

# Gibberellic acid induces $\alpha$ -amylase expression in adipose-derived stem cells

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**Abstract.** Salivary  $\alpha$ -amylase is the most important enzyme for oral digestion of dietary starch. Therefore, regeneration of the salivary glands via a tissue engineering approach is clearly required for patients with salivary gland dysfunction. Early during seed germination, the embryo synthesizes gibberellic acid ( $GA_3$ ), a plant hormone that activates the synthesis and secretion of  $\alpha$ -amylase. The purpose of this study was to explore an approach for differentiation of stem cells into salivary glands using  $GA_3$ . We isolated adipose-derived stem cells (ASCs), which are positive for mesenchymal stem cell markers (CD73, CD90 and CD105) and possess pluripotency to osteoblasts, adipocytes and neural cells, from human buccal fat pads, which are a readily available source for dentists and oral surgeons. In addition, we investigated the cytotoxicity of  $GA_3$  for human ASCs.  $GA_3$  neither affects cell morphology nor cell viability in a dose- or time-dependent manner. ASCs were incubated with  $GA_3$  to assess mRNA and protein expression of  $\alpha$ -amylase by reverse transcriptase-polymerase chain reaction and western blot analyses.  $\alpha$ -amylase mRNA expression on 21 days after treatment with  $GA_3$  (1 mM) was seven times greater than that in resting condition (Day 0). While we did not detect  $\alpha$ -amylase bands on Day 0,  $\alpha$ -amylase protein was detectable 7 days after treatment with  $GA_3$ , reaching a maximal level on Day 21. Our results indicated that  $GA_3$  can increase cellular  $\alpha$ -amylase expression and that our induction method would be useful for therapeutic application for salivary gland regeneration.

## Introduction

In human saliva,  $\alpha$ -amylase is the most abundant protein (1), accounting for 40-50% of salivary protein (2), and has the

important capacity to rapidly alter the physical properties of starch in the oral cavity (3). Aging or radiation therapy for head and neck cancer leads to severe salivary gland dysfunction and consequential xerostomia (dry mouth syndrome), resulting in hampered speech, dental problems, difficulties with swallowing and food mastication, impaired taste, and nocturnal oral discomfort (4-6).

Mesenchymal stem cells (MSCs) have been isolated from various tissues, such as bone marrow (7), muscle (8), skin (9), and adipose tissue (10). Among them, adipose tissue contains 100- to 300-fold more MSCs than the bone marrow (11). Recent studies have identified adipose-derived stem cells (ASCs) that can differentiate along multiple pathways, including into osteogenic, adipogenic, myogenic, and chondrogenic lineages, if an appropriate environment is provided (12-17). Thus, ASCs have increasingly gained importance due to their abundance in tissues and easy availability for extraction (1).

Plant hormones are small organic molecules commonly used to increase grain production (18,19). Among the hormones, gibberellic acid ( $GA_3$ ), a plant growth regulator, is used worldwide to increase the growth of fruits, such as strawberries, grapes, and date palm (20) and of some vegetables, such as tomatoes, cabbages, cauliflower, peppers, and olives (21-23). Signal transduction pathways of  $GA_3$  enable aleurone cells to modulate hydrolase production, mainly  $\alpha$ -amylase, in response to hormonal and environmental stimuli. These enzymes digest the stored starch and other nutrients in the endosperm to support the growth of young seedlings.

Although  $GA_3$  is widely used in agriculture, its effects on human health have not been well explored. Thus, we focused on the potential effects of  $GA_3$  and demonstrated a novel induction approach that buccal fat pad (BFP)-derived ASCs differentiate into salivation cells with  $GA_3$  treatment.

## Materials and methods

**Primary culture of human ASCs.** BFPs were obtained from healthy donors at Chiba University Hospital, Chiba, Japan. All donors provided written informed consent for a protocol reviewed and approved by the institutional review board of Chiba University. To isolate ASCs, we performed the centrifuge methods described previously (12). Briefly, the adipose tissues were harvested, washed extensively with PBS, minced

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for 10 min with fine scissors, and enzymatically digested at 37°C for 40 min with 0.1% collagenase (Wako, Osaka, Japan). An equal volume of control medium (Dulbecco's modified Eagle's medium/F-12; Sigma-Aldrich Co., St. Louis, MO) containing 10% fetal bovine serum (FBS; Sigma Aldrich Co.) and 50 U/ml penicillin and streptomycin (Sigma Aldrich Co.) was then added to neutralize the collagenase. The cell suspension was centrifuged at 1,300 rpm (260 x g) for 5 min to obtain a high-density ASC pellet, which was resuspended in control medium. After being counted using trypan blue, the cells were plated at a concentration of 5x10<sup>5</sup> cells/100-mm cell culture dishes (BD Biosciences, Franklin Lakes, NJ) and kept in the control medium at 37°C in 5% CO<sub>2</sub>.

