3) increased experimental reproducibility by offering the same cells and the same donor (genetic background); 4) possibility of accessing genetic differences that could be the cause of clinical variability of disease symptoms; 5) production of unlimited amount of cells and adaptation to high throughput drug screening platforms.

Taking all the information together, we can conclude that human iPSCs and cardiomyocytes derived from iPSCs are excellent research tools that should be supported by not only the Brazilian scientific community but also by its regulatory framework. As mentioned before, we are dealing with human biological material (cells) that have undergone extensive laboratory

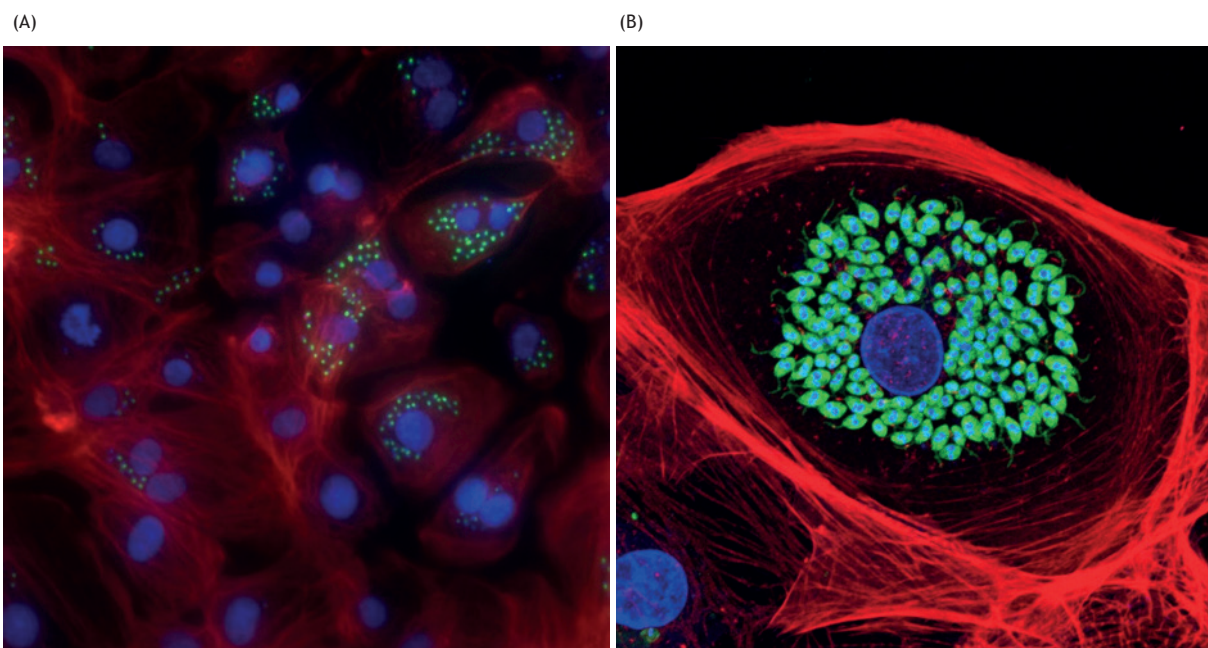


**Figure 4.** Upper panel; (A) Flow Cytometry of cardiac Troponin T (cTnT) for different batches of differentiation and (B) Biosynthesis' MTT and Neutral Red analysis of doxorubicin toxicity Doxo 1 and Doxo 10 stands for 10 μM and 10 μM of doxorubicin treatment. Lower panel; (C) Flow Cytometry of cardiac Troponin T for different batches of differentiation; (D) CIEnP's MTT and Neutral Red analysis of doxorubicin toxicity; and (E-H) CIEnP's MTT and Neutral Red analysis for possible new drugs not related with cardiac diseases. For flow cytometry, numbers inside the charts in upper left position indicate lot, in upper right position indicate percentage of positive cells.

manipulations to acquire the specific properties discussed in this publication. Internationally, these biological reagents are faced by regulatory agencies as RESEARCH USE ONLY (RUO) products, that is, they do not need any market registration or clearance from the regulatory agency to be commercialized. RUO label carries the understanding that the product is not allowed for human or diagnostic use. It is the seller's responsibility to provide that information and the buyer's responsibility to abide in it as well and make correct use of the product. In Brazil, the Brazilian Health Regulatory Agency (Anvisa) has already issued a Director Collegiate Resolution (RDC) that allows RESEARCH USE ONLY products to be commercialized in the country without the need for registration<sup>16</sup>. It is of clear understanding that the biological reagents reported in this publication fits exactly the aforementioned description.

It is important to highlight the importance of facing these biological reagents (human cells) as a commercial product as well. It may be argued that cells cannot be commercialized in Brazil for our constitution has prohibited such endeavor (art 199, #4). We suggest here that this understanding of the constitution had been surpassed and should be updated to reflect later advances in the scientific field, such as the advent of iPSCs, discussed in this publication. The Federal Attorney General's Office (Advocacia Geral da União) has publicly stated that it is not forbidden to commercialize biological products (human cells) that have undergone extensive manipulations in the laboratory (as to exclude the intention of commodification of body parts) because, due to technological advances, the commercialization of these biological products could lead, in the future, to outstanding medical and therapeutic applications ([http://bit.ly/AGU\\_parecer](http://bit.ly/AGU_parecer)). We





**Figure 5.** (A) Cardiomyocytes infection with *T. cruzi* in the trypomastigotes form, zoom 20X. (B) Cardiomyocytes infection with amastigotes form, zoom 100X. Red stain Actin, green are modified *T. cruzi* and Blue stain nuclei and kinetoplast.

enforce and echo this rationale, as this would open the gates for great biotechnological progress to our country, bringing legal certainty and investment opportunities.

It is well known by the Brazilian scientific community that there are no impediments (either ethical nor regulatory) to import human cells from abroad. Being the only requirement for the researcher, to have the Term of Consent of the donor of the biological material (cells) being used presented in his/her project to the Ethics Research Committee System and the National Commission on Research (*Sistema Comitê de Ética em Pesquisa/Comissão Nacional de Ética em Pesquisa - CEP/Conep*). In a commercial interaction (purchase of the cells), this document is given to the researcher (buyer) by the company (seller) selling the cells proving that the company accessed the biological material under an ethically approved Term of Consent. We suggest that by applying the

same reasoning to the Brazilian companies, we are, in fact, enforcing the principle of isonomy, a basic and highly appreciated principle of our constitution.

## CONCLUSIONS

In this publication, we have shown that we have the capacity to produce technically advanced and reliable biological reagent for the scientific community in Brazil and that the regulatory framework is already established to support commercialization of such biological products under addressable ethical requirements (Term of Consent). This is an important step towards biotechnological independence and shows that Brazilian technological competence has been increasing. Results reported here are in accordance to those reported elsewhere in the literature and more data are being collected using these cells to be reported in future scientific peer review publications.

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#### Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.



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