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Original article

Functional peptide KP24 enhances submandibular gland tissue growth *in vitro*

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ABSTRACT

Introduction: Salivary gland hypofunction, also known as xerostomia, occurs as a result of radiotherapy for head and neck cancer, autoimmune diseases, or aging. Xerostomia leads to oral health problems and thus affects the quality of life. Biological salivary gland tissue generated *in vitro* would provide an alternative mode of treatment for this disease.
Methods: To develop a novel method for modulating salivary gland tissue growth *in vitro*, we prepared a KP24 peptide-immobilized hydrogel sheet, wherein the peptide comprised repeating proline and lysine sequences, and evaluated the effect of this peptide on salivary gland tissue growth.
Results: We found that the KP24 peptide has the potential to enhance glandular tissue growth *in vitro*. This enhancement is associated with neurite outgrowth and increasing neural innervation.
Conclusion: KP24 peptide modified material would be a promising material for the modulation of salivary gland tissue growth *in vitro*.

1. Introduction

Saliva has multiple functions in the mouth, including an antibacterial effect, mucous protection, pH buffering, mastication of food, and re-calcification of teeth. Previous research has indicated that the number of patients with xerostomia increased in recent years [1]. Salivary hyposecretion is caused by aging, autoimmune diseases, and salivary gland damage due to malignant tumors and radiotherapy [2,3]. Since xerostomia can cause the induction and progression of dental caries, periodontitis, oral candidiasis, and dysgeusia and also cause a burning sensation in the mouth, it leads to a decline in quality of life (QOL). For example, xerostomia patients may have severe problems performing everyday tasks such as eating or holding a conversation [1]. Palliative care, such as gargling, parasympathomimetic drugs, and artificial saliva substitutes, are currently used for xerostomia treatment [4,5]. However, these approaches provide relief for very short periods of time. Therefore, a radical alternative treatment such as the development of tissue regenerative therapy is required.

Recently, the fabrication of salivary gland tissue *in vitro* has been paid attention as an alternative approach for tissue regeneration. Wei et al. demonstrated that submandibular gland (SMG) epithelial cells can self-organize into a tissue in a synthesized environment [6]. Furthermore, Ogawa et al. have established an *in vitro* method for the generation of SMG-like tissue, making use of the condensation of mesenchymal and epithelial cells in type I collagen gel [7]. These studies demonstrated that SMG tissue generation could occur *in vitro*; however, the tissue generated in this manner still exhibited several problems concerning the size and morphology necessary for the transplantation. Hence, developing a method for modulating tissue growth *in vitro* is required. Previously, we have aimed to develop an original substrate that can be used for modulating SMG tissue growth *in vitro*, and prepared hydrogels with different mechanical stiffness [8]. Also, we have prepared a substrate modified by the Arginine-Glycine-Aspartic-acid (RGD) peptide, which is a fibronectin motif. In both case, the developed materials were valuable to modulate SMG tissue growth *in vitro* [9].

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Recently, a peptide called KP24, composed of repeating proline and lysine sequences, was shown to be a trigger for cell aggregate formation in mesenchymal cell culture [10]. This repeating motif was important for cell aggregation not for cell adhesion caused by lysine repeating. Therefore, to investigate the effect of KP24 modified substrate on SMG tissue growth *in vitro*, we attempted to culture SMG tissue on KP24 immobilized alginate hydrogel sheets in this study.

2. Materials and methods

2.1. Preparation of alginate hydrogel sheet

The preparation of alginate hydrogel sheets has been described elsewhere [8]. Briefly, sodium alginate solution (4 wt.%, Wako Pure Chemical, Japan) was poured into a porous alumina mold. The mold was soaked in calcium chloride solution (5wt.%) for 1.5 h to obtain the alginate hydrogel sheet, which was then washed with ethanol and Milli-Q ultrapure water (Millipore, MA, USA), and stored in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM-F12) (Wako Pure Chemical) supplemented with 1% penicillin/streptomycin(PS) (Nacalai Tesque, Japan), for at least 24 h (gel size: 10 × 10 × 1.5 mm). We had previously found that stiffer alginate hydrogel (Young's modulus of 184 kPa) attenuates SMG growth *in vitro*. Therefore, in the current study, we used this stiffer hydrogel as a control, in order to evaluate the effect of the peptide-immobilized substrate on SMG tissue growth.

