Articles

JNK1 downregulation confers fibroblasts with a mammary epithelial cell fate

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The fate of cells is fundamentally determined by their specific transcription patterns, which dictate cellular identity and differentiation pathways. Previously, in our research, we demonstrated that chemical cocktails, including the single small molecule TGFβR1 inhibitor RepSox(R), can induce significant changes in transcription patterns and drive the reprogramming of somatic cells into chemically induced mammary epithelial cells (R-iMECs) by effectively downregulating TGFβR1 expression. However, the underlying molecular mechanism of chemical induction for such reprogramming remains unclear and poorly defined, limiting its broader application. Here, to address this gap, we employed comprehensive single-cell sequencing (scRNA-seq and scATAC-seq), to systematically uncover RepSox-induced reprogramming events, map detailed molecular trajectories. Importantly, our integrated analysis revealed that JNK1 expression, a critical downstream gene in the TGFβR1 signaling pathway, plays an indispensable role in R-iMECs conversion. Thus, the TGFβR1-JNK1 signaling cascade may serve as a key regulator of fibroblast-to-mammary epithelial cell fate decision, orchestrating cellular transitions through transcriptional modulation. These findings provide valuable mechanistic insights into the complex processes governing mammaryepithelial cell fate decisions and may pave the way for developing novel, efficient strategies to generate large-scale, functional milk-secreting mammary cells in vitro, while also facilitating targeted mammary gland regeneration and repair in vivo for therapeutic advancements.

Introduction

During fertilization, oocytes fuse with sperm to form zygotes. Subsequently, they undergo division and differentiationto gener ate an embryo consisting of three germ layers, which eventual ly develops into the entire organism[1,2]. Hence, all specialized cells in an organism originate from a single cell. In most org -anisms, particularly mammals,embryonic cells irreversibly tran-sform into terminally differentiated cells that ultimately underg -o programmed cell death[1,3]. Although some stemcells exist i-n organisms and can differentiate into functional cells to com-pensate for cellular loss, these stem cells are scarce or beco-me significantly depleted over time[4]. The loss of self-regener-ation ability may serve as a protective mechanis against oncog -enicity since uncontrolled cell division could lead tocancer fo -rmation[5,6,7]. However, under pathological cond-itions where s-pecific tissues lose their functionality due to disease or injury, reprogramming terminally differentiated functional cells back i-nto undifferentiated state holds great pote-ntial for tissue regen -eration by enabling them to differentiatein to other normal fu -nctional cell types[8].

Previous study has demonstrated that somatic cells can be rep -rogrammed into induced pluripotent stem cells (iPSCs), which possess the ability to differentiate into various cell types deriv -ed from three germ layers[9].However, the utilization of iPSCs for obtaining terminally differentiated functional cells may pos -e potential oncogenic risks due to the challenge in ensuring c -omplete segregation between differentiated and undifferentiated iPSCs[10,11].To circumvent this issue, researchers have employed transdifferentiation, also known as direct lineage reprogrammin -g, to generate differentiated functional cell types directly fro-m somatic cells[12].Transdifferentiation offers a lower risk of o -ncogenicity since it does not involve the induction of pluripot -ency. Various functional cell types such as myoblasts[13], neur -ons[14,15], cardiomyocytes[16], neural stem/progenitor cells[17], an -d hepatocytes[18] have been successfully generated through tra-nsgene-mediated transdifferentiation in vitro using fibroblastsor other somatic cell sources. Unfortunately, this approach stillrai-ses concerns regarding oncogenicity due to the use of transge-nes[19]. In recent years, novel strategies involving chemical-me-diated transdifferentiation have effectively addressed some of t -hese concerns by enabling the successful generation of a wid -e range of induced cell types from somatic cells including in -duced neural stem cells[20,21],induced neurons[22,23], and induced cardiomyocytes[16] under chemical induction conditions.These ch -emically-induced cells hold promising clinicalapplications in ti -ssue regeneration and provide valuable insights into the mech -anisms underlying cellular fate conversion from somatic cells.

The mammary gland is a vital organ in mammals, playing a crucial role in milk secretion. Damage to the mammary gland can have severe consequences for mammals. Mammary epithel ial cells are capable of restoring the structure and functions of mammary glands both in vivo and in vitro, making them valu -able for various applications[24].Therefore, the generation of m -ammary epithelial cells from nonmammary cells holds great p -otential for both in vivo and in vitro purposes. Our previous studies have shown that small molecule cocktail induction or TGFβR1 inhibitor RepSox alone can induce nonmammary cell-s to acquire a mammary cell fate[8]. In this study, we conduct -ed a comprehensive investigation into the reprogramming eve-nts and tracked the conversion of chemically induced mammar -y epithelial cells (CiMECs) from somatic cells using single-ce -ll sequencing analysis, aiming to elucidate the similarities and differences between iMECs generated through chemical cocktai -ls induction or TGFβR1 inhibitor RepSox induction. Addition-ally,we discovered that TGFβR1 downregulation induces this r -eprogramming event through its regulatory effects on JNK1 d -ownregulation. These findings provide insights into the role o -f the TGFβR1-JNK1 signaling pathway in somatic cell reprog

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-ramming and decision-making processes related to mammary epithelial cell fate determination. Furthermore, they establish a novel technical platform for generating abundant functional ma -mmary epithelial cells in vitro, which could be applied to va -rious bioapplications and potentially contribute to mammalian gland regeneration in vivo.

Materials and methods

Cell culture

Goat ear fibroblasts (GEFs) were cultured in high-glucose DM -EM(GIBCO) supplemented with 10% fetal bovine serum (FB-S,GIBCO). Once the GEFs approached approximately 80%-90 % confluence, they were passaged. GEFs were seeded 24 hou -rs later, and the medium was replaced with R-induction medi -um (N2B27 and 10μM RepSox). The culture was continued f -or 8 days, and the induction medium was refreshed every 2d -ays.The specific experimental methods used were described pr -eviously[8].

Immunofluorescence staining

The procedure was performed according to Zhang's procedure[8]. The primary antibodies used were EpCAM(1:100), KRT19(1:200), ITGA6(1:100), and KRT8(1:1,000) at 4°C overnight.Th -e secondary antibodies used were (1:250) Alexa Fluor 488 D -onkey Anti-rabbit, Alexa Fluor 555 Donkey Anti-rabbit,and A -lexa Fluor 488 Donkey Anti-mouse for 1h in the dark at roo -m temperature.The nuclei were stained with Hoechst 33342 f-or 15 minutes.The antibody details are listed in the Key Reso -urces Table.

qRT–PCR

Total RNA was extracted using TRIzol reagent (Life Technolo -gies, 149112), and cDNA synthesis was performed using Sup -erMix (Vazyme, R223-01). The cDNA samples were subjecte-d to real-time quantitative PCR using a SYBR Green real-tim-e PCR Master Mix Kit (Vazyme, Q711-02). The sequences of primers used for qPCR in this study are listed in the Key Re -sources Table. The expression of the genes was normalized t-o that of the internal control gene GAPDH, and the fold chan -ges in the samples were calculated relative to those in the fi-broblasts.

Western Blot

Add lysis buffer (RIPA) containing 10% protease inhibitor(PM -SF) to the cell pellet in proportion. Lyse on ice for 20 minu -tes, and then centrifuge at 1200 rpm for 10 minutes at 4°C. Use the BCA detection kit to measure the protein concentratio -n. After preparing the separating gel and stacking gel, load t-he samples and perform electrophoresis. Transfer the proteins to the membrane,and then block with 5% non-fat milk at roo-m temperature for 1 hour (or overnight at 4°C). Dilute the pr -imary antibody in TBST according to the concentration reco-mmended by the manufacturer, and incubate at room temperat -ure for 2 hours. After washing with TBST, dilute the second -ary antibody according to the manufacturer's instructions, and incubate on a shaker at room temperature for 40 minutes. Fi -nally, after washing with TBST, place the membrane in the i -mager, add the substrate for imaging and recording.

