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The specificity of chitosan in promoting branching morphogenesis of progenitor salivary tissue

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ABSTRACT

Chitosan has been shown to be effective in regulating progenitor salivary tissue morphogenesis, however, the specificity of chitosan effects remains unclear. To assess the regulatory ability of chitosan in salivary gland morphogenesis, progenitor salivary tissue from embryonal submandibular gland (SMG) was cultured in chitosan-containing medium. It was found that soluble chitosan was able to promote SMG branching in a dose-dependent manner. The effect was chitosan-specific and was not reproduced by substrates with similar chemical structures or other polymeric molecules of natural or synthetic origin. Furthermore, the branch-promoting effects were molecular weight-dependent. In addition, following digestion with lysozyme, chitinase, or chitosanase, digested chitosan was unable to reproduce the similar effects. In all, this study clarifies the specificity and preferential activity of chitosan in enhancing branching morphogenesis of progenitor salivary tissue and highlights its potential utility for application in salivary tissue regeneration.

Branching morphogenesis is a fundamental process in the embryogenesis of vascular, aerodigestive, and excretory tissues to form ramified structures. By forming complex and branching networks, the efficacy and capacity of numerous metabolic exchanges efficiently increase, thereby meeting the physiological demands of multicellular organisms to sustain life. The salivary glands are an example of organs formed by the reiteration of similar branching processes. They are important glandular organs responsible for regulating saliva secretion to maintain oral hygiene, aid with digestion, and control infection. During organogenesis, the glands begin from a single epithelial bud of oral epithelium [1]. Subsequently, by reciprocal and dynamic interaction with the surrounding mesenchyme, these epithelial cells start to proliferate and ramify, accompanied by the appearance of clefts and branches. This process is repeated several times, and a tree-like complex structure is finally formed and organized [1].

Chitosan is a linear polysaccharide consisting of β (1–4) linked D-glucosamine residues with a variable number of *N*-acetyl-glucosamine groups in its structure. As it is biodegradable, biocompatible, and bioadhesive, chitosan has been highlighted as a useful biomaterial for tissue engineering [2]. Chitosan mediates biological functions by interacting electrostatically with other anionic molecules including glycosaminoglycans (GAGs), proteoglycans, and many proteins that play key morphogenetic roles during development [2,3]. As such, chitosan-containing biomaterials that are capable of cooperating with essential morphogenetic factors might be more useful in tissue engineering than other substrates, owing to their ability to provide morphogenetic guidance for engineered tissue.

To regenerate a functional salivary gland, it is necessary to recapitulate the tissue branching process. The formation of a branched structure might reduce the cell number and tissue volume needed to fulfill physiological requirements. Previously, our group showed that the chitosan membrane was bioactive and competent in promoting salivary gland branching by providing an interactive environment for progenitor salivary tissues [4]. Another study by our group showed that the soluble form of chitosan was also capable of facilitating branching morphogenesis of SMG [5]. We demonstrated that chitosan might cooperatively interact with mesenchyme-derived morphogens to enhance SMG branching. These studies indicated a novel function and a potential application of chitosan in regulating progenitor salivary tissue morphogenesis.

To date, however, the unique properties of chitosan that enable branch-promoting, as well as the specificity of its effects, remain unclear. Therefore, this study aimed to investigate the properties of chitosan responsible for mediating the SMG branch-promoting effects by using different approaches, such as comparison with other similar chemicals and administration of digestive enzymes for specific polymeric linkages. It was found that the effect was

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unique to chitosan, and was dependent on molecular weight and specific chemical linkage. The current study yields key information on the unique regulatory properties of chitosan, which would be useful for the future investigation and application of chitosan in salivary gland regeneration.

