

CELL BIOLOGY

Conversion of mouse embryonic fibroblasts into neural crest cells and functional corneal endothelia by defined small molecules

Shao-Hui Pan^{1†}, Ning Zhao^{2,3†}, Xiang Feng¹, Ying Jie², Zi-Bing Jin^{2,3*}

Reprogramming of somatic cells into desired functional cell types by small molecules has vast potential for developing cell replacement therapy. Here, we developed a stepwise strategy to generate chemically induced neural crest cells (ciNCCs) and chemically induced corneal endothelial cells (ciCECs) from mouse fibroblasts using defined small molecules. The ciNCCs exhibited typical NCC features and could differentiate into ciCECs using another chemical combination in vitro. The resulting ciCECs showed consistent gene expression profiles and self-renewal capacity to those of primary CECs. Notably, these ciCECs could be cultured for as long as 30 passages and still retain the CEC features in defined medium. Transplantation of these ciCECs into an animal model reversed corneal opacity. Our chemical approach for direct reprogramming of mouse fibroblasts into ciNCCs and ciCECs provides an alternative cell source for regeneration of corneal endothelia and other tissues derived from neural crest.

INTRODUCTION

Corneal disorders are the vital leading cause of blindness, with 12.7 million patients suffering from corneal blindness globally (1). Corneal transplantation, which replaces the damaged cornea with healthy donated corneal tissue, is the primary therapy. However, fewer than 1.5% of patients requiring corneal grafts can receive allotransplants due to a global shortage of donor corneas. Corneal endothelial keratoplasty has grown exponentially and is currently indicated for close to half of all corneal transplantations (2). The corneal endothelium, a monolayer of dedicated cells essential for maintaining the cornea's transparency and useful visual function, is naturally nonrenewable in vivo but is lost with age or in various disease states. Therefore, the massive global demand for corneal endothelia allograft cannot be fulfilled at the current rate of cornea donation. Recently, success with surgical grafting of in vitro cultured corneal endothelial cells (CECs) provided a critical proof of principle for a strategy to develop CEC-based cell therapies (3). However, it remains challenging to obtain sufficient CECs from healthy donor tissues.

Direct lineage reprogramming facilitates the generation of functional cell types independent of the donor organ for applications in cell replacement therapy (4). Terminally differentiated cells can be converted to other cell types in vitro by the introduction of lineage-specific transcription factors (TFs), bypassing the pluripotent state (5–10). Similarly, these conversions can also be induced by the overexpression of specific TFs in vivo (11–13). In addition, direct lineage reprogramming mediated by TFs has been used in disease modeling (14), implying its potential for practical applications. Recently, the small molecule-based conversion of fibroblasts to other functional cells represents an attractive reprogramming strategy

and has attracted much interest because of its safety and efficiency (15–18).

Developmentally, the corneal endothelia originate from neural crest cells (NCCs) (19, 20), which are a population of transient and multipotent cells giving rise to diverse differentiated cell types, including peripheral neurons, glia, melanocytes, and several types of ocular cells (21). During ocular development in vertebrates, NCCs delaminate from the roof plate upon closure of the neural tube and migrate into the eye, where they form the periorcular mesenchyme; this tissue further differentiates into diverse cell lineages, including the corneal endothelium, stroma, trabecular meshwork, and others (20, 22, 23).

In this study, we developed a two-step lineage reprogramming strategy to generate chemically induced CECs (ciCECs) from fibroblasts using defined small molecules. We screened a new cocktail of small molecules that could efficiently convert mouse fibroblasts into chemically induced NCCs (ciNCCs) bypassing the pluripotent state. The ciNCCs exhibited typical NCC features and could be further differentiated into ciCECs using another combination of small molecules in vitro. Through lineage tracing in Wnt1-Cre/*ROSA*^{tdTomato} and *Fsp1-Cre/ROSA26*^{tdTomato} fibroblasts, we confirmed that the ciNCCs and ciCECs were converted from fibroblast cells. The ciCECs showed similar gene expression profiles and self-renewal capacity to those of primary CECs (pCECs). Transplantation of the ciCECs into an animal model reversed corneal opacity, yielding clear tissue. Our findings provide a new approach to the generation of neural crest–like cells and functional corneal endothelial–like cells, providing a different alternative cell source for regeneration of corneal endothelia and other tissues derived from neural crest.

RESULTS

Conversion of fibroblasts into ciNCCs by chemically defined conditions

To identify the chemicals sufficient for ciNCC generation from mouse fibroblasts, we used a lineage tracing strategy to monitor the conversion process and excluded any NCCs from the starting mouse

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¹Institute of Stem Cell Research, The Eye Hospital, Wenzhou Medical University, Wenzhou 325027, China. ²Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Ophthalmology and Visual Science Key Laboratory, Beijing 100730, China. ³Beijing Advanced Innovation Center for Big Data-Based Precision Medicine, Beihang University and Capital Medical University, Beijing Tongren Hospital, Beijing 100730, China.

*Corresponding author. Email: jinzb502@ccmu.edu.cn

†These authors contributed equally to this work.

embryonic fibroblasts (MEFs) (Fig. 1A and fig. S1A). Wnt1-Cre transgenic mice have been validated as a lineage-tracing reporter model for NCC development (24, 25). In Wnt1-Cre/ROSA26^{tdTomato} mice, tdTomato protein was faithfully expressed in NCCs. MEFs were isolated from Wnt1-Cre/ROSA26^{tdTomato} mice at embryonic day 13.5 (E13.5). Because the NCC population was marked with tdTomato, we performed fluorescence-activated cell sorting (FACS) to collect the tdTomato⁺ population to exclude any NCCs or progenitors (the purified cells are hereinafter referred to as Wnt1-tdTomato⁺ MEFs; fig. S1B). We confirmed that the Wnt1-tdTomato⁺ MEFs were also negative for other NCC markers, including Sox10, P75, Pax3, HNK1, and AP2 α (fig. S1, C and D).

To generate ciNCCs from mouse fibroblasts, we hypothesized that small molecules shown to target NCC lineage-specific signaling would facilitate NCC reprogramming. Therefore, we selected 16 small molecules as candidates based on (i) the epigenetic regulation and signaling modulation of NCC development (26–29) and (ii) enhanced neural lineage reprogramming. The small molecules for NCC reprogramming were as follows: valproic acid (VPA), SB431542, RepSox, LDN193189 (LDN), CHIR99021, Y-27632, retinoic acid (RA), Forskolin, A8301, EPZ004777 (EPZ), RG108, 5-azacytidine (5-Aza), SMER28, AM580, Parnate, and BMP4 (table S1). For initial screening, the Wnt1-tdTomato⁺ MEFs were cultured in a 24-well plate and treated with small molecules. After testing various small-molecule conditions, we found that a combination of SB431542, CHIR99021, and Forskolin yields consistent tdTomato expression ($0.53 \pm 0.03\%$) (Fig. 1B). SB431542 is an inhibitor of transforming growth factor- β (TGF- β) signaling, which is important for NCC differentiation (30–32). Similarly, CHIR99021 is an inhibitor of glycogen synthase kinase 3 (GSK3) signaling, which is involved in promoting NCC fate (31, 33, 34). Forskolin, an adenosine 3',5'-monophosphate (cAMP) agonist, is crucial in early reprogramming for mesenchymal-to-epithelial transition (MET) (35). For subsequent screening and optimization,

we used the combination of SB431542, CHIR99021, and Forskolin as induction basal condition. We found that VPA (a histone deacetylase inhibitor), EPZ004777 [Disruptor of telomeric silencing 1-like (DOPTiL) inhibitor], and 5-Aza (a DNA methylation inhibitor) further enhanced the induction of Wnt1-tdTomato⁺ cells (Fig. 1C). Therefore, we used a chemically defined medium combined with a cocktail of six small molecules (VPA, CHIR99021, SB431542, Forskolin, 5-Aza, and EPZ004777) (hereafter termed M6) to reprogram mouse fibroblasts into ciNCCs (Fig. 1D). Expression of Wnt1-tdTomato in individual cells was observed as early as day 3 with M6 medium treatment (Fig. 1E). The M6 reprogramming medium effectively induced Wnt1-tdTomato⁺ cells at $3.67 \pm 0.21\%$ (Fig. 1F). On days 5 to 7, induced Wnt1-tdTomato⁺ colonies were observed in the M6 reprogramming medium (Fig. 1G and fig. S1E). These Wnt1-tdTomato⁺ cells and colonies had molecular feature similar to those of primary NCCs (pNCCs). The Wnt1-tdTomato⁺ cell number increased notably in small colonies at approximately day 12 (Fig. 1H). Although the generation of Wnt1-tdTomato⁺ NCCs was only as efficient as the conversion of human fibroblasts using TFs (33), merely 2 to 5% of cells were positive for Wnt1-tdTomato in this study. The results were reproducible in different batches of MEFs ($n = 8$), and MEFs with different genetic backgrounds (C57BL/6, 129 $\times$ C57BL/6, and 129) could also be converted into ciNCCs via M6 conditions (fig. S2). Together, these results indicate that M6 can reprogram MEFs into ciNCCs. Furthermore, M6 could induce neonatal mouse tail-tip fibroblasts (TTFs) into ciNCCs, albeit at a lower reprogramming efficiency (fig. S3).

