Activation of Wnt signaling reduces the population of cancer stem cell in ameloblastoma

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Jin Hoo Park

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This certifies that the Doctoral Dissertation of
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The Graduate School
Yonsei University

June 2020

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항상 사랑과 믿음으로 지켜봐 주시고 지원을 아끼지 않으시는 아버지, 어머니와 멀리 떨어져 있지만 항상 격려해 주시는 형에게 진심으로 감사의 마음을 전하고 싶습니다.

2020년 6월

박 진 후

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Activation of Wnt signaling reduces the papulation of cancer stem cells in ameloblastoma

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Cancer stem cells (CSCs) are a self-renewing subpopulation of tumor cells that contribute to the heterogeneity of tumors by asymmetric division. Clinically, CSCs are believed to be responsible tumor recurrence, displaying resistance to radiotherapy and chemotherapy. Ameloblastoma, a tumor of the odontogenic epithelium, is classified as a benign tumor owing to its slow growth rate and non-metastatic features. However, local recurrence of ameloblastoma is very common, and malignant changes and metastases are observed in about 1%. It has been reported that ameloblastoma is difficult to treat with radiation therapy or drug therapy. Because of this, treatment of ameloblastoma requires extensive surgical resection, including the periphery of the lesion, leaving a large defect in the patient's jaw. The purpose of this study is to identify the CSCs of ameloblastoma using AM-1 cells and to investigate the effect of Wnt signal activator on the CSCs. First, AM-1 cells, a well-known human ameloblastoma cell line, were analyzed using flow cytometry.

Immunostaining was performed to identify cells suspected of CSCs among the obtained cells. The effect of applying Wnt signal activator to the CSCs was investigated by immunostaining. Through the above study process, the following results were obtained.

1. AM-1 cells exhibited significant heterogeneity in cell morphology. The largest cell was 12 times larger than the smallest cell, and various shapes of cells, from round to amorphous, were observed.
2. Immunocytochemistry analysis showed that small-sized and round-shaped cells were proliferative and expressed a marker of dental epithelial stem cells (DESCs), SRY related HMG box 2 (Sox2).
3. Sox2 expression in cells was negatively correlated with activation of Wnt signaling.
4. *In vitro* spheroid-forming assay and the orthotopic graft of the cell-sheet into first molar extracted region of nude mice confirmed the stem cell-like characteristics of AM-1 cells.
5. Exogenous activation of Wnt signaling using lithium chloride (LiCl) and valproic acid (VPA) increased the cell size and decreased proliferation and expression of Sox2.

This study confirmed the putative CSCs population in AM-1. This confirmed the possibility of using AM-1 as an *in vitro* and *ex vivo* model for the study of CSCs in ameloblastoma. And reduction of CSCs cell population and reduction of spheroid in AM-1 were confirmed through activation of Wnt signaling. Through this, the possibility of activating Wnt signaling as an agent for treatment of ameloblastoma and suppression of recurrence was confirmed.

Keywords: cancer stem cells, ameloblastoma, Wnt signaling, AM-1

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I. INTRODUCTION

The cancer stem cells (CSCs) model is a prominent concept and explains heterogeneity in tumors (van Niekerk *et al*., 2017). The CSC model began in the study of hematological malignancies, and now CSCs are described in solid tumors, CSCs are becoming models that can explain all neoplastic systems (Wang and Dick, 2005). Over the past decade, a number of researchers have identified CSCs in several types of tumors, including brain tumors, melanoma, breast cancer, liver cancer, pancreatic cancer, and colon cancer (Duchartre *et al*., 2016). CSCs have been revealed to be a self-renewing subpopulation in tumors that generate various differentiated cell populations in tumors. Characterization of CSCs has indicated that they are remarkably resistant to conventional radio- and chemotherapy. Clinically, the remaining populations of CSCs are responsible for metastasis and recurrence in cancer patients, which can lead to the disease becoming chronic and incurable. Their static state and specific interaction with the microenvironment play an important role in drug resistance properties. All of these characteristics make it

possible to explain why CSCs are strongly correlated with cancer establishment, progression, drug resistance, and poor outcomes in clinical studies. Therefore, elimination of CSCs is an important goal of cancer treatments (Carnero *et al*., 2016).

