



Single-cell RNA sequencing reveals the transcriptional heterogeneity of Tbx18-positive cardiac cells during heart development

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Abstract

The T-box family transcription factor 18 (Tbx18) has been found to play a critical role in regulating the development of the mammalian heart during the primary stages of embryonic development while the cellular heterogeneity and landscape of Tbx18-positive (Tbx18+) cardiac cells remain incompletely characterized. Here, we analyzed prior published single-cell RNA sequencing (scRNA-seq) mouse heart data to explore the heterogeneity of Tbx18+ cardiac cell subpopulations and provide a comprehensive transcriptional landscape of Tbx18+ cardiac cells during their development. Bioinformatic analysis methods were utilized to identify the heterogeneity between cell groups. Based on the gene expression characteristics, Tbx18+ cardiac cells can be classified into a minimum of two distinct cell populations, namely fibroblast-like cells and cardiomyocytes. In terms of temporal heterogeneity, these cells exhibit three developmental stages, namely the MEM stage, ML_P0 stage, and P stage Tbx18+ cardiac cells. Furthermore, Tbx18+ cardiac cells encompass several cell types, including cardiac progenitor-like cells, cardiomyocytes, and epicardial/stromal cells, as determined by specific transcriptional regulatory networks. The scRNA-seq results revealed the involvement of extracellular matrix (ECM) signals and epicardial epithelial-to-mesenchymal transition (EMT) in the development of Tbx18+ cardiac cells. The utilization of a lineage-tracing model served to validate the crucial function of Tbx18 in the differentiation of cardiac cells. Consequently, these findings offer a comprehensive depiction of the cellular heterogeneity within Tbx18+ cardiac cells.

Keywords ScRNA-seq · Transcriptional heterogeneity · Tbx18 · Cardiac cell · Heart development

Abbreviations

Tbx18+	Tbx18-positive	SCENIC	Single-cell regulatory network inference and clustering
E	Embryonic	PCR	Polymerase chain reaction
P	Postnatal	GRN	Gene regulatory network
ScRNA-seq	Single-cell RNA sequencing	t-SNE	T-distributed stochastic neighbor embedding
ECM	Extracellular matrix	DEGs	Differentially expressed genes
EMT	Epithelial-to-mesenchymal transition	GO	Gene Ontology
CPC	Cardiac progenitor cell	KEGG	Kyoto Encyclopedia of Genes and Genomes
TF	Transcription factor	EPDCs	Epicardial-derived cells
		hiPSCs	Human-induced pluripotent stem cells

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Introduction

The heart is the first organ to form and function during mammalian embryonic development (Buckingham et al. 2005). Although there exists a comprehensive understanding of the morphological modifications that transpire in the mammalian heart during embryonic development, additional refinement is necessary for the exploration of gene regulation

during the progression of cardiac development. Cardiogenesis encompasses the cooperative endeavors of diverse cell types, such as cardiomyocytes, fibroblasts, and immune cells, which engage in synergistic actions (Gancz et al. 2020; Filosa and Sawamiphak 2023). Moreover, throughout the course of heart development, discernible disparities in the gene expression patterns of particular cell types are also evident.

The relationship between heterogeneity in gene expression and variations in cellular developmental processes and functional properties has been thoroughly investigated in recent years through numerous studies that have analyzed gene expression patterns during heart development (Sahara et al. 2019; Litvinukova et al. 2020; Churko et al. 2018; Carter and Zhao 2020). Concurrently, single-cell RNA sequencing (scRNA-seq) has emerged as an innovative approach for conducting high-throughput sequencing and analysis of RNA at the individual cell level (Zhu et al. 2022). This technique offers novel and distinct possibilities for characterizing the cellular composition and transcriptional heterogeneity in various cell types during heart development (Xiong et al. 2019; Xiong and He 2020; Long et al. 2023).

Studies utilizing scRNA-seq have investigated the spatial and temporal dynamics of gene expression during heart development in both human and experimental animal models, thereby enhancing our comprehension of the intricate gene expression network underlying organogenesis (Xiong et al. 2019; Jia et al. 2018; Liu et al. 2019). For example, Litvinukova et al. conducted a study that focused on examining the cellular heterogeneity of cardiomyocytes, pericytes, and fibroblasts in the adult human heart. Their findings revealed distinct cellular compositions in various regions of the heart, which could potentially be attributed to their diverse developmental origins (Litvinukova et al. 2020). DeLaughter et al. utilized scRNA-seq to discern the transcriptional heterogeneity that occurs during the development of cardiomyocytes (DeLaughter et al. 2016). Similarly, Lescroart et al. investigated the region specificity of *Mesp1*-expressing cardiovascular progenitors (Lescroart et al. 2018). In 2022, Red-House et al. conducted a study that utilized pulse-chase lineage tracing and scRNA-seq to investigate the involvement of *Bmp12* and *CXCL12/CXCR4* signaling in endocardial angiogenesis (D'Amato et al. 2022). Their findings revealed that the differentiation of the endocardium into coronary arteries predominantly occurs between embryonic days 10.5 to 13.5. Additionally, Anderson et al. employed scRNA-seq analysis of fixed cardiomyocytes from developing mouse hearts at different stages (E16.5, P1, and P5) to identify *ZEB1* as a crucial factor in both proliferation and endoreplication processes (Bak et al. 2023).

It is worth noting that the heart originates from a diverse range of cell lineages that are determined by cardiac progenitor cells (CPCs), which play a crucial role in maintaining the cellular and

functional integrity of the heart (Lalit et al. 2016; Srivastava 2006). These CPCs exhibit a heterogeneous nature and can be found in various regions of the heart, including the ventricles, atria, epicardium, and pericardium (Le and Chong 2016). The differentiation process of CPCs is contingent upon the expression of specific transcription factors (TFs) and genes (Yan and Jiao 2016). Among these genes, T-box family transcription factor 18 (*Tbx18*) has been identified as a crucial marker gene for the epicardium and plays a vital role in regulating heart development during the early stages of embryogenesis in mammals (Ma et al. 2013; Dai and Weber 2018). Additionally, *Tbx18* has been observed to be expressed in the interventricular septum, left ventricular myocardium, and sinus horns (Christoffels et al. 2009). A comprehensive understanding of the cellular heterogeneity of cardiac cells expressing the *Tbx18* gene is essential for further investigation into its expression characteristics. However, there is currently a lack of a relevant systematic overview on this topic.

To date, scRNA-seq has been accompanied by a plethora of robust analytical techniques that find extensive application in cell type identification, trajectory inference, regulatory network inference, and analysis of single-cell transcriptome dynamics (Cao et al. 2019; Qiu et al. 2017a; Aibar et al. 2017a). Notably, the utilization of single-cell regulatory network inference and clustering (SCENIC) has proven instrumental in uncovering significant biological insights pertaining to the mechanisms governing cellular heterogeneity (Sande et al. 2020). Additionally, the employment of trajectory analysis through single-cell transcriptomics serves as a valuable tool for comprehending the temporal progression of cell states (Li et al. 2023a). The utilization of these analysis methods grounded in independent modalities has significantly advanced our comprehension of cellular diversity and developmental landscapes.

