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Transcriptome analysis in cardiomyocyte-specific differentiation of murine embryonic stem cells reveals transcriptional regulation network

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ABSTRACT

The differentiation to cardiomyocytes is a prerequisite and an important part of heart development. A good understanding of the complicated cardiomyocyte differentiation process benefits cardiogenesis study. Embryonic stem cells (ESCs), cell lines with infinite ability to proliferate and to be differentiated into all cell types of the adult body, are important research tools for investigation of differentiation and meanwhile good models for developmental research. In the current study, genome-wide gene expression of ESCs is profiled through high throughput platform during cardiomyocyte-specific differentiation and maturation. Gene expression patterns of undifferentiated ESCs and ESC-derived cardiomyocytes provide a global overview of genes involved in cardiomyocyte-specific differentiation, whereas marker gene expression profiles of both ESC-related genes and cardiac-specific genes presented the expression pattern shift during differentiation in a pure ESC-derived cardiomyocyte cell culture system. The differentiation and maturation process was completed at day 19 after initiation of differentiation, according to our gene expression profile results. Functional analysis of regulated genes reveals over-represented biological processes, molecular functions and pathways during the differentiation and maturation process. Finally, transcription factor regulation networks were engineered based on gene expression data. Within these networks, the number of identified important regulators (Trim28, E2f4, Foxm1, Myc, Hdac1, Rara, Mef2c, Nkx2-5, Gata4) and possible key co-regulation modules (Nkx2-5 – Gata4 – Tbx5, Myc – E2F4) could be expanded. We demonstrate that a more comprehensive picture of cardiomyocyte differentiation and its regulation can be achieved solely by studying gene expression patterns. The results from our study contribute to a better and more accurate understanding of the regulation mechanisms during cardiomyocyte differentiation.

Heart development is one of the earliest and most important processes during embryonic development. The morphology of vertebrate heart development has been intensively studied over a very long period of time. Recent studies of heart development have put more emphases on molecular mechanisms involved in this process. Mouse embryonic stem cells (ESCs) (Evans, 2011) are promising cell sources for drug discovery (McNeish, 2004), toxicity tests (Denecke and Schwengberg, 2010; Kuske et al., 2012), disease mechanism research (Pardal et al., 2003), as well as for basic biological research (Solter, 2006). The advances of ESC research enabled new accessible ESC models for cardiac development and disease studies (Doetschman et al., 1985). As cardiomyocytes cannot divide, ESCs are also believed to be potential important source for therapeutical purpose of cardiovascular diseases (Passier et al., 2008). Differentiation of ESCs is a comprehensive and highly coordinated process involving multiple stimuli. It is challenging to control the differentiation process and achieve pure cultures of a specific cell type, which is an essential prerequisite for biological/clinical research studies and therapeutical applications. So far, there is no direct and efficient way to generate pure populations of ESC-derived cardiomyocytes (Uosaki et al., 2011). One method to solve this problem is to enrich cardiomyocytes by using a resistance marker under control of a cardiomyocyte specific promoter (Klug et al., 1996; Kolossov et al., 2005). With this system, pure populations of dif-

Abbreviations: BP, biological process; ChIP, Chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; EB, embryoid bodies; EGFP, enhanced green fluorescence protein; ESC, Embryonic stem cells; FCS, fetal calf serum; GO, gene ontology; IMDM, Iscove's modified Dulbecco's Medium; ITFP, Integrated Transcription Factor Platform; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; PCA, principal component analysis; TF, transcription factors; α-MHC, α-myosin heavy chain.

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ferentiated cardiomyocytes can be generated and applied for a variety of research purposes including gene expression profiling during cardiomyocyte-specific differentiation.

Controlled gene expression is not the only decisive factor, but the basis of the manifestation of a certain phenotype. The elucidation of the gene expression profile of ESCs during differentiation is therefore important to understand the molecular mechanisms in developmental processes. High throughput microarray technology provides the possibility to survey transcriptional profile of undifferentiated ESCs and ESC-derived cells at specific differentiation stages. In this field, many exciting gene profiling results of ESCs have already been reported (Lee et al., 2010; Palmer et al., 2012; Pashai et al., 2012; Sperger et al., 2003). Thus, genome-wide expression profiling is a powerful tool to analyse changes during the cardiomyocyte-specific differentiation of ESCs.

There is an increasing interest in not only the expression pattern, but also in the regulation, i.e. the trigger, maintenance, and coordination of gene expression within the differentiation processes. Regulation of expression acts at both transcriptional and post-transcriptional levels. The differentiation of ESCs is a comprehensive process regulated by many factors, with transcription factors (TFs) being one of the most prominent players in ESC differentiation (Kashyap et al., 2009; Lee et al., 2010; Pashai et al., 2012). TFs are proteins, which bind to specific DNA sequences (motif) to promote or repress the recruitment of RNA polymerase and thus enhance or suppress the transcription of target genes. TFs together with other factors, e.g. miRNA, react to external and internal stimuli and regulate gene expression in a comprehensive manner.

