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Stage specific transcriptome profiles at cardiac lineage commitment during cardiomyocyte differentiation from mouse and human pluripotent stem cells

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Cardiomyocyte differentiation occurs through complex and finely regulated processes including cardiac lineage commitment and maturation from pluripotent stem cells (PSCs). To gain some insight into the genome-wide characteristics of cardiac lineage commitment, we performed transcriptome analysis on both mouse embryonic stem cells (mESCs) and human induced PSCs (hiPSCs) at specific stages of cardiomyocyte differentiation. Specifically, the gene expression profiles and the protein–protein interaction networks of the mESC-derived platelet-derived growth factor receptor-alpha (PDGFRα)$^+$ cardiac lineage-committed cells (CLCs) and hiPSC-derived kinase insert domain receptor (KDR)$^+$ and PDGFRα$^+$ cardiac progenitor cells (CPCs) at cardiac lineage commitment were compared with those of mesodermal cells and differentiated cardiomyocytes. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses revealed that the genes significantly upregulated at cardiac lineage commitment were associated with responses to organic substances and external stimuli, extracellular and myocardial contractile components, receptor binding, gated channel activity, PI3K–AKT signaling, and cardiac hypertrophy and dilation pathways. Protein–protein interaction network analysis revealed that the expression levels of genes that regulate cardiac maturation, heart contraction, and calcium handling showed a consistent increase during cardiac differentiation; however, the expression levels of genes that regulate cell differentiation and multicellular organism development decreased at the cardiac maturation stage following lineage commitment. Additionally, we identified for the first time the protein–protein interaction network connecting cardiac development, the immune system, and metabolism during cardiac lineage commitment in both mESC-derived PDGFRα$^+$ CLCs and hiPSC-derived KDR$^+$PDGFRα$^+$ CPCs. These findings shed light on the regulation of cardiac lineage commitment and the pathogenesis of cardiometabolic diseases. [BMB Reports 2021; 54(9): 464-469]

INTRODUCTION

Cardiomyocyte differentiation from pluripotent stem cells (PSCs) involves complex processes that are tightly regulated (1). For example, it was found that mesodermal cells that give rise to cardiomyocytes could also give rise to cells of endothelial, hematopoietic, and mural lineage (2). Cardiomyocyte differentiation from mesodermal cells generally involves cardiac lineage commitment (or fate determination) and maturation, which is a continuous process and of which the timing is difficult to define (3). Although cardiac lineage commitment, cell proliferation, and maturation occur simultaneously during the early period of cardiomyocyte differentiation, cell proliferation is the dominant action during this period. On the other hand, the proliferative capacity of the cells decreases and the maturation process becomes dominant during the late stage of differentiation (3, 4).

It has been reported that cells expressing both the kinase insert domain receptor (KDR) and platelet-derived growth factor receptor-alpha (PDGFRα) are populations of cardiac progenitor cells (CPCs) with cardiomyogenic potential (5, 6). Our group also previously described a novel population of PSC-derived PDGFRα$^+$ cardiac lineage-committed cells (CLCs), which while being actively proliferating cells, were also morphologically and functionally immature compared to differentiated cardiomyocytes (7, 8). However, the detailed biological processes and molecular mechanisms involved in

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cardiac lineage commitment have still not yet been fully determined (9). Therefore, to elucidate the genome-wide characteristics of cardiac lineage commitment, in this study, we compared the transcriptome profiles and the protein–protein interaction networks of highly upregulated genes in mouse embryonic stem cell (mESC)-derived PDGFRα$^+$ CLCs and human induced PSC (hiPSC)-derived KDR$^+$PDGFRα$^+$ CPCs with those of mesodermal cells and differentiated cardiomyocytes.

