


# Conjunctival reconstruction via enrichment of human conjunctival epithelial stem cells by p75 through the NGF-p75-SALL2 signaling axis

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## Funding information

Science and Technology Commission of Shanghai Grant, Grant/Award Numbers: 19JC1411703, 17411963800; Shanghai Municipal Education Commission-Gaofeng Clinical Medicine Grant Support, Grant/Award Number: 20161421; National Natural Science Foundation of China, Grant/Award Numbers: 31500835, 31800809, 81800873, 81770888; National Key R&D Program of China, Grant/Award Numbers: 2018YFC1106101, 2018YFC1106100

## Abstract

Severe conjunctival diseases can cause significant conjunctival scarring, which seriously limits eye movement and affects patients' vision. Conjunctival reconstruction remains challenging due to the lack of efficient methods for stem cells enrichment. This study indicated that p75 positive conjunctival epithelial cells (CjECs) were mainly located in the basal layer of human conjunctival epithelium and showed an immature differentiation state *in vivo*. The p75 strongly positive (p75++) CjECs enriched by immuno-magnetic beads exhibited high expression of stem cell markers and low expression of differentiated keratins. During continuous cell passage cultivation, p75++ CjECs showed the strongest proliferation potential and were able to reconstruct the conjunctiva *in vivo* with the most complete structure and function. Exogenous addition of NGF promoted the differentiation of CjECs by increasing nuclear localization of SALL2 in p75++ CjECs while proNGF played an opposite role. Altogether, p75++ CjECs present stem cell characteristics and exhibit the strongest proliferation potential so can be used as seed cells for conjunctival reconstruction, and NGF-p75-SALL2 signaling pathway was involved in regulating the differentiation of CjECs.

## KEYWORDS

conjunctival epithelial stem cells, conjunctival reconstruction, NGF, p75, proNGF, SALL2

## 1 | INTRODUCTION

The structural and functional integrity of the conjunctiva plays a crucial role in maintaining ocular surface homeostasis.<sup>1,2</sup> Many common conjunctival disorders, such as acid-base chemical injury, thermal burn, Stevens-Johnson syndrome, and mucous membrane pemphigoid, can lead to conjunctival deficiency and severe symblepharon.<sup>3-6</sup>

Significant conjunctival scarring caused by conjunctival deficiency can result in limited eye movement and even ocular surface failure, which seriously affect the vision of patients.<sup>7-9</sup> Currently, surgical reconstruction of the conjunctiva is the main treatment method, but it is still faced with several problems such as graft restriction and incomplete functional substitution.<sup>10-12</sup> Therefore, there is an urgent need to develop improved conjunctiva reconstruction methods.

Current conjunctival reconstruction techniques mainly focus on the available substrates, including biological substrates, such as

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amniotic membrane and acellular conjunctival matrix, biosynthetic substrates, such as collagen and fibrin, and synthetic substrates, such as PLGA (poly(lactic-co-glycolic acid), PLGA) and hydrogel.<sup>13-15</sup> However, there is still a problem of insufficient seed cells for conjunctival reconstruction. Autologous conjunctival epithelial stem cells are considered to be the most suitable seed cells for conjunctiva reconstruction as they are easy to access, have strong amplification ability and are not rejected by the immune system.<sup>16</sup> Studies have shown that conjunctival epithelial stem cells were distributed in many parts of the conjunctiva, including the fornix, bulbar conjunctiva, palpebral conjunctiva, and the palpebral margin,<sup>17-19</sup> especially in the medial canthal and inferior forniceal areas.<sup>20</sup> Although various stem cell markers of conjunctival epithelium have been identified including ABCG2, p63, and integrins,<sup>21-23</sup> no effective methods have been developed for the enrichment of conjunctival epithelial stem cells. The lack of methods for this purpose has limited the development of conjunctival reconstruction and retarded the advancement of the treatment process of patients with severe conjunctival defects.

The low-affinity neurotrophic factor receptor, p75, is a member of the tumor necrosis factor receptor superfamily.<sup>24</sup> p75 can not only participate in the regulation of cell growth and apoptosis, but also characterize stem cells in various epithelial tissues.<sup>25-28</sup> In conjunctival epithelium, the expression of p75 has been detected in the basal layer rather than in the upper layers, suggesting the potential localization of putative stem cells in human conjunctival epithelium.<sup>19</sup> The potential of p75 as a marker for the enrichment of human conjunctival epithelial stem cells is yet to be determined. In addition, the mechanism of p75 in regulating the growth and differentiation of conjunctival epithelial stem cells has not yet been delineated.

p75 can bind to a variety of neurotrophic factors including NGF, BDNF, and NT3/4,<sup>29-31</sup> and acts to regulate survival, differentiation, and apoptosis.<sup>32</sup> Similar to many other neurotrophic factors, NGF is also first synthesized as an immature precursor called proNGF, which is then proteolytically cleaved intracellularly by furin, or extracellularly by matrix metalloproteinase, before finally being released as mature ligands.<sup>33</sup> Both NGF and proNGF can act as ligands for p75; however, they perform opposite biological functions under certain physiological conditions.<sup>34</sup> It has been found that NGF may have a positive impact on the proliferation and differentiation of CjECs (conjunctival epithelial cells).<sup>35</sup> To date, the effect of proNGF on CjECs has not been reported.

SALL2 is a downstream transcription factor of p75 that has been reported to play important roles in neurogenesis and eye development.<sup>36</sup> It has been confirmed that SALL2 is conservatively expressed in the cornea, lens, and retina at different developmental stages, and mutations of SALL2 can result in recessive ocular coloboma.<sup>37</sup> Recently, the role of SALL2 in regulating the fate of stem cells has been the subject of much interest.<sup>38</sup> Pincheira et al found that after the activation of p75 signaling pathway by NGF, SALL2 dissociates from the intracellular domain of p75, and then enters the nucleus to initiate transcription, consequently affecting the differentiation of nerve cells.<sup>39</sup> Currently, no relevant studies have explored the role of SALL2 in the growth and differentiation of CjECs.

### Significance statement

Severe conjunctival diseases can cause significant conjunctival scarring, which seriously limits eye movement and affects patients' vision. Currently, surgical reconstruction of the conjunctiva is the main treatment method, but remains challenging due to the lack of efficient methods for stem cells enrichment. In this study, the potential of p75 as a stem cell marker to enrich conjunctival epithelial stem cells for conjunctival reconstruction was investigated. This simple and effective method of enriching conjunctival epithelial stem cells provides the potential to obtain sufficient seed cells for functional conjunctival reconstruction and may promote the treatment of conjunctival deficiency in clinical practice.

In this study, the potential of p75 as a stem cell marker to enrich conjunctival epithelial stem cells for conjunctiva reconstruction was investigated along with investigation of the related molecular mechanisms. Conjunctival reconstruction in vitro and animal models were used to verify whether p75++ CjECs are a rich source of seed cells for conjunctival defects repair. This study aims to provide a simple and effective method for the enrichment of conjunctival epithelial stem cells that has the potential to provide a good source of seed cells for conjunctival reconstruction. Our approach has the potential to promote the treatment of patients with large conjunctival defects.

## 2 | MATERIALS AND METHODS

### 2.1 | Human conjunctival epithelial cell isolation and culture

Human conjunctival tissues were obtained from the Eye Bank of the Ninth People's Hospital affiliated to Shanghai Jiao Tong University, School of Medicine. A total of 15 conjunctival samples came from nine different voluntary donors under informed consent. All of the remaining samples were used for cell culture and enrichment, excluding the 20 sections selected from 10 conjunctival samples used for immunofluorescence staining. Each conjunctival sample has been fully used. Conjunctival samples were obtained mainly from superior/inferior fornix and bulbar conjunctiva. The size of the conjunctival sample was approximately 2 cm × 2 cm. During the operation, the sclera, limbus, and meibomian glands should be carefully handled to avoid damage. Also, it is important to be careful with sterility to prevent bacterial or fungal contamination. The methods of cell isolation and cultivation have been described previously.<sup>40,41</sup> Briefly, conjunctiva was rinsed and soaked several times in phosphate buffered saline (PBS) containing penicillin, and incubated in Dispase II (neutral protease, grade II) (5 mg/mL, Roche Diagnostics GmbH, Mannheim, Germany) at 4°C for 14 to 16 hours. The detached epithelial layer was dispersed into single cells by digestion with

Collagenase A (0.001 mg/mL, Roche Diagnostics GmbH) and trypsin (Gibco, Fremont, California) for 15 to 20 minutes at 37°C. Cells were seeded on type I collagen-coated culture dishes and cultured with DMEM/F-12 (dulbecco's modified eagle medium, Gibco) supplemented with human keratinocyte growth factor (Peprotech, Rocky Hill, New Jersey), Y27632 (Sigma-Aldrich, St. Louis, Michigan), and B27 (Thermo Fisher Scientific, Waltham, Massachusetts).