Animal experiments

In vivo administration of R-CiMEC, followed by hematoxylin-eosin (HE) staining and immunohistochemical staining of paraf -fin sections (IHC-P), was conducted in accordance with estab -lished literature[8].

Statistical analysis

All the values are presented as the means ± SEMs. The value -s were considered to be significantly different at $p < 0.05$ ac -cording to two-tailed tests assuming equal variance.

Bulk RNA-seq bioinformatic analysis

For the bulk RNA-seq of RepSox reprogramming samples, a t -ranscriptome library was prepared following the instructions o -f the TruSeq RNA Sample Preparation Kit from Illumina® (San Diego, CA) using 1 μg of total RNA. The bulk RNA-seq data were analyzed on the Majorbio Cloud Platform. The Maj -orbio Cloud Platform (www.majorbio.com) was used for addit -ional details.

Single-cell multiome data generation

ScRNA-seq was performed on 0d, 4d and 8d cell samples usi ng a 10× Genomics system. Briefly, dissociated cells (~10,000 cells per sample) were loaded into a 10× Genomics Chromiu m Single Cell system using Chromium Single Cell 3' Reagent Kits v3.1 (10× Genomics, Pleasanton, CA). ScRNA libraries were generated by following the manufacturer's instructions. T -he libraries were pooled and sequenced on an Illumina Nova-Seq 6000. The sequencing reads were processed through the Cell Ranger 4.0.0 pipeline (10× Genomics) using the default p -arameters.

ScATAC-seq was performed on 0d, 4d and 8d cell using a 1 0× Genomic Single Cell ATAC Reagent v1.1 Kit following th -e manufacturer's instructions. The libraries were pooled and s -equenced on Illumina NovaSeq 6000. The sequencing data w-ere processed through the Cell Ranger ATAC 1.1.0 pipeline (10x Genomics) using the default parameters.

Single-Cell RNA-Sequencing (scRNA-seq) Data Analysis

Single-Cell RNA-Seq Data Pretreatment

Preprocessing of scRNA-seq data of R-0d,R-4d and R-8d was performed using the Seurat package in R4.4.1. This included r -emoving low-quality cells, normalizing read counts, and select -ing highly variable genes.

Dimensionality Reduction and Clustering

UMAP and t-SNE algorithms from the Seurat package were u -sed for dimensionality reduction of single-cell data to uncover potential cell population structures.

Automatic Cell-Type Annotation

The SingleR package was used for automatic annotation of ce -ll clusters. By comparing gene expression traits of unknown cell populations with reference datasets, specific cell type labe -ls were assigned to each cell subpopulation.

Functional Enrichment Analysis

GO/KEGG enrichment analyses of the target gene set were pe rformed using the clusterProfiler package in R4.4.1.

Pseudotime analysis

Pseudo-time ordering of the cell populations was performed us -ing the monocle 3 package with default parameters.

Single-CellATAC-Sequencing (scATAC-seq) Da ta Analysis

For theATAC data, All the analyses (UMAP dimension reduct -ion, cluster identification, and identification of differentially a-ccessible regions) were performed in Signac and the default p -arameter settings were used to construct cell trajectories with Monocle 3.

Results

The transcriptional dynamics of induced mammary epithelial cells generated by the TGFβR1 inhibitor RepSox induction.

Our previous studies[8] have demonstrated that BFRTV cocktail (1 μM TTNPB (B), 10 μM forskolin (F), 10 μM RepSox (R), 10 μM tranylcypromine (T) and 500 μg/ml VPA (V)) or Rep-Sox alone all can induce the conversion of fibroblasts into in-duced mammary epithelial cells (referred to as BFRTV-CiME Cs or R-CiMECs). However, it remains unclear how the trans -criptional profiles of cell fates change under RepSox inductio -n. To address this question, we collected cells at different ti-me points (0, 1, 2, 3, 4, 6, and 8 days post-induction) for bu -lk RNA sequencing analysis. Firstly,the correlation coefficient graph (Figure 1A) revealed significant differences between day 0 and other time points. Furthermore, during the induction pro -cess, fibroblast marker genes (FBN1/VIM/TWIST2) were cons -istently downregulated while mammary epithelial cell marker genes (CDH1/EPCAM et al.), hormone-related gene PRLR and milk fat synthesis-related gene DGAT2 showed gradual upregu -lation over time (Figure 1B).

We subsequently conducted a comprehensive analysis of transc -riptional trends during the transdifferentiation process using fu -zzy clustering. In total, we identified four distinct gene subse -ts that exhibited a consistent expression pattern under specific conditions (Figure 1C). These findings indicate a progressive downregulation of somatic programs followed by an upregulati -on of genes involved in embryonic development as well as activation of programs related to epithelial cell proliferation an -d mammary epithelial cell development. Notably, genes associ -ated with cell cycle regulation and epigenetics exhibited a flu -ctuating pattern of change over time (Figure 1D-E). Moreover, GSEA plot analysis also demonstrated that compared to day-0 samples,the mammary epithelial cell lineage fate was activated in the day-8 sample (Figure 1F). Therefore, this comprehensiv -e transcription profile data provides evidence that R-CiMECs exhibit typical characteristics associated with mammary epitheli -al cell fate.

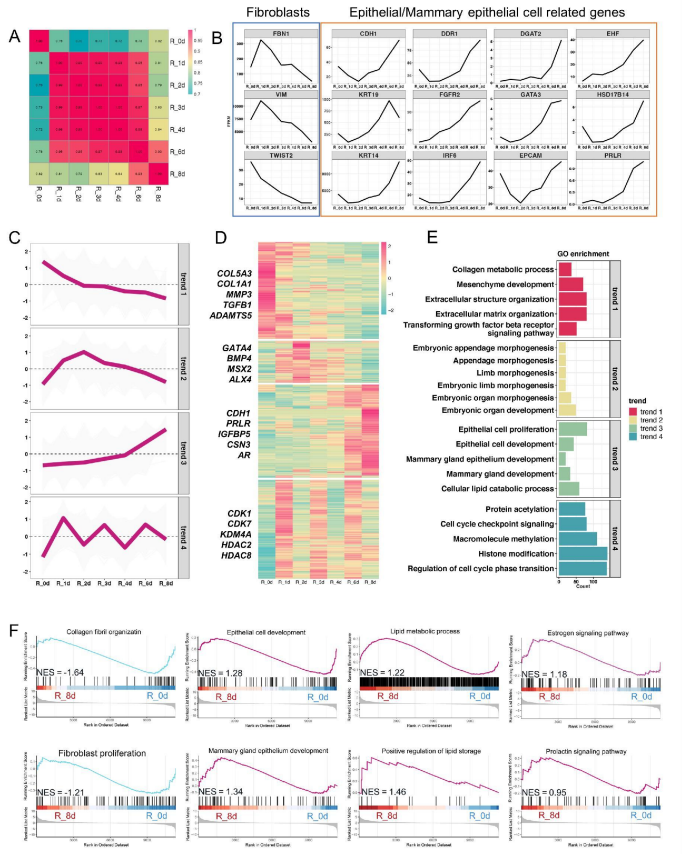

Figure1 The transcriptional dynamics of induced mammary epithelial cells generated by the TGFβR1 inhibitor RepSox induction. A.Heatmap illustr ati -ng the correlation coefficients of gene expression among R-0d,R-1d,R-2d,R-3d, R-4d,R-6d,andR-8d in the mRNA-seq dataset. B.Line chart depicting the expre -ssion patterns of fibroblast and epithelial/ma-mmary epithelial cell-related mar-ker genes obtained from bulk mRNA-seq analysis of sam-ples collected at diff -erent time points(R-0d,R-1d,R-2d,R-3d,R-4d,R6-d and 8-d). C.Fuzzy clustering approach applied to bulk RNA-seq data for investigating gene expression dyna -mics during specific time periods under defined conditions. D.Heatmap displa-ying relative gene expression levels across different conditions and time point-s with each gene group associated with a corresponding trend as described in(C). E.Gene Ontology(GO)analysis revealing enriched terms linked to each iden tified trend. F.Gene Set Enrichment Analysis(GSEA)demonstrating differential e xpression of lactation-related signaling pathways between cells collected at day 8(R-8d)and day 0(R-0d).