2.2. Fabrication and analysis of KP24-modified alginate gel

H-(Lys-Pro)12-OH is a linear peptide synthesized using the Fmoc peptide synthesis method. Carbodiimide chemistry was used for the fabrication of KP24-conjugated alginate solution. Briefly, 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride (3×10^{-3} mol/l, EDC, Watanabe Chemical, Japan) was dissolved in cooled ultrapure water, and agitated for 10 min. N-hydroxysulfosuccinimide (3×10^{-3} mol/l, Sulfo-NHS, Thermo Fisher Scientific, IL, USA) and KP24 peptide (7.5×10^{-4} mol/l) were added to the solution and agitated overnight at room temperature.

The solution was purified for 4 days by membrane dialysis (Biotech CE, Spectrum, CA, USA), and lyophilized before storing. To confirm the immobilization of KP24 peptide, the powder obtained after lyophilization was dissolved in citrate buffer solution (pH 2.2), and amino acid analysis was performed.

2.3. Animal experiments

ICR mice were purchased from Charles River Laboratories (MA, USA). All animal procedures strictly adhered to the Guidelines for Animal Experiments at Okayama University. All experimental protocols were approved by Okayama University Animal Care and Use Committee (OKU-2013033 and OKU-2013341).

2.4. SMG tissue culture on KP24-modified gel sheets

SMG tissue was extracted at ICR mouse embryonic day (E) 12.5, and was placed directly on non-modified alginate hydrogel sheets with a mechanical stiffness of 184 kPa, on the KP24-immobilized alginate hydrogel sheets with a mechanical stiffness of 184 kPa, or on tissue culture dishes. The SMG culture was maintained at 37 °C in a humidified atmosphere with 5% CO_2 for 3 days, in DMEM-F12/PS medium. Growth and morphological changes of the SMG tissue in culture were observed by using a microscope (TE2000, Nikon, Japan). The number of buds in the developing SMG tissue was counted at 0, 24, 48, or 72 h using Image J software (NIH, MD, USA). Terminal end bud numbers were obtained from five SMG tissue samples per group. Also, the SMG tissue size was measured by using Image J software at 72 h. Each experiment was repeated at least three times.

2.5. Immunofluorescence staining

The cultured SMG tissues were fixed with 4% paraformaldehyde (PFA) and incubated with fluorescein isothiocyanate (FITC)-conjugated PNA (1:200; Sigma–Aldrich, MO, USA). The fixed tissues were treated with anti-β III-tubulin (1:1000; R&D Systems, MN, USA), Antibody binding was detected with Alexa Fluor-conjugated secondary antibodies (1:200; Life Technologies, NY, USA), and the samples were imaged by confocal microscopy (C1; Nikon).

2.6. MC3T3-E1 cell and mesenchymal stem cell culture on KP24-modified gel sheets

Osteoblast-like MC3T3-E1 cells (Riken BRC, Japan) and mouse mesenchymal stem cells (MSCs) were used for cell culture study. The SMG development is greatly regulated by mesenchymal cells. Since both 3T3E1 cells and mesenchymal stem cells were derived from mesenchymal tissue, we decided to use these cells in this study. MC3T3-E1 cells that had undergone 5–6 passages were harvested and used in this study. These cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere for 7 days, in the α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 1% PS. MC3T3-E1 cells were seeded on KP24 modified alginate gels or non-modified alginate gels at initial densities of 5000 cells/cm^2. Mesenchymal stem cells (MSCs), isolated from the femurs of BALB/c mice (6–10 weeks of age, Shimizu Laboratories, Japan), were cultured as well. Cell adhesion and proliferation on the sheet were observed under a microscope (TE2000, Nikon, Japan).

