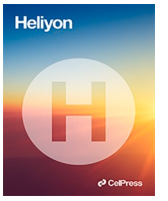
Heliyon 10 (2024) e33506

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

TGF-β1 maintains the developmental potential of embryonic submandibular gland epithelia separated with mesenchyme

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ARTICLE INFO

Keywords:
Salivary glands
Morphogenesis
Transforming growth factor beta
Embryonic development

ABSTRACT

Objective: The objective of this study was to investigate the impact of transforming growth factor β1 (TGF-β1) on epithelial development using an ex vivo model of submandibular gland (SMG) epithelial-mesenchymal separation.

Materials and methods: The ex vivo model was established by separating E13 mouse SMG epithelia and mesenchyme, culturing them independently for 24 h, recombining them, and observing branching morphogenesis. Microarray analysis was performed to evaluate the transcriptome of epithelia treated with and without 1 ng/ml TGF-β1. Differential gene expression, pathway enrichment, and protein-protein interaction networks were analyzed. Quantitative real-time polymerase chain reaction, Western blot, and immunofluorescence were employed to validate the mRNA and protein levels.

Results: Recombined SMGs using separated epithelia and mesenchyme that were cultured for 24 h showed a significant inhibition of epithelial development compared to SMGs recombined immediately after separation. The level of TGF-β1 decreased in the SMG epithelia after epithelia-mesenchyme separation. Epithelia that were separated from mesenchyme for 24 h and pretreated with 1 ng/ml TGF-β1 continued to develop after recombination with mesenchyme, while epithelia without 1 ng/ml TGF-β1 treatment did not. Microarray analysis suggested pathway enrichment related to epithelial development and an upregulation of *Sox2* in the 1 ng/ml TGF-β1-treated epithelia. Further experiments validated the phosphorylation of SMAD2 and SMAD3, upregulation of SOX2 and genes associated with epithelial development, including *Prol1*, *Dcpp1*, *Bhlha15*, *Smgc*, and *Bpifa2*. Additionally, 1 ng/ml TGF-β1 inhibited epithelial apoptosis by improving the BCL2/BAX ratio and reducing cleaved caspase 3.

Conclusions: The addition of 1 ng/ml TGF-β1 maintained the developmental potential of embryonic SMG epithelia separated from mesenchyme for 24 h. This suggests that 1 ng/ml TGF-β1 may partially compensate for the role of mesenchyme during the separation phase, although its compensation is limited in extent.

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https://doi.org/10.1016/j.heliyon.2024.e33506
Received 6 March 2023; Received in revised form 11 June 2024; Accepted 21 June 2024
Available online 25 June 2024

1. Introduction

Submandibular glands (SMGs) are exocrine glands located in the head and neck region, and they play a crucial role in the secretion of saliva, which is essential for maintaining the oral microenvironment and human oral health [1–4]. There are various factors that can cause damage to salivary glands, including radiotherapy for head and neck tumors, aging, Sjogren's syndrome, gland surgery, and drug side effects. These conditions can lead to problems oligoptyalism and xerostomia, which in turn can cause dental caries and adversely affect chewing and swallowing abilities, significantly impacting the quality of life of affected individuals [5–7]. While conventional treatments like artificial saliva are available, tissue engineering holds great potential for regenerating SMGs and addressing xerostomia [8]. However, the precise mechanism underlying salivary gland development is not yet fully understood, which poses a challenge to the advancement of salivary gland tissue engineering [9].

Salivary gland development is a complex process that involves epithelial-mesenchymal interaction and branching morphogenesis, with transforming growth factor β1 (TGF-β1) playing a pivotal role [10]. TGF-β1, a member of the TGF-β superfamily, plays a dual role in salivary gland development. It is an essential promoter of salivary gland fibrosis and reduction in secretion [11]. Conversely, aging and pathological factors can increase the levels of TGF-β1 in salivary glands, leading to fibrosis and reduced salivary flow [12,13]. Interestingly, TGF-β1 expression can be detected in both the epithelial and mesenchymal components during the initial bud stage of submandibular gland (SMG) development, while it is primarily localized in the branching epithelia in later stages [14]. Knockout mice lacking TGF-β1 exhibit impaired development of SMGs, highlighting the indispensable role of TGF-β1 as a regulatory signal during salivary gland development [15]. However, despite these findings, the dual nature of TGF-β1 in salivary gland development emphasizes the need for further research to fully understand its effects.

Salivary gland development involves intricate interactions between epithelial and mesenchymal cells. However, the current study of salivary gland development mainly relies on 2D cell culture, which lacks the ability to accurately recreate the complex extracellular matrix crucial for salivary gland development. The emergence of organoid culture technology has improved our ability to simulate in vivo conditions, but it still faces challenges in faithfully replicating the complex extracellular matrix [16]. To overcome this limitation, we utilized an ex vivo model of submandibular gland (SMG) epithelial-mesenchymal separation [17,18] to investigate the impact of TGF-β1 on epithelial development.

