Transcriptomic Optimization of Embryonic Mouse Cell Differentiation For Producing Ventricular Cardiomyocytes

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Introduction

Pluripotent stem cells (PSCs) hold immense promise for regenerative medicine and disease modeling due to their ability to differentiate into various cell types. In this report, we present an experimental protocol aimed at generating ventricular cardiomyocytes from PSCs. Ventricular cardiomyocytes form the muscular walls of the heart and generate contractile force of the heart, allowing the heart to function properly[1]. Since it makes up the ventricular wall of the heart, it suggests an important therapeutic target population to model and treat cardiovascular diseases. As the heart develops, its cardiac performance increases and the ventricular cardiomyocytes adapt to its more demanding tasks by growing in diameter and becoming more structurally compact[2].

The ontogeny of ventricular cardiomyocytes we are studying follows this pathway: epiblast/primitive streak, nascent mesoderm, lateral plate mesoderm, first heart field, ventricular cardiomyocyte. Several factors for signaling pathways which have been previously established to promote cardiac mesoderm specification include fibroblast growth factor, bone morphogenetic protein, and sonic hedgehog. Other ligands, such as Wnt1, Wnt3a, and Wnt8, have been shown to inhibit specification during development. Mesp1 has also been noted to be a key transcription factor in this process[3].

As such, in this study, our protocol focuses on optimizing the purity of our target cell population as well as maximizing transcriptomic similarity through applications of several computational techniques such as trajectory and pseudotime inference as well as gene regulatory network (GRN) reconstruction. Leveraging knowledge of different signaling pathways, we discovered necessary transcription factors that were to be upregulated at strategic stages in order to steer differentiation. Additionally, we identified several marker genes which aim to further

guide protocol optimization. By utilizing transcriptomic data across mouse development, we hope to refine and validate our protocol, ultimately advancing the field of cell differentiation and paving the way for applications in regenerative medicine and beyond.

Methods

Datasets

Multiple datasets were used to trace the mice ontogeny. The first dataset used for early embryonic development originally comprised the profiles of 116,312 single cells isolated from mouse embryos spanning a developmental timeline from 6.5 to 8.5 days post-fertilization[4]. For the purposes of this project, extraembryonic cell types were removed, and annotations were revised. The refined dataset contains 20 cell types each with 100 cells for a total of 2000 cells with their associated raw counts.

The second dataset covering later embryonic development contains the transcriptional states of 12.4 million nuclei from 83 embryos. The counts were sampled at two to six hour intervals spanning late gastrulation (embryonic day 8) to birth (postnatal day 0). Hundreds of cell types exploring the ontogenesis of the posterior embryo during somitogenesis and of kidney, mesenchyme, retina and early neurons were originally included[5].

Analysis

Each dataset was first subjected to quality control filtering and cleaning for <20% mitochondrially encoded gene total unique molecular identifiers. Additionally, cells were filtered by a minimum of 500 genes detected per cell and having a maximum 30,000 counts. Genes detected in at least 3 cells were filtered/kept as well. Next, the datasets were normalized through library size scaling and log transform. Highly variable genes were then determined, with principal component analysis (PCA) then performed. K-nearest neighbors with 20 neighbors and 10 PCs followed, with Leiden used as the community detection method. Finally, PAGA and UMAP were then applied to infer dynamic relationships and optimization.

From both the Early Stage dataset and Late Stage dataset, relevant cell types from our target pathway were isolated such that each transition stage could be analyzed individually. Using scFates, a principal tree was generated with 100 nodes for the early stages and 200 nodes for the late stages. Branches with a length of less than 10 were cut for the early stages and less than 50 for the late stages. Tree roots were then identified and set, and pseudotime was computed. Finally, genes or features significantly associated with the trajectories were determined, expression levels were modeled based on tree positions, and features were then clustered with 5 PCs for the early stages and 20 PCs for the late stages.

