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Expression of Cardiac Specific Cell Marker in *Ex Vivo* Differentiated Canine iPSC

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ABSTRACT

Induced Pluripotent stem cells (iPSC) have a high ability to renew and differentiate themselves into various lineages and as vehicles of cell based therapy. Stem cell can differentiate under appropriate *in vitro* and *in vivo* conditions into different cell types. This study described the establishment of condition for *in vitro* expression of alpha MHC gene in cardiac differentiated canine iPSC (ciPSC). *In vitro* differentiation of canine iPSCs via embryoid bodies (EBs) were produced by 'Hanging Drop' method. EB's were differentiated by using IMDM differentiation media: FBS – 10%, NEAA (100X) – 0.5%, B-Mercaptoethanol- 100mM, Gentamycin- 5µg/ml supplemented with Azacytidine- 0.5µM. During differentiation, EBs were collected on day 4, 6, 8, 12, 16, 20 and 24 for characterization of cardiomyocytes specific marker expression. Total RNA from EBs were extracted by using Trizol method and subsequently cDNA were synthesized. The differentiated cells expressed cardiac specific gene (Alpha MHC) which started from day 6 of differentiation upto day 24 Immunocytochemistry and relative expression of cardiac specific genes revealed that ciPSC have the potential to differentiate into cardiomyocytes which can be used for cardiac tissue regeneration and as disease models for pharmaceutical testing.

Key words: Canine iPSC, Cardiac, Differentiation.

INTRODUCTION

For many years, the prevailing dogma of cardiac biology was that there is no renewal of cardiomyocytes during adult life and also studies reported that the mammalian heart exhibits a capacity, albeit limited, to generate new cardiomyocytes (Bergmann *et al*., 2009; Kajstura *et al*., 2010; Senyo *et al*., 2013). In adults, the turnover rate is low at around 1% per annum, which decline with age (Ali *et al*., 2014; Garnern and Lee, 2013; Malliaras *et al*., 2013; Mollova *et al*., 2013; Senyo *et al*., 2014; Walsh *et al*., 2010). As adult cardiomyocytes (CMs) have limited regenerationability to compensate myocardial infarction-induced loss of CMs, the necrotic CMs are progressively replaced by fibroblasts to form scar tissues. Cellular cardiomyoplasty is an ultimate strategy to repair necrotic CMs and to advance cardiac function, but critical donor remains to be a great limitation. Due to this limitation, recently, stem cell based therapy is gaining ground. Several research groups have produced functional cardiomyocytes (CMs) *in vitro* from murine and human pluripotent stem cells (PSC) including induced PSC (iPSC). Cardiomyocytes produced from iPSC are very similar in characteristics (morphology, marker expression, electrophysiological features and sensitivity to chemicals) to the CMs of cardiac muscle and to CMs produced from differentiated ESCs. The pluripotent stem cells generation by iPSC technology offers a potential strategy to generate patient-specific pluripotent stem cells. iPSC are a type of pluripotent stem cell artificially derived from a non-pluripotent cell, typically from an adult somatic cell, by inducing a "forced" expression of specific factors. These cells have the capacity to differentiate into all cell types (Hayashi *et al*., 2012; Yanagimachi *et al*., 2013) and, therefore, have significant potential for autologous stem cell therapies (Hanna *et al*., 2007; Alipio *et al*., 2010. This also provides a fascinating route to generate patient-specific pluripotent cells as disease models and drug-testing systems (Yu *et al*., 2007). Myosin, the protein that couples ATPase activity with mechanical work, is expressed in two myosins heavy chain (MHC) isoforms, α and β. The expression of cardiac MHC isoforms changes during development in a species-dependent manner. During fetal development, β-MHC transcription decreases and is replaced by α-MHC (Lompre *et al*., 1984; Lyons *et al*., 1990), which ultimately leads to an adult ventricular myocardium that expresses mostly the α isoform (~90%) (Lompre *et al*., 1979). Dogs provide a more clinically relevant model of human disease than rodents. Thus, the availability of canine stem cells will greatly facilitate the use of the dog in the development of stem cell-based gene therapies and regenerative medicine. Domestic dogs offer numerous significant advantages as animal models for stem cell therapy evaluation, including the spontaneous

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development of diseases that closely act as human disease, a shared environment with humans and the availability of an outbred population with strong immune systems and exposure to diverse pathogens (Hoffman and Dow, 2016).

