A new method for identification and isolation of human embryonic stem cell-derived cardiac progenitors and cardiomyocytes

Theses of Ph.D. dissertation

Kornélia Szebényi

Semmelweis University
Doctoral School of Molecular Medicine

Doctoral supervisor: Dr. Balázs Sarkadi, member of HAS
Scientific consultant: Dr. Ágota Apáti, Ph.D.

Official opponents: Dr. Gábor Földes, Ph.D.
Dr. Melinda Pirity, Ph.D.

President of the examination committee: Dr. Erzsébet Ligeti, member of HAS
Members of the examination committee: Dr. Szabolcs Sipeki, Ph.D.
Dr. László Hiripi, Ph.D.

Budapest
2014

Introduction

Stem cells are capable of renewing themselves through cell division for unlimited times, while under certain physiological or experimental conditions, they can be induced to become tissue-specific cells with special functions through differentiation. Human embryonic stem cells (hESCs) are derived from human blastocyst-stage embryos and can provide an unlimited source for differentiated cells through their unlimited self-renewal ability as well as the capacity to generate all cell types of the body (pluripotency).

However, directed differentiation protocols are needed to allow the enrichment of the cell type of particular interest, enabling the use of hESC-derivatives in large-scale applications such as drug screening or therapeutic approaches. Applicability of hESC-derivatives depends not only on the large number of cells needed to be produced, but also on the purity of the cell population obtained at the end of differentiation. Therefore, the development of methods, allowing distinction between different types of cells is at least as relevant as the directed differentiation protocols.

Isolation of living cells can be based on the use of genetically engineered reporter systems or cell surface markers. Genetically modified features are specially required when markers of the cell type of interest are not located on the cell surface (e.g. transcription factors or sarcomeric proteins) and therefore selection would not be possible without disruption of the cell membrane. This was the case for cardiomyocytes (CMs) for a long time, previously to the identification of surface markers (SIRPA, VCAM1 and ALCAM) co-expressing with cardiac-specific transcription factors (*ISL1, TBX5, NKX2.5* and *GATA4*) and sarcomeric proteins (troponin I and T).

Increased yield of CMs as well as purification could also be achieved by isolation of progenitors (e.g. mesodermal, cardiac mesodermal, cardiovascular or cardiac progenitors), since progenitors possess high proliferation capacity and restricted differentiation potential allowing first the expansion of these cells by providing stage-specific renewal signals and then CM differentiation by cardio-inductive signals.

Our research group joined the field of stem cell biology with the aim to address the generation of large amounts of pure cardiomyocytes and progenitors through identifying and isolating cell types at different stages of cardiac commitment.

Aims

The goal of the research presented in the dissertation was to establish a novel *in vitro* method for the purification of human embryonic stem cell-derived cardiomyocytes and cardiac progenitors. In order to achieve this objective, the aims of the present study were:

1. To demonstrate that the CAG promoter provides the opportunity to identify cardiomyocytes in spontaneous differentiation cultures of human embryonic stem cells.
2. To induce cardiac differentiation of human embryonic stem cells in order to enrich the differentiation cultures for cardiac progenitors.
3. To demonstrate that the CAG promoter provides the possibility to identify and isolate cardiac progenitors during directed differentiation of human embryonic stem cells.
4. To find optimal culture conditions for isolated cardiac progenitors to maintain cardiac commitment during further differentiation.
5. To demonstrate that the isolated human embryonic stem cell-derived cardiac progenitors can be differentiated into relatively pure population of cardiomyocytes.
6. To optimize reaggregation and survival properties of isolated human embryonic stem cell-derived cardiac progenitors to enhance cardiomyocyte yield.

Methods

Human ESC culture and differentiation

The original HUES9 human embryonic cell line was kindly provided by Dr. Douglas Melton from the Harvard University, while BG01V was purchased from ATCC. The Sleeping Beauty (SB) transposon based gene delivery method was applied to genetically modify HUES9 and BG01V hESCs with a plasmid containing the cDNA of the fluorescent protein EGFP, under the control of a specific variant of the CAG promoter (SB-CAG-EGFP construct). This specific variant contains a CMV enhancer region, two sequences from the chicken β-actin promoter and one short part of the rabbit β1-globin promoter.

HUES9-CAG-EGFP and BG01V-CAG-EGFP hESC colonies were cultured on mitomycin-C treated mouse embryonic fibroblast (MEF) feeder cells. For spontaneous differentiation the embryoid body (EB) formation method was used: differentiation was initiated via EB formation under suspension culture conditions in the presence of foetal bovine serum (FBS), and 6 days later EBs were seeded to an adherent surface. During

directed differentiation instead of FBS a mix of defined growth factors was used to provide the necessary differentiation signals.

