

Differentiation of Adipose Tissue-Derived Mesenchymal Stem Cells Into Cardiomyocytes

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Abstract

Background: Cardiomyocytes have small potential for renovation and proliferation *in vivo*. Consequently, the heart muscle has limited capacity of self-renewal. Mesenchymal stem cells (MSC) therapy, as well as MSC differentiated into cardiomyocytes, has been used in the attempt to minimize the effects of ischemic-hypoxic lesions and those affecting the electrical conduction system of the heart.

Objective: The present study compared three distinct protocols for induced differentiation of MSC into cardiomyocytes aimed at finding a viable method for producing a large number of functional cells expressing cardiomyogenic phenotype.

Methods: Mesenchymal stem cells were obtained from the adipose tissue of young transgenic Lewis rats expressing green fluorescent protein (GFP), and submitted to three distinct differentiation-inducing media: 1) Planat-Béarnard, 2) 5-azacytidine, and 3) Planat-Béarnard + 5-azacytidine; further, these cells were identified based on the expression of cardiac cell markers.

Results: All three protocols detected the expression of sarcomeric- α -actinin protein in the exoskeleton of cells, expression of connexin-43 in the nuclear and cytoplasmic membrane, and formation of gap junctions, which are necessary for electrical impulse propagation in the myocardium. However, no spontaneous cell contraction was observed with any of the tested protocols.

Conclusion: Induction with 5-azacytidine provided an effective cardiomyogenic cellular differentiation similar to that obtained with Planat-Béarnard media. Therefore, 5-azacytidine was the method of choice for being the simplest, fastest and lowest-cost protocol for cell differentiation. (Arq Bras Cardiol. 2012; [online].ahead print, PP.0-0)

Keywords: Somatic stem cells; 5-azacytidine; cellular therapy; cardiomyocytes; cell differentiation.

Introduction

Cardiomyocytes have small potential for renovation and proliferation *in vivo*. Consequently, the muscle has a limited capacity for replacing cell lost under hypoxic-ischemic conditions¹ or lesions in the specialized electrical conduction path of the heart^{2,3}. Depending upon the extension of these injuries, the necrotized cardiomyocytes are progressively replaced by fibroblastic tissue resulting in contractile dysfunction and congestive heart failure⁴.

In an attempt to minimize these cardiac damages and as an alternative to heart transplant, cellular therapy has been proposed as a method for repairing infarcted areas and to restore the electrical conduction system⁵. Due to its availability, feasibility, plasticity, and most recently, its capability for expressing cardiomyocyte phenotypes at specific differentiation media, the MSC originated from a variety of tissues have been utilized^{6,7}.

Valiunas et al⁸ and Kehat et al² demonstrated *in vitro* that MSC possess the ability to form functional gap junctions and generate spontaneous rhythm, while Plotnikov et al⁹ and Potapova et al³ verified *in vivo* that differentiated cells exhibit electrophysiological function similar to cells originated from cardiac tissue, which support the expectations around the therapeutic applications of MSC.

A variety of protocols regarding MSC differentiation into cardiomyocytes have been described, such as stimulus using demethylating agents^{2,6,10-12}, pool of growth factors¹³, co-culture with normal cardiomyocytes⁷, and specific gene insertions⁴. Despite satisfactory results, the *in vitro* differentiation into functional cardiomyocytes remains questionable. Little is known about the mechanisms underlying MSC differentiation^{11,14} furthermore, only a small proportion of MSC exhibit cardiomyogenic phenotype and the clinical benefits for cardiac repair remain limited¹⁵.

The present work aimed at identifying a viable method to produce cells for pre-clinical applications. In order to accomplish this, three distinct protocols to induce differentiation of adipose tissue-derived MSC into cardiomyocytes were compared.

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Methods

Animals

Animals were housed in individual cages and maintained at 23°C, 12-h light/dark cycle, fed with commercial chow and tap water *ad libitum*. This study followed the standards established in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, D.C., 1996) and respected the Ethical Principles in Animal Experiments of the Brazilian Society of Laboratory Animal Sciences (SBCAL). All procedures with animals were approved by the Ethics Committee on Animal Experimentation (CEEAA), under the protocol number 14/2011, at the Universidade Federal de Viçosa (UFV).

Transgenic rats were obtained by transfecting Lewis rats with lentiviral vectors expressing GFP under the control of ubiquitin C promoter (LEW-Tg eGFP F455/Rrc). The *in vitro* experiments were carried out using three transgenic, 4 weeks old male rats.

