International Journal of Cardiology 165 (2013) 341–354

Contents lists available at SciVerse ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Cardioprotective molecules are enriched in beating cardiomyocytes derived from human embryonic stem cells

Jung-Il Chae [a,1], Jumi Kim [b,1], Mi-Young Son [c,1], Young-Joo Jeon [a], Dong-Wook Kim [a], Hye Eun Kim [b], Min Ji Lee [b], Joohyun Ryu [d], Byoung Chul Park [d], Dong-Seok Lee [e], Kang Seok Seo [f], Hak Kyo Lee [g], Nag-Jin Choi [h], Yong-Kook Kang [c,*], Hyung Min Chung [b,i,**]

[a] Department of Oral Pharmacology, School of Dentistry and Institute of Oral Bioscience, BK21 Project, Chonbuk National University, Jeonju, Republic of Korea
[b] CHA Bio & Diostech Co., Ltd. 606-16 Yeoksam 1 dong, Gangnam gu, Seoul 135-907, Republic of Korea
[c] Development and Differentiation Research Center, KRIBB, 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea
[d] Medical Proteomics Research Center, KRIBB, Daejeon, Republic of Korea
[e] College of Natural Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
[f] Department of Animal Science and Technology, Sunchon National University, Suncheon 540-742, Republic of Korea
[g] Genomic Informatics Center, Hankyong National University, 67 Sukjong-dong, Ansung-city, Kyeonggi-do 456-749, Republic of Korea
[h] Department of Animal Science, College of Agricultural & Life Science, Chonbuk National University, Jeonju, Republic of Korea
[i] Graduate School of Life Science, CHA Stem Cell Institute, College of Medicine, CHA University, 605-21 Yeoksam 1 dong, Gangnam gu, Seoul 135-907, Republic of Korea

ARTICLE INFO

Article history:
Received 12 January 2012
Received in revised form 16 July 2012
Accepted 21 July 2012
Available online 13 November 2012

Keywords:
Human embryonic stem cell
Embryonic body
Cardiomyocytes
Proteomic analysis

ABSTRACT

Background: Cardiomyocytes derived from human embryonic stem cells (hESC-CMs) have attracted attention because of their cardiac regenerative potential in vivo. Differentiated CMs can be distinguished into two different phenotypic populations: beating and non-beating CMs. A thorough understanding of the different molecular conditions of beating and non-beating CMs would provide valuable information for other potential applications and cell therapy.
Methods: In this study, we generated a comparative protein profiles using proteomic analysis and western blotting, to compare the specific protein expression patterns of beating and non-beating hESC-CMs.
Results: Abundantly 72 upregulated proteins are involved in different biological processes such as stimulus response, cellular catabolism and cell motility. Among these proteins, such as HSPs and other antioxidant molecules, are known to be proteins that potentially play an important role in cardioprotection through the enhancement of cell survival in hypoxic and ischemic conditions present in the injured heart.
Conclusion: As a first step toward understanding the different molecular conditions of beating and non-beating hESC-CMs, we sought to study their differential expression patterns and discuss their relevance to in vivo functioning in cardiac injury repair. Thus, the results of this study could provide further evidence supporting a cardiac regenerative approach using an optimized cell source derived from hESCs.

1. Introduction

The heart is the organ responsible for supplying blood throughout the whole body and becomes functional during vertebrate embryonic cardiac development, which is accomplished by the differentiation of mesoderm germ-layer cells into mesothelium, endothelium and myocardium [1].

Because adult heart tissue has a restricted regenerative capacity, any essential cell loss or dysfunction, such as that which occurs during cardiac infarction, is mostly irreversible and may lead to progressive heart failure, a leading cause of morbidity and mortality [2,3].

Thus, several approaches have been pursued to cure heart failure events, such as myocardial infarction and cardiomyopathy. The methods include drug treatment, surgery, cardiac organ transplantation and stem cell transplantation [4–7].

Recently, stem cell transplantation using adult cell sources such as cord blood or bone marrow is commonly preferred. However, the insufficient availability of adult cellular sources is a major problem [8–12]. Thus, pluripotent stem cells, such as hESCs, or induced pluripotent stem cells (iPSCs) have drawn attention in cell replacement therapy for injured tissue because of their unique capacities of unlimited proliferation and pluripotency [8]. Cardiomyocytes derived from hESCs could thus have great potential for cardiac regenerative medicine [13,14].