2. Materials and methods

2.1. Ex vivo culture of SMGs

This study was approved by the Ethics Committee of West China Stomatology Hospital, Sichuan University (WCHSIRB-D-2017-233). Eight-week-old ICR mice were obtained from Chengdu Dossy Experimental Animal Co. Ltd. The submandibular glands (SMGs) were harvested from E13 embryos following the procedure described by Liu et al., 2020 [19]. The SMGs were cultured on Nuclepore Track-Etch membranes using a medium composed of DMEM/F12 (1:1), 100 U/ml penicillin, 100 μg/ml streptomycin, 150 μg/ml L-ascorbic acid, and 50 μg/ml transferrin. The cultures were maintained at 37 °C with 5 % CO2. After incubation, the cultured SMGs were observed for 72 h. The number of buds formed during the 72-h culture period was counted using microscopy, and the relative epithelial area was quantified using ImageJ software.

2.2. Epithelia-mesenchyme separation and recombination of SMGs

Mouse embryonic SMGs were cultured ex vivo for 2 h. After this initial culture period, the SMGs were subjected to digestion using neutral protease (1.5 U/ml) for 5 min. Subsequently, to separate the epithelial and mesenchymal components, a 1 ml syringe needle was carefully used to puncture the epithelial-mesenchymal junction under a microscope. This process facilitated the removal of the superficial mesenchyme, resulting in the isolation of the epithelial tissue.

The isolated epithelial and mesenchymal components were then cultured independently for a specified duration, such as 24 h, with or without the addition of 1 ng/ml TGF-β1 (7754-BH, R&D Systems Company, Minneapolis, USA), as well as non-specific IgG (AB-105-C, R&D Systems Company, Minneapolis, USA). Following the independent culture period, the isolated epithelia were reintroduced into the mesenchyme, resulting in the formation of recombined SMGs. These recombined SMGs were subsequently cultured on Nuclepore Track-Etch membranes in a culture medium. The cultures were maintained at 37 °C with 5 % CO2, and observations were made 72 h after incubation.

2.3. Microarray analysis

Microarray analysis was performed on three samples of fetal mouse SMGs, which were treated with 1 ng/ml TGF-β1 and IgG, respectively, to identify differentially expressed mRNAs. The samples were labeled using the 3′ IVT Pico Kit reagent (902,930, Affymetrix, California, USA) and hybridized onto microarrays. The microarrays underwent washing procedures, followed by data normalization using the RMA normalization method. Data summarization was performed for each probe set, resulting in signal intensity values for all probe sets across the samples. The limma package (version 3.50.3) in R software (version 4.1.3) was utilized to identify the differentially expressed mRNAs. Heatmaps were generated, with a specific focus on genes associated with salivary gland development. Enrichment analysis of the differentially expressed genes was performed using the clusterProfiler package (version

4.2.2). Gene sets related to apoptosis were obtained from MSigDB. The GSVA package (version 1.42.0) was employed to score the gene sets of the samples. The list of differentially expressed genes was input into the Lisa Transcription Factor Database to extract the top 30 most significant differentially expressed transcription factors. These extracted transcription factors were then input into the String protein interaction database to construct a protein interaction network. Network node information was exported and imported into Cytoscape software. Degree values between nodes were calculated, and Hub genes were identified through filtering in Cytoscape.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction was carried out using an RNA isolation kit (4992858, Tiangen, Beijing, China). Reverse transcription was performed using the PrimeScriptTM RT reagent kit (RR037B, Takara Bio Inc., Kusatsu, Japan). This step involves converting RNA into complementary DNA (cDNA). qPCR was conducted using the SYBR Green Premix Ex TaqTM kit (RR820A, Takara Bio Inc., Kusatsu, Japan). This technique is employed to quantify the levels of specific RNA molecules within the cDNA samples. The primers used in the qPCR experiments were previously described or designed using the NCBI Primer-BLAST design tool [20]. Subsequently, these primers were synthesized by TsingKe Biological Technology in Beijing, China.

Tgf-β1	Forward Primer: 5'-CTACTATGCTAAAGAGGTCACC-3'
	Reverse Primer: 5'-TTTCTCATAGATGGCGTTGTTGC-3'
Prol1	Forward Primer: 5'-AGATGTGCCCTCCAGGAACTAC-3'
	Reverse Primer: 5'-GTGCTGGTGTGGTTGCATTTGG-3'
Dcpp1	Forward Primer: 5'-ACCAAGCTTATGTTCCAGCTGGAGG-3'
	Reverse Primer: 5'-CCCTCTAGAATGCCTGAGTGGCCATATT-3'
Bhlha15	Forward Primer: 5'-GAGCGAGAGAGGCAGCGGATG-3'
	Reverse Primer: 5'-AGTAAGTATGGTGGCGGTCAG-3'
Smgc	Forward Primer: 5'-ACAGTCTCTACACTTAGGTCCCA-3'
	Reverse Primer: 5'-ACTTGTGATCGGTTCGACTATT-3'
Bpifa2	Forward Primer: 5'-CTCCATTTCCTTGTTGGGAA-3'
	Reverse Primer: 5'-AGTTTTGCAGGACGGTTGAC-3'
Sox2	Forward Primer: 5'-AAAAAGCAGGCTTGTATAACATGATGGAGACGG-3'
	Reverse Primer: 5'-AGAAAGCTGGGTTTCACATGTGCGACAGGGGCAGT-3'
Gapdh	Forward Primer: 5'-TGCTGAGTATGTCGTGGAGTCTA-3'
	Reverse Primer: 5'-AGTGGGAGTTGCTGTTGAAATC-3'