Cell culture

To obtain MSC, animals were euthanized in an anesthetic chamber by passive inhalation of isoflurane until overdose. Prior to placing animals in a laminar flow chamber, hair was clipped from the abdominal area. An incision was made in the Linea Alba to expose the peritoneum and remove the inguinal fat. The material collected was cut in small pieces, treated twice with PBS solution 0.15M, pH = 7.2 containing penicillin, streptomycin, and amphotericin B (PSA - Gibco, Paisley, UK), then maintained in Dulbecco's Modified Eagle Medium solution (DMEM - Gibco) supplemented with 10% fetal bovine serum (FBS - Gibco) and PSA.

The adipose tissue was digested with type I collagenase solution (Sigma, St Louis MO, USA) in a 5% CO₂ incubator at 37°C for 60 minutes while shaking every 15 minutes. This mixture was spun at 1500 rpm for 10 minutes, the supernatant removed and the pellet resuspended in complete DMEM. Initially, cells were plated at a concentration of 5x10⁶ in 75 mm² culture flasks (Sarstedt, Nümbrecht, Germany), and maintained in a 5% CO₂ incubator at 37°C for four to five passages. Prior to the differentiation phase, MSC were characterized by flow cytometry.

Cellular characterization

Cells were characterized by flow cytometric analysis of the expression of cell surface molecules CD 73 (anti-CD73 clone 5 F/B9 mouse — AbCam Cambridge, Massachusetts, USA), CD 54 (anti-CD54 clone 1A29 mouse — AbCam), CD 90 (anti-CD90 clone Ox-7 mouse - AbCam), and CD 45 (anti-CD45 clone 69 mouse — BD Bioscience, San Jose, Califórnia, USA), using flow cytometry FACScan and CellQuest® software, acquiring 30,000 events/sample, in the fourth passage.

Osteogenic differentiation

After the fifth passage, adherent cells were detached using trypsin, counted and replated in 6-well plates (TPP —

Zollstrasse, Trasadingen, Switzerland). These were covered by 22 mm diameter coverslips (Sarstedt), and maintained with DMEM enriched with FBS 10%, 10⁻⁸mol/mL of dexamethasone (Sigma), 5,0 µg/mL of ascorbic acid-2-phosphate (Sigma), and 10,0 mmol/L of β-glycerophosphate (Sigma) media, following incubation at 37°C for four weeks. On the 30^o day, the coverslips were washed with PBS and observed for calcium deposition after Von Kossa staining.

Cardiogenic differentiation

Cells from the fifth passage were detached with trypsin, counted and replated at a concentration of 1x10⁴ cells, maintained with culture media DMEM enriched with FBS 10% in 6-well plates covered by 22 mm diameter coverslips (Sarstedt) in the incubator.

Twenty-four hours later, the culture media was removed, the cells washed with PBS solution, and the plates submitted to distinct protocols of cardiogenic differentiation as depicted in Table 1. This process was carried out three times.

Plates were incubated in a 5% CO₂ incubator at 37°C for 15 days. During this period, cultures were observed and evaluated regarding morphological alteration and spontaneous contraction of the cells using an inverted optical microscope.

Immunofluorescence

After induction to differentiation, plates were washed with PBS solution and cells fixed with 4% paraformaldehyde in phosphate buffer for 15 minutes. After that, the cells were washed twice with PBS for 10 minutes and permeabilized with 0,25% Triton x-100 (Roche, Germany), followed by three washes with PBS only. The coverslips containing treated cells were blocked with 1% bovine serum albumin (BSA) and 5% goat serum in PBS. Then, they were incubated with the primary antibody for 2 hours in a humid chamber at 4°C.

Cardiogenic differentiation was carried out using primary antibodies: rabbit anti-sarcomeric-α-actinin (AbCam), 1:100 dilution, and rabbit anti-connexin 43, clone GJA1(CX-43 - AbCam), 1:400 dilution, followed by goat anti-rabbit and goat anti-rat Alexa 555 fluorochrome conjugated secondary antibodies.

The nucleus was stained with Hoescht (0.2 µg/mL). The coverslips were mounted over the slide with the aid of one drop of Hydromount (National Diagnostics, USA) and protected from light.