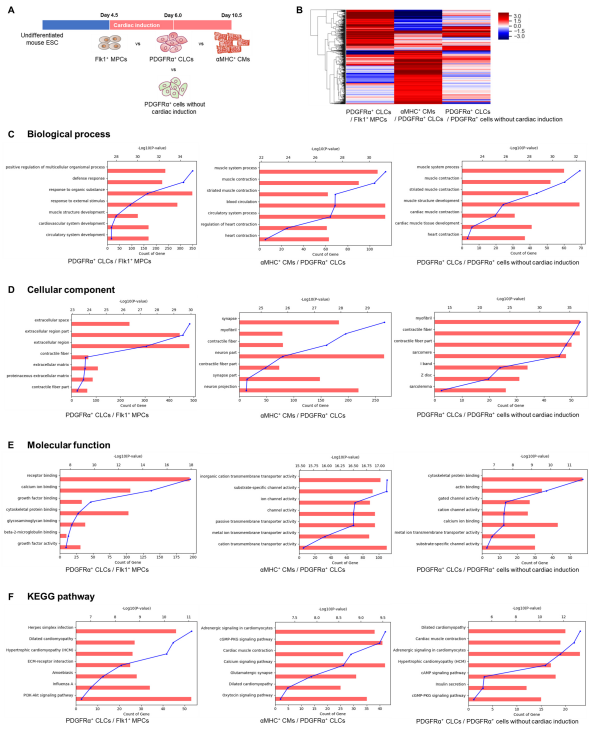

Fig. 1. Microarray transcriptome analysis of stage-specific cells during mESC-derived cardiac differentiation. (A) Sampling time points of mESC-drived cells: Flk1$^+$ MPCs at day 4.5, PDGFRα$^+$ CLCs and PDGFRα$^+$ cells without cardiac induction at day 6.0, and αMHC$^+$ cardiomyocytes at day 10.5. mESC: murine embryonic stem cell; Flk1$^+$ MPCs: vascular endothelial growth factor receptor 2-expressing mesodermal precursor cells; PDGFRα$^+$ CLCs: platelet-derived growth factor receptor-alpha-expressing cardiac lineage-committed cells; αMHC: alpha-myosin heavy chain. (B) Heatmap of the hierarchical clustering comparison of each developmental stage of cells derived from mESCs. (C-E) Gene Ontology analysis of the significantly upregulated genes at each developmental stage in cells derived from mESCs, showing the top 7 GO terms in the (C) Biological Process, (D) Cellular Component, and (F) Molecular Function categories. (F) Kyoto Encyclopedia of Genes and Genomes pathway analysis of the significantly upregulated genes in each developmental stage of cells derived from mESCs; the top 7 pathways are shown.

RESULTS

Clustering heatmap, Gene Ontology, and KEGG pathway analyses

The differentially expressed gene (DEGs) patterns of the various transcriptomes were depicted using hierarchical clustering analysis in order to distinguish each developmental stage of the cells derived from the mESCs (Fig. 1B) and hiPSCs (Fig. 2B). Significantly upregulated genes in the mESC-derived cells were defined as those having a fold change $\geq$ 3.5, normalized data (log2) of 4, and a P-value $<$ 0.05 and significant upregulation in the hiPSC-derived cells was defined as a fold change $\geq$ 2.0, normalized data (log2) of 4, and a p-value of $<$ 0.05. To determine the stage-specific role of the upregulated genes, Gene Ontology (GO) and Kyoto Encyclopedia

