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Aza-induced cardiomyocyte differentiation of P19 EC-cells by epigenetic co-regulation and ERK signaling

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ABSTRACT

Stem cells in cell based therapy for cardiac injury is being potentially considered. However, genetic regulatory networks involved in cardiac differentiation are not clearly understood. Among stem cell differentiation models, mouse P19 embryonic carcinoma (EC) cells, are employed for studying (epi)genetic regulation of cardiomyocyte differentiation. Here, we comprehensively assessed cardiogenic differentiation potential of 5-azacytidine (Aza) on P19 EC-cells, associated gene expression profiles and the changes in DNA methylation, histone acetylation and activated-ERK signaling status during differentiation. Initial exposure of Aza to cultured EC-cells leads to an efficient (55%) differentiation to cardiomyocyte-rich embryoid bodies with a threefold (16.8%) increase in the $cTnI^+$ cardiomyocytes. Expression levels of cardiac-specific gene markers i.e., Isl-1, BMP-2, GATA-4, and α-MHC were up-regulated following Aza induction, accompanied by differential changes in their methylation status particularly that of BMP-2 and α-MHC. Additionally, increases in the levels of acetylated-H3 and pERK were observed during Aza-induced cardiac differentiation. These studies demonstrate that Aza is a potent cardiac inducer when treated during the initial phase of differentiation of mouse P19 EC-cells and its effect is brought about epigenetically and co-ordinatedly by hypo-methylation and histone acetylation-mediated hyper-expression of cardiogenesis-associated genes and involving activation of ERK signaling.

1. Introduction

Regulation of gene expression during stem cell differentiation is governed by a number of mechanisms at a transcriptional level, including epigenetics. Aberrations in this are thought to be responsible for a number of disease and disorders. Prominent among them is cardiovascular disorder i.e., myocardial infarction. To alleviate this disease, stem cell transplantation is potentially exploited for regeneration of cardiac tissues (Abdallah et al., 2006). Stem cells being experimented include mesenchymal stem cells: MSCs (Hakuno et al., 2002; Rangappa et al., 2003), adult cardiogenic cells (Murry et al., 2004; Nygren et al., 2004) and pluripotent stem cells (PSCs) i.e., embryonic stem cells (ES-cells) (Kehat et al., 2001), induced pluripotent stem cells (iPS-cells) (Mummery et al., 2012) and embryonal carcinoma cells (EC-cells) (van der Heyden and Defize, 2003). However, differentiation potential of stem cells to cardiac progenitors and cardiomyocytes has been of concern both in-vitro (Zimmermann et al., 2002) and in-vivo (Antonitsis et al., 2007; Vidarsson et al., 2010) in terms of efficiency of differentiation and enrichment capability of differentiated cardiac progenitors. In-vitro differentiation efficiencies achieved so far from MSCs and PSCs have only been ~10% and 30%, respectively (Antonitsis et al., 2007; Fujiwara et al., 2011). Moreover, enrichment of cardiac progenitors and their purity status continue to limit their possible use in cell transplantation (Fujiwara et al., 2011). In view of these, a number of studies involving various culture systems are being extensively carried out. In this regard, EC-cell culture system is an excellent in-vitro model system to study cardiac differentiation. EC-cells qualify as a typical PSCs, they are easy to maintain in culture and, unlike PSCs, they do not require LIF supplementation and fibroblast-feeders (van der Heyden and Defize, 2003). Moreover, they are easily amenable to exogenous gene integration and genetic manipulation studies. In view of these attributes, EC-cells are highly versatile and are exploited to study cardiomyocyte differentiation and the accompanying molecular and (epi)genetic regulatory mechanisms.