2.5. Western blot (WB) analysis

WB analysis was performed using the method previously described [21,22]. Total proteins from SMG tissues were extracted using RIPA lysis buffer (89,900, Thermo Fisher Scientific, Waltham, USA) following standard procedures. The extracted proteins were subjected to WB analysis, as described in our methodology. Primary antibodies were used at a 1:1000 dilution and included the following: SMAD2/3 (sc-8332, Santa Cruz, Dallas, USA), *p*-SMAD2/3 (sc-11769, Santa Cruz, Dallas, USA), SOX2 (WL03767, Wanlelbio, Shenyang, China), BCL-2 (bs-4563R, Bioss, Beijing, China), BAX (bs-0127R, Bioss, Beijing, China), cleaved Caspase-3 (9661, Cell Signaling Technology, Danvers, USA), Caspase-3 (4372, Cell Signaling Technology, Danvers, USA), and GAPDH (GB15002-100, Servicebio, China). Secondary antibodies were purchased from ZSGB-BIO (ZF-0512, ZF-0317, ZF-0316, Beijing, China) and were used at a 1:5000 dilution.

2.6. Immunofluorescence

The immunofluorescence was performed by the method as previously described [23]. SMG tissues were fixed by 4 % paraformaldehyde solution for 24 h and transferred in 70 % ethanol at 4 °C before analysis. The specimens were sliced into 4-μm-thick sections. Sections were incubated with specific primary antibodies for SOX2 (WL00982a, Wanlelbio, Shenyang, China) and cleaved Caspase-3 (9661, Cell Signaling Technology, Danvers, USA) at a 1:200 dilution. Secondary antibody of Alexa Fluor® 594-injugated (ab150080, Abcam, Cambridge, UK) was used followed by primary antibodies at a 1:1000 dilution. The nuclei were stained with DAPI (C0065; Solarbio). Then the sections were observed on an Olympus microscope.

2.7. Statistical analyses

Statistical analyses were performed using GraphPad Software (version 9.4, GraphPad Software, San Diego, CA, USA). For the comparison of two groups, unpaired two-tailed Student t-tests were used for continuous variables. A p-value below 0.05 was considered statistically significant.

3. Results

3.1. TGF-β1 decreases in the SMG epithelia after epithelia-mesenchyme separation

Following the separation of epithelia from mesenchyme, immediate recombination of salivary gland tissues (SMGs) allowed for the continuous process of branching morphogenesis. However, when the epithelia and mesenchyme were cultured separately for 24 h and then recombined, a significant decrease in both the number and surface area of epithelial buds in the recombined SMGs was observed compared to those recombined immediately (Fig. 1a–d). Notably, the level of TGF-β1 in the epithelia decreased significantly 24 h after separation compared to the initial time point, suggesting the involvement of TGF-β1 in epithelial development (Fig. 1e). However, the addition of exogenous TGF-β1 (1 ng/ml) to the entire SMG inhibited branching morphogenesis (Fig. 1f–h). These results suggest that the expression of TGF-β1 decreased as the recombined SMGs regressed, while the addition of TGF-β1 appeared to accelerate their regression. The role of TGF-β1 in this process seems paradoxical, warranting further investigation to determine if TGF-β1 exerts a specific effect only during the recombination process.

3.2. Separated epithelia pretreated with TGF-β1 continues development after recombination with mesenchyme

Considering the inhibitory effect of exogenous TGF-β1 on SMG development, we aimed to investigate the potential of TGF-β1 administration during the separation phase. Regrettably, treatment with 1 ng/ml TGF-β1 alone for 24 h resulted in the loss of epithelial and mesenchymal morphology, with no significant differences compared to the control (Fig. 2a). Subsequently, we recombined the TGF-β1-treated epithelia and mesenchyme, as well as IgG-treated counterparts (Fig. 2b). Surprisingly, treatment of the epithelia with 1 ng/ml TGF-β1 led to continued development, characterized by an increased number of epithelial buds and an overall larger size of the SMGs. However, there were no noticeable differences in the branching morphogenesis of recombined SMGs when using mesenchyme treated with either 1 ng/ml TGF-β1 or IgG (Fig. 2c–e). Importantly, no significant difference was observed between recombined SMGs using 1 ng/ml TGF-β1-treated epithelia alone and those using 1 ng/ml TGF-β1-treated epithelia/mesenchyme. These results suggest that during the separation phase, 1 ng/ml TGF-β1 may partially compensate for the role of the mesenchyme.

