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Dynamic regulation of small RNAome during the early stage of cardiac differentiation from pluripotent embryonic stem cells

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ABSTRACT

Embryonic stem cells (mESCs), having potential to differentiate into three germ-layer cells including cardiomyocytes, shall be a perfect model to help understanding heart development. Here, using small RNA deep sequencing, we studied the small RNAome in the early stage of mouse cardiac differentiation. We found that the expression pattern of most microRNA (miRNA) were highly enriched at the beginning and declined thereafter, some were still insufficiently expressed on day 6, and most miRNAs recovered in the following days. When pluripotent embryonic stem cells are differentiating to cardiomyocytes, targeted genes are concentrated on TGF, WNT and cytoskeletal remodeling pathway. The pathway and network of dynamically changed target genes of the miRNAs at different time points were also investigated. Furthermore, we demonstrated that small rDNA-derived RNAs (srRNAs) were significantly up-regulated during differentiation, especially in stem cells. The pathways of srRNAs targeted genes were also presented. We described the existence and the differential expression of transfer RNA (tRNA), Piwi-interacting RNA (piRNA) and Endogenous siRNAs (*endo*-siRNAs) in this process. This study reports the genome-wide small RNAome profile, and provides a uniquely comprehensive view of the small RNA regulatory network that governs embryonic stem cell differentiation and cardiac development.

1. Introduction

Small RNAs are master regulators controlling proliferation and differentiation, particularly in regulating stem cell biology and cardiac development. The switch from pluripotent to lineage-specified cells is accompanied by the up-regulation of many small regulatory RNAs, such as microRNAs (miRNAs) [7] — noncoding endogenous RNAs with approximately 18–25 nucleotides in length, which regulate gene expression mainly by inhibiting translation or promoting degradation of target mRNAs by base paring with specific mRNA targets [11].

Using combinations of next-generation sequencing (NGS) technologies, small RNA population was profiled in numerous areas of biological research, including health and diseases [10].

Lots of heart disease are associated with decreased functional cardiomyocytes. It is well established that embryonic stem cells (ESCs) are able to generate *bona fide* cardiomyocytes. ESCs derived from the inner cell mass (ICM) of preimplantation embryos can be propagated *in vitro* in an undifferentiated state and, when allowed to differentiate, can form endodermal, ectodermal, and mesodermal derivatives *in vitro* and *in vivo*. ESCs thus can be a potential source of donor cardiomyocytes (or alternatively, donor cardiomyogenic progenitors) for therapeutic interventions targeting heart diseases.

Cellular differentiation is a sequential commitment process achieved through multiple intermediate states, and is tightly regulated by different levels of gene expression program. Cardiac differentiation from ESCs represents a paradigm for studying cell fate determination in both cellular and molecular levels. Spontaneous cardiac differentiation from mESCs can mimic the *in vivo* cardiac development. Thus, understating how the cell fate of cardiac cells is determined not only holds unlimited potential for the cell replacement therapies, but also yields general principles of cell fate determination and organ development [22].

In vitro ESC differentiation typically requires an initial aggregation step to form structures, termed embryoid bodies (EBs), which further

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differentiate into cardiomyocytes [1]. Thus, *in vitro* cardiac differentiation involves several distinct processes, including stem cell aggregation to form embryoid bodies, stem cell commitment and final functional cardiogenesis, which entails maturation to form beating cardiomyocytes. It has been shown that miRNAs can promote cell cycle exit and differentiation, and are required for proper ESCs differentiation [7]. However, the mechanism through which small RNA contribute to stepwise differentiation is currently unknown, and a global view of small RNA repertoire is still lacking.

To comprehensively understand the dynamic changes of small RNA expression during cardiac differentiation, we applied next-generation sequencing to analyze the small RNA fraction on day 0 (D0), day 2 (D2), day 6 (D6) and day 9 (D9) of differentiation. These time points afforded a view of critical transcriptomic changes occurring both before and during cardiac differentiation from pluripotent stem cells.

Our results provide a global view of RNAome, including miRNAs, srRNAs, piRNAs, *endo*-siRNAs and tRNAs, as well as their expression pattern during differentiation. We also provide a potential resource for the regulatory role of miRNAs and srRNAs that target signaling pathway during the differentiation of ESCs to cardiomyocytes.

2. Materials and methods

2.1. mESCs culture and differentiation

E14T mESCs were maintained under feeder-free conditions as previously described [19]. Briefly cells were grown in 0.1% gelatin-coated dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplied with 15% ESC-qualified FBS (Invitrogen), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1000 units/ml leukemia inhibitory factor (LIF; Chemicon), 100 U/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen) at 37 °C, 5% CO_2.