5-azacytidine (5-aza) is a cytosine analog, it is an effective DNA hypomethylating agent and it is capable of altering the expressions of certain genes (Mohandas *et al*., 1981; Branch *et al*., 1996) regulating cell differentiation (Jones and Taylor, 1980; Bartolucci *et al*., 1989). Several studies have found that mesenchymal stem cells can be transformed into cardiomyocytes after an exposure to 5-aza (Makino *et al*., 1999; Hakuno *et al*., 2002; Rangappa *et al*., 2003). In addition, human ES cells (Xu *et al*., 2002) and stem cell antigen-1 (Sca-1) + cardiac progenitorcells (Oh *et al*., 2003) were differentiated into cardiomyocytes in response to 5-aza or 5-aza-2'-deoxycytidine treatment. In this study, we exposed reprogrammed canine induced pluripotent stem cells (ciPSCs) to *in vitro* cardiomyocyte differentiation process using 5-azacytidine and analysed the differentiated cells for expression of cardiac specific gene.

MATERIALS AND METHODS

Culture and cardiomyocytes differentiation from canine iPSCs

The ciPSCs generated by pLentG-KOSM plasmid vector (Cell Biolabs, Cat no. LTV 700) was used for this experiment, ciPSCs were cultured with Knock Out DMEM/F12 (GIBCO: 10829-018) supplemented with 20% fetal calf serum, 1% non-essential amino acids, 2 mmol/l L-glutamine and 100 µmol/l β-ME, LIF and BFGF. The differentiation ability of ciPSC were examined by hanging drop culture. Briefly, a single cell suspension of ciPSCs (2.5 × 10^4 cells/ml) was prepared in differentiation media consisting of IMDM supplemented with 10% FBS, 0.5% NEAA (100X), 100µMB-Mercaptoethanol and 0.1 µM azacytidine. From this cell suspension, 20 µl was pipetted into the lid of a tissue culture dish and inverted over its bottom dish containing 5 ml phosphate-buffered saline. On day 3, the embryo bodies (EBs) were transferred on a gelatin coated cover slip in 6 well tissue culture dishes and allowed to grow till day 24, with periodical changing of the media. During differentiation, EBs were collected on day 4, 6, 8, 12, 16, 20 and 24 for characterization of cardiomyocytes specific marker expression.

Molecular characterization

Total RNA was extracted and cDNA was prepared using a cDNA Synthesis kit (QuantiTect® Reverse Transcription Kit), from the collected samples. Reverse transcriptase-polymerase chain reaction (RT-PCR) and semi-quantitative gene expression analysis was carried out using Real-Time PCR system (Bio-Rad, USA) with SsoFast™ Eva Green supermix (Bio-Rad, USA) and the canine specific primers for alpha MHC: F-CGGCAGACGCGGACCATCA, R-CCGGCCTCGCTCT GGTTGTAGTA, annealing temperature of 57°C. The endogenous house keeping control gene was considered as the glyceraldehyde 3-phosphate dehydrogenase *GAPDH* F-CCATCTTCCAGGAGCGAGAT, R- TTCTCCATGGTGG TGAAGAC annealing temperature of 55°C. Each PCR product was size-fractionized by 2% agarose gel electrophoresis and bands were visualized with a UV trans-illuminator (Bio Rad).

Cytochemical staining

Immunocytochemistry was done as per the methods of National Institutes of Health, resource for stem cell research, USA. For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS (Sigma-Aldrich). The cells were then blocked with 2% bovine serum albumin in PBS and incubated with rabbit polyclonal primary antibody *viz* alpha MHC. The cells were washed and then incubated with fluorescence-labeled anti rabbit secondary antibodies (Cloud-Clone Crop; 1:200) and DAPI to stain the nuclei. The negative control was performed following the entire procedure, except adding primary antibodies.

