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RESEARCH ARTICLE

Specific complexes derived from extracellular matrix facilitate generation of structural and drug-responsive human salivary gland microtissues through maintenance stem cell homeostasis

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Abstract

Three-dimensional cultured salivary glands (SGs) microtissues hold great potentials for clinical research. However, most SGs microtissues still lack convincing structure and function due to poor supplementation of factors to maintain stem cell homeostasis. Extracellular matrix (ECM) plays a crucial role in regulating stem cell behavior. Thus, it is necessary to model stem cell microenvironment in vitro by supplementing culture medium with proteins derived from ECM. We prepared specific complexes from human SG ECM (s-Ecx) and analyzed the components of the s-Ecx. Human SG epithelial and mesenchymal cells were used to generate microtissues, and the optimum seeding cell number and ratio of two cell types were determined. Then, the s-Ecx was introduced to the culture medium to assess its effect on stem cell behavior. Multiple specific factors were presented in s-Ecx. s-Ecx promoted maintenance of the stem cell and formation of specific structures resembling that of salivary glands and containing mucins, which suggested stem cell differentiation potential. Moreover, treatment of the microtissues with s-Ecx increased their sensitivity to neurotransmitters. On the basis of the analysis of components, we believed that the presented growth factors are able to interact with stem cell they encountered in vivo, which promote the capacity to maintain stem cell homeostasis. This work provided foundations to study molecular mechanism of stem cell homeostasis in SGs and develop novel therapies for dry mouth through new drug discovery and disease modeling.

KEYWORDS

complexes, dry mouth, extracellular matrix, human salivary gland microtissues, stem cell homeostasis

1 | INTRODUCTION

Dry mouth is a common symptom and can develop from salivary glands diseases. Patients with reduced saliva production have symptoms of impaired oral health, difficulties in swallowing and digestion food and other complications, which significantly decreased their quality of life and general health status (Foraida, Kamaldinov, Nelson, Larsen, & Castracane, 2017; Frank, Herdly, & Philippe, 1965; Valdez, Atkinson, Ship, & Fox, 1993). However, due to the lack of ideal models to investigate disease mechanisms and screening efficient drugs, treatment of this disease is insufficiently efficacious (Konings, Coppes, & Vissink, 2005).

Recently, three-dimensional (3D) cultured microtissues have emerged as a potential tool for both fundamental research and regeneration medicine because of its near-physiological microenvironment (Lombaert, Movahednia, Adine, & Ferreira, 2017; Rowe & Daley, 2019). Many studies focused on the generation of 3D cultured salivary microtissues using stem cells for this purpose. In native salivary glands (SGs), stem cells act as "reserve army" to repair injured acinar and duct cells to ensure the physiological function. In order to generate SG microtissues that could mimic structures formation and function differentiation, stem cell pool needs to be maintained. (Clevers, 2016). Because of the limited understanding of salivary specific stem cell niche, current salivary organoid culture systems still lack effective niche factors to mimic the interaction of stem cells with their niches, which hamper the differentiation of specific structures and function (Lombaert et al., 2017). In order to overcome these limitations, supplementation of factors function on maintaining stem cell homeostasis is required for generation mature microtissues from human salivary epithelium and mesenchymal cells. Studies have concentrated on finding new signaling factors involving in a certain pathway to enhance proliferation and differentiation capacity of salivary stem cells (Maimets et al., 2016). However, multiple proteins covered a wide range of signaling was needed to mimic the signaling interactions network that stem cells encounter in vivo.

Extracellular matrix (ECM) regulates cell behavior of specific organ by its biophysical and biochemical properties. The ECM is not just scaffolds to support cells. Many ECM proteins (e.g., proteoglycans and fibronectins), by binding to growth factors, establish a microenvironment to maintain stem cell homeostasis. Such ECM proteins act as reservoirs for growth factors to assist in regulating cell behavior through releasing factors during degradation of themselves (Hynes, 2009). Therefore, ECM is an ideal source of specific factors for maintaining stem cell pool in vitro. However, current studies engineer ECM to mimic the native microenvironment based on its biophysical properties. Procedures involved in biomaterials preparation may impact the activity of growth factors binding to the ECM proteins (Gattazzo, Urciuolo, & Bonaldo, 2014). Currently, studies have established protocols for extracting ECM supplement from decellularized tissue improved the proliferation and differentiation of skin, liver, and muscle cells (Yi et al., 2017; Zhang et al., 2009). Therefore, based on current research, we attempted to extract s-Ecx from human salivary gland ECM through a novel method to avoid loss of biological molecular. We hypothesis that by supplementation such s-Ecx to culture medium, salivary microtissues cultures can model such interactions that stem cell encounter in vivo.

In this study, we first isolated epithelial and mesenchymal cells, contained stem/progenitor cells, from human salivary glands and prepared specific s-Ecx derived from salivary ECM. Such cells hold great proliferation capacity so that we could harvest enough cells for 3D culture. Protein array results showed that such s-Ecx contained a series of growth factors specific for salivary. After screening two cell types in optimized ratio and suitable initial cell numbers of salivary microtissues, s-Ecx was supplemented to culture medium, and the results suggested that microtissues cultured with s-Ecx showed an increased tendency of structural and functional differentiation and more sensitive to drugs. We established a specific supplement depot for in vitro salivary microtissues cultures for the first time, which held essential potentials to model the balance between maintenance and differentiation. By supplementing of s-Ecx, we could harvest SG microtissues with more similar structure and function to physiology. This study gives foundations for studying salivary stem cell biology. Moreover, we provided novel tools for the treatment of dry mouth.

2 | METHODS AND MATERIALS

2.1 | Isolation and culture of human submandibular gland cells

The collection of human submandibular gland (SMG) tissues was approved by the Institutional Review Board of Peking University. All of the patients who participated in this study provided written informed consent, in accordance with ethical guidelines. Fresh human SMG samples were collected from patients (20–40 years old) with ameloblastoma. For removing the entire tumor, healthy SMGs need to be resected to perform the intraoral microvascular anastomosis. Sample isolation was performed as described previously (Lu et al., 2015).