Materials and methods

SMG ex vivo organ culture. SMG explants were retrieved from E12 ICR mice. Animal protocols were approved by the National Taiwan University Animal Care and Use Committee. The explants were cultured as described [5]. The SMG medium was composed of DMEM/F12 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 150 μg/ml vitamin C, and 50 μg/ml transferrin [4,5]. The SMG explants were cultured at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. The SMG explants were photographed and measured at the indicated time-points of culture. SMG branching numbers were presented as the ratio change in budding, obtained by dividing the buds measured at the indicated experimental condition by those in the corresponding control after 48 h of culture. Each experimental set was repeated at least three times. The statistical analyses of the ratio change of buds were evaluated by *t*-test and paired *t*-test where appropriate.

Preparation of chitosan-containing culture medium. The chitosan culture medium was prepared from a 2 wt.% (w/v) chitosan solution by dissolving chitosan (448869, Mn:612 kD, Sigma–Aldrich Chemical Co. St. Louis, MO, USA) in 1 M acetic acid, mixing it with SMG medium and neutralizing it with sodium hydroxide [5,6]. It was prepared in concentrations ranging from 0.01 mg/ml to 0.4 mg/ml. Mock SMG medium was prepared in the same way as that of chitosan-containing SMG medium, mixing the same amount of acetic acid and sodium hydroxide without adding chitosan. Our previous study found that mock SMG medium and SMG medium had similar effects on the branching morphogenesis of SMG explants without significant differences [5]. Therefore, the representative control of SMG medium was used for comparison in the following assays.

Preparation of chitosan monomers, analogues, and related polymeric substrates. Chitosan monomers and analogues, including *N*-acetyl-D-glucosamine (GluNAc), *N*-acetyl-D-mannosamine (ManNAc), *N*-acetyl-D-galactosamine (GalNAc), and D-glucosamine (GluN) were obtained from Sigma (St. Louis, MO). The oligomeric forms of glycosaminoglycans such as heparan sulfate (HS, H7640) and chondroitin sulfate (CS, C9819) were also purchased from Sigma. Chitosan with different molecular weights (C-3646, Mn:810 kD, degree of deacetylation: 75–85%; 448869, Mn:612 kD, degree of deacetylation: 75–85%) and chitosan oligosaccharide lactate (523682, Mn < 5 kD, degree of deacetylation: 90%) were also purchased from Sigma. Type I collagen and laminin, obtained from BD Biosciences (San Jose, CA), were prepared in the culture medium of the SMG explants with the same concentration of chitosan. Poly-lysine was purchased from Sigma. These substrates were dissolved in SMG medium and used for culture as described above.

Enzyme digestion assay. In the enzyme digestion assay, chitosan was digested using the indicated enzyme prior to SMG explant culture. For each enzyme reaction, lysozyme (Sigma, L6876, 1 mg/ml), chitinase (Sigma, C6137, 1 mg/ml), and chitosanase (Sigma, C9830, 1 mg/ml) were used to digest chitosan overnight in accordance to the manufacturer's protocol. After digestion, the enzyme-treated chitosan was used to prepare the chitosan-containing SMG medium for explant culture in the same way as those described above. Similarly, equal amounts of enzyme were also used to treat the control. In addition, the enzyme-digested chitosan was separated using a membrane with specific molecular weight pore sizes (Scientific, Cellu Sep T4: MW: 12000–14000; Scientific, Cellu Sep T2, MW: 6000–8000). The filtrate was subsequently used to prepare chitosan-containing medium for SMG explant culture.

Results

Chitosan promotes SMG branching morphogenesis in a dose-dependent manner

The branching morphogenesis of cultured SMG explants with different concentrations of chitosan ranging from 0.01 to 0.4 mg/ml is shown in Fig. 1. When chitosan was added in the culture medium, the branching numbers of SMG explants increased. The increasing tendency of budding correlated with the concentration of chitosan, suggesting that chitosan promoted SMG branching morphogenesis in a dose-dependent manner. The effect of chitosan in regulating SMG explant branching was the most significant at a concentration of 0.3 mg/ml among all experimental conditions. Therefore, 0.3 mg/ml of chitosan was used in the following assays for the investigation of chitosan effects.