Samples were analyzed using confocal microscopy Zeiss 510 Meta and LSM Image Browser software (Carl Zeiss, Stuttgart, Germany). Cells incubated with secondary antibody only were used as negative controls. Images were viewed and individually captured using 420 to 460 nm (blue), 510 to 560 nm (green), and 560 to 660 nm (red) filters.

Results

Cell Culture

During the first two days, the culture was heterogeneous containing round and non-adherent cells with lipid micelles in

Table 1 - Distribution of the groups of adipose tissue-derived mesenchymal stem cells submitted to differentiation into cardiomyocytes

Group	Name	Composition	Protocol
G1	Control	DMEM media + FBS10%	Constantly maintained in the same media, changed every 3 to 4 days.
		DMEM media + FBS 15% + bovine serum albumin 1% + 10 ⁻⁴ mol/L of 2-mercaptoethanol + 2mmol/L of L-glutamine	
G2	Planat-Bérnard*	+ 10µg/mL of human recombinant insulin + 200 µg/mL of human transferrin + 10 ng/mL of recombinant IL-3 + 10 ng/mL of recombinant IL-6 + 50 ng/mL of recombinant stem cell factor (SCF recombinant).	Constantly maintained in the same media, changed every 3 to 4 days.
G3	Planat-Bérnard + 5-azacytidine	G2 + 5µM 5-Aza	Maintained in Planat-Bérnard media with 5-Aza for 24 hours, then maintained in Planat-Bérnard media.
G4	5-azacytidina	DMEM media + FBS 10% + 5µM 5-Aza	Maintained in the media for 24 hours, then in complete DMEM.

* Planat-Bérnard et al (2004)¹³.

the supernatant. After the 10th day, the cell population became more homogenous presenting an adherent fibroblastoid appearance and formed cell colonies.

Changing the culture media removed non-adherent cells along with lipid micelles. Plates containing 80% of confluent cells required trypsinization to generate new subcultures.

Adherent cells were phenotypically stable demonstrating no signs of senescence throughout the culture period.

Cellular characterization

Flux cytometry assay from adipose tissue-derived eGFP cells demonstrated that approximately 87.2% of cells expressed CD90, 92.1% CD73, 93.2% CD54, and 2.17% CD45 (Figure 1). This set of markers allowed the assumption that the isolated and cultured cell population was homogenous and clearly distinct from the hematopoietic lineage.

Osteogenic differentiation

Cells induced to osteogenic differentiation presented calcification in the matrix after the 10th day. These cells changed from fibroblastoid and elongated appearance to rounder shape, similarly to bone marrow lineage. Differentiation of MSC into osteoclasts was certified by the presence of an extracellular matrix rich in calcium, which was evident as brownish and blackish nodules of mineralization stained by Von Kossa staining, on the 30th day. These nodules were not found in the control group; neither did they present any morphological alteration, and maintained fibroblastoid characteristics.

Cardiogenic differentiation

Cells submitted to G2-differentiation protocol did not present morphological alteration compared to the control group (G1), and maintained fibroblastoid nature and its adherence to the plate during the 15-day evaluation period. Groups G3 and G4 presented an area 20% larger than the control group and fibroblastoid cells slightly round.

Regardless of the cardiogenic differentiation protocol, no binucleated cells or spontaneous contraction were observed.

In the 5-azacytidine groups (G3 and G4), 30% mortality of adhered cells was observed 24 hours after being in this differentiation media. Following that, the media was changed as described in the protocol. As this process removed the dead cells, the ones left behind were expanded in number five days later.

The MSC presented sarcomeric-alpha-actinin and connexin 43 expressions in all differentiation groups. However, this was not true for the control group (Figures 2 and 3). Expression of sarcomeric-alpha-actinin protein was observed in the exoskeleton of cells in all three differentiation protocols; this protein is typical of cardiac muscle cells. Connexin 43 expression was found in both nuclear and cytoplasmic membrane, mainly in the interaction points with neighboring cells, demonstrating on these the formation of gap junctions, which are required for propagation of the electrical impulse.