2.2 | Preparation of s-Ecx

s-Ecx was prepared as described in our previous work (Yi et al., 2017). Briefly, after removing fat and connective tissues, SMG tissues was cut into small cubes (1 mm^2). Then, tissues were made into fine fibers using homogenizer. For decellularization, the tissue fibers were treated with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin in Dulbecco's phosphate-buffered saline (Hyclone, USA) for 3 days at 4 °C, and solution was changed daily. After being decellularized for 1 day, the decellularized tissue fibers were washed three times to remove remaining fetal bovine serum. Then, the decellularized tissue fibers were lyophilized for 48 hr. After lyophilization, tissues were homogenized to powder. Equal amount of pepsin containing 3,400 units of protein and 0.1 N HCL was mixed with ECM powder and incubated at 37°C on a shaking bed for 2 days. For negative control, lyophilized tissues were replaced with NaCl. After digestion for 2 days, most of the lyophilized ECM powders have been dissolved and less precipitate remained. Thus, we harvested the supernatant by centrifugation at 3,500 rpm for 5 min. The supernatant was adjusted to pH 7.0 using NaOH, filtered (0.22 μm), and added to the medium. The decellularized human SMG supernatant was used as 100% s-Ecx.

2.3 | Analyses of s-Ecx

We determined the efficiency of decellularization by measuring the DNA concentration. DNA extraction was performed following the

manufacturer's instructions (QIAGEN, DNeasy, blood and tissue kit). We analyzed potent growth factors of s-Ecx using a Human Growth Factor Array (RayBiotech, Norcross, GA). Three s-Ecx samples from different human SGs (hSGs) tissues were sent on dry ice to the company for growth factor assays. After subtracting background and normalized to positive control, we analyzed the amount of proteins according to the densities of signals on blotting membranes by Image J software.

2.4 | Formation of hSG microtissues in 3D culture

Microtissues were cultured using the Gravity Plus 3D hanging-drop culture system (InSphero). Briefly, mesenchymal cells and epithelial cells were dissociated with 0.25% trypsin-EDTA in 37°C for 2 and 4 min and resuspended in 3D culture medium with or without s-Ecx, and 40 μl of each well (3,000, 5,000, 7,000, and 9,000 cells) were transferred to the GravityPlus microtissue platform. After hanging for 5 days, the cell aggregates were transferred to a 96-well low-attachment U-bottom plate (GravityTRAP) for long-term culture. Microtissues were cultured in DMEM/F12 (Gibco) supplemented with 20-ng/ml epidermal growth factor (EGF), 20-ng/ml fibroblast growth factor 2, 1% N-2 (serum-free supplement for culturing neural cells), 10-μg/ml insulin, and 1-μM dexamethasone with or without 200-μM carbachol (Sigma). To culture hSG microtissues with supplements, 1%, 10% s-Ecx, or 1% Matrigel was added to the 3D culture medium.

2.5 | Determined the effect of s-Ecx in 2D culture

SGs epithelium and mesenchymal cells in a ratio of 9:1 with 5,000 starting cells were seeded in 96 wells tissue culture treated plate (40 μl per well). In order to prevent the medium from drying, 40-μl fresh medium was changed every 2 days.

2.6 | Quantitative real-time polymerase chain reaction

Total RNA was isolated from microtissues using TRIzol reagent (Invitrogen). RNA (2 μg) was reverse-transcribed to complementary DNA using the RevertAid First Strand complementary DNA Synthesis Kit (Thermo Fisher). Gene expression levels were determined by quantitative real-time polymerase chain reaction. Quantitative real-time polymerase chain reaction was performed as follows: predenaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Table 1 lists the sequences of the primers used.

2.7 | Immunohistochemistry

Microtissues were fixed in 4% (w/v) paraformaldehyde overnight at 4°C or 30 min in room temperature and equilibrated in 30% sucrose overnight at 4°C. The cell pellets were embedded in optical coherence tomography and cryosectioned at a thickness of 5–10 μm. Tissue sections were stained with hematoxylin and eosin following a standard protocol. For immunohistochemistry staining, sections were blocked with 3% bovine serum albumin (BSA) for 2 hr in room temperature, primary antibody incubation was overnight at 4°C. For immunohistochemistry, biotinylated goat anti-rabbit secondary antibody was used, followed by Vectastain ABC-HRP reagent and developed with diaminobenzidine substrate. Sections were then counterstained with hematoxylin.

TABLE 1 Sequence for primers used for quantitative real-time polymerase chain reaction

Gene	Forward primer sequence	Reverse primer sequence
28sr RNA	CCCAGTGCTCTGAATGTCAA	AGTGGGAATCTCGTTCATCC
Ki67	ACGCCTGGTTACTATCAAAAGG	CAGACCCATTTACTTGTGTTGGA
Zo-1	CGGTCCTCTGAGCCTGTAAG	GGATCTACATGCGACGACAA
Ascl3	TGATCTGCCTGCCTCGGCCT	ACTCCTCCCCACCCCTCCCA
α-amylase	AATTGATCTGGGTGGTGAGC	CTTATTTGGCGCCATCGATG
AQP5	CCGCTCACTGGGTTTTCTGG	TTTGATGATGGCCACACGCT
MUC1	GCATCAGGCTCAGCTTCTACT	GTCTCTCTGCAGCTCTTGGTA
K5	CCGCTCACTGGGTTTTCTGG	GAGGAATGCAGACTCAGTGGA
K19	TGAGGAGGAAATCAGTACGCT	CGACCTCCCGGTTCAATTCT
K14	ATCCAGAGATGTGACCTCCTC	GTCTGCCTCCAAGGTCCTCTGA
K7	TCCGCGAGGTCACCATTAAC	GCTCTGTCAACTCCGTCTCAT
Mist1	CGGATGCACAAGCTAAATAACG	GCCGTCAGCGATTTGATGTAG
Sox2	TGGCGAACCATCTCTGTGGT	GGAAAGTTGGGATCGAACAAAAGC
Sox10	TCATCCCTTCAATGCCCCCT	TGCGTCTCAAGGTCATGGAGG
CD44	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT

2.8 | Immunofluorescence staining and confocal microscopy

For immunofluorescence staining, tissue sections were treated with 0.1% Triton X-100 for 15 min, blocked in 3% BSA for 2 hr at room temperature and incubated with the primary antibodies in 0.1% Triton X-100 and 3% BSA at room temperature for 2 hr. Following incubation with the appropriate secondary antibodies, the blots were washed three times with PBS. 4′,6-Diamidino-2-phenylindole was used to stain nuclei. Sections were imaged using the NIKON A1R-si Confocal microscope.

2.9 | Alcian blue periodic acid Schiff staining

To assess the formation of saliva, the tissue sections are stained with alcian blue and periodic acid Schiff. Tissue sections were first incubated with alcian blue for 10 min, rinsed in distilled water for three times, and then oxidized with periodic acid for 5 min. Next, tissue sections were treated with Schiff reagent for 15 min to reveal carbohydrates.