Characterization of the converted ciNCCs

In the reprogramming process of ciNCCs, Wnt1-positive cells appeared as early as day 3. Small cell colonies emerged toward day 5. With the extension of induction time, the Wnt1-positive cells proliferated gradually.

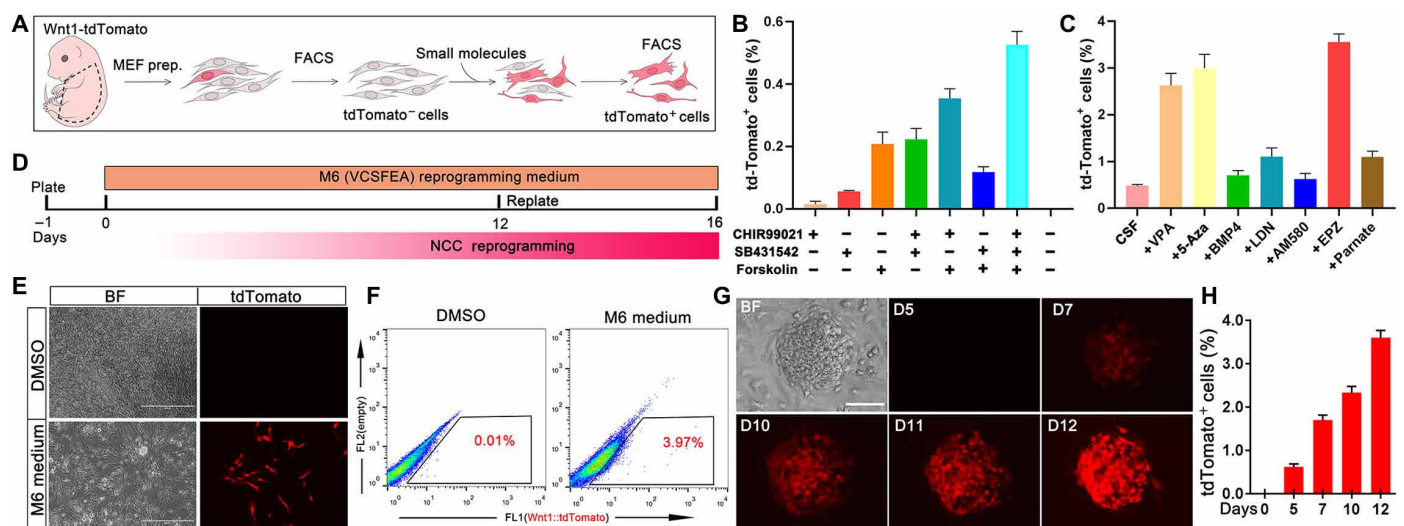


Fig. 1. Conversion of MEFs to ciNCCs by small-molecule induction. (A) Schematic diagram of the reprogramming of NCCs from MEFs. (B) Effects of individual chemicals on ciNCC generation. Data are means $\pm$ SD; $n = 3$ independent experiments. (C) Enhancement effects of individual chemicals on the generation of ciNCCs. Data are means $\pm$ SD; $n = 3$ independent experiments. (D) Schematic illustration of our strategy to convert MEFs into ciNCCs. (E) Generation of Wnt1⁺ ciNCCs from MEFs using a cocktail of small molecules. DMSO, dimethyl sulfoxide. (F) Quantification of Wnt1⁺ cells induced by candidate cocktails at day 12. Independent experiments, $n = 3$. (G) Morphological changes on distinct days during the induction process of ciNCCs. Scale bar, 50 μ m. (H) Percentages of Wnt1-tdTomato⁺ cells induced by candidate cocktails on distinct days. Independent experiments, $n = 3$.

To obtain the ciNCCs, we performed FACS to collect the Wnt1-tdTomato⁺ cells on days 12 to 16. Established ciNCCs were serially propagated in NCC expansion medium containing N2, B27, basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF). Morphologically, M6-induced cells at P3 maintained typical NCC features in monolayer culture (Fig. 2A). After passaging, the ciNCCs became morphologically homogeneous. To further characterize the M6-induced Wnt1-tdTomato⁺ cells, we sought to examine their gene expression. Our results showed the ciNCCs expressed multiple NCC markers, including P75, HNK1, AP2 α , and Nestin (Fig. 2B). Furthermore, we tested if the ciNCCs have differentiation potential toward peripheral neurons, Schwann cells, and others. For differentiation of ciNCCs, these cells were cultured in different lineage differentiation media. After cultured for 2 to 4 weeks, the differentiated cells were examined by assessing the expression of the markers by immunostaining. Notably, the ciNCCs could also give rise to cells expressing specific markers for neuron, including Tuj1 and Peripherin (Fig. 2C). For melanocyte differentiation, we observed melanocytes after 2 to 3 weeks of induction (Fig. 2C). Our results of immunostaining showed that the ciNCCs could differentiate to Schwann cells. The induced Schwann cells were GFAP⁺ and S100 β ⁺ cells (Fig. 2D). Further differentiation of these ciNCCs in vitro gave rise to mesenchymal lineages, resulting in typical mesenchymal cell morphology. Our results showed that these ciNCC-derived mesenchymal cells could give rise into osteogenic, adipogenic, and chondrogenic cells (Fig. 2E). Collectively, these data indicated that our ciNCCs could be induced to differentiate toward peripheral nervous system lineages and mesenchymal lineages.

Small molecules facilitate the induction of ciNCCs into ciCECs

The mechanisms prompting the differentiation of NCCs to CECs remain unclear (36, 37). The niche surrounding NCCs, for example, lens epithelial cells (LECs), determines their ultimate fate during differentiation (38). To generate mouse CEC-like cells from ciNCCs, we sought small molecules based on their importance in CEC organogenesis and maintenance in vitro. SB431542 (an inhibitor of TGF- β signaling) was capable of inducing CEC-like cells from human pluripotent stem cell (PSC) (39, 40). A previous study has reported that the Wnt signaling inhibitor Dkk2 promotes NCC differentiation into CECs (39). We selected the chemical inhibitor CKI-7 as a substitute, which has been shown to block Wnt signaling by inhibiting casein kinase I (41).

We decided to use the medium containing SB431542 and CKI-7 for CEC differentiation from ciNCCs (Fig. 3A). After 7 to 15 days of culture in this differentiation medium, a small population outside or inside the clusters displayed typical tight aggregates with homogeneous and polygonal morphology (Fig. 3B). We also observed that these colonies rapidly expanded, and the small clusters merged into larger ones by days 12 to 15 (Fig. 3B and movie S1). These CEC-like cells grew rapidly and had strong proliferation ability. The ciNCC-derived CEC-like cells formed a monolayer of hexagonal and pentagonal cells. To confirm that the CEC-like cells were derived from ciNCCs, we differentiated tdTomato⁺ ciNCCs with CEC differentiation medium containing 5 μ M SB431542 and 5 μ M CKI-7. These tdTomato⁺ ciNCCs could also be subsequently induced to differentiate toward polygonal cells (Fig. 3C). We next assessed CEC gene expression including Na⁺/K⁺-ATPase (Na⁺- and K⁺-dependent adenosine triphosphatase), AQP1, ZO-1, and N-cadherin by immunofluorescence staining. The results showed that these

cells expressed CEC-specific markers (Fig. 3D). The TTF-derived ciNCCs also could be induced into CEC-like cells and expressed multiple CEC markers (fig. S3, A and C). In this study, we identified the function of the CEC-like cells by Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) uptake (Fig. 3E). Global gene expression analysis by RNA sequencing (RNA-seq) showed that the CEC-like cells shared a similar gene expression pattern to that of pCECs, but this pattern was distinct from that of the initial MEFs (Fig. 3F). The presence of tight junctions in the CEC-like cells was observed by transmission electron microscopy (TEM) (Fig. 3G). To further monitor the reprogramming process, we confirmed the expression of a panel of NCC and CEC markers at distinct times by using quantitative reverse transcription polymerase chain reaction (qRT-PCR). By day 12, robust expression of NCC genes was detected in cells, including *Hnk1*, *P75*, *Sox10*, *Sox9*, *Pax3*, and *Ap2* (Fig. 4A). In addition, similar kinetics of gene activation for CEC genes, such as *Slc4a1c*, *Col8a1*, *Na⁺/K⁺-ATPase*, *Aqp1*, and *N-cadherin*, were also detected by qRT-PCR (Fig. 4B). The genes that are known to be enriched in pCECs were greatly up-regulated in ciCECs. To validate the transition process from fibroblast to CECs, we analyzed the transcriptome by using RNA-seq (Fig. 4C). The expression of CEC signature genes in ciCECs was substantially up-regulated, consistent with that of pCECs, whereas fibroblast signature genes were markedly down-regulated. Notably, a panel of NCC marker genes was initially up-regulated and subsequently down-regulated during the induction process (16, 18, 40, 42, 43). Principal components analysis revealed that M6-treated cells were distinct from the original MEFs, indicating that chemical reprogramming led to marked transcriptional changes (Fig. 4D). These results indicate that the CEC-like cells obtained CEC identity. Collectively, these data suggest that the combination of SB431542 and CKI-7 effectively promoted the generation of CECs within 10 to 15 days in the ciNCC culture. Those fibroblast-originated CEC-like cells are thereafter referred to as ciCECs.