Discussion

The adherent cells observed in the initial phase of the culture did not necessarily represent MSC. Other cells such as fibroblasts, macrophages, and some other hematopoietic

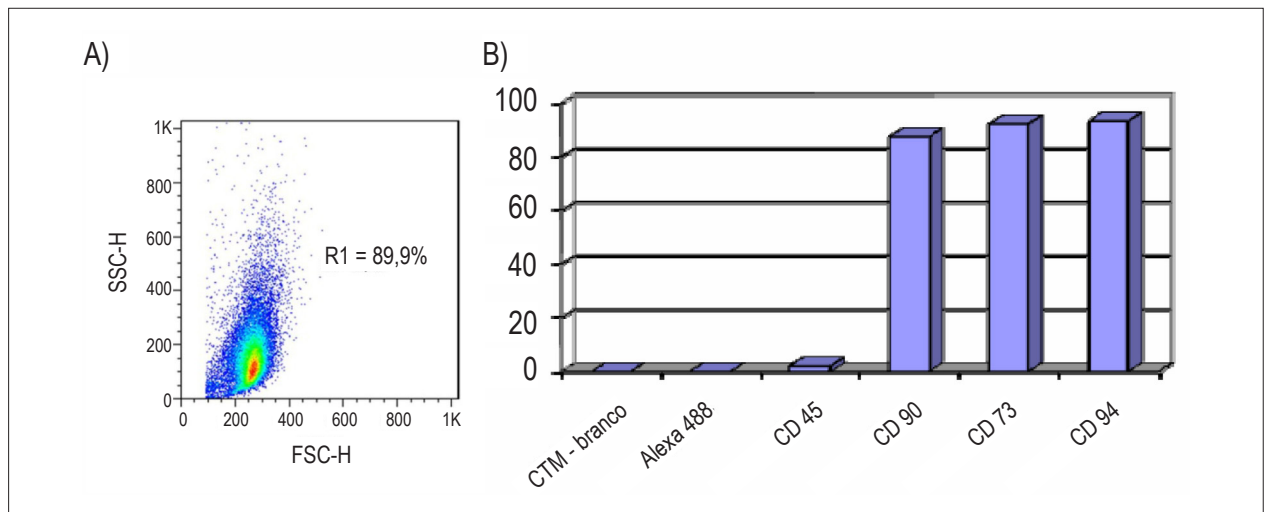


Figure 1 – Phenotypical evaluation of adipose tissue-derived MSC of Lewis eGFP rats, by flux cytometry. A) A plot of granulosity (SSC) versus size (FSC), demonstrating the cell population selected for the study (R1). B) Graphic representation showing the percentage of fluorescent cells inside the selected population (R1), using the antibodies anti-CD45, anti-CD54, anti-CD73, and anti-CD90. SSC – granulosity, FSC - size

cells may adhere to the culture plate and grow with similar morphological characteristics of MSC^{16,17}; therefore, the property to adhere to culture plates is not exclusive of MSC¹⁸, hence, this highlights the importance of performing other stages of characterization.

The phenotypic characterization of adipose tissue-derived MSC from Lewis eGFP transgenic rats showed lack of CD45 in 98% of the cells, and expression of CD73, CD54, and CD90 in 92.1; 93.2; and 87.2% of the cells, respectively. The phenotypic characterization of cells prior to differentiation allows inferring the culture's purity level. Other cell types found in bone marrow and adipose tissue may present morphological characteristics similar to MSC *in vitro*, such as hematopoietic cells^{19,20} and fibroblasts²¹. Hematopoietic cells express among other molecules CD45^{20,22}, which may be expressed in fibroblasts as well (Ishii et al., 2005). The molecule CD73 may be expressed in both fibroblasts and in MSC²¹. However, the molecules CD90²⁰ and CD54²³ expressed in more than 90% of the cells in this study are exclusively expressed on MSC, and not expressed in fibroblast or hematopoietic cells. Therefore, the phenotypical characterization was capable of confirming that more than 90% of the culture was indeed MSC, demonstrating the high level of purity of these cells.

The capability for osteogenic differentiation shown by adipose tissue-derived cells supports that the cells in the study were unquestionably MSC^{19,20,24}. The MSC changed from a fibroblastoid to a rounder appearance resembling osteoblasts^{22,25,26}. In addition, a pattern of mineralization nodules was observed, confirming that these cells, in fact, differentiated into osteoblasts²⁵⁻²⁸.

The 5-azacytidin, the Planat-Bérnard media, and the association of both were able to induce molecular modifications in adipose tissue-derived MSC from Lewis eGFP transgenic rats, generating cells capable of expressing cardiac markers similar to cardiomyocytes, as found by Planat-Bérnard et al¹³, Martin-Rendon et al¹⁵ and Bianco et al¹⁹.