Transcription factors were identified and isolated in the data, and using pySingleCellNet, a classifier was built. oneSC was then utilized to construct GRNs, and together with the classifier, it was then utilized to simulate perturbations which yield the purest populations of our desired targets. Finally, differential expression analysis (DEA) was performed to identify genes that could potentially serve as markers. To further determine signaling pathways and marker genes to be used, an extensive literature review was conducted and the STRING database was referenced.

Our original code can be found in the supplementary section at the end of this report.

Results

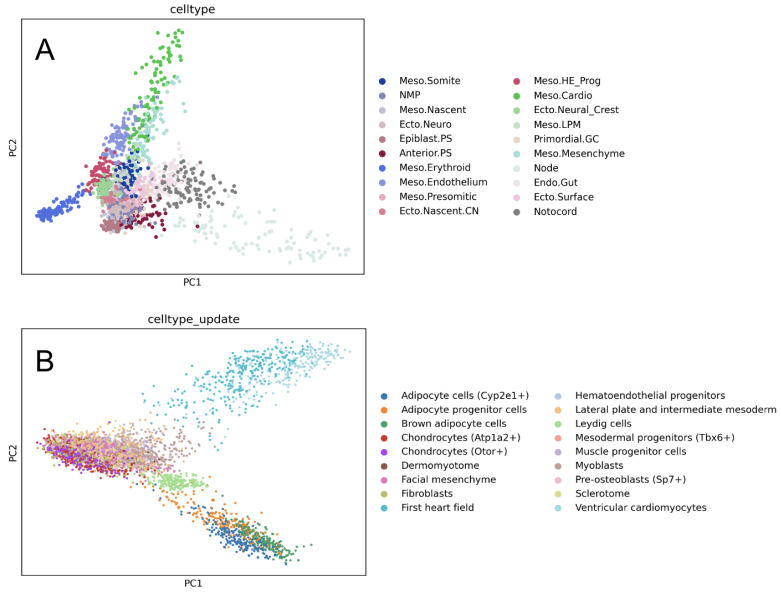

Figure 1. PCA Visualization of All Cell Types Found Within Derived Data Sets. A) displays the cells which appear in the Early Stages dataset while B) displays the cells which appear in the Late Stages dataset.

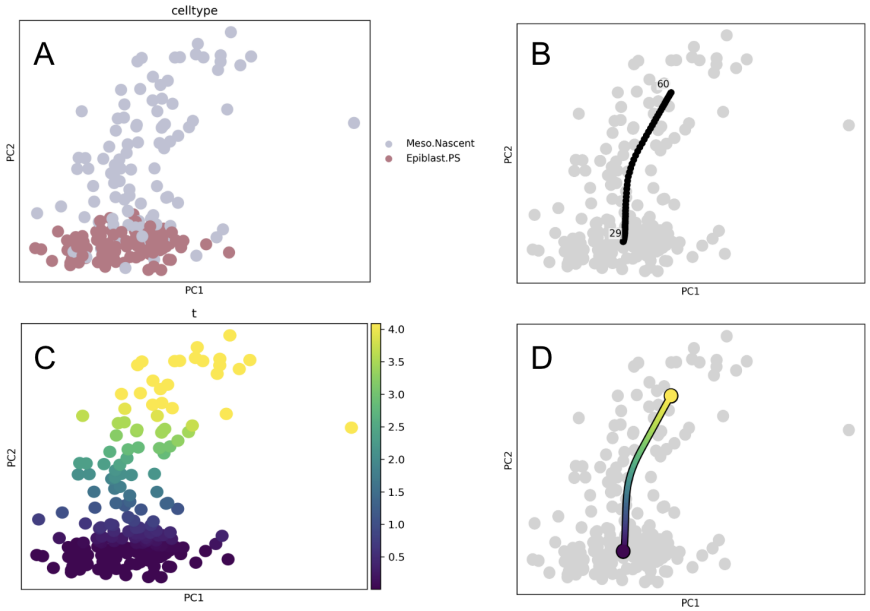

Figure 2. Visualizations for Transition from Epiblast/Primitive Streak to Nascent Mesoderm. A) PCA of Isolated Cell Types from the Early Stages Dataset. B) Trajectory Inference of Transition. Milestone 29 represents the initial stage while milestone 60 represents the terminal stage. C) Pseudotime mapped onto PCA. D) Pseudotime mapped onto TI.