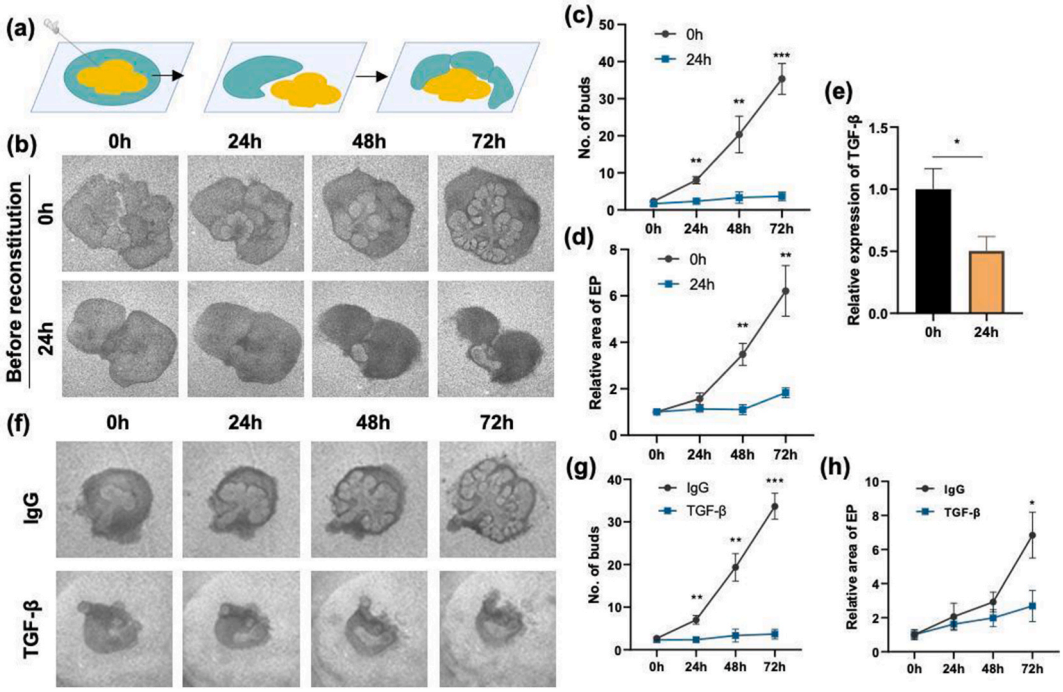

Fig. 1. TGF-β1 decreases in the SMG epithelia after epithelia-mesenchyme separation. (a) Illustration of separation and recombination of SMG epithelia and mesenchyme. (b) Representative images of SMGs cultured for 0 h–72 h, which was recombined immediately after separation or 24 h after separation. (c) The number of buds of SMGs recombined immediately after separation or 24 h after separation (n = 3 per group). (d) The relative area of epithelia in the SMGs recombined immediately after separation or 24 h after separation (n = 3 per group). (e) TGF-β1 expression level of SMG epithelia measured by qRT-PCR immediately after separation and 24 h after separation (n = 3 per group). (f) Representative images of SMGs after 24 h treatment with 1 ng/ml TGF-β1 or IgG. (g) The number of buds of SMGs after 24 h treatment with 1 ng/ml TGF-β1 or IgG (n = 3 per group). (h) The relative area of epithelia in the SMGs after 24 h treatment with 1 ng/ml TGF-β1 or IgG (n = 3 per group). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Scale bars = 250 μm.