The molecular cardiomyocyte markers connexin 43 and sarcomeric- α -actinin protein were expressed in groups G2, G3 and G4, and similar findings were observed by other authors^{2,6,29,30}.

Martin-Rendon et al¹⁵ demonstrated that 5-azacytidin can be used at either 5 μ M or 10 μ M concentration, offering no difference in the induction of MSC to cardiogenic differentiation. Additionally, varying exposure intervals to 5-Aza (24h, 24h-3 days, 3 days, or 1 week) did not promote an increase in the number of differentiated cells¹⁵. Conversely, increasing time to 5-azacytidin exposure can further increase the genotoxic effect of this demethylating agent; thus the recommendation is to use the shortest time of exposure and lowest concentration to induce differentiation as this reduces costs and time to acquire the desired cells. The 3 μ M of 5-Aza concentration was sufficient to induce cardiogenic differentiation of MSC^{10,31}.

The only morphological alteration exhibited by cells in the G3 and G4 groups was an increase in cell size. This had been previously described by Makino et al⁶. However, Haghani et al²⁹ observed morphologically round cells, larger than the fibroblastoid cells initially present in the culture, and binucleated cells. Group G2 did not show evidence of morphological alteration.

Spontaneous contraction was not observed in any treatment group, as opposed to previous studies, which used 5-Aza^{6,15} and Planat-Bérnard media¹³. This finding corroborates the results obtained by Balana et al³², where only a few contractile cells were observed *in vitro* while using 5-Aza in human bone-marrow-derived MSC; and the results demonstrated by Martin-Rendon et al¹⁵, in which the expression of myogenic markers occurs in approximately 0.07% of the MSC treated with 5-Aza, hence spontaneous contraction is not always observed.