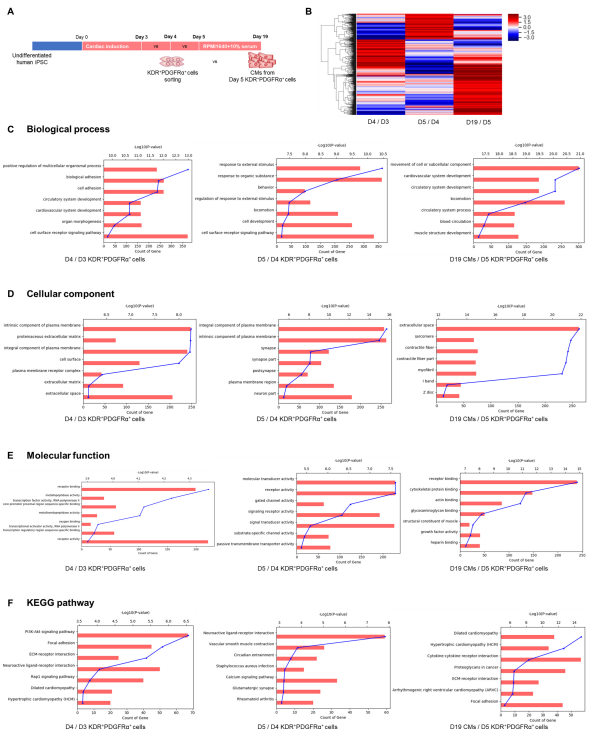

Fig. 2. Microarray transcriptome analysis of stage-specific cells during hiPSC-derived cardiac differentiation. (A) Sampling time points of hiPSC-drived cells: KDR$^+$PDGFRα$^+$ cells at days 3.0, 4.0, and 5.0 and cardiomyocytes at day 19.0. hiPSC: human induced pluripotent stem cell; KDR: kinase insert domain receptor; PDGFRα: platelet-derived growth factor receptor-alpha. (B) Heatmap of the hierarchical clustering comparison of each stage of cells derived from hiPSCs. (C-E) Gene Ontology analysis of the significantly upregulated genes in each developmental stage of cells derived from hiPSCs, showing the top 7 GO terms in the (C) Biological Process, (D) Cellular Component, and (E) Molecular Function categories. (F) Kyoto Encyclopedia of Genes and Genomes pathway analysis of the significantly upregulated genes in each stage of cells derived from hiPSCs; the top 7 pathways are shown.

of Genes and Genomes (KEGG) pathway analyses were carried out to compare each stage of development in mESCs (Fig. 1C-F) and hiPSCs (Fig. 2C-F); the seven most high-ranking GO terms and KEGG pathways were selected. In the GO Biological Process category (Fig. 1C and 2C), the significantly upregulated genes at cardiac lineage commitment were enriched in terms associated with responses to organic substances and external stimuli, as well as muscle and cardiovascular system development. Genes related to the heart contraction and circulatory system processes were significantly upregulated in the differentiated cardiomyocytes. In the Cellular Component category (Fig. 1D and 2D), the significantly upregulated genes were associated with extracellular region and plasma membrane components at cardiac lineage commitment. Genes related to myocardial contractile components, such as the sarcomere, Z disc, I band, and neuron parts, were found to be gradually upregulated during cardiomyocyte differentiation. In the Molecular Function category (Fig. 1E and 2E), genes related to receptor binding and activity, gated channel activity, and cytoskeletal protein binding were significantly upregulated at cardiac lineage commitment and during cardiomyocyte differentiation. Pathway analysis (Fig. 1F and 2F) revealed that the significantly upregulated genes at cardiac lineage commitment and during cardiomyocyte differentiation were associated with phosphatidylinositol 3-kinase and protein kinase B (PI3K-AKT) signaling, extracellular matrix (ECM)–receptor interactions, calcium signaling, and cardiac hypertrophy and dilation pathways.

Protein–protein interaction networks during cardiac differentiation

We conducted protein–protein interaction analysis to gain insight into the regulation of cardiac lineage commitment, fate determination, and maturation during cardiac differentiation. In the mESC-derived cells, commonly expressed genes that showed over a 3-fold increase in expression levels in PDGFRα$^+$ CLCs were compared to those of vascular endothelial growth factor receptor 2-expressing (Flk1$^+$) mesodermal precursor cells (MPCs) and PDGFRα$^+$ cells without cardiac induction; these genes were selected first for analysis. Among the selected genes, genes that showed over 3-fold increased or decreased expression levels in the alpha-myosin heavy chain (αMHC)$^+$ cardiomyocytes were defined as the genes that induced cardiac maturation or the genes in control of cardiac linage commitment, respectively. When compared to the Flk1$^+$ MPCs and PDGFRα$^+$ cells without cardiac induction, 425 genes showed over a 3-fold increase in expression levels in the PDGFRα$^+$ CLCs, of which 162 genes showed consistently increased expression levels in the αMHC$^+$ cardiomyocytes and 89 genes showed decreased expression levels (Fig. 3A, Supplementary Table 1). The protein–protein interaction network was constructed using these 251 genes and the final mESC-derived cardiac differentiation network included 208 genes (Fig. 3B and C). When compared to the Flk1$^+$ MPCs and