Effects of GAGs on SMG branching morphogenesis

In light of the structural and functional resemblance of chitosan to GAGs [2], the effects of GAGs on SMG branching morphogenesis were examined. When HS was added to the culture medium, shape deformation of cultured SMG explant was detected. Not only did the branching number decrease, the phenotypes of the cultured explants were seemingly underdeveloped (Fig. 2A). On the other hand, when CS was added, the cultured SMG explants showed similar phenotypes to explants with added chitosan, and the quantitative results showed no significant increase in SMG branching numbers ($p > 0.05$) (Fig. 2A). These results indicate that the manner in which chitosan facilitates SMG branching varies.

Effects of chitosan monomers and analogues on SMG branching morphogenesis

To elucidate the underlying properties of chitosan effects, monomers and analogues of chitosan were prepared. Chitosan is composed of a combination of GluNAc and GluN moieties. Therefore, to determine whether the effects of chitosan were derived from these constituents, the two monomers were prepared separately in the cultured medium. When cultured for 48 h, chitosan monomers showed no activity on the cultured SMG explants. The explants demonstrated similar phenotypes compared to those of control, and the branching number was statistically insignificant ($p > 0.05$) (Fig. 2B). Moreover, to determine whether the effect of chitosan was a general phenomenon of chemicals with similar structures, analogues of chitosan, including ManNAc and GalNAc, were examined. Fig. 2 shows that, when compared to the control group, the chitosan analogues added in culture at the same concentration did not exhibit significant effects on SMG branching ($p > 0.05$) (Fig. 2B). This suggested that the branch-promoting effects of SMG were specific to chitosan, rather than being a general effect of analogues with similar chemical structures.

The effects of natural and synthetic polymers on SMG explant branching

Because chitosan was a polymer with intricate crosslinking networks, natural and synthetic polymers that had similar polymeric structures, including type I collagen, laminin, and poly-lysine, were examined, respectively. Type I collagen and laminin are endogenous natural polymers produced in the developing salivary gland and are essential components of salivary extracellular matrix [1]. In addition, polylysine is a polypeptide that is commonly used to enhance cell adhesion to the culture surface during *in vitro* cultivation [7]. When type I collagen was supplemented in culture, the SMG explants exhibited branching activity.

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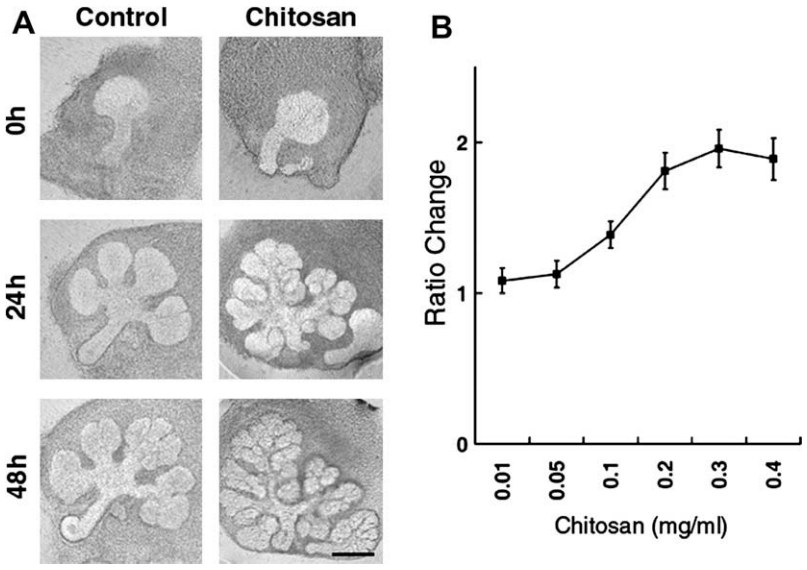

Fig. 1. Effects of chitosan on salivary branching morphogenesis (A) SMG explants cultured in control medium (control) and chitosan medium (chitosan) (0.3 mg/ml) for 48 h. Scale bar = 100 μm. (B) Quantification was performed at 48 h and was presented as the ratio change of buds.