2.10 | Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling assay

To assay cell death within the microtissues, we used the TMR Red In Situ Cell Death Detection Kit (Roche) following the manufacturer's instructions. Briefly, tissue sections were permeated in 0.1% Triton X-100 for 2 min on ice and incubated with terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling reaction mixture (10% enzyme solution in labeling solution) for 1 hr at 37°C. Tissue sections were observed using a fluorescence microscope with an excitation wavelength of 543 nm. Areas of positive staining indicated apoptotic cells.

2.11 | Statistical analysis

All quantitative values are expressed as $M \pm SD$ of three or more biological repeat samples, and results were a conclusion of at least two independent experiments unless otherwise stated. We used one-way analysis of variance with Tukey's multiple comparison test to determine statistical significance. Differences were considered significant at a p value of <.05 and.01.

3 | RESULTS

3.1 | s-Ecx contain multiple specific growth factors involving in SGs regeneration and development

The procedures of preparing s-Ecx were followed as described previously (Figure 1a). First, we extracted DNA from fresh SG tissues and decellularized ECM with the same quality. The DNA concentration of treated ECM significantly decreased compared with that of fresh tissues (Figure 1b) and did not decrease during long-time decellularization. Thus, cells were removed from ECM, and we determined the time of decellularization as 1 day. For investigation of the components of s-Ecx, a growth factor protein array was used to

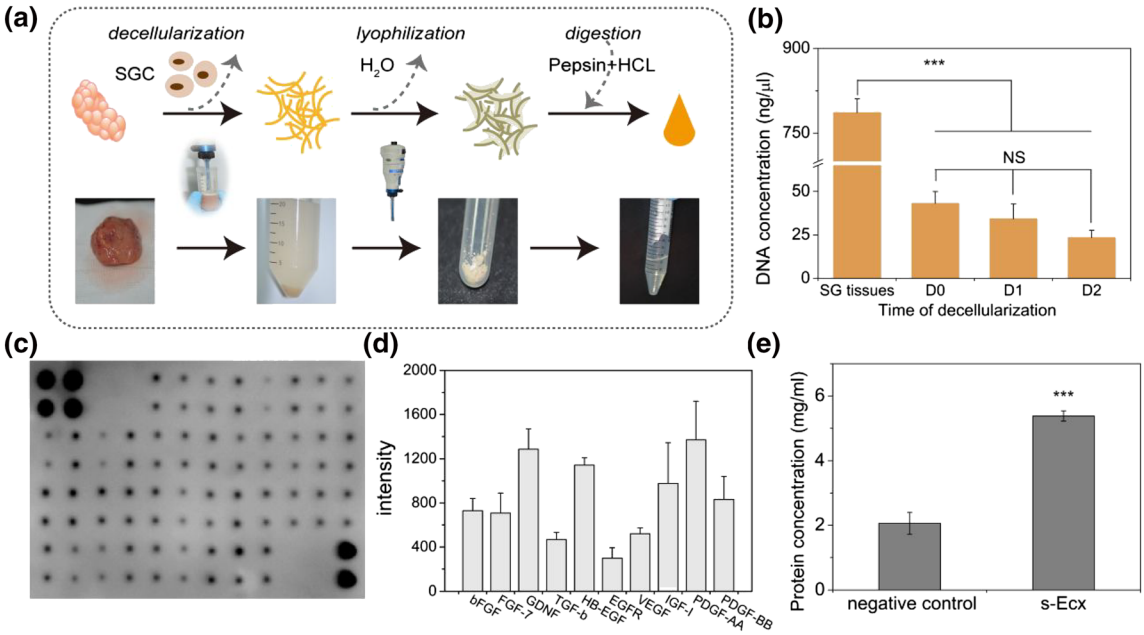

FIGURE 1 Preparing procedures of s-Ecx. (a) Photographs showing the s-Ecx preparing methods. (b) DNA concentration of fresh SG tissues and decellularized tissues. (c and d) A growth factor protein array was used to analyze the components of s-Ecx (c) and quantification of important factors related to salivary glands development after background subtraction (intensity) was shown (d). (e) Protein concentration of s-Ecx and negative control. Data are $M \pm SD$ of three independent experiments. NS, no-significant; SGC, salivary gland cell. $^{*}p < .05$, $^{***}p < .01$

characterize the salivary gland stem cell homeostasis related factors in s-Ecx. The results showed that several important growth factors were included in s-Ecx. Growth factors that are related to salivary glands acinar and duct development such as bFGF, FGF7, GDNF, TGF-β, HB-EGF, and EGFR were found to be presented in s-Ecx (Figures 1c,d and S1b). Other important factors that specific to embryonic development were also present, included, IGF-1, IGFBP-1, and VEGF. Additionally, we measured the protein concentration of s-Ecx and negative control (Figure 1e). The protein in negative control (about 2 mg/ml) was pepsin. Complexes without lyophilized SGs tissues showed decreased protein concentration, which suggested that these proteins were extracted from SG ECM.

3.2 | Formation of hSG microtissues in vitro

In order to screen the optimized culture conditions, a novel hanging-drop culture system was used. This platform allowed us to generate relative uniform microtissues in a high-throughput way (Figure S1a). We first evaluated microtissues with different initial numbers of epithelial cells in terms of cell morphology, apoptosis, and proliferation. The average diameter of microtissues was positively correlated with the initial cell number (Figure 2a,b). Microtissues with 5,000, 7,000, and 9,000 cells generated compact structures (Figure 2b). Histologic staining revealed that microtissues with 5,000 cells had a compact structure and a distinct outer boundary (Figure S2a). The 5,000-cell microtissues exhibited the highest Ki67 expression, indicating the greatest proliferation capacity (Figures 2c and S3a). Results showed that cultures of larger microtissues tended to be less healthy. Additionally, TUNEL staining revealed that the cells in microtissues had a low frequency of apoptosis (Figure S2a). Therefore, the 5,000-cell microtissues were used in subsequent experiments.

