Orignal Article

Analysis of Protein-Protein Interaction Network Based on Transcriptome Profiling of Human Induced Pluripotent Stem CellsIdentifies Candidate Genes

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) offer an exceptional opportunity for generating disease-specific models to study and explore the underlying mechanisms. The present study aims to perform bioinformatics analysis to explore the potential hub genes, cluster as well as functional pathways depended upon the differentiation of induced pluripotent stem cells. Differentially expressed genes (DEGs) between induced pluripotent stem cells (hiPSCs) and their differentiated cells (cardiomyocytes, CMs) were identified by TAC software. Subsequently, the protein-protein interaction (PPI) network was built using the differentially expressed genes (DEGs) and NetworkAnalyst tool, which then analyzed by Cytoscape software. The results disclosed that 3654 DEGs (1699 [46.49%] upregulated and 1955 [53.50%] downregulated) are primarily implicated in the CMs differentiated from hiPSCs. The component of the main constructed PPI network comprised of 975 nodes with 2472 edges. Six hub genes were recognized (MCM3, MCM5, CDC6, EP300, RPS27A, CDKN1A) by overlap of the top fifty genes as stated by five calculation methods in CytoHubba. In addition, five significant modules were generated using MCODE application in Cytoscape. The result of GO analysis for the five modules A-E, respectively, disclosed that DNA replication origin binding, structural constituent of ribosome, RNA binding, actin binding, exodeoxyribonuclease activity, producing 5'-phosphomonoesters are the most significantly enriched in molecular function term; DNA replication initiation, translational elongation, ribosome biogenesis, muscle filament sliding, ERBB2 signaling pathway are the most significantly enriched in biological process term; chromosome, telomeric region, large ribosomal subunit, nucleolus, striated muscle thin filament, basal plasma membrane are the most enriched in cellular component term. The KEGG pathway analysis disclosed that the genes of five modules A-E, respectively, were enriched in Cell cycle, Ribosome, Ribosome biogenesis in eukaryotes, Cardiac muscle contraction, ErbB signaling pathway.In conclusion, the analysis of DEGs and hub genes analysis play significant role in hiPSCs differentiation into CMs and could improve our understanding their differentiation.

Keyword: Bioinformatics analysis, Cardiomyocytes differentiation, Human induced pluripotent stem cells, Protein-protein network

Introduction

The inability to proliferate of matured cardiomyocytes makes the functional of these cells to end permanently following their injury.[1] The applying of new technologies to human cells were rapidly grown and gave promise with the successful creation of human induced pluripotent stem cells (hiPSCs) from human fibroblast by introducing pluripotency factors using either retroviral transduction of four transcription factors (OCT3/4, SOX2, KLF4, and c-MYC) or partially different collection of transcription factors (OCT4, SOX2, NANOG, and LIN28). This method has been recently applied

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to create disease- relevant induced pluripotent stem cell lines from patients suffering from different diseases.[2,3] *In vitro* studies have been characterized derivatives of three primary germ layers following differentiation of several human iPS cell lines.[4,5] although detailed characterization of the ability of hiPSCs to generate functionally characterized specific cell linages are generally inadequate.[2] During the past years, substantial progress in the cardiomyocyte generation from induced pluripotent stem cells, technology and methods have been improved, including modulation of GSK and Wnt pathways using minor molecule inhibitors.[6,7] using of Activin A and BMP in addition to the matrigel sandwich technique.[7]

Large group gene expression analysis facilitates identification of hundreds of differentially expressed genes (DEGs) simultaneously from a provided data set.[8,9] As all the biochemical processes in the human cells are administered by theproteins, we propose that proteins encoded by the genes derived from transcriptome expression profiling affect other proteins derived from other genes in a network, and these processes are critical in human induced pluripotent stem cell differentiation.[9] Computational and bioinformatics approaches with the nominee genes derived from studying the transcriptome expression further help in the identification and validation of derived biological processes to nominee genesconcerning to the pathophysiology of the disease.[9] Therefore, in the current work, microarray data were utilized from public microarray data, the Gene Expression Omnibus (GEO), to recognize the differentially expressed genes (DEGs) between hiPSCs and their differentiated cells (CMs). Functional enrichment analysis was achieved and the regulatory pathways were discovered through topological analysis of protein-protein interaction (PPI) network following construction of it by introducing the DEGs to the PPI data. This biological information with the relevant biological knowledge may facilitate to understand the underlying biological mechanisms of stem cells during the differentiation.