Nonetheless, the increase in branching number was not significant compared to that of the control ($p > 0.05$) (Fig. 2C). Similar results were shown in the SMG explant culture supplemented with laminin ($p > 0.05$) (Fig. 2C). Furthermore, when poly-lysine was added to the cultured medium, the SMG explants not only decreased in branching number but also demonstrated different phenotypes with decreasing sizes of epithelial components (Fig. 2C). These results suggested that the branch-promoting effects of chitosan did not simply result from the common effects of crosslinking structures, but that the constituents comprising the network might also play a role.

The effect of chitosan molecular weight on SMG explants branching

Because the molecular weight of chitosan has been shown to be important in determining its biological response [8,9], the role of molecular weight in regulating SMG explant branching activities was further examined. Chitosan culture medium was prepared by adding chitosan with different molecular weights. When SMG explants were cultured in medium supplemented with chitosan with molecular weight of 612 kD (kiloDaltons) and 810 kD, the explants sprouted significantly more branches than the control after 48 h in culture ($p < 0.01$) (Fig. 3). In contrast, when SMG explants were cultured in the presence of chitosan oligomers that were less than 5 kD, the effects disappeared (Fig. 3). The explants cultured with chitosan oligomers did not exhibit more budding than the control. Furthermore, the effects of chitosan's molecular weight were further confirmed by applying chitosan filtrate that had been prepared by enzyme digestion and membrane filtration. The results showed that chitosan filtrate less than 14 kD was unable to affect the branching efficiency (data not shown). This suggested that the effects existed only in the presence of chitosan with a higher molecular weight, and not in the presence of chitosan with smaller molecular weights or chitosan oligomers.

The effect of lysozyme, chitinase, and chitosanase on the chitosan-mediating SMG branching morphogenesis

Based on the previous results, linkages between chitosan monomers, which are essential for molecular weight accumulation, might be pivotal for the effects of chitosan to occur. To investigate this possibility, we used enzymes that were able to hydrolyze the intrinsic linkage within the chitosan, including lysozyme, chitinase, and chitosanase [10]. Lysozyme is capable of hydrolyzing the linkage between GluNAc residues in chitodextrin. When it was added to the control medium, no differences in the phenotypes or branching numbers of SMG explants were found compared to those of the control group, implying that lysozyme itself was not detrimental to the branching morphogenesis of cultured SMG explants. On the other hand, when the lysozyme-treated chitosan medium was used, the effect of chitosan was abolished. The branching numbers of SMG explant showed no difference when compared to the control group, suggesting that the linkage between the GluNAc moieties within the chitosan polymer might responsible for its effects (Fig. 4).

Chitinase has also been reported to be able to cleave the linkage between GluNAc monomer subunits within chitosan. As such, chitinase was used to further confirm the importance of the GluNAc linkage in the effects of chitosan. When chitinase was added to the culture system, no alteration of SMG branching phenotypes and numbers were observed, suggesting that the presence of chitinase was not deleterious to the morphogenesis of SMG explants. When the chitinase-treated chitosan was prepared for culture, the effects of chitosan could not be detected (Fig. 4). Accordingly, the results further confirmed that the linkage between the GluNAc monomer was required for the effects mediated by chitosan.

Chitosan consists of linked moieties of GluNAc and GluN. Chitosanase has been shown capable of hydrolyzing the linkage between these subunits. Therefore, it was used to further verify the role of this linkage in mediating chitosan effects. When chitosanase was applied to the control medium, no change in the phenotypes or branching numbers of the explants were found, suggesting that the presence of chitosanase did not adversely affect cultured salivary tissue. When chitosan was treated with chitosanase, the effect of chitosan was absent in culture, indicating that the chitosan branch-promoting effects were affected by chitosanase (Fig. 4). These results showed that the linkage between GluNAc and GluN was also critical to the chitosan effects in branch promotion.