2.7. PC12 cell culture on KP24-modified gel sheets

PC12 cells were used as a model for nerve cells (ATCC, VA, USA). PC12 cells were seeded at a density of 5000 cells/cm^2 and cultured on KP24-modified and non-modified alginate gels with High Glucose-DMEM (Wako Pure Chemical) supplemented with 10% horse serum (Life Technologies), 5% FBS, 1% PS, and nerve growth factor (NGF, 50 ng/ml, Millipore) at 37 °C in a humidified 5% CO_2 atmosphere for 7 days. Cell number was measured under a

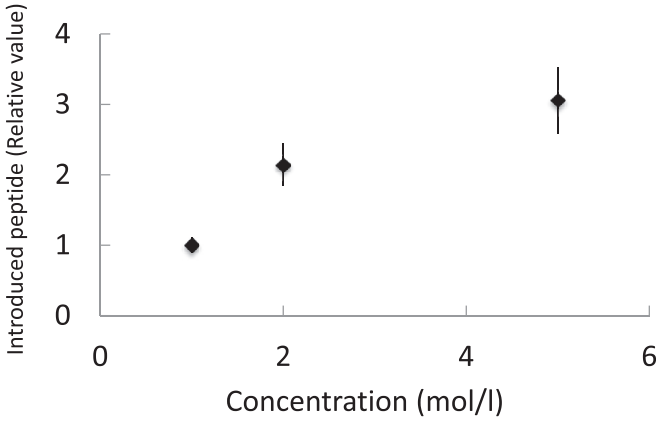

Fig. 1. Different concentrations of peptide introduced into sodium alginate. The amounts introduced into the gel increased in proportion to the initial concentration of peptide in the reactive solution.

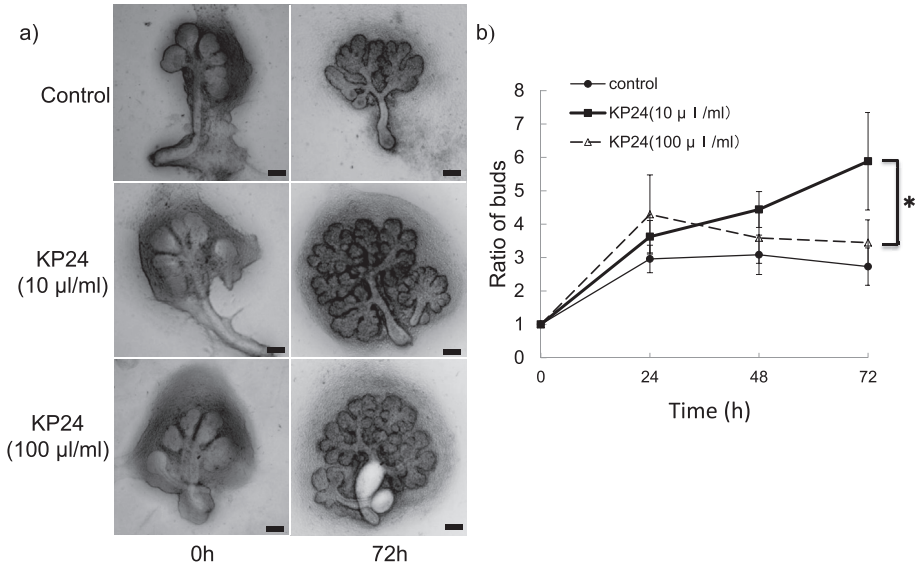

Fig. 2. a,b) SMG on 184 kPa hydrogel sheets cultured in medium containing various KP24 concentrations (0–100 μl/l, Bar = 100 μm, *:$p < 0.05$).

microscope (TE2000, Nikon, Japan). We also measured the length of the neurites growing out from the cell body. The length was measured by using Image J software from obtained images. Five neurites from single cells were found to be the average neurite length in this study.

3. Results

3.1. Effect of KP24 on SMG tissue culture

Amino acid analysis results showed that, depending on the concentration of the peptide added, the amount of immobilized peptide in the sodium alginate gels increased (Fig. 1).

Firstly, KP24 was solely dissolved in media and added to the isolated SMG tissue cultured on the stiff hydrogels. This promoted tissue culture growth suggested that KP24 probably has a specific influence on SMG tissue growth (Fig. 2).