## 2.2 | Rabbit conjunctival epithelial cell isolation and culture

Rabbit conjunctival tissues were obtained from New Zealand white rabbits weighing approximately 1.5 kg. All animal procedures were approved by and carried out in accordance with the Experimental Animal Ethics Committee. Nine rabbits were anesthetized and then killed by air embolism. Sterile instruments were used to separate all conjunctival tissues from both of the rabbit's eyes. Next, rabbit conjunctival epithelial cells were isolated and cultured in a manner similar to human conjunctival epithelial cells mentioned above.

## 2.3 | p75++ CJECs enrichment

p75++ CJECs were enriched using an EasySep Human FITC Positive Selection Kit (STEMCELL Technologies, Canada). The suspensions of CJECs were mixed with an FcR blocker for 15 minutes and then labeled with an FITC-conjugated p75 primary antibody (Novus Biologicals) for another 15 minutes. Cells were then washed with EasySep buffer (STEMCELL Technologies), incubated with a selection cocktail for 15 minutes and magnetic particles for another 10 minutes. Cells bound to the magnetic beads were isolated under a strong magnetic field and treated as p75++ CJECs. To increase the sorting efficiency, a second screening among the remaining cells was performed. A sub-population of cells could not be adsorbed and were treated as p75+ CJECs, while those still left were treated as p75- CJECs.

## 2.4 | Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from different subsets of CJECs and the relative mRNA expression level was analyzed by the Pfaffl method.<sup>42</sup> cDNA was synthesized from 2000 ng total RNA using a high capacity reverse transcription kit for RT-PCR (Takara, Japan). Real-time PCR was carried out on an ABI Prism 7000 instrument using SYBR Green PCR Master Mix (Life Technologies). The primers sequences used are shown in Table 1.

## 2.5 | Western blot analysis

Total protein lysates were extracted from different subgroups of CJECs using Lysis Buffer (Thermo Fisher Scientific) and added to

**TABLE 1** Primer sequences

p75	Forward primer	TGAGTGCTGCAAAGCCTGCAA
	Reverse primer	TCTATCCTGGTAGTAGCCGT
ABCG2	Forward primer	GACTTATGTTCCACGGGCT
	Reverse primer	GGCTCTATGATCTCTGTGGCTTT
p63	Forward primer	ATTGCTTTTAGCCTCCCGGC
	Reverse primer	GGGTTTCTACGAAACGCTGG
K4	Forward primer	GAAGCAGTGCCAGACTCTTCA
	Reverse primer	GGTAGGTGGCGATCTCGATG
K13	Forward primer	TGGTTCCACACCAAGAGTGC
	Reverse primer	TGCTGGTCTGAATCATGGCA
GAPDH	Forward primer	GGTCGGAGTCAACGGATTT
	Reverse primer	CCAGCATCGCCCACTTGA

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Takara). Proteins were separated on a 10% polyacrylamide gel (Bio-rad laboratories, Hercules, California) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, Massachusetts). Membranes were blocked with 5% bovine serum albumin (BSA) in TBS buffer before being incubated with primary antibodies (p75, Cell Signaling Technology, Danvers, Massachusetts; K4, Abcam, Cambridge, Massachusetts; K13, Abcam; SALL-2, Santa Cruz Biotechnology, Santa Cruz, California;  $\beta$ -actin, Abcam) at 4°C overnight, and then treated with fluorescent-conjugated secondary antibody for 1 hour at room temperature.

## 2.6 | Immunofluorescence staining

To detect the in vivo distribution pattern of p75++ CJECs, a total of 20 sections were selected from 10 conjunctival samples of five different donors for analysis. After fixation with 4% paraformaldehyde (PFA) samples were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 30 minutes, and then blocked with 5% donkey serum for 1 hour at room temperature. Primary antibodies against p75 (Cell Signaling Technology) and K4 (Abcam) were used to stain both cell and tissue samples. ABCG2 (Abcam), p63 (Santa Cruz), and SALL2 (Santa Cruz) were used in the cell samples. After incubation at 4°C overnight, samples were then incubated with Alexa Fluor-conjugated secondary antibodies. After extensive washing, nuclei were stained with DAPI for 10 minutes.

## 2.7 | Colony-forming efficiency assay

Cells were plated onto a six-well culture dish at a density of 2000 per well and cultured for 7 days. Three replicates were set for each subgroup. Colonies were fixed with 4% PFA and stained with 0.1% crystal violet. After extensive washing, colonies were counted independently by three investigators. Colony-forming efficiency (CFE) was calculated by the number of colonies/number of cells seeded  $\times$  100.

## 2.8 | CCK8 assay

Different subgroups of CjECs were seeded onto a 96-well culture dish at a density of 1000 per well. Six replicates were set for each subgroup. Ten microliters of the Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan) was added into each well and incubated for 4 hours at 37°C. The absorbance at 450 nm was measured every 24 hours until day 8.

## 2.9 | Cell proliferation assay

A total of  $10^6$  cells of each subset were seeded in triplicate onto 10 cm culture dishes for continuous cell passage cultivation. Culture medium was changed every 2 days. Cell passage was carried out in a ratio of 1:3 when they became confluent. A hemocytometer was used to count each subgroup of cells every 5 days.

## 2.10 | Multilayer conjunctival reconstruction in vitro

In order to remove epithelial cells and prepare amniotic basement membrane, human amniotic membrane was first digested by mild trypsin at 37°C for 5 to 10 minutes, and then followed by mechanical curettage of the epidermis with a sterile scraper. Each group of conjunctival epithelial cells was inoculated on the amniotic basement membrane, respectively, and cultured continuously for 7 to 10 days in vitro until the multilayer conjunctival epithelium was formed. The reconstructed human multilayer conjunctiva was used for immunohistochemical detection, while the rabbit multilayer conjunctiva was used for transplantation of rabbit conjunctiva defects.

## 2.11 | Rabbit conjunctival defect model

Eighteen New Zealand white rabbits were anesthetized by intramuscular injection of ketamine and xylazine for the construction of rabbit conjunctival defect model. Among them, 12 eyes of three rabbits were used in the control group, including six eyes in the negative control group (no transplanted tissue) and six in the positive control group (normal eyes). The experimental group included the amniotic membrane transplantation group (AM), the total group, the p75++ group, and the p75– group. Each group also included six eyes. All animal procedures were approved by and carried out in accordance with the Experimental Animal Ethics Committee. Round conjunctival defects were produced at the bulbar conjunctiva on both eyes. A 7 mm diameter vacuum trephine was used to mark the extent of the defect, and then scissors were used to completely remove the conjunctiva. The area of conjunctival defect was stained with Lissamine Green B (Sigma-Aldrich).

## 2.12 | Histology and immunohistochemical staining

Histomorphology was detected by H&E staining as previously described.<sup>43,44</sup> Briefly, tissue samples were cut into 8  $\mu$ m sections and stained with H&E. Immunohistochemical staining also followed the previously described method. Briefly, the sections were incubated with 5% donkey serum at room temperature for 1 hour and stained with primary antibodies (K13, Abcam; MUC5AC, Invitrogen; ZO-1, Santa Cruz). Then, sections were incubated with appropriate secondary antibodies (1:2000, Abcam) at room temperature for 1 hour and examined under a fluorescence microscope.

## 2.13 | Statistical analysis

Data are presented as mean  $\pm$  SD. All experiments were performed with at least three repeats. Two-sample equal variance t test was used to analyze the significant differences. A value of  $P < .01$  was considered statistically significant.