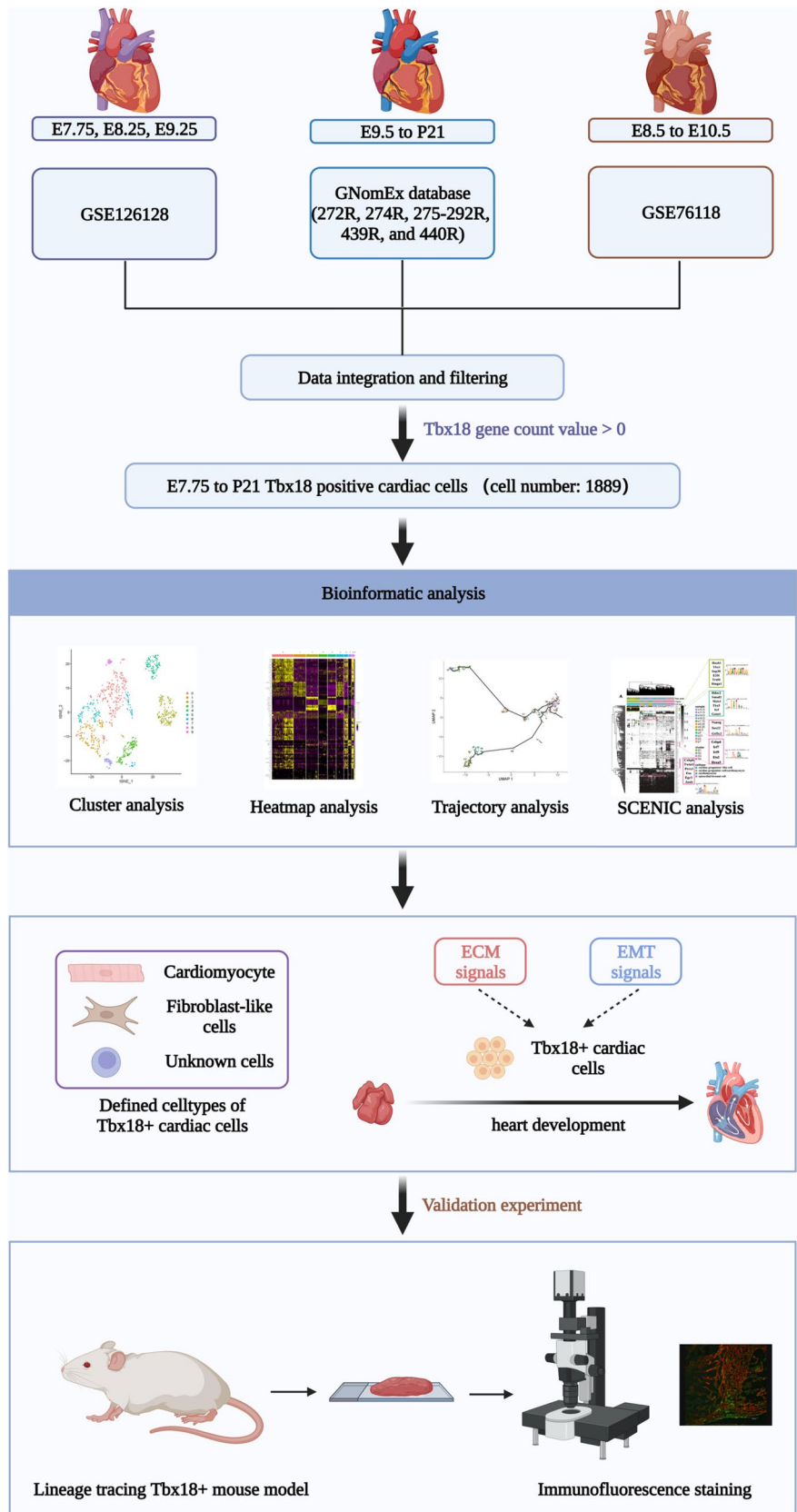
In this study, we adopted an unbiased, systematic approach to dissecting mouse *Tbx18*-positive (*Tbx18*+) cardiac cells at single-cell resolution and provided the reference map of differentiating and mature *Tbx18*+ cardiac cellular transcriptional states in embryonic (E) 7.75 day to postnatal (P) 21 day. The potential regulatory role of extracellular matrix (ECM) and epithelial-to-mesenchymal transition (EMT) signals on *Tbx18*+ cardiac cell development was investigated by performing the bioinformatic analysis based on the integrated scRNA-seq datasets. A brief flow of the study in this paper is shown in Fig. 1.

Materials and methods

Animals

All procedures involving living mice were approved by the Commission of Chongqing Medical University for the Ethics of Animal Experiments. The *Tbx18*-Cre mice were donated

Fig. 1 A brief flow chart of this study



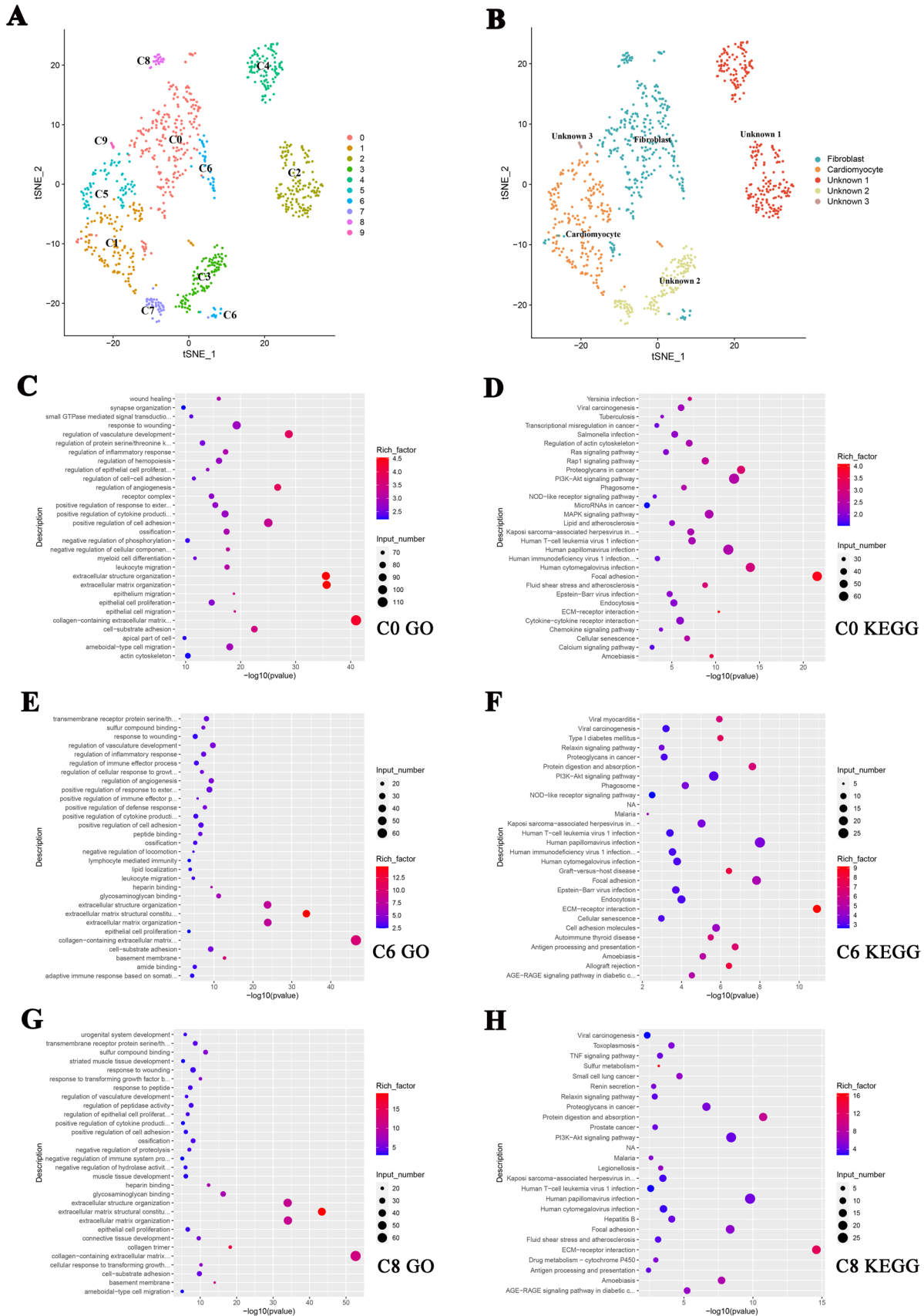


Fig. 2 Overview of single-cell landscape in developing and postnatal mouse Tbx18+ cardiac cells. **A** The t-SNE plot representation of 1889 Tbx18+ cardiac cells was colored by cluster. A total of 10 different clusters were identified after unsupervised clustering. **B** The identity of each cluster was determined by matching expression profiles of clusters with established cell-specific marker genes of different cells, including fibroblasts and cardiomyocytes. **C, D** The GO and KEGG enrichment analysis of the C0 cluster. **E, F** The GO and KEGG enrichment analysis of the C6 cluster. **G, H** The GO and KEGG enrichment analysis of the C8 cluster. The “Rich_factor” is the ratio of differentially expressed gene numbers annotated in this pathway term to all gene numbers annotated in this pathway term. The greater the Rich factor, the greater the degree of pathway enrichment. The values of rich factors are expressed as percentages (%)

by Sylvia M. Evans (Cai et al. 2008). The Rosa26-EYFP Cre reporter mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) (Cai et al. 2008; Srinivas et al. 2001). The methods of mouse genotyping and primer design were as previously described (Srinivas et al. 2001). The Tbx18 probe plasmids were donated by A. Kispert (Kraus et al. 2001). Homozygous Tbx18-Cre mice were identified by routine polymerase chain reaction (PCR). The Tbx18-Cre/Rosa26-EYFP heterozygous mice were generated by crossing single-heterozygous mice. As previously described, immunofluorescence was performed on cultured cells or heart cryosections to determine lineage specification (Jing et al. 2016). The investigator was not blinded to the group allocation during the experiment.