Several TFs have been characterized as key regulators for pluripotency of ESCs. For example, a combination of TFs, like Pou5f1, Sox2, Klf4 and Myc, is sufficient to reprogram adult differentiated cells into induced pluripotent ESC-like cells (Okita et al., 2007). Pou5f1, a member of the POU family, is found to control the pluripotency of stem cells in a quantitative manner. It is determined that high levels of Pou5f1 expression drives ESCs to endoderm and mesoderm lineages, while ESCs with low levels of Pou5f1 differentiate into trophoectoderm. Only a certain level of Pou5f1 can retain stem cells in a pluripotent state (Niwa et al., 2000). Also the TF Sox2, a member of the SRY-high mobility box TF family, has been implicated in controlling differentiation in many ways, including e.g. (i) maintenance of self-renewal and pluripotency in ESCs and neural retinal stem cells (Kiernan et al., 2005), (ii) differentiation of tongue taste buds and ear sensory cells from progenitors (Okubo et al., 2006), and (iii) induction of reprograming (Taranova et al., 2006). The TFs Nanog and Pou5f1 share substantial overlap of target genes. Nanog also regulates the expression of Pou5f1 and Sox2. One likely mechanism is that Nanog sustains self-renewal and the undifferentiated state through the modulation of Pou5f1 and Sox2 levels, and these two TFs in turn control the downstream genes (Loh et al., 2006).

In recent years, several cardiac-related TFs were discovered: Mef2 proteins possess similar structure, with a MADS-box at their N-termini mediating homo- and hetero-dimerization, and binding to the DNA sequence CTA(A/T)$_4$TAG/A. Adjacent to the MADS-box domain is a Mef2-specific domain that influences DNA-binding affinity and cofactor interactions. Mef2 proteins contain C-terminal transcriptional activation domains and were found to play an important role in skeletal and cardiac muscle differentiation programs (Edmondson et al., 1994; McKinsey et al., 2002).

Gata TF family (Gata4, 5, and 6), a group of zinc-finger cardiac TFs, are essential TFs in cardiomyocyte differentiation of ESCs (Boheler et al., 2002). Nkx2-5 is a co-activator of Gata4 and elicits the transcription of cardiac restricted genes (Sachinidis et al., 2003).

TF binding sites determine the primary TF targets and therefore the resulting regulation. Chromatin immunoprecipitation (ChIP) can provide the real binding information of TFs in a specific biological context (Weinmann, 2004), while computational ("in silico") methods can be used to predict potential TF binding sites (Mathelier and Wasserman, 2013). Due to the multiple targeting of TFs, the complexity of these networks increases rapidly with increasing number of TFs involved. Much effort has been dedicated to modelling gene regulation networks (Karlebach and Shamir, 2008). Several models (e.g. logical model (Thomas, 1973), Boolean networks (Glass and Kauffman, 1973), and probabilictic Boolean networks (Shmulevich et al., 2002)) have been introduced in this research field. Gaussian graphical Markov model is a simple and useful tool for estimating correlation of expression data (Castelo and Roverato, 2009). This model can be fitted to evaluate connections between TFs and regulated genes on transcription level, and to identify possible connections. The correlation information achieved from the Gaussian graphical model in combination with TF target knowledge enables the engineering of TF regulation networks for further in-depth studies concerning transcription regulation.

In the current study, pure cell populations of ESCs and ESC-derived cardiomyocytes at various differentiation and maturation stages were applied in transcriptome analysis in order to characterize cardiomyocyte-specific differentiation in the view of the transcriptional level, targeting the identification of TF regulatory mechanisms during this process.

1. Results

1.1. Pure cardiomyocyte culture were successfully generated by specific differentiation of mouse ESCs

In order to profile samples of pure cell populations, transgenic mouse ESCs were cultured, and differentiated into cardiomyocytes. Resulting cardiomyocytes were purified using Puromycin selection, and further cultured to obtain mature phenotypes. At start time point day (d) 0, typical stem cell colonies were observed (Fig. 1a). The Puromycin selection was started at d9 for a total of 3 days. At d12 after initiation of differentiation, reporter gene expression (EGFP under control of the α-MHC promoter) was observed in all remaining, spontaneously beating cardiomyocytes, with a complete overlay to phase contrast microscopy photo (Fig. 1b, c). Cells without active α-MHC promoter were successfully eliminated through the 3 days of puromycin selection (d9-d12). Cardiac α-actin staining at d19 showed mature cardiomyocytes with cardiomyocyte morphology and sarcomeric banding pattern (Fig. 1d). The resulting cardiomyocyte cultures have a high purity of over 99% (Kolossov et al., 2006).

1.2. Gene expression reflects cardiomyocyte specific differentiation and maturation process

Gene expression of mouse ESCs (d0) and ESC-derived cardiomyocytes at various stages of differentiation and maturation (d12, d19, and d26) were characterized genome-wide with Affymetrix GeneChip® Mouse Exon 1.0 ST Arrays. In principle component analysis (PCA), the gene expression of differentiated mouse ESCs showed significant distance to undifferentiated mouse ESCs (Fig. 2a) at PC1, which represent 63.86% of variance. d12 samples (differentiated cardiomyocytes at early stage) were distinctively separated from d19 and d26 samples (mature cardiomyocytes) at PC2 (9.81% variance), while no clear separation was observed between d19 and d26 samples. These results indicate that at d19 the maturation process of cardiomyocyte was almost completed in terms of gene expression.

For further analysis, genes whose expression values were over threefold changed with a p-value lower than 0.01 were defined as regulated genes at different stages of cardiomyocyte differentiation and maturation. According to these stringent criteria, in total 652 genes were up-regulated and 722 genes were down-regulated

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