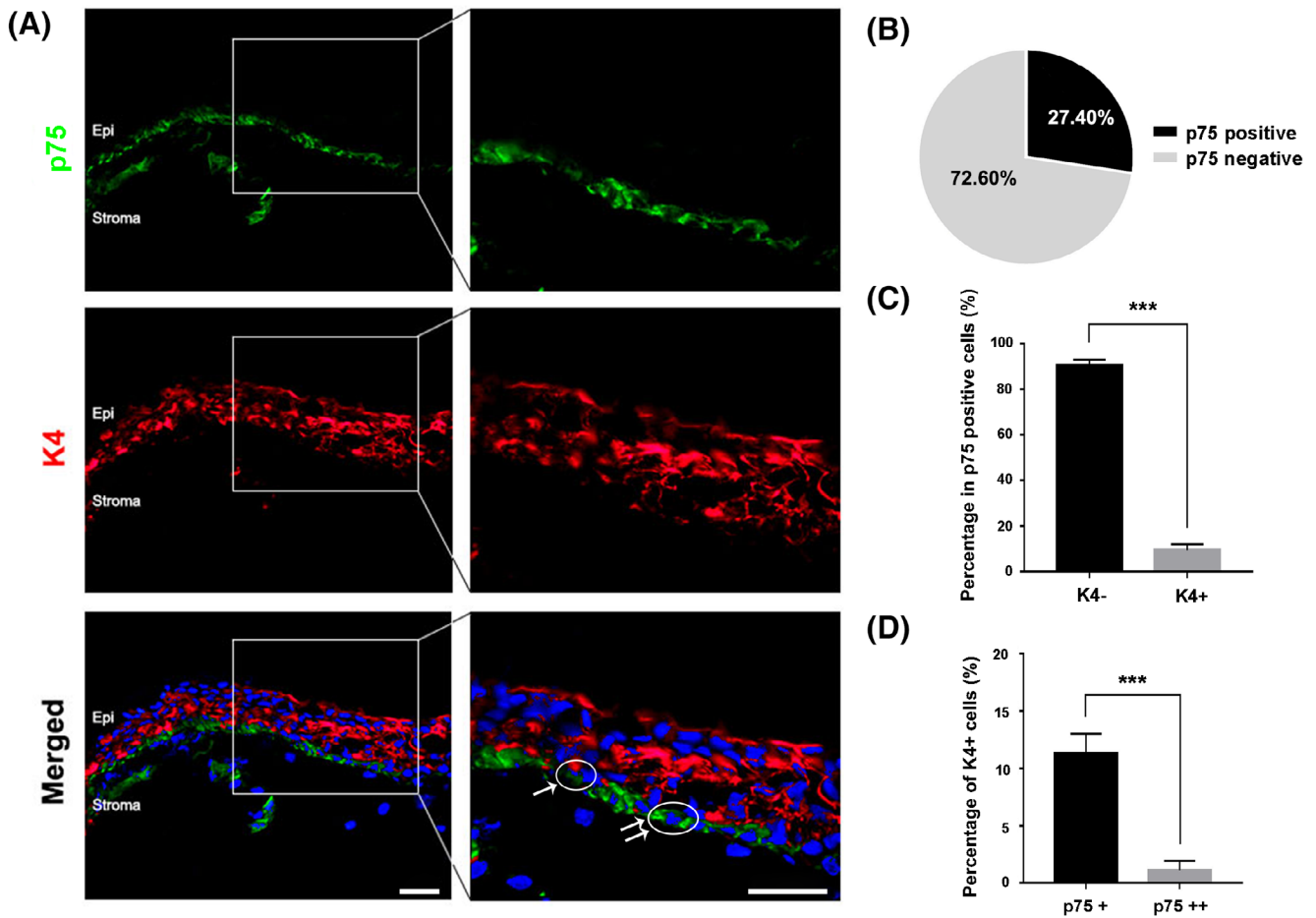
# 3 | RESULTS

## 3.1 | Expression pattern of p75 in human conjunctival epithelium

The expression pattern of p75 in human conjunctival epithelium was analyzed by immunofluorescence staining. The results showed that in the multilayer conjunctival epithelium, a proportion of cells showed p75 positive expression (Figure 1A). These p75 positive cells made up a relatively small fraction of the total number of cells at approximately  $27.40\% \pm 7.42\%$ , while the remaining majority were negative for p75 expression (Figure 1B). It was worth noting that almost all of the p75 positive cells were located in the basal layer of the laminated conjunctival epithelium, where epithelial stem cells were thought to exist.<sup>45</sup>

To further explore the possibility of p75 as a characteristic of conjunctival epithelial stem cells in vivo, we costained with K4, a differentiation-associated keratin of CjECs, to analyze the differentiation status of the p75 positive cells. After analyzing a total of 20 sections from five different donors, we found that CjECs with high expression of p75 showed low expression of K4. K4 positive CjECs were mainly located in the upper layers of the conjunctival epithelium and infrequently colocalized with p75 positive CjECs (Figure 1A). Among the p75 positive CjECs, K4 negative CjECs accounted for the majority ( $90.51\% \pm 2.45\%$ ), which was significantly higher than the number of K4 positive CjECs ( $9.49\% \pm 2.45\%$ ) ( $***P < .001$ , t test) (Figure 1C).

To further analyze the in vivo expression patterns of p75 and K4, we divided the p75 positive CjECs into strong and weak positive subsets according to the expression level of p75 by fluorescence intensity. Statistical analysis showed that among p75 strongly positive CjECs, K4 positive CjECs only accounted for  $1.11\% \pm 0.84\%$ , which



**FIGURE 1** In vivo expression pattern of p75 in human conjunctival epithelium. A. Representative immunofluorescence images of p75 (green), K4 (red), and DAPI (4',6-diamidino-2-phenylindole, blue) in human conjunctival epithelium. The p75 strongly positive conjunctival epithelial cells (CjECs) are shown by the double arrows and the p75 weakly positive CjECs are denoted by the single arrow. Scale bar = 50  $\mu$ m. B. The percentages of p75 positive and p75 negative CjECs (based on total 20 sections from five different donors). C. Statistics of the K4+ and K4- CjECs among p75 positive CjECs. D. The percentages of K4+ CjECs, respectively, among p75 strongly positive (p75++) and p75 weakly positive (p75+) CjECs. Data are presented as the mean  $\pm$  SD and statistically tested by a two-tailed *t* test, \*\*\**P* < .001; *n* = 5

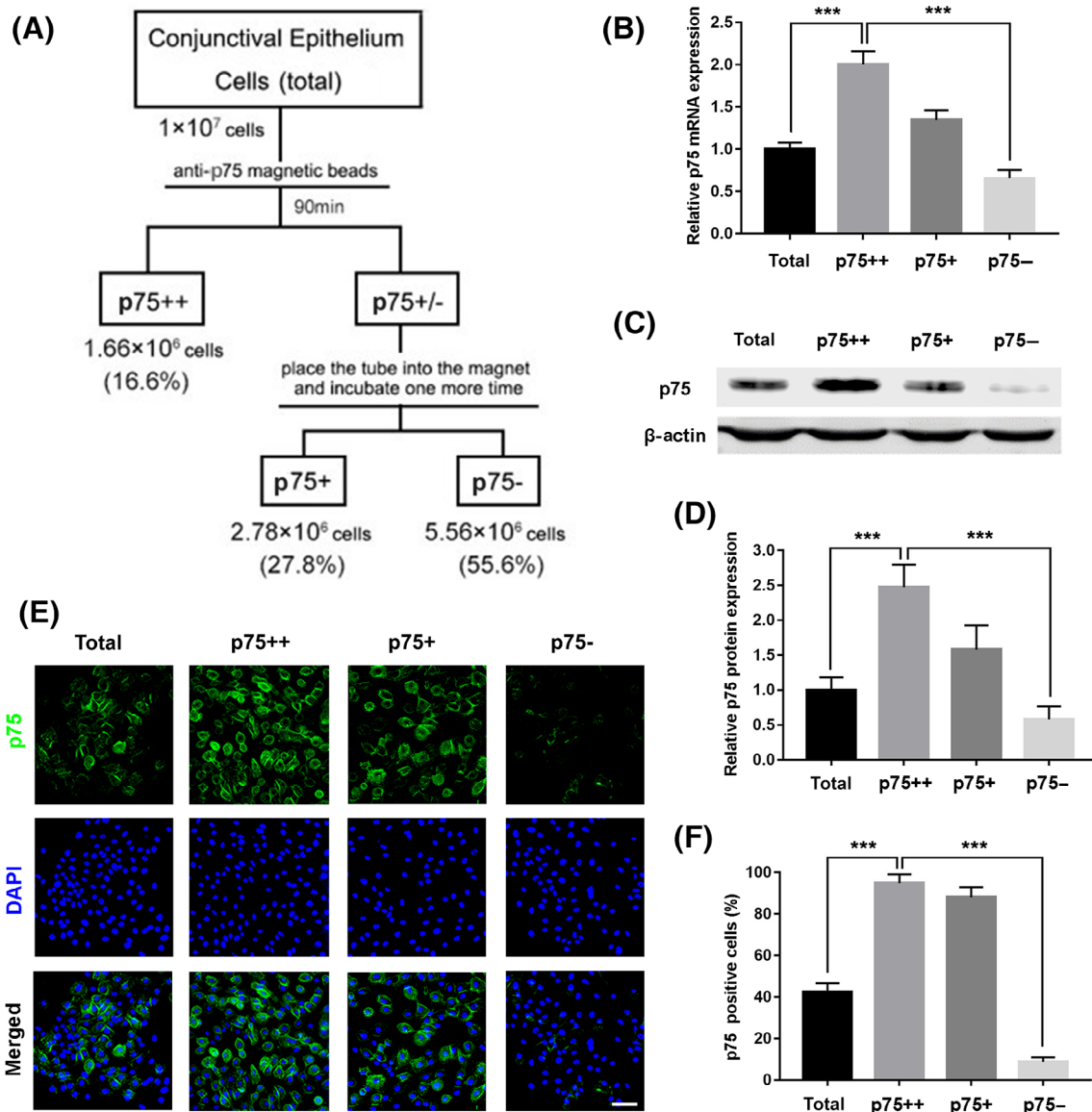
was significantly lower than that in p75 weakly positive CjECs ( $10.60\% \pm 3.29\%$ ) (\*\**P* < .001, *t* test) (Figure 1D). This expression pattern indicated that p75 was able to mark a small subpopulation of CjECs with a low-differentiation state in vivo and so the stronger the expression of p75, the lower the level of differentiation. The p75 strongly positive cells were likely to be putative conjunctival epithelial stem cells, while the weakly positive cells could be in a transition state.