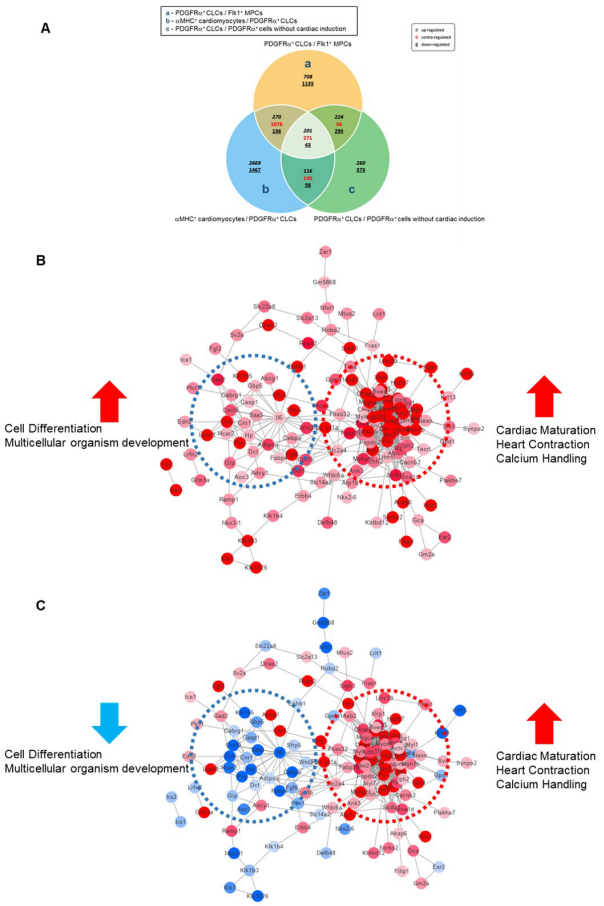

Fig. 3. Protein–protein interaction networks of mESC-derived cardiac differentiation, based on STRING analysis. (A) Venn diagrams depicting overlap of genes at each developmental stage of cells derived from mESCs during cardiac differentiation. mESC: murine embryonic stem cell; (B) Protein–protein interaction networks of commonly increased genes in the PDGFRα$^+$ CLCs as compared to Flk1$^+$ MPCs and PDGFRα$^+$ cells without cardiac induction. Flk1$^+$ MPCs: vascular endothelial growth factor receptor 2-expressing mesodermal precursor cells; PDGFRα$^+$ CLCs: platelet-derived growth factor receptor-alpha-expressing cardiac lineage-committed cells; αMHC: alpha-myosin heavy chain. (C) Protein–protein interaction networks of consistently increased genes (red) and decreased genes (blue) in αMHC$^+$ cardiomyocytes among the commonly increased genes in PDGFRα$^+$ CLCs.

PDGFRα$^+$ cells without cardiac induction, the genes that commonly showed increased expression levels in the PDGFRα$^+$ CLCs (Fig. 3B) were divided into genes with either increased (red) or decreased (blue) expression in the αMHC$^+$ cardiomyocytes (Fig 3C). The genes with increased expression levels were mainly involved in regulating cardiac maturation, heart contraction, and calcium handling, and the genes with decreased expression levels were involved in regulating processes such as cell differentiation and multicellular

organism development. Notably, these analyses revealed, for the first time, the cardiac developmental-immune-metabolic protein–protein interaction networks during cardiac lineage commitment. In the mESC-derived PDGFRα$^+$ CLCs, the proteins Wnt family member 2 (Wnt2) and secreted frizzled related protein (Sfrp5) (related to cardiac development), interleukin 6 (Il6) (related to immune system) and adiponectin (Adipoq), fatty acid-binding protein 4 (Fabp4) and hydroxycarboxylic acid receptor 2 (Hcar2) (related to metabolism) were connected.

In the hiPSC-derived cells, genes with consitently increased expression levels (over 2-fold) in the KDR$^+$PDGFRα$^+$ cells at day 4.0 as compared to those on day 3.0 and the genes with increased expression in the KDR$^+$PDGFRα$^+$ CPCs compared to those of the KDR$^+$PDGFRα$^+$ cells at day 4.0 were selected for analyses depending on the time of differentiation. Among the selected genes, genes that showed over a 2-fold increase or decrease in expression levels in the cardiomyocytes were defined as the genes that induced cardiac maturation and the genes that controlled cardiac linage commitment, respectively. When compared to the KDR$^+$PDGFRα$^+$ cells at day 3.0 and 4.0, 296 genes showed over a 2-fold increase in expression level in the KDR$^+$PDGFRα$^+$ CPCs, of which 104 genes showed continuous overexpression in the cardiomyocytes and 123 genes showed underexpression (Fig. 4A, Supplementary Table 2). The protein–protein interaction network was constructed using these 227 genes, and finally, an hiPSC-derived cardiac differentiation network composed of 191 genes was constructed (Fig. 4B and C). When compared to the KDR$^+$PDGFRα$^+$ cells at day 3.0 and 4.0, the genes commonly overexpressed in the KDR$^+$PDGFRα$^+$ CPCs (Fig. 4B) were divided into genes that were consistently overexpressed (red) or underexpressed (blue) in the cardiomyocytes (Fig 4C). Similar to the mESC-derived network, the genes showing increased expression levels were mainly involved in regulating cardiac maturation, heart contraction, and calcium handling, and the genes showing decreased expression levels were involved in regulating processes such as cell differentiation and multicellular organism development. In addition, the proteins fibroblast growth factor 10 (FGF10) (related to cardiac development), Toll-like receptor 4 (TLR4) (related to immune system), and alpha-2-HS-glycoprotein (AHSG) (related to metabolism) showed interactions in the hiPSC-derived KDR$^+$PDGFRα$^+$ CPCs and these data were similar to those of the mESC-derived network.