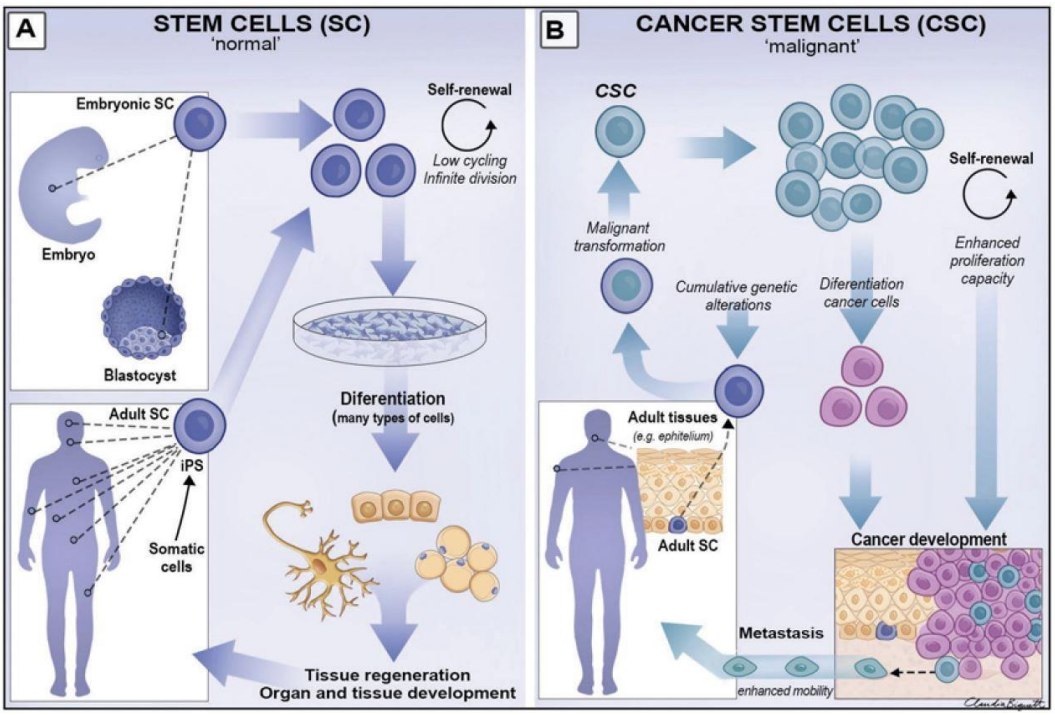


Figure 1. Schematic diagram of A. stem cells (SCs) and B. cancer stem cells (CSCs).
A: Normal SCs retain properties of indefinite division through self-renewal and generation of differentiated cells under appropriate conditions.
B: After malignant formation of SCs to CSCs. CSCs retain properties of the self-renewal and generation of differentiated cancer cells, leading to cancer development and metastasis (Rodini *et al.*, 2017).

Ameloblastoma is a rare odontogenic neoplasm of the mandible and maxilla with a prevalence of 0.92 person per 1 million people, and has multiple histologic variants and high recurrence rates if improperly treated (Hendra *et al*., 2020). The classification of ameloblastomas includes four subtypes. Solid/multicystic is the most common type, comprising 91 % of ameloblastomas in the largest series (Reichart *et al*., 1995). This type is followed by the unicystic type, comprising 6 %; extra osseous ameloblastoma, comprising 2 %; and desmoplastic type, comprising 1%. The most aggressive clinical/pathologic association is observed in the solid/multicystic type, which is associated with the highest recurrence rate (up to 90 %) with conservative operations, such as enucleation and curettage. The current standard of treatment is wide resection with appropriate margins and immediate reconstruction, which is associated with significant patient morbidity (McClary *et al*., 2016). However, due to the nature of the jawbone located on the face, problems may occur in appearance and function when wide resection including margin of lesions is performed (Laborde *et al*., 2017). For this reason, clinicians are trying to prevent the recurrence of ameloblastoma at the same time while performing the most conservative surgery. In order to prevent recurrence of ameloblastoma, such as the use of carnoy's solution or Cryotherapy using liquid nitrogen, additional treatment with conservative surgery is performed (Pogrel and Montes, 2009). However, there are many disagreements as to whether these additional procedures are effective in preventing recurrence of ameloblastoma. Though radiotherapy has been attempted to suppress the recurrence rate, its efficacy is not clearly (McClary *et al*., 2016; Mendenhall *et al*., 2007). Recently, target therapy with BRAF / MEK inhibitors (dabrafenib, trametinib) has been reported to treat some metastatic or difficult surgery ameloblastoma, but treatment with drugs is still limited (Kaye *et al*., 2015). In order to overcome this situation, there is a need to study more conservative treatment methods for preventing recurrence of ameloblastoma and restoring aesthetics and function due to tumor resection.