3.2. Effect of KP24 immobilized hydrogel on SMG tissue growth

We then cultured SMG explants on KP24 peptide-immobilized hydrogel (184 kPa) for 72 h. In a previous study, we had observed that stiff hydrogel attenuated SMG growth. However, in the current study, we found that SMG tissues cultured on KP24 immobilized hydrogel exhibited enhanced growth even when the stiff hydrogel was used. The number of buds in the control group did not increase

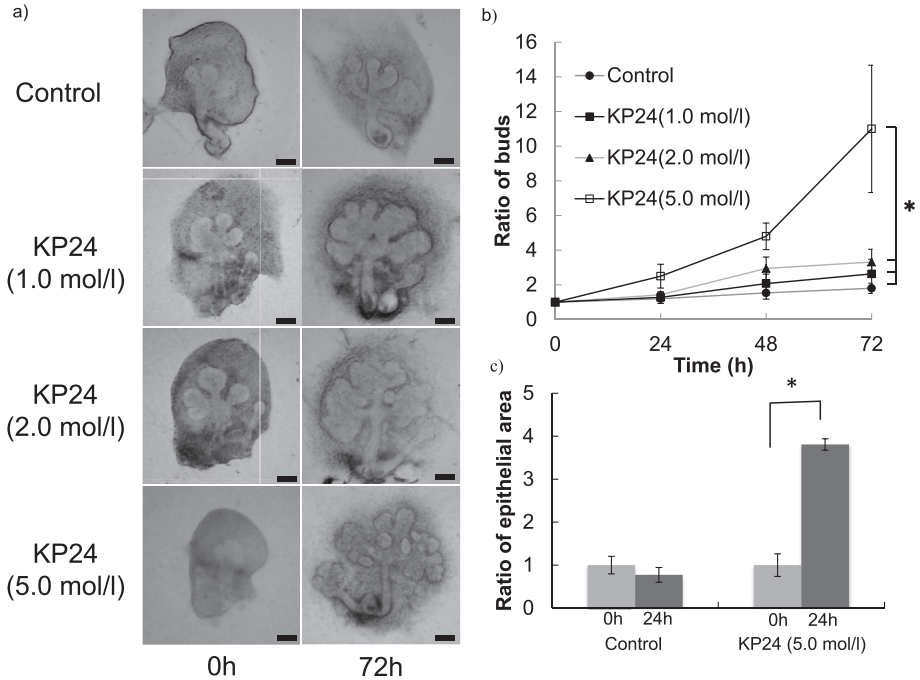

Fig. 3. a–c) SMG cultured on hydrogel sheets modified by introducing various concentrations of KP24 (0–5.0 mol/l, Bar = 100 μm). The SMG growth changed in accordance with the introduced amount of KP24 (*: $p < 0.05$).

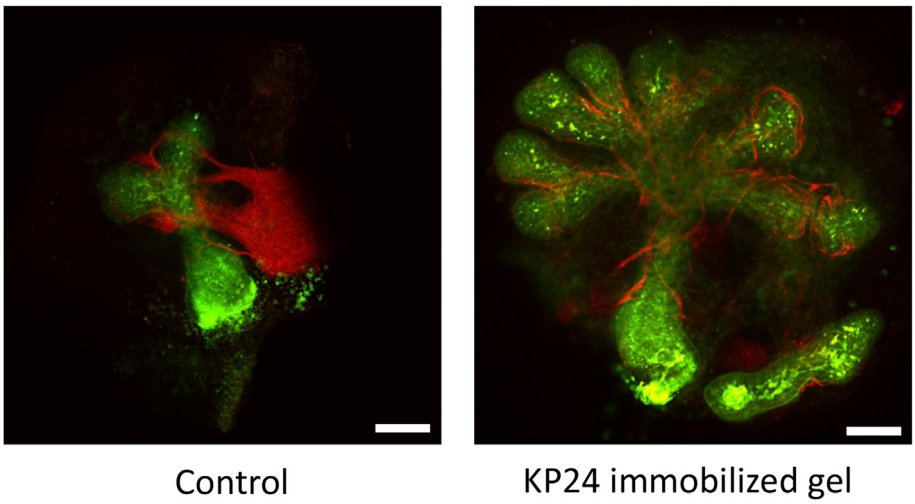

Fig. 4. Neural growth in SMG tissue cultured for 72 h on hydrogel sheets with or without KP24 modification (red: anti-βIII tubulin, green: PNA, bar = 100 μm).

with increase in the duration of SMG tissue culture. However, in the SMG tissue samples cultured on the peptide-immobilized hydrogels, the bud numbers increased on an average from 3 to 30 during 72 h of culture (Fig. 3). Moreover, the epithelial area of SMG tissue was greatly enhanced when it's cultured on KP24 immobilized surface (Fig. 3).