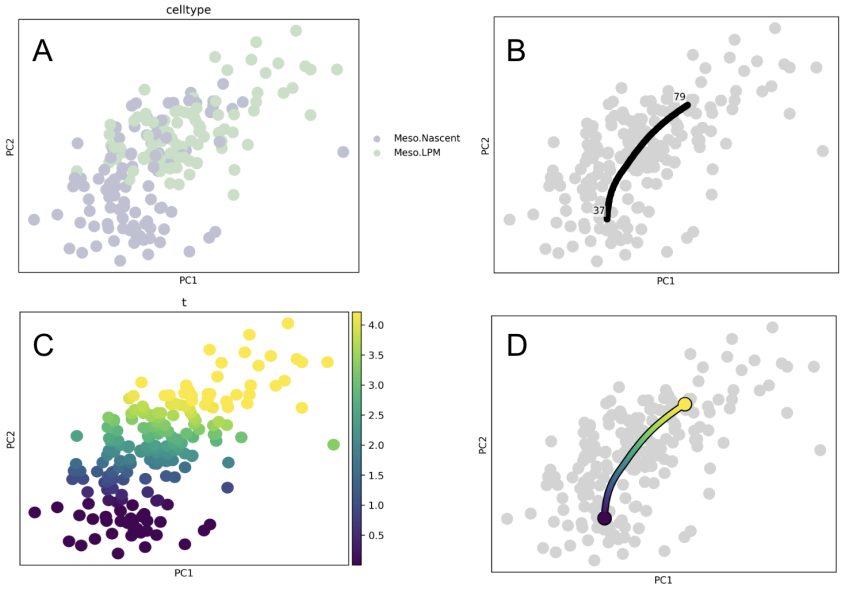

Figure 3. Visualizations for Transition from Nascent Mesoderm to Lateral Plate Mesoderm. A) PCA of Isolated Cell Types from the Early Stages Dataset. B) Trajectory Inference of Transition. Milestone 37 represents the initial stage while milestone 79 represents the terminal stage. C) Pseudotime mapped onto PCA. D) Pseudotime mapped onto TI.