Lineage tracing to confirm the induction of ciCECs from fibroblasts

To confirm the origin of the initial fibroblasts for the small molecule-based reprogramming, we sought a genetic lineage-tracing strategy to purify fibroblast-specific protein 1 (Fsp1)-tdTomato⁺ fibroblasts (Fig. 5A). Fsp1-Cre has been validated as a specific fibroblast marker for lineage tracing (44); thus, Fsp1-Cre mice were crossed with ROSA26^{tdTomato} mice. MEFs were isolated from transgenic mice (Fsp1-Cre/ROSA26^{tdTomato}) at E13.5, and the fibroblasts expressed tdTomato specifically; these cells are hereafter named tdMEFs (fig. S4A). To avoid possible contamination of the MEFs with NCC progenitor cells, we carried out FACS to collect the tdTomato⁺/p75⁻ population (Fig. 5B and fig. S5G). These tdMEFs were negative for all NCC markers, including P75, HNK1, Sox10, and AP2 α (Fig. 5C).

Then, these tdMEFs were induced with the abovementioned M6 medium. Epithelial clusters expressing tdTomato were observed during ciNCC induction (Fig. 5D and fig. S4B). We passaged the Fsp1-tdTomato⁺ ciNCCs and cultured them in NCC expansion medium for further experiments (after 2 weeks of induction). Immunofluorescence analysis confirmed that these Fsp1-tdTomato⁺ ciNCCs were positive for the NC markers P75, HNK1, Sox10, and AP2 α , indicating that the colonies of Fsp1-tdTomato⁺ ciNCCs had the differentiation potential toward CEC-like cells (fig. S4C). Furthermore, the Fsp1-tdTomato⁺ ciNCCs could differentiate into tdTomato⁺

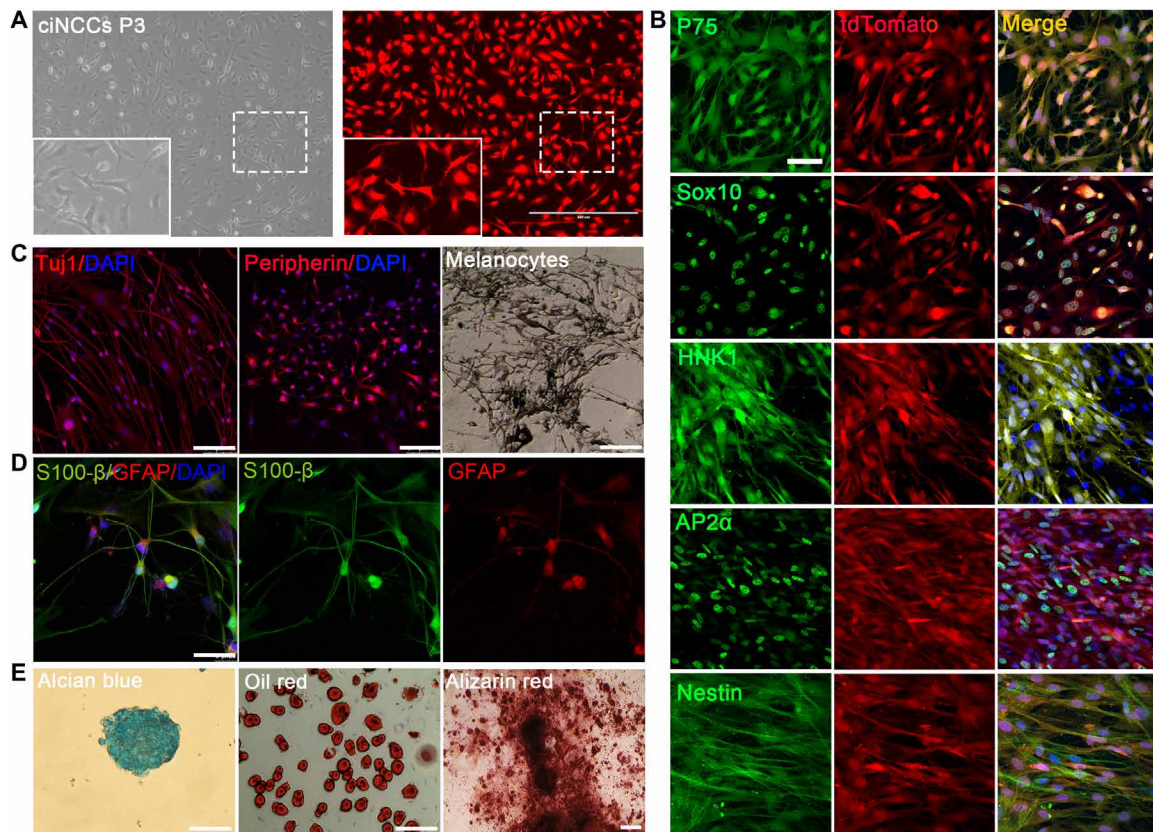


Fig. 2. Characterization of M6-induced ciNCCs. (A) Morphology of M6-induced ciNCCs. Scale bar, 400 μm . (B) Immunostaining showing that MEF-derived ciNCCs express P75, Sox10, HNK1, AP2 α , and Nestin. Scale bar, 50 μm . (C) Representative images of melanocytes and differentiated ciNCCs stained with peripheral neuron markers. Scale bars, 50 μm . (D) Differentiation of ciNCCs into Schwann cells and melanocytes with marker expression. Scale bar, 50 μm . (E) ciNCCs were differentiated into mesenchymal lineages and further into adipocytes, chondrocytes, and osteocytes. Scale bars, 100 μm .

ciCECs (fig. S4D). By immunostaining, we found that differentiated CEC-like cells coexpressed Na^+/K^+ -ATPase, AQP1, laminin, ZO-1, Na^+/K^+ -ATPase, and tdTomato (Fig. 5E). Notably, all these ciCECs also expressed tdTomato, demonstrating a conversion from fibroblasts. The results confirmed that the ciNCCs and ciCECs were converted by two-step lineage reprogramming from fibroblasts.

ciCECs generated with chemicals bypass the iPSC stage

Because the mechanism of our lineage reprogramming might be similar to that of chemically reprogrammed induced PSCs (iPSCs) (9, 16), we sought to assess whether the ciCECs went through an iPSC stage. We performed a comparison between chemical iPSC reprogramming and ciCEC induction from MEFs derived from mice harboring an Oct4 promoter-driven green fluorescent protein (GFP) (OG2) reporter (35, 45). The M6-treated MEFs morphologically underwent a characteristic MET, and small cell colonies gradually emerged toward day 6 (fig. S5A). These cell colonies expressed the NCC marker Sox10 (fig. S5B). In contrast, we did not observe any Oct4-GFP-positive cells during the entire process from MEFs to ciCECs based on our method (fig. S5, C and D). Furthermore, the ciCECs maintained a normal karyotype during 10 continuous passages in vitro (fig. S5E). To evaluate the potential risk of tumorigenesis, a total of 5×10^6 ciCECs and 2×10^6 mouse embryonic stem cells (mESCs) were transplanted subcutaneously into nonobese diabetic mice (NOD)/severe combined immunodeficient (SCID) mice. Notably,

no tumors formed over 6 months after transplantation with ciCECs, whereas large teratomas developed in the mice transplanted with mESCs after 4 to 8 weeks (fig. S5F). This result suggests that the ciCECs have no tumorigenic potential. To better understand their differentiation in vivo, we transplanted ciCECs into the anterior chambers of the eyes of NOD/SCID mice. After 4 to 8 weeks, the transplanted ciCECs did not form tumors over 6 months after transplantation. These results demonstrated that our approach could directly reprogram MEFs to ciNCCs and eventually ciCECs while bypassing the iPSC stage.

Long-term expansion capacity of ciCECs in vitro

The maintenance of cultured CECs with morphology and normal physiological function in vitro has proven challenging (46). We aimed to test whether large numbers of functional ciCECs could be generated from fibroblasts to enable the large-scale application of ciCECs. On the basis of our observations that ciCECs cultured in medium with SB431542 (5 μM) and CKI-7 (5 μM) were small, polygonally shaped cells (fig. S6A), we hypothesized that SB431542 and CKI-7 would promote ciCEC growth in vitro. We evaluated the long-term expansion capacity of ciCECs in vitro by continuously passaging ciCECs at a 1:6 ratio and found that the phenotype was similar between P3 and P30 (Fig. 6, A and B). This result showed that SB431542 and CKI-7 strongly promoted ciCEC expansion. These ciCECs sustained themselves as a homogeneous cell population for