Materials and Methods

Microarray Dataset

The mRNA expression profile GSE35108 based on the GPL6244 platform of Affymetrix Human Gene 1.0 ST Array, deposited by Sun N et al. (2012), was taken from the Gene Expression Omnibus (GEO) repository of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo). In this microarray experiment, the expression of genes in 4 samples of induced pluripotent stem cells following reprogramming skin fibroblast of patients with dilated cardiomyopathy (DCM iPSCs) was compared to that in 2 samples of generated cardiomyocytes by differentiation of induced pluripotent stem cells that originated from patients with dilated cardiomyopathy (DCM iPSCs-CMs).[10]

Identification of Differentially Expressed Genes (DEGs)

To find the differentially expressed genes (DEGs), raw Affymetrix data (.CEL files) were downloaded and introduced to Transcriptome Analysis Console (TAC) software (version 4.0.1.36) for analysis. Log 2 fold change > 2 or < -2 and P- value < 0.05 were considered as threshold values for the DEGs.

Construction of Protein-protein Interaction Network

To obtain insights into the interaction among DEGs linked to DCM iPSCs differentiation, a zero-order PPI network was built via Search Tool for the Retrieval of Interacting Genes (STRING), a database of known and anticipated protein interactions, within the NetworkAnalyst tool (http//www.networkanalyst.ca/). PPIs that possessed a medium confidence score of 400 were decided to be statistically significant when generating network in STRING. The PPI network was subsequently visualized by Cytoscape software (version 3.6.1) for further analyses.

Identification of Hub Gene and Analysis of Modules

The Cytoscape Molecular Complex Detection (MCODE) plug-in was used to analyze the zero-order PPI network and search clustered subnetworks (modules); MCODE scores ≥ 4 and the number of nodes > 5 were used as cutoff criteriawith the default parameters (degree cutoff ≥ 2, node score cutoff ≥0.2, K-Core≥2, and Max. depth =100). Then, the zero- order PPI network was further analyzed using Cytoscape CytoHubba plug-in to explore the network hub genes; five calculation methods were employed: MCC, MNC, EPC, Degree, EcCentricity. The top fifty genes derived from each method had been analyzed by Venn diagram using Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/) to obtain the intersected genes among these methods which may serve as candidate genes with crucial biological control functions.

Gene Ontology (GO) and Pathway Enrichment Analysis

The genes of the modules were uploaded to web-based ToppGene (http://toppgene.cchmc.org) to perform gene ontology including the biological process (BP), molecular function (MF), and cellular component (CC) and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway enrichment analysis. P-value < 0.05 was weighed as an indication of statistical significance.

Results

Identification of differentially expressed genes (DEGs)

After removing each probe that mapped to more than one gene and the probes that could not assign gene name, whereas select only a single probe per gene of those multiple probe sets per the same gene. A total of 3654 DEGs were obtained based the cut-off criteria (Log 2 fold change > 2 or < -2 and P-value < 0.05) when comparing DCM iPSCs with DCM iPSCs-CM. Among these, 1699 upregulated genes and 1955 downregulated genes were recognized.

The result has been represented as the volcano plot in figure 1.

Construction of Protein-protein Interaction (PPI) of DEGs and Hub Gene Selection

The PPI network was built by using NetworkAnalyst. Because of too many seed proteins (i.e. >3000) that will lead to create too large network, a zero-order network was created to conserve only seed proteins that affect each other and simplify a dense network. The zero-order network with 975 nodes and 2472 edges is presented in figure 2.