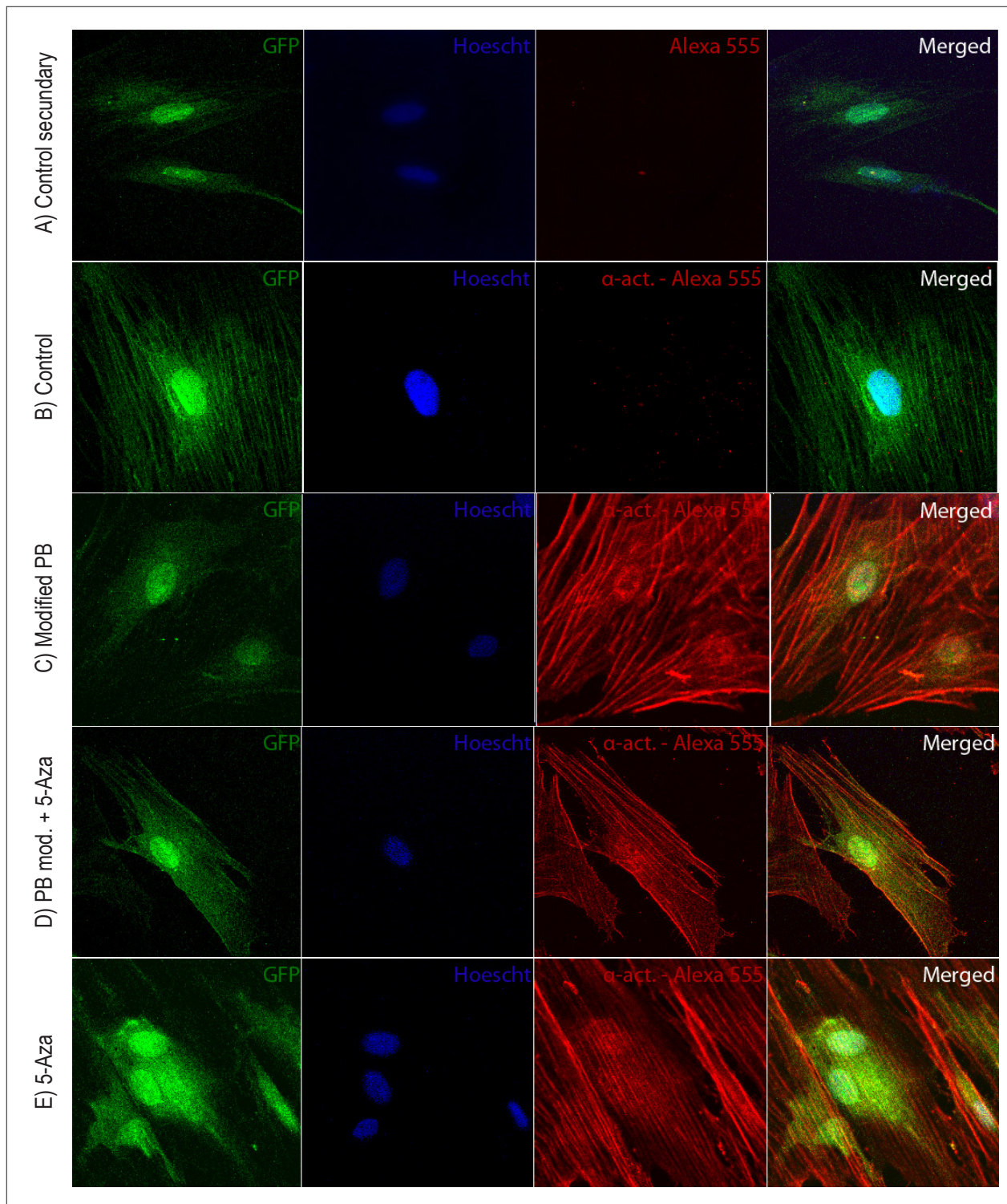


Figure 2 – Immunofluorescence analysis of adipose tissue-derived MSC. The result represents sarcomeric-alpha-actinin expression in MSC submitted to three protocols for cardiogenic differentiation (C, D, and E). The undifferentiated MSC (control) did not present expression of the evaluated protein (A and B). Panel A shows the secondary antibody test, demonstrating the lack of unspecific reaction with MSC. The nuclei is marked with Hoechst (blue), the MSC in green (GFP), and the tested antibody (anti-sarcomeric-alpha-actinin) in red (Alexa 555). The panel in the far right illustrates the superposition of images individually obtained with each filter (merged).