*Flow cytometric analysis of ASCs.* Cultured ASCs were washed twice in cold PBS supplemented with 2% FBS (Sigma-Aldrich Co.) and resuspended to a concentration of about 1x10<sup>6</sup> cells/antibody test and labeled with anti-human CD73-PE, CD90-FITC, CD105-PerCP, CD31-PE, CD34-PerCP, and CD45-FITC antibodies for 20 min at room temperature in the dark (BD Biosciences). The labeled cells were analyzed using a fluorescence-activated cell sorter (FAC; BD Biosciences). Negative control stains were performed using FITC-, PE- and PerCP-conjugated mouse IgG1  $\kappa$  isotypes (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

*Differentiation culture conditions.* To induce osteogenic differentiation, ASCs were cultured in an osteogenic differentiation basal medium containing osteogenic supplement (Invitrogen, Carlsbad, CA). After 3 weeks, osteogenic differentiation was evaluated with alkaline phosphatase (ALP) staining (Primary Cell Co., Ltd., Hokkaido, Japan). Adipogenic differentiation of ASCs was induced by adipocyte differentiation basal medium containing an adipogenic supplement (Chemicon International, Inc., Temecula, CA) for 4 weeks. After induction, the cells were stained with Oil Red O (Sigma). To induce neural differentiation, ASCs were grown in neural differentiation medium (Thermo Fisher Scientific, Rockford, IL) for 3 days. The induced cells were subjected to immunocytochemical analysis to assess the expression of nestin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a neural marker.

*GA<sub>3</sub> cytotoxicity.* ASCs were seeded at a density of 1x10<sup>4</sup> cells/60-mm cell culture dishes (BD Biosciences) in the control medium with the indicated concentrations of GA<sub>3</sub> for the indicated time points. The effect of GA<sub>3</sub> cytotoxicity on the numbers of ASCs was determined using phase-contrast microscopy and a trypan blue exclusion test.

*Treatment of ASCs with GA<sub>3</sub>.* The ASCs at 80% confluence were incubated in the control medium with the indicated concentrations of GA<sub>3</sub>. ASCs were harvested for extraction of total-RNA and protein at 0, 7, 14, 21 and 28 days after 1 mM GA<sub>3</sub> treatment.

*Preparation of cDNA.* Total-RNA was isolated using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. cDNA was generated from 5  $\mu$ g of total-RNA using Ready-To-Go You-Prime First-Strand Beads (GE

Healthcare, Buckinghamshire, UK) and oligo(dt) primer (Sigma-Genosys, Ishikari, Japan), according to the manufacturer's instructions.

*mRNA expression analysis.* To evaluate the expression levels of  $\alpha$ -amylase in ASCs, real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed. qRT-PCR was carried out with one method using a LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). The PCR reactions using the LightCycler apparatus were performed in a final volume of 20  $\mu$ l of a reaction mixture consisting of 2  $\mu$ l of FirstStart DNA Master SYBR-Green I mix, 3 mM MgCl<sub>2</sub>, and 1  $\mu$ M primers, according to the manufacturer's instructions. The reaction mixture was loaded into glass capillary tubes and subjected to an initial denaturation at 95°C for 10 min, followed by 45 rounds of amplification at 95°C (10 sec) for denaturation, 62°C (10 sec) for annealing, and 72°C (10 sec) for extension, with a temperature slope of 20°C/sec. Amplified products were analyzed by 3% agarose gel electrophoresis to ascertain size and purity. The transcript amounts for the target genes were estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript amount determined in corresponding samples. The following primers were used:  $\alpha$ -amylase, forward, 5'-ATTTTCATGTGCCCCGTTGT-3' and reverse, 5'-CCCATGTGATGGACCAATGTC-3'; GAPDH, forward, 5'-CATCTCTGCCCCCTCTGCTGA-3' and reverse, 5'-GGATGACCTTGCCCACAGCCT-3'.