The progression of R-CiMEC fate acquisition and reprogramming is revealed through single-cell sequencing analyses.

To further investigate the reprogramming trajectories of R-CiM ECs, we performed scRNA-seq and scATAC-seq analyses. The analysis results revealed that the R-0d, R- 4d, and R-8d cells were segregated into seven distinct clusters (Figure 2A-B) , a s well as the cell numbers of each sample and cluster (Figure 2C). The comparison of GO and KEGG between day 4 and day 0 showed that the pathways related to the fate of mamm ary epithelial cells were activated (Figure 2D-E). The compari son of GO and KEGG between day 8 and day 0 showed that not only the pathways related to the fate of mammary epithe lial cells were activated, but also the pathways related to lipid synthesis and protein secretion were activated (Figure 2F-G). Furthermore, the scATAC-seq results further prove d that the day 8 cells had obvious characteristics of mammary epithelial cells (Figure 2H-I). At the same time, GO analysis was performed on 7 clusters to define the identity of each cl

uster (Figure 3A-G), as well as the UMAP display of marker genes of each cluster (Figure 3H).

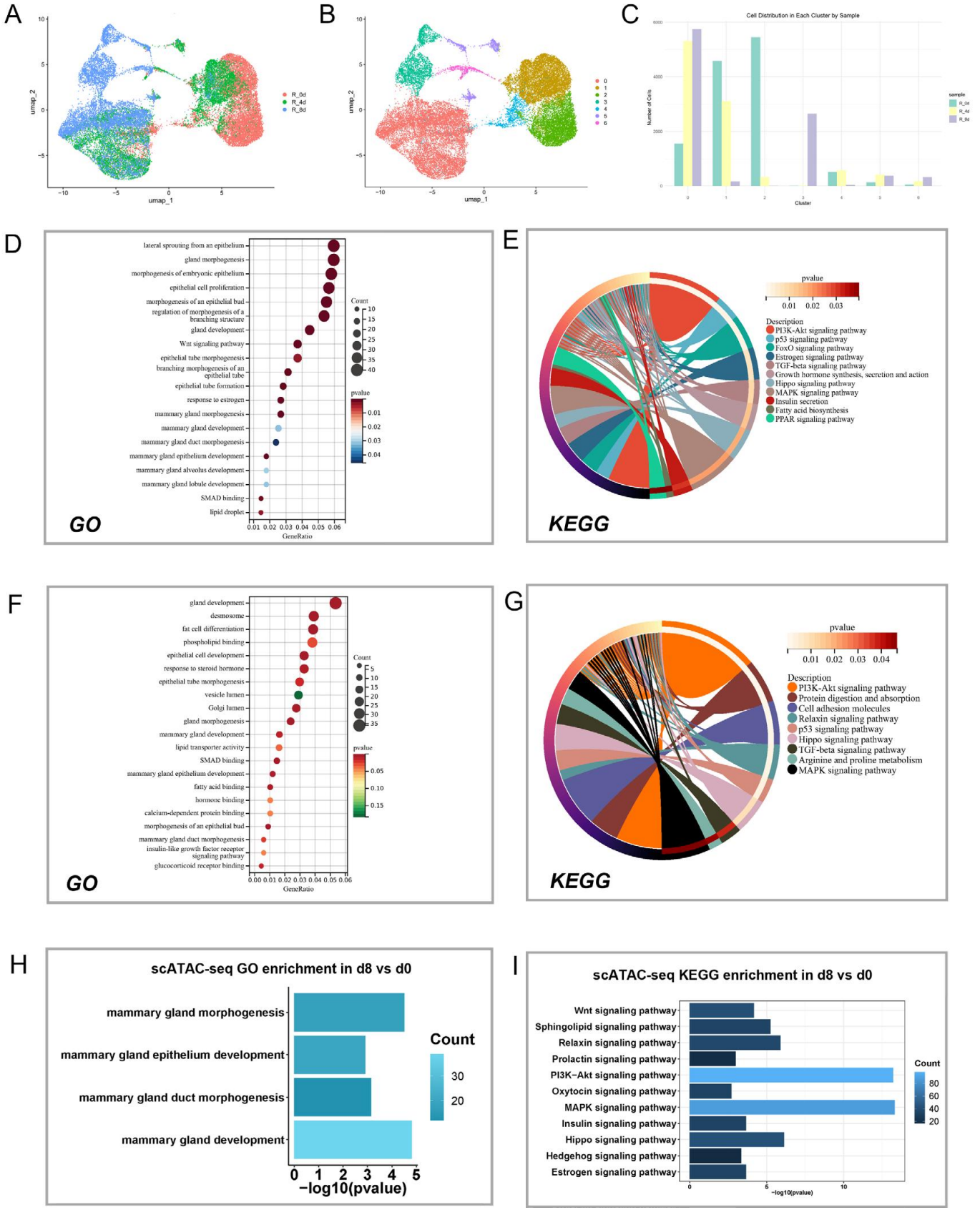

Figure 2 Reprogramming of fibroblasts into mammary epithelial cells examined by single cell sequencing analysis. A. UMAP visualization illustrating the distribution of samples among groups (R-0d, R-4d, R-8d) in scRNA-seq data. B. The UMAP plot shows that the samples can be divided into seven different clusters. C. Bar chart shows the cell distribution in each cluster by sample. D. GO enrichment analysis of differentially expressed genes (DEGs) between 0d and 4d in scRNA-seq data. E. KEGG enrichment analysis of differentially expressed genes between 0d and 4d in scRNA-seq data. F. GO enrichment analysis of differentially expressed genes between 0d and 8d in scRNA-seq data. G. KEGG enrichment analysis of differentially expressed genes between 0d and 8d in scRNA-seq data. H. GO enrichment analysis of differentially expressed genes between 0d and 8d in scATAC-seq data. I. KEGG enrichment analysis of differentially expressed genes between 0d and 8d in scATAC-seq data.