A zero-order network was analyzed to identify the hub genes via CytoHubba, a Cytoscape plug-in. The top fifty

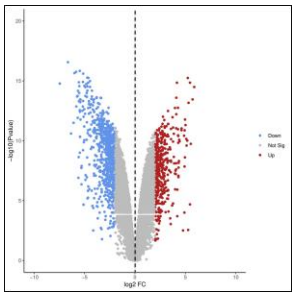

Figure 1: Volcano plot of differentially expressed genes (DEGs). Upregulated genes indicated by red dots and downregulated genes indicated by blue dots with statistical significant log 2 fold change > 2 or < -2 and P-value < 0.05, grey dots fail to meet these statistical criteria. The x-axis represent the log2 FC (fold change), and the y-axis represent the –log10 (P value). This volcano plot was constructed using galaxy platform (https://usegalaxy.org).

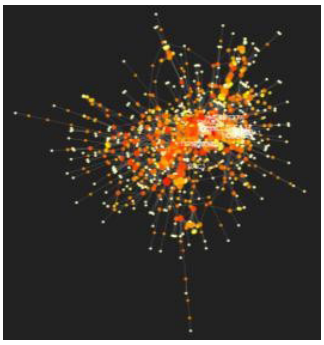

Fig. 2: PPI network (zero-order network) of DEGs in microarray dataset. Proteins are represented as nodes and interactions between them are represented as edges.

genes evaluated by five CytoHubba methods (MCC, MNC, EPC, Degree, EcCentricity) and the Venn diagram (figure 3) showed the intersection of these five CytoHubba methods and identified significant hub genes. The six most significant hub genes (the overlapping genes) were (MCM3, MCM5, CDC6, EP300, RPS27A, CDKN1A).

Module Identification and Enrichment Analysis

Further analysis of zero-order network by MCODE plug-in revealed 26 modules. Of these, five modules passed the cut-off criterion (MCODE score ≥4; number of nodes ≥5). All selected modules are represented in figure 4. The first module consisted of DBF4, MCM2, POLA1, ORC4, CHEK1, MCM6, ORC1, MCM4, POLE, MCM9, CDC7, MCM7, ORC2,

MCM3, POLE2, MCM5, CDC6, and RPS27A (figure 4A), the second consisted of SEC61G, MRPL13, RPL7, MRPL16, RPS24, RPL21, MRPL15, RPL39L, RPL24, MRPL22, RPL22, RPL9, RPL11, and RPL27A (figure 4B), the third consisted of FBL, DHX37, WDR3, TBL3, UTP15, UTP20, PUM3, NAT10, CEBPZ, DKC1, and RBM28 (figure 4C), the fourth consisted of MYL2, TNNI1, ACTN2, TNNT3, TNNI3, and TNNC1 (figure 4D), the fifth consisted of KRAS, POLD1, BLM, TIMELESS, RAD1, NRG1, BRCA1, SMAD3, NOTCH1, RFC4, AR, EGF, ERBB2, DNA2, ERBB4, EGFR, and ERBB3 (figure 4E).

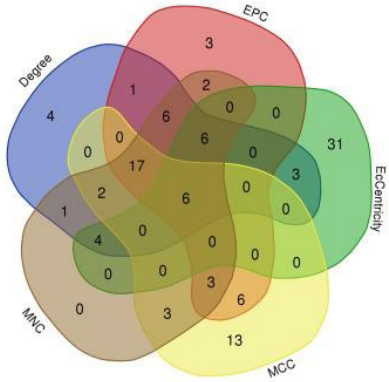

Fig. 3: Venn diagram of intersecting genes among five methods (MCC, MNC, EPC, Degree, EcCentricity) to generate identify significant hub genes. Areas with dissimilar colors represent different methods. The intersection area indicates theaccumulated genes. The genes in concurrent area are the 6 hub genes (MCM3, MCM5, CDC6, EP300, RPS27A, and CDKN1A).

