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Short Communication

Differentiation of Embryonic Stem Cells into Cardiomyocytes on Polymeric Substrate

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Abstract

Among the current biomaterial applications, the use of biopolymers as cell scaffolds could aid the development of stem cell-based therapies aimed at replacing tissue loss due to injuries or diseases, such as during myocardial infarction. Embryonic stem cells (ESC) provide a great therapeutic potential for cardiac tissue. In this study, the interaction of mouse embryonic stem cells (mESC) with poly L-co-D, L lactic acid-co-trimethylene carbonate (PLDLA-co-TMC) was assessed. The characterization of this material was made by means of SEM, DSC, TGA, GPC, Tensile Test, Viability Assay, and stem cells behavior by qPCR and Immunofluorescence techniques. From the obtained results, we conclude that the TMC elements added to PLDLA influenced the material proprieties, providing greater flexibility to the biopolymer and showed weight loss throughout the hydrolytic degradation process. mEScell growth and differentiation on PLDLA-co-TMC membrane, demonstrated the cyto compatibility of this polymer, and provided evidence of minimal to no toxicity on the cells, allowing adhesion and cell proliferation, as well as differentiation into cardiomyocytes. Overall, these results present the polymer as a potential scaffold to be exploited in cardiac tissue regeneration.

ABBREVIATIONS

CPC: Cardiac Progenitor Cell; DSC: Differential Scanning Calorimetry; mESC: Mouse Embryonic Stem Cell; GPC: Gel Permeation Chromatography; IF: Immunofluorescence; Isl-1: Insulin Gene Enhancer Protein 1; LIF: Leukemia Inhibitory Factor; MHC: Myosin Heavy Chain; MI: Myocardial Infarct; MPa: Megapascal; MTT: 3-(4:5-Dimethyl-2-Thiazolyl)-2:5-Diphenyl-2H-Tetrazoliumbromide; Nkx2.5: Homeobox Protein Nkx2.5; PCR: Polymerase Chain Reaction; PLDLA: Poly L-*co*-D: L Lactic Acid; PTMC: Poly Trimethylene Carbonate; SEM: Scanning Electron Microscopy; TGA: Thermogravimetric Analysis

INTRODUCTION

According to the World Health Organization, heart disease, especially myocardial infarction, (MI) is the first cause of morbidity and mortality worldwide [1]. Furthermore, MI represent a meaningful problem for health and economy in the western countries [2].

Because of the impaired self-renewal capacity of cardiomyocytes and the limited number of cardiac progenitor cells, the human heart lacks the ability to restore its function after injury [3]. Currently, surgical intervention combined with pharmacological treatment remains the only avenue aimed at improving patient outcome [4]. However, despite the benefits of conventional interventions in the treatment of MI symptoms, the challenge of repair of the damaged tissue remains unresolved. Therefore, tissue engineering with cell therapy emerges as a promising alternative to remedy these problems [5,6].

Cell therapies would greatly enhance and extend the lives of patients with muscle wasting conditions due to diseases and/or aging. Embryonic stem cells (ESCs) have unlimited proliferation potential and evidence thus far suggests that embryonic stem (ES) cell-derived cardiomyocytes or cardiomyocyte progenitor cells can repair damaged myocardium in animals [7]. Mouse ES (mES) cells pre-differentiated into cardiomyocytes contributed to cardiac repair in a rat infarct model, forming new cardiac tissue within the myocardial scar [8,9].

Basically, the four main steps required for cardiomyocytes generation from pluripotent stem cells are: (i) mesoderm formation, (ii) mesoderm patterning toward cardiogenic mesoderm and anterior mesoderm, (iii) formation of cardiac mesoderm and (iv) maturation of primary cardiomyocytes [10].

Induction of mES cells to differentiate into cardiac cells following these steps can be characterized by the expression of transcription factors such as, Brachyury-T for primitive mesoderm, Isl-1 for cardiogenic mesoderm, Nkx2.5 for cardiac mesoderm (CPCs – cardiac progenitor cells) and MHC6(Myosin Heavy Chain – isoform 6) for cardiomyocytes [11,12].

Hence, the field of cardiovascular tissue engineering is receiving growing attention as a new therapeutic approach to treat heart injuries, combining cells and biopolymer scaffolds capable of restoring or replacing organs and damaged tissues [13]. An underlying principle in this approach is that biomaterial or cell-biomaterial constructs degrade concomitantly with tissue regeneration.