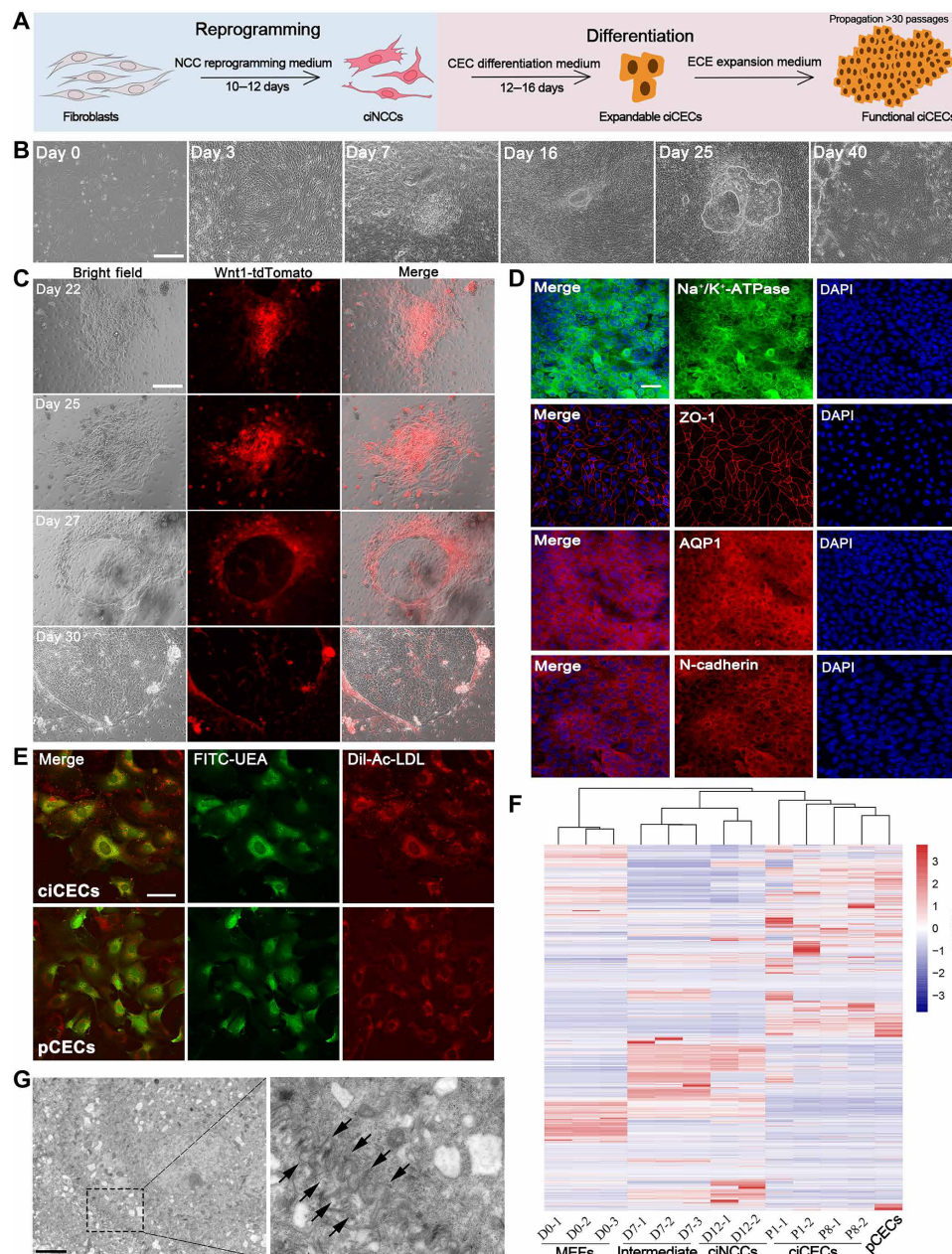


Fig. 3. Generation of mouse ciCECs from fibroblasts by small-molecule induction. (A) Schematic diagram for the chemical reprogramming of ciCECs from MEFs. (B) Bright-field images of initial MEFs, reprogrammed ciNCC colonies, and ciCECs. Scale bar, 400 μ m. (C) Morphological changes on different days during the induction process of ciCECs from Wnt1-tdTomato⁺ ciNCCs. Scale bar, 400 μ m. (D) Immunofluorescence staining of ciCECs for the corneal endothelium markers Na⁺/K⁺-ATPase, AQP1, vimentin, N-cadherin, laminin, and ZO-1. Scale bar, 50 μ m. (E) LDL uptake function in ciCECs at P2 and pCECs at P3. Scale bar, 50 μ m. (F) Heatmap of differentially expressed genes among the samples at the indicated time points. These numbers below the heatmap indicate independent biological replicates. Red and blue indicate up-regulated and down-regulated genes, respectively. (G) TEM of ciCECs showing the tight junctions. Scale bar, 5 μ m.

at least 30 passages (P30) with hexagonal morphology in small molecule-based medium. In addition, we successfully clonogenically cultured these ciCECs to 10 passages and demonstrated consistent morphologies. Our results of immunostaining showed that the rate of Ki67-positive cells was higher in ciCECs at P3 than in pCECs at P3 (fig. S6B). ciCECs were highly proliferative, as 24.6, 37.8, and 48.1% of these cells showed incorporation of ethynyl deoxyuridine (EdU) at P1, P3, and P6 (fig. S6C). The results of FACS analysis with propidium iodide (PI) staining showed that the cell cycle distribution

(G₀-G₁, S, and G₂-M phases) was 46.30, 45.11, and 8.59% for ciCECs at P3 and 67.30, 21.50, and 11.20% for pCECs at P3 (fig. S6D). They rapidly expanded into large, homogeneous colonies with population doubling times of 22.3 ± 3.7 hours (Fig. 6C). Large “vacuole-like” structures were found on the surface of the ciCECs at P2 to P10 (Fig. 6D). These vacuole-like structures disappeared at P20 when they were serially propagated. Notably, the ciCECs at P30 also expressed typical CEC markers, including Na⁺/K⁺-ATPase, AQP1, and ZO-1 (Fig. 6E). When assayed by imaging for the ability to

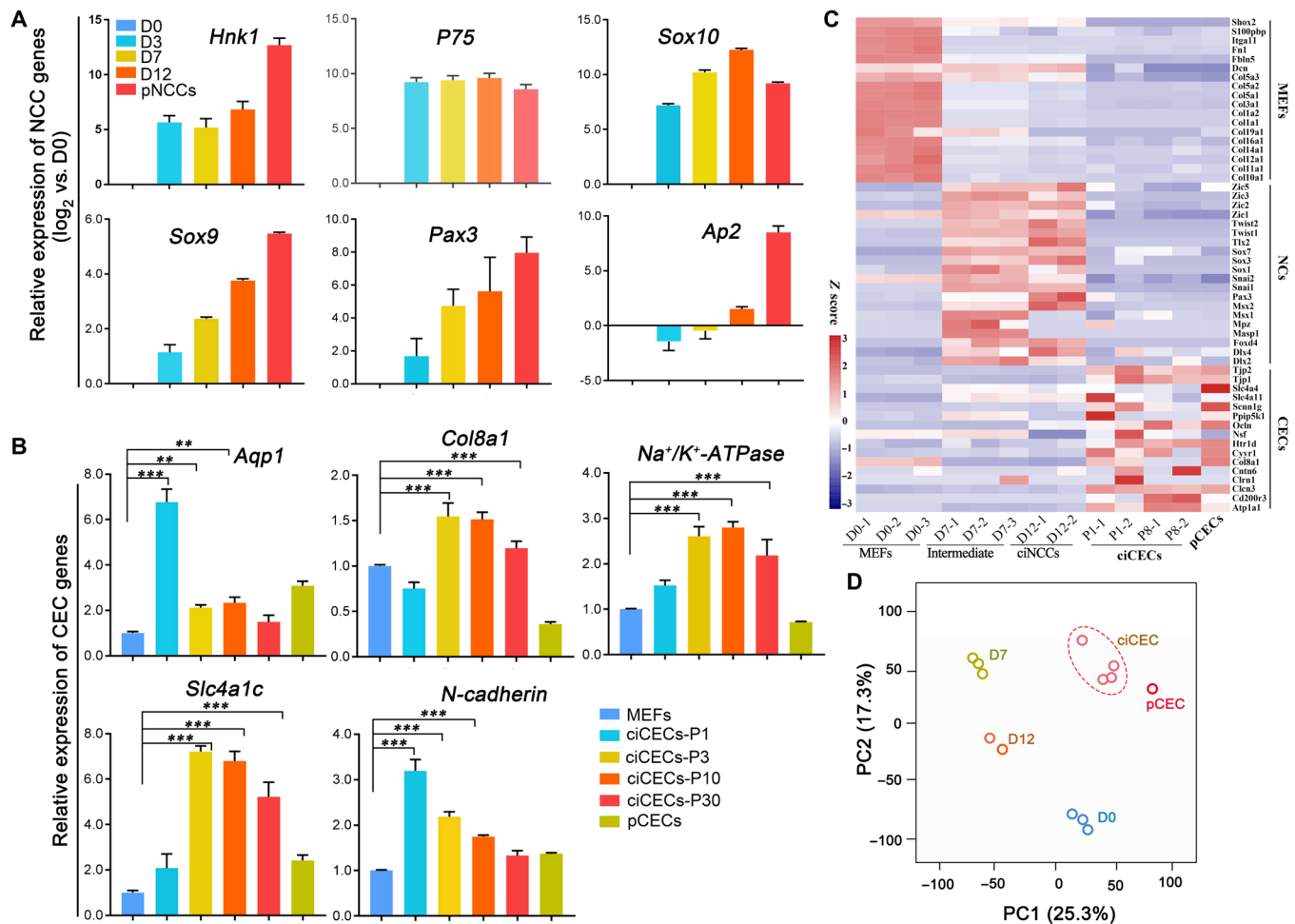


Fig. 4. Gene expression profiling of ciNCCs and ciCECs. (A) qRT-PCR analysis showing the expression of NCC genes at the indicated time points. Gene expression (log₂) was normalized to that in MEFs. (B) qRT-PCR analysis of the expression of the indicated CEC genes in MEF-derived ciCECs at different passages, MEFs, and pCECs. (C) Heatmap of differentially expressed genes among samples at the indicated time points. The number below the heatmap indicates independent biological replicates ($n = 12$). Red and blue indicate up-regulated and down-regulated genes, respectively. (D) Principal components analysis of samples from day 0 (D0), day 7 (D7), and day 12 (D12) of reprogramming, ciCECs, and the control pCECs.

migrate into the space created by a scratch wound, ciCECs cultured in medium with SB431542 and CKI-7 at different passages showed a stronger capability of proliferation and migration as compared to that of pCECs (fig. S6, E and F). Collectively, these results demonstrated that SB431542 and CKI-7 had a robust and general effect on long-term expansion of ciCECs in vitro.