Mesenchymal cells also play an important role during SG development and regeneration. The growth factors secreted by mesenchymal cells interact with adjacent epithelium and help to regulate SG formation. Therefore, we tested different proportion of mesenchymal cells in microtissues. Microtissues comprising 10% mesenchymal cells and 90% epithelial cells had the most compact structure, smallest

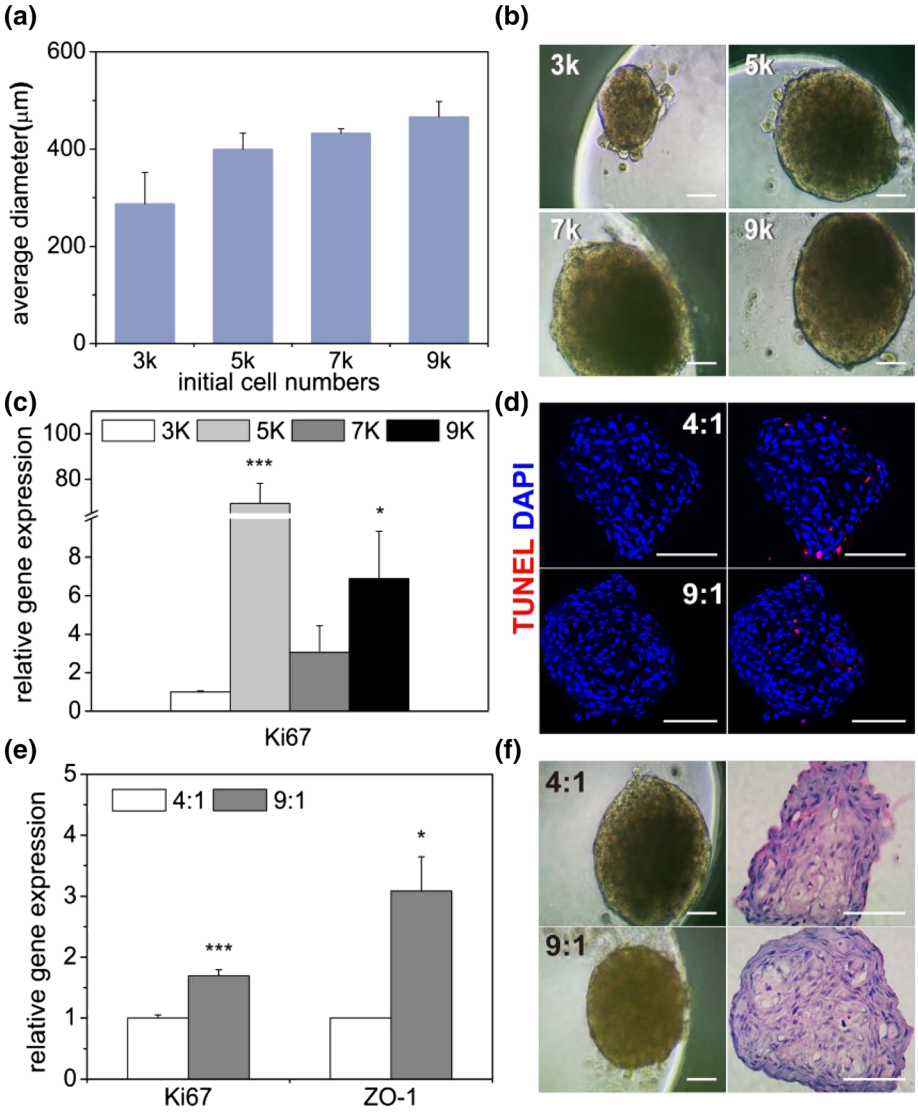

FIGURE 2 Optimization of salivary glands microtissues culture conditions. The optimum number (3,000, 5,000, 7,000, and 9,000 cells per well), and the ratio of epithelial and mesenchymal cells (4:1 and 9:1) were evaluated. (a) The average microtissues diameter increased as the initial number of cells increased morphology at Day 7 of microtissues according to initial number of cells. (b) Morphology at Day 7 of microtissues according to initial number of cells. (c) Expression levels of genes related to proliferation compared with 3,000-cell microtissues; 5,000-cell microtissues had the highest proliferation rate. (d) TUNEL staining of microtissues based on different cell ratio. (e) Cell proliferation rate and expression levels of genes related to tight junctions according to the epithelial: mesenchymal cell ratio compared with the ratio of 4:1. (f?????????) Microtissues morphology, histology according to the epithelial: mesenchymal cell ratio. Scale bar indicates 100 μm. Data are $M \pm SD$ of three independent experiments. $^{*}p < .05$; $^{***}p < .01$

diameter, and highest expression of the gene encoding the tight-junction protein, ZO-1 (Figure 2e,f????????). We assumed that these may result from the successful combination of epithelium and mesenchymal cells. Moreover, these microtissues had a higher proliferation capacity and a lower frequency of apoptosis (Figures 2d–f, S2b, and S3b). Therefore, we used microtissues comprising epithelial: mesenchymal cells at a 9:1 ratio. In addition, we tested the localization of mesenchymal cells marker in microtissues. Positive staining of CD73 was observed in microtissues, which suggested mesenchymal cells were successfully organized in microtissues (Figure S3c). After culture for 21 days, hematoxylin and eosin staining revealed that the structure of the microtissues became disrupted (Figure S2c).

3.3 | Proper concentration of s-Ecx was selected

To investigate the effect of s-Ecx on stem cells within hSG microtissues, we assessed the effect of 0% (control), 1%, and 10% s-Ecx on the structural and functional differentiation of hSG microtissues.

Compact microtissues with clear borders resulted from the use of 0% and 1% s-Ecx (Figure S4a). However, 10% s-Ecx resulted in microtissues resembling a mass of cell debris without clear borders (Figure S4b); excess ECM proteins may have prevented the formation of cell pellets. At Day 14, several cavities and a large quantity of cell debris were present within the inner portion of the microtissues cultured with 0% s-Ecx (Figure S4c). However, the structure of microtissues cultured with 1% s-Ecx was intact at Day 14 (Figure S4c). Furthermore, although at Day 7 there was no difference between microtissues cultured with 0% and 1% s-Ecx, at Day 5, microtissues cultured with 0% s-Ecx were large-diameter transparent spheroids (Figure 3a) and not self-organized as well as which cultured with 1% s-Ecx (Figure 3b), which indicate that cells cultured with 1% s-Ecx held increasing efficiency of forming compact microtissues. We thus used 1% s-Ecx to assess its effect on salivary microtissues formation.