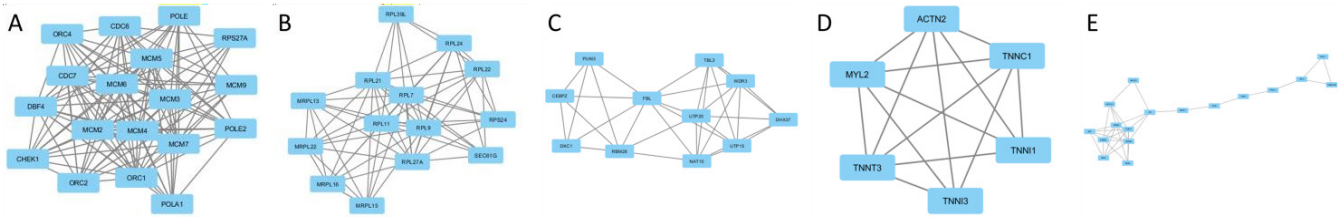

Fig. 4: The five significant modules identified from the zero-order protein-protein interaction network. (A) The first module consists 18 proteins. (B) The second module consists of 14 proteins. (C) The third module consists of 11 proteins.

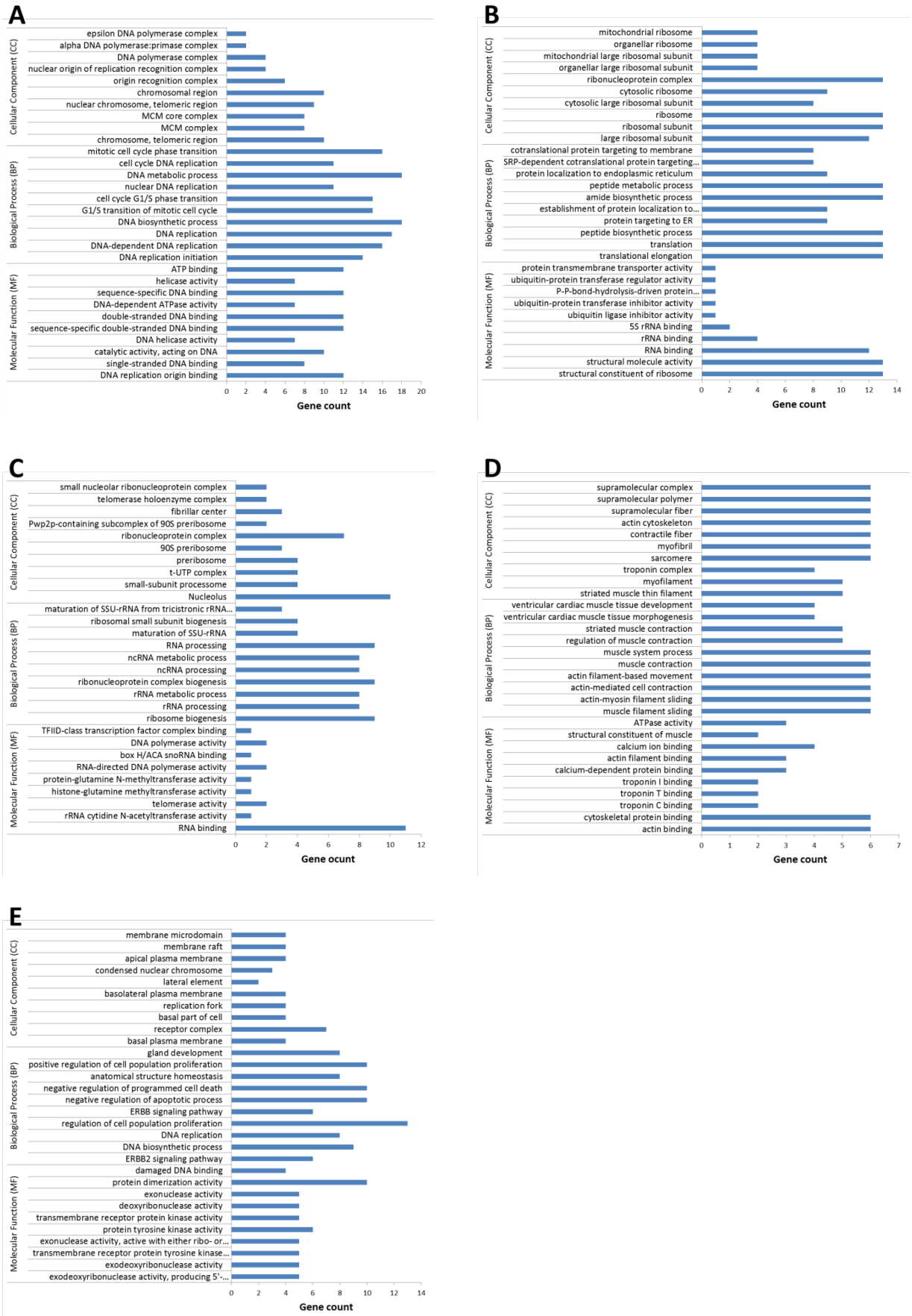

Fig. 5: The top ten enriched gene ontology terms for genes in the five significant modules (A-E).

