Roles of MicroRNAs and Transcription Factors in the Maturation of mESC-Differentiated Cardiomyocytes

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Abstract
ESC-derived cardiomyocytes receive increasing attention because of their value in seeking healing methods for cardiovascular diseases. For many of these applications, mature cardiomyocytes are a prerequisite. Therefore, it is essential to understand the maturation process of ESC-derived cardiomyocytes. In this study, we profiled the genome wide mRNA and microRNA expression during the cardiomyocyte-specific differentiation of murine ESCs in order to identify cardiomyocyte maturation related genes and their functions. In total, we found 63 genes specifically related to the analysed maturation process. Functional analysis of these genes suggested an important role of cell adhesion in the cardiomyocyte-specific maturation process. Deciphering of the gene expression regulatory mechanisms during the maturation process is an important aspect. Various bioinformatics sources for target information of transcription factors and microRNAs combined with the detected gene expression patterns were applied to distinguish regulators involved in the gene expression regulation of maturation related genes. Overall 14 transcription factors were identified. Focusing on cell adhesion related pathways, the three transcription factors Ctcf, Trp53, Creb3 and two groups of microRNAs could be highlighted as core regulators for Mapk7, Itgb1, Col4a1 and Bcl2, which are part of these pathways. This study presents an overview of the gene expression changes at the early maturation phase of ESC-derived cardiomyocytes. The function of maturation related genes indicates the importance of cell adhesion during the cardiomyocyte maturation. These results suggest the possibility of getting more control of cardiomyocyte maturation by interfering cell adhesion, i.e. through identified transcription factors and microRNAs.

ABBREVIATIONS
TF: Transcription Factor; miRNA: microRNA; ESC: Embryonic Stem Cell; mESC: murine Embryonic Stem Cell; ECM: Extra Cellular Matrix; vs.: versus

INTRODUCTION
Cardiovascular diseases, especially myocardial infarction, are the leading cause of death in industrialized countries [1]. As it is challenging to isolate pure cardiomyocytes from heart tissues [2], ESC-derived cardiomyocytes became valuable models for basic research, pharmaceutical tests as well as potential source for therapeutic applications for cardio diseases [3]. It has been demonstrated that the cell fate commitment of ESCs to cardiomyocytes is a key point in the ESC differentiation scenario [4]. So it is not surprising to see a lot of intensive research activities focusing on the differentiation process of ESCs from both, human and mouse, in various aspects [5,6]. ESC-derived cardiomyocytes were found comparable to fetal cardiomyocytes [7]. Preclinical and clinical trials using cardiac progenitor cells or ESC-derived cardiomyocytes showed variable results [8,9]. This makes it important to understand the maturation process of ESC-derived cardiomyocytes to ensure their safe and efficient applications. Mature cardiomyocytes have not only increased contractile force, but also specialized cell-cell junctions for the transmitting of electrochemical signals and mechanical forces. Deficiency in electrical coupling between cells could explain the low efficiency of transplanted immature ESC-derived cardiomyocytes [10]. Recently Aratyn-Schaus et al., found out that synchronized contraction of ESC-derived myocytes was achieved through coupling with mature cardiomyocytes. The establishment of such coupling based on interactions between cells and the extracellular matrix (ECM) as well as a redistribution of cell-cell adhesions [11]. This aspect indicates the important

role of cell adhesion pathways during the cardiomyocyte maturation process. The maturation process of ESC-derived cardiomyocytes is highly coordinated by many factors. The regulation of the gene expression during this process, apart from other biological processes, is delegated by transcription factors (TFs) and microRNAs (miRNAs). The elucidation of regulatory mechanisms involving TFs and miRNAs will shed more light in understanding the cardiomyocyte maturation. In this paper, we triggered a cardiomyocyte-specific differentiation of murine ESCs to study the cardiac differentiation of mESCs and thereafter also the maturation of mESC-derived cardiomyocytes. Cell samples at four different time points (day0, day12, day19 and day26) after the start of differentiation were collected for mRNA and miRNA expression analysis with microarrays. Regulatory relations between TFs, miRNAs and affected genes were explored for the maturation process of ESC-derived cardiomyocytes.