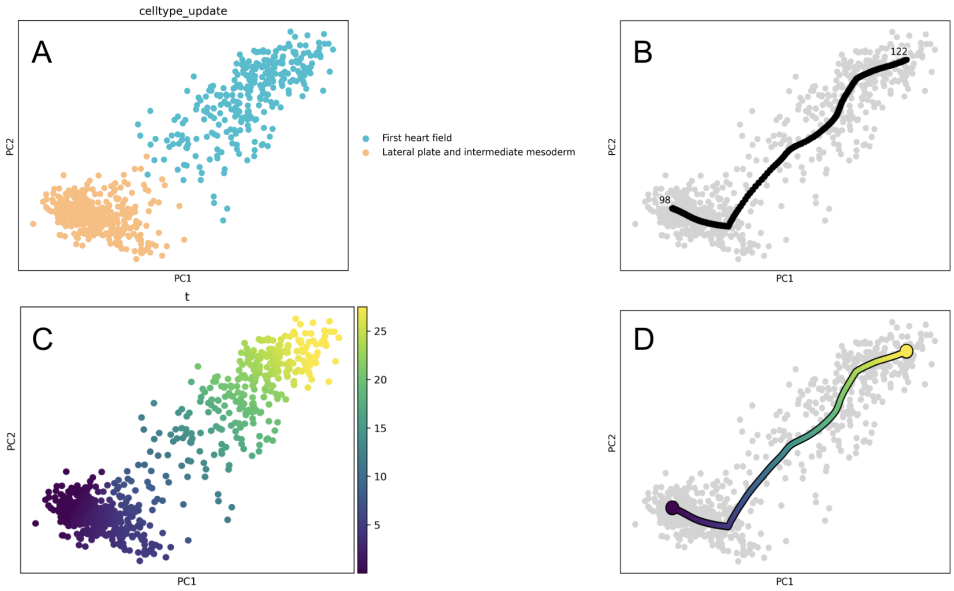

Figure 4. Visualizations for Transition from Lateral Plate Mesoderm to First Heart Field. A) PCA of Isolated Cell Types from the Late Stages Dataset. B) Trajectory Inference of Transition. Milestone 98 represents the initial stage while milestone 122 represents the terminal stage. C) Pseudotime mapped onto PCA. D) Pseudotime mapped onto TI.

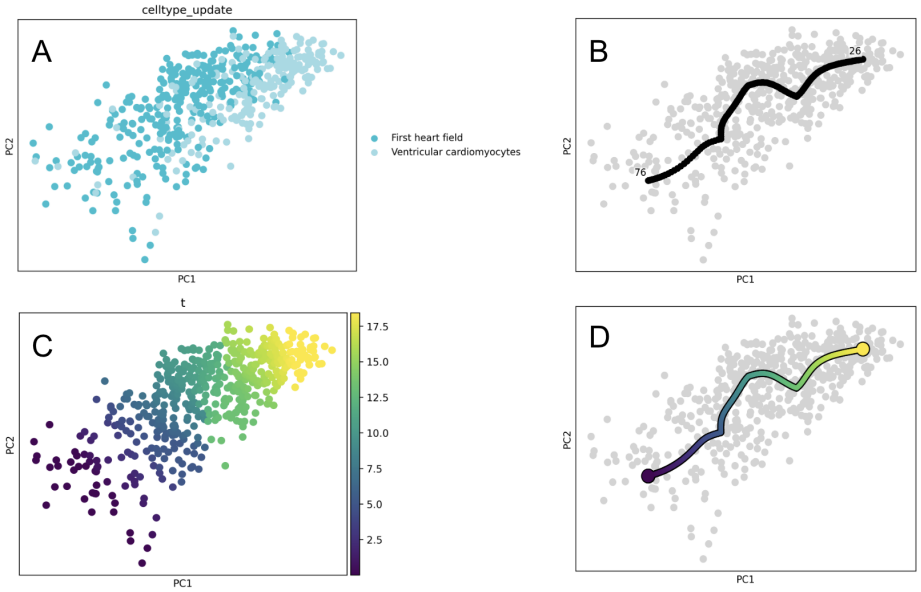

Figure 5. Visualizations for Transition from First Heart Field to Ventricular Cardiomyocytes. A) PCA of Isolated Cell Types from the Late Stages Dataset. B) Trajectory Inference of Transition. Milestone 76 represents the initial stage while milestone 26 represents the terminal stage. C) Pseudotime mapped onto PCA. D) Pseudotime mapped onto TI.