(D) The fourth module consists of 6 proteins. (E) The fifth module consists of 17 proteins.

The functions and pathway enrichment of the five modules of zero-order network genes were evaluated at Toppgene website. The result of GO analysis is established in the following three functional categories: (1) molecular function (MF),

(2) biological process (BP), and (3) cellular component (CC). For GO term MF, the genes of modules A-E were enriched in 50, 12, 9, 32, 165 terms respectively. For GO term BP, the genes of modules A-E were enriched in 137, 69, 103, 107, 200 terms respectively. For GO term CC, the genes of modules A-E were enriched in 35, 25, 19, 16, 62 terms respectively. The top 10 GO terms for each category in which the modules were enriched are represented in figure 2. For further exploration of the dysregulated pathways affected by genes of the five modules, the KEGG pathways in which the genes were significantly enriched for modules A-E are represented in figure 6.

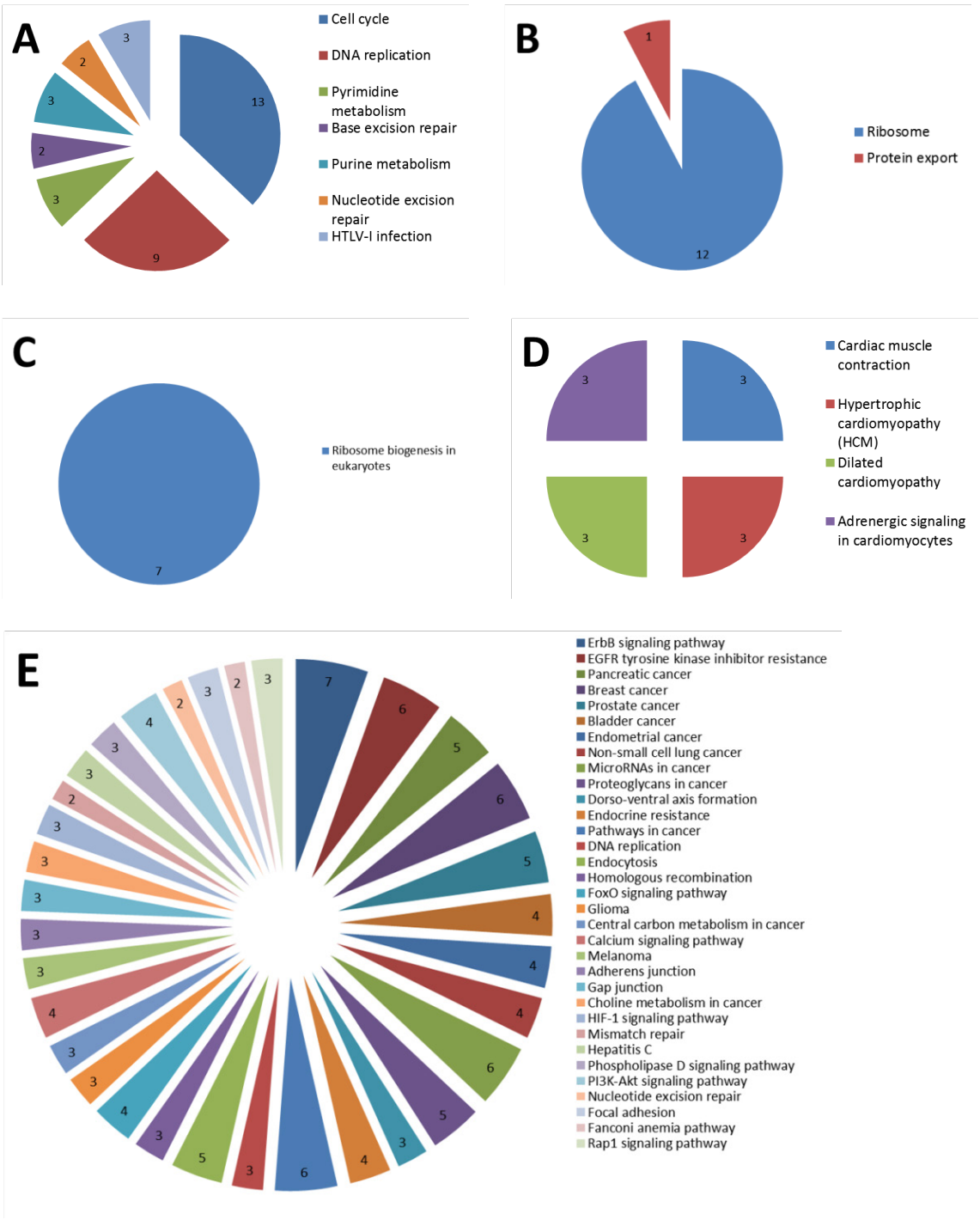

Fig. 6: KEGG pathway enrichment analysis for genes in the five significant modules (A-E).

Discussion

In the current study, protein-protein interactions (PPIs) - associated genes responsible for differentiation of DCM iPSCs into DCM iPSCs-CMs using microarray expression data are studied. Multiple analyses including alterations in gene expression, PPI network, hub genes as well as modules identification GO/KEGG pathway enrichment were performed to identify potential key genes/pathways that can discriminate DCM iPSCs from DCM iPSCs-CMs.

Gene expression profiling analysis showed genes with abnormal expression concerned with DCM iPSCs differentiation. In this study, a total of 3654 DEGs including 1699 upregulated genes and 1955 downregulated genes were recognized from microarray data analysis.

The hub gene analysis of the PPI network showed that the majority of the hub genes were downregulated including minichromosomemaintenancecomplexcomponent3(MCM3), minichromosome maintenance complex component 5 (MCM5), cell division cycle 6 (CDC6), E1A binding protein p300 (EP300), and ribosomal protein S27a (RPS27A), while one hub gene was upregulated (Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A)). MCM is known as the central constitute of the replicative helicase, and origin licensing is labeled to the process of MCM loading. The total level of MCM throughout the phases of cell cycle remains constant, but the level of DNA-loaded MCM continues increases while cells proceed through the G1 phase of cell cycle until reaching a maximum point at G1/S transition.