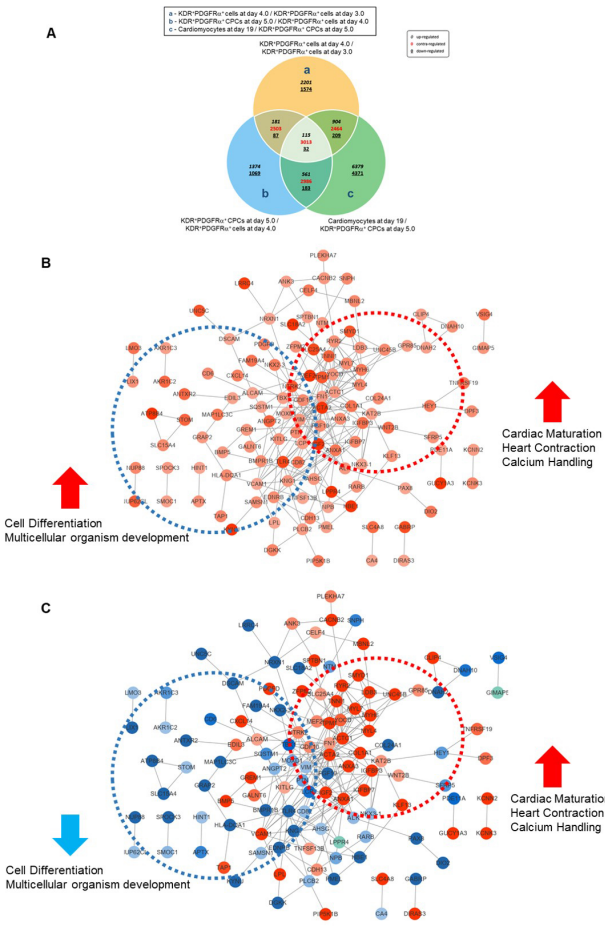

Fig. 4. Protein–protein interaction networks of hiPSC-derived cardiac differentiation, based on STRING analysis. (A) Venn diagrams depicting overlap of genes at each developmental stage of cells derived from hiPSCs during cardiac differentiation. hiPSC: human induced pluripotent stem cell. (B) Protein–protein interaction networks of commonly increased genes in KDR$^+$PDGFRα$^+$ CPCs at day 5.0 as compared to KDR$^+$PDGFRα$^+$ cells at day 3.0 and 4.0. KDR: kinase insert domain receptor; PDGFRα: platelet-derived growth factor receptor-alpha. (C) Protein–protein interaction networks of consistently increased genes (red) and decreased genes (blue) in cardiomyocytes at day 19 as they relate to the commonly increased genes in KDR$^+$PDGFRα$^+$ CPCs at day 5.0.

DISCUSSION

In this study, GO and KEGG pathway analyses revealed that genes related to the responses to organic substances and external stimuli, extracellular and myocardial contractile components, receptor binding, gated channel activity, PI3K-AKT signaling, and cardiac hypertrophy and dilation signaling pathways were commonly upregulated at cardiac lineage commitment. Protein–protein interaction network analysis revealed that genes that regulate cardiac maturation, heart contraction, and calcium handling were consistently increased during cardiac differentiation, while genes regulating cell differentiation and multicellular organism development were decreased at cardiac maturation after lineage commitment. Additionally, we identified for the first time the cardiac developmental-immune-metabolic protein–protein interaction networks in both the mESC-derived PDGFRα$^+$ CLCs (involving Wnt2, Sfrp5, Il6, Adipoq, Fabp4, and Hcar2) and hiPSC-derived KDR$^+$PDGFRα$^+$ CPCs (involving FGF10, TLR4, and AHSG).

To induce the differentiation of cardiomyocytes from PSCs, treatment with various compounds and growth factors at specific times and in specific doses is essential for directing mesodermal differentiation toward a more specific cardiac fate (1). Therefore, the cellular responses to organic substances and external stimuli, such as hormones, chemokines, cytokines, as well as cell adhesion-associated genes, may be significantly upregulated at cardiac lineage commitment. In order to receive signals from organic substances and external stimuli, the development of cell membrane structures and receptors is crucial. Indeed, our GO and KEGG pathway analyses revealed that genes associated with plasma membrane components and receptor binding and activity were significantly upregulated at cardiac lineage commitment.