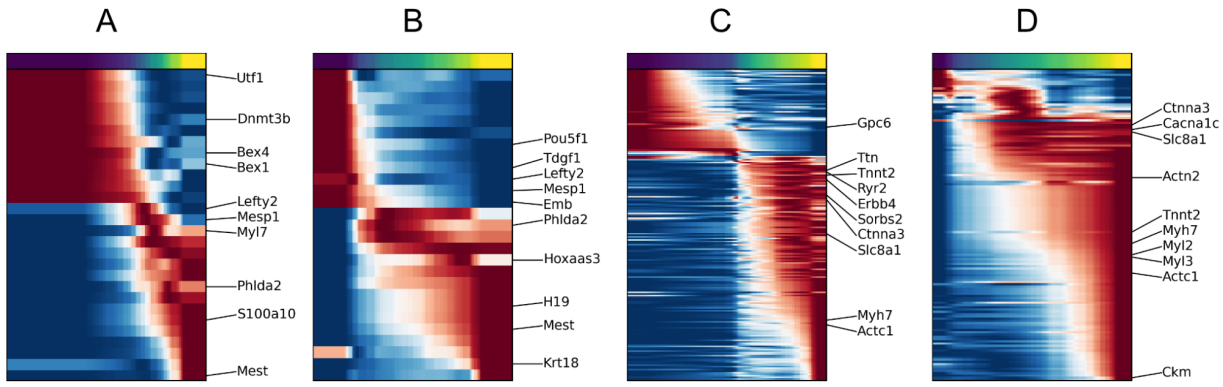

Figure 6. Heatmaps of Fitted Features for Each Developmental Transition Along Pseudotime. A) Epiblast/Primitive Streak to Nascent Mesoderm. B) Nascent Mesoderm to Lateral Plate Mesoderm. C) Lateral Plate Mesoderm to First Heart Field. D) First Heart Field to Ventricular Cardiomyocytes.