Transplantation into an animal model revealed ciCEC function

To evaluate whether the ciCECs had the capacity to regenerate the corneal endothelia in vivo, we transplanted them into a well-established rabbit model with bullous keratopathy by mechanically scraping corneal endothelia from Descemet's membrane (47, 48). A previous study found that injection of human pCECs supplemented with a ROCK inhibitor restored endothelial function (49). Corneal edema decreased much earlier after ciCEC transplantation in the grafted eyes than in the untreated eyes. Compared to the untreated eyes, which showed that the clarity of the cornea was unchanged, we noted that the clarity of the cornea of the grafted eyes increased gradually after transplantation (Fig. 7A). After 7 days, the corneas of the

grafted eyes became transparent, while corneal opacity and stromal oedema remained poor in the untreated eyes. The results of slit-lamp examination showed that the clarity of the cornea of the grafted eyes was also notably improved after injection, and the pupil and iris texture could be clearly observed (Fig. 7B and fig. S7A).

Next, we investigated ciCEC survival in the grafted eyes. Eyeballs were enucleated at postsurgical day 28 to evaluate the transplanted ciCECs. Fluorescence microscopy examination confirmed the existence of tdTomato-tagged grafted cells. Visante optical coherence tomography (OCT) of the anterior segment also showed that the corneal thickness decreased after ciCEC injection (Fig. 7C and fig. S7C). Confocal microscopy confirmed full coverage of polygonal endothelial cells on Descemet's membrane in the ciCEC-grafted disease models, while this could not be detected in the untreated models due to their severe corneal opacity (Fig. 7D). Under magnification, the grafted ciCECs tightly adhered to the posterior surface of the cornea in a monolayer, whereas the Descemet's membranes in the untreated model were bare and had no detectable CECs on day 28. Immunohistochemistry demonstrated ZO-1 expression, indicating the adhesion of injected cells onto the corneal tissue (Fig. 7E and fig. S7D).

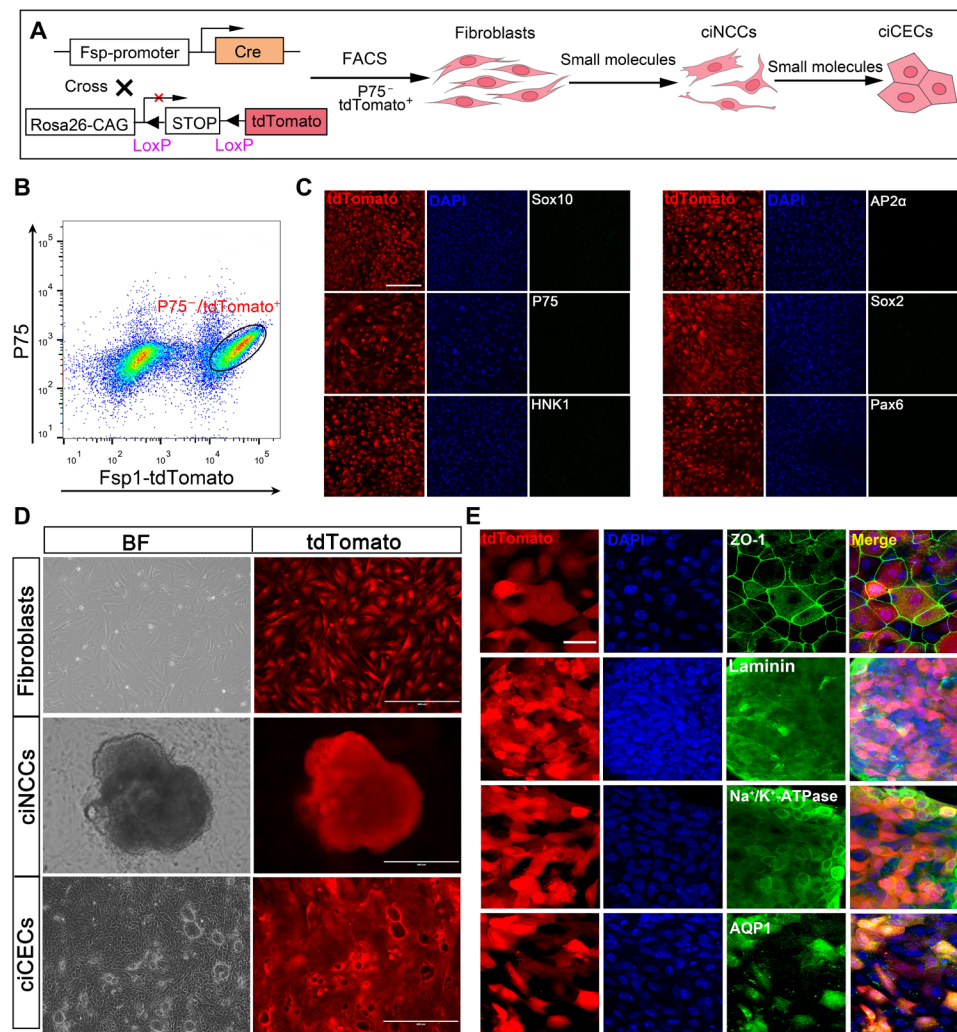


Fig. 5. Lineage tracing to confirm the induction of ciCECs from fibroblasts. (A) Schematic diagram illustrating the genetic lineage-tracing strategy. MEFs were obtained by sorting for $p75^+/tdTomato^+$ cells from MEFs derived from E13.5 mouse embryos of the $Fsp1-Cre/ROSA26^{tdTomato}$ background. (B) FACS result showing the $p75^+/tdTomato^+$ cells from MEFs with a genetic background of $Fsp1-Cre/ROSA26^{tdTomato}$. (C) Immunostaining analysis showing negative results for $Sox10$, $P75$, $HNK1$, $AP2\alpha$, $Sox2$, and $Pax6$ in the $p75^+/tdTomato^+$ cells. Scale bar, 100 μm . (D) $p75^+/tdTomato^+$ cell differentiation toward $ciNCCs$ and $ciCECs$. Scale bars, 400 μm . (E) Immunostaining analysis showing positive results for $ZO-1$, $laminin$, $Na^+/K^+-ATPase$, and $AQP1$ in the $Fsp1-tdTomato$ -MEF-derived $ciCECs$. Scale bar, 50 μm .

There was a rapid decrease in corneal thickness within 4 weeks after ciCEC injection, followed by a more gradual decrease over the next 2 weeks (Fig. 7F and fig. S7E). In the untreated group, mean corneal thickness was approximately 1200 μm throughout the 42 days of observation. In contrast, it decreased rapidly in the grafted group, being significantly less than that in the untreated group. We observed that the mean corneal thickness at postoperative days 14 ($P < 0.01$), 21, 28, 35, and 42 ($P < 0.001$) in the ciCEC-grafted group was significantly less than that in the control group, indicating that corneal edema was markedly reversed. Meanwhile, corneal clarity gradually increased after ciCEC transplantation, and grafted corneas had higher corneal transparency compared to untreated controls (Fig. 7G). These results strongly suggest that ciCEC transplantation repopulated and self-organized on the posterior surface of the cornea and has the capacity to regenerate the corneal endothelium. In this study, the ciCECs were injected combined with a ROCK inhibitor (Y-27632) into the anterior chambers of the eyes

(Fig. 7H and movie S2). Each recipient received 1×10^6 ciCECs. Fellow eyes (normal) and untreated eyes [phosphate-buffered saline (PBS) injection] were used as experimental controls.