*Protein extraction.* The cells were washed twice with cold PBS and centrifuged briefly. The cell pellets were incubated at 4°C for 30 min in a lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, and 10 mM Tris pH 7.4) with a proteinase inhibitor cocktail (Roche Diagnostics). The protein concentration was measured with the BCA Protein Assay kit (Thermo Scientific).

*Evaluation of  $\alpha$ -amylase protein expression by western blot analysis.* Protein extracts were electrophoresed on 4-12% Bis-Tris gels, transferred to nitrocellulose membranes (Invitrogen), and blocked for 1 h at room temperature in Blocking One (Nacalai Tesque, Kyoto, Japan). The membranes were washed three times with 0.1% Tween-20 in Tris-buffered saline and incubated with anti-human  $\alpha$ -amylase (1:100 dilution) and  $\beta$ -actin (1:1,000 dilution) monoclonal antibodies (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membranes were washed again and incubated for 1 h at room temperature with a 1:2,500 of goat anti-mouse IgG (H+L) HRP conjugate (Promega, Madison, WI) as a secondary antibody. Finally, the membranes were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) and immunoblotting was visualized by exposing the membranes to ATTO Light-Capture II (ATTO, Tokyo, Japan). Signal intensities were quantitated using the CS Analyzer version 3.0 software (ATTO).

## Results

*Isolation of ASCs from human BFPs.* FACS analysis of BFP-derived ASCs at the fifth passage showed that the cells