Polymer membranes are devices that are used to deliver drugs, cells and genes into the desired target. They provide a suitable substrate for cell attachment, proliferation and differentiation. Poly(α-hydroxyacids) is a biodegradable, resorbable material with good biocompatibility. The combination of these features makes it a desired candidate for tissue engineering. Among lactide-derived polymers, only poly(L-lactide) and poly(D,L-lactide) have been extensively studied and considered for useas biomaterials [14]. Malleability is a key property of biomaterials used in myocardial regeneration studies and thus the presence of units in the polymer chain which increase it's suppleness is of high importance, since this property can influence many aspects such as differentiation, adhesion, contractility, cell migration and scattering [15]. The suitability of poly trimethylene carbonate (TMC) for the preparation of biomedical implants has also been evaluated [16]. Consequently, poly(TMC) has been used as a unstiffening uniting rigid and frail polymers [17].

Therefore, this study proposes to evaluate mouse embryonic stem cell differentiation into cardiomyocyteson PLDLA-*co*-TMC membranes to verify the promising potential of this biopolymer.

MATERIALS AND METHODS

PLDLA-co-TMC membrane fabrication

The PLDLA-*co*-TMC was synthesized in the Biomaterials Laboratory of PUC São Paulo – Brazil, as described in [17].

Polymer characterization

PLLA-*co*-TMC membranes were placed in phosphate buffered saline (PBS) pH 7.4 at 37 ± 1ºC and removed from the solution after 0, 30, 60, 90 and 120 days, washed with distilled water and vacuum dried. After drying the samples were characterized.

Morphological analysis of the scaffolds was carried out by scanning electron microscopy (SEM) using a JEOL JXA-840A scanning electron microscope after gold coating (Balzer SCD 050) the samples.

Differential scanning calorimetry (DSC) measurements were carried out on a DSC 2920 thermal analyzer (TA Instruments, USA). The samples were exposed to temperatures ranging from −50ºC to 200ºC at increments of 10°C.min^{-1}, with nitrogen atmosphere flushed at 5mL.min^{-1}. The membranes thermal stability was tested in a STA 409C thermo gravimetric analyzer (Netzsch) in which the samples were heated at 10°C.min^{-1} up to 600°C, in an inert nitrogen atmosphere.

Gel permeation chromatography (GPC) analyses were performed using a Waters instrument (Waters Corporation, USA) equipped with Waters Styragel columns (HR 4E andHR 5E). Tetrahydrofuran was used as the mobile phase at a flow rate of 1 mL.min^{-1}.

The tensile test was performed in an 858 MTS instrument at room temperature. Each sample was stretched at a rate of 50 mm.min^{-1} with a load cell of 150 kgf and the operational equipment limit was 100 mm.

Characterization of cell/polymer interaction

Cell viability assay: The cell viability was determined by the 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay performed with Vero cells. The controls included native cells and medium alone. The spectrophotometric absorbance was measured at 550 nm wavelength using a microplate reader (ELx 800, Bio-Tek Instruments Inc., USA). The cytotoxicity as percentage of cell death was calculated by the following formula: (1 − [absorbance of experimental wells/absorbance of control wells]) × 100%.

Mouse embryonic stem cell (mESC) culture and differentiation

D3 mouse ES cells (ATCC, #CRL-1934) were maintained at 37°C and 5% CO_2 in mESC complete medium containing DMEM High Glucose Medium (Invitrogen) supplemented with 13% FBS, 1X MEM non-essential amino acids (Invitrogen), 50 μM Gentamycin (Invitrogen), 8 mg/L 2-Mercaptoethanol (J.T.Baker) and 1000 U/mL leukemia inhibitory factor (LIF) (Millipore). Cells were fed every 2 days and passed using Trypsin-EDTA before reaching confluence.

For differentiation, the "hanging drops" protocol adapted from Schulpen was implemented[18]. After culturing aggregates in suspension for an additional 5 days, they were plated on tissue-culture grade adherent plates or the PLDLA-*co*-TMC polymer for the remaining 2 days of the 9-day differentiation protocol.

Total RNA was extracted using an RNeasy Micro Kit (Qiagen). RNA from each sample was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). qPCRwas carried out using the Master-cycler Realplex and analyzed with Realplex software (Eppendorf). For real-time PCR, 1/40 of the total first strand synthesis product was used as a template for PCR amplification using Kapa SYBR Fast qPCR kit (KapaBiosystems). Each reaction was carried out in duplicate, and fold changes were calculated using the comparative Ct method[19]. The resulting Ct values of target genes were normalized to those of β-Actin.