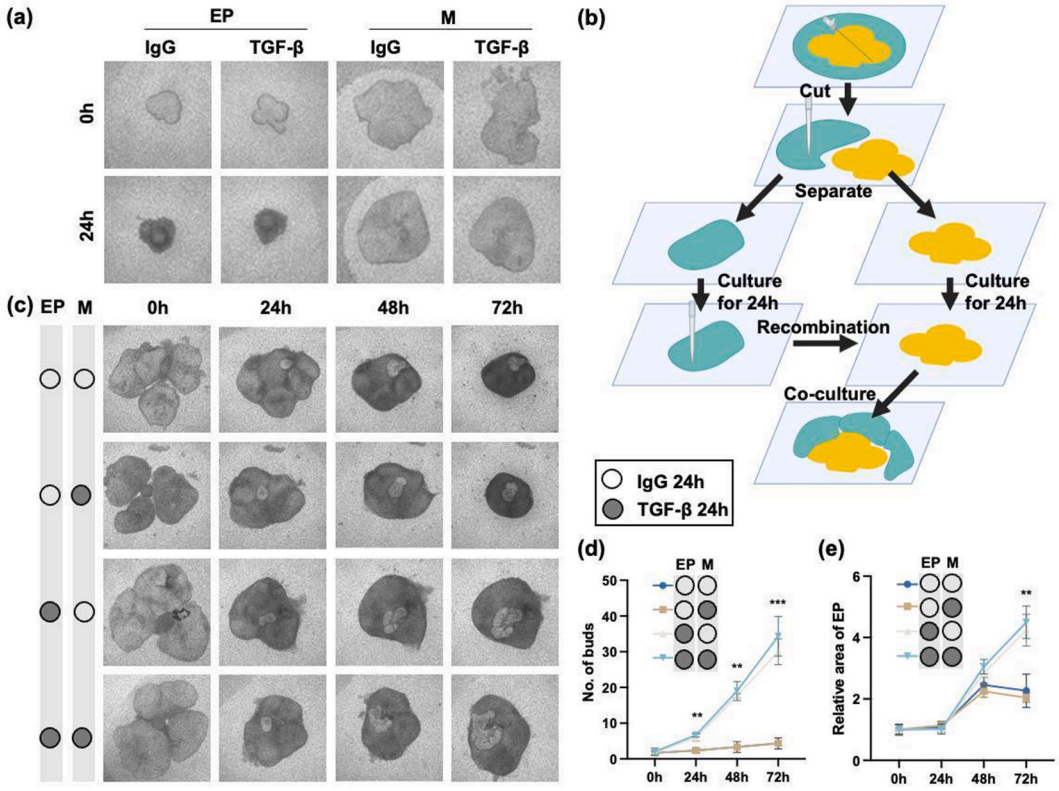

Fig. 2. Separated epithelia pretreated with TGF-β1 continues development after recombination with mesenchyme. (a) Representative images of the separated epithelium and mesenchyme before and after 24 h treatment with 1 ng/ml TGF-β1 or IgG. (b) Illustration of the workflow of recombination of TGF-β1 or IgG-treated epithelia and mesenchyme. (c) Representative images of the recombined SMGs after culture of 0 h–72 h, in which the epithelia and mesenchyme were pretreated with 1 ng/ml TGF-β1 or IgG for 24 h. (d) The number of buds of the recombined SMGs after culture of 0 h–72 h, in which the epithelia and mesenchyme were pretreated with 1 ng/ml TGF-β1 or IgG for 24 h (n = 3 per group). (e) The relative area of epithelia in the recombined SMGs after culture of 0 h–72 h, in which the epithelia and mesenchyme were pretreated with 1 ng/ml TGF-β1 or IgG for 24 h (n = 3 per group). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Scale bars = 250 μm.

3.3. *TGF-β1 maintains the potential of epithelial development due to increased SOX2 expression*

To further elucidate the mechanism of TGF-β1, we conducted microarray analysis comparing 1 ng/ml TGF-β1-treated epithelia with IgG-treated epithelia. *Dcpp1* and *Prol1* are markers of ductal cells, while *Bpifa2, Smgc,* and *Bhlha15* are markers of acinar cells [24–28]. The results revealed significant increases in the expression of genes associated with acinar development (*Prol1* and *Dcpp1*) and ductal development (*Bhlha15, Smgc,* and *Bpifa2*) in 1 ng/ml TGF-β1-treated epithelia compared to the IgG group (Fig. 3a). The findings from the microarray were validated using PCR (Fig. 3d).

Additionally, the enrichment analysis demonstrated that the upregulated genes were enriched in pathways related to epithelial cell development and gland development in the Kyoto Encyclopedia of Genes and Genomes (KEGG), as well as in pathways associated with the cell cycle in the Gene Ontology (GO) (Fig. 3b). The protein-protein interaction (PPI) network revealed *Sox2* as one of the key genes among the differentially expressed genes (Fig. 3c). Subsequently, we performed additional confirmatory experiments using PCR, WB, and immunofluorescence to validate the mRNA and protein expression of SOX2 (Fig. 3d–g). SOX2 is a transcription factor that is closely associated with cell stemness and proliferation [29]. As a result, these findings strongly suggest that 1 ng/ml TGF-β1 may enhance epithelial development potential by upregulating SOX2. We also observed the upregulation of SMAD2/3 phosphorylation after adding 1 ng/ml TGF-β1 (Fig. 3e).

3.4. *TGF-β1 inhibits epithelial apoptosis by improving BCL2/BAX ratio and reducing cleaved caspase 3*

Despite its impact on epithelial development, our hypothesis was that 1 ng/ml of TGF-β1 may also inhibit cell apoptosis. The microarray analysis revealed a significant decrease in the apoptosis score in epithelial cells after treatment with 1 ng/ml of TGF-β1 (Fig. 4a). BCL-2 is known for its anti-apoptotic effect, while BAX is a protein that promotes apoptosis. In the epithelial cells treated with 1 ng/ml of TGF-β1, the expression of BCL-2 was upregulated, while the expression of BAX was downregulated, resulting in an increased BCL2/BAX ratio (Fig. 4b–d). Caspase-3 is a critical component of the apoptotic cascade, and cleaved Caspase-3 represents its active