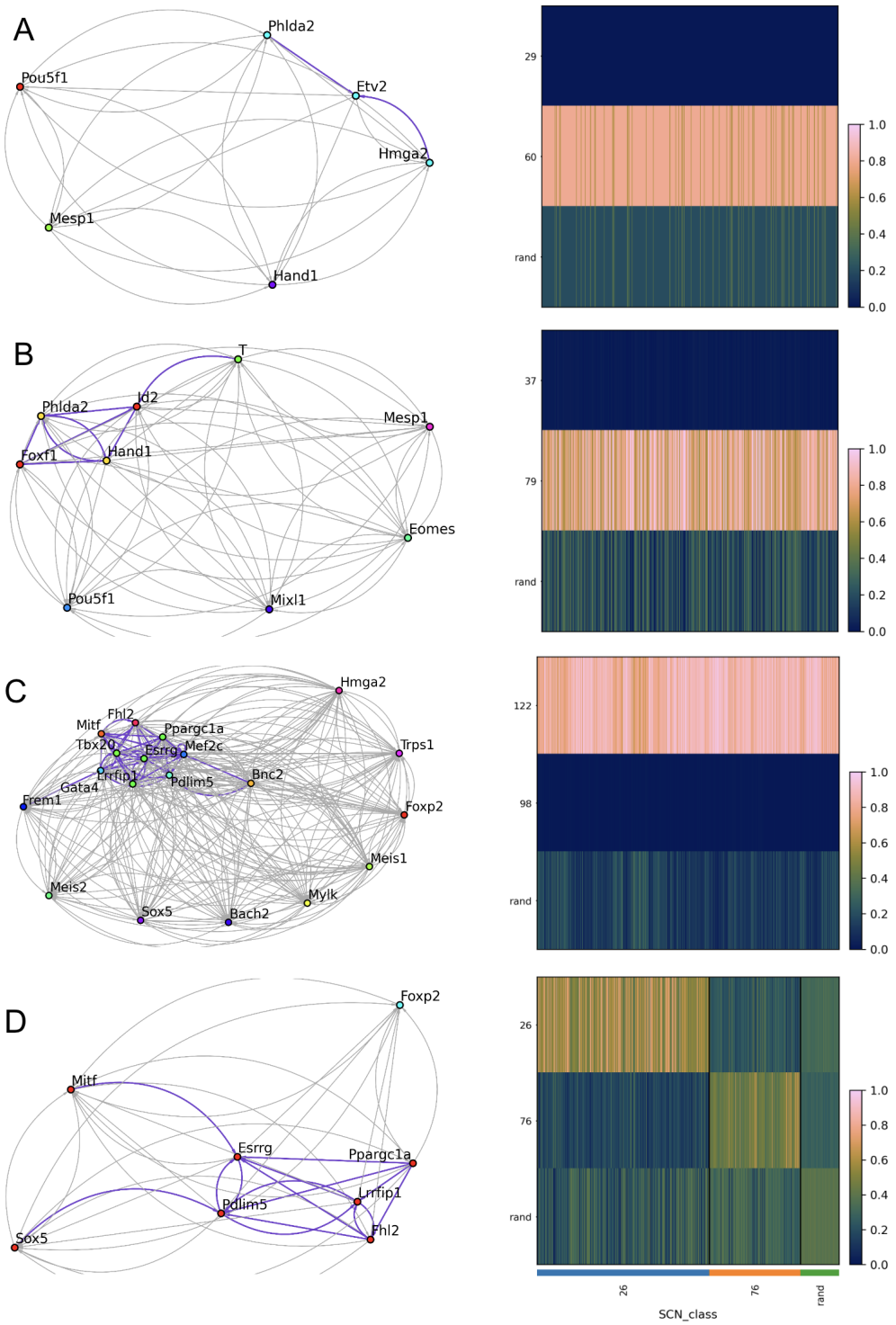

Figure 7. GRNs for Each Developmental Transition Alongside Heatmaps of Perturbations Yielding Purest Populations. A) Epiblast/Primitive Streak to Nascent Mesoderm. Phlda2 was perturbed through upregulation. Milestones align with those characterized in **Figure 2**. B)

Nascent Mesoderm to Lateral Plate Mesoderm. Foxf1 was perturbed through upregulation. Milestones align with those characterized in **Figure 3**. C) Lateral Plate Mesoderm to First Heart Field. Gata4 was perturbed through upregulation. Milestones align with those characterized in **Figure 4**. D) First Heart Field to Ventricular Cardiomyocytes. Ppargc1a was perturbed through upregulation. Milestones align with those characterized in **Figure 5**.

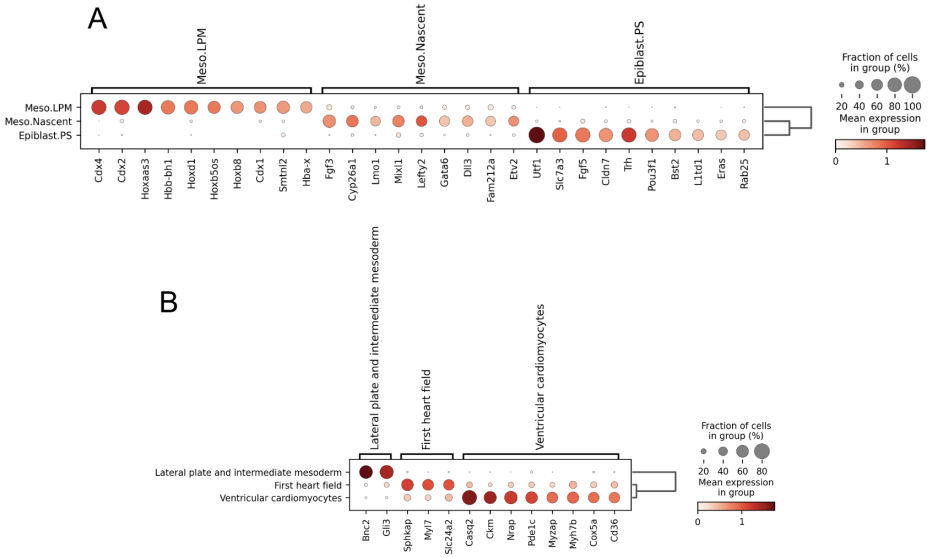

Figure 8. DEA of Each Cell Type in Target Developmental Pathway. A) displays the relevant cells isolated from the Early Stages dataset while B) displays the relevant cells isolated from the Late Stages dataset.