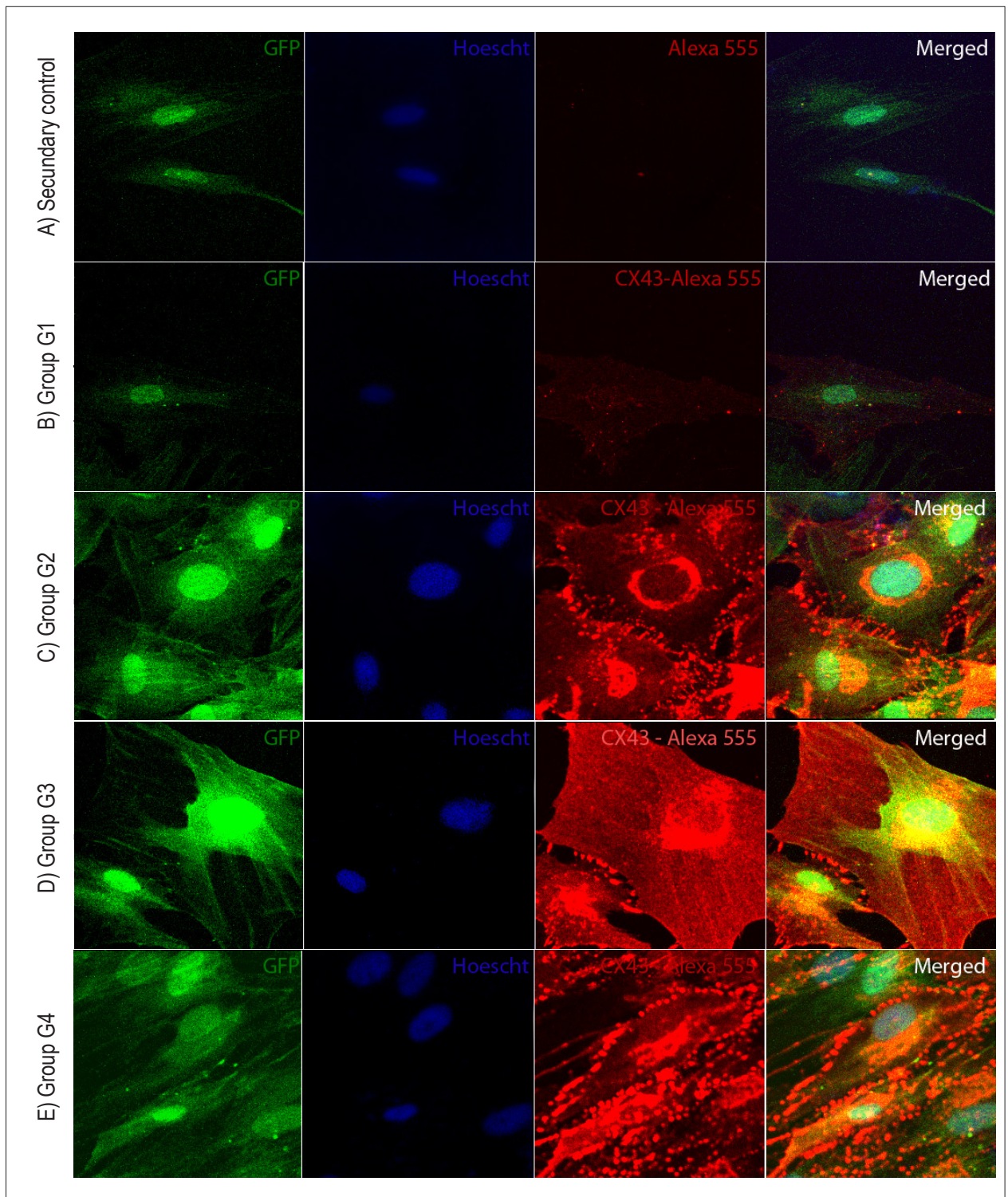


Figure 3 – Immunofluorescence analysis of adipose tissue-derived MSC. The result represents connexin 43 expression in MSC submitted to three protocols for cardiogenic differentiation (C, D, and E). The undifferentiated MSC (control) did not present expression of the evaluated protein (A and B). Panel A shows the secondary antibody test, demonstrating the lack of unspecific reaction with MSC. The nuclei is marked with Hoechst (blue), the MSC in green (GFP), and the tested antibody (CX43) in red (Alexa 555). The panel in the far right illustrates the superposition of images individually obtained with each filter (merged). Objective: 63x oil immersion.

Although Planat-Bénard et al¹³ demonstrated that 5-Aza exposition is required to induce contraction, group G3 presented no cells with spontaneous contraction. However, according to Valiunas et al^{4,8}, the lack of spontaneous contraction does not necessarily rule out the possibility of using these cells for cardiac disease therapy. The reasoning is that those cardiomyocytes differentiated from MSC showed molecular expressions of sarcomeric- α -actinin, connexin 43, and gap junctions, which allow cell contraction and electrical impulse propagation to other myocardial cells.

Data obtained *in vivo* by Toma et al³⁰ and Dai et al³³ demonstrated differentiation of the transplanted MSC into functional cardiomyocytes, differing from *in vitro* observations, in which voluntary contraction was not noted. This suggests that treatment with demethylating agents (5-Aza) or with other inducing methods may not be sufficient to differentiate adult MSC into cardiogenic line and that there are possibly other factors supplied by the "cardiac niche" affecting differentiation *in vivo*^{14,15}.

Induction with 5-Aza is a simpler and lower cost method of differentiation compared to the media developed by Planat-Bénard et al¹³. Additionally, the mechanisms of action of 5-Aza are better known^{11,12} and its use in research is more widespread^{6,10,15,34}.

For presenting molecular modifications similar to cardiomyocytes, but not showing any spontaneous functional activity, it is admitted that the MSC were pre-induced to cardiomyocytes, and presented cell characteristics to carry out cardiomyocyte functions¹¹, and will possibly accelerate their action in heart disease therapy compared to undifferentiated MSC. However, in order to confirm their functionality

in the myocardium, to prove their differentiation into cardiomyocytes and their propagation of physiologic electrical impulses in a synchronized manner, it is necessary to evaluate these cells *in vivo*.

Conclusion

Based on these findings, we concluded that adipose tissue-derived MSC subject to three distinct protocols of cardiogenic differentiation were capable of causing pre-induction to differentiated cells. This was documented by expression of gap junctions and sarcomeric- α -actinin protein, necessary for electrical impulse propagation and contraction of the heart. Five-Azacytidin is an effective agent in promoting cardiomyogenic differentiation similar to that found using the Planat-Bénard media. Furthermore, 5-Aza is a simpler, faster and lower-cost method of differentiation. Therefore, it is the recommended method to achieve MSC differentiation into cardiomyocytes.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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