3.4 | s-Ecx promotes the formation of structural salivary microtissues

Histologic staining revealed that the microtissues contained several cavities. Unlike cell debris, these structures were integrated and with smooth borders (seen as dashed boxes, Figure 3c, yellow arrows indicated duct-like structures, and black asterisk indicated cavities caused by cell death). Therefore, we speculated that these were likely cystoid structures containing saliva secreted by surrounding cells or duct-like structures. More duct-like structures were noted in microtissues cultured with mesenchymal cells and s-Ecx (Figure 3d). Also, small buds were observed in the duct-like structures, which may mimic acinar development. Alcian blue periodic acid Schiff staining revealed that neutral mucins (magenta) and acidic mucins (blue) are positive in microtissues surrounding duct-like structures (Figure 4a). Therefore, more duct-like structures secrete more saliva. Especially, acidic mucins mainly secreted within the small buds, which may suggest that s-Ecx

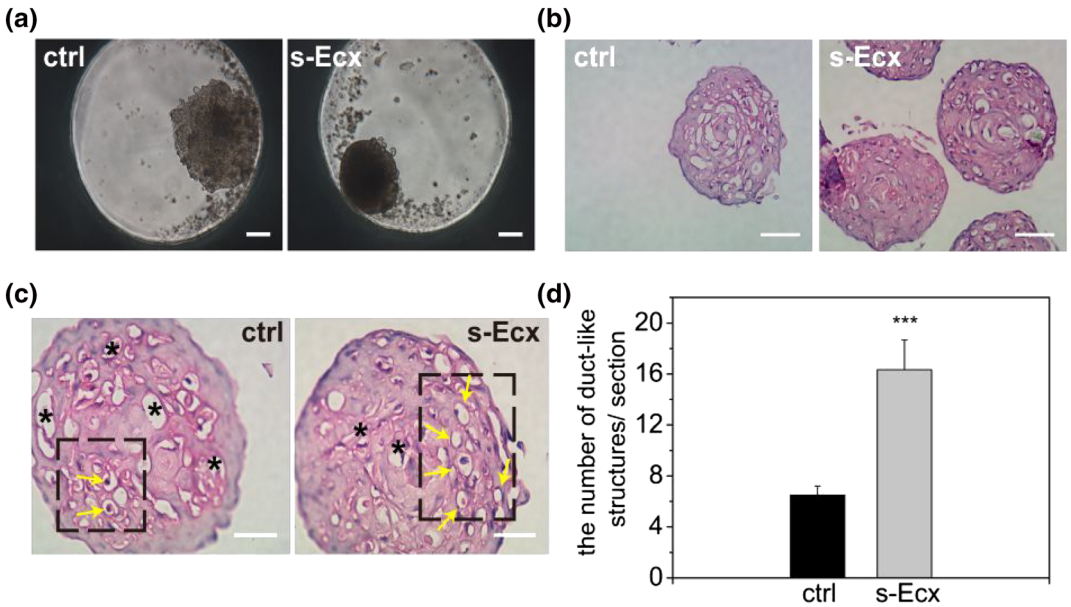

FIGURE 3 s-Ecx promotes the structural differentiation of salivary glands microtissues. (a) Morphology and histological staining of microtissues treated with or without (control) s-Ecx at Day 5. (b) Morphology and histological staining of microtissues treated with or without (control) s-Ecx at day 5. (c) Hematoxylin and eosin staining of microtissues treated with or without (control) s-Ecx at Day 7, yellow arrows indicated duct-like structures and black asterisk indicated cavities caused by cell death. (d) Number of duct-like structures treated with or without (control) s-Ecx at Day 7. Scale bars indicate 50 μm (b,b') and 100 μm. ctrl, control. Data are *M* ± *SD* of three independent experiments. $^{*}p < .05$; $^{***}p < .01$

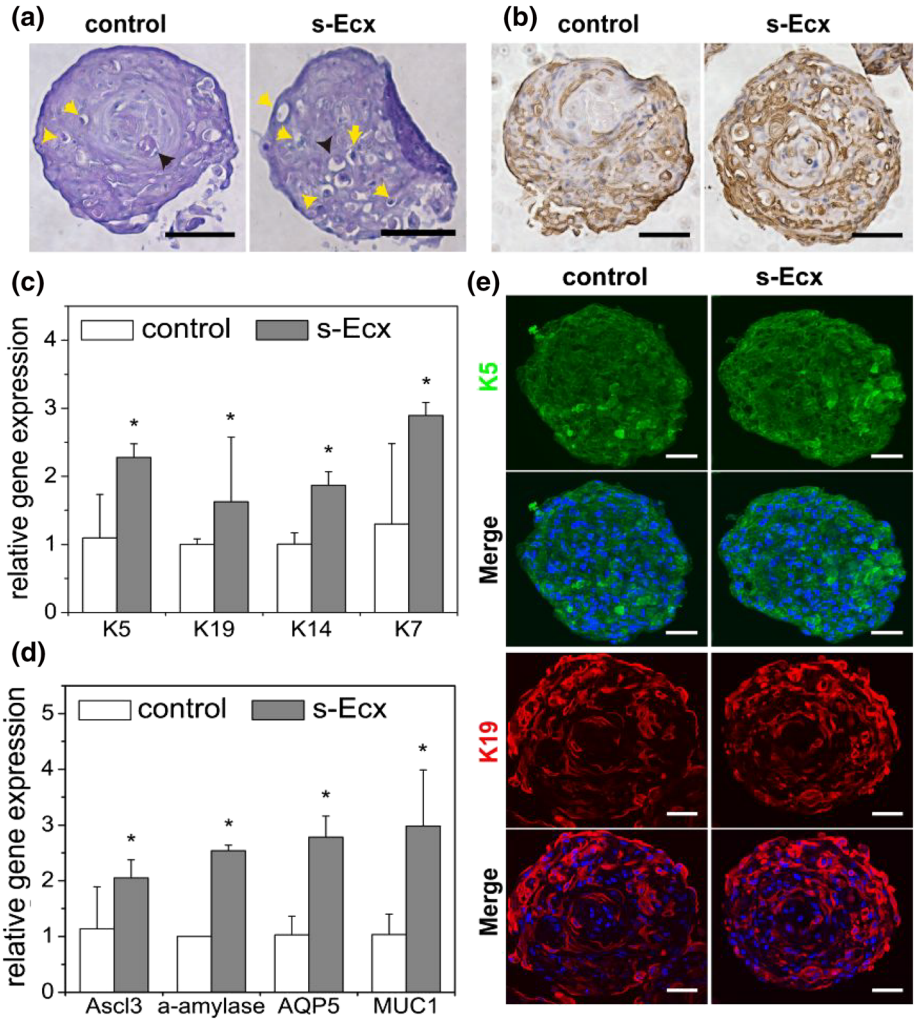

FIGURE 4 s-Ecx promotes specific gene expression and functional differentiation of salivary glands microtissues. (a) Alcian blue periodic acid Schiff staining of microtissues treated with or without s-Ecx (control) at Day 7(yellow arrows indicate acidic mucins; black arrows indicate neutral mucins). (b) Immunohistochemistry for K19 of microtissues treated with or without s-Ecx (control) at Day 7. (c) Quantitative real-time polymerase chain reaction analysis of the expression of salivary stem/progenitor cell genes treated with or without s-Ecx (control). (d) Quantitative real-time polymerase chain reaction analysis of the expression of salivary mature cell genes treated with or without s-Ecx (control) at Day 7. (e) Immunofluorescence staining of K5 (green) and K19 (red) following treatment with s-Ecx. Scale bars indicate 50 μm. Data are $M \pm SD$ of three independent experiments. $^{*}p < .05$; $^{***}p < .01$

promoted the structural and functional differentiation of salivary gland microtissues by regulating the balance of stem cell maintenance and differentiation.