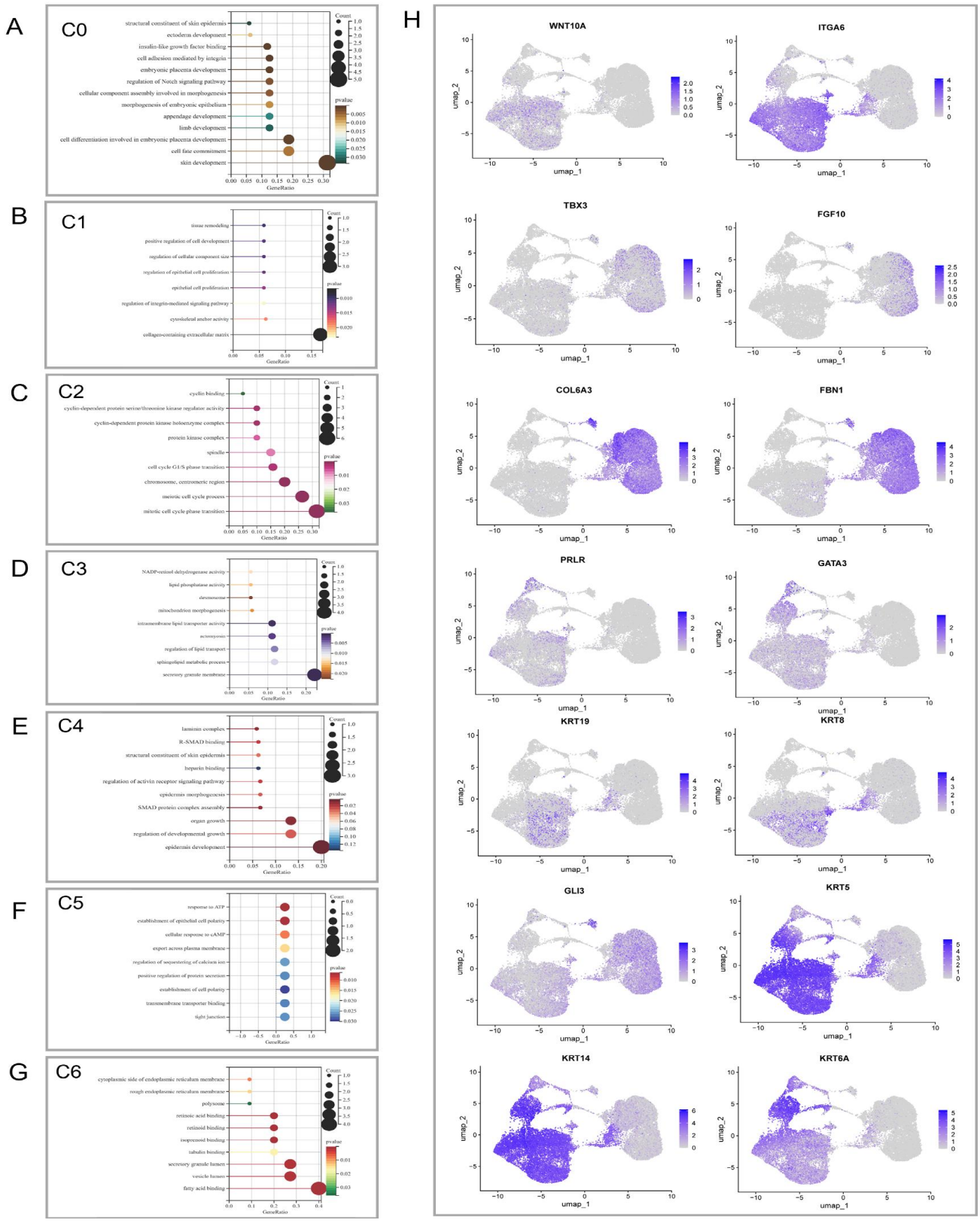

Figure 3 Identification of the identities of each cluster in scRNA-seq data and presentation of the expression of marker genes. A-G: GO enrichment of 7 different clusters (C0, C1, C2, C3, C4, C5, C6) in scRAN- seq. H: UMAP plot depicting expression patterns offibroblasts, development-related genes, and mammary genes across various clusters.

Furthermore, we reconstructed the transdifferentiation trajectory of R-CiMECs. The cells were ordered and color-coded based on t-heir predicted pseudotime (Figure 4A). Additionally, the genes were further categorized and clustered into three distinct modules a -ccording to their coexpression levels (Figure 4B). Module 1 (in red) exhibited enrichment in fibroblast gene sets,including FBN1, COL1A1, and COL6A3,with significant involvement in "extracellular structure organization" and "extracellular matrix organization" GO terms (Figure 4C-D). In Module 2 (in green), there was an enrichment of GO terms related to "ATP biosynthetic process,""n cRNA processing," and "protein folding." This suggests that the ongoing transdifferentiation process may be actively regulated at t -he transcriptional level (Figure 4C). Furthermore, expression levels of early embryonic development-and epithelial-related genes su

-ch as GLI3, KRT14 and ITGA6 significantly increased over time (Figure 4D). In Module 3 (in blue), GO terms associated with "hormone-mediated signaling pathway" and "mammary gland epithelium development" were enriched. Notably, representative genes like ELF5, PRLR, and DGAT2 showed increasing expression along the pseudotime trajectory (Figure 4C-D), indicating a transitio-n towards mammary cell lineage differentiation. Moreover, scATAC data revealed a decrease in activity for fibroblast marker gen-es COL6A1, MAPK8, and MAPK9 with increasing induction days (Figure 4E-F), while markers for epithelial development (GAT-A4, OVOl, KRT5) and lactation-related genes (ELF5,XDH,DGAT2) gradually became more activ e(Figure 4G-H). Collectively, the -se findings provide insights into the sequential molecular progression underlying successful reprogramming offibroblasts into mam -mary epithelial cells.

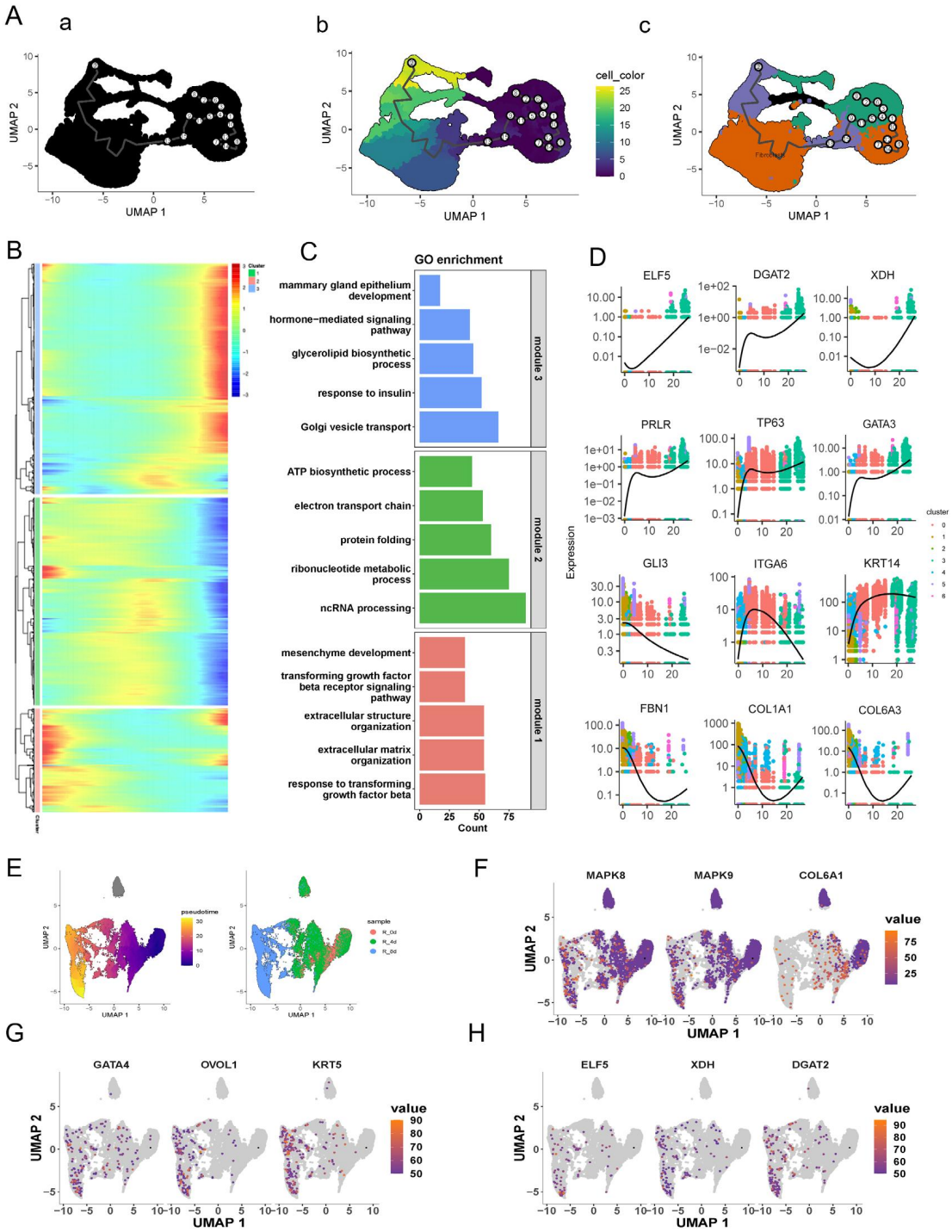

Figure 4 Reconstruction of the RepSox-induced reprogramming trajectory in a pseudotime manner was performed to elucidate the temporal progression of cellular transformations. A.scRNA-seq data reveals trajectory reconstruction at three time points in a sample of single cells.a,Trajectory plot colored by Seurat clust -er (starting point:cluster 1), and the gray lines represent the cell differentiation trajectories constructed by Monocle3.b, Trajectory plot colored by pseudotime.c, Traj-ectory plot colored by manually annotated cell types. B.A heatmap illustrates the expression patterns of pseudotime-dependent genes along the inferred pseudotime a-xis, which have been hierarchically categorized into three modules. C.The gene modules show enrichment for representative Gene Ontology terms. D.The dynamic e-xpression trends offibroblast,development and differentiation,and mammary epithelial marker genes during the cell differentiation process(pseudotime).The horizontal ax -is (pseudotime) represents the time course of cell differentiation (calculated by Monocle3), with larger values indicating that cells are closer to the end of differenti -ation. The vertical axis represents gene expression levels. E.Pseudotime analysis of scATAC-seq data (R-0d,R-4d,and R-8d samples) throughout RepSox-induced repr -ogramming is presented on the left side, while a Uniform Manifold Approximation and Projection (UMAP) plot shows sequential changes during this process on th -e right side. F.UMAP visualization demonstrates fibroblast gene activity in scATAC-seq data during sequential changes. G.UMAP visualization depicts epithelial cell gene activity in scATAC-seq data during sequential changes.H.UMAP visualization showcases mammary gene activity in scATAC-seq data during sequential changes.