Discussion

In this study, the preferential properties of chitosan that are effective in stimulating branching morphogenesis of progenitor

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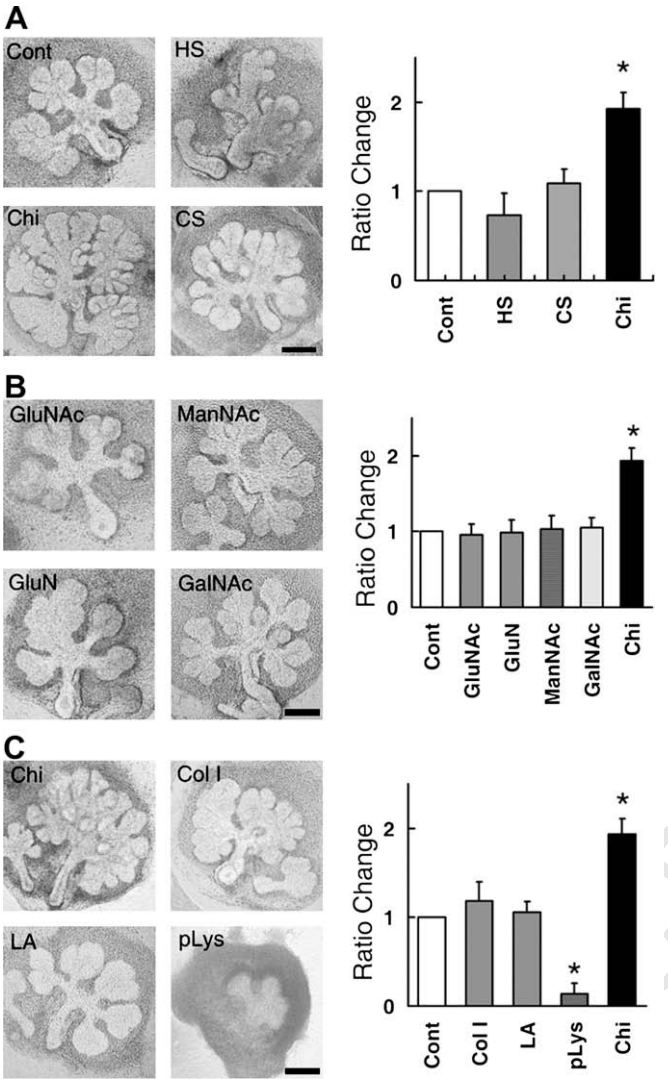

Fig. 2. Effects of GAGs (A), chitosan monomers and analogues (B), and polymeric molecules (C) on salivary branching morphogenesis. Quantification was performed at 48 h and was presented as the ratio change of buds. (Cont, control; Chi, chitosan; Col I, type I collagen; LA, laminin; pLys, poly-lysine). Asterisks (*) denote significant differences ($p < 0.01$) compared with control. Scale bar = 100 μm.

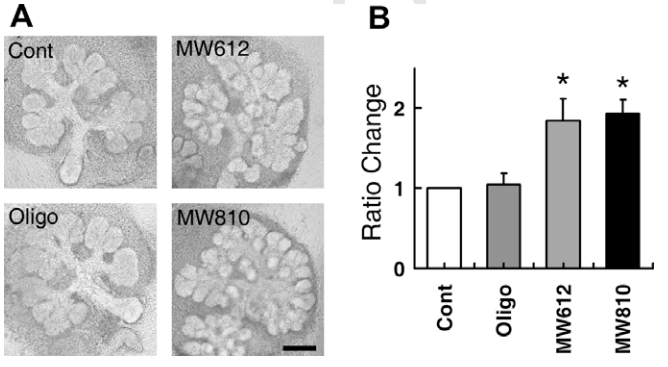

Fig. 3. Effects of chitosan molecular weights on salivary branching morphogenesis. (A) SMG explants cultured with chitosan of different molecular weights (MW612: 612 kD; MW810: 810 kD) and oligochitosan (Oligo) for 48 h. (Cont: control) Scale bar = 100 μm. (B) Quantification was performed at 48 h and was presented as the ratio change of buds. Asterisks (*) denote significant differences ($p < 0.01$) compared with control.