DISCUSSION

Previous studies have reported that NCCs can generate from fibroblasts by introducing NCC-specific TFs (33, 42). In this study, we demonstrated that $ciNCCs$ could be generated from MEFs via a small-molecule reprogramming method in vitro. The generated $ciNCCs$ resemble NCCs in the expression profile of signature genes, capacity for self-renewal, and the differentiation potential into derivative neurons, Schwann cells, and mesenchymal lineages. These finding demonstrated that direct lineage-specific conversion to NCCs from MEFs could be achieved by the manipulation of signaling pathways with small molecules. In addition, given the broad spectrum of NCC derivatives, our approach for developing $ciNCCs$

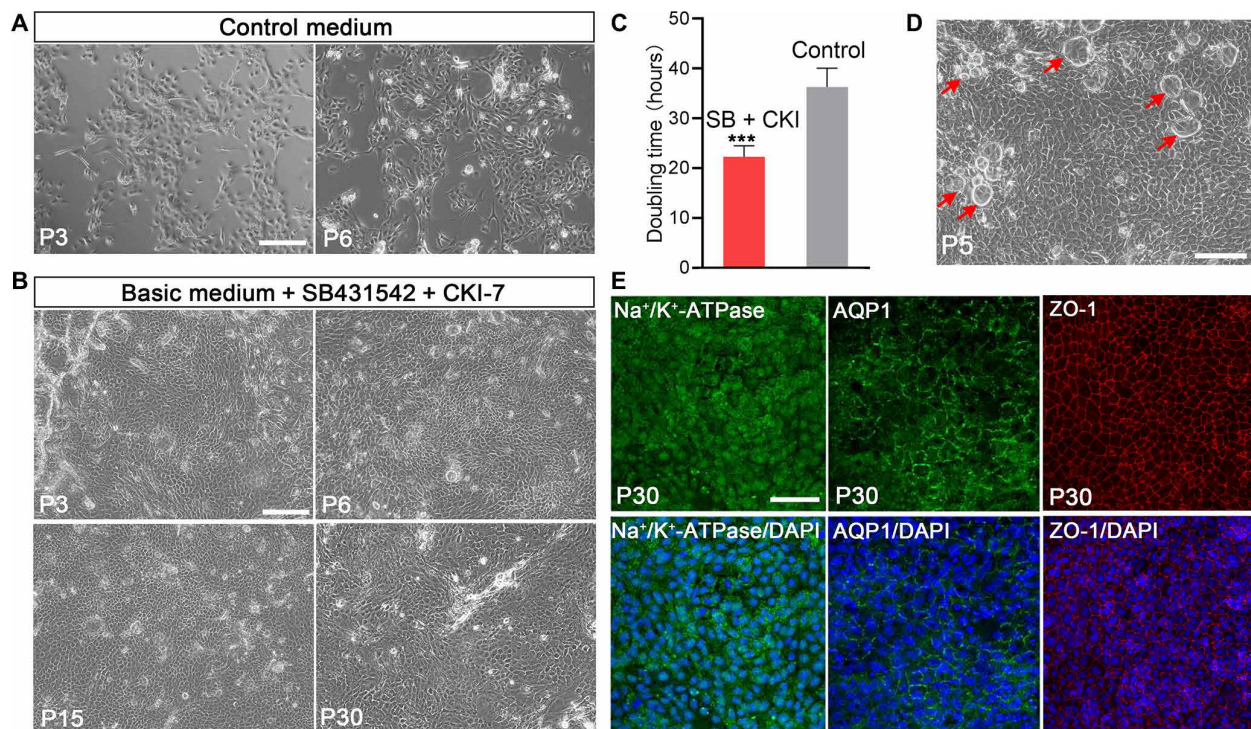


Fig. 6. Small molecules facilitate long-term expansion of ciCECs. (A) ciCECs were expanded for 3 days in serum-free control medium. Scale bar, 200 μ m. (B) Serial expansion of ciCECs in the serum-free medium with addition of SB431542 and CKI-7. Scale bar, 200 μ m. (C) Average population doubling times (means $\pm$ SD, $n = 3$; *** $P < 0.001$) of the ciCECs cultured in medium with and without SB431542 and CKI-7. (D) Bright-field image of ciCECs at P5, which were expanded for 3 days in culture condition with addition of SB431542 and CKI-7. Arrows indicate the vacuole-like structures. Scale bar, 50 μ m. (E) These ciCECs at P30 were fixed and stained for Na⁺/K⁺-ATPase, AQP1, and ZO-1. Scale bar, 50 μ m.

holds great potential as a different source to generate other NCC-derivative cell types (50).

Furthermore, we successfully generated functional ciCECs from ciNCCs. We explored defined differentiation conditions including SB431542 and CKI-7 to achieve this process in vitro. ciCECs showed an expression profile and function close to those of mature CEC. These ciCECs were found to expand further and maintain contact-inhibited hexagonal phenotype in the defined serum-free chemical medium. The maintenance of pCECs in vitro has been proven challenging. When grown under normal culture conditions in vitro, pCECs often show morphological fibroblastic changes and lose their physiological function (43, 51). A TGF- β inhibitor maintains the CEC phenotype during the process of ciCEC generation. A previous study showed that SB431542 assisted in the maintenance of cultivated CECs with a normal polygonal and functional phenotype (52). We found that the ciCECs could maintain normal polygonal morphology and function in the medium with SB431542 and CKI-7 over the long term. Notably, these cells could be serially expanded up to 30 passages and maintained contact-inhibited hexagonal phenotype. The contact-inhibited plays important roles in keeping the monolayer structure of ciCECs. Thus, it does not allow ciCECs to overproliferate in vitro and in vivo. During the passage, main genes related to maturation were increased, indicating that they were mature ciCECs.

Corneal endothelium is derived from periocular NCCs (53). Previous reports have shown that PSCs can differentiate into CECs with a conditioned medium (36, 37, 39). Defined medium containing

small molecules promoted PSC to differentiate into CEC-like cells (39). Most approaches for the generation of CECs from stem cells in vitro were stepwise procedures according to the developmental process. Consistent with previous studies, the lineage conversion from mouse fibroblasts is also a stepwise procedure. The induction strategy for ciCECs is composed of two major steps, including an initial chemical conversion of ciNCCs from mouse fibroblasts, followed by lineage-specific differentiation into ciCECs. The ciCECs have advantages over the PSC-derived CECs in certain aspects. The small molecule-based conversion from fibroblasts makes the generation of ciCECs safe, which have no tumorigenic potential. Moreover, ciCECs can be produced from individual patients, thus developing individualized cell therapy (40).

The generation of large numbers of functional CECs for cell-based approaches to corneal endothelial dysfunction is an important goal. Direct reprogramming of fibroblasts to CECs could offer a solution to this problem. The ciCECs exhibited a monolayer of hexagonal shaped cells in vitro. In vivo engraftment of ciCECs substantially reversed the corneal opacity in the rabbit corneal endothelial dysfunction model, indicating their therapeutic effect for corneal endothelial deficiency. A previous study has shown that cultivated rabbit CECs injected with Y-27632 were successful in recovering complete transparency of the corneas (47). However, further commenting is needed on the immunologic rejection of xenotransplantation that occurs in this study.

It has been known that MEFs contain a heterogeneous population of nonfibroblast precursor cells (16). To avoid possible

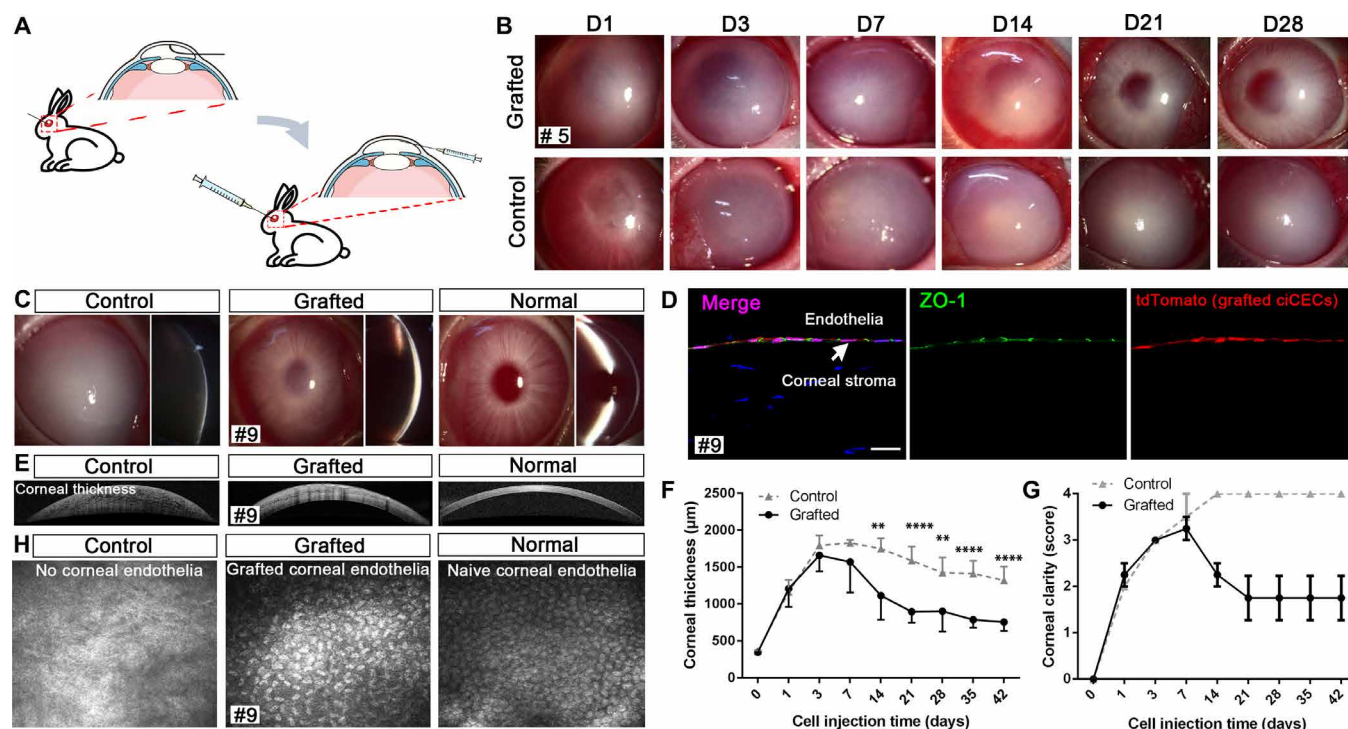


Fig. 7. Transplantation of ciCECs in vivo. (A) Diagram depicting the transplantation of ciCECs with the ROCK inhibitor into model rabbits. (B) Corneal transparency in the grafted eyes was notably improved after transplantation, while corneal opacity and stromal oedema were still serious in the untreated controls. (C) Slit-lamp microscopic images showing that the clarity of the grafted corneas was substantially improved after transplantation, while corneal opacity and stromal oedema remained in the untreated controls. (D) Immunohistochemistry showing that surviving tdTomato⁺ ciCECs were attached to Descemet's membrane. Scale bar, 100 μm. (E) Visante OCT showing ameliorated corneal oedema (reduced corneal thickness) in a grafted eye. (F and G) Trend in corneal thickness and corneal clarity after transplantation. There were significant differences in corneal thickness and transparency between the untreated controls and the grafted groups. The results are means and SEM for biological replicates ($n = 9$). (H) Live confocal imaging of corneal endothelia confirmed full coverage of polygonal cells on Descemet's membrane in the grafted eyes. Photo credit: Zi-Bing Jin, Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Ophthalmology & Visual Science Key Laboratory, Beijing, 100730 China.