MATERIALS AND METHODS

ESC differentiation to cardiomyocytes

Mouse ESCs (D3, ATCC CRL 1934), stably transfected with the α-MHC-Pac-IRE-EGFP vector containing the EGFP gene and the PuromycinR (Pac) cassette under control of the cardiac α-myosin heavy chain promoter (clone aPIG 44) were cultured and differentiated resulting in Cor.At® cells. Cell samples were collected for undifferentiated ESCs (at day0) and selected differentiated cardiomyocytes (at day12, day19 and day26 after the initiation of differentiation) [12].

RNA isolation, quantification, and quality control

Total RNAs were extracted using PeqGold RNA pure (Peqlab Biotechnology, Erlangen, Germany) according to the manufacturer’s instructions. Quantity of total RNA was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Optical density values at 260/280 were consistently between 1.9 and 2.0. Total RNA quality was assayed on an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with intact, distinct ribosomal peaks were chosen for further analysis.

mRNA and miRNA expression profiling

Transcriptome profiling was performed using Affymetrix GeneChip® Mouse Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) as described in previous paper [13]. The complete dataset is deposited in Gene Expression Omnibus database with the accession number GSE58300. Genes were considered to be not expressed if none of their expression values is 2-fold or higher than the mean of the negative controls. Expressed genes, which have expression values changed more than 3 fold between time points with a raw p-value less than 0.01, were-defined as regulated genes. p-values were calculated with the moderated t-test from AltAnalyze [14].

The identical samples used in mRNA expression analysis were applied on Gene Chip® miRNA 1.0 Array (Affymetrix) for miRNA expression profiling and verified with a Geniom Real time Analyzer (GRTA, Febit GmbH, Heidelberg, Germany) using the Geniom Biochip miRNA mus musculus [12].

Identification of maturation related genes and their function

Genes were identified as cardiomyocyte maturation related when they fulfill following criteria: i) identified as regulated between day26 and day0 sample; ii) not regulated in comparisons day12 vs. day0 and day19 vs. day0; iii) the expression changes in comparisons day12 vs. day0 and day19 vs. day0 were less than 3-fold.

Pathway analyses were applied to the selected maturation related gene set with Web Gestalt [15] for top involved Wiki Pathways [16] and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways [17].

Generation of sequence based regulation network involving regulated TFs, miRNAs and genes

Predicted miRNA target information was retrieved from AltAnalyze [14] and miRWalk 2.0 database [18], which distinguish miRNA targets on 3’UTR, 5’UTR and CDS regions. Validated miRNA targeting information was also achieved from miRWalk 2.0 database.

TFs are defined as genes in GO term GO: 0003700 “sequence-specific DNA binding transcription factor activity” [13]. In addition, TFs were also retrieved from Riken TF Database [19] and mouse TF list of FANTOM5 database [20]. Predicted target information of TFs to genes were summarized from FANTOM TF interaction map [21] and TF binding site scan results from Enrichr [22] with Pairwise Weight Matrix (PWM) from TRANSFAC [23] and JASPAR [24]. Additional target information for TFs and miRNAs from RegNetwork [25] were also considered during the network construction.

Correlation network based on transcriptome profile and miRNA expression analysis results

We defined all miRNAs as transcription repressors while define TFs’ regulation effect as activator, repressor, or unknown based on literature mining. Correlation network was generated based on changes in the expression level of the regulators and their targets in the sequence based regulation network. Valid connections are defined for transcription activators with their targets changing the expression into the same direction and repressors with their targets changing the expression into the opposite direction.