Furthermore, it has been reported that cardiac ECM composition and structure, soluble factors, and extracellular vesicles may contribute to cardiomyocyte specification and maturation (10, 11). Interestingly, it was recently demonstrated that significant changes in the secretome profile occur during cardiac progenitor specification, and the complexity of the ECM increases (12). Consistent with these observation, our GO and KEGG pathway analyses revealed that genes associated with the extracellular region, ECM, and ECM–receptor interactions were highly upregulated at cardiac lineage commitment. Additionally, several studies have demonstrated that the PI3K–AKT signaling pathway contributes to cardiomyocyte differentiation, proliferation, and survival under developmental and pathological conditions (13-15). Similarly, our pathway analysis indicated several genes significantly upregulated at cardiac lineage commitment that were associated with PI3K–AKT signaling.

Our data also show that genes related to muscle and cardiovascular system development, blood circulation, myocardial contractile components (e.g., sarcomere, Z disc, and I band), cytoskeletal protein binding, and the cardiac hypertrophy and dilation pathways were consistently upregulated not only at cardiac lineage commitment, but also during further cardiomyocyte differentiation from both mESCs and hiPSCs. However, the genes associated with synapses, neuron parts, and calcium signaling pathways were expressed earlier in the hiPSC-derived $KDR^+PDGFR\alpha^+$ CPCs; these genes were highly expressed in the mESC-derived differentiated αMHC^+ cardiomyocytes.

Notably, protein–protein interaction network analysis revealed two separate gene clusters, of which genes with consistently increased expression levels were involved in regulating cardiac maturation, heart contraction, and calcium handling during cardiac differentiation, whereas genes with decreased expression levels were involved in regulating cell differentiation and multicellular organism development at cardiac maturation following lineage commitment. These data suggest that the genes with decreased expression levels at cardiac maturation could contribute to cardiac lineage commitment and fate determination. Furthermore, we identified for the first time the cardiac developmental-immune-metabolic protein–protein interaction networks in both the mESC-derived $PDGFR\alpha^+$ CLCs (involving Wnt2, Sfrp5, Il6, Adipoq, Fabp4, and Hcar2) and hiPSC-derived $KDR^+PDGFR\alpha^+$ CPCs (involving FGF10, TLR4, and AHSG). These networks might have developed because $PDGFR\alpha^+$ CLCs and $KDR^+PDGFR\alpha^+$ CPCs share some characteristics of other mesodermal lineage cells, such as immune cells and adipocytes. Although a few studies have reported that Il6 contributes to cardiomyocyte-like cell differentiation from mesenchymal stem cells (16, 17), Il6 and TLR4 are primarily inflammatory, immune-related proteins, and are mainly associated with cardiovascular diseases such as heart failure and atherosclerosis (18, 19). Similarly, Adipoq, Fabp4, Hcar2, and AHSG are associated with cardiometabolic diseases, including diabetes, obesity, heart failure, and coronary atherosclerosis (20-23). Therefore, we hypothesize that these cardiac developmental-immune-metabolic protein–protein interaction networks established at cardiac lineage commitment likely contribute to the future development of cardiometabolic diseases. Further studies using cardiometabolic disease- and patient-specific iPSC-derived cardiomyocytes are warranted to verify this hypothesis.

In the present study, we did not focus on the transcriptomic differences between mESC- and hiPSC-derived cardiomyocytes. Notably, a recent study showed that in terms of transcriptomes, mouse hearts at P0-3 and human hearts at 18-19 weeks post conception showed the closest proximity, and ribosomal genes showed differential expression patterns between mice and humans (24). Therefore, it may be worthwhile to analyze the transcriptomic differences between mESC- and hiPSC-derived cardiomyocytes across specific time points.

In conclusion, we analyzed the transcriptome-wide profiles of mESC-derived $PDGFR\alpha^+$ CLCs and hiPSC-derived $KDR^+PDGFR\alpha^+$ CPCs with a focus on gene expression at cardiac lineage commitment. In this study, our group identified, for the first time, the cardiac developmental-immune-metabolic protein–protein interaction networks of these cells.

MATERIALS AND METHODS

The detailed methods are described in the "Supplementary Materials and Methods".

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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