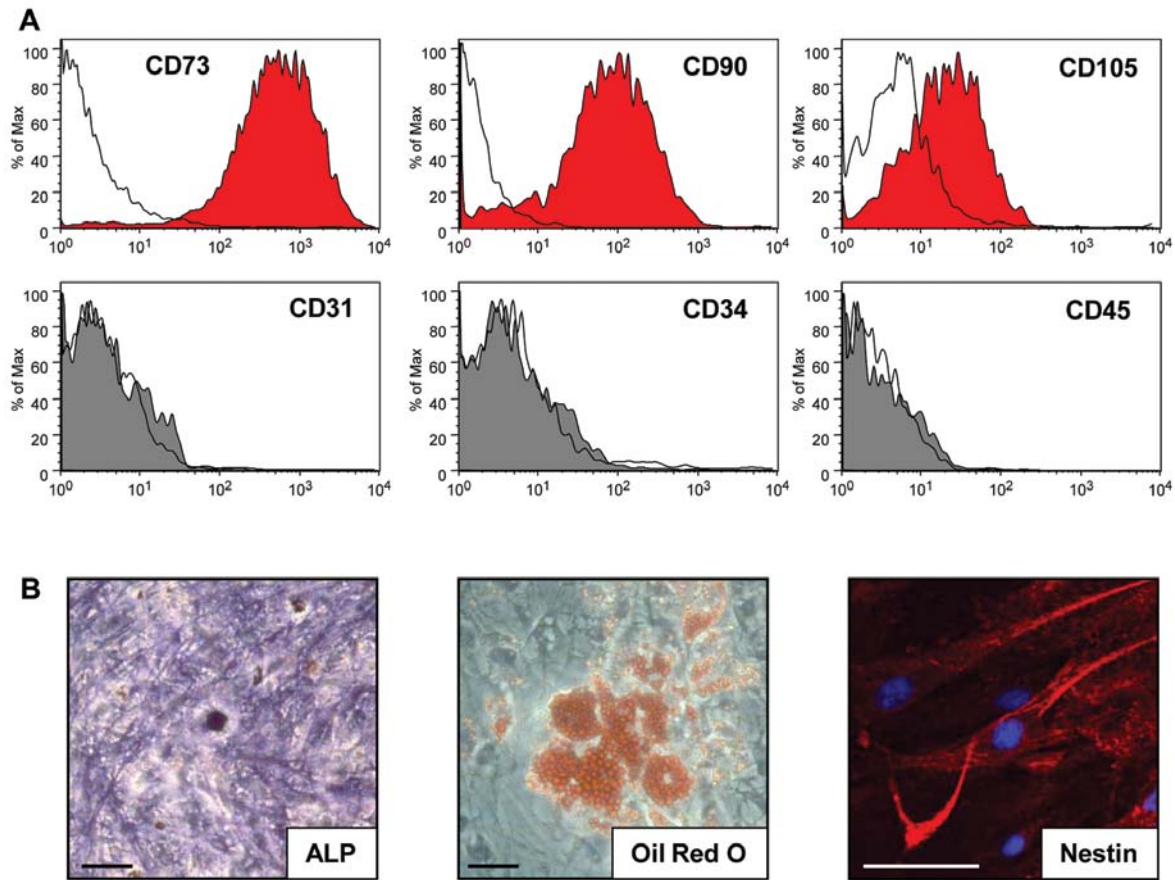


Figure 1. Isolation and characterization of ASCs. (A) The ASCs isolated from BFPs are labeled with antibodies specific to CD31, 34, 45, 73, 90 and 105. The surface phenotype is analyzed by FACS. The ASCs are positive for CD73, CD90 and CD105, cell surface markers for MSCs but do not express hematopoietic stem cell markers including CD31, CD34 and CD45. (B) The cells are cultured for 3 weeks in osteogenic differentiation media. Osteogenic differentiation is seen with ALP staining (left). The cells are cultured for 4 weeks in adipogenic differentiation media. Adipogenic differentiation is shown with Oil Red O (middle). The cells are cultured for 3 days in neural differentiation media. Neural differentiation is seen with immunocytochemistry for nestin (right). Scale bars, 50  $\mu$ m.

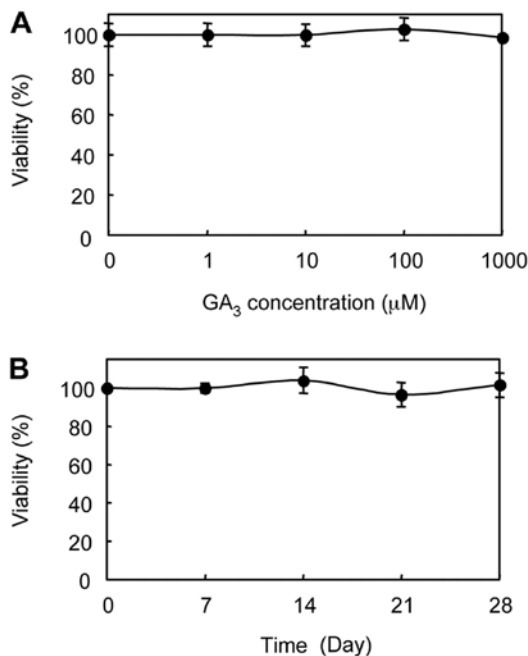


Figure 2. Effect of GA<sub>3</sub> on cell viability. (A) The ASCs are incubated with the indicated concentrations of GA<sub>3</sub> for 72 h. (B) The ASCs are incubated with 1 mM GA<sub>3</sub> for the indicated times. GA<sub>3</sub> does not affect ASC cell viability in a time- or dose-dependent manner. Data are expressed as the means of the percentage of cell viability (%) and  $\pm$  SEM of the mean.

expressed the cell surface markers, CD73, CD90, and CD105 but not CD31, CD34 and CD45 (Fig. 1A). These results are consistent with the definition that MSCs must express CD73, CD90 and CD105, as suggested by Dominici *et al* (24). ASCs did not spontaneously differentiate during culture expansion. To determine whether ASCs from BFPs can differentiate into various cell types, such as osteoblasts, adipocytes, and neural cells *in vitro*, ASCs were cultured in specific selection media. After 3 weeks in the osteogenic medium culture, the cells differentiated into osteoblasts, which were confirmed with strong ALP staining (Fig. 1B). After 4 weeks in the adipogenic differentiation culture, the cells differentiated into lipid-laden cells that were stained with Oil Red O (Fig. 1B). After 3 days of neural differentiation culture, the ASCs differentiated into neural cells, which were confirmed with immunocytochemistry for nestin (Fig. 1B). These results showed that ASCs from BFPs can multidifferentiate.