Single-cell RNA data source and preparation

Publicly available scRNA-seq data on embryonic and postnatal mouse hearts was obtained from multiple sources. The following datasets were included: E7.75 (cardiac crescent region from E7.75 mouse embryo, 4326 cells), E8.25 (heart tube region from E8.25 mouse embryo, 5664 cells), E9.25 samples (looped heart and pharyngeal arch region, 11,376 cells) (De Soysa et al. “GSE126128,” totally 21,366 cells) (Soysa et al. 2019), E9.5 to P21 samples (DeLaughter et al. “GNomEx database 272R, 274R, 275 to 292R, 439R, and 440R,” atria and ventricle from E9.5 to P21 mouse heart, totally 1200 cells) (DeLaughter et al. 2016); and E8.5 to E10.5 samples (Li et al. “GSE76118,” atria, ventricle, outflow tract from E8.5 to E10.5 mouse heart, totally 2233 cells) (Li et al. 2016). Seurat’s standard process was conducted (Butler et al. 2018; Stuart et al. 2019). In brief, we removed low-quality cells according to the standard, which are cells with fewer than 300 unique molecular identifiers (UMIs) or mitochondrial gene expression exceeding 25% (Koenig et al. 2022; Osorio and Cai 2021; Wang et al. 2022). The transcriptome data were normalized using the “SCTransform” function. The batch effects for samples from different sources were removed by the Harmony R package. Screening conditions for Tbx18 positive cells were defined as count values greater than 0. A total of 1889 cells were used for subsequent bioinformatic analysis. The operating environment included a Linux

operating system, 128G RAM, 16 threads, and R language 4.02 with installed packages Seurat, SingleR, Celltalk, Tidyverse, and Ggplot2.

Bioinformatic analysis

Principal component analysis clustering was used for the dimensional reduction. Cluster analysis of samples was performed with the R Seurat FindClusters method. The t-SNE algorithm for all selected cells was run (Butler et al. 2018; Stuart et al. 2019). According to the clustering results, singleR was used to identify the cluster’s cell type, and Monocle3 was used for the cell trajectory analysis (<https://cole-trapnell-lab.github.io/monocle3>). The computational method of SCENIC was used to identify the key transcription factors (TFs) and regulatory network cell states (Sande et al. 2020; Aibar et al. 2017b) (<http://scenic.aertslab.org>). Gene regulatory network (GRN) construction and cell-state identification were performed on sc RNA-seq data. A volcano plot was used to visualize the results with the R ggplot package, and the R pheatmap package was used for heatmap construction (Chen et al. 2020). The GRN was visualized using the Network Analyzer available in the Cytoscape software (version: 3.9.1).

Immunofluorescence staining

All immunofluorescence staining experiments were performed as previously described (Yuan et al. 2021). Cells obtained in culture and tissues were soaked in 4% paraformaldehyde for fixation. Tissues were embedded and then cut by cryo-sectioning to 10 μ m. Nonspecific sites were blocked. The following primary antibodies were used, including mouse monoclonal anti-cardiac troponin T (MSZ-295-P0, Thermo Fisher Scientific, Waltham, MA, USA) and rabbit polyclonal anti-Perilipin antibody (1:50, ab3526; Abcam, Cambridge, UK). The primary antibodies were also rabbit anti-Periostin (ab215199; Abcam), rabbit polyclonal anti-Myh11 (ab224804, Abcam) diluted 1:50, mouse monoclonal antibody to Snai1 (sc-271977; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse monoclonal antibody to Twist (ab175430, Abcam).

The sections were incubated with Alexa Fluor 594- and 488-conjugated secondary antibodies. Microscopy was performed with a laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Germany). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 1–5 min.

Results

Single-cell RNA-seq landscape of Tbx18+ cardiac cells in fetal and neonatal mouse

To investigate the single-cell transcriptomic characteristics and subcluster heterogeneity of Tbx18+ cardiac cells

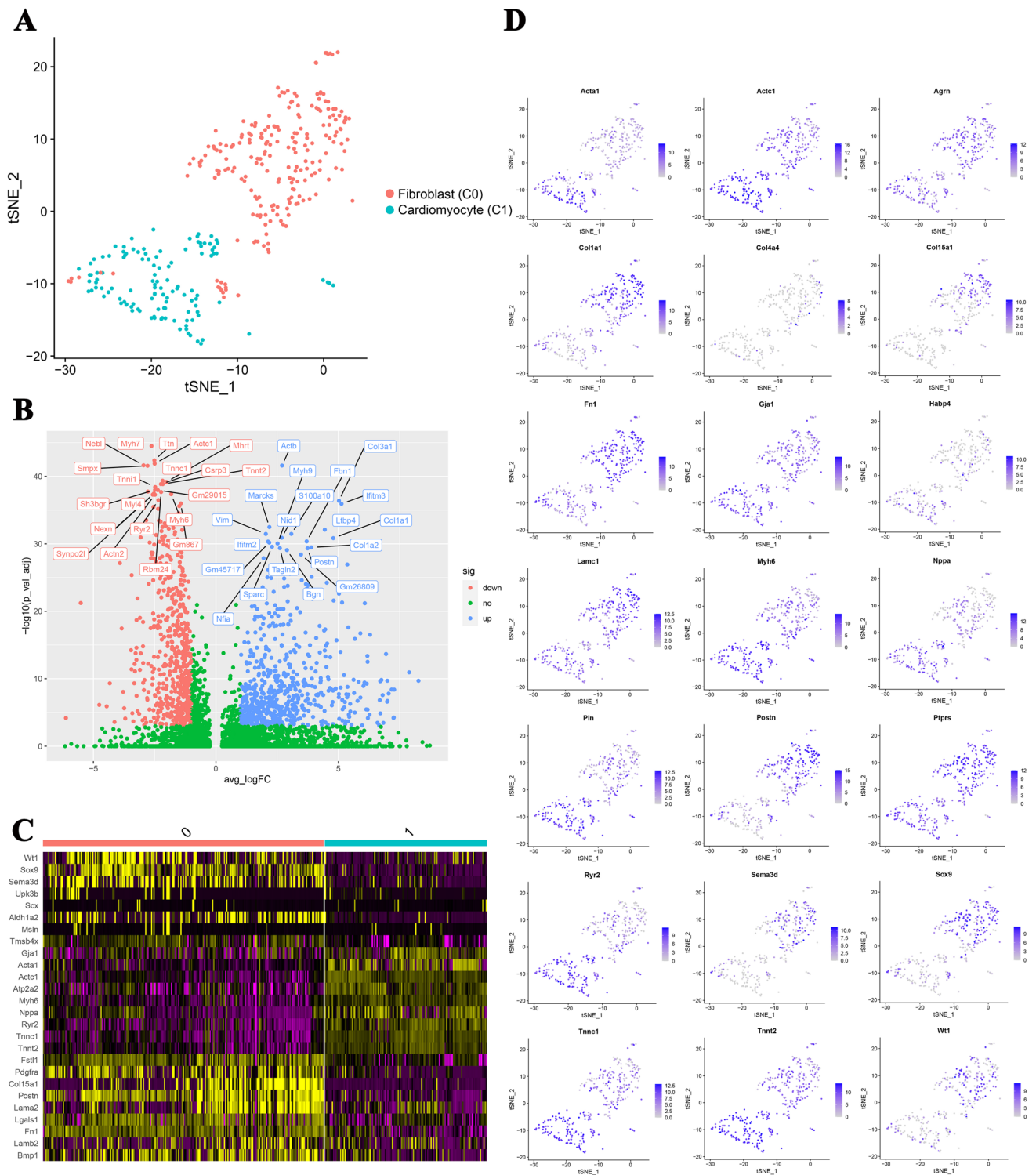


Fig. 3 Characterization of C0 and C1 clusters. **A** The t-SNE plot of C0 and C1. **B** The volcano plot showed DEGs between C0 and C1 clusters. DEGs upregulated in C0 were marked with blue, and DEGs