On day 9 of mES cell differentiation, cultures were fixed and incubated with MF20 (Developmental Studies Hybridoma Bank, USA) monoclonal antibody to detect expression ofMHC6 protein. Cy3-conjugated secondary antibodies (Jackson Immuno Research

Laboratories, USA) were used to detect immunofluorescence (IF). Hoechst dye was used as a nuclear marker. Indirect IF was captured using a Leica DMI6000B microscope (Leica Microsystems GmbH, Germany), captured with a Hamamatsu Orca AG camera (Hamamatsu Photonics, Germany) and processed with the Velocity 4.3.2 software (Perkin Elmer, Canada).

Statistical analysis

Viability and qPCR assays were repeated five times. Numerical data were reported as mean ± standard deviation (SD). Statistical differences between means were calculated using two-tailed Student's t test. *P* values <0.05 were considered significant.

RESULTS AND DISCUSSION

Morphology, thermal and chemical characterization

SEM analysis was the first step to evaluate the membrane morphology according to the degradation time *in vitro*. Before degradation the PLDLA-*co*-TMC membrane is dense, smooth and compact on its surface. After 30 and 60 days, the degradation study showed little alteration in surface morphology. After 90 days, degradation was observed resulting in a clear variation on the membranes' external morphology, with the appearance of wrinkles and cracks. The membranes' fracture showed little or no morphological variation. After 120 days the material was totally degraded which precluded SEM analysis.

It was observed that the PLDLA-*co*-TMC membrane degradation occurred slower than that of other PLDLA-*co*-TMC structures. PTMC studies demonstrate that the polymer degrades extremely slowly in aqueous solution [20,21]. Due to the high degradation rate of the material, it was not possible to perform SEM analysis for the 90 and 120-day degradation periods.

The influence of *in vitro* degradation on thermal properties of PLDLA-*co*-TMC membranes is presented in Table (1) (DSC) and Table (2) (TGA).None of the results indicated any presence of crystallinity in the membrane, presenting it completely amorphous. Also, as shown in DSC, there is no significant difference inglass transition(T_g) temperature between 0, 30, and 60 days of degradation. T_g becomes significantly different only after 90 and 120 days. Table (2) shows typical trace of weight loss as a function of temperature over the degradation time, demonstrating that this polymer decomposed in single-stage and the onset temperature of PLDLA-*co*-TMC decreased from 310°C to 245°C along the first and last day of degradation.

The fact that the PLDLA-*co*-TMC is amorphous makes its degradation time lowest. The presence of TMC in the polymer chains is responsible for making the PLDLA-*co*-TMC more flexible as well as reducing its T_g, when compared to PLDLA alone [14]. Also, comparing the T_{onset} of pure PLDLA [22,23] or even the PLLA a crystalline polymer, to the results obtained by TGA in this paper, a reduction in mass loss onset temperature was observed. This is due to PTMC incorporation into PLDLA-*co*-TMC synthesis, consequently giving the polymer a lower thermal stability [24,25].

Tensile test, presented in Figure (1), was performed only for 0 and 30 days of degradation since after this period the samples were too degraded, making impossible to perform the test. The

Table 1: Thermal properties of polymer membranes of PLDLA-*co*-TMC determined via DSC analysis for different *in vitro* degradation times showing the T_g.

Degradation Time (days)	T_g(ºC)
0	17
30	16
60	15
90	12
120	8

Abbreviations: T_g: Glass Transition Temperature.

Table 2: Thermogravimetric analysis of the PLDLA-*co*-TMC membrane.

Degradation time (days)	T_{onset} (ºC)	T_d (ºC)
0	310	334
30	307	324
60	298	320
90	269	287
120	245	272

Abbreviations: T_{onset}: Initial Decomposition Temperature. T_d: Decomposition Temperature

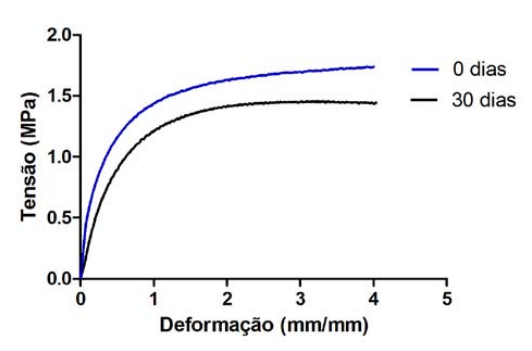

Figure 1 Tensile vs. Deformation curves for PLDLA-co-TMC membranes on 0 and 30 days of degradation, since after this period the samples were much degraded, making it impossible to test. Data showed initial elastic behavior, with no lasting deformation and after this initial stretch, i.e., after the elastic region, there is the beginning of plastic region or material flow, in which the permanent deformations are permanent.