Immunofluorescence staining of the cultured tissue showed that the neurons were well distributed among the proliferating epithelial tissue in the experimental SMG tissue cultures. In contrast, in the control samples, attenuated neuronal growth without cell proliferation was observed (Fig. 4).

3.3. Isolated cell activity on peptide immobilized substrate

In order to understand the mechanism underlying these observations, MC3T3-E1 cells and isolated primary MSCs were cultured on the hydrogels with or without peptide modification. The results indicated that even when peptide-modified hydrogel was used, decrease in cell adhesion was observed, which suggested that extent of cell adhesion depended on the cell type (Fig. 5). On the other hand, when we cultured PC12 cells on the non-modified or modified hydrogels, we found that cell adhesion maintained on the peptide-modified substrate. Moreover, the neuronal outgrowth was significantly higher in PC12 cells cultured on the peptide-modified substrate, which suggested that this peptide was able to influence neuronal cell behavior (Fig. 6).

4. Discussion

In vitro generation of biological tissues has been paid attention in these days [7,11–17]. A specific morphological change called branching morphogenesis is observed in the SMG tissue [18,19]. Since such branching morphogenesis can also be observed in other glandular-like tissues, including the lung, kidney, pancreas, and mammary gland tissues [20,21], methods developed for SMG tissue

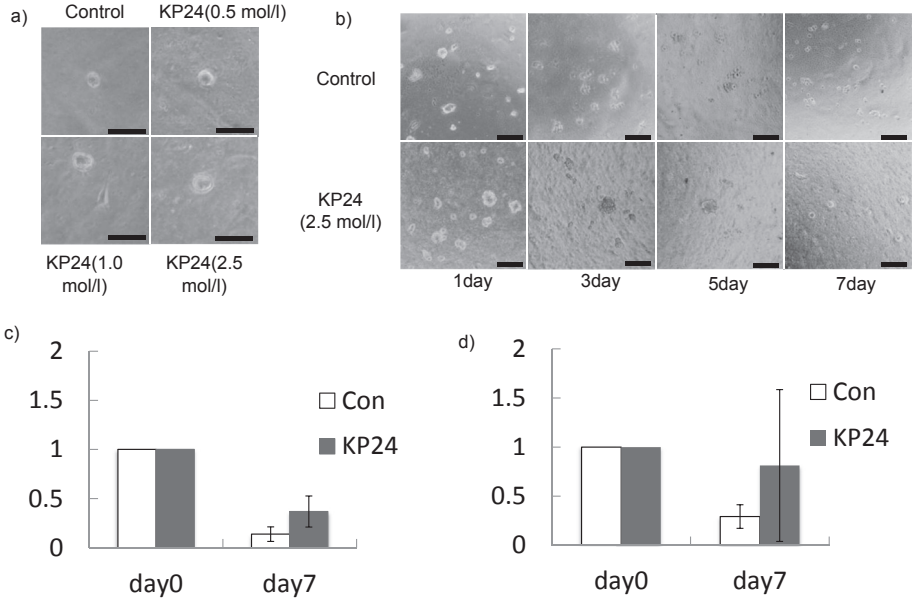

Fig. 5. a) MC3T3-E1 cells (osteoblast-like cell) cultured for 7 days on hydrogel modified by KP24 (0–2.5 mol/l, Bar = 100 μm). b) MSC (Mesenchymal Stem Cells) cultured for 7 days on hydrogel modified by KP24 (2.5 mol/l, Bar = 100 μm). c) Ratio of MC3T3-E1 cell adhesion on hydrogel with or without KP24 modification. d) Ratio of MSC adhesion on hydrogel with or without KP24 modification.