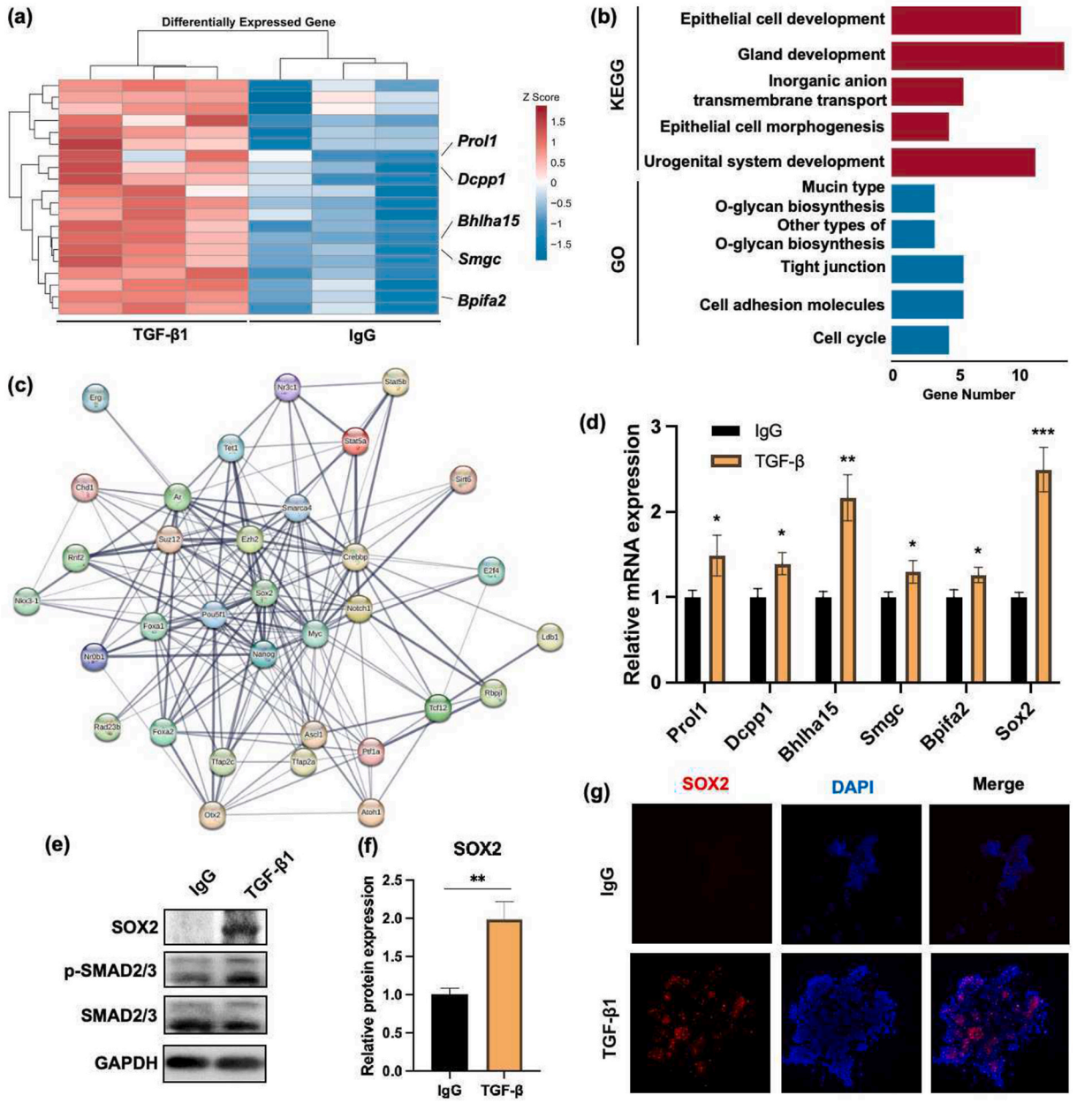

Fig. 3. TGF-β1 maintains the potential of epithelial development due to increased SOX2 expression. (a) Heatmap of the top 20 differentially expressed genes between the 1 ng/ml TGF-β1-treated and IgG-treated epithelia (n = 3 per group). (b) The results of enriched GO and KEGG pathways for upregulated differentially expressed genes ($p < 0.05$) in the 1 ng/ml TGF-β1-treated epithelia. (c) The significantly upregulated differentially expressed genes in the TGF-β1 group were input into the String database to generate a PPI network, with Sox2 located at the core position. (d) Relative mRNA expression of Prol1, Dcpp1, Bhlha15, Smgc, Bpifa2, and Sox2 between the 1 ng/ml TGF-β1-treated and IgG-treated epithelia (n = 3 per group). (e) Representative WB of Sox2, *p*-SMAD2/3, and SMAD2/3 of the 1 ng/ml TGF-β1-treated and IgG-treated epithelia. (f) Relative protein expression of Sox2 between the 1 ng/ml TGF-β1-treated and IgG-treated epithelia (n = 3 per group). (g) Representative images of Sox2 immunofluorescent staining of the 1 ng/ml TGF-β1-treated and IgG-treated epithelia. *$p < 0.05$, **$p < 0.01$.

form. After administration of 1 ng/ml of TGF-β1, both Caspase-3 and cleaved Caspase-3 were downregulated, leading to a decrease in the proportion of cleaved Caspase-3 in the total Caspase-3 (Fig. 4b–d). Immunofluorescence analysis further confirmed these observed trends in protein expression (Fig. 4e). In summary, these findings indicate that 1 ng/ml of TGF-β1 could inhibit apoptosis in epithelial buds, which is crucial for promoting branching morphogenesis.