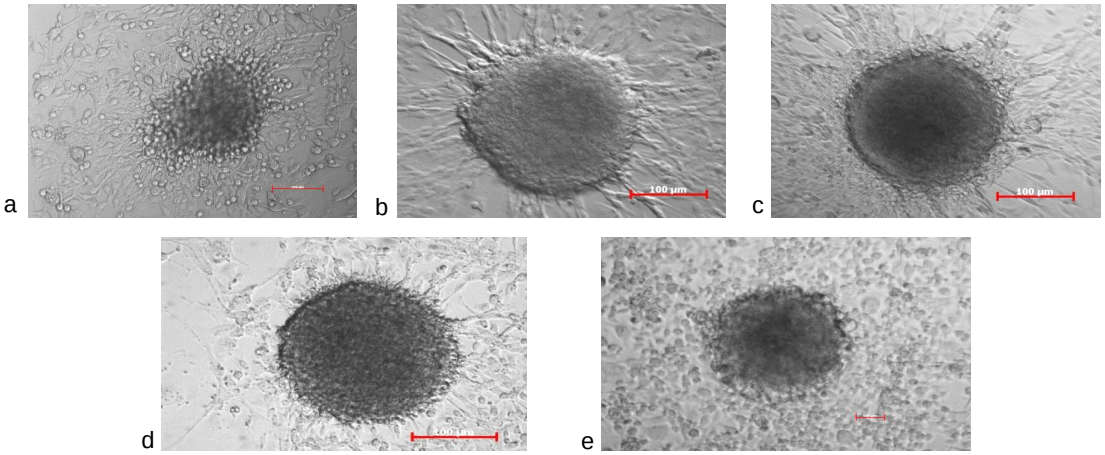

Fig 1: ciPSCs derived from adipose tissue colonies at different Passage: (a) P6, (b) P17 (c) P19, (d) P75, (e) P102 (4x magnification).

RESULTS AND DISCUSSION

In the present study, ciPSCs (Fig 1) were differentiated into cardiomyocyte by using azacytidine. It was observed that the EBs when treated with differentiation media, the morphology of cells changed and spreading of EBs was extensive at the periphery (Fig 2). Cardiac specific marker expression studies by RTPCR and gel electrophoresis revealed that alpha MHC expression started from day 4, and continued till day 24 of differentiation (Fig 3). It was observed that initially the expression was low but over the time of differentiation, the expression level was enhanced and higher expression was observed on day 24 of differentiation. The differentiated cells were found to be immunopositive for alpha MHC (Fig 4). However, no beating clusters were observed during the entire period of differentiation.

Several groups have reported that 5-azacytidine, a demethylating agent, induced the differentiation of mesenchymal stem cells into cardiomyocytes *in vitro* (Makino *et al*., 1999; Hakuno *et al*., 2002; Fukuda, 2003). Treatment of human ESCs with 0.1 mM of 5- azacytidine for 1–3 days significantly increased the number of beating cells and simultaneously enhanced the expression of cardiac-specific markers (Yoon *et al*., 2006). 5'- azacytidine can cause extensive demethylation of 5-methylcytosine and reduce DNA methyltransferase activity in the cell (Haaf and Schmid, 2000).

Study on murine iPSC differentiation by media containing beta-mercaptoethanol have shown the expression of marker genes for cardiac mesoderm, and cardiomyocytes including Mesp1, GATA2 (FOG-2), GATA4, Nkx2.5, Tbx5, Tbx20, atrial natriuretic factor (ANF), MLC2a,

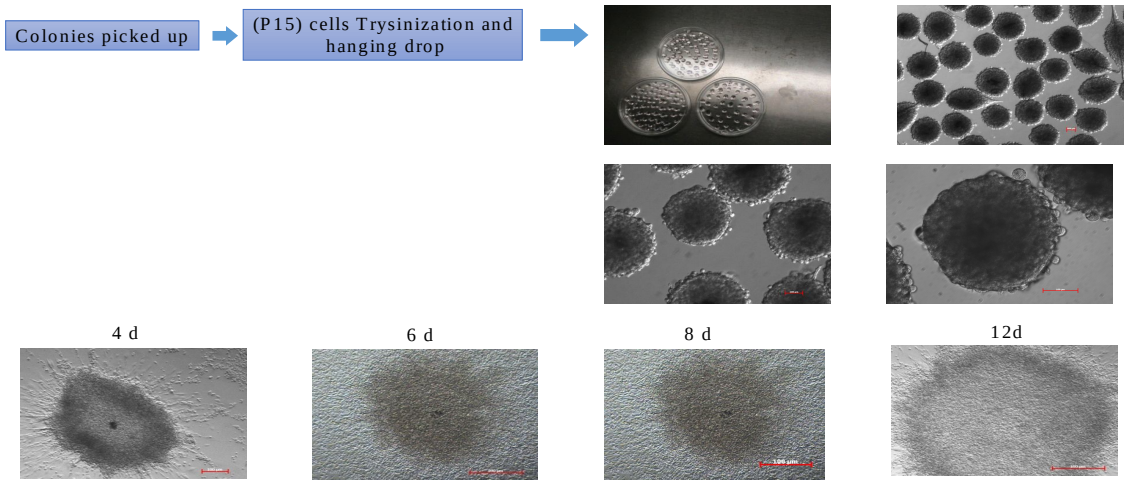

Fig 2: *In vitro* differentiation of canine iPSCs via embryoid bodies (EBs) produced by 'Hanging Drop' method and different day morphology.