### 3.2 | Enrichment and identification of p75++ CjECs

To further verify the possibility of marking conjunctival epithelial stem cells by p75, immune-magnetic beads were used to enrich p75 strongly positive (p75++) CjECs. Here, CjECs were coincubated with p75 indirectly labeled immunomagnetic beads and screened through magnetic adsorption (see Section 2 for details). Cells adsorbed at the first screening had a stronger washing intensity

and were treated as P75++ cells. Then, a second screening was performed among the remaining cells for a further 5 minutes to isolate p75 weakly positive (P75+) CjECs. The cells, which finally remained, were treated as p75 negative (p75-) CjECs. The sorting effectiveness was further verified in the following experiments. Based on this approach, the total CjECs were separated into three different subsets according to the expression levels of p75: p75++ CjECs, accounting for approximately  $16.6\% \pm 1.28\%$ ; p75+ CjECs, accounting for  $27.8\% \pm 3.09\%$ ; and p75- CjECs, accounting for  $55.6\% \pm 4.43\%$  (Figure 2A).

To ensure the effectiveness of enrichment, we used RT-PCR, Western blot, and immunofluorescence staining to test the efficiency of separation. RT-PCR showed that the expression levels of p75 in p75++ CjECs were significantly higher than the other subgroups, followed by p75+ and total CjECs, while p75- CjECs were below the unsorted level (Figure 2B). Western blot and immunofluorescence staining both confirmed the expression of p75 at the protein level (Figure 2C-F).

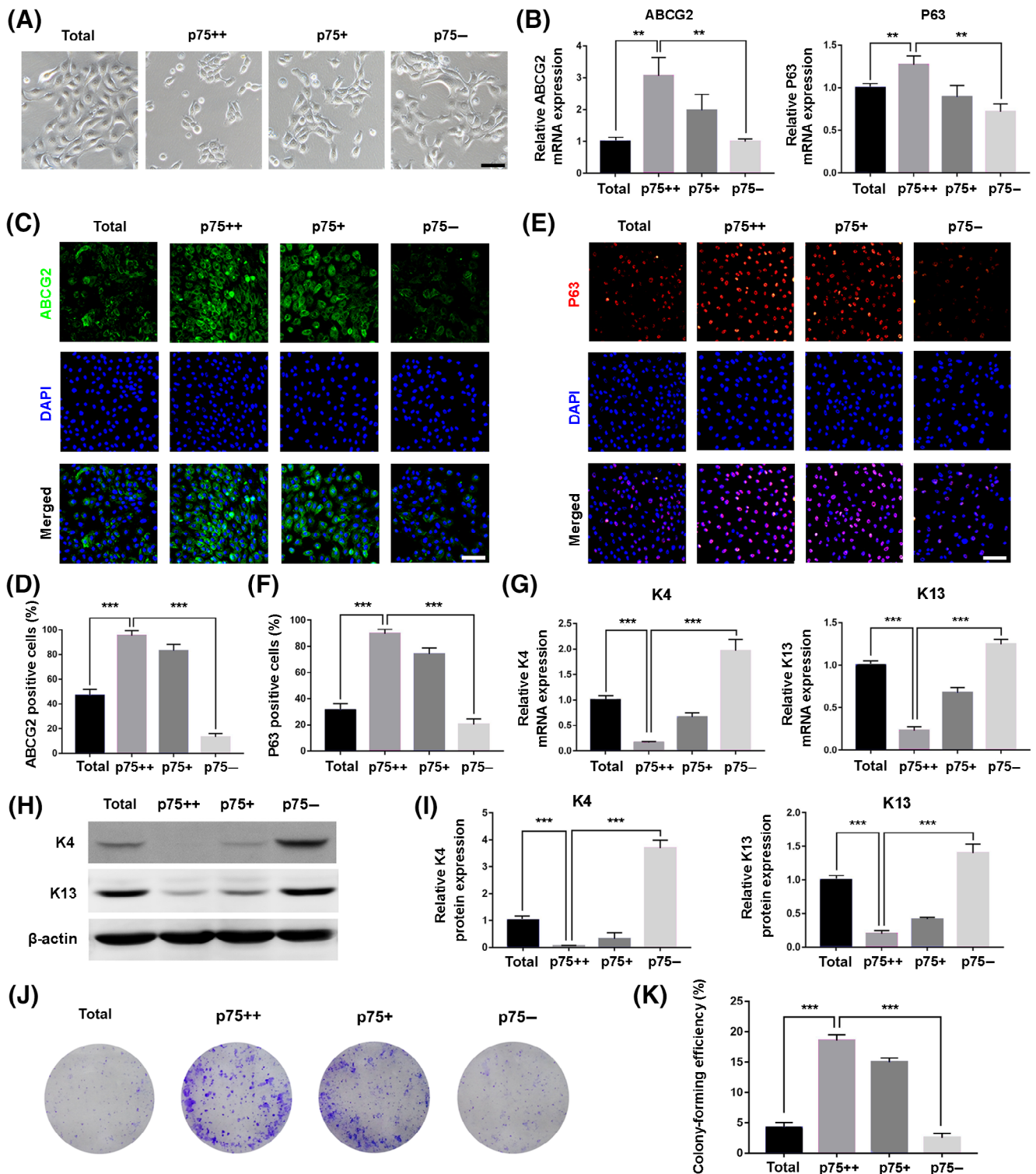


**FIGURE 2** Enrichment and identification of p75<sup>++</sup>, p75<sup>+</sup>, and p75<sup>-</sup> conjunctival epithelial cells (CjECs). A, Schematic diagram of cell sorting by immunomagnetic beads and the statistics of cell numbers in each subgroup. During the process, cells absorbed through the magnetic field after the first strong elution were recorded as p75<sup>++</sup> CjECs. In the second sorting of the remaining CjECs, cells absorbed after a weak elution were recorded as p75<sup>+</sup> CjECs, while those that were still unable to be adsorbed were recorded as p75<sup>-</sup> CjECs. The percentages of the three cell subsets were 16.6%, 27.8%, and 55.6%, respectively, which were consistent in multiple rounds of sorting. B, mRNA expression level of p75 in each cell subgroup analyzed by reverse transcription-polymerase chain reaction (RT-PCR). GAPDH (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. C, Representative Western blot analysis of p75 in each subgroup.  $\beta$ -actin was used as an endogenous control. D, Quantification of relative p75 protein expression. The gray values of the p75 bands in each group were compared to those of  $\beta$ -actin. Statistical results were standardized to the total. E, Representative immunofluorescence images of p75 (green) and DAPI (4',6-diamidino-2-phenylindole, blue) in each cell subset when attached to the dish 6 hours after isolation. Scale bar = 50  $\mu$ m. F, Quantification of the percentage of p75 positive cells. Data are presented as the mean  $\pm$  SD and statistically tested by a two-tailed *t* test, \*\*\**P* < .001; *n* = 3

To test whether the p75<sup>++</sup> CjECs had the features of conjunctival epithelial stem cells, we undertook characterization experiments. We first observed the morphology of each subgroup at 6 hours after separation. The results showed that p75<sup>++</sup> CjECs appeared to be morphologically the smallest (Figure 3A), which was consistent with the general smaller size of stem cells.<sup>46</sup>

Then, we analyzed the expression levels of multiple markers in each subgroup by RT-PCR, Western blot, and immunofluorescence

staining. ABCG2 (surface marker) and P63 (nuclear marker), which have been widely recognized as stem cell markers of conjunctival epithelium, were significantly highly expressed in p75<sup>++</sup> CjECs (Figure 3B-F). The expressions of K4 and K13, which serve as differentiation-associated keratins of the conjunctiva, were significantly lower at both the mRNA and protein levels (Figure 3G-I), demonstrating an immature differentiation state of p75<sup>++</sup> CjECs.