downregulated in C0 were marked with red. **C** The heatmap showed the expression of the ECM-related genes of C0 and C1. **D** Feature plots displaying the marker gene expression of C0 and C1

during cardiogenesis, we integrated the scRNA-seq datasets of Tbx18+ cardiac cells from mouse heart tissue spanning embryonic day (E) 7.75 to 18.5 and postnatal day (P) 1 to 21 for further analysis. In a global view, we identified 1889 Tbx18+ cells grouped into ten major clusters with t-distributed stochastic neighbor embedding (t-SNE) analysis of the integrated dataset (Fig. 2A). We then generated cluster-specific marker genes by performing differential gene expression analysis to define the identity of each cell cluster. The heatmap of the top differentially expressed genes (DEGs) for each cluster is shown in Fig. S1. We then performed the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs belonging to each cluster. We combined the expression level of known markers and the DEG enrichment analysis to determine the cell identity of each cluster. We identified two major cell types: fibroblast-like cells and cardiomyocytes (Fig. 2B).

Specifically, the clusters C0, C6, and C8 were defined as fibroblast-like cells, which highly expressed the cardiac fibroblast marker gene *Col3a1*; the stromal cell marker genes *Thbs2*, *Thbs1*, *Cxcl1*, *Tcf4*, and *Sparcl1* (<http://bio-bigdata.hrbmu.edu.cn/CellMarker/index.html>); and the ECM-related genes such as *Col14a1*, *Col15a1*, *Col6a3*, *Postn*, *Aspn*, and *Fndc1* (Fig. S1). The GO and KEGG enrichment analysis of the C0, C6, and C8 showed enrichment in the extracellular matrix organization, collagen-containing extracellular matrix, and focal adhesion (Fig. 2C–H). The other predominant cell population consisted of cardiomyocytes, specifically C1 and C5, exhibiting high expression of the myosin heavy chain genes *Myh7* and *Myh4*, both of which are widely recognized as conventional markers for cardiomyocytes. The GO and KEGG enrichment analysis of the C1 and C5 clusters showed enrichment in the sarcomere, myofibril, contractile fiber, and cardiac muscle contraction (Figs. S2B to E). Since there was no known marker gene expression, the C2 and C4 were defined as “unknown 1” cells with high expression of *Pdcd5*, *Mir99ahg*, and *Actg*, which related to cell death and movement (Han et al. 2021; Xiao et al. 2021). The C3 and C7 were defined as “unknown 2” cells with high expression of the ribosomal protein-encoding genes *Rps19* and *Rpl27*. C9 was defined as “unknown 3” cells, which specifically expressed the genes *Dmrta1*, *Fgr*, *Pof1b*, *Stoml3*, *Rasgef1a*, and *Cpa5*.

Based on the single-cell transcriptomic data, we determined the grouping characteristics of these Tbx18+ cardiac cells. Our results showed that Tbx18+ cells have the properties of at least two cell populations, fibroblast-like cells (C0, C6, and C8) and cardiomyocytes (C1 and C5). Meanwhile, the expression of the ECM genes, such as fibronectin 1 (*Fn1*), intracellular hyaluronic acid-binding protein 4 (*Habp4*), protein tyrosine phosphatase sigma (*Ptprs*), agrin protein (*Agrn*), Collagen 1A1, 4A4 (*Col1a1*, *Col14a4*), laminin subunit gamma 1 protein (*Lamc1*), and periostin (*Postn*), was shown in the t-SNE plot (Fig. S2A). This result

suggested that heterogeneity exists within Tbx18+ cardiac cells, and the ECM genes were potentially involved in the formation of cellular heterogeneity. Cluster analysis identified that C0 and C1 clusters account for the largest and second largest proportion of all Tbx18+ cardiac cells during cardiogenesis (477 and 364 cells, respectively), defined as the fibroblast-like cells and cardiomyocytes (Fig. 2A, B).

Characterization of C0 and C1 clusters

To further investigate the differences between Tbx18+ cardiomyocytes and non-cardiomyocytes, we analyzed the transcriptional characteristics of fibroblast cluster C0 and cardiomyocyte cluster C1 and explored their roles in cardiac development. The t-SNE plot showed the clusters C0 and C1 (Fig. 3A). The volcano plot revealed the top twenty DEGs between the C0 and C1 clusters in Fig. 3B. The marker genes of the epicardial cell, cardiomyocyte, and fibroblast were selected, with the heatmap in Fig. 3C constructed to represent the expression of these marker genes in clusters C0 and C1. Interestingly, cluster C0 displayed the molecular signature of epicardial cells and fibroblasts. The marker genes of epicardial cells (i.e., *Wt1*, *Sox9*, *Upk3b*, and *Aldh1a2*) and fibroblasts (i.e., *Fstl1*, *Col15a1*, *Bmp1*, *Lgals1*, and *Lama2*) were both highly expressed in cluster C0 (Fig. 3C, D). Among these markers, *Wt1*, *Sox9*, and *Pdgfra* were demonstrated to participate in the EMT of the epicardium (Wagner and Wagner 2021; Hong et al. 2022; Xia et al. 2022). The GO analysis showed that cells in this cluster were associated with ECM organization, epithelial cell proliferation, epithelial cell migration, focal adhesion, and regulation of angiogenesis (Fig. S2B and C). Since previous studies have confirmed that epicardial cells can differentiate into coronary vascular smooth muscle cells and cardiac fibroblasts through EMT and cell migration, the deduction was made that this cluster could potentially be the epicardial-derived cells (EPDCs) undergoing EMT (Sanchez-Fernandez et al. 2022).

Meanwhile, cluster C1 expressed the cardiomyocyte marker genes (i.e., *Acta1*, *Actc1*, *Tnnt2*, *Tnnc1*, *Ryr2*, *Nppa*, and *Myh6*) (Lupu et al. 2020) (Fig. 3C, D), and the DEG analysis of C1 showed enrichment in muscle cell development and differentiation, cardiac muscle contraction, and cardiomyopathy (Fig. S2B and C). Thus, it could be potentially identified as the typical cardiomyocytes. This result suggested that Tbx18+ cardiac cells may play diverse roles by exhibiting specific transcriptional features during cardiogenesis.