Flow cytometry

Undifferentiated HUES9-CAG-EGFP colonies were harvested from mouse feeder cells by enzymatic digestion with 0.05% trypsin-EDTA, HUES9-CAG-EGFP EBs were dissociated with 0.25% trypsin-EDTA. Single cell suspension was washed with PBS containing 0.5% bovine serum albumin and incubated for 30 min at 37 °C with the directly labelled monoclonal antibodies. For intracellular staining the cells were fixed and permeabilized with 4% paraformaldehyde (PFA) in PBS, subsequently the indirect staining was performed in PBS with 2% BSA and 0.75% Saponin (Sigma). Control staining with appropriate isotype-matched antibodies or background levels of fluorescence of the fluorochrome-conjugated secondary antibody was included. Dead cells were gated out based on 7AAD (Sigma) staining. Samples were analyzed by a FACSCalibur flow cytometer (Beckton-Dickinson, San Jose, CA) with BD CellQuest acquisition software (BDIS) or by FACSAria High Speed Cell Sorter (Beckton-Dickinson, San Jose, CA) with BD FACSDiva software.

Cell sorting

After HUES9-CAG-EGFP EBs were enzymatically dissected, single cell suspension was sorted into artificial fractions (low, mid and high) based on EGFP fluorescent signal intensity. Precision of the sorting procedure was monitored by EGFP expression profiling of the sorted fractions with flow cytometry, immediately after sorting. Cells obtained from the different fractions were either recultured as reaggregated EBs (rEBs) or monolayers; or propagated to further gene expression analysis.

Fluorescence plate reader measurements

Fluorescence plate reader measurements was carried out 3 and 20 days after sorting by using the VICTORX3 2030 Multilabel Reader (Perkin Elmer). For detection of EGFP fluorescence excitation wavelength was 490 nm, and a F535 emission filter was used. For propidium iodide (PI) staining the rEBs were fixed with methanol on ice. After incubation with PI for 15 minutes the rEBs were transferred into fresh PBS in a 96 well plate. Excitation wavelength was 540 nm, and a F660 emission filter was used for detection of PI fluorescence.

Immuncytochemistry

For immunostaining EBs and rEBs were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing steps with DPBS, nonspecific antibody binding was blocked for 1 h at room temperature in DPBS containing 2mg/ml bovine serum albumin, 1% fish gelatin, 5% goat serum and 0.1% Triton-X 100. The samples were incubated for 1 h at room temperature with both the primary and the secondary antibodies. DAPI (Invitrogen, Madison, WI) was used for nuclear staining. Samples were either examined by an Olympus FV500-IX confocal laser scanning microscope or by fluorescence microscopy.

Real-time quantitative PCR analysis

Total RNA was isolated from cells using TRIzol™ Reagent (Invitrogen) following the manufacturer's instructions. cDNA samples were prepared from 0.2 μg total RNA using the Promega Reverse Transcription System Kit as specified by the manufacturer. For real-time quantitative PCR (QPCR) Pre-Developed TaqMan® assays were purchased from Applied Biosystems. QPCR analyses were carried out using the StepOne™ Real-Time PCR System (Applied Biosystems). The fold change of mRNA in experimental and control cells was determined using the 2-ΔΔCt method. Relative mRNA levels were presented as mean values ± S.E.M.of 3 independent experiments.

Results

1. The CAG promoter allows the identification of cardiomyocytes in spontaneous differentiation cultures of human embryonic stem cells

EGFP expressing HUES9 and BG01V hESCs were generated by transfection with the SB-CAG-EGFP construct (see Methods). Transgenic hESC lines (HUES9-CAG-EGFP and BG01V-CAG-EGFP) were established through the enrichment of EGFP expressing hESCs by sorting or cloning. The transgenic hESC lines maintained pluripotency during long-term culture and during spontaneous differentiation loss of pluripotency and differentiation into the three germ layers could be confirmed by QPCR and immunostaining studies. The presence of cardiomyocytes was indicated by the emergence of spontaneously contracting areas after 14 days of differentiation. At the same time an exceptionally high EGFP signal was observed onsite of the contracting areas, while in other tissues the EGFP expression remained low. This cardiomyocyte reporter feature proved to be a specific property of the CAG promoter that was used (a specific variant of the original artificial CAG promoter) and provided a method for recognition and separation of CMs. The CAG promoter based system was designated as a "double feature" system, since it allows the detection and isolation of transgene expressing

hESCs and their cardiomyocyte progeny based on the CAG promoter driven EGFP expression.