3.5 | s-Ecx promotes the expression of genes specific to progenitor/stem cells and mature cells

To further investigate the effect of s-Ecx on stem cell maintenance and determine the characteristics of these duct-like structures, we next evaluated the expression levels of SG duct and acinus marker genes involving in the development and mature stage. The expression of K19, a progenitor duct-cell marker (Szymaniak et al., 2017), was reduced when cultured without s-Ecx (Figure 4b–c and e). Immunohistochemistry staining further showed that K19 was almost expressed around the duct-like structures. We also assessed the expression levels of Krt5, a marker of epithelium progenitor cells, and K14 and K7, which are highly expressed during SG duct development (Emmerson et al., 2017; Szymaniak et al., 2017). The expression levels of these genes were enhanced in microtissues treated with s-Ecx (Figure 4c,e). The increased expression of markers involved in early SG development inspired us to investigate the expression of mature SG stem cell marker, Ascl3 (Bullard et al., 2008). The expression of Ascl3 exhibited a similar tendency (Figure 4d). Moreover, genes specific to mature acinar (AQP5 and α-amylase for functional acinar cells and MUC1 for mature duct cells) were upregulated by culture with s-Ecx (Figures 4d and S5a). The s-Ecx promoted the expression of genes involved in early SG development and then up-regulated mature gene expression. Thus, we put forward that the duct-like structures may be derived from stem/progenitor cell differentiation and were salivary gland specific lineage. Moreover, these results also demonstrated the function of s-Ecx on salivary microtissues stem cell maintenance.

Matrigel (MG), a commercially available ECM matrix extracted from mouse teratoma, has been reported better capacity to mimic in vivo microenvironments for two-dimensional (2D) and 3D cell culture. We compared the effect of s-Ecx with MG (1% final concentration) on formation of SGs microtissues. The messenger RNA expression level of mature genes showed no significant difference in two groups (Figure 5a). For progenitor/stem cell markers, MG did not promote the expression of K5, K19, and Ascl3 compared with microtissues cultured with s-Ecx. Although the expression of K14 was increased in MG group, only K14 was insufficient to regular differentiation of SGs microtissues. These data indicated that s-Ecx showed similar functions to s-Ecx.

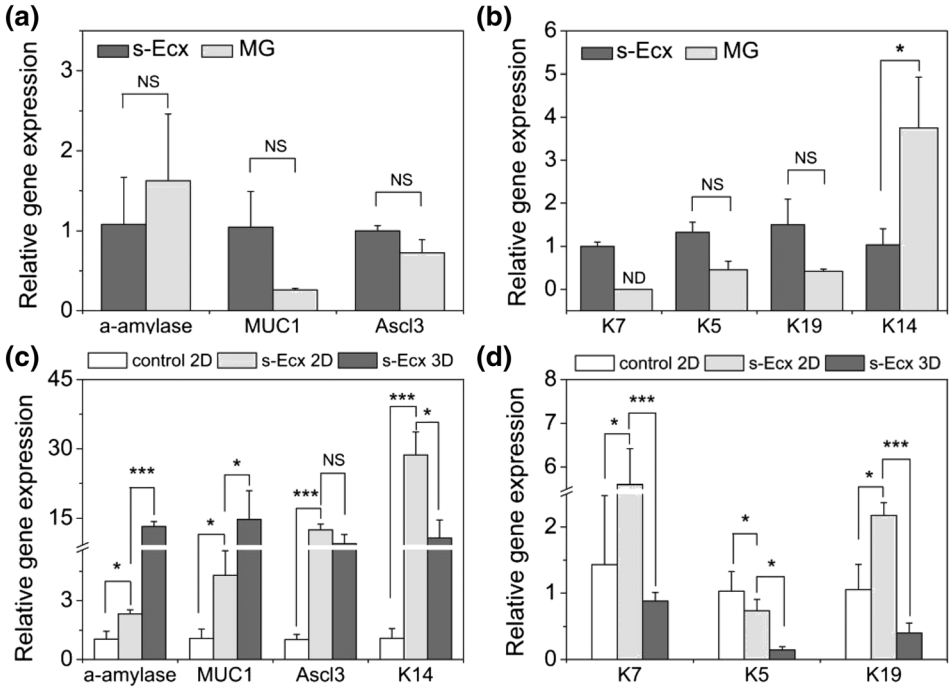

FIGURE 5 s-Ecx showed the same effect to matrigel and also promote gene expression in 2D culture. (a, b) Quantitative real-time polymerase chain reaction analysis of the expression of salivary mature and progenitor/stem cell genes treated with s-Ecx and MG at Day 7. (c, d) Quantitative real-time polymerase chain reaction analysis of the expression of salivary mature and progenitor/stem cell genes of 2D cultures treated with or without s-Ecx (control 2D). Data are $M \pm SD$ of three independent experiments. MG, matrigel; ND, no-detected; NS, no-significant. $^{*}p < .05$; $^{***}p < .01$

3.6 | s-Ecx also facilitated mature and progenitor/stem cell gene expression in 2D cell cultures.

We next determine whether s-Ecx could improve the 2D cell culture. Similarly, s-Ecx also promoted the expression of mature genes (α-amylase and MUC1) and most of the progenitor markers (K7, K14, K19, and Ascl3) in 2D cultures (Figure 5c,d). 2D culture limits the interaction between two kinds of cells, which may decrease the drug sensitivity. Thus, messenger RNA expression of K5 was decreased when cultured with s-Ecx. In addition, we further compared the effect of s-Ecx between 2D and 3D cell culture. Mature genes showed higher expression levels in 3D cultures, but the expression of progenitor/stem cell genes decreased. This may indicate that 3D cell culture holds potentials to promote cell differentiation.

3.7 | s-Ecx promoted the response to neurotransmitters

The behavior and development of SGs are controlled by the peripheral nervous system. To investigate the effect of the s-Ecx on their response to neurotransmitters, the microtissues were treated with the muscarinic receptor agonist carbachol (CCh) for 24–48 hr. Cell-surface muscarinic receptors on SG epithelial cells are activated by acetylcholine or CCh. After 24 hr of treatment, the s-Ecx promoted the expression of the mature acinar markers α-amylase and Mist1 (Figure 6a). Additionally, the expression of K19, Sox2, and Sox10 was increased in microtissues cultured with s-Ecx (Figures 6a–d and S5b). CCh also promoted cell proliferation in microtissues treated with s-Ecx, as indicated by Ki67 expression (Figures 6a and S5c). Furthermore, s-Ecx also increased the saliva secretion of microtissues treatment with CCh (Figure 6e). After treatment with CCh for 48 hr, the expression of K19, Sox2, and Ki67 was similar to that after 24 hr and that of K5 and CD44 was upregulated in microtissues cultured with s-Ecx (Figures 6b–d and S5b). However, the expression of mature acinar markers was unchanged (data not shown). Most genes showed no significant change when treated with CCh in microtissues without s-Ecx. Therefore, microtissues cultured with s-Ecx had a greater response to CCh than control.