contamination of NCCs in the starting MEFs, we carried out a lineage-tracing experiment to track the origin of the ciNCCs and ciCECs. We verified that the Wnt1⁺ ciNCCs were generated from non-NCC fibroblasts. To further confirm the origin of the ciNCCs, we confirmed that the tdTomato-positive ciNCCs generated from fibroblasts and engrafted Fsp1-tdTomato ciCECs in the animal model by using a fibroblast-specific Fsp1-Cre lineage-tracing reporter in MEFs.

In conclusion, our study presents a new strategy to generate functional CECs through induction of NCCs with chemically defined small-molecule cocktails, providing a new source of neural crest derivatives for the purpose of corneal engineering and regeneration.

MATERIALS AND METHODS

Animals

The Wnt1-Cre [Tg(Wnt1-Cre)11Rth], Fsp1-Cre [BALB/c-Tg(S100a4-cre)1Egn/YunkJ], and ROSA26-tdTomato [Gt(ROSA)26Sortm14 (CAG-tdTomato)Hze] mice were obtained from The Jackson Laboratory; the Oct4-GFP transgenic allele-carrying mice (CBA/Ca×C57BL/6J) were also obtained from The Jackson Laboratory; and the 129Sv/Jae and C57BL/6 mice were obtained from Shanghai Vital River Laboratory. The rabbits used in this study were of the New Zealand white strain and were obtained from JOINN

Laboratories (Suzhou) Inc., Suzhou, China. All the animals were housed under stable conditions ($21^\circ \pm 2^\circ\text{C}$) with a 12-hour dark/light cycle. All animal experiments were approved by the Animal Ethics Committee of Wenzhou Medical University, Wenzhou, China.

Cell isolation and culture

MEFs were isolated from E13.5 embryos as previously described (54). Briefly, the neural tissues (including head, spinal cord, and tail), limbs, gonads, and visceral tissues of the E13.5 mouse embryos were carefully removed and discarded before MEF isolation. The remaining tissues were sliced into small pieces, trypsinized, and plated onto 10-cm dishes in fibroblast medium. Mouse NCCs were isolated from the neural tube of E8.5 embryos under a dissection microscope as previously described (55). The mouse meninges and vessels were removed and discarded. The remaining brain tissues were sliced into small pieces, dissociated with 0.25% trypsin (Gibco) for 15 min at 37°C , washed with Dulbecco's modified Eagle's medium (DMEM)/F12 twice, and then plated in a T25 flask bottle in NCC medium, which is composed of DMEM/F12 (Gibco) supplemented with $1 \times \text{N2}$ (Gibco), $1 \times \text{B27}$ (Gibco), bFGF (20 ng/ml) (PeproTech), and EGF (10 ng/ml) (PeproTech). Mouse pCECs were isolated from postnatal day 30 (P1)–2 pups following a published protocol (56). They were cultured in DMEM containing 10% fetal bovine

serum (FBS) (Gibco), 0.1 mM nonessential amino acids (NEAAs) (Sigma-Aldrich), 2 mM GlutaMAX (Gibco), and 2 mM penicillin-streptomycin (Gibco). The mESCs were maintained in ESC medium, which is composed of DMEM with 1× N2 (Gibco), 1× B27 (Gibco), leukemia inhibitory factor (LIF), 0.1 mM nonessential amino acids (Sigma-Aldrich), 2 mM GlutaMAX (Gibco), 2 mM penicillin-streptomycin (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), CHIR99021 (3 mM), and PD0325901 (1 mM).

FACS of Wnt1⁺ MEFs and Fsp1⁺ MEFs

Primary MEFs were isolated from E13.5 mouse embryos with a genetic background of Wnt1-Cre/ROSA26^{tdTomato} (Wnt1-Cre mice × ROSA26^{tdTomato} mice) and Fsp1-Cre/ROSA26^{tdTomato} (Fsp1-Cre mice × ROSA26^{tdTomato} mice). The MEFs at P2 were dissociated with 0.25% trypsin at 37°C for 5 min and neutralized with MEF medium. To prepare the Fsp1-MEFs, the resulting fibroblasts were sorted to obtain tdTomato⁺/p75⁺ cells by FACS. These MEFs were stained with a specific antibody against p75 and subjected to FACS for tdTomato⁺/p75⁺ cells. During FACS, a Matrigel-coated 24-well plate was prewarmed at 37°C for at least 30 min before seeding the tdMEFs. The tdMEFs were planted immediately after FACS into the prewarmed Matrigel-coated 24-well plate at 1.5×10^4 cells per well in MEF medium in 5% CO₂ and 20% O₂ at 37°C for 5 hours to allow the cells to attach to the plate.

Small-molecule compounds and libraries

Small molecules, including the GSK3 inhibitor CHIR99021, the TGF-β inhibitor SB431542, the cAMP inducer Forskolin, and CKI-7, were acquired from Sigma-Aldrich. bFGF was acquired from PeproTech. All chemical components are described in table S1.

Detailed protocol for the generation of ciCECs from mouse fibroblasts in chemically defined medium

M6 reprogramming medium preparation

The basal medium contained knockout DMEM (Gibco), 10% KSR (KnockOut serum replacement) (Gibco), 10% FBS (Gibco), 1% NEAA (Gibco), and 0.1 mM 2-mercaptoethanol (Gibco) supplemented with the small molecules CHIR99021 (3 μM), SB431542 (5 μM), Forskolin (10 μM), VPA (500 μM), EPZ004777 (5 μM), and 5-Aza (0.5 μM). The medium was shaken for 30 min to ensure that all components were fully dissolved.

CEC differentiation and medium preparation

DMEM/F12/GlutaMAX (Gibco), 10% KSR (Gibco), 1% NEAA (Gibco), and 0.1 mM 2-mercaptoethanol (Gibco) were supplemented with SB431542 (5 μM) and CKI-7 (5 μM).

Chemical conversion of NCCs from mouse fibroblasts

The MEFs and TTFs were plated at 5×10^4 cells per well on six-well tissue culture plates in fibroblast medium. The culture plates were precoated with fibronectin or laminin for more than 2 hours. After overnight culture, the medium was exchanged with M6 chemical medium, which was refreshed every 2 days. NCC-like cells appeared and increased at days 3 to 5. After 7 to 10 days of induction, FACS was performed to collect Wnt1⁺ cells.

Generation of ciCECs from mouse ciNCCs

On days 12 to 16, the M6 chemical medium was replaced with CEC differentiation medium, which was refreshed every 2 days. Endothelial-like cell clusters appeared as early as day 20. During days 30 to 35, these CEC-like cell colonies were counted or further detected.

Immunocytochemistry

Cells were washed once with 1× PBS and then fixed with 4% paraformaldehyde at room temperature for 10 to 15 min, followed by permeabilization with 0.2% Triton X-100 in 1× PBS for 10 min and blocking with 7.5% bovine serum albumin (BSA) for at least 1 hour. All primary antibodies were diluted in 7.5% BSA, and the primary antibody reactions were incubated at 4°C overnight. Then, the cells were washed with 1× PBS for 10 min five times at room temperature. The secondary antibodies with Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647, purchased from Invitrogen, were diluted in 7.5% BSA, and incubation was performed for 1 hour at room temperature, followed by five 10-min washes with 1× PBS. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The antibodies used in this study are listed in table S2.

RNA preparation and RT-PCR

Total RNA was extracted using an RNeasy Plus Mini kit (Qiagen). In brief, 1 μg of total RNA was used for reverse transcription with an iScript cDNA synthesis kit (Bio-Rad), and the resulting complementary DNA (cDNA) was diluted five times in H₂O for PCR. For semiquantitative PCR, 1 μl of one-fifth diluted cDNA was used as template for the following PCR program: 95°C for 5 min and 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. Quantitative PCR was performed following the FAST SYBR Green Master Mix (ABI) protocol. All PCR was performed in triplicate, and the expression of individual genes was normalized to that of *Gapdh*. The primer sequences are listed in table S3.

RNA sequencing and analysis

Total RNA for each sample was isolated with TRIzol reagent and purified using the RNeasy 23 Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quality and quantity were assessed using NanoDrop 2000, Agilent 2100 Bioanalyzer, and Agilent RNA 6000 Nano Kit. RNA library construction and RNA-seq were performed by the Annoroad Gene Technology. Sequencing libraries were generated using the NEB Next Ultra RNA Library Prep Kit for Illumina24 (NEB), and library clustering was performed using HiSeq PE Cluster Kit v4-cBot-HS (Illumina) following the manufacturer's recommendations. After cluster generation, the libraries were sequenced on an Illumina platform and 150-base pair paired-end reads were generated. The initial data analysis was performed on BMKCloud (www.biocloud.net/).