**FIGURE 3** Expression levels of stem cell markers and differentiation-associated keratins in each subgroup. A, Representative images of cell morphology in each subgroup when attached to the dish 6 hours after isolation. Scale bar = 50  $\mu$ m. B, mRNA expression levels of ABCG2 and p63 in each subgroup analyzed by reverse transcription-polymerase chain reaction (RT-PCR). C, Representative immunofluorescence images of ABCG2 (green) and DAPI (4',6-diamidino-2-phenylindole, blue) of each cell subgroup when attached to the dish 6 hours after isolation. Scale bar = 50  $\mu$ m. D, Quantification of the percentage of ABCG2 positive cells. E, Representative immunofluorescence images of p63 (red) and DAPI (blue) of each cell subgroup when attached to the dish 6 hours after isolation. Scale bar = 50  $\mu$ m. F, Quantification of the percentage of p63 positive cells. G, mRNA expression levels of K4 and K13 in each subgroup analyzed by RT-PCR. H, Representative Western blot analysis of K4 and K13 in each subgroup. I, Quantification of relative K4 and K13 protein expressions. The gray values of K4 and K13 bands in each group were compared to the gray values of  $\beta$ -actin. Statistical results were standardized with the total. J, Representative images of colonies formed by each subgroup 7 days after isolation. K, Quantification of CFE. CFE was calculated as the number of clones formed compared to the cells initially seeded. Data are presented as the mean  $\pm$  SD and statistically tested by a two-tailed *t* test, \*\**P* < .01, \*\*\**P* < .001; *n* = 3. CFE, colony-forming efficiency

Since the strong colony-forming ability is one of the most important characteristics of stem cells, we further compared the CFE of p75<sup>++</sup> and p75<sup>-</sup> CJECs. The CFE assay exhibited that p75<sup>++</sup> CJECs had the highest colony-forming ability. p75<sup>+</sup> CJECs showed a relatively lower ability to form clones, but were higher than the total CJECs, while p75<sup>-</sup> CJECs had the lowest colony-forming ability (Figure 3J,K).

### 3.3 | Proliferative ability of p75<sup>++</sup> CJECs during continuous cell passage cultivation in vitro

Stem cells often show high proliferation potential, which is conducive to the rapid repair of tissue damage.<sup>45,47-49</sup> We used CCK8 assay to detect the proliferation rate of each cell subgroup during short-term cultivation. The continuous cell-counting assay was used to detect the maximum proliferation potential during the continuous cell passage cultivation process in vitro. The results showed that p75<sup>++</sup> CJECs gradually acquired the fastest proliferation rate after entering the in vitro culture process, which was significantly faster than the other three subgroups (Figure 4A).

During the process of continuous cell passage cultivation, each subgroup was continuously amplified at the same initial number and counted every 5 days. It was demonstrated that the proliferation potential of p75<sup>++</sup> CJECs was also significantly higher than the other three subsets. At the 15th day of cultivation, p75<sup>++</sup> CJECs had expanded to  $13.90 \pm 0.90$  times the initial number which was significantly higher than the total CJECs ( $4.09 \pm 0.43$  times) ( $***P < .001$ , *t* test). As expected, p75<sup>-</sup> CJECs exhibited the lowest amplification ability (Figure 4B). Ki67 staining of each cell subgroups on the 10th day showed that p75<sup>++</sup> CJECs were still in a relatively active proliferation state as they had the highest ki67 positive rate ( $16.71\% \pm 1.96\%$ ). The proliferative activity of the total CJECs decreased significantly with the positive rate at  $5.37\% \pm 0.89\%$ , and in p75<sup>-</sup> CJECs, only a few cells were found to be in the proliferating state ( $2.69\% \pm 0.77\%$ ) ( $***P < .001$ , *t* test) (Figure 4C,D).

### 3.4 | Conjunctival reconstruction ability of p75<sup>++</sup> CJECs

Since the proliferative ability of p75<sup>++</sup> CJECs during the continuous cell passage cultivation in vitro was significantly higher than other cell subsets, these cells might play an important role in conjunctival epithelium reconstruction. To explore whether p75<sup>++</sup> CJECs are able to construct the laminated conjunctival epithelium, we incubated each cell subgroup on an amniotic basement membrane and cultured the cells for 10 days. H&E staining demonstrated that the conjunctival epithelium formed by p75<sup>++</sup> CJECs appeared to be the most complete and homogeneous in morphology, which had, on average,  $9.50 \pm 1.29$  layers of cells. In contrast, the total CJECs could form a relatively weaker conjunctival epithelial tissue with  $4.25 \pm 0.65$  layers of cells. p75<sup>-</sup> CJECs could only form a single layer ( $1.63 \pm 0.48$ ) of discontinuous conjunctival epithelium, lacking the ability to construct a complete

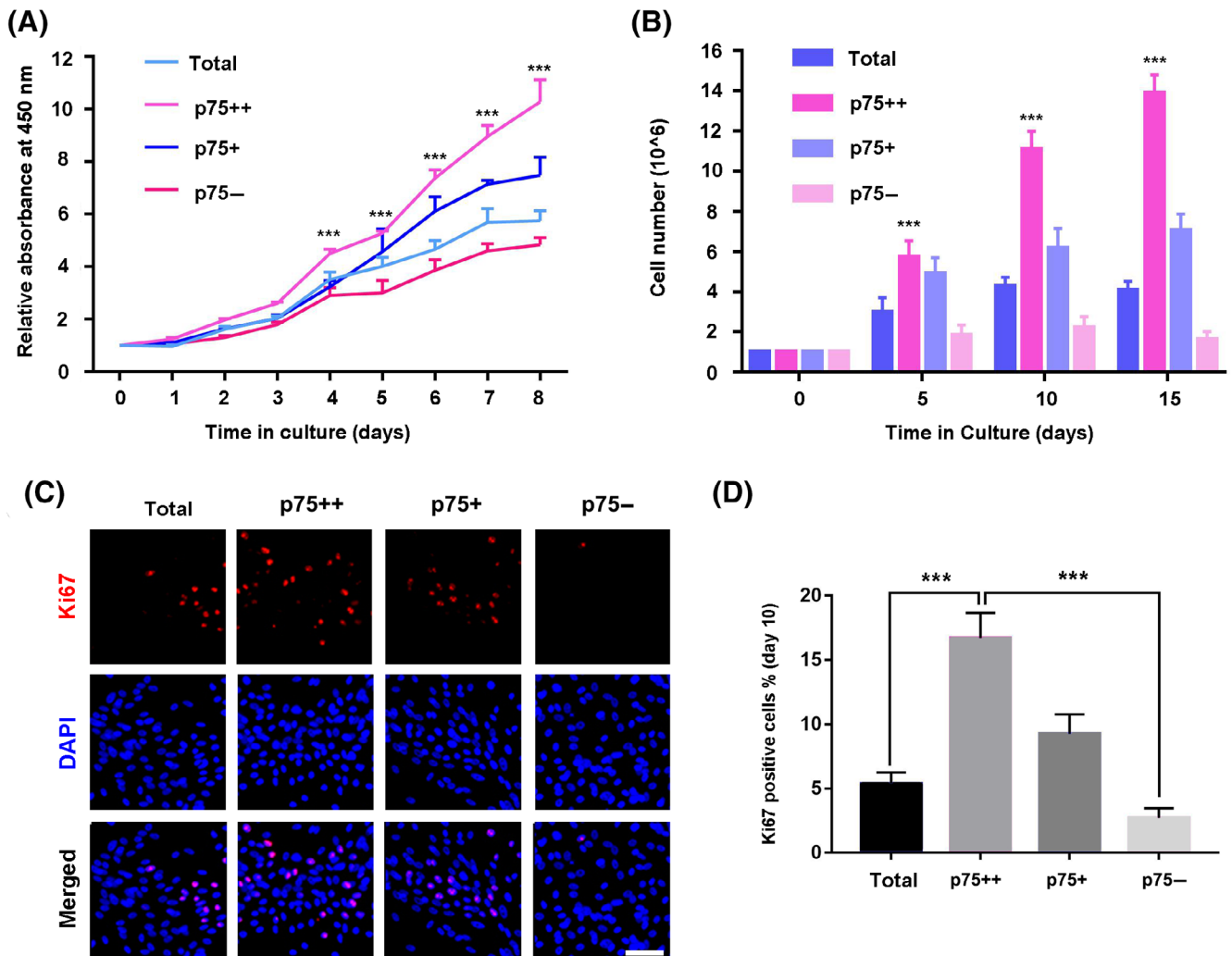
multilayer structure (Figure 5A,B). To detect the differentiation and functional status of conjunctival epithelial tissues formed by various subsets of cells, immunohistochemical staining was used to analyze the expressions of differentiated keratin K13, functional MUC5AC mucin, and tight-junction-associated protein ZO-1. The results showed that the expression levels of K13, MUC5AC, and ZO-1 in p75<sup>++</sup> CJECs formed in multilayer tissues were all higher than the total CJECs. These differentiation and functional indicators were almost not expressed in p75<sup>-</sup> CJECs formed tissues (Figure 5C), suggesting the possibility for future clinical applications of p75<sup>++</sup> CJECs in conjunctival reconstruction.