4 | DISCUSSION

Currently, 3D cultured SGs microtissues are emerging as a promising model to study common salivary diseases and to apply in regenerative therapy (Kagami, 2015). Progenitor/stem cells play essential roles in maintain organ function in vivo and formation of structural and functional microtissues in vitro. However, difficulty to maintain stem cell homeostasis due to lack of specific proteins in culture medium still precluded application of salivary gland microtissues. Followed by advancement understanding of salivary stem cell molecular mechanism, several factors were introduced to hold the potential to regulate the balance between stem cell maintenance and differentiation. However, a culture medium containing one or two factors was not enough to mimic the complex native microenvironment. Thus, supplements containing multiple stem cell related factors are needed to regulate the structural and functional differentiation of salivary microtissues.

A series of ECM proteins participant in maintenance the balance between self-renewal, differentiation, and maturation of the whole organ through releasing growth factors bound to them (Akita et al., 2009; Nakamura-Ishizu et al., 2012; Sirko, von Holst, Wizenmann, Götz, & Faissner, 2007). Thus, in order to mimic the native

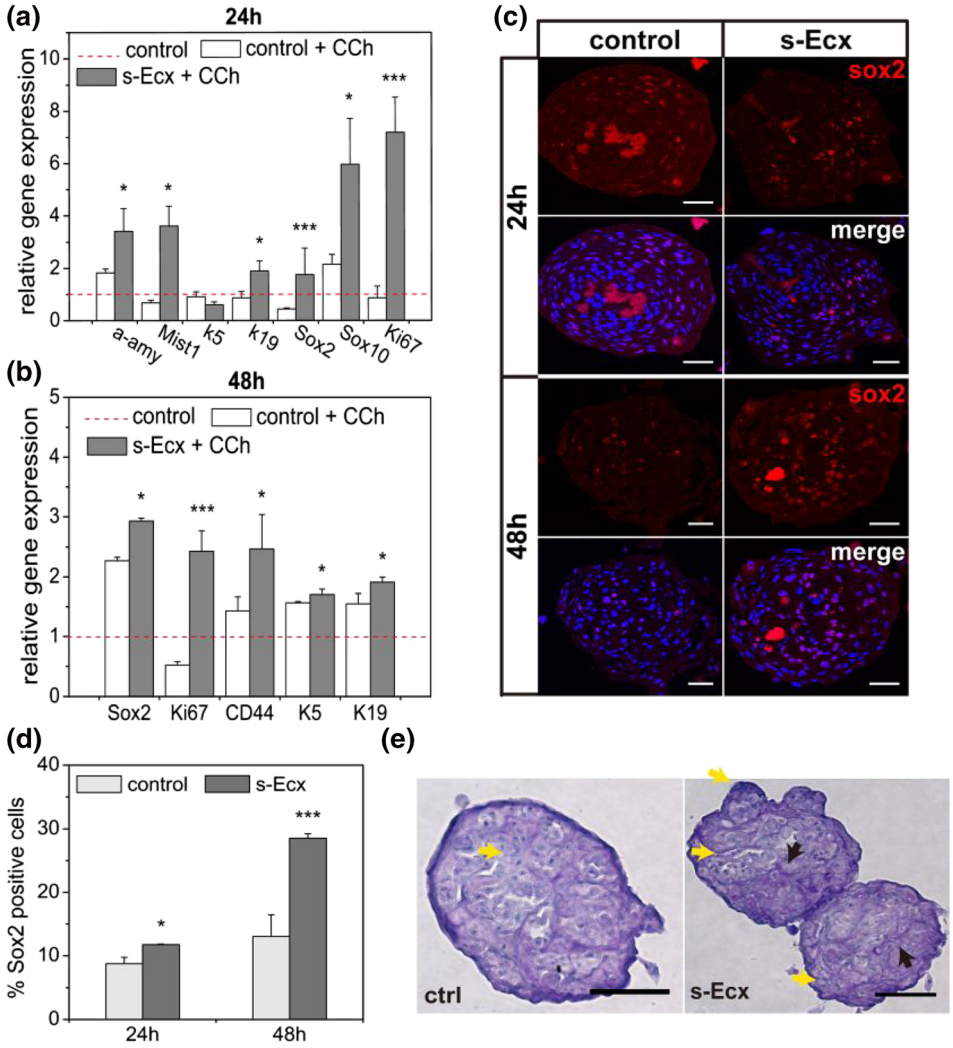

FIGURE 6 s-Ecx promotes the response to neurotransmitters of SG microtissues. (a) Quantitative real-time polymerase chain reaction analysis of the expression of SG-related genes treatment for 24 hr; (b) Quantitative real-time polymerase chain reaction analysis of the expression of SG-related genes treatment for 48 hr; (c and d) immunofluorescence staining and quantification of the embryonic stem cell marker Sox2 treatment for 24 and 48 hr. (e). Alcian blue periodic acid Schiff staining of microtissues treated for 24 hr (yellow arrows indicate acidic mucins and black arrows indicate neutral mucins). Scale bar indicates 50 μm (c) and 100 μm (e). Data are *M* ± *SD* of three independent experiments. Values differ significantly from control (without s-Ecx). ctrl, control; SG, salivary glands. $^{*}p < .05$; $^{***}p < .01$

microenvironment of salivary gland microtissue cultures, we extracted s-Ecx from human salivary gland ECM using a safety method. The method allows large ECM proteins to degrade and release soluble growth factors. Components analysis showed that multiple growth factors were presented in the s-Ecx. Especially, several growth factors identified in the s-Ecx were previously reported involved in maintenance salivary gland stem cell homeostasis.