Discussion

In **Figure 1**, the various cell types found within the data were identified and explored through PCA, with **Figures 2~5** showcasing individual assessments of the necessary cell types for our identified pathway extracted based on their transition. Here, we can also explore their trajectory and pseudotime relationship, which aided in our exploration of fitted features of such transitions as highlighted in **Figure 6**. Notable features found included Mesp1 and Phlda2. This data then led to the development of the GRNs displayed in **Figure 7**, which served as the main source of analysis for identifying signaling pathways which could be utilized in a protocol to develop these cells. Additionally, **Figure 7** also presents the results of perturbing certain transcription factors for each transition which seemed to give the purest resultant populations. Finally, the DEA conducted in **Figure 8** presents some potential markers for the components of our chosen pathway; however, further research suggested that these were not surface markers and thus may not be suitable for optimization. Therefore, others had to be chosen based on literature.

From the data we collected, the presence of the transcription factor Phlda2 seemed to produce the purest population of nascent mesoderm cells. Phlda2 has long been associated with the regulation of embryonic development and placenta growth in mice[6]. To target Phlda2, we can target the Pou5f1 gene, which inhibits Phlda2 as seen in our inferred gene regulatory network. In turn, Pou5f1 is regulated by the Wnt signaling pathway[7]. In the canonical pathway, Wnt proteins bind to Frizzled receptors and allow for the stable formation of β-catenin. Then, β-catenin travels to the nucleus to bind to T-cell factors (TCF) and lymphoid enhancer factors (LEF) to form the TCF/LEF complex, initiating the transcription of Phlda2. Relevant antagonists include the Dickkopf (DKK) protein family, Wise/SOST, and IGFBP-4[8]. Based on the literature, other

signaling pathways that could influence the transition from epiblast cells to nascent mesoderm cells include the TGFβ and Hippo pathways. For the former, there is a clear correlation between the TGFβ and Wnt pathways, but it is unclear whether one causes or merely amplifies the other[9]. In the latter, it is suggested that Hippo signaling directly inhibits the Wnt pathway[10]. As confirmed using fluorescence-activated cell sorting (FACS) analysis, Tbx6 transduced cells expressed cell-surface markers for nascent mesoderm from pluripotent stem cells[11].

After identification of nascent mesoderms, the Hedgehog Pathway can be activated by the Hedgehog agonist protein (Hh-Ag), and signal transduction is initiated. This pathway is vital to animal development because it mediates the differentiation of multiple cell types during embryogenesis[12]. The ligands that are most relevant to triggering the Hedgehog Pathway are Shh and Ihh, with the main transmembrane receptors including Patched 1 (Ptch1) and Patched 2 (Ptch2). After the initial signal transduction, the pathway follows with Shh binding and inactivating Ptch transmembrane protein. Ptch then goes into the cell and the proteasome degrades leading Smoothened (Smo) to move from the vesicle and embed itself in the membrane. Lastly, these activate the Gli protein effectors; in this case, we will be focusing on Gli3 since it interacts with Foxf1, the transcription factor that seems to produce the purest population of lateral plate mesoderm based on our data[13]. The Gli protein migrates into the nucleus and acts as a transcription factor, turning on genes that promote development and turning off others[14]. Foxf1 is then a mediator of the Hh (endoderm) to mesoderm signaling pathway[15]. It encodes a transcription factor expressed in extraembryonic and lateral plate mesoderm[16]. As verified and identified using flow cytometry, the surface receptor Frizzled 4 (FZD4) can be used as a marker gene for lateral plate mesoderm, and in conjunction with FLK1 and PDGFRA, FZD4 increases cardiomyocyte enrichment[17].