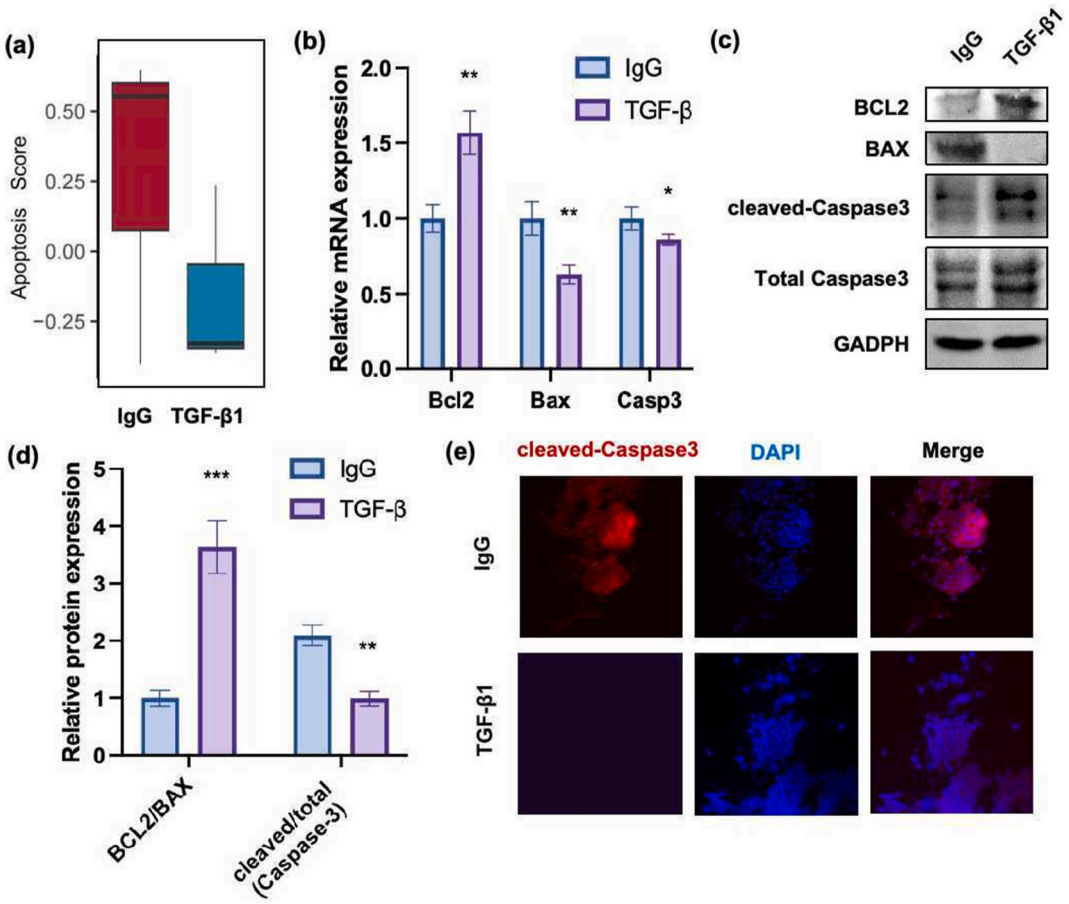

Fig. 4. TGF-β1 inhibited epithelial apoptosis by improving Bcl2/Bax ratio and reducing cleaved Caspase-3. (a) The apoptosis score between the 1 ng/ml TGF-β1-treated and IgG-treated epithelia. (b) Relative mRNA expression of Bcl-2, Bax, and Caspase-3 between the 1 ng/ml TGF-β1-treated and IgG-treated epithelia (n = 3 per group). (c) Representative WB of Bcl-2, Bax, cleaved Caspase-3, and total Caspase-3 of the 1 ng/ml TGF-β1-treated and IgG-treated epithelia. (d) Relative protein expression of Bcl-2/Bax and cleaved Caspase-3/total Caspase-3 between the 1 ng/ml TGF-β1-treated and IgG-treated epithelia (n = 3 per group). (e) Representative images of cleaved Caspase-3 immunofluorescent staining of the 1 ng/ml TGF-β1-treated and IgG-treated epithelia. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4. Discussion

TGF-β has long been recognized for its role in promoting mesenchymal fibrosis, which inhibits epithelial differentiation and promotes extracellular matrix deposition. This process can ultimately lead to excessive extracellular matrix and the replacement of epithelial cells [30]. Conditional overexpression or the exogenous administration of 10 ng/ml TGF-β1 has been shown to have detrimental effects on glandular development, impairing branching morphogenesis and increasing fetal mortality rates [31,32]. Consistent with these observations, our findings also indicate that TGF-β1 treatment inhibits gland development. However, our study uncovered a surprising aspect of TGF-β1's effects. Specifically, we found that 1 ng/ml TGF-β1 can also have a positive impact by preserving the developmental potential of epithelial cells when separated from the mesenchyme for a prolonged period (24 h).

Previous studies have indicated the significance of TGF-β1 in the epithelial component as a critical factor for SMG branching morphogenesis [33]. It has been demonstrated that disruption of TGF-β1 gene expression in mice leads to abnormal SMG development and the infiltration of inflammatory factors [15]. However, existing literature suggests that the effects of TGF-β1 are mainly limited to the epithelial component. During the initial stages of SMG development, when only a single epithelial bud is present, TGF-β1 is observed in both the epithelia and mesenchyme. However, as development progresses, particularly during epithelial branching morphogenesis, TGF-β1 is only detectable within the epithelia [34]. Interestingly, our study, utilizing an ex vivo model of epithelial-mesenchyme separation and recombination, suggests that 1 ng/ml TGF-β1 may partially compensate for the role of the mesenchyme during the separation phase. Nonetheless, the extent of this compensation appears to be limited, as indicated by the smaller size of the recombined SMG buds.