FACS cytometry

For MEF preparation, fibroblasts with the desired genotype were cultured in MEF medium until they reached more than 80% confluence. The cells were washed twice with 1× PBS and treated with 0.25% trypsin at 37°C for 5 min. After harvesting, the cells were passed through a 70-μm filter, washed twice with PBS, and resuspended in precooled buffer (1× PBS, 1.5% FBS, and 0.5% BSA). The cells were incubated with either fluorescein isothiocyanate (FITC)-conjugated P75 antibody (Abcam) or isotype control (BD) at the suggested concentrations on ice for 30 min or room temperature for 45 min, followed by six washes with FACS buffer. Cells were then resuspended in FACS buffer and sorted with BD FACSaria II.

ciNCC differentiation

Approximately 5×10^3 ciNCCs were seeded on laminin-coated 24-well tissue culture plates and cultured in N2B27 medium, which

contained $1 \times N2$, $1 \times B27$, EGF (10 ng/ml), and bFGF (10 ng/ml) in Neurobasal medium. After 24 hours, cells were subjected to differentiation conditions.

For peripheral neuron differentiation, the medium was switched to neuron differentiation medium [NCC medium without bFGF and EGF, with the addition of 200 μ M ascorbic acid, 2 μ M dibutyryl cAMP (db-cAMP), brain-derived neurotrophic factor (BDNF) (25 ng/ml), NT3 (25 ng/ml), and glial cell line-derived neurotrophic factor (GDNF) (50 ng/ml)]. Half of the medium was changed every 2 to 3 days. Specific neuron markers were analyzed by day 10 to day 20 after differentiation.

For Schwann cell differentiation, ciNCCs were cultured in N2B27 medium for at least 2 weeks. Differentiation was then induced by culturing in NCC medium without FGF2 and EGF and supplemented with ciliary neurotrophic factor (10 ng/ml), neuregulin (20 ng/ml), and 0.5 mM db-cAMP for 3 to 4 weeks. Media were changed every 2 to 3 days. Cells were then examined for the expression of Schwann cell protein markers by immunostaining.

To differentiate into melanocytes, ciNCCs were cultured in the presence of 5 μ M RA and Shh (Sonic hedgehog) (200 ng/ml) for 1 day and platelet-derived growth factor-AA (PDGF-AA) (20 ng/ml), bFGF (20 ng/ml), and Shh (200 ng/ml) for 3 to 5 days; then, they were cultured in differentiation medium containing T3 (40 ng/ml), Shh (200 ng/ml), 1 nM LDN193189, 5 mM db-cAMP, and NT3 (10 ng/ml) for 8 to 12 days. The medium was refreshed every other day.

For mesenchymal differentiation, ciNCCs were cultured for 3 weeks in α -MEM containing 10% FBS, as previously described (57). The differentiation potential of ciNCC-derived mesenchymal stem cells was achieved by incubation with adipogenesis medium, osteogenesis medium, and chondrogenesis medium, respectively (Cyagen Biosciences). The differentiation medium was refreshed every 3 days. The induced cells were stained with Oil Red O stain kit (Solarbio), alizarin red S (Sigma-Aldrich), and Alcian blue (Sigma-Aldrich) after 3 weeks of induction.

Cell cycle analysis

Cells were carefully dissociated into single-cell suspensions by trypsin (Gibco), washed twice with PBS, and then fixed overnight with cold 70% ethanol. Fixed cells were washed twice with PBS, followed by ribonuclease (100 μ g/ml; Sigma-Aldrich) treatment and PI (50 μ g/ml; Sigma-Aldrich) staining for 30 min at 37°C. Approximately 1×10^6 cells were analyzed using FACSCanto II (Becton Dickinson) to determine the cell cycle distribution pattern. The percentages of cells in the G₁, S, and G₂-M phases of the cell cycle were analyzed using ModFit 4.1 (Verity Software House).

Teratoma formation

For generation of teratoma in vivo, 5×10^6 ciCECs were subcutaneously injected into each recipient NOD/SCID mouse ($n = 5$). Control NOD/SCID mice ($n = 3$) were injected with 2×10^6 mESCs, and teratoma formed from 4 to 8 weeks. Then, images of mice were captured with the cell phone imaging system.

Transplantation

All rabbits weighing 2.0 to 2.5 kg were anesthetized intramuscularly with ketamine hydrochloride (60 mg/kg) and xylazine (10 mg/kg; Bayer). The rabbits were divided into two groups ($n = 10$ each group), and the right eye was used for this experiment. After disinfection and sterile draping of the operation site, a 6-mm corneal

incision centered at 12 o'clock was made with a slit knife, and a viscoelastic agent (Healon; Amersham Pharmacia Biotech AB) was infused into the anterior chamber. After the corneal surface had been ruled with a marking pen (Devon Industries Inc.), a 6.0-mm-diameter circular aperture for descemetorhexis was created in the center of the cornea with a 30-gauge needle (Terumo), and Descemet's membrane was removed from the anterior chamber of the eye. The corneal endothelium was mechanically scraped from Descemet's membrane with a lacrimal passage irrigator (Shandong Weigao) as previously described. Fsp-ciCECs were dissociated using 0.25% trypsin-EDTA, resuspended in basic medium at a density of 1×10^7 cells/ml, and kept on ice. The anterior chamber was washed with PBS three times. After this procedure, a 26-gauge needle was used to inject 1×10^6 cultured ciCECs suspended in 100 μ l of basic DMEM containing 10 μ M ROCK inhibitor Y-27632 (Selleck) into the anterior chamber of the right eye. Thereafter, rabbits in the ciCEC transplantation groups were kept in the eye-down position for 24 hours under deep anesthesia so the cells could become attached by gravitation. Each surgical eye was checked two or three times a week by external examination, and photographs were taken on days 3, 7, 14, and 28 after injection. Central corneal thickness was measured using the Spectralis BluePeak OCT unit (Heidelberg Engineering, Heidelberg, Germany), and CECs were imaged with confocal scanning laser ophthalmoscopy (HRT3, Heidelberg Engineering, Heidelberg, Germany) on days 0.5, 1, 3, 7, 14, 21, 28, 35, and 42 after surgery. An average of three readings was taken. Corneal transparency was scored using a scale of 0 to 4 as previously described (58), where 0 = completely clear; 1 = slightly hazy, iris and pupils easily visible; 2 = slightly opaque, iris and pupils still detectable; 3 = opaque, pupils hardly detectable; and 4 = completely opaque with no view of the pupils. Photographs of ocular surface were taken with slit-lamp microscopy (SLM-8E, KANGHUA, China) at each time point.

Cell proliferation assay

The proliferation rate of ciCECs cultured in differentiation medium was determined by the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen) according to the manufacturer's instructions. Briefly, passaged CECs were seeded onto a slide at a lower density of 5×10^3 cells per cm² and cultured for 24 hours.

TEM analysis

For TEM analysis, cells were fixed in 2.5% EM-grade glutaraldehyde (Servicebio) for 2 to 4 hours at 4°C, washed with 0.1 M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide for 2 to 4 hours at 4°C, and washed and then dehydrated in an ethanol series (50 to 100%) to a final rinse in 100% acetone, followed by 2-hour incubations in 1:1 acetone/Pon 812 (SPI) and overnight incubation in 1:2 acetone/Pon 812. The samples were embedded in Pon 812, polymerized for 48 hours at 60°C, and then sectioned (60 to 80 nm) with a diamond knife (Daitome). Sections were stained with 2% uranyl acetate, followed by lead citrate, and visualized using an HT7700 transmission electron microscope (HITACHI).

Dil-Ac-LDL uptake and FITC-lectin staining assay

ciCECs and pCECs were cultured for 24 hours and then incubated with Dil-Ac-LDL (10 μ g/ml) (Invitrogen) in culture medium at 37°C for 6 hours. Cells were washed three times with PBS and stained with FITC-lectin (10 mg/ml) (Sigma-Aldrich) at 37°C in the dark for 2 hours. Thereafter, cells were fixed with 4% paraformaldehyde

for 15 min. The cells were imaged using an inverted fluorescence microscope.

Karyotype analysis

Cells were treated with colcemid (0.1 µg/ml) (Gibco) at 37°C for 2 hours, trypsinized, resuspended, and incubated in 0.075 M potassium chloride for 15 min at 37°C, fixed with 3:1 methanol:acetic acid, and then dropped onto slides to spread the chromosomes. The chromosomes were visualized by Giemsa (Solarbio) staining.

Statistical analysis

All experiments were independently performed at least three times. The results are expressed as means ± SD. The data were analyzed by unpaired two-tailed Student's *t* tests for comparisons of two groups and by one-way analysis of variance (ANOVA) with Tukey's test or Dunnett's multiple comparisons test for comparisons of multiple groups. All analyses were performed using SPSS Statistics 19.0 software. *P* < 0.05 was considered significant. The accession number for the RNA-seq data reported in this paper is National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO): GSE162889.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/7/23/eabg5749/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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Shao-Hui Pan, Ning Zhao, Xiang Feng, Ying Jie and Zi-Bing Jin

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