Wnt signaling has been studied as involved in numerous basic processes essential for embryonic development and normal adult homeostasis (Willert and Jones, 2006). The Wnt

signaling is generally classified into three sub-paths, the canonical, non-canonical planar cell polarity (PCP) pathway and non-canonical Wnt/calcium pathway. The three different pathways aid in the understanding of complex signaling systems, but in reality Wnt signaling is a combination of these three paths, all of which must be considered (Florian *et al.*, 2013). Dysfunctional Wnt signaling can play an important role in CSCs metastasis and resistance to chemotherapy (Ksiazkiewicz et al., 2012). Generally, activation of Wnt signaling has been detected in various type of cancer cells. However activation of Wnt signaling has been reported to inhibit cancer in cancers such as medulloblastoma (Salaroli *et al*., 2015). Many components of the Wnt signaling have been identified, and their effects on the disease have been extensively reported. In the past few years, some Wnt signaling modulators targeting other major phases of the Wnt pathway (Wnt secretion, signaling or ß-catenin transcriptional activity) have been used clinically (Duchartre *et al*., 2016). Wnt signaling is a highly evolutionarily conserved pathway involved in many important homeostatic functions, and targeting this pathway can cause serious side effects, such as changes in bone density. In addition, many potential targets such as ß-catenin are also involved in other important functions (cell-cell adhesion, development, self-renewal). In order to balance the anti-tumor efficacy and adverse events by utilizing Wnt signaling, it is necessary to control the exact level of the Wnt pathway and research is needed.