RESULTS AND DISCUSSION

Cardiomyocyte specific differentiation was almost complete at day19

To gain a pure population of mESC-derived cardiomyocytes, the differentiated cardiomyocytes were selected by puromycin resistance under control of the α-myosin heavy chain promoter [26]. This selection system ensures a high purity of the cells (over 99%) but also removes the possibility to collect cardiomyocytes samples before day12. The advantage of using a pure cell population is that the contamination from other types of coexisting cells can be minimized for expression profiling study. The morphological and immunohistochemical observations of mESCs and mESC-derived cardiomyocytes at different time
points as well as the gene expression profile data during this process indicated that the cardiomyocyte differentiation process was completed before day 19 [13]. Based on this result, we consider the maturation of our cardiomyocytes started at day 19. The expression ratio between Myh6 (α-MHC) and Myh7 (β-MHC) (Myh6:Myh7) is an important indicator for cardiomycytse maturation [27]. Myh7 was known as fetal specific, while Myh6 more for adult cardiomycytes. Our data demonstrated that both, Myh6 and Myh7, increased significantly (see supplement table 1 for fold changes and p-values) during the differentiation and maturation process but showed an increase in the ratio since day 19 due to the reduced expression of Myh7 (Myh6:Myh7 ratios were 2.73 (day0), 2.45 (day12), 9.24 (day19) and 12.15 (day26)) (Figure 1a).

Ieda et al. reported that embryonic cardiomycocytes express abundant Itga1, Itga5 and Itgb1 while adult cardiomycocyte express more Itga6, Itga7 and Itgav [28]. In our dataset, Itga5, Itga6 and Itgb1 were highly expressed since undifferentiated state. Itgb1 was up regulated, started to increase at day 12 and reached the highest expression level at day 26. In contrast to Itgb1, Itga5 and Itga6 didn’t show significant increase. Itga1 increased significantly at day 12 and Itga7 increased slowly through the whole process. (Figure 1b, supplement Table 1). The increase of the Myh6:Myh 7 ratio at day 19 and suggested the switch from embryonic to adult cardiomycytes. But the expression patterns of integrin markers, on the contrary, still indicated that there were lot of embryonic cardiomycocyte markers at day 26. For this reason, we believe that cardiomycocytes at day 26 are still at the early phase of ESC-derived cardiomycyte maturation.

Differentiated expressed genes after day 19 and their functions

Morphological changes coupled of course with the gene expression. The genome-wide gene expression profiling after the start of differentiation showed significant expression changes during the differentiation and maturation process [13]. In our experiment, 1342 genes were identified as regulated in the differentiation and maturation process of the ESC-derived cardiomycocytes. The majority of the regulated genes were identified before day 19. This again support the assumption that the cardiomycyte differentiation was completed before day 19 (Figure 2a). The genes, which were identified as regulated only after day 19, are highly possible to be involved in the maturation process of the completely differentiated ESC-derived cardiomycocytes.

In total, 180 genes were identified as regulated only after day 19 (Figure 2a). A large part of these genes was also regulated at day 12 or day 19 over 3-fold but with less plausible p-values. Another part of these genes, 63 out of 180 genes, was regulated in less than 3-fold at day 19 or day 12. These genes were selected as maturation related genes for our further study (Figure 2 b). In the selected gene group, Creb3, Tnaiq3, Zeb2, Baz2a, Pou2f1, Zfp787 and Rbm14 are TFs according to our definition.

Function analyses unveiled the top 10 Wiki Pathways and KEGG pathways in which the selected 63 genes were involved (Table 1). Three cell adhesion related pathways (“focal adhesion”, “ECM-receptor interaction” and “integrin-mediated cell adhesion”) could be identified in these top pathways, which involved the genes Mapk7, Itgb1, Col4a1 and Bcl2.

<table>
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Denecke et al. (2016)

Figure 1 a) Mean expression values with standard deviation of fetal specific Myh7 (black bars) and adult specific Myh6 (grey bars) during the cardiomyocyte specific differentiation and maturation. The significance levels for the comparisons between differentiated samples (day12, day19 and day26) and undifferentiated samples (day0) are presented with stars. Detailed fold changes and p-values are listed in the supplement table 1.