2. Directed cardiac mesoderm differentiation of CAG-EGFP expressing human embryonic stem cells

To facilitate the examination of the ability of the CAG promoter to identify cardiac progenitors, spontaneous differentiation was needed to be replaced by a differentiation protocol which is able to enrich the output of the differentiation for cells with mesoderm origin. A protocol obtained from the literature was optimized for transgenic hESCs, resulting in more synchronized and hastened differentiation of individual cells. Compared to spontaneously differentiating cells, cardiac directed differentiation resulted in increased mesoderm differentiation, as confirmed by flow cytometry analysis. Efficient cardiac differentiation was confirmed by the higher number of contracting areas (and higher percentage of troponin I positive cells) generated during directed differentiation compared to spontaneous differentiation.

3. The CAG promoter allows identification and isolation of human embryonic stem cell-derived cardiac progenitors

In order to assess whether the CAG-EGFP signal intensity allows the identification and isolation of cardiac progenitors, cell sorting was performed from the trypsinized cultures of differentiating HUES9-CAG-EGFP cells and transcriptional profile of the isolated samples were compared by QPCR. It was revealed that the population with exceptionally high EGFP signal (CAG-EGFP^{high}) expressed significantly higher levels of the cardiac specific genes than the population with low EGFP signal (CAG-EGFP^{low}), while early ecto- and endodermal marker genes were significantly higher expressed in the CAG-EGFP^{low} samples. An exception was the early cardiac-specific transcription factor *NKX2.5*, with significantly lower mRNA levels in CAG-EGFP^{high} samples compared to CAG-EGFP^{low} samples.

To identify the maturity status of CAG-EGFP^{high} progenitors, we compared the CAG-EGFP system to recently discovered cardiomyocyte- and cardiac precursor cell surface markers such as SIRPA, VCAM1 and ALCAM. Based on our findings, CAG-EGFP^{high} cells emerge earlier than SIRPA positive cells, but during further differentiation CAG-EGFP^{high} progenitor cells upregulate these cardiomyocyte markers.

4. Examining different culture conditions for isolated CAG-EGFP^{high} cardiac progenitors

To further investigate CAG-EGFP^{high} cells, culture conditions needed to be tested allowing re-culture and further differentiation of the isolated cells. Two different monolayers and a 3D suspension culture were tested in combination with two different culture media to compare their ability for supporting cardiac differentiation of CAG-EGFP^{high} cells. QPCR analysis revealed that the cardiac differentiation potential can be best supported when CAG-EGFP^{high} cells are cultured as 3D aggregates in conventional differentiation medium.

5. CAG-EGFP^{high} cardiac progenitors give rise to a relative pure population of cardiomyocytes

In order to further examine the potential of the CAG-EGFP^{high} cells, isolated CAG-EGFP^{high} and CAG-EGFP^{low} cells were plated in suspension cultures either on day 10 or day 12 of the differentiation, and maintained as rEBs for several weeks in conventional differentiation medium. Spontaneous contractile activity was first detected at 25 days after the formation of CAG-EGFP^{high} rEBs (approx. 30% of CAG-EGFP^{high} rEBs contracted), while CAG-EGFP^{low} rEBs did not show any contractile activity. Moreover, CAG-EGFP^{high} rEBs expressed significantly higher levels of cardiac specific genes than CAG-EGFP^{low} rEBs, and these transcriptional levels were several fold higher than those of the initially isolated CAG-EGFP^{high} cells, implying maturation of the CAG-EGFP^{high} cardiac progenitors into CMs.

6. Enhanced culture conditions for supporting the growth of CAG-EGFP^{high} rEBs without losing cardiac commitment

Reaggregation properties of isolated cells were relatively poor, since one day after the sorting procedure a large amount of single cells was detectable, floating around the forming CAG-EGFP^{high} rEBs. Reaggregation and survival properties of CAG-EGFP^{high} progenitors could be enhanced by treatment with Thiazovivin, a ROCK inhibitor or by the use of a medium conditioned by END-2. END-2 is a mouse visceral endoderm cell line, known for secreting cardioinductive molecules and direct the differentiation of hESCs into the cardiac lineage. However, in our hands the cardioinductive effect of END-2 was not detectable and long-term treatment with Isoproterenol, a beta-adrenerg agonist was needed to support the cardiogenic potential of CAG-EGFP^{high} progenitors when cultured in END-2 conditioned medium as 3D aggregates.