PLDLA-*co*-TMC displayed an initial elastic behavior, since after removal of the applied tension the polymers' dimensions returned to those before the application of tension, thus demonstrating no permanent deformation. After the elastic region, there is the initial of plastic region or material flow, wherein the deformation is permanent [26]. Therefore, it can be inferred that the PLDLA-*co*-TMC has the behavior of an amorphous polymer with an elastic modulus of 2.6 MPa.

The fabrication of devices with mechanical properties that mimic the target tissue is taken into great consideration, together with the properties of degradation and cell maintenance [27,28]. The use of PLDLA-*co*-TMC for the regeneration of soft tissues with

low elastic modulus such as cardiac muscle (~0.5 MPa) [29,30] would be very relevant. Furthermore, the main characteristics of pure PLDLA are controllable and possess relatively rapid degradation rates. Furthermore, PLDLA does not generate crystalline fragments, it is amorphous and has low mechanical strength with an elastic modulus of about 35 MPa [31]. Additionally, despite the PTMC being an elastomer and having a very low elastic modulus (approximately 2.9 MPa), previous works discouraged the practical application of pure PTMC because of its very hydrophobic nature [32,33]. Nevertheless, PTMC has been widely used as a component, which loosens the polymer chain [32]. Thus, for the desired application, the addition of PTMC into the molecular chain proves to be indispensable for the modification of the mechanical properties of the biopolymer, making it more flexible.

Table (3) shows molar mass of PLDLA-*co*-TMC measured by GPC. As shown by the analysis, the polymer was gradually degraded exhibiting molar mass loss during *in vitro* degradation. It can be observed that the reduction of the chain size (Mw) is followed by the decrease in chains number (Mn) during the degradation process, suggesting loss of mechanical proprieties.

As shown by GPC analysis the polymer was gradually degraded exhibiting molar mass loss during *in vitro* degradation. Size reduction of the chains (Mw) is accompanied by a reduction in the number of chains (Mn) during degradation process, suggesting a loss of mechanical proprieties. This behavior is expected since in an aqueous medium, the polymer absorbs water and begins the hydrolytic cleavage of the bond, causing a decrease in Mw. The main products of ester bond hydrolysis are CO_2 and H_2O.

Cell culture and differentiation

The cell viability assay presented in Figure (2), was performed for a previous evaluation of cell interaction with the polymer and showed that the cell viability increased over time, an indication of cell proliferation on the membrane. After 21 days, cells grown on PLDLA-*co*-TMC showed greater viability than those grown in control wells (P<0.05).

Cell viability assay is important for biomaterial sciences to assess the interaction between the polymer and the cell. Even after the slow adhesion in synthetic materials the PLDLA-*co*-TMC presented a satisfactory result for cell adhesion and growth [34].

After checking cell viability on the PLDLA-*co*-TMC polymer, D3 mouse ES cells were used to evaluate their interaction and differentiation on the PLDLA-*co*-TMC polymer. mESC were differentiated using the hanging drop protocol. RNA was collected on days 0, 4, 6 and 9, for time point data along the differentiation

Table 3: PLDLA-*co*-TMC molar mass related to degradation time.

Degradation time (days)	Mn ($g.mol^{-1}$)	Mw ($g.mol^{-1}$)	(Mw/Mn)
0	83120	138368	1,66
30	41059	76241	1,85
60	24805	38380	1,55

Abbreviations: Mn: Molar Mass Averages of the Number; Mw: Molar Mass Averages of the Weight; Mw/Mn: Polydispersity Index

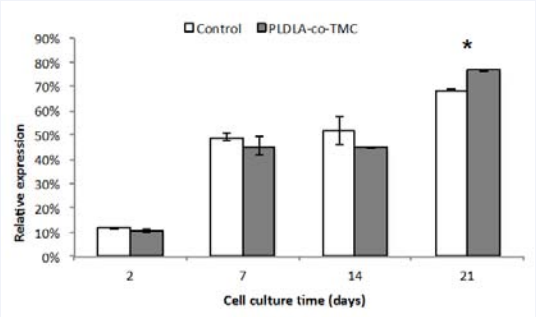

Figure 2 After a period of 2, 7, 14 and 21 days, cell viability was measured by comparison with the respective control. PLDLA-co-TMC allowed cell growth over cultivation time and consequently, present itself as nontoxic. Error bars represent the mean of 5 independent experiments ± SD. *P<0.05.

process and qPCR was used to assess the expression levels of cardiomyogenesis markers, such as Isl-1, Nkx2.5 and MHC6. *In vitro* cardiomyogenesis was characterized by means of qPCR analysis and corroborated by immunofluorescence assay (Figure 3) with the end differentiation marker MHC6.