The regenerative function and reprogramming trend of R-CiMECs exhibit similarities to those of BFRTV-CiMECs.

To further elucidate the similarities and discrepancies between R-CiMECs and our previously reported BFRTV-CiMECs[8], we conducted additional investigations to address these pertinent issues. First, we confirmed the regenerative capacity of R-CiMECs in vi -vo. The results demonstrated that hematoxylin and eosin staining clearly revealed well-defined acinar structures (Figure S1A). Sta -ining with goat-specific casein antibodies (CSN2 and CSN3) revealed positive results in the structures of mammary glands derive -d from transplanted R-CiMECs; however, negative staining was observed in paraffin sections from untreated nude mouse mamma -ry glands which confirmed the specificity of these antibodies for detecting goat antigens (Figure S1B). These findings strongly su -ggest that similar to BFRTV-CiMECs', R-CiMCEs are capable of forming functional mammary gland structures.

Subsequently, we conducted a comparative analysis of gene expression patterns revealed by mRNA-seq data between R-CiMECs a -nd BFRTV-CiMECs. The hierarchical clustering divided RepSox- and BFRTV-treated cells into two distinct groups, both of whic -h exhibited transcriptional differences from the parent R-0d/BFRTV-0d (GEFs) (Figure 5A). However, both induction environment -s demonstrated that as the induction time increased,the expression of fibroblast marker genes gradually decreased while genes pro -moting mammary epithelial cell formation and lactation gradually increased (Figure 5B and S2A). The fuzzy clustering analysis o -f gene transcription trends during transdifferentiation also revealed that R-CiMECs (Figure S2B) exhibited similar transcriptional p -rofiles to BFRTV-CiMECs (Figure S2C), with the distinction being that R-induced reprogramming processes, cell cycles, and epi-genetic signaling were more actively engaged.

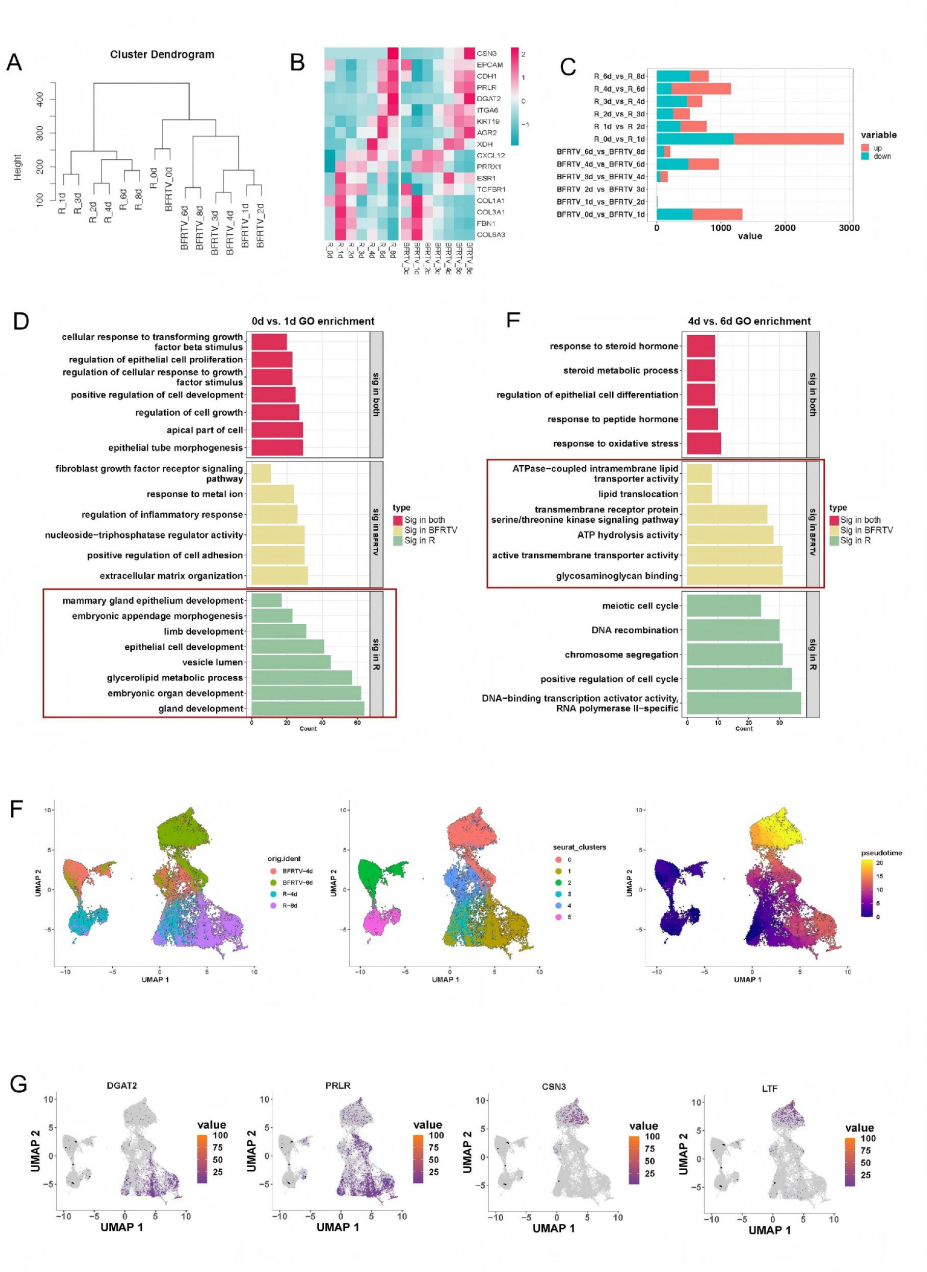

Figure 5 Discrepancies in the maturation status between R-CiMECs and BFRTV-CiMECs. A.A hierarchical clustering analysis was performed on RepSox- and BFRTV-treated cells using unsupervised methods in bulk mRNA-seq data. B.The expression of fibroblast genes and mammary genes in these cells was visualized thr -ough a heatmap in bulk mRNA-seq data. C.Bar plot illustrating the differential expression of genes (DEGs) associated with the R-induced environment and BFRTV -induced environment in bulk mRNA-seq data. D.The GO enrichment map was generated to identify differentially expressed genes (DEGs) between R and BFRTV(5 i)on the first day of induction in bulk mRNA-seq data. E.The GO enrichment map was generated to identify differentially expressed genes (DEGs) between R and BFRTV (5i) after 4 days of induction in bulk mRNA-seq data. F.UMAP visualization demonstrates the clustering(left and middle panels)and pseudotime distribution(right panel) of the combined scRNA-seq data for R-CiMECs-4d, R-CiMECs-8d, BFRTV-CiMECs-4d, and BFRTV-CiMECs-8d. G.The UMPA illustrates the spatial di

-stribution of gene expression related to lactation (DGAT2,PRLR,CSN3 and LTF) of the combined scRNA-seq data for R-CiMECs-4d, R-CiMECs-8d, BFRTV-CiMECs-4d, and BFRTV-CiMECs-8d.