Towards achieving enhanced efficiency of cardiac differentiation of stem cells, small molecules such as DMSO (McBurney et al., 1982; Skerjanc et al., 1998), retinoic acid (Wobus et al., 1997), and growth factors such as BMP (Sachinidis et al., 2003), FGF (Rosenblatt-Velin et al., 2005), and Wnt (Nakamura et al., 2003; Pandur et al., 2002)

Abbreviations: α-MHC, α-Myosin heavy chain; Aza, 5-Aza cytidine; BMP, Bone morphogenetic protein; cTnI, cardiac Troponin I; COBRA, Combined bisulfite restriction analysis; DMSO, Dimethyl sulfoxide; DNMT, DNA methyl transferase; EBs, Embryoid bodies; EC-cells, Embryonal carcinoma cells; ES-cells, Embryonic stem cells; ERK, Extracellular receptor kinase; FGF, Fibroblast growth factor; H, Histone; hESC, Human embryonic stem cells; Ig, Immunoglobulin(s); LIF, Leukemia inhibitory factor; MSP, Methylation specific PCR; MSCs, Mesenchymal stem cells; MLC 2v, Myosin light chain 2v; PSCs, Pluripotent stem cells; SDS, Sodium dodecyl sulfate; TRED, Transcriptional regulatory element database.

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are supplemented in culture and also manipulation of culture conditions (Heng et al., 2004). These approaches have yielded a variable degree of success. A few of the above molecules are thought to bring about epigenetic modifications and they regulate gene expression dynamics of cardiogenesis-associated genes during stem cell differentiation (Ehrlich, 2003). In this regard, the specific DNA methyl transferase (DNMT) inhibitor, 5-azacytidine (Aza), is reported to regulate gene expression of stemness genes as well as differentiation-associated genes (Branch et al., 1996; Mohandas et al., 1981). Because Aza is used clinically as an anti-cancer drug for acute myelogenous leukemia (Lemaire et al., 2005) and because it is involved in cell growth and differentiation in general, Aza is potentially used to achieve cardiac differentiation of MSCs (Fukuda, 2002, 2003), hESC (Yoon et al., 2006), mouse EC-cells (Choi et al., 2004) and endothelial cell differentiation of mouse ES-cells (Banerjee and Bacanamwo, 2010).

However, the possible mechanism of Aza-induced cardiomyocyte differentiation of PSCs, i.e., EC-cells, is not completely understood and this needs to be investigated. Discreet experimental approaches, carried out previously, have shown that Aza-mediated differentiation of EC-cells, cultured in monolayer, to cardiomyocytes is accompanied by an up-regulation of cardiac marker genes and BMP-signaling molecules (Choi et al., 2004). Using various cardiogenic small molecules such as cardiogenol (Jasmin et al., 2010; Wu et al., 2004), oxytocin (Jasmin et al., 2010; Paquin et al., 2002) and retinoic acid (Edwards and McBurney, 1983; Wobus et al., 1997) cardiomyocyte differentiation of EC-cells was demonstrated. Despite the use of various cardiac differentiation induction protocols and molecular analysis associated with cardiac differentiation, till date, there is no single comprehensive and correlative study demonstrating Aza-induced cardiac differentiation of EC-cells, accompanied by DNA methylation status gene expression regulatory networks and cell signaling systems. In this study, we show that when Aza is applied during the early culture phase of EC-cells it results in efficient and increased cardiomyocyte differentiation and this differentiation is comprehensively and epigenetically associated with methylation status-dependent expressions of cardiac differentiation-associated genes, accompanied by (H3) histone acetylation and the involvement of activation of ERK signaling system.

2. Materials and methods

2.1. Culture of P19 EC-cells

The P19 EC-cells (kind gift from Dr. A. Wobus) were used for the study. EC-cells were plated on 0.1% gelatin-coated Petri dishes (Griener) in KO-DMEM (Invitrogen) supplemented with 15% heat-inactivated fetal bovine serum (ES-FBS), L-glutamine (2 mM), non-essential amino acids (NEAA, 100 μM), (all from Invitrogen), penicillin (100 U/ml; Sigma), streptomycin (100 μg/ml, Sigma), and β-mercaptoethanol (100 μM, Sigma). All the cells passed after 24 h interval when they attained ~90% confluency.