Transcript levels of Brachyury-T, an early mesoderm marker[35] (Figure 4), were upregulated on day 4 of differentiation and then fades as differentiation progresses.

The next step towards cardiomyogenesis is cardiac progenitor cell (CPC) formation with the expression of cardiac precursor factors, Isl-1 and Nkx2.5 [36,37] presented in Figure (5A and 5B). Both transcription factors were used as an intermediate differentiation marker and were detected at high levels on days 6 and 9 when compared to the early days of differentiation [38].

Finally, mature cardiomyocytes are identified by the expression of MHC6shownin Figure (6). As predicted in the literature, MHC6 expression levels were observed on day 9 of mESC differentiation [39,40].

The cells presented a rounded or elongated morphology with a large nucleus, this result is consistent with other studies in which we used polymeric substrates to differentiate mESC [41,42].

Our results showed that Brachyury-T expression has its peak in the fourth day, which is significantly higher compared to subsequent days, suggesting that the expression remained highest during the early phase of differentiation and reduced drastically along the later stage [36]. Both transcription factors, Isl-1 and Nkx2.5 were used as intermediate differentiation markers and according to the literature these factors are observed on 7 and 8 days of differentiation respectively [38]. Finally, mature cardiomyocytes can be identified by the expression of MHC6, consistent with previously published work,MHC6 expression levels were observed to be highest on day 9 [43]. According to our qPCR analysis there is no difference in the transcript expression levels of cells differentiated on the PLDLA-*co*-TMC membrane versus the control. Consequently, it may be inferred that the polymer did not interfered with cardiomyogenesis pathway of mESC.

Indirect immunofluorescence of MHC6 on day 9 of mESC differentiation indicated that PLDLA-*co*-TMC membrane supported mESC cultivation and adherence, as well the differentiation into cardiomyocytes, which can be verified by the presence of viable and differentiated cells. The cells feature a rounded or elongated morphology with a large nucleus, a normal characteristic when cells are grown on polymeric substrates [41,42].

Perhaps, the most striking evidence for differentiation of these cardiomyocytes over polymer, is observed in culture, as soon that the cardiomyocytes form layers, the cells spontaneously start beating with synchronized contraction waves propagating through the culture.

CONCLUSION

Based on our results, TMC units added to PLDLA influenced molar mass, thermal and mechanical properties, providing greater flexibility to the biopolymer.

Additionally, culture assays on the PLDLA-*co*-TMC membrane, presented the material as cytocompatible and non-toxic to the cells allowing adhesion and proliferation of two different cell lines. Moreover, PLDLA-*co*-TMC permitted the differentiation of mESCs intocardiomyocytes at a similar efficiency when compared to the positive control, suggesting that this polymer can be used in regenerative cardiac therapy and recommended for use in

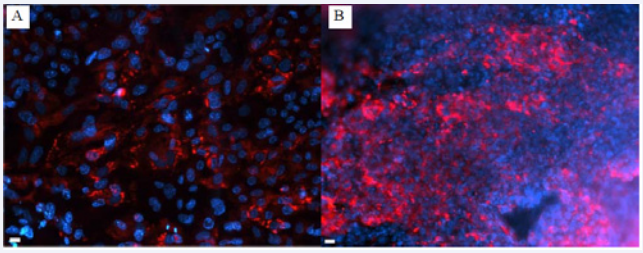

Figure 3 MHC6 protein (red) was visualized in corresponding day 9 differentiating cells by indirect immunofluorescence with a DMI6000B Leica inverted microscope. (A) – Control, (B) – PLDLA-co-TMC. Hoechst (blue) was used to identify nuclei. Scale bar (20 µm).