Comparing the differentially expressed genes between two consecutive days, it was observed that the up-and down-regulated genes at 0d vs 1d and 4d vs 6d exhibited the most pronounced changes under both induction environments (Figure 5C). Further analysi -s revealed a significant enrichment of genes and items associated with embryonic development and mammary epithelial cell devel -opment during the early stage of transdifferentiation (0d vs 1d) induced by RepSox (R) (Figure 5D and Figure S2D-E). In contr-ast,in the middle and late stages of transdifferentiation (4d vs 6d), there was a notable enrichment of signals promoting lactation,s -uch as lipid droplet transport and ATP utilization,in the BFRTV-induced environment (Figure 5E). The single cell transcription d-ata of R-CiMECs-4d, R-CiMECs-8d, BFRTV-CiMECs-4d and BFRTV-CiMECs-8d were integrated and analyzed, and it was found that the early lactation genes DGAT2 and PRLR were highly expressed under RepSox induction system.The key genes of milk pr -otein LTF and CSN3 were highly expressed in BFRTV induction system (Figure 5F-G). These findings suggest that RepSox-indu -ced transdifferentiation facilitates rapid epithelialization and entry into the cellular plasticity stage and that BFRTV-CiMECs repre-sent mature mammary epithelial cells that are more closely associated with the lactation stage.The discrepancy may be attributed t -o the heightened activity of epigenetic genes in R-CiMECs (Figure S2F).

The JNK signaling pathway governs the determination of mammary epithelial cell fate.

To further explore the downstream molecular mechanisms following repsox treatment,we analyzed the bulk RNA-seq data from th-e early induction stage. The results revealed a transient EMT-MET process on the first day of induction (Figure 6 A-B). Quantita -tive PCR results also confirmed this finding (Figure 6C). The differentially expressed genes between day 0 and day 1 were enric -hed in the MAPK and JNK signaling pathways (Figure 6D). We hypothesized that the downregulation of TGFβR1 may modulate the JNK/MAPK signaling pathway to regulate the reprogramming process. To investigate this regulatory mechanism, we conducted further experiments based on our previous single-cell sequencing analyses. Surprisingly, screening for small molecule compounds t -argeting the JNK/MAPK signaling pathway revealed that SP600125(SP), a specific inhibitor of JNK1/MAPK8,induced goat ear fi-broblasts (GEFs) to form epithelial cell colonies similar to R-CiMECs (Figure 7A-B). Additionally, we performed knockout experi -ments on JNK1/MAPK8 to determine its effects on reprogramming. The results demonstrated that downregulation of MAPK8 can induce iMEC generation (Figure 7C). The immunofluorescence staining results revealed that SP600125-induced mammary epithelial cells (SP600125-8d cells) and KO-MAPK8-GEFs-8d cells exhibited a similar expression pattern to R-8d cells (positive control), de -monstrating the presence of epithelium-specific marker antigens EpCAM,ITGA6, KRT8, and KRT19 (Figure 7D).The WB (Figure 7E) and qRT-PCR (Figure 7F) results further confirmed that the inhibition of TGFβR1 or JNK1/MAPK8 significantly increased th -e expression levels of genes related to mammary gland development, differentiation and lactation (TP53,LTF,CSN2,DGAT2).These findings suggest that inhibiting JNK1 is crucial for converting nonmammary-derived stromal fibroblasts into mammary epithelial ce -lls and indicate a potential role for the TGFβR1-JNK1 signaling pathway in mediating this reprogramming event.

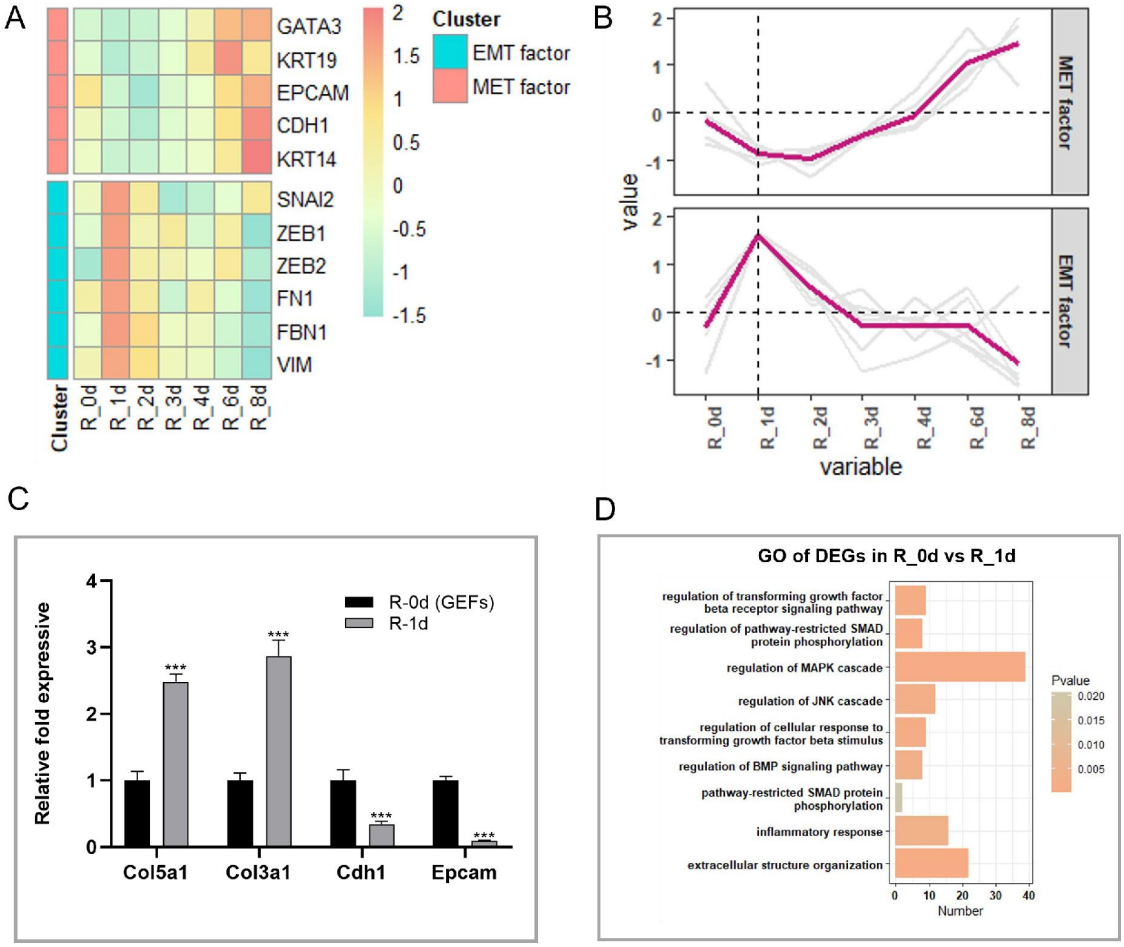

Figure 6 Identification of key nodes in the RepSox-induced reprogramming process through bulkRNA-seq data analysis. A.Heat map of representative genes showing a sequential EMT-MET during the chemical conversion process. B.The corresponding expression dynamic of EMT- and MET-related genes at the indicated

time points during chemical reprogramming. Red lines indicate the average gene expression patterns in each cluster. C.qRT-PCR results show the expression of stromal and epithelial cell marker genes. (mean ± SEM, n=3 biological replicates, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA). D.GO analyses of DEGs i -n d1.