A rabbit conjunctival defect model was used to detect the ability of p75<sup>++</sup> CJECs to repair large conjunctival defects in vivo. Rabbit CJECs were cultured and used in animal experiments. The multilayer conjunctival epithelial tissue constructed by each subgroup of cells in vitro was transplanted and sutured to the defect of rabbit bulbar conjunctiva and the effect was observed. The conjunctival defect area was detected by Lissamine Green staining during continuous observation for 10 days. The results showed that among all the groups, the rate of conjunctiva repair by p75<sup>++</sup> CJECs was the fastest (Figure 5D), and was significantly better than that of p75<sup>-</sup> CJECs from day 3 (Figure 5E). By day 10, the p75<sup>++</sup> CJECs had almost completely repaired the large defect of the conjunctiva. The rabbits were killed on day 10, and the tissue within the defect area was taken for histomorphological analysis (Figure 5F). H&E staining demonstrated that tissue structure of the defect site repaired by p75<sup>++</sup> CJECs was more complete than that of the other groups, and the number of layers of the repaired conjunctiva was the most similar to that of the positive control group. periodic acid-schiff (PAS) staining showed that there were abundant goblet cells in normal conjunctival tissue, and the number of goblet cells in the p75<sup>++</sup> CJECs group was significantly higher than that of the other groups. As for the p75<sup>-</sup> CJECs group, goblet cells were almost absent. Masson staining showed that after the conjunctival defect was repaired, the organization of conjunctival stroma fibrillar in the p75<sup>++</sup> CJECs group was more consistent with the uninjured tissue. These results demonstrated that p75<sup>++</sup> CJECs were able to reconstruct the conjunctiva in vivo with the most complete structure and function.

### 3.5 | The role of the NGF-p75-SALL2 signaling pathway in the regulation of p75<sup>++</sup> CJECs

Lambiase et al reported that NGF played a key role in promoting the differentiation of CJECs,<sup>35</sup> and that p75, as a low affinity receptor of NGF, was essential in this process. It is also known that the precursor form of NGF, proNGF, can also activate the signaling pathway by binding to p75 with a relatively high affinity.<sup>33</sup> Therefore, we explored whether NGF/proNGF played different roles in p75<sup>++</sup> and p75<sup>-</sup> CJECs. Western blot showed that exogenous addition of NGF at 100 ng/mL could promote the differentiation of CJECs. The expression of K13 in the total CJECs increased by  $1.45 \pm 0.20$ -fold. In contrast, the addition of proNGF at 1000 ng/mL inhibited the





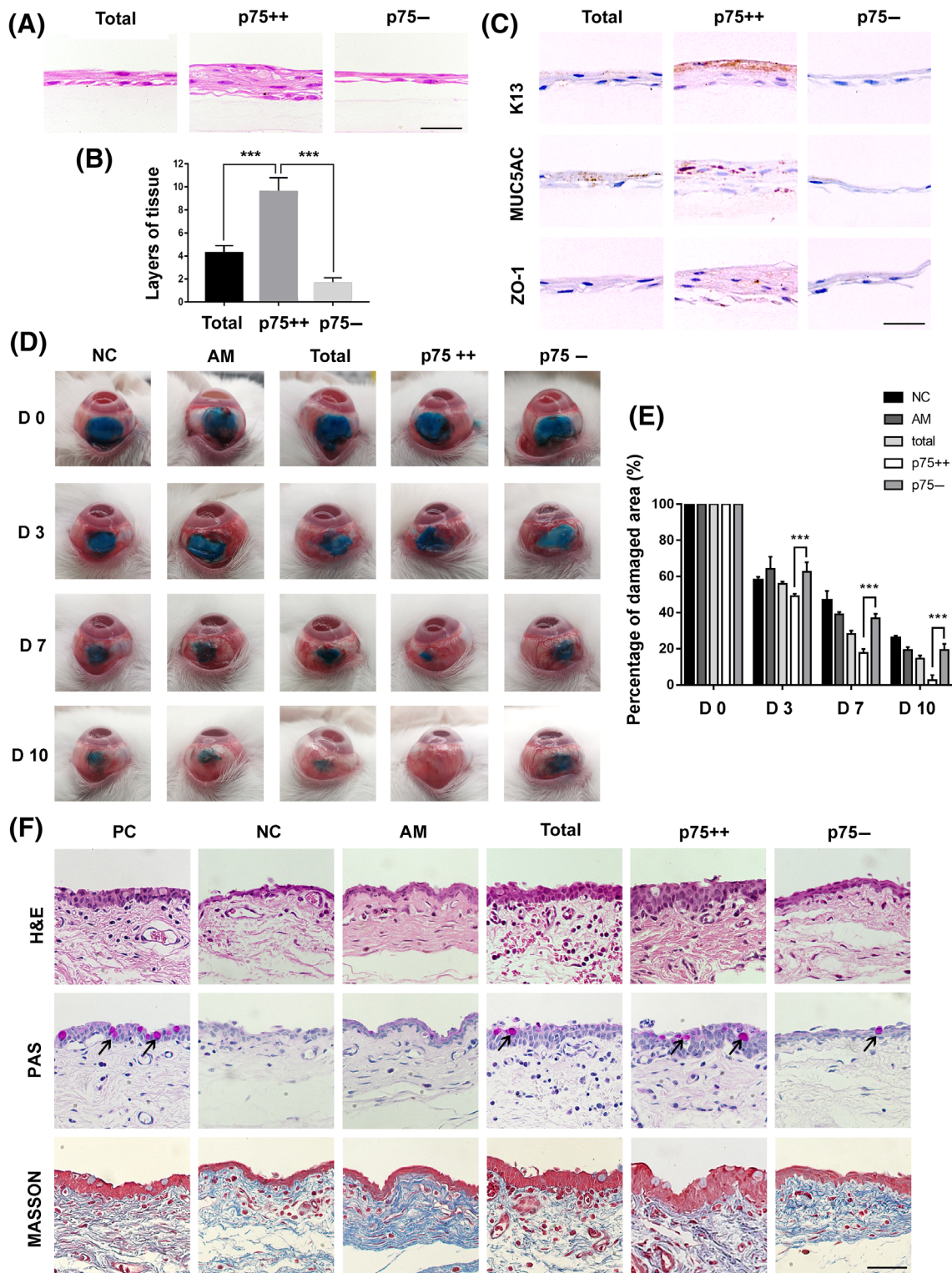
**FIGURE 4** Proliferative ability of each subgroup conjunctival epithelial cells (CjECs) during short and continuous cell passage cultivation in vitro. A, The short-term proliferative rate of each cell subgroup analyzed by CCK8 assay and quantification of relative absorbance at 450 nm. B, Proliferation potential of each cell subset. A total of 106 cells were seeded in a 10 cm culture dish and cultured continuously in vitro. Cells were passaged in a ratio of 1:3 when they became confluent. Cell counts were performed every 5 days. C, Representative immunofluorescence images of ki67 (red) and DAPI (4',6-diamidino-2-phenylindole, blue) of each cell subset on the 10th day during continuous cell passage cultivation. Scale bar = 50 μm. D, Quantification of the percentage of ki67 positive cells. Data are presented as the mean ± SD and statistically tested by a two-tailed *t* test, \*\*\**P* < .001; *n* = 3

differentiation of CjECs ( $0.81 \pm 0.03$  times) (\*\**P* < .01, \*\*\**P* < .001, *t* test) (Figure 6A,B). Interestingly, these effects were most significant in p75++ CjECs where the expression of K13 increased by  $2.37 \pm 0.12$ -fold. Neither exogenous addition of NGF nor proNGF could further influence the expression level of K13 in p75- CjECs, indicating that without the presence of p75, NGF/proNGF could not regulate the differentiation of CjECs. Therefore, NGF/proNGF regulates the differentiation status of CjECs through p75.