The temporal transcriptional heterogeneity of Tbx18+ cardiac cells

The classification of heterogeneity within a cell population encompasses two distinct categories, namely cellular heterogeneity and temporal heterogeneity. In the context of heart

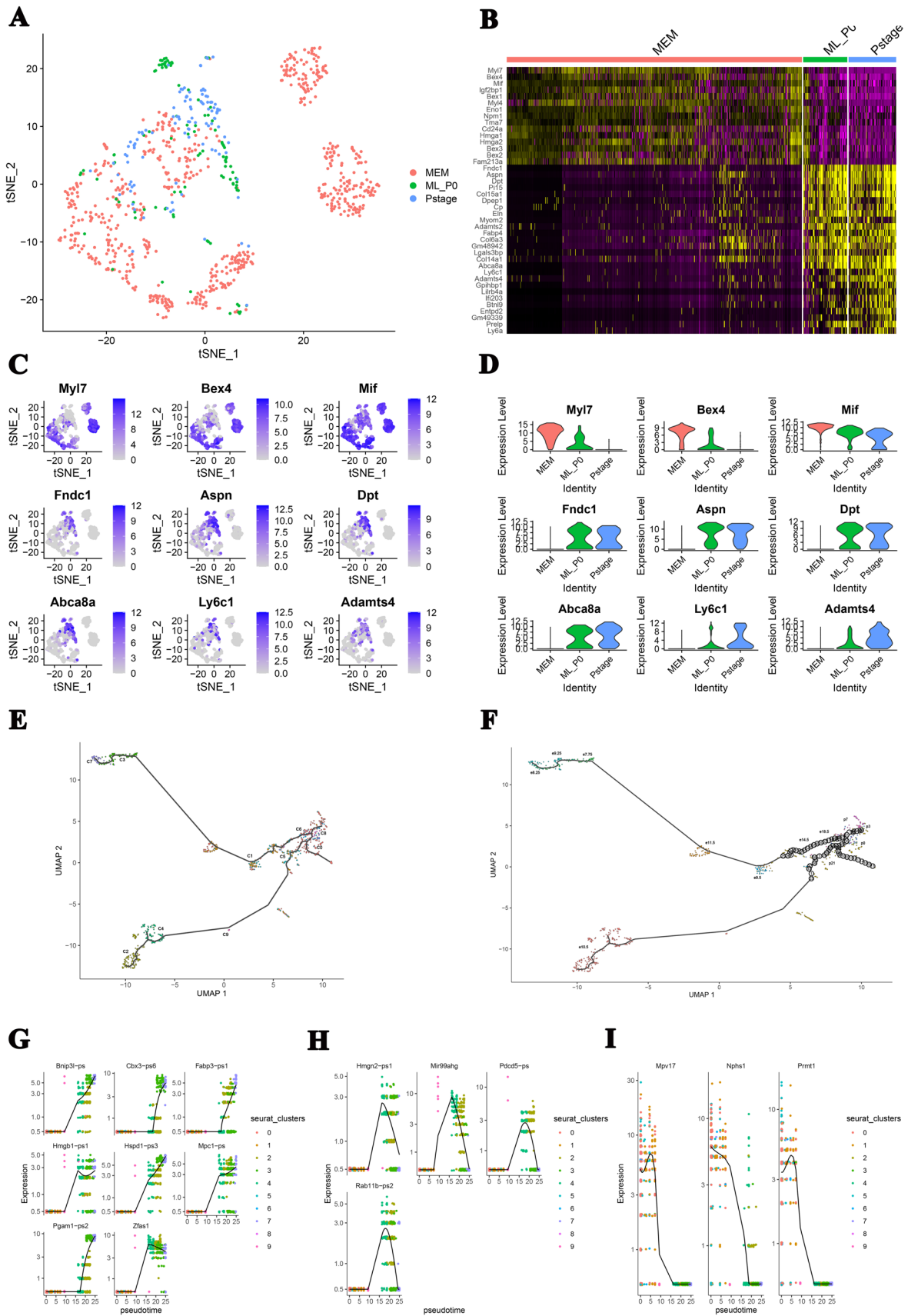


Fig. 4 The temporal transcriptional heterogeneity of single-cell Tbx18+ cardiac cells. **A** The t-SNE plot representation of 1889 Tbx18+ cardiac cells was colored by stages. **B** The heatmap showed the expression of the cardiac and ECM marker genes per stage for all Tbx18+ cardiac cells. **C** Feature plots displayed the cardiac and ECM marker gene expression per stage for all Tbx18+ cardiac cells. **D** Violin plots displayed the cardiac and ECM marker gene expression per stage for all Tbx18+ cardiac cells. **E, F** The single-cell trajectory of Tbx18+ cardiac cells constructed by Monocle3. **E** was colored by identified clusters. **F** was colored by real-time points. **G–I** Expression trend of selected DEGs along the pseudo-timeline

development, the expression of numerous genes occurs in a time-specific manner, resulting in variations in the transcriptional state of cells belonging to the same population at different time points, commonly referred to as temporal heterogeneity (Kwon et al. 2019). It is important to note that the presence of both cellular and temporal heterogeneity within a cell population does not preclude their simultaneous occurrence. To identify the temporal-specific genes and temporal heterogeneity of Tbx18+ cardiac cells, we partitioned the samples into three subtypes according to the embryonic developmental stage from which they originated. The Tbx18+ cardiac cells were grouped into three distinct clusters: MEM stage represented the early and middle stage of Tbx18+ cardiac cells from E7.75 to E14.5; ML_P0 stage represented the late embryonic and early postnatal stage of Tbx18+ cardiac cells from E18.5 to P0; P stage represented late postnatal Tbx18+ cardiac cells from P3 to P21 (Fig. 4A). According to prior research, the timepoints of E14.5 and E18.5 corresponded to the formation of the primary structures of the heart and the maturation of cardiomyocyte and fibroblast functionality, respectively (Gu et al. 2022; Cui et al. 2019).

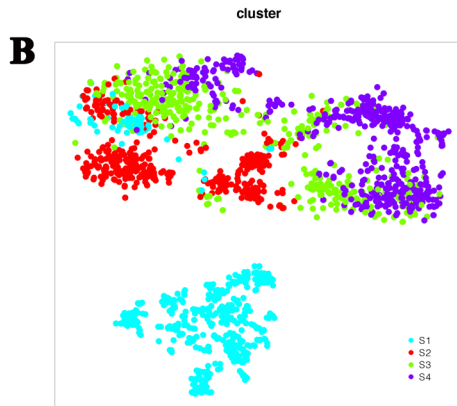
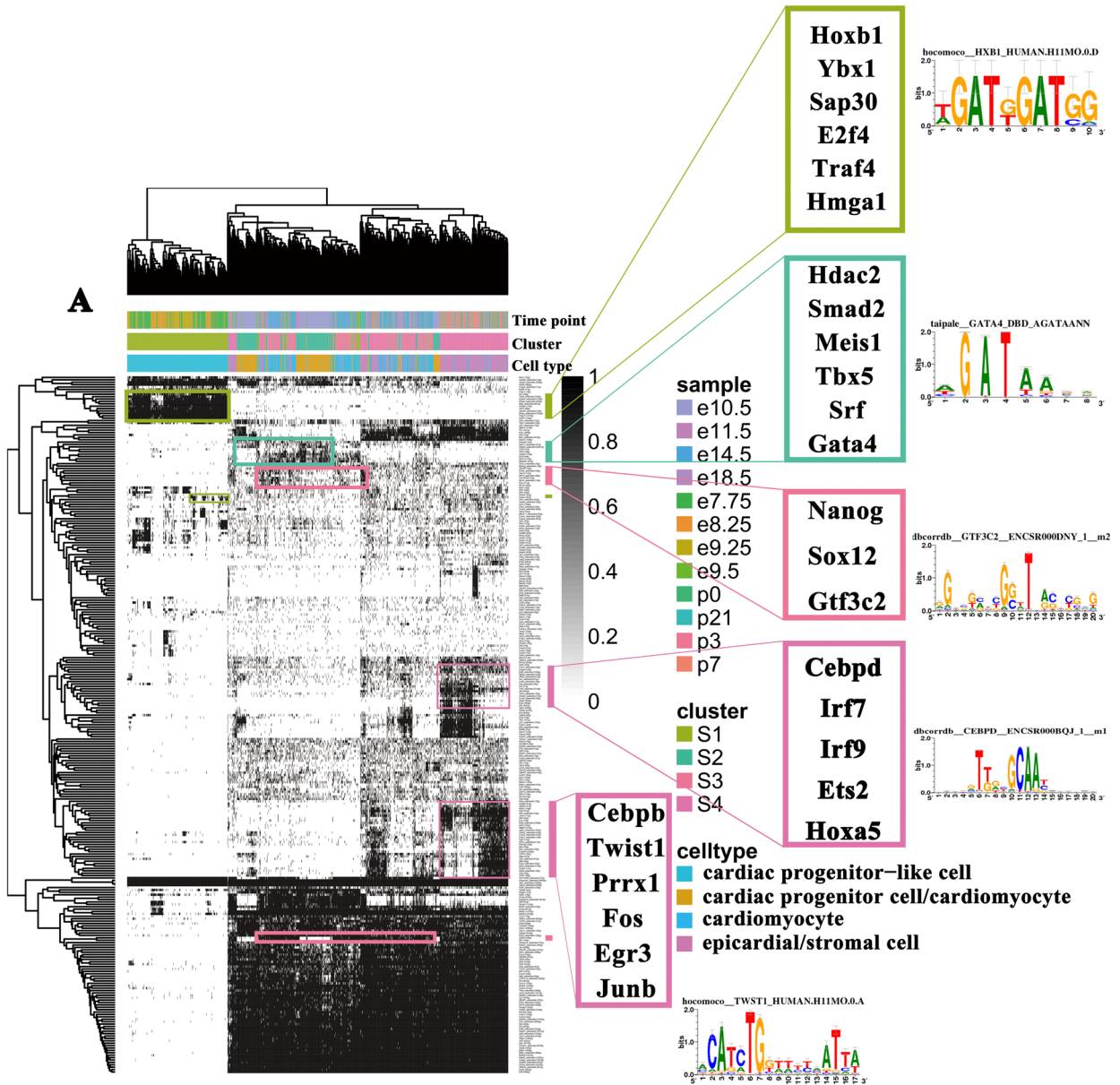
Heatmap analysis of the top DEGs expressed in different subgroups demonstrated huge differences in gene expression among these subgroups (Fig. 4B). The GO and KEGG enrichment analyses revealed that the DEGs of MEM stage cells were enriched in muscle tissue development, muscle cell differentiation, constituent of ribosome, and metabolic process, which were concerning cardiomyocyte development and metabolism (Fig. S3A and B). DEGs of the ML_P0 stage cells were enriched in extracellular matrix structural constituent, collagen-containing extracellular matrix, and ECM-receptor interaction (Fig. S3C and D). DEGs of the P stage cells were enriched in the collagen-containing extracellular matrix, focal adhesion (Fig. S3E and F). Based on the results obtained from heatmap analysis, as well as GO and KEGG enrichment analysis, it is noteworthy that the gene expression profiles of ML_P0 stage and P stage cells exhibit a remarkable similarity. Conversely, discernible disparities in gene expression levels were observed between the MEM stage and the aforementioned ML_P0 stage and P stage. These findings suggest that the time interval spanning from