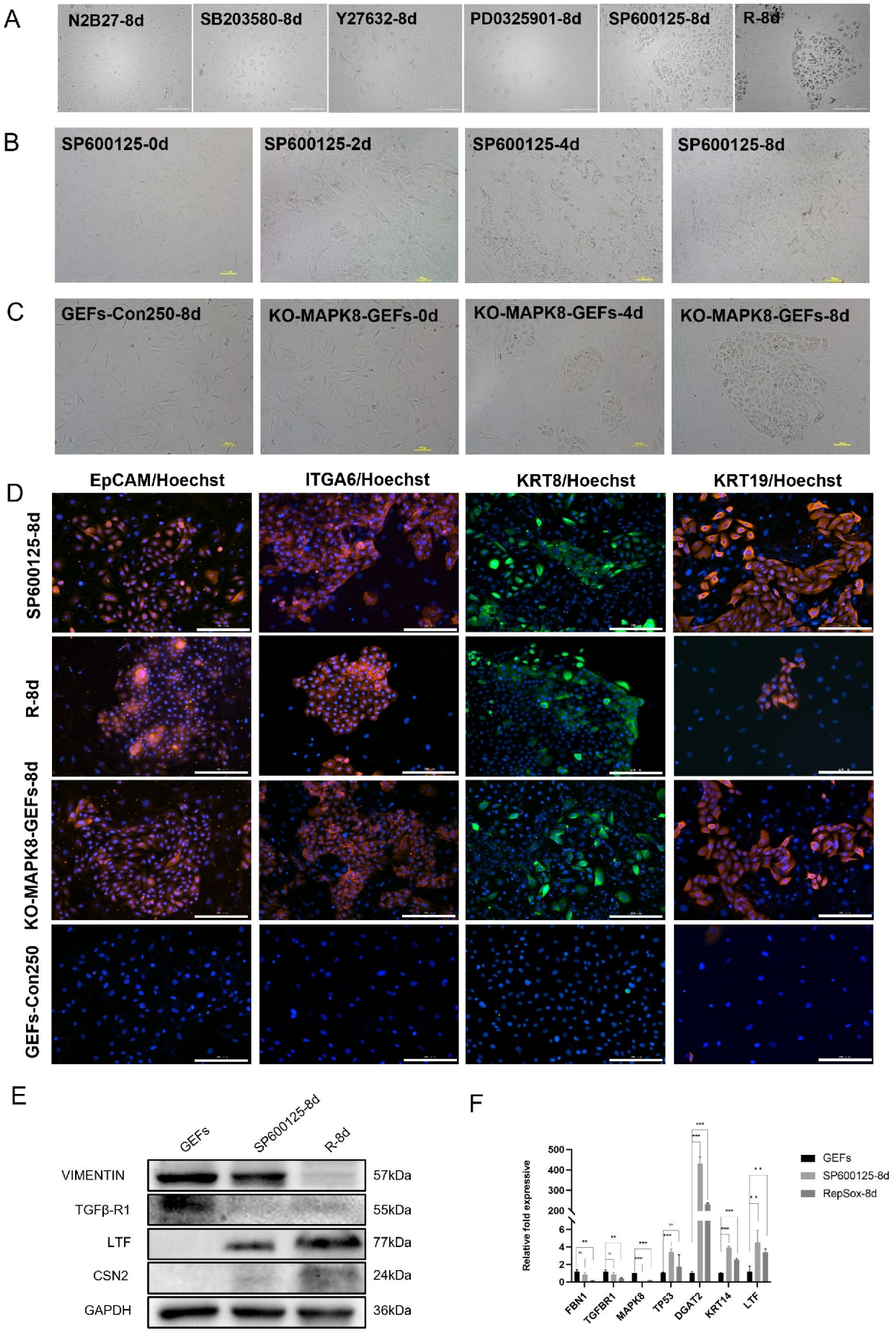

Figure 7 The downregulation of JNK1/MAPK8 gene expression plays a crucial role in the formation of induced mammary epithelial cells.A.Reprogramming c -ellular morphology through the application of diverse small molecule compounds to regulate JNK/MAPK8 signaling.Scale bars, 200μm. B.The cellular morphology o -f mammary epithelial cell formation induced by SP600125 at different time points is illustrated.Scale bar, 100μm. C.The morphological landscape of induced mamm -ary epithelial cell formation triggered by MAPK8 knockout was examined. GEF-Con250-8d cells were utilized as negative controls.Scale bar, 100μm. D.Immunofluo -rescence was performed to assess the expression of MEC marker proteins (EpCAM,ITGA6,KRT8,and KRT19) in SP600125-8d cells and KO-MAPK8-GEFs-8d cells. GEFs-Con250-8d cells were utilized as negative controls, while R-8d cells served as positive controls.Scale bar, 200μm. E.Western blot results showed the expressio-

n changes of fibroblast marker proteins and lactation-related proteins in cell samples of cells treated with GEFs,SP600125-8d and R-8d. F.qRT–PCR analysis was co -nducted to investigate alterations in the expression of fibroblast-related marker genes and MEC marker genes in samples from GEFs, SP600125-8d cells, and RepSo -x-8d cells. Three biological replicates were included for each group. The data are presented as means±SEMs with statistical significance denoted by**p<0.01,***p<0. 001 based on one-way ANOVA.

Discussion

Transdifferentiation offers an alternative approach to derive var -ious functional cell types from somatic cells and holds great potential in clinical applications for tissue regeneration[25]. Prev -iously, we demonstrated that the downregulation ofTGFβR1 c -an induce the conversion of fibroblasts into mammary epitheli -al cells[8]. In this study, we further elucidate the mechanism by which goat ear fibroblasts (GEFs) can be reprogrammed in to milk-secreting mammary epithelial cells through the suppres -sion of TGFβR1 expression. Additionally, we establish an eff -icient platform for generating a large number of induced ma-mmary epithelial cells in vitro. Given that goats possess two breasts similar to humans, they serve as suitable model animal -s for investigating human mammarygland development and re -generation. Moreover, goats are commonly utilized as mamma -ry bioreactors for producing valuable recombinant human prot -eins used in disease treatment. Therefore, our findings may p -ave the way for a novel strategy to generate abundant functi-onal mammary epithelial cells and mammary bioreactors capab -le of producing "culture dish milk" and recombinant proteins at a potentially lower cost compared to traditional methods.

Moreover, based on the results of single-cell sequencing analy -sis, we investigated reprogramming events and tracks. Our fin -dings revealed that RepSox induction showed a trend similar to our previously reported small-molecule cocktail induction m ethod. The results indicated that the small molecule cocktail (BFRTV) erased somatic cell epigenetic memory and subsequen -tly induced cellsto enter the mammary epithelial cell fate. Ho -wever, R induction directly induced somatic cells to enter the mammary epithelial ce-ll fate without significant signals for er -asing epigenetic memory. Our previous studies[8] have shown that BFTV functions includeepigenetic modification and promo -tion of mammary development. Therefore, under R induction, epigenetic barriers may force som-atic cell reprogramming to r -esemble a "wavy" transcriptional wave which requires additio-nal time for induced cells under R induction to reach lactatio-n functionality. These findings indicate that TGFβR1 inhibitor RepSox plays an indispensable role in cocktails and further e-mphasize downregulating TGFβR1 as key in mammary epithel -ial cell fate decisions while other small moleculesin cocktails(BFTV) may facilitate smoother reprogramming progress leadin-g to faster secretion stages.

Importantly, our findings demonstrate that the downregulation of TGFβR1 may exert regulatory effects by suppressing JNK1 /MAPK8 signaling, suggesting that the TGFβR1-JNK1 pathway mediates these reprogramming events. JNKs play crucial role in processes such as proliferation, migration, apoptosis, and embryonic development[26]. Previous studies have shown that J -NK negatively influences somatic cell reprogramming and its downregulation can enhance reprogramming efficiency[27]. In th -is study, we discover-ed that the downregulation of JNK1 fol -lowing TGFβR1 downregulation directly initiates somatic cell reprogramming rather than merely promoting it. Recent resear -ch indicates[28] that the JNK pathway acts as a major obstacl-e to chemical reprogramming. Suppression of JNK is essentia-l for inducing cellular plasticity and a regeneration-like progr-am by inhibiting proinflammatory pathways. During the induct -ion of human cells into pluripotent stem cells using combinat -ion small molecule compounds, researchers[29] foundthat the c-Jun N-terminal kinase inhibitor (JNKIN8) is a critical small m -olecule capable of inducing plasticity characteristics andactivat -ing SALL4 expression along with genes related to embryonic development. Furthermore, addition of proinflammatory factors TNF-αand IL-1βimpairs the generation of plasticity features, fu -rther confirming that the JNK pathway serves as a significant barrier in recreating plasticity in human somatic cells through regulation of inflammation-related pathways.