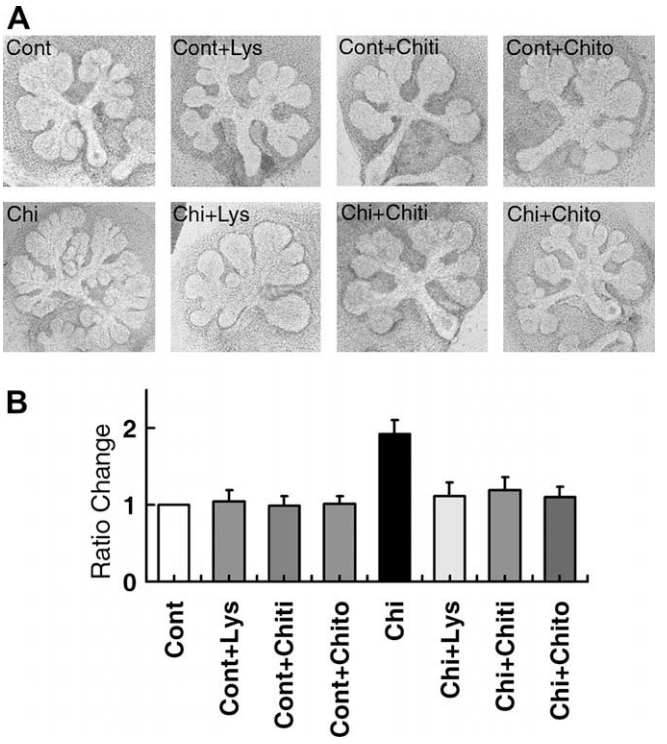

Fig. 4. Effects of lysozyme, chitinase, and chitosanase in chitosan-mediated SMG branching morphogenesis. (A) SMG explants cultured in the indicated conditions for 48 h. (Cont, control; Cont + Lys, Cont + Chiti, Cont + Chito: control treated with lysozyme, chitinase, and chitosanase, respectively; Chi, chitosan; Chi + Lys, Chi + Chiti, Chi + Chito: chitosan treated with lysozyme, chitinase, and chitosanase, respectively) Scale bar = 100 μm. (B) Quantification was performed at 48 h and was presented as the ratio change of buds. Asterisk (*) denotes a significant difference ($p < 0.01$) compared with control.

salivary tissue are explored. Previously, we showed that soluble chitosan was able to promote salivary tissue branching morphogenesis by cooperating with mesenchyme-derived morphogens [5]. In the current study, we provide further evidence that the branch-enhancing effect is unique to chitosan. The higher molecular weight and the presence of chitosan-specific linkage are essential in facilitating the development of salivary branching structures.

Chitosan is a natural polysaccharide derived from partially deacetylated chitin. The molecular weight of chitosan is important in determining its biological response [8,9,11]. It has been reported that chitosan with a molecular weight above 40–80 kD is able to enhance peptide absorption [12]. Moreover, chitosan with a higher molecular weight can increase the loading capacities of protein [13]. Similarly, in this study, high molecular weight chitosan demonstrated branch-promoting properties of progenitor salivary tissue, while others did not. In the enzyme digestion assay, the chitosan effects disappeared, and the chitosan filtrate smaller than 14 kD failed to demonstrate similar effects. These results provide further evidence that the effects of chitosan are correlated to its molecular weight. One of the possible underlying mechanisms accounting for the impact of molecular weight on chitosan effects might stem from the formation of specific structural complexes, which in turn influence the biological responses [8,9,14].