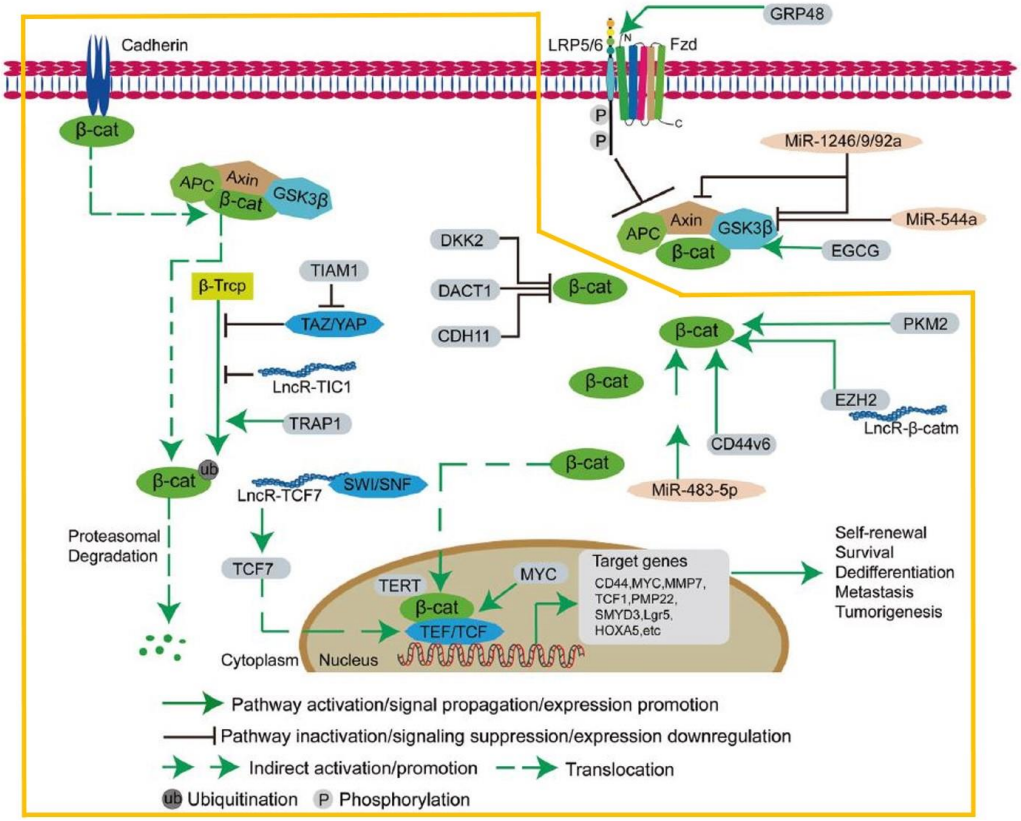


Figure 2. Wnt signaling in CSCs.

The canonical Wnt/β-catenin pathway regulates the pluripotency of CSCs and determines the differentiation fate of CSCs. In the absence of Wnt signaling, β-catenin is bound to the Axin complex, which contains APC and GSK3β, and is phosphorylated, leading to ubiquitination and proteasomal degradation through the β-Trcp pathway. However, the complex (TAZ/YAP), the long noncoding RNA TIC1 and proteins (TRAP1 and TIAM1) regulate the β-Trcp pathway. (Yang *et al.*, 2020).

AM-1 is an ameloblastoma cell line which is immortalized using human papillomavirus type-16 (Harada *et al.*, 1998). The established ameloblastoma cells exhibit similar characteristics with *in situ* ameloblastoma cells in marker expression and invasive properties, which shows that this cell-line is an appropriate model system of ameloblastoma (Harada *et al.*, 1998; Sandra *et al.*, 2005; Takebe *et al.*, 2016; Yoshimoto *et al.*, 2016). Studies using this cell-line revealed that the Akt, MAPK, and Sonic hedgehog signaling pathways are related to proliferation and apoptosis of AM-1 (Hendarmin *et al.*, 2005; Kanda *et al.*, 2013; Nakao *et al.*, 2013; Sandra *et al.*, 2004; Sandra *et al.*, 2006). Interestingly, the Wnt pathway, an osteogenic signaling pathway, is suppressed in this cell line (Sathi *et al.*, 2009; Sathi *et al.*, 2012). The early progeny of Sox2-postive dental epithelial stem cells (DESCs) are known to transiently express a Wnt inhibitor, secreted frizzled related protein (Sfrp) 5 (Juuri *et al.*, 2012). AM-1 also expresses the Wnt antagonist Sfrp2 (Sathi *et al*., 2009), and osteogenic genes related to Wnt signaling are suppressed in this cell-line (Sathi *et al.*, 2012). A recent study showed that Wnt signaling is important for enamel formation by facilitating ameloblast differentiation and movement (Guan *et al.*, 2016).

The purpose of this study was to identify the CSCs of ameloblastoma using AM-1 cells. Samples of AM-1 cells are acquisitioned using flow cytometry. Immunostaining is performed to identify cells suspected of CSCs among the obtained cells. To investigate the effect of the Wnt signal activator on cells identified as CSCs by immunostaining, the Wnt signal activator is applied to the CSCs. By confirming this, it is confirmed that the Wnt signal activator can be introduced into new treatments for ameloblastoma.

II. MATERIALS & METHODS

Cell culture, spheroid formation, and cell sheet generation

AM-1 was a gift from Dr. H. Harada (Iwate Medical University, Japan). AM-1 cells were cultured in Keratinocyte growth medium supplemented with pituitary extract (Gibco, Grand Island, NY, 17005-042) at 37 ℃ and 5% CO_2 in a humidified incubator. For spheroid formation, 1.0×10^5 or 2×10^5 cells were plated onto Ultra-Low Attachment Surface Costar 6 Well Plates (Corning Inc., Corning, NY, 4371) with Keratinocyte growth medium or Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 11995-065) supplemented with 10% fetal bovine serum (FBS, Gibco, 12484-020) and 1% penicillin/streptomycin (Gibco, 15140-112) solution at 37 ℃ for 1 week. For cell sheet generation, AM-1 cells were plated onto temperature-responsive dishes (Nunc UpCell 3.5-cm dish, Thermo Fisher Scientific, Somerset, NJ, NUN-174904) and cultured with Keratinocyte growth medium until fully confluent. The confluent cells were detached in the form of a cell sheet as described in the manufacturer's instructions.