E14.5 to E18.5 may serve as a transitional stage, potentially facilitating alterations in gene expression patterns within Tbx18+ cardiac cells.

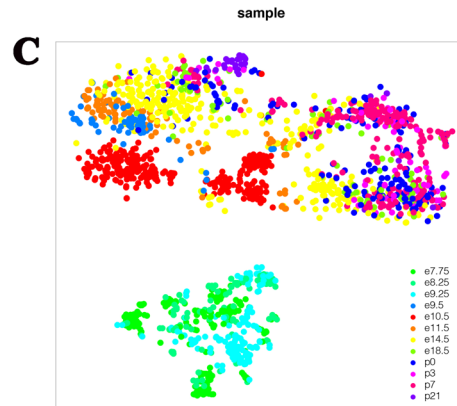
We next plotted the expression profile of the ECM and cardiomyocyte marker genes in clusters of different stages. The feature plots showed that cardiomyocyte marker genes *Myl7*, *Bex4*, and *Mif* were expressed at higher levels in the cluster of MEM stage. ECM genes *Fndc1*, *Aspn*, *Dpt*, *Abca8a*, *Ly6c1*, and *Adamts4* were expressed at higher levels in the cluster of ML_P0 and P stage (Fig. 4C). The trends of marker genes in each cluster were plotted as shown in the violin plot (Fig. 4D). The expression levels of *Myl7*, *Bex4*, and *Mif* decreased with the development of the mouse heart, while the expression levels of ECM genes increased with the development of the mouse heart.

To further explore the temporal dynamics of Tbx18+ cardiac cells during heart development, we arranged these individual cells from the identified ten clusters by Monocle 3 (Qiu et al. 2017a, b) (Fig. 4E). When combined with the real development time point (Fig. 4F), the result demonstrated that the subgroup proportions of Tbx18+ cardiac cells changed dynamically with heart development. To elucidate the molecular dynamics in each cluster, we further analyzed the gene expression patterns of all genes along the trajectory of cardiac development and identified fifteen genes with dynamic expression changes. The trends and distribution of single gene expression in each cluster were plotted (Figs. 4G–I and S4A to C). The genes related to CPC differentiation, cardiomyocyte development and function, and angiogenesis (*Bnip2l*, *Cbx3*, *Fabp3*, *Hmgb1*, *Hspd1*, *Mpc1*, *Pgam1*, and *Zfas1*) gradually increased along pseudotime progression, highly expressed in cells at E7.75 to E9.25. The genes related to cell apoptosis and movement, cell migration, and epithelial cells (*Hmgn2*, *Mir99ahg*, *Pdcd5*, and *Rab11b*) reached the peak in the middle stage of pseudotime, highly expressed in cells at E10.5. The genes related to epicardial EMT, cardiac angiogenesis, and mitochondrial function (*Mpv17*, *Nphs1*, and *Prmt1*) gradually decreased along pseudotime progression, highly expressed in cells at E11.5 to P21. When combined with the gene expression profiles and DEG analysis of three defined developmental stages, it was suggested that Tbx18+ heart cells have multiple cell biological processes in mouse heart development. The early stage involved CPC differentiation and cardiomyocyte development, while the late stage involved ECM gene enrichment and epicardial EMT signals.

This result demonstrated that the transcriptional profile of Tbx18+ cardiac cells differed at different developmental stages. Tbx18+ cardiac cells in the early embryonic stage exhibited CPC and cardiomyocyte transcriptional properties, while Tbx18+ cardiac cells in the late embryonic stage exhibited EPDC transcriptional properties. Furthermore, the ECM genes and epicardial EMT genes had similar



t-SNE on Binary 388 regulons (50PCs, 50 perplexity)



t-SNE on Binary 388 regulons (50PCs, 50 perplexity)

Fig. 5 Transcription factor network analysis of cellular heterogeneity in Tbx18+ cardiac cells. **A** The binary active heatmap (left) showed the activity of regulons in each cell. Black indicated active, and white indicated inactive. The selected regulons were shown in different colored rectangular frames (middle). The enriched motif logos were shown on the right. **B** The t-SNE plot showed cells colored by cell states. **C** The t-SNE plot showed cells colored by developmental stages

expression trends during Tbx18+ cardiac cell development, suggesting that the two may be synergistic in the specific developmental period, which was consistent with the former results in Fig. 3.

Transcription factor network analysis of cellular heterogeneity in Tbx18+ cardiac cells

We used the SCENIC algorithm to map the GRNs governing the Tbx18+ cell states (Bak et al. 2023; Lalit et al. 2016). The regulon activity analysis of heart tissue samples from the E7.7.5 to P21 delineated four transcription-factor-driven regulon groups. Ultimately, 388 regulons were identified according to the binary regulon activity and showed distinct differences among Tbx18+ cardiac cells from subcellular clusters. The results of the SCENIC analysis provided further evidence that these Tbx18+ cells were divided into distinct cell states and identified possible candidate transcription factors (TFs) that may maintain transcription programs during cardiac development (Fig. 5A). Furthermore, based on the identified temporal-specific genes' expression characteristics, the S1–S4 correspond to different periods of cell development, respectively (S1: e7.75–e9.5; S2: e10.5, e11.5; S3: e14.5, e18.5; S4: P0–P21) (Fig. 5B, C).