**Cytotoxicity of GA<sub>3</sub>.** Ishii *et al* (25) reported that plant hormones are closely related to anticancer therapy. We treated the ASCs with GA<sub>3</sub> to determine the cytotoxic effect. GA<sub>3</sub>, up to 1 mM, did not affect the cell viability of ASCs in a dose- or time-dependent manner (Fig. 2). In addition, there were no morphologic changes when we challenged the ASCs with GA<sub>3</sub> (data not shown).

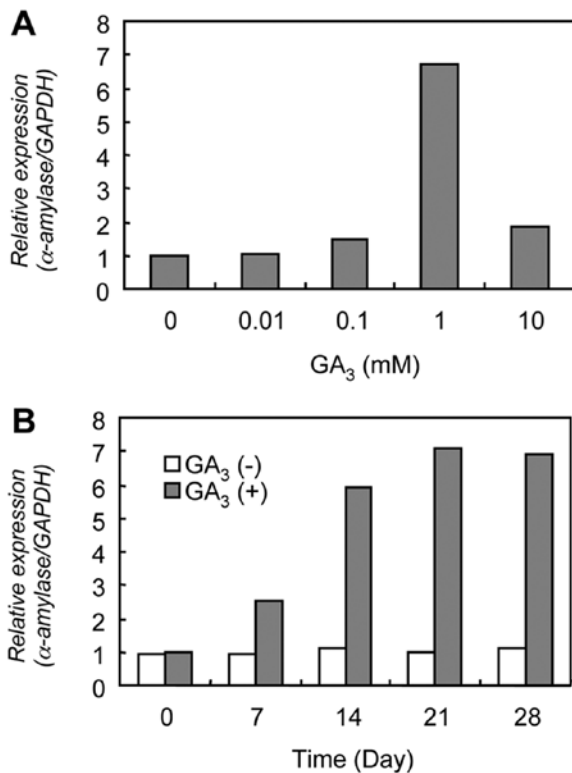


Figure 3. Typical results of expression of  $\alpha$ -amylase mRNA in GA<sub>3</sub>-treated ASCs. (A) The ASCs are treated with indicated concentrations of GA<sub>3</sub> for 14 days. (B) The ASCs are treated with GA<sub>3</sub> for the indicated periods. Untreated cultures are controls (0 day).  $\alpha$ -amylase mRNA reaches a maximum level on Day 21. Quantification of mRNA levels in ASCs by qRT-PCR. The experiments repeated three times with similar results.

**Evaluation of  $\alpha$ -amylase mRNA expression.** The result of qRT-PCR analysis for  $\alpha$ -amylase mRNA expression is shown in Fig. 3. Higher  $\alpha$ -amylase mRNA expression was found after treatment with 1 mM GA<sub>3</sub> for 14 days.  $\alpha$ -amylase mRNA expression reached its maximum on 21 days after 1 mM GA<sub>3</sub> treatment, which was 7-fold than that of resting conditions (0 day).

**Evaluation of  $\alpha$ -amylase protein expression.** We performed western blot analysis to determine the  $\alpha$ -amylase protein expression status in the GA<sub>3</sub>-treated ASCs. Representative results of western blot analysis for  $\alpha$ -amylase protein expression are shown in Fig. 4. We did not detect any  $\alpha$ -amylase protein bands under resting conditions (0 day).  $\alpha$ -amylase protein became evident 7 days after treatment with GA<sub>3</sub>, reaching a maximal level on Day 21.

## Discussion

The current study showed that GA<sub>3</sub>, a plant growth regulator, plays an important role in regulating  $\alpha$ -amylase in BFP-derived ASCs and that the induction method could be an emerging potential therapeutic approach for regenerating salivary glands.

ASCs have been recognized as an efficient source of adult stem cells because of their easy accessibility, minimal morbidity upon harvesting, and abundance of stem cells compared with bone marrow-derived MSCs (11). Moreover,