For the transition of lateral plate mesoderm cells to the first heart field, the presence of the transcription factor Gata4 seemed to produce the purest population. Gata4 has been implicated in cardiac development, promoting cardiomyocyte gene expression while down-regulating endocardial and endothelial gene expression[18]. The TGFβ pathway appears to be the most relevant for this stage, since although it has been known to induce heart hypertrophy and apoptosis, it has also been found to specifically upregulate Gata4 through its inhibition. The TGFβ pathway begins with the binding of TGFβ to TGFβ/activin receptors, which allows for the phosphorylation of receptor-activated SMADs (r-SMADs). r-SMADs undergo oligomerization with common-partner SMADs (co-SMADs) and enter the nucleus to act as transcription factors[19]. In this role, they bind to and prevent Gata4 from interacting with JMJD3/BRG1, necessary transcription factors for cardiomyogenesis[20]. In this situation, the main (non-BMP related) antagonists that would encourage differentiation are follistatin (FST) proteins. Other relevant pathways include the aforementioned Wnt, FGF (AKT), and FGF (MAPK) pathways. Unlike the TGFβ pathway, the activation rather than inhibition of these pathways would induce differentiation and growth. As verified using in-situ hybridization, RT-PCR, and alignment testing, DM-GRASP is a surface marker of first heart field cells[21].

Lastly, our analysis concluded that Ppargc1a would likely produce the purest population of ventricular cardiomyocytes. In literature, Ppargc1a—referred to as PGC-1a for short—is known for its role in regulating key mitochondrial genes necessary for metabolism and adaptive thermogenesis (heat production)[22]. In order to meet the increased demand for ATP and work output post-birth, ventricular cardiomyocytes experience a boost in mitochondria biogenesis and oxidative metabolism, which is preceded by a rise in PGC-1a levels. An important signaling pathway for PGC-1a is FGF. FGF can cause multiple signaling cascades, so we will be referring

specifically to the PI3-K and AKT pathway[23]. The binding of FGF ligands to the FGF receptor (FGFR) causes the enzyme PI3-K to phosphorylate another enzyme AKT. AKT affects multiple downstream targets such as Foxol, and research has shown that this interacts with PGC-1a[13, 24]. Therefore, potential agonists to upregulate PGC-1a are FGF proteins. Other relevant pathways include the FGF (STAT)[25] and Notch[26] pathways, both of which play similar roles in regulating the final stages of heart growth. As verified using immunostaining, western blot analysis, and fluo-4 AM (a green-fluorescent calcium indicator), CD200 and JAK2 are identified as potential surface markers for ventricular cardiomyocytes[27].

Thus, to summarize based on our results, a protocol that we could develop would start with inhibiting the Wnt pathway using the DKK protein family, Wise/SOST, or IGFBP-4 in order to produce nascent mesoderms identified through Tbx6. Then, Hh-Ag could be used to activate the Hedgehog Pathway in order to produce lateral plate mesoderm identified through FZD4. First heart field could then be developed through inhibiting the TGFβ pathway using FST proteins and be identified through DM-GRASP. Finally, ventricular cardiomyocytes could be produced by activating the PI3-K/AKT pathway using FGF proteins and then identified using CD200 and JAK2.

Conclusion

Through harnessing the power of computational transcriptomics, we were able to identify several parameters for optimizing a protocol to develop ventricular cardiomyocytes from PSCs. Based on our data and cross-referencing literature and other databases, we were able to highlight several signaling pathways which could be used to stimulate differentiation at each stage, and we were also able to provide certain marker genes which could aid in characterization. Some limitations to our study could include our approach in conducting individual analyses of transitions rather than an overall one as well as the omission of other cell types found and not found in the dataset during analysis which could have better characterized differentiation if there were in. For future iterations, we would also like to incorporate additional computational techniques in order to improve the accuracy and precision of our analysis of transcription factors as well as the construction of our GRNs. Additionally, our approach towards the identification of marker genes for the various stages of development could be honed a bit further and align better with computational results. Utilizing resources such as ChIP-Atlas and more carefully researching protein-protein interactions could also prove beneficial to refining the pathways which could be leveraged for development.

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