In vitro differentiation of mESCs to cardiomyocytes was performed using the hanging drop method [20]. Briefly, ESCs were trypsinized and resuspended in complete growth medium without LIF, and plated in hanging drops at around 400 cells. They were dropped per 20 μl on the lids of petri dishes filled with PBS and cultivated in hanging drops for two days. EBs then were collected and plated on low-attachment cell culture plates for two days [9]. They were further plated separately onto gelatin coated 24-microwell on D6. For the determination of cardiac differentiation, spontaneously beating cardiomyocytes were investigated from the outgrowth of EBs cultured in 24-microwell plates. The well harboring beating cells were marked and cells in it were picked up on D9. Cells were collected on D0, D2, D6 and D9.

2.2. Small RNA cloning and sequencing

To obtain high quality total RNA samples, embryoid bodies, spontaneously and rhythmically contracting cardiomyocytes were manually picked up under compound microscope. Total RNA from D0, D2, D6, and D9 were extracted using Trizol (Invitrogen). The quality and integrity of the total RNA was evaluated by electrophoresis on 1.2% agarose gel and Agilent 2100 BioAnalyzer (Agilent). Small RNAs ranging from 18 to 30 nt were gel-purified and ligated to the 3′ adaptor and 5′ adaptor oligonucleotides. Samples were allowed for deep sequencing on the Illumina GAII platform.

2.3. Bioinformatics analysis of small RNA data

In order to make statistics of miRNA reads, mirTools [23] was used. Reads were mapped to the University of California at Santa Cruz (UCSC) mm10 assembly of the murine genome by using megablast. miRNAs profiling was according to miRNA (miRBase version 18).

Normalization formula is by using transcripts parts per million (TPM): normalized expression = actual miRNA count / total count of clean reads × 1,000,000. Here, unique reads refer to different types of reads, and redundant reads refer to total reads.

Mapped miRNA which has reads in at least three samples were further analyzed using Gene Cluster 3.0. Gene Tree view which can display hierarchical as well as *k*-means clustering results was used.

The miRNA targets prediction software was composed of validated and the computationally predicted targets. The validated target data sets were selected from TarBase, miRecords, whereas miRanda, TargetScan, PicTar, PITA, MirTarget2, RNAhybrid, RNA22 were used to predict the targets of miRNAs. The majority rule voting was selected to predict the targets. After that, the validated and the predicted targets are combined as miRNA targeted gene pool [11]. PACCMIT-CDS, RNAhybrid, miRanda and MicroTar were used in srRNAs targets prediction.

2.4. Functional annotation of target genes

To group miRNA targets that are functionally related into known functional categories and pathways, metaCore-GeneGo Pathway Maps were used [11]. For highly enriched miRNAs, their targets were predicted and uploaded to GeneGo. Targets were analyzed with the Pathway Maps [11]. Regulatory networks were drawn using Cytoscape (http://www.cytoscape.org).

2.5. Fetch RNA sequences and gene annotation

The *M. musculus*, sequences were retrieved from UCSC (http://hgdownload.soe.ucsc.edu/downloads.html#mouse) Dec. 2011 (GRC-m38/mm10).

The sequence of mouse 5S, 5.8s, 18s, 28s ribosomal RNA and tRNA were downloaded from http://www.ncbi.nlm.nih.gov/nuccore/M31319.1, http://www.ncbi.nlm.nih.gov/nuccore/175404, http://www.ncbi.nlm.nih.gov/nuccore/NR_003278.3, http://www.ncbi.nlm.nih.gov/nuccore/53988, http://gtrnadb.ucsc.edu/download.html. piRNA sequences were downloaded from NCBI Nucleotide database. Some reads mapped to more than one type of annotation. The following priority rule was used: miRNA > mRNA > srRNA > piRNA > *endo*-siRNAs.

2.6. Novel miRNA and endo-siRNA prediction

Small RNA sequences were mapped to the genome and fetch each exact sequence match along with 100 bases flanking either side. For predicting the secondary structure, mfold was utilized (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form).

Reads were aligned themselves by using megablast. We only fetched the plus/minus paired reads. Candidates were mapped to the transcriptome SRR1028908. By using mfold, the secondary structure of transcriptome was predicted. The paired reads so as to leave 3′, two-nucleotide overhangs, and on the long double-stranded transcriptomes was identified as novel *endo*-siRNAs.

3. Results

3.1. High-throughput sequencing and annotation of mouse small RNA sequences during cardiac differentiation of mESCs

3.1.1. Summary of small RNA-Seq reads counts

To obtain a comprehensive overview of small RNA dynamics during cardiac differentiation, a feeder-free murine embryonic stem cells (mESCs)-derived cardiomyocyte differentiation program was used. Samples were collected from four time points: pluripotent embryonic