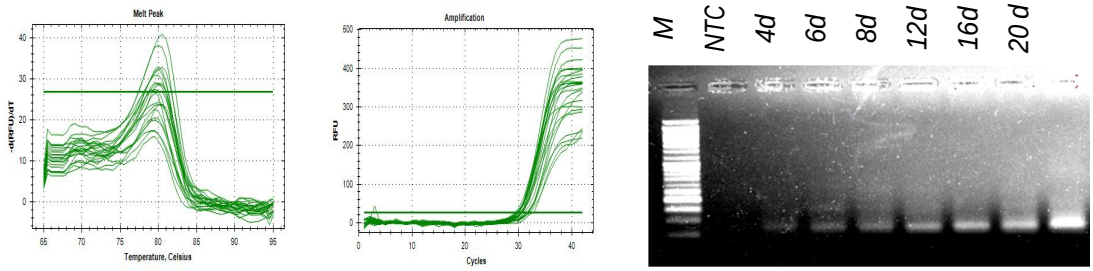

Fig 3: Molecular characterization of alpha MHC in Embryoid bodies made from ciPSC colonies.

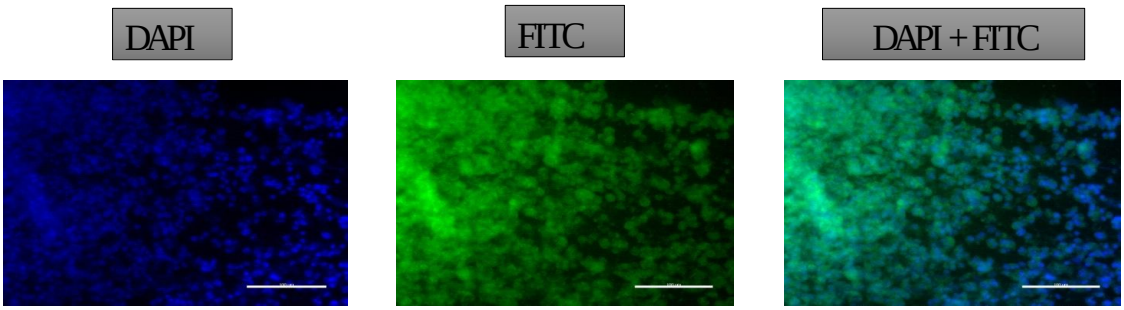

Fig 4: Immunolocalization of cardiac markers: alpha MHC in Embryoid bodies made from ciPSC colonies.

alpha-MHC and cardiac troponin T in differentiation cultures of iPS cells. Immunocytology also confirmed the expression of cardiomyocyte-typical proteins including sarcomeric alpha-actinin, titin, cardiac troponin T, MLC2v (Mauritz *et al*., 2008). Several research groups have produced functional cardiomyocytes (CMs) *in vitro* from murine and human iPSCs (Medvedev 2010). Cardiomyocytes produced from iPSC are very similar in characteristics (morphology, marker expression, electrophysiological features and sensitivity to chemicals) to the CMs of cardiac muscle and to CMs produced from differentiated ESCs. Moreover, murine iPSCs, when injected, can repair muscle and endothelial cardiac tissues damaged by cardiac infarction (Medvedev 2010). In case of canine iPSCs, Joseph Wu's group has demonstrated the preclinical potential of ciPSCs by treating immunodeficient mouse models of myocardial infarction and hind limb ischemia with transplanted endothelial cells derived from ciPSCs (Lee *et al*., 2011). ciPSCs grown on feeder as well as functionalized CNT have been differentiated into cardiomyocytes (Mahalakshmi, 2018).

Since there was no standard protocol for differentiation of induced pluripotent of canine, the protocol was tested based on the protocol used for cardiomyocytes generation from mouse/human ESC or iPSC. In the present study there was no beating clusters generated indicating that other methods may be tested for generation of beating cardiomyocytes from ciPSCs.

CONCLUSION

The results suggested that canine iPSCs were readily differentiated into cardiomyocytes as evidenced by the expression of cardiac specific marker. This finding demonstrates the potential for using canine induced pluripotent stem cells for cardiac tissue regeneration and as disease models for pharmaceutical testing.

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