The regulon activity matrix revealed their differential expression of TFs in distinct cell states. TFs Hoxb1, Ybx1, Sap30, E2f4, Traf4, and Hmga1 were found to be key transcriptional regulators of the S1 cluster. Hoxb1 played a crucial role in patterning CPCs, and the TFs Ybx1, Sap30, E2f4, Traf4, and Hmga1 were essential for cardiomyocyte proliferation during cardiac development (Stefanovic et al. 2020; Stefanovic et al. 2020; Varma et al. 2023; Teittinen et al. 2012; Dingar et al. 2012; Li et al. 2023b). These TFs of S1 were in accordance with the expression characteristics of cardiac progenitor-like cells. TFs Hdac2, Smad2, Meis1, Tbx5, Srf, and Gata4 were enriched in the S2 cluster. Hdac2 and Gata4 coordinately regulated cardiomyocyte proliferation during embryonic development (Trivedi et al. 2010). Smad2 was related to Ca²⁺ signaling in cardiomyocytes (Duran et al. 2018). Meis1 was identified as a critical transcriptional regulator of cardiomyocyte proliferation (Mahmoud et al. 2013). Srf played a vital role in the structural and transcriptional maturation of cardiomyocytes (Guo et al. 2020). Tbx5 was required in embryonic cardiomyocytes for proliferation (Trivedi et al. 2010). The TFs Gtf3c2,

Nanog, and Sox12 were the key transcriptional regulators of the S3 cluster. Nanog was positively expressed in myocardial cells, Gtf3c2 was associated with acute myocardial infarction, and Sox12 was involved in cardiac development (Gao et al. 2016; Luo et al. 2014). Their differential expression of TFs in S2 and S3 corresponded to the characteristics of CPCs and cardiomyocytes. TFs Fos, Egr3, Cebpd, Cebpb, Irf7, Irf9, Hoxa5, Prrx1, Twist1, Junb, and Ets2 were enriched in the S4 cluster. C/EBP transcription factors mediated epicardial activation during heart development and injury (Huang et al. 2012). Twist was associated with the epicardial EMT process (Yang et al. 2016). Fos, Egr3, Ets2, Irf7, and Prrx1 were associated with cardiac fibrosis and fibroblasts (Palomer et al. 2020; Teng et al. 2020; Islas et al. 2012; Jiang et al. 2014; Wang et al. 2022). Junb, Irf9, and Hoxa5 were related to vascularization (Yoshitomi et al. 2021; Zhang et al. 2014; Jing et al. 2021). These transcription characteristics of the S4 cluster were mostly similar to those of epicardial and stromal cells. To further analyze and validate the transcriptional regulators and their target genes, we constructed the regulon specificity score (RSS) map to show each stage's top five specific regulons in Fig. S5. By combining regulon activity heatmap (Fig. 5A), RSS maps (Fig. S5A to D), regulon expression maps (Figs. S5E, F, and S6), and previous research, the TFs Hoxb1 (S1), Gata4 (S2), Gtf3c2 (S3), Cebpd (S4) and Twist1 (S4) were chosen to construct representative GRNs of each stage in Fig. 6A–E. Based on the GRNs, Aldh1a2, C1ql1, Cacng7, Cdx1, Fgf15, Hoxa1, Osr1, Tbx1, Tead3, Zic3, and Sfrp5 were enriched in Hoxb1 GRN, and these genes participated in the cardiovascular formation during embryogenesis (Stefanovic et al. 2020; Beecroft et al. 2021; Liu et al. 2017; Toran et al. 2019; Chu et al. 2014; Vincentz et al. 2005; Zhou et al. 2015; Martucciello et al. 2020; Han et al. 2020; Bellchambers and Ware 2021; Fujii et al. 2017). The genes Gata5, Gata6, Tbx20, Tbx5, Wnt2, Igfbp5, Myl7, Wnt2b, Hey2, EphA3, Sfrp5, Casz1, Hand2, Bmp10, and Meis1 were enriched in the Gata4 GRN, and these genes participated in the cardiomyocyte proliferation and the cardiomyocyte cell cycle (Mahmoud et al. 2013; Singh et al. 2010; Lu et al. 2017; Gharibeh et al. 2021; Jia et al. 2019; Onizuka et al. 2012; Song et al. 2013; Sun et al. 2020, 2022a; Steimle et al. 2018; Gibb et al. 2018; Stephen et al. 2007; Qiu et al. 2017c; Xia et al. 2019; Qu et al. 2019). Mark4, Myl6b, Mef2b, Ipo7, Tesk2, Plcd4, Nek6, Nfatc2, and Nol3 were enriched in Gtf3c2 GRN, and these genes were involved in cardiomyopathy and myocardial injury (Yu et al. 2021; Galindo et al. 2022; Zhong et al. 2021; Dang et al. 2020; Berg et al. 2017; Charron et al. 2015; Qin et al. 2014; Bourajaj et al. 2008; Xu et al. 2022). The genes Adamts9, Fosl1, Fosl2, IL-6, Osmr, Ptx3, Junb, and Zfp3612 were enriched in the Cebpd GRN, and these genes were involved in the cardiac remodeling, cardiac fibrosis, vascularization, and ECM remodeling

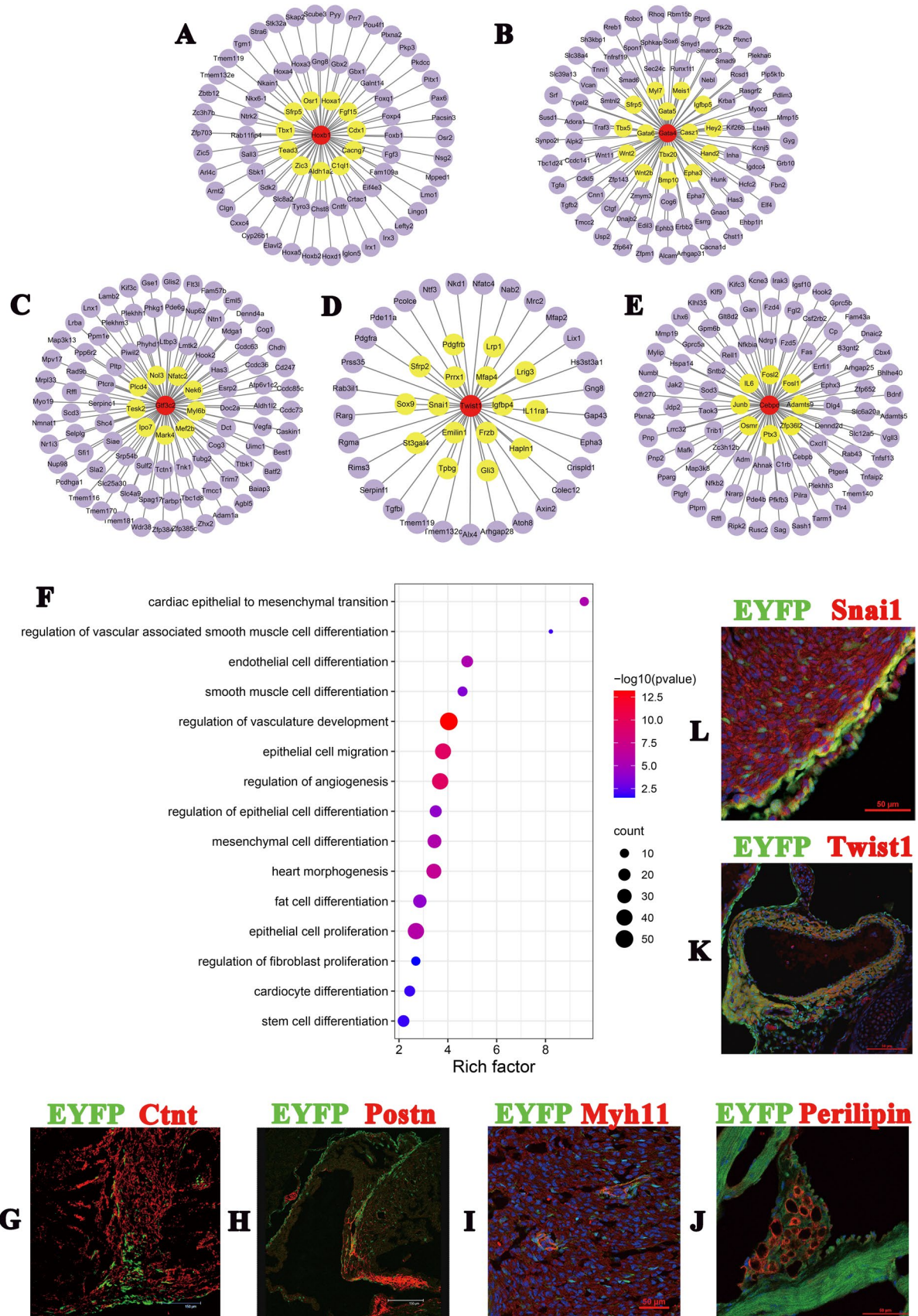


Fig. 6 The gene regulatory network and immunofluorescence results. **A–E** The gene regulatory network analysis using SCENIC identifies critical nodes driving the S1–S4 stages. **F** The GO enrichment analysis of the C0 cluster at the MEM stage. **G–L** EYFP and Cntn (**G**), Postn (**H**), Myh11 (**I**), Perilipin (**J**), Twist1 (**K**), and Snail1 (**L**) expression, detected by immunofluorescence in the heart of Tbx18-Cre/Rosa26-EYFP heterozygous mouse