To be functional during tissue development, many molecules involved in endogenous morphogenesis are presented in the polymerized form. For example, polymerization of collagen is required for its bioactivity. Many diseases might result from abnormal degradation and crosslinking of collagen [15]. During branching morphogenesis of salivary tissue, the alignment and deposition of collagen fibers provide guiding information and physical forces

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that delineate the boundary of the epithelium and mesenchyme. The application of collagenase largely disrupts collagen function by dissembling the collagen structure and network. This results in abnormal and underdeveloped branching phenotypes in salivary tissue [16]. Similarly, the basement membrane, a component critical to salivary tissue morphogenesis, is composed of an intricate polymeric network that is predominantly composed of crosslinked laminin and collagen [15,17]. The digestive enzyme that disintegrates the network of basement membrane leads to an alteration in the developing phenotypes of salivary tissue [17]. Therefore, in agreement with the idea that the polymeric structure might play a major role in mediating biological activities during salivary tissue morphogenesis, it is logical to presume that biomaterials that are used for tissue engineering should provide a network structure resembling that of endogenous tissue. In the current study, this is compatible with the concept that the preservation of polymeric structure is indispensable for chitosan to act as the branch promoter for progenitor salivary tissue.

In biomedical applications, chitosan has been extensively used for the preparation of nontoxic polyelectrolyte complex products [14]. Its polycationic property enables it to directly interact with numerous natural polyanionic molecules, such as collagen, dextran sulfate, heparin, hyaluronic acid, etc., to develop crosslinking network structures. It has also been demonstrated that chitosan can interact with GAGs directly by forming a complex [14,18]. These polyanionic molecules fundamentally constitute the extracellular matrix, which is important in mediating cell functions. By forming polyelectrolyte complexes, chitosan is able to enhance the absorption and immobilization of these molecules, which are beneficial to tissue reconstruction [12]. Generally, the mechanism of polyelectrolyte complex formation is molecular weight-dependent, and the molecular weight may be varied by the structural change of constituting polymers [9]. Hence, the enzymatically hydrolyzed chitosan might provide fewer polymeric chain entanglements, resulting in the alteration of the physicochemical properties of polymeric complex, and affecting the related biological functions [9].

It is well known that chitosan might affect cellular function in the same manner as that of GAGs [2]. During salivary tissue development, GAGs are capable of regulating morphogen activities [19]. However, their effects could be eliminated if the intrinsic linkages are interrupted or if GAGs are to be provided in unlinked forms [19,20], indicating that the polymeric structure of GAGs is essential. Likewise, Chitosan can directly interact with morphogens, which protects morphogens from degradation, activates the associated signaling, and finally, directs tissues to perform desired functions [21]. In our previous work, the synergistic effects between chitosan and morphogens on salivary tissue have been shown [5]. The ineffectiveness of chitosan oligomers and the functional impairment of chain-interrupted chitosan in current work provide evidence that maintaining the chain structure of chitosan is the key factor which contributes to the effectiveness of this phenomenon on progenitor salivary tissue.

In the current study, the branch-promoting effects of SMG are only observed in chitosan but are not seen in other chitosan-related analogues or polymeric molecules. The results exclude the possibility that the morphogenesis-regulating effect of chitosan is simply derived from the chemical structure of its subunits, or from the general effects of a polymeric network. Though natural polymers like collagen and laminin are essential in the endogenous development of salivary tissue, it has been shown that the exogenous supplementation of these polymers in culture systems does not confer any advantages in promoting salivary tissue morphogenesis. Therefore, chitosan is specific in regulation of branch promotion.

In conclusion, this study demonstrates the specificity of the branch-promoting effects of chitosan in regulating progenitor salivary tissue morphogenesis. By providing specific polymeric linkages and an appropriate molecular weight, chitosan is capable of promoting progenitor salivary tissue to establish ramified structures more efficiently. This work suggests possible unique properties accounting for chitosan-promoting effects in salivary gland morphogenesis, and provides useful information for chitosan to be selected as a promising substrate for salivary gland investigations and regeneration.

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