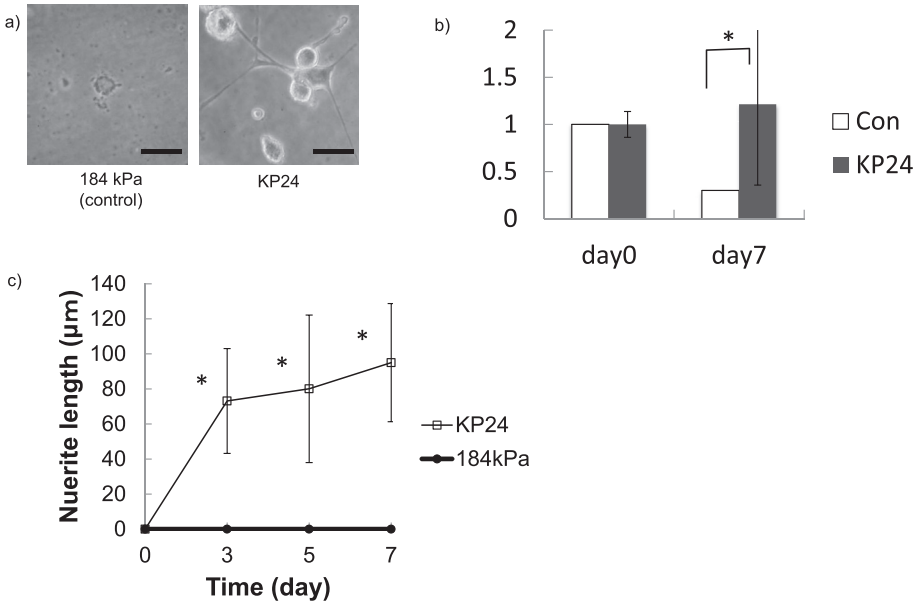

Fig. 6. a) Neural cell culture for 7 days on hydrogel sheets with or without KP24 modification. b) Cell adhesion number on the KP24-modified sheet showed better than do cells on the sheet without KP24 modification (*p: <0.05). c) Cells on the KP24-modified sheet showed better neurite growth than do cells on the sheet without KP24 modification (Bar = 100 μm, *p: <0.05).

growth modulation may be used for the growth modulation of other tissues as well.

In this study, we investigated the ability of a functional peptide to modulate SMG tissue growth *in vitro*. SMG tissue culture results showed that tissue growth is enhanced when the peptide solution is added to the medium. In addition, the peptide-immobilized substrate enhanced the SMG growth significantly in a dose-dependent manner. KP24 is a linear peptide with a repeating lysine (basic amino acid) and proline (neutral amino acid) motif. This peptide is composed of H-$(Lys\text{-}Pro)_n$-OH sequence and promotes cell aggregate formation when it is added to the medium [10]. KP24 (n = 12) exhibits more cell agglutination capacity than KP20 (n = 10) or KP 28 (n = 14). In order to understand the obtained phenomena further, different types of cells were cultured on the peptide-modified, and unmodified substrate. MC3T3-E1 osteoblast-like cells and isolated primary MSCs did not attach well, even to the peptide-modified substrate. These results indicated that the enhanced SMG growth is not related to the interaction between KP24 and general mesenchymal cells. In contrast, PC12 neuron-like cells adhered to the substrate and showed better neurite outgrowth on the peptide-modified substrate when compared to the unmodified substrate.

The importance of neuronal growth in SMG tissue development has been previously reported. For example, removal of the parasympathetic ganglions in mouse explant organ culture decreased the number of epithelial buds and slowed down SMG morphogenesis [22,23]. In one of our recent reports, we demonstrated that attenuation of neurturin (NRTN), a neurotrophic factor, inhibits *ex-vivo* SMG growth even when cultured on the RGD-modified alginate hydrogel. We have also shown that neurite outgrowth is crucial for increasing SMG growth as well [9]. Therefore, we propose herein that the KP24 peptide-immobilized substrate plays a role in SMG tissue growth *in vitro* by promoting neuronal survival and growth, which leads to the growth and expansion of the SMG tissue in culture.

Since peptide synthesis method is well-established, this peptide-based approach would be a promising method for the modulation of SMG tissue growth *in vitro*.

5. Conclusion

KP24 peptide enhanced SMG tissue growth by increasing neuronal growth and enhancing neural innervation. The peptide-modified substrate would be a promising tool for the modulation of SMG tissue growth *in vitro*.

Conflict of interest

All authors declare no conflict of interest associated with this manuscript.

Acknowledgment

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