* Correspondence to: Y. K. Kang, Development and Differentiation Research Center, KRIBB, 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea. Tel.: +82 42 860 4427; fax: +82 42 860 4608.
** Correspondence to: H. M. Chung, CHA Bio & Diostech Co., Ltd. 606-16 Yeoksam 1 dong, Gangnam gu, Seoul 135-907, Republic of Korea. Tel.: +82 2 3468 3391; fax: +82 2 3468 3373.
E-mail addresses: ykkang@kribb.re.kr (Y.-K. Kang), hmchung@cha.ac.kr (H.M. Chung).
[1] The authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.ijcard.2012.07.013

To induce the differentiation of hESCs into functional CMs, leading to the spontaneous formation of embryoid bodies (EBs), treatments with chemical or growth factors and stromal cell co-culture methods have frequently been used. During the differentiation of hESCs into CMs, two cell populations, which are clearly distinguished by their phenotypic outcomes, appeared in differentiated CMs: beating and non-beating hESC-CMs [15–17].

Although both cell populations retain similar cardiac characteristics, they represent a significant physiological difference, the spontaneous beating capacity. Furthermore, the use of hESC-CMs in the field is contingent largely on understanding the molecular conditions in these cells.

Previous studies have investigated the morphology, ultrastructure, electrophysiology and cardiac marker expression of differentiated CMs. The molecular levels have also been analyzed by homologous recombination, gene mutation, and transgenic studies. However, the fundamental differences at the molecular level and the differing roles of beating and non-beating CMs in vivo are largely unknown [18].

The differences between the proteomic profiles of beating and non-beating hESC-CMs have yet to be discovered. A two-dimensional map of beating and non-beating hESC-CMs could provide further evidence of different beneficial roles of beating and non-beating hESC-CMs when they are transplanted in vivo, based on their differing molecular conditions.

In the present study, we attempted to further characterize and identify the proteomic profiles of beating and non-beating hESC-CMs using a high-throughput proteomics tool.

We employed proteomic approaches using two-dimensional gel electrophoresis (2-DE), followed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) to investigate differentially expressed proteins in beating and non-beating hESC-CMs. The 72 proteins that were identified as upregulated in beating hESC-CMs are involved in several different biological processes. Some identified proteins, such as heat shock proteins and antioxidant molecules, including HSP 90-α, HSP 90-β, HSP70, GST and Prx1, are related to the cardioprotection mechanism by reducing apoptosis in hypoxic and ischemic conditions present in an infracted heart. Heat shock proteins (HSPs, also known as molecular chaperones) were originally discovered under high-temperature conditions but also play an important role in stressful conditions [19]. Thus, specific cellular sources of upregulated protein expression, including those of HSPs and antioxidant molecules, may have more therapeutic potential when transplanted into stressful conditions, such as those arising during ischemia or heart failure. As the first step to understanding the different molecular conditions of beating and non-beating hESC-CMs, we sought to discover the most suitable cellular source for cardiac regeneration and its relevance to the in vivo mechanism of cardiac injury repair. Thus, this protein expression profiling of beating hESC-CMs will be applied to elevate the cardiac regeneration capacity.

2. Materials and methods

2.1. hESC culture and in vitro differentiation into CMs

The hESC line H9 (NIH Code, WA09; WiCell Research Institute, Madison, WI) was propagated on irradiated mouse embryonic fibroblast feeder layers using hESC medium consisting of DMEM/F12 (Invitrogen, Carlsbad, CA), 20% Knockout Serum Replacement (KSR, Invitrogen), 1% non-essential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 1% penicillin/streptomycin (Invitrogen), and 4 ng/ml basic fibroblast growth factor (bFGF, Invitrogen) as previously described [66]. hESC colonies were passaged once a week by mechanical dissociation. Our research was performed under ethical approval from the Institutional Review Board (IRB) at KRIBB. To induce embryoid body (EB) formation, hESCs were mechanically dissected into small clumps using a sterile needle, followed by collagenase IV (1 mg/ml; Invitrogen) treatment. These cell clumps were seeded onto plastic petri dishes and cultured in suspension for 5 days in differentiation medium consisting of knockout-DMEM (Invitrogen), 20% FBS (Invitrogen), 1% non-essential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% penicillin/streptomycin. Five-day-old EBs were subsequently plated onto gelatin-coated cell culture dishes in the differentiation medium. The medium was changed every 2 days. At day 5+2 (cultured in suspension for 5 days and as attached EBs for 2 days), spontaneously beating clusters were observed by microscopic observations. To compare the beating and non-beating areas, beating areas of attached EBs at day 10 were dissected with a sterile blade, and non-beating areas were similarly collected.