(Kern et al. 2010; Wu et al. 2021a, b, c; Kumar et al. 2019; Farini et al. 2020; Seidenberg et al. 2021). The genes Snai1, Prrx1, Mfap4, Igfbp4, Frzb, Emilin1, Sox9, St3gal4, Tpbp, Gli3, Hapln1, IL11ra1, Lrig3, Lrp1, Pdgfrb, and Sfrp2 were enriched in the Twist1 GRN, and these genes were associated with EMT, cardiac morphogenesis, cellular differentiation, cardiovascular fibrosis, angiogenesis, cardiac development, cardiac regeneration, ventricular remodeling, epicardium, and intramyocardial adipocytes, respectively (Wang et al. 2022; Al-Hattab et al. 2018; Jiang et al. 2021a, b; Sun et al. 2022b; Dorn et al. 2021; Wu et al. 2020). The transcription factor regulatory network of Tbx18+ heart cells was time-specific and existed a transition from CPC properties to epicardial/stromal cell characteristics during cardiogenesis. At the same time, this result confirmed the existence of epicardial EMT in Tbx18+ cardiac cells at the late developmental stage from the perspective of transcriptional regulation.

Lineage tracing of Tbx18+ cardiac cells

In mice, genetic lineage studies can assess precursor cells' contribution to a mature tissue or organ. For the proepicardium/epicardium, such an effort has now been undertaken by Cai et al. based on the observation that the transcription factor gene Tbx18 was expressed at high levels in these embryonic tissues (Cai et al. 2008).

The scRNA-seq showed that the Tbx18+ cardiac cells had CPC and epicardial cell properties during heart development. To further assess and characterize the Tbx18+ cardiac cells in cardiogenesis, we performed lineage tracing following Tbx18 gene expression of the heart tissue in the Tbx18-Cre/Rosa26-EYFP heterozygous mice model after E10.5, in which Tbx18-Cre mice crossed with Rosa26-EYFP reporter mice were used. The cardiomyocyte marker cTnT and EYFP were co-expressed (Fig. 6G). The vascular smooth muscle cell marker Myh11 and EYFP were co-expressed in the blood vessels (Fig. 6I). The adipocyte marker Perilipin and EYFP were co-expressed in the epicardial adipose tissue (Fig. 6J). The fibroblast marker Postn and EYFP were co-expressed in the fibrous tissue of the heart (Fig. 6H). The regulon Twist1 and EYFP were co-expressed in the blood vessels. The EMT signaling molecules Snail and EYFP were co-expressed in the epicardium. The immunofluorescence result suggested that the Tbx18+ cardiac cells could differentiate into vascular smooth muscle cells, fibroblasts,

adipocytes, and a few working cardiomyocytes, and the process may be related to EMT. The Tbx18+ cardiac cells exhibited the characteristics of epicardial progenitor cells in the lineage tracing model, in line with the GO analysis of EPDCs C0 at the MEM stage (Fig. 6F). The DEGs were enriched in cardiac EMT, smooth muscle differentiation, angiogenesis, and fat cell differentiation (Fig. 6F).

Discussion

The cardiac cellular heterogeneity may be caused by the spatiotemporal expression heterogeneity of genes among cardiac cell subsets (Galdos and Wu 2019). Asp et al. constructed a comprehensive transcriptional landscape of cell types during the developmental stages of the embryonic heart by combining three different technologies, spatial transcriptomics (ST), scRNA-seq, and in situ sequencing (ISS), and they investigated spatiotemporal gene expression patterns of the developing human heart at the cellular level (Asp et al. 2019). Therefore, the differential activation and repression of related genes were indispensable for cardiac cell development.

In the past 10 years, there have been several controversies about the gene Tbx18. The Tbx18+ epicardial cells used to be considered the progenitor pool except for the first and second heart field progenitor cells, but some researchers found defects in this view (Christoffels et al. 2009; Cai et al. 2008). There is no dispute about the vital role of Tbx18 in cardiovascular development (Ma et al. 2013). Recent studies have found that the Tbx18 gene can conduct human-induced pluripotent stem cells (hiPSCs) to differentiate into pacemaker-like cells, and spatiotemporal analysis showed that the Tbx18 expression was found in mural cells of both epicardial and non-epicardial origin (Dai and Weber 2018; Jing et al. 2016; Gorabi et al. 2019). Asp et al. (2019) found the Tbx18 expressed in the epicardial cells and EPDCs using the ISS. These findings suggested that the cardiac cells expressing Tbx18 have different cellular compositions.

This study investigated the transcriptional and temporal heterogeneity in developing Tbx18+ cardiac cells by scRNA-seq. We found that the Tbx18+ cardiac cells could be divided into at least two cell types with distinct gene expression signatures: fibroblast-like cells and cardiomyocytes. An in-depth study of two representative clusters compared the transcriptional profiles between cardiomyocytes and non-cardiomyocytes. The transcriptional heterogeneity was demonstrated to exist in the Tbx18+ cardiac cells during cardiogenesis, and the ECM and EMT genes were potentially involved in the process. The Tbx18+ cardiac cells' temporal heterogeneity was next studied, and the transcription factor network analysis was constructed. Dynamic changes in gene expression levels and transcriptional

regulatory networks contributed to the temporal heterogeneity of Tbx18+ cardiac cells. For example, the ECM and EMT genes showed increased expression in the late developmental stage of Tbx18+ cardiac cells. The lineage-tracing model showed that Tbx18 participated in heart cell differentiation into vascular smooth muscle cells, fibroblasts, adipocytes, and cardiomyocytes. The Tbx18+ cardiac cells exhibited the characteristics of epicardial progenitor cells during cardiogenesis, consistent with the previous study (Sanchez-Fernandez et al. 2022).

There are also several limitations to the present study. For example, although the original plan was to include as many datasets as possible, only three datasets met the criteria and were analyzed. Undoubtedly, more scRNA-seq datasets will improve the accuracy of current results. In addition, results in this study were obtained through integrated analysis of scRNA-seq data and lineage-tracing model; the identified temporal-specific genes during cardiogenesis need to be further validated.

Conclusion

In this paper, we provided a reference map of Tbx18+ cardiac cellular transcriptional states between E7.75 and P21 to understand better the temporal transcriptional heterogeneity and the properties of Tbx18+ cardiac cells in cardiovascular development. Moreover, these findings indicated that ECM and EMT signals potentially played a key role during Tbx18+ cardiac cell development and may also partially explain the regulatory mechanisms underlying heart cell differentiation.

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Availability of data and materials The datasets used and analyzed during the current study are available from the corresponding author Jianlin Du.

Declarations

Ethics approval and consent to participate All animal experiments were maintained according to the Regulation for the Administration of Affairs Concerning Experimental Animals (State Council of China, 2017 Revision), and our study was approved by the Commission of Chongqing Medical University for the Ethics of Animal Experiments.

Competing interests The authors declare no competing interests.

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