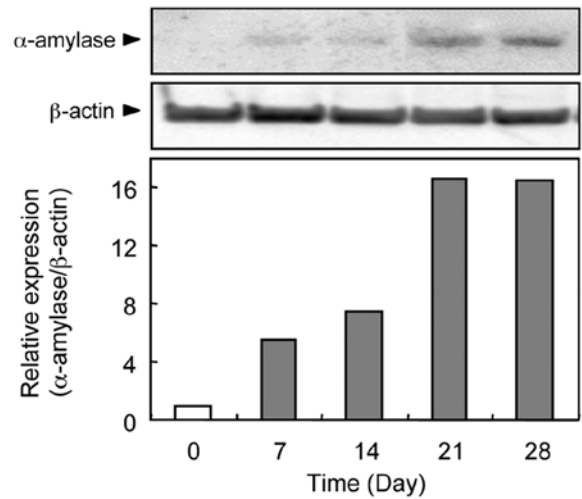


Figure 4. Representative results of expression of  $\alpha$ -amylase protein in GA<sub>3</sub>-treated ASCs. To investigate  $\alpha$ -amylase protein expression in untreated and GA<sub>3</sub>-treated ASCs, we performed western blot analysis. Untreated cultures are controls (0 day).  $\alpha$ -amylase protein is evident 7 days after treatment with GA<sub>3</sub>, reaching a maximal level on Day 21. The experiments were repeated three times with similar results.

ASCs can be propagated more rapidly, and they retain their mesenchymal pluripotency after multiple passages (15). We isolated ASCs from BFPs, adipose-encapsulated masses in the oral cavity, and revealed that BFP-derived ASCs showed positive MSC markers and pluripotency. BFPs are an easy source for dentists and oral surgeons who treat patients for dry mouth syndrome.

The digestion of dietary starch in humans is initiated by salivary  $\alpha$ -amylase, an endo-enzyme that hydrolyzes starch into maltose, maltotriose, and larger oligosaccharides. Salivary  $\alpha$ -amylase accounts for 40 to 50% of protein in human saliva and rapidly alters the physical properties of starch. This amyolytic digestion begins during mastication in the oral cavity and continues in the stomach (1-3).

Gibberellins were identified initially in the 1930s as a product of a fungus, which caused excessive shoot elongation. Further studies found that gibberellins are also involved in other processes, e.g., promoting flowering and seed germination (18). One gibberellin, GA<sub>3</sub>, accelerates and improves the yield of a wide variety of plants by increasing cell division (18,26). Early in seed germination, the embryo synthesizes GA<sub>3</sub>, which diffuses to the aleurone cells in which GA<sub>3</sub> acts as a signal to activate synthesis and secretion of  $\alpha$ -amylases and other hydrolases. While GA<sub>3</sub> is widely used in agriculture, only a few experiments have examined the possible toxic effects in mammals. A previous study reported that gibberellin derivatives had strong anticancer activities by inhibiting topoisomerase I activity in rodents (27). To determine the effect of GA<sub>3</sub> on cell viability in ASCs, we carried out a cytotoxic assay of ASCs using several concentrations of GA<sub>3</sub> for a maximum of 28 days. GA<sub>3</sub> never affected cell viability or cell morphology up to 1 mM. However, some groups reported that exposure of GA<sub>3</sub> induced oxidative stress and histopathological changes to rats (28,29). Therefore, further studies with more *in vivo* samples are needed to address the status of  $\alpha$ -amylase expression after GA<sub>3</sub> treatment in greater detail.

The aleurone layer of cereal grains is the most widely studied and best characterized system for studying the activity of GA<sub>3</sub>. To date, at least one GA<sub>3</sub> receptor is present in the plasma membrane (30) and there is evidence of a number of other components of the pathways, including Ca<sup>2+</sup> (31,32), lipases (33), cGMP (34), protein phosphatases (35), an endoplasmic reticulum-located Ca<sup>2+</sup>-ATPase, inositol-1,4,5-triphosphates, and Ca<sup>2+</sup>/calmodulin (36) at the early stage of GA<sub>3</sub> signal transduction. The GA<sub>3</sub>-regulated myb gene, GAmyb, may be a component of the GA<sub>3</sub> response pathway and has been shown to transactivate the  $\alpha$ -amylase promoter (37). In the present study, we found that GA<sub>3</sub> regulated  $\alpha$ -amylase expression in human ASCs, suggesting that mammalian cells also may have a GA<sub>3</sub> response pathway. Since the mammalian signal transduction pathways of GA<sub>3</sub> are unknown, further studies are required to reveal the pathway for  $\alpha$ -amylase expression.

The potential effects of GA<sub>3</sub> on human health have not been explored. This is the first report to show that GA<sub>3</sub> treatment can increase the expression of cellular  $\alpha$ -amylase and that our induction method might be a useful therapeutic application for salivary gland regeneration.

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