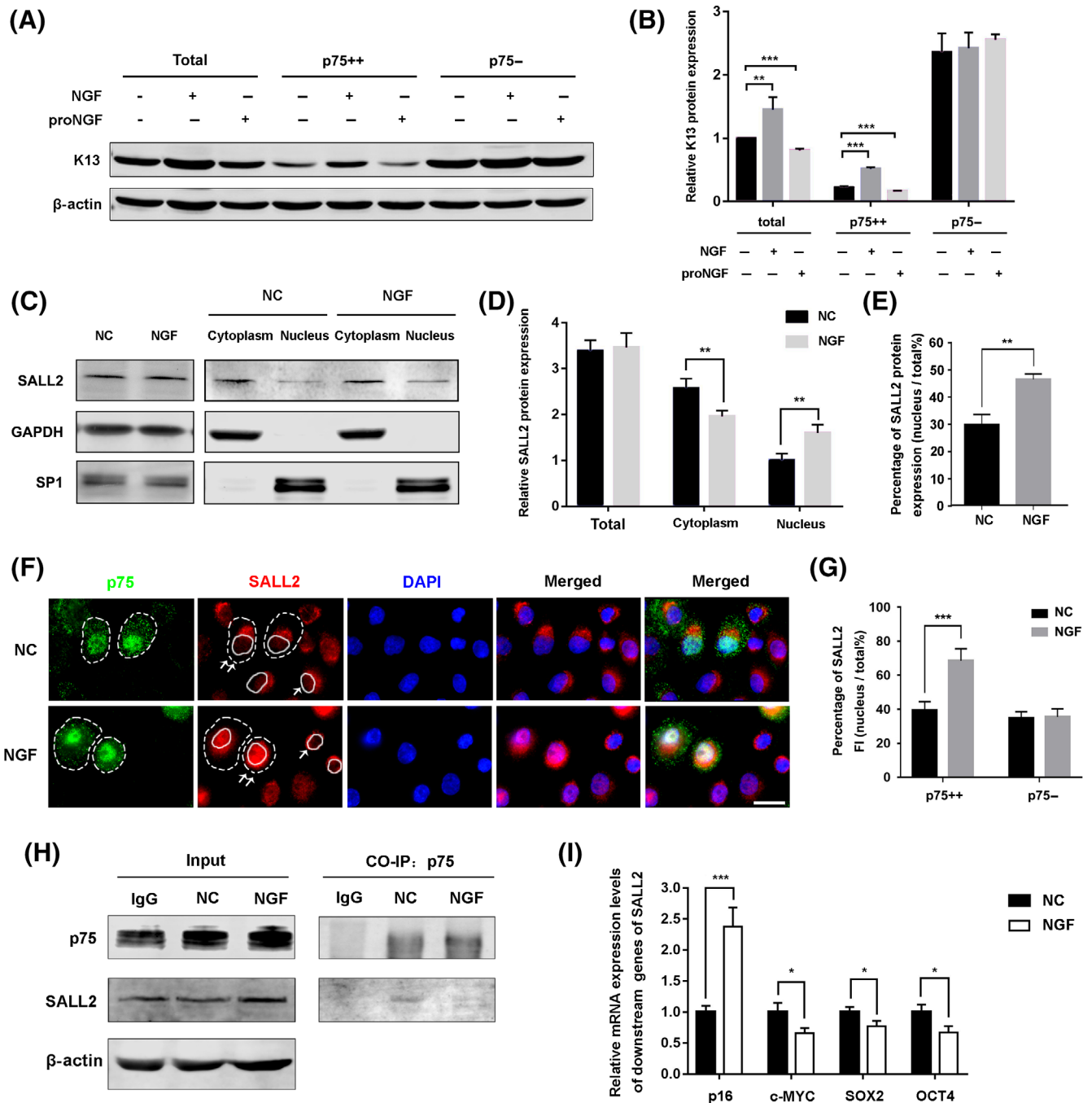
As SALL2 has been reported to participate in the regulation of neural cell differentiation as a downstream molecule of p75,<sup>39</sup> we investigated whether SALL2 could regulate CjECs differentiation after activation by NGF. As a transcription factor, the nuclear localization of SALL2 plays an important role for its biological function by controlling the transcription of downstream genes. We respectively analyzed

the expression levels of SALL2 in the nucleus and cytoplasm of CjECs before and after the treatment with NGF. Our results showed that although the total protein expression of SALL2 was not affected, NGF treatment could increase the nuclear expression of SALL2 (Figure 6C, D). The nucleus/total ratio of SALL2 after treatment with NGF increased significantly (from  $29.54\% \pm 3.85\%$  to  $46.18\% \pm 2.16\%$ ; \*\**P* < .01, *t* test) (Figure 6E).

To verify this result, immunofluorescence staining was used to detect the localization of SALL2 in CjECs with or without the addition of NGF. We costained p75, to determine the differences between p75++ and p75- CjECs in the influence of NGF on the subcellular localization of SALL2. The results showed that after the addition of NGF, the increased nuclear localization of SALL2 was accompanied by a decrease in cytoplasmic localization. This specifically occurred in



**FIGURE 5** Conjunctival reconstruction ability of each subgroup conjunctival epithelial cells (CjECs). A, Representative H&E staining images of laminated conjunctival epithelium constructed by each subgroup of cells on an amniotic basement membrane cultured for 10 days. B, Quantification of tissue layers. C, Representative immunohistochemical staining images of the laminated conjunctival epithelium constructed by each subgroup of cells. The expression levels of differentiated keratin K13, functional protein MUC5AC, and ZO-1 were analyzed. Scale bar = 50  $\mu$ m. D, Repair of conjunctival defect in rabbits by each subgroup of cells. The initial size of the conjunctival defect is 7 mm in diameter. The area of conjunctival defect was stained with Lissamine Green B. E, Quantification of the percentage of damaged area. F, Histological analysis of conjunctival defect reconstruction. The goblet cells are shown by the black arrow. Scale bar = 50  $\mu$ m. Data are presented as the mean  $\pm$  SD and statistically tested by a two-tailed t test, \*\*\* $P < .001$ ; n = 3



**FIGURE 6** The role of the NGF-p75-SALL2 signaling pathway in the regulation of p75<sup>++</sup> conjunctival epithelial cells (CjECs). A, Representative Western blot analysis of different cell subsets treated with nerve growth factor (NGF) at 100 ng/mL and pro-NGF at 1000 ng/mL for 72 hours. NGF promoted differentiation of CjECs while proNGF inhibited the differentiation, which was more obvious in p75<sup>++</sup> CjECs but not in p75<sup>-</sup> CjECs. B, Quantification of relative K13 protein expression in each subgroup. The gray values of the K13 bands in each group were compared to the gray values of  $\beta$ -actin. Statistical results were standardized with the blank control group. C, Representative Western blot analysis of the distribution of SALL2 in the nucleus and cytoplasm before and after treated with NGF at 100 ng/mL for 2 hours. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for cytoplasm and SP1 for the nucleus. The addition of NGF could increase nuclear localization of SALL2. D, Quantification of relative SALL2 protein expression. The gray values of the SALL2 bands were compared with the gray values of GAPDH and SP1. E, Percentage of SALL2 protein expression in the nucleus accounted for the total before and after NGF treatment. F, Representative immunofluorescence images of SALL2 (red), p75 (green), and DAPI (4',6-diamidino-2-phenylindole, blue) in CjECs, before and after treatment with NGF at 100 ng/mL for 2 hours. The p75 positive CjECs are shown by double arrows, and the p75 negative CjECs are shown by a single arrow. The nuclear localization of SALL2 was increased by NGF in p75<sup>++</sup> CjECs but not in p75<sup>-</sup> CjECs. Scale bar = 20  $\mu$ m. G, Percentage of SALL2 FI in the nucleus accounting for total cells before and after NGF treatment. H, Representative co-immunoprecipitation (CO-IP) analysis of the interaction of SALL2 with p75 before and after treated with NGF at 100 ng/mL for 2 hours. p75 was coimmunoprecipitated from cell lysates and Western blot was probed with antibodies to p75 and SALL2.  $\beta$ -actin was used as an endogenous control. The addition of NGF reduced the interaction of SALL2 with p75. I, mRNA expression level of downstream genes of SALL2 (p16, c-MYC, SOX2, and OCT4) before and after treated with NGF analyzed by reverse transcription-polymerase chain reaction (RT-PCR). GAPDH was used as an endogenous control. NGF treatment significantly increased the expression level of p16 and decreased the expression level of c-MYC, SOX2, and OCT4. Data are presented as the mean  $\pm$  SD and statistically tested by *t* test, \**P* < .05, \*\*\**P* < .001; *n* = 3. FI, fluorescence intensity

p75<sup>++</sup> CJECs, while in p75<sup>-</sup> CJECs, there was no significant change in SALL2 nuclear localization after NGF treatment (Figure 6F,G). These results indicated that NGF activated p75 signaling pathway, leading to the relocalization of SALL2, and thus regulated the differentiation status of p75<sup>++</sup> CJECs.