In summary, our findings demonstrate that the suppression of the TGFβR1-JNK1 signaling plays a crucial role in facilitating the entire reprogramming process. Chemical reprogramming lea -ds to the transformation from fibroblast characteristics to ma-mmary epithelial cell characteristics, providing valuable insight -s into fate determination of mammary epithelial cells. Therefo -re, our study notonly sheds light on the novel regulatory effe -cts of the TGFβR1-JNK1 signaling pathway in somatic cell r -eprogramming but also establishes an alternative research plat -form for investigating mammary gland development and regen -eration.

Reference

1. Daley GQ(2015)Stem cells and the evolving notion o -f ellular identity.Philos Trans R Soc Lond B Biol Sci 370:20140376.
2. Valcourt JR,Huang RE,Kundu S,Venkatasubramanian D,Kingston RE,et al.(2021)Modulating mesendoderm co mpeten-ce during human germ layer differentiation.Cell Rep 37:109990.
3. Morris SA,Daley GQ(2013)A blueprint for engineering c-ell fate:current technologies to reprogram cell identity. Cell Res 23:33-48.
4. Bacakova L,Zarubova J,Travnickova M,Musilkova J,Pa -jorova J,et al.(2018)Stem cells:their source,potency a-nd use in regenerative therapies with focus on adip-osederivedstem cells a review.Biotechnol Adv 36:1111-1126.
5. Ahmad A,Ahsan H(2020)Ras-Mediated Activation of N F-kappaB and DNA Damage Response in Carcinogenes -is.Can-cer Invest 38:185-208.
6. Qayum A,Shah SM,Singh SK(2022)Divergent Signalin -g Pathways May Lead to Convergence in Cancer The -rapy-A Review.Cell Physiol Biochem 56:180-208.
7. Zhang C,Cao S,Toole BP,Xu Y(2015)Cancer may be a pathway to cell survival under persistent hypoxia and elevated ROS:a model for solid-cancer initiation and early development.Int J Cancer 136:2001-2011.
8. Zhang D,Wang G,Qin L,Liu Q,Zhu S,et al.(2021) Restoring mammary gland structures and functions wit -h autogenous cell therapy.Biomaterials 277:121075.
9. Malik N,Rao MS(2013)A review of the methods for human iPSC derivation.Methods Mol Biol 997:23-33.
10. Scesa G,Adami R,Bottai D(2021)iPSC Preparation a -nd Epigenetic Memory:Does the Tissue Origin Matte -r?Cell-s 10.

11. Tan Y,Ooi S,Wang L(2014)Immunogenicity and tumor -igenicity of pluripotent stem cells and their derivatives: geneticand epigenetic perspectives.Curr Stem Cell Res Ther 9:63-72.

12. Chambers SM,Studer L(2011)Cell fate plug and play:d -irect reprogramming and induced pluripotency.Cell 145: 827-830.

13. Abdel-RaoufK,Rezgui R,Stefanini C,Teo J,Christoforou N (2021)Transdifferentiation of Human Fibroblasts into Skeletal Muscle Cells:Optimization and Assembly int -o Engineered Tissue Constructs through Biological Li gands.Biology(Basel)10.

14. Han F,Liu Y,Huang J,Zhang X,Wei C(2021)Current A pproaches and Molecular Mechanisms for Directly Re-programming Fibroblasts Into Neurons and Dopamine Neurons.Front Aging Neurosci 13:738529.

15. Wapinski OL,Vierbuchen T,Qu K,Lee QY,Chanda S,et al.(2013)Hierarchical mechanisms for direct reprogram ming of fibroblasts to neurons.Cell 155:621-635.

16. Cao N,Huang Y,Zheng J,Spencer CI,Zhang Y,et al.(20 16)Conversion of human fibroblasts into functional cardi -omyocytes by small molecules.Science 352:1216-1220 .

17. Kim J,Efe JA,Zhu S,Talantova M,Yuan X,et al.(2011) Direct reprogramming of mouse fibroblasts to neural pro genitors.Proc Natl Acad Sci U S A 108:7838-7843.

18. Chen YF,Tseng CY,Wang HW,Kuo HC,Yang VW,et al.(2012)Rapid generation of mature hepatocyte-like c ells from human induced pluripotent stem cells by an efficient three-step protocol.Hepatology 55:1193-12 03.

19. Scesa G,Adami R,Bottai D(2021)iPSC Preparation an -d Epigenetic Memory:Does the Tissue Origin Matter? Cells10.

20. Han YC,Lim Y,Duffieldl MD,Li H,Liu J,et al.(2016)Direct Reprogramming of Mouse Fibroblasts to Neural Stem C-ells bySmall Molecules.Stem Cells Int 2016:4304916.

21. Wang YY,Sun TT,Yang P,Xu JJ,Liang Y,et al.(2022)[V CR,a Small Molecule Compound,Induces Reprogram-ming of Rat Fibroblasts into Neural Progenitor C-ells under Hypoxic Condition].Sichuan Da Xue Xue B -ao Yi Xue Ban 53:790-797.

22. Qin H,Zhao AD,Sun ML,Ma K,Fu XB(2020)Direct conversion of human fibroblasts into dopaminergic neuron-like cells using small molecules and protein fa -ctors.Mil Med Res 7:52.

23. Zhao AD,Qin H,Sun ML,Ma K,Fu XB(2020)Efficient an-d rapid conversion of human astrocytes and ALS mous -e model spinal cord astrocytes into motor neuronli -ke cells bydefined small molecules.Mil Med Res 7:4 2.

24. Macias H,Hinck L(2012)Mammary gland development. Wiley Interdiscip Rev Dev Biol 1:533-557.

25. Xie X,Fu Y,Liu J(2017)Chemical reprogramming and tr -ansdifferentiation.Curr Opin Genet Dev 46:104-113.

26. 26.Semba T,Sammons R,Wang X,Xie X,Dalby KN,et al. (2020)JNK Signaling in Stem Cell Self-Renewal and D -ifferentiation.Int J Mol Sci 21.

27. Yao K,Ki MO,Chen H,Cho YY,Kim SH,et al.(2014)JN-K1 and 2 play a negative role in reprogramming to pl-uripotent stem cells by suppressing Klf4 activit -y.Stem Cell Res 12:139-152.

28. Guan J,Wang G,Wang J,Zhang Z,Fu Y,et al.(2022)Che-mical reprogramming of human somatic cells to pluripo -tent stem cells.Nature 605:325-331.

29. Guan J,Wang G,Wang J,Zhang Z,Fu Y,et al.(2022)Che-mi-cal reprogramming of human somatic cells to plurip otent s-tem cells.Nature 605:325-331.

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Authors' contributions

D.D.Z.developed the chemical induction protocol, performed the e -xperiments, analyzed the data, and wrote and revised the manusc -ript. L.S.Q.contributed to the bulk RNA-seq sequencing data anal -ysis. Q.H.L.contributed to animal experiment. S.D.and L.F.S.contr -ibuted to the cell culture. Z.G.L.and H.P.contributed to molecular biology experiments. Z.Q.W and X.Y. revised the manuscript. G. D.W analyzed single-cell data and revised the manuscript. B.H.co-nceptualized the study, supervised the entire project,and revised t-he manuscript.

Ethical issues

The animal experiments were approved and monitored by the ani mal experiments ethical review committee of Guangxi University, Nanning, China.

Conflict of interest statement

The authors declare no competing interests.

SUPPORTING INFORMATION

Additional supplementary information is available for downloa-d and review in the supplementary information section located on the right-hand side of this article's HTML page.

Figure S1.

Figure S2.