[11] Previous study revealed that rapid MCM loading to license replication origins is an intrinsic pluripotent cells property and MCM loading rate increase when somatic cells were reprogrammed into induced pluripotent stem cells.[11] About CDC6 gene, previous study reported that the common of the regulatory factors including CDC6 is rather constitutively expressed throughout the cell cycle of embryonic stem cells than being restricted to a particular phase in cell cycle.[12] EP300 gene as reported previously, is important in different biological functions including cellular proliferation, DNA damage repair, apoptosis, cell cycle regulation, cell fate determination and stem cell pluripotency.[13] EP300, as a histone acetyltransferase, regulate transcription by chromatin remodeling.[14] EP300 colocolizes with OCT4, SOX2, and NANOG at many enhancer regions in embryonic stem cells,[15,16] it works together with OCT4 and SOX2 to promote transcriptional activation from an FGF4 enhancer which is active in ES cells, as well as both NANOG and OCT4 are essential for total employment of EP300 to SOX2 sites.[17] It is also required for embryonic development and differentiation of a broad range of cell types.[18] The result of this study is in agreement with previous results of a study that showed a decreased expression of EP300 during hiPSCs differentiation into induced cardiomyocytes (iCMs).[19] It is reported that RPS27A play a role in the regulation of cell cycle and promote cell proliferation.[20] This was consistent with the result of the present study that shows the expression of RPS27A was downregulated during the differentiation of hiPSCs, which in turn that the differentiation affects cell proliferation. Cell cycle regulation is interlinked in the mechanisms of maintaining the pluripotency of induced pluripotent stem cells. In induced pluripotent stem cells, the G1 phase (growth phase) is rapid when compared to other cell types.[21] In agreement with previous study, normally CDKN1A gene sustains low level in pluripotent cells, and its expression has been reported to increase upon differentiation which linked to a lengthened G1 phase.[21]