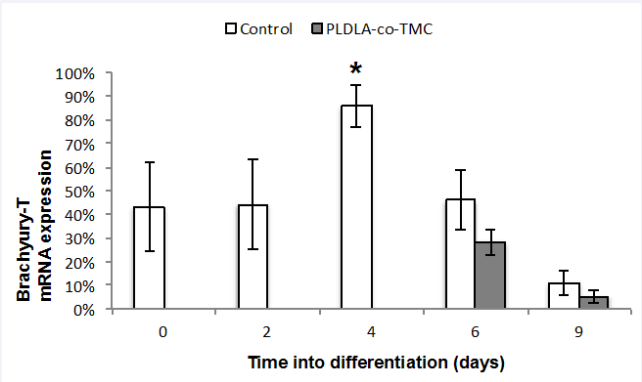

Figure 4 Transcription levels of Brachyury-T in corresponding differentiating cells were quantified using quantitative PCR analysis. Expression levels were first normalized to β-actin, then normalized to the expression levels on day 0 of mESC (Control) and finally presented as a percentage of the highest transcriptional expression for Brachyury-T. Error bars represent the mean of 5 independent experiments ± SD of mRNA expression. *P< 0.05.

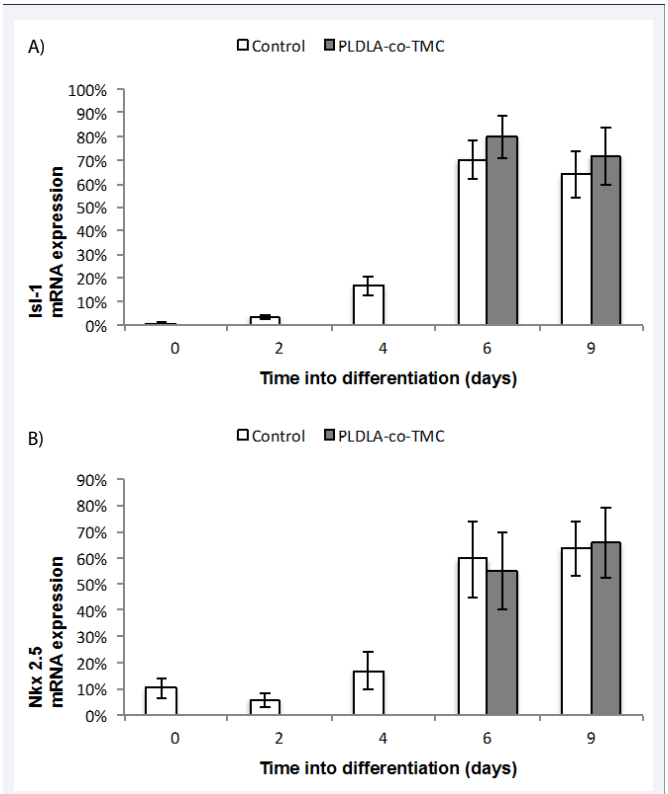

Figure 5 Transcription levels of Isl-1(A) and Nkx2.5(B) in corresponding differentiating cells were quantified using quantitative PCR analysis. Expression levels were first normalized to β-actin, then normalized to the expression levels on day 0 of mESC (Control) and finally presented as a percentage of the highest transcriptional expression for Isl-1 and Nkx2.5 respectively. Error bars represent the mean of 5 independent experiments ± SD of mRNA expression. *P< 0.05.

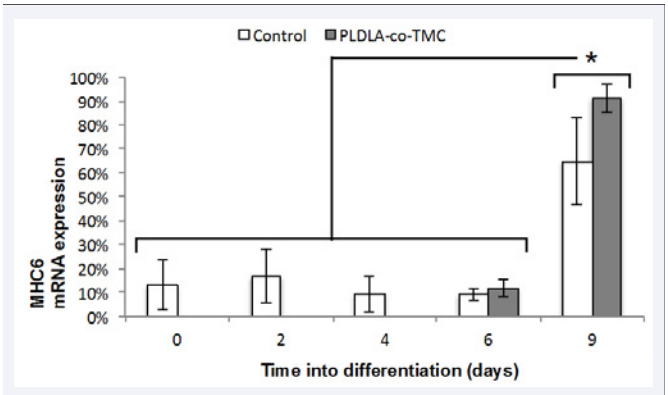

Figure 6 Transcription levels of MHC6 in corresponding differentiating cells were quantified using quantitative PCR analysis. Expression levels were first normalized to β-actin, then normalized to the expression levels on day 0 of mESC (Control) and finally presented as a percentage of the highest transcriptional expression for MHC6. Error bars represent the mean of 5 independent experiments ± SD of mRNA expression. *P< 0.05.

tissue engineering. Further in-depth research is still required,but the results obtained in this study contribute to encourage more studies in the field of regenerative cardiac therapy.

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