We used protein co-immunoprecipitation (CO-IP) to examine whether p75 interacts with SALL2 in human conjunctival epithelial cells. The results of CO-IP analysis showed that SALL2 interacted with p75 in conjunctival epithelial cells, and the level of SALL2 binding to p75 decreased significantly after NGF treatment (Figure 6H), suggesting that SALL2 and p75 could be dissociated by NGF activation. The results were consistent with previous studies that SALL2 bound to the intracellular domain of p75 in mouse brain lysate, and their interaction could be reduced after NGF treatment.<sup>39</sup>

In order to detect the transcriptional level of the downstream target genes of SALL2 after NGF treatment, RT-PCR was used to detect the changes of mRNA expression levels of p16, c-MYC, SOX2, and OCT4, four of the downstream target genes of SALL2 reported in previous studies.<sup>36,50-52</sup> The results showed that the expression levels of p16 and c-MYC, closely related to the regulation of cell cycle, were upregulated and downregulated, respectively. The expression levels of stemness indicators, SOX2 and OCT4, were significantly reduced after treatment of NGF (Figure 6I). Changes in the expression levels of these downstream target genes might affect the differentiation status of conjunctival epithelial cells, reducing their stemness and proliferation ability, and gradually leading to the terminal differentiation.

## 4 | DISCUSSION

Although previous study has shown that human conjunctival epithelial stem cells are more plentiful in the medial canthal and inferior forniceal areas,<sup>20</sup> to date, no studies have applied stem cell markers to the enrichment of conjunctival epithelial stem cells. Recognizing the characteristics of p75 as a membrane protein and combining it with immunomagnetic beads, we achieved the enrichment of human conjunctival epithelial stem cells *in vitro* for the first time. Immunofluorescence staining of human conjunctival tissues showed that p75 positive CJECs were mainly located in the basal layer of conjunctival epithelium and did not colocalize with K4 (Figure 1), indicating that they are in an immature differentiation state *in vivo*. We used p75 indirectly labeled immune-magnetic beads to enrich p75<sup>++</sup> CJECs (Figure 2), and found that the CJECs enriched by p75 showed excellent stem cell characteristics. The cells expressed high levels of conjunctival epithelial stem cell markers ABCG2 and p63 (Figure 3B-F), and showed a low level of differentiation-associated keratin K4 and K13 (Figure 3G-I). In addition, CFE assay indicated that p75<sup>++</sup> CJECs had the strongest colony-forming ability (Figure 3J,K). These features of p75<sup>++</sup> CJECs were all consistent with the characteristics of stem cells that have been reported previously.<sup>53,54</sup> These data further verified p75 as a stem cell marker to enrich conjunctival epithelial stem cells.

Conjunctival reconstruction methods, such as transplantation of the amniotic membrane, oral mucosa, and autologous or allogeneic

conjunctiva,<sup>55-57</sup> encounter various problems including limited sources and being easily dissolved.<sup>58,59</sup> In contrast, the cultivation of autologous conjunctival epithelial stem cells can be used as an effective method to reconstruct the conjunctiva. We observed that during a short proliferative period and continuous cell passage cultivation *in vitro*, p75<sup>++</sup> CJECs showed the fastest amplification rate and strongest proliferation potential of all the cell subsets (Figure 4). In the process of simulating the reconstruction of laminated conjunctival epithelial tissue on an amniotic basement membrane, p75<sup>++</sup> CJECs could reconstruct conjunctival epithelial tissues with the most complete multilayer structure and most appropriate expression of differentiation markers (K13) and functional markers (MUC5AC, ZO-1) (Figure 5). MUC5AC is the major mucin secreted by conjunctival goblet cells, and is an important component of tears which play a significant role in the function of conjunctival epithelium in maintaining ocular surface homeostasis.<sup>60</sup> In the future, we will continue to explore effective methods to improve the expression of MUC5AC during the differentiation of p75<sup>++</sup> CJECs and the construction of functional laminated conjunctival tissues. Taken together, these observations suggest that the enriched p75<sup>++</sup> CJECs have the characteristics of conjunctival epithelial stem cells and could be used as seed cells for the repair of conjunctival defects.

To further improve the proliferative ability and conjunctival reconstruction properties of conjunctival epithelial stem cells, it is necessary to explore the relevant molecular mechanisms underlining the process in maintaining the balance between stemness and cell differentiation. With the addition of exogenous NGF and proNGF, we found they could promote or inhibit differentiation of CJECs respectively. These findings are consistent with previous reports showing they often play opposing roles in the same physiological process.<sup>37</sup> Furthermore, the regulatory function of NGF/proNGF was particularly evident in p75<sup>++</sup> CJECs compared to the total CJECs, but had little effect on p75<sup>-</sup> CJECs (Figure 6A,B). These observations suggest that the p75 signaling pathway is involved in the regulation of the differentiation of conjunctival epithelial stem cells, and to some extent, the ratio of proNGF/NGF may determine the degree of differentiation. We speculate that p75<sup>++</sup> CJECs barely express K4/K13 and maintain the stem cell state of low-differentiation *in vivo*, which may be due to the inhibition of differentiation by proNGF. As CJECs grew and became mature physiologically, or under pathological conditions such as the repair of conjunctival defects, the role of NGF in promoting differentiation gradually became dominant, mediating part of p75<sup>++</sup> CJECs transition to the differentiation state. This was accompanied by a decrease in p75 expression and increased expression of differentiation-related keratin, finally presenting as a terminal differentiation state of p75<sup>-</sup> CJECs that can repair damaged tissues or replace aging cells.

As a downstream signaling molecule of p75, SALL2 has been reported to be regulated by NGF and affects the survival and differentiation of neurocytes.<sup>39</sup> Here, we first demonstrated the role of SALL2 in CJECs. The addition of NGF regulates the subcellular localization of SALL2, increasing nuclear localization and decreasing distribution in cytoplasm (Figure 6C-E). This pattern of regulation specifically occurred in p75<sup>++</sup> CJECs rather than p75<sup>-</sup> CJECs (Figure 6F,G). The interaction between SALL2 and p75 in conjunctival epithelial cells was

confirmed by CO-IP, and NGF diminished their interaction (Figure 6H). After NGF treatment, the expression levels of SALL2 downstream target genes, cell cycle regulatory genes p16 and c-MYC, as well as the stemness indicators SOX2 and OCT4, were all regulated accordingly (Figure 6I), resulting in the changes of low proliferation and reduced stemness in conjunctival epithelial cells. Therefore, we consider that after the activation of the p75 signaling pathway by NGF, SALL2 dissociates from the intracellular domain of p75 and enters the nucleus to initiate the downstream transcription process, affecting the expression of differentiation-associated markers of CJECs. Our investigation showed that the NGF-p75-SALL2 signaling pathway is involved in maintaining the stemness of conjunctival epithelial stem cells and regulating differentiation status.

Our studies show that NGF/proNGF could be involved in regulating the differentiation state of conjunctival epithelial stem cells by regulating the nuclear localization of SALL2 through p75. These observations revealed the mechanism of maintaining the stemness and regulating the differentiation of conjunctival epithelial stem cells. In addition, we enriched conjunctival epithelial stem cells by p75, and verified their proliferative ability and conjunctival reconstruction in vivo. This simple and effective method of enriching conjunctival epithelial stem cells provides the potential to obtain sufficient seed cells for functional conjunctival reconstruction and may promote the treatment of large conjunctival defects in clinical practice.

## ACKNOWLEDGMENTS

This study was supported by the National Key R&D Program of China (2018YFC1106100, 2018YFC1106101), the National Natural Science Foundation of China (Grant 81770888, 81800873, 31800809, and 31500835), the Shanghai Municipal Education Commission-Gaofeng Clinical Medicine Grant Support (Grant 20161421), the Science and Technology Commission of Shanghai Grant (Grant 17411963800 and 19JC1411703).

## CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

## AUTHOR CONTRIBUTIONS

Y.F., H.S.: experiment design, data analysis, manuscript preparation; N.W., C.Y.: experiments execution (most), data analysis, original draft writing; J.C., Q.Y., Y.L., F.Y.: experiments execution (part), data analysis.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**How to cite this article:** Wu N, Yan C, Chen J, et al. Conjunctival reconstruction via enrichment of human conjunctival epithelial stem cells by p75 through the NGF-p75-SALL2 signaling axis. *STEM CELLS Transl Med*. 2020; 9:1448-1461. <https://doi.org/10.1002/sctm.19-0449>