2.2. Reverse-transcription (RT)–polymerase chain reaction (PCR)

Total RNA was obtained from the hESCs, their differentiated EBs at day 5, and the manually dissected beating and non-beating areas at day 10 after the EBs were plated. The RNA was extracted using an RNeasy kit and reverse-transcribed (1 μg) using the Superscript II First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. PCR was performed using the Taq PCR Master Mix kit (Qiagen) under the following conditions: 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a 10-min extension at 72 °C. The primers used in this study are listed in Table 1.

2.3. Immunocytochemistry

For immunostaining, we attached 5-day-old EBs on 0.1% gelatin-coated plastic coverslips in a 4-well plate. After 10 days, the EBs were examined for the presence of beating areas and were recorded on video. The EBs were fixed in 4% formaldehyde for 15 min at room temperature and then washed twice in PBS. The EBs were rinsed with 0.05% Tween-20 in PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and rinsed with 0.05% Tween-20 in PBS three times. The EBs were then blocked with 4% normal donkey serum in PBS for 1 h at room temperature and stained overnight at 4 °C with α-actinin (1:100, Chemicon), ANP (1:100, Chemicon), MHC (1:100, Chemicon), and Nkx2.5 (1:100, Chemicon). Also, to confirm the expression pattern of 2-DE analysis, we stained beating and non-beating cardiomyocytes with HSP 90 α (1:500, Santa Cruz Biotechnology), HSP 90 β (1:500, Santa Cruz Biotechnology), HSP 70 (1:500, Santa Cruz Biotechnology) and HSP 27 (1:500, Santa Cruz Biotechnology). The EBs were then washed five times with 0.05% Tween-20 in PBS and then incubated with the appropriate secondary antibodies for 45 min at room temperature. The EBs were rinsed with 0.05% Tween-20 in PBS five times. The nuclei were stained with DAPI for 5 min and rinsed. The coverslips were then mounted in the mounting solution.

2.4. Proteomic analysis

Total protein extracts were prepared from beating and non-beating hESC-CM samples using a protein extraction solution (1.0 mM PMSF, 1.0 mM EDTA, 1 M pepstatin A, 1 M leupeptin, and 0.1 M aprotinin). 2-DE was performed using an IPGphor IEF unit as described previously [20] and above. The silver-stained gels were scanned with an ImageScanner (Amersham, USA) and analyzed with Phoretix Expression software (ver. 2005; Nonlinear Dynamics, UK). Destaining and in-gel tryptic digestion of the protein spots were performed as described [21]. Xcise (Shimadzu Biotech Co., Japan), an automatic sample preparation system, was used for in-gel digestion, desalting, and plating onto a MALDI-TOF plate. Desalting was performed with ZipTipC^{18} (Millipore, Bedford, MA, USA), and plating was accomplished using a 4-hydroxy-α-cyano-cinnamic acid (HCCA) matrix solution. The in-gel-digested peptides were analyzed using an ultraflex-TOF/TOF (Bruker Daltonics, Germany) mass spectrometer. The mass spectra were calibrated and processed using Flex Analysis and BioTool 2.2 software (Bruker Daltonics, Germany). Peptide mass fingerprinting (PMF) ion searches were performed using Mascot 2.1 software (http://www.matrixscience.com) integrated with BioTool 2.2. The MSDB (version 20060831: 3239079 sequences) and NCBInr (version 20080125: 5872070 sequences) protein databases were searched using the following Mascot settings: taxonomy: Homo sapiens, one incomplete tryptic cleavage allowed, peptide tolerance: 50–100 ppm, fragment tolerance: 0.5 Da, monoisotopic mass, 1+ peptide charge state as HCCA protonation, alkylation of cysteine by carbamidomethylation as a fixed modification, and oxidation of methionine as a variable modification. For each search, statistically significant ($p<0.05$) matches were regarded as correct hits. The threshold score for the MSDB was 67, and the threshold score for the NCBI database was 67–78.

Table 1
Primers used in this study.

Gene	Primer (forward)	Primer (reverse)
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
Oct4	AGTGAGAGGCAACCTGGAGA	CAAAAACCCTGGCACAAACT
Nanog	CAAAGGCAAACAACCCACTT	ATTGTTCCAGGTCTGGTTGC
Nkx2.5	GGTCTATGAACTGGAGCGGC	ATAGGCGGGGTAGGCGTTAT
αMHC	GTCATTGCTGAAACCGAGAATG	GCAAAGTACTGGATGACACGCT
βMHC	AGATGGATGCTGACCTGTCC	GGTTTTTCCTGTCCTCCTCC
cTnT	GGCAGCGGAAGAGGATGCTGAA	GAGGCACCAAGTTGGGCATGAACGA
ANP	GAACCAGAGGGGAGAGACAGAG	CCC TCAGCTTGCTTTTTAGGAG