Figure 2 a) Venn diagram for regulated genes between ESC-derived cardiomyocytes (samples day12, day19 and day26) and undifferentiated ESCs (day0). b) Heatmap of expression changes (log2) of 63 selected maturation related genes in three comparisons (day12 vs. day0, day19 vs. day0 and day26 vs. day0): red colour means up regulation and blue colour represents down regulation.

TF and miRNA co-regulate the maturation related genes

Pre-knowledge of TF and miRNA targets from various sources as well as the mRNA/miRNA expression profiles were employed to identify possible regulating TFs and miRNAs for the 63 maturation related genes. This resulted in a regulatory network with regulators (TFs and miRNAs) and maturation related genes. Fourteen TFs (Ctcf, Creb3, Trp53, Meis1, Myb, Mcm2, Nlx2-5, Rarg, Zic3, Myc, HmgA1, Pou2F1, Mcm3) were found to be involved in this regulatory network (supplement table 2). All 50 as regulated defined miRNAs had their targets in the maturation related gene set. This is i.e. due to the fact that the miRNA target predicting process has generally high false positive rate.

Among the identified top involved pathways of our maturation genes, cell adhesion related pathways were over-represented. It is also of interest to find key regulators at the early phase of cardiomyocyte maturation by detecting regulators involved in these pathways. Down regulated Mapk7 together with up regulated Itgb1, Col4a1, Bcl2 from the maturation related gene set were found in the three cell-adhesion related pathways. The regulatory mechanism of these four genes with identified TFs and miRNAs was illustrated in Figure 3. A group of miRNAs regulated concurrently Itgb1, Col4a1 and Bcl2. Besides this group of miRNAs, Bcl2 was also regulated by the TFs Ctcf, Trp53 and Creb3, which is also only regulated after day 19 (Figure2b).

Mapk7 was found to be a target of a large group of miRNAs but no target of TF(s) (Figure 3). Some of these regulatory mechanisms have been reported under different contexts, which will support our results. For example, miR-24, identified as regulatory miRNA in our study for Mapk7, was found experimentally repressing Mapk7 in human adipocyte differentiation [29]. The protein family Bcl-2 was known as one of the p53 dependent cell survival factors and a negative regulation of Bcl-2 expression by p53 was reported in hematopoietic cells [30]. Similar regulatory mechanism between Trp53 and Bcl2 was observed in mouse cardiomyocytes [31]. In our results, a negative regulation of Trp53 on Bcl2 on the transcriptional level was also observed.
CONCLUSIONS

The maturation of ESC-derived cardiomyocytes is essential for both, research and clinical applications. To gain more control over this process, a thorough understanding of the regulatory mechanisms involved in the cardiomyocyte maturation process is an important premise. In this paper, we studied the early maturation process of cardiomyocytes derived from mESCs with genome wide expression analysis and bioinformatics tools. In total, we could identify 63 maturation related genes and 14 TFs involved in the maturation process (Ctcf, Creb3, Trp53, Mef2a, Meis1, Myb, Mcm2, Nkx2-5, Rarg, Zic3, Myc, Hmga1, Pou2f1, Mm43). Functional analysis of the gene set involved in cardiomyocyte maturation showed an emphasis on cell adhesion function. Three TFs (Ctcf, Trp53 and Creb3) and two groups of miRNAs (Figure 3) were highlighted as regulators responsible for the expression of genes involved in the cell adhesion related pathways (namely: Mapk7, Itgb1, Col4a1 and Bcl2). Beyond single regulatory mechanisms, our results present a good overview of the complete regulation scenario concerning TFs and miRNAs in the context of maturation process of ESC-derived cardiomyocytes, which will surely be a sound fundament for further studies with this theme.

REFERENCES


Cite this article