2.2. Differentiation

For differentiation, P19 EC-cells (n = 400) were allowed to form embryoid bodies (EBs) by a hanging drop method (Desbaillets et al., 2000) in KO-DMEM with 20% FBS, L-glutamine, NEAA and penicillin, streptomycin as described above. At day 2, EBs were transferred into bacteriological 60 mm Petri dishes and cultured in suspension up to day 5. Different concentrations of Aza (0.1–10 μM) were added to the cells at the time of EB formation for 2 days. At day 5, EBs were transferred to 0.1% gelatin-coated 60 mm tissue culture dishes (Griener) and cultured in 20% FBS medium. Derived EBs and differentiated cell types were observed morphologically and analyzed by RT-PCR, immunofluorescence and FACS. To determine the sizes of EBs at days 2 and 5, photomicrographs of EBs were captured by a digital camera (JVC, TK 1085 E, Victor company of Japan Ltd, Yokohama, Kanagawa, Japan) connected to stage-top of the Olympus inverted microscope (IX70; Olympus Optical Co Ltd, Tokyo, Japan) with the Image Pro Imaging System (Media Cybernetics, Silver Spring, MD, USA), and images were then analyzed with measurements using micrometer. EB-outgrowths having cardiac contracting phenotypes of differentiation cell patches were video-recorded as described above. Real-time continuous recordings of specified areas were made for about 2–5 min using the VCR (JVC) with the SP mode VHS-videotapes. Grabbed video clippings were played-back, selected and converted to digital mode and the specified recordings were shown in the Results section.

2.3. RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from the undifferentiated cells and the EBs by Trizol (Sigma) method. Isolated RNA was digested by 1 U of DNAse I (Fermentas) as recommended by the manufacturer. The first-strand cDNA was synthesized from 2 μg of DNase-treated total RNA using 50 ng random hexamers (Fermentas) and 20 U of MMLV reverse transcriptase (Fermentas) and the reaction was carried out at 42 °C for 60 min in a volume of 20 μl. The prepared cDNA (50 ng) was used for PCR amplification in a 10 μl reaction mixture with –RT and water controls. Various samples at different phases of EB differentiation starting from day 0 to days 2, 5, 9, 11 and 13 were used for analysis. PCR was performed under the following conditions: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s, with an additional 7-min incubation at 72 °C after cycle completion. The PCR reaction was carried out for 35 cycles with β-actin for semi-quantitative and quantitative analyses (the sequences for all primers used for the analysis will be provided on request). The PCR products were electrophoretically separated on 2% agarose gels containing ethidium bromide and gels were illuminated with UV light, stored analyzed by alpha DigiDoc software. Later qPCR was performed with ABI 7300 Real-Time PCR System (Applied Biosystems) for the same samples using 10 ng of cDNA. The data of target genes were plotted as fold changes in relation to the expression of the house-keeping gene, β-actin. The transcript level of β-actin was used as normalization control, for all samples analyzed. For each transcript 2 to 3 independent experiments were analyzed and obtained values subjected to statistical analysis, described below (Wobus et al., 1997).

2.4. Immunocytochemistry

EBs were plated in 4-well plate on 0.1% gelatin coated coverslips. They were then fixed for 20 min in 4% paraformaldehyde (Sigma) in DPBS. After washing with DPBS twice, fixed cells/EB outgrowths were permeabilized for 10 min in DPBS containing 0.2% Triton X-100, and later were blocked with 5% BSA in DPBS for 2–4 h after DPBS washes. Day 11 EB outgrowths were then incubated with the primary antibodies for cTnI (1:200, Millipore) at 37 °C for 60 min or overnight at 4 °C. Samples were washed 2–3 times in DPBS and incubated with fluorescence-labeled Alexa Fluor 488 or FITC-conjugated secondary anti-mouse IgG antibody (Molecular Probes, Invitrogen) at a dilution of 1:300; diluted in 0.5% BSA in DPBS at 37 °C for 30 min. For nuclei staining, cells were incubated with PI for 15 min at 37 °C after 10 min of RNAse treatment. After washing twice with DPBS samples were mounted in prolong gold antifade (Invitrogen) and viewed under Zeiss LSM confocal microscope (Carl Zeiss Micro imaging, GmBH, Germany) for fluorescent signal (Wobus et al., 1997).

2.5. Western blotting

Cells were lysed with RIPA as lysis buffer and samples containing 25–50 μg of total protein lysates (after Bradford estimation) were separated by SDS-PAGE and blotted onto a PVDF membrane (Millipore).