Through pathway enrichment analysis, we identified SOX2 as a key protein involved in this process. SOX2 is a transcription factor closely associated with cellular stemness, exhibiting high expression in the inner cell mass during early embryonic development as well

as in embryonic and adult stem cells [29]. Notably, SOX2 is closely linked to cell proliferation and is significantly upregulated in various cancer cells [35,36]. As an end-effector protein, SOX2 is regulated by multiple signaling pathways, including the Wnt/β-catenin and PI3K-Akt pathways [37,38]. Previous studies have demonstrated that TGF-β can upregulate SOX2 in melanoma and non-small-cell lung cancer [39,40]. Consistent with these findings, our study suggests that TGF-β upregulates SOX2, contributing significantly to SMG development. Further investigations can be conducted to elucidate the mechanisms underlying SOX2 upregulation.

Smad transcriptional complexes, downstream effectors of TGF-β, play a regulatory role in several apoptotic target genes [41]. Administration of 1 ng/ml TGF-β1 to epithelial buds notably suppressed the expression of pro-apoptotic proteins such as BAX and reduced Caspase-3 cleavage, indicating a decrease in apoptosis. Without support and stimulation from the mesenchyme, epithelial buds activate apoptosis-related signals. However, exogenous TGF-β1 appears to counteract this effect by inhibiting epithelial apoptosis. The impact of TGF-β on apoptosis can vary depending on the cell type, with tissue-specific, organ-specific, and phase-specific effects [41]. For example, 0.5 ng/ml TGF-β1 promotes apoptosis by increasing Caspase-3 levels in hepatocyte homeostasis [42], while 10 ng/ml TGF-β downregulates Fas and Caspase-8 expression, preventing Fas-mediated programmed cell death in follicular dendritic cells [43]. The differential impact on apoptosis by TGF-β is closely related to the activation of the phosphatidylinositol-3-kinase/Akt (PI3K-Akt) pathway. The PI3K-Akt signaling pathway promotes the survival of mammary gland epithelial cells when activated by 2 ng/ml TGF-β [44]. Nevertheless, PI3K-Akt signaling is primarily activated in mesenchymal cells such as fibroblasts [45]. These findings strongly suggest that the effects of TGF-β on apoptosis are tissue-specific, organ-specific, and phase-specific. In our study, we observed that 1 ng/ml TGF-β1 effectively inhibited epithelial apoptosis during the branching morphogenesis phase when separated from the mesenchyme. However, it is important to note that these conclusions may not be generalizable to the entire salivary gland, as the effects of TGF-β on apoptosis can vary depending on specific contextual factors.

This study has several limitations that should be acknowledged. Firstly, the effects of TGF-β1 appear to be concentration-dependent, with low concentrations promoting epithelial differentiation and maintaining stemness. However, we did not investigate the effects of high concentrations of TGF-β1 in this study. Secondly, we did not investigate the specific cell type in which TGF-β1 exerts its effects. Future studies should consider utilizing genetic manipulations to address this issue and gain a more detailed understanding of cell-specific responses to TGF-β1. Thirdly, we used IgG as a control in TGF-β1 experiments that may impact cellular responses. Fourthly, we observed that although epithelial buds treated with TGF-β1 continued to develop and branch after recombination, they were smaller in size compared to normal glands. Further investigations are needed to understand and address this discrepancy. Acknowledging these limitations is essential for a comprehensive understanding of the study's scope and for guiding future research directions.

5. Conclusion

1 ng/ml TGF-β1 maintains the developmental potential of embryonic SMG epithelia separated with mesenchyme for 24 h. 1 ng/ml TGF-β1 may partially compensate for the mesenchyme's role during the separation phase but the extent of this compensation appears to be limited.

Patients and public involvement

Not applicable.

Consent for publication

All authors provided written informed consent to publish this study.

Funding

This study was supported by the National Natural Science Foundation of China (81870782, 82270994, 82301106), Natural Science Foundation of Sichuan Province (2024NSFSC1585), the Fundamental Research Funds for the Central Universities, Research and Develop Program of West China Hospital of Stomatology Sichuan University (RD-02-202409), and Sichuan University Innovation Spark Library (2018SCUH0055).

Data availability statement

All data used in the generation of the results presented in this manuscript will be made available upon reasonable request from the corresponding author.

CRediT authorship contribution statement

Honglin Li: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Guanru Wang:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Guile Zhao:** Writing – original draft, Visualization, Software, Conceptualization. **Huabing Liu:** Formal analysis, Data curation. **Liu Liu:** Methodology, Data curation. **Yubin Cao:** Writing

– review & editing, Conceptualization, Supervision, Funding acquisition.**Chunjie Li:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used language AI (ChatGPT version 3.5) to edit the whole manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

List of abbreviations

cDNA	complementary DNA
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PI3K-Akt	phosphatidylinositol-3-kinase
PPI	protein-protein interaction network
qRT-PCR	Quantitative real-time polymerase chain reaction
SMG	submandibular gland
TGF-β1	transforming growth factor β1
WB	Western blot

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