A classical factor that function on promoting endbud formation during salivary gland development, FGF7 was identified in the s-Ecx. FGF7 was also supplemented to medium to maintain epithelium maintenance and promote differentiation of salivary microtissues (Tanaka et al., 2018). TGF-β and basic FGF were also expressed in s-Ecx. BMP4 and BMP7 are numbers of TGF-β superfamily. BMP4 inhibits epithelium development and BMP7 increases it. Studies reported that this balance was modulated by FGFs (Steinberg et al., 2005). During lumen formation and maturation, $K5^{+}$ progenitors proliferate in response to HB-EGF through EGFR and then generate maturing $K19^{+}$ cells (Knox et al., 2010). Additionally, HB-EGF and EGFR were identified in the s-Ecx. It was surprising to find GDNF expression in the s-Ecx. GDNF is a neurotrophic factor that reported to promote the survival of salivary stem cells and functional innervation of the developing epithelium (Xiao et al., 2014). Other growth factors involving in salivary gland repair and regeneration were also presented in s-Ecx, such as VEGF, IGF-I, and PDGF. Because protein array can only detect limited proteins, therefore, we assume that the beneficial effects were not only caused by these identified proteins, but the other unidentified proteins derived from SG ECM.

By supplementing culture medium with the s-Ecx, we found that s-Ecx increased the number of duct-like structures, which were putatively determined as immature ductal and acinar combination structures through the analysis of saliva secretion and relative gene expression. Genes specific for progenitor cells (K5, K19, K7, and K14; Emmerson et al., 2017) and adult stem cells (Ascl3; Bullard et al., 2008) were up-regulated by supplementation of s-Ecx, suggesting a function in maintaining homeostasis of the progenitor/stem-cell pool. Additionally, the up-regulation of several mature genes (AQP5, α-amylase, and MUC1) and increased secretion of mucins suggested a function in promoting differentiation capacity. Furthermore, we found that microtissues cultured with s-Ecx and MG showed similar expression level of both mature genes and progenitor/stem markers. Although MG hold potential in 3D cultures, this result may suggest that s-Ecx contains multiple specific factors that mimic SGs microenvironment in

vitro. We also determined the effect of s-Ecx on 2D cultures. The expression of such genes showed similar tendency to 3D cultures, which further demonstrated the potential of s-Ecx on applying in treatment of dry mouth.

In adult SGs, the parasympathetic nervous system stimulates saliva production and secretion. Developmentally, parasympathetic ganglia function in maintaining the K5, K19, and Sox2 progenitor cells in early stages and selectively preserve those progenitors specifically to the acinar cell lineage (Emmerson et al., 2017; Knox et al., 2010; Nedvetsky et al., 2014). The increasing expression of mature and progenitor genes by short-term CCh stimulation suggested increased drug sensitivity of microtissues treated with the s-Ecx. These results further demonstrated the increased capacity of differentiation of salivary microtissues cultured with the s-Ecx.

We found that the s-Ecx promoted specific structure forming and functional differentiation in vitro through regulating the balance between maintenance and differentiation of stem cells within SG microtissues, which played a similar role to native salivary ECM. Although not all cells in microtissues are stem cells, the existed stem cell is enough to interact with stem-cell-related factors. We supposed that different factors in the s-Ecx played different roles in microtissues. Some act as modulators to maintain the stem cell pool, and others triggered differentiation. Therefore, the s-Ecx and salivary microtissues built an interaction network culture system to recapitulate the native homeostasis of the organ. A common treatment made in patient with dry mouth to stimulate saliva secretion is using parasympathomimetic drugs, although these drugs have side effects. In our culture system, we can generate mucin-secretive and drug-responsive salivary microtissues. Additionally, sufficient salivary epithelium and mesenchymal cells can be harvested from human tissues. We, therefore, suggested that this culture system will allow further studying of dry mouth by modeling disease and can be used as tools to discover more effective and safety drugs. Furthermore, this system allowed us to further investigate the stem cell homeostasis of salivary gland, which will promote the study of generating more native salivary gland microtissues. In order to meet the clinical requirement, further research should focus on development this s-Ecx to safe and chemically defined supplement, which may result in more cell death when long-term culture in this study.

In conclusion, in order to model stem cell homeostasis in the SG microtissues cultures, we prepared human salivary gland ECM to growth factors-riched s-Ecx based on its biochemical properties. The addition of s-Ecx to the medium increased the specific structural formation and gene expression level of both progenitor markers, mature markers, and adult stem cell markers. Then, the supplements also enhanced the drug sensitivity of microtissues. We, for the first time, put forward that introducing specific s-Ecx helped maintain the stem cell pool and promoted structural and functional differentiation in vitro, which is similar to the in vivo functions of ECM. Our culture system helped us to generate more structural and functional salivary glands microtissues. We provided a new tool that has potential applications for pathology researches and drug discovering as disease models toward treatment of patients suffered from dry mouth. The effect of s-Ecx on stem cell for salivary gland microtissue cultures allows us to investigate the mechanism of ECM to pattern organ homeostasis. Moreover, such salivary gland cultures also give us the possibility to study common salivary disease.

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CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Figure S1. Optimization of SG microtissues culture conditions. a) schematic diagram of a novel hanging drop 3D culture platform and uniform microtissues were generated; b) growth factor protein array map corresponding to figure 1c. POS: positive control of protein array assay.

Figure S2. Optimization of SG microtissues culture conditions. a) Structural aspects of SG microtissues (5000-cells) with a low frequency of apoptosis, as assessed by TUNEL and DAPI staining. b) Number of apoptotic cells according to the epithelial: mesenchymal cell ratio compared to the ratio of 9:1. c) Disrupted structure of microtissues with a mesenchymal:epithelial cell ratio of 1:9 at day 21. Scale bar indicates 100 μm. Data are means ± standard deviations (SD) of three independent experiments. *, $p < 0.05$; ***, $p < 0.01$.

Figure S3. Optimization of SG microtissues culture conditions. a-f????????) Immunofluorescence staining for ki67 of microtissues with different staring number and the ratio of two cell types. g) Immunofluorescence staining for CD73 of microtissues with cell ratio of 9:1. Scale bar indicate 10 μm (a'), 25 μm (b' and c'), 50 μm (a, d', e', f') and 100 μm (b-g)

Figure S4. Optimization of the s-Ecx concentration. a) Morphology of microtissues treated with 0% (control) or 1% s-Ecx at day 7. b) 10% s-Ecx did not generate SG microtissues. c) Morphology and histological staining of microtissues treated with 0% (control) and 1% s-Ecx at day 14. Scale bar indicates 100 μm.

Figure S5. Identify of microtissues with or without s-Ecx. a) Immunofluorescence staining of AQP5 following treatment with s-Ecx. b) Immunofluorescence staining of the SG progenitor cell markers K5 and K19 treatment for 24 h and 48 h. c-e) Immunofluorescence staining of ki67 treatment for 24 h and 48 h. Scale bar indicate 25 μm (c'-e') and 50 μm (a-e).

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