Conclusions

1. The CAG promoter-driven EGFP reporter system enables the selection of transgene-expressing hESCs. Moreover, cardiomyocytes differentiated from CAG-EGFP expressing hESCs can be easily identified based on their extremely high EGFP expression due to the cardiac tissue-specific transcriptional upregulation of the CAG promoter. These two features of this CAG promoter variant led to the designation as a "double feature promoter".

2. Directed differentiation of human embryonic stem cells results in enrichment of CAG-EGFP^{high} progenitors.

3. The CAG-EGFP system offers the advantage to isolate *NKX2.5*^{low} cardiac progenitor cells. During differentiation CAG-EGFP^{high} cardiac progenitor cells upregulate cardiomyocyte markers, such as SIRPA, VCAM1 and ALCAM.

4. Isolated CAG-EGFP^{high} cardiac progenitors are able to maintain their cardiac commitment during differentiation when cultured as 3D aggregates in conventional differentiation medium.

5. CAG-EGFP^{high} progenitor cells can generate spontaneously contracting 3D aggregates. These rEBs contain more than 90% of mature cardiomyocytes and show enhanced *NKX2.5* and *MYL7* transcription. CAG-EGFP^{high} cells possess a more restricted differentiation potential than previously described cardiovascular progenitor cells, since CAG-EGFP^{high} cardiac progenitor cells give rise to smooth muscle cells and atrial myocytes, but fail to differentiate into endothelial cells under 3D culture conditions.

6. Reaggregation and survival properties of CAG-EGFP^{high} progenitors can be enhanced by Thiazovivin or by the use of an END-2 conditioned medium, resulting rEBs with larger size than under control conditions. In our hands the cardioinductive effect of END-2 was not detectable and Isoproterenol, a beta-adrenerg agonist was needed to support the cardiogenic potential of CAG-EGFP^{high} progenitors when cultured in END-2 conditioned medium as 3D aggregates.

The application of this ''double-feature'' promoter provides a unique opportunity to examine the selective markers, including cell surface proteins of the cardiac lineage from early cardiac progenitors to late CMs and allows purification and enrichment of cardiac cells.

Publications related to this thesis

1 Szebenyi K, Péntek A, Erdei Z, Varady G, Orban TI, Sarkadi B, Apati A. (2014) Efficient Generation of Human Embryonic Stem Cell-Derived Cardiac Progenitors Based on Tissue-Specific EGFP Expression. Tissue Eng Part C Methods, Online Ahead of Print. IF: 4.254*

2 Orbán TI, Apáti Á, Németh A, Varga N, Krízsik V, Schamberger A, Szebényi K, Erdei Z, Várady G, Karászi É, Homolya L, Német K, Gócza E, Miskey C, Mátés L, Ivics Z, Izsvák Z, Sarkadi B. (2009) Applying a "double-feature" promoter to identify cardiomyocytes differentiated from human embryonic stem cells following transposon-based gene delivery. Stem Cells, 27:(5) pp. 1077-1087. IF: 7.747

Publications not directly related to this thesis

1 Erdei Z, Lőrincz R, Szebényi K, Péntek A, Varga N, Likó I, Várady Gy, Szakács G, Orbán TI, Sarkadi B, Apáti Á. (2014) Expression pattern of the human ABC transporters in pluripotent embryonic stem cells and in their derivatives. Cytometry B Clin Cytom, 86: 299-310. IF: 2.283*

2 Apáti A, Pászty K, Hegedus L, Kolacsek O, Orbán TI, Erdei Z, Szebényi K, Péntek A, Enyedi A, Sarkadi B. (2013) Characterization of calcium signals in human embryonic stem cells and in their differentiated offspring by a stably integrated calcium indicator protein. Cell Signal, 25:(4) pp. 752-759. IF: 4.471

3 Erdei Z, Sarkadi B, Brozik A, Szebenyi K, Varady G, Makó V, Pentek A, Orban TI, Apati A. (2013) Dynamic ABCG2 expression in human embryonic stem cells provides the basis for stress response. Eur Biophys J, 42:(2-3) pp. 169-179. IF: 2.474

4 Nagy L, Milano F, Dorogi M, Agostiano A, Laczko G, Szebenyi K, Varo G, Trotta M, Maroti P. (2004) Protein/lipid interaction in the bacterial photosynthetic reaction center: Phosphatidylcholine and phosphatidylglycerol modify the free energy levels of the quinones. Biochemistry, 43:(40) pp. 12913-12923. IF: 4.008