According to gene ontology (GO) enrichment analysis, a total of five modules analysis revealed that each module represent a different gene expression pattern. The molecular function of the five modules DEGs were mainly within DNA replication origin binding, catalytic activity, acting on DNA, sequence-specific double-stranded DNA binding, double- stranded DNA binding, sequence-specific DNA binding, ATP binding, structural constituent of ribosome, structural molecule activity, RNA binding, RNA binding, protein dimerization activity. Significant physiological changes are observed during differentiation and development, when stem and progenitor cells initiate fate courses and go through terminal differentiation; however a population with self-renewing possession is maintained.[22] DNA replication, an essential process for all living organism, is required to activate the genes of pluripotency.[23] while cell cycle is essential for the reprogramming of somatic cells.[24] The biological process of the five modules DEGs were mainly within DNA replication initiation, DNA-dependent DNA replication, DNA replication, DNA biosynthetic process, G1/S transition of mitotic cell cycle, cell cycle G1/S phase transition, nuclear DNA replication, DNA metabolic process, cell cycle DNA replication, mitotic cell cycle phase transition, translational elongation, translation, peptide biosynthetic process, amide biosynthetic process, peptide metabolic process, regulation of cell population proliferation, negative regulation of apoptotic process, negative regulation of programmed cell death, positive regulation of cell population proliferation. The crucial activity of the S phase (synthesis phase) in which DNA is replicated, introduce a unique opportunity during the life cycle of cells for epigenetic control and maintaining genome stability which may be involved in stabilizing the pluripotent state. One of the numerous options is the proteins can regulate DNA replication directly and may also support pluripotency.[25] Previous research work presented that the processes of cell differentiation are closely linked with DNA replication. Furthermore, induction of pluripotent stem cells differentiation specifically in G1 phase, proposing that the balance among the phases of cell cycle influence differentiation potential.[11] The cellular components of the five modules DEGs were mainly within chromosome, telomeric region, chromosomal region, large ribosomal subunit, ribosomal subunit, ribosome, ribonucleoprotein complex, and nucleolus. The genes involved here showed enrichment in cardiomyocyte differentiation gene ontology term, indicating that spatial genome organization definitely play a role in governing cellular identity direction.

Furthermore, KEGG pathway enrichment analysis showed that the five modules were mainly enriched in cell cycle, DNA replication, nucleotide excision repair, ribosome, ribosome biogenesis in eukaryotes, ErbB signaling pathway, EGFR tyrosine kinase inhibitor resistance, pancreatic cancer, breast cancer, prostate cancer, microRNAs in cancer, proteoglycans in cancer, pathways in cancer, endocytosis. Interestingly, it is notable that both DNA replication pathway and nucleotide excision repair pathway appeared twice in the KEGG pathway analysis of the five modules, accounting for 12 and 4 genes, respectively. Human induced pluripotent stem cells have a shortened G1 phase as well as have inactive G1/S chechpoint control in the cell cycle profile.[26] Hence, the majority of pluripotent stem cells are in the S phase and therefore these cells are prone to replication-induced DNA damage.[27] In addition, survival of stem cells for long term as compared with somatic cells put them in a high risk of accumulating exogenous and endogenous-induced DNA damages. Moreover, mutations that related replication are more frequent in stem cells as they reproduce themself more times than somatic cells.[28]

Conclusion

This study has some restriction as the data utilized in this study, downloaded from GEO database not generated by me, consisted of 4 samples of induced pluripotent stem cells generated from patient suffering from dilated cardiomyopathy (DCM iPSCs) and 2 samples of generated cardiomyocytes by differentiation of induced pluripotent stem cells that originated from patients suffering from dilated cardiomyopathy (DCM iPSCs-CMs). In addition, the results were web-basedand the specific genes regulations in different time point of differentiation were not included in this study; hence, further biological experiments are need verification by biological experiments.

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