Real-time PCR analysis

Total RNA of AM-1 cells cultured with conventional cell culture methods or cultured as spheroids were extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA, 16596026) as described in the manufacturer's instructions. The extracts were reverse-transcribed using Maxime RT PreMix (iNtRON, Seongnam, Korea, 25081). The products were subjected to real-time PCR analyses with primer sets (*Oct3/4*, F 5'-CTGGGCTCTCCCATGCAT-3', R 5'-CCTGTCCCCCATTCCTAGAAG-3'; *Sox2*, F 5'-ACAGCAAATGACAGCTGCAAA-3', R 5'-TCGGCATCGCGGTTTTT-3'; *CD49f*, F5'GATCCCGGCCTGTGATTAATATT-3', R 5'-CTGGCGGAGGTCAATTCTGT-3'; *Bcl11b*, F 5'-GCTGGGTCCAGGTGAAGTGA-3', R 5'-CGAAAGGTCCTGGCTGTGAT-3'; *GAPDH*, F 5'-GAAGGTGAAGG

TCGGAGT-3', R 5'-GAAGATGGTGATGGGATTTC-3') using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific).

Flow cytometry analysis of AM-1 cells

Fluorescence-activated cell sorting (FACS, BD Biosciences, San Jose, CA) was used for flow cytometry.

Immunoblot analysis

AM-1 cells were lysed in protein extraction buffer with a protease inhibitor cocktail (cOmplete Mini, Roche, Indianapolis, IN, 11836170001). The lysates were subjected into immunoblot analyses using anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, sc-7199) and anti-α-tubulin (Sigma-Aldrich, St Louis, MO, T6199). For visualization, anti-mouse or rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, sc-2005 and sc-2004, respectively) was applied and visualized with ECL (GE Healthcare, Piscataway, NJ, USA, RPN2232) using a chemiluminescence imaging system (Davinch-chemi, Core Bio, Seoul, Koera).

Animal experiments

Female nude (nu/nu BALB/c/Bkl) mice (Narabiotech Co., Seoul, Korea) were housed in a temperature-controlled room (22℃) under artificial illumination (lights on from 05:00 to 17:00) and 55% relative humidity. Mice had access to food and water ad libitum. For orthotopic grafts, the upper first molars of 8-week-old nude mice were extracted, and a hole was prepared using a portable drill with a 0.75-mm tip in the extraction site under deep anesthesia. Subsequently, a properly sized AM-1 cell sheet was grafted into the hole using forceps. AM-1 cell sheet-grafted mice were housed for 1 week for healing and subsequently sacrificed with CO_2 for histological and immunohistological analyses.

Immunohistochemical and immunohistochamical analysis

Cells were fixed in 4% paraformaldehyde (PFA) and permeabilized with 0.02% Triton X-100 in phosphate buffered saline. Spheroids or decalcificated tissues were fixed in 4% PFA. Staining was performed on 4-μm paraffin-embedded sections. After deparaffinization, the slides were incubated with pepsin (Digest-All™ 00-3009, Invitrogen) for 15 minutes at 37℃. After blocking with 5% bovine serum albumin, cells were incubated with the following primary antibodies: anti-β-catenin (Santa Cruz Biotechnology, sc-7199), anti-Sox2 (Santa Cruz Biotechnology, sc-20088), and anti-Ki67 (Spring Bioscience Corp., Fremont, CA, M3060). For visualization, anti-mouse or rabbit IgG conjugated with Alexa Fluor 488 or 555 dye (Invitrogen) was applied and observed under a confocal microscope (LSM700, Carl Zeiss, Jena, Germany). The cytoskeleton or nucleus was stained using phalloidin conjugated with Alexa Fluor 488 dye (Invitrogen) or 4',6-Diamidino-2-Phenylindole (ThemoFisher Scientific, D1306), respectively. The cell size and staining intensity were measured using the image analyzer software ImageJ 1.51g (National Institutes of Health, Bethesda, MD).

Statistical analysis

Pearson's correlation coefficient was used to analyze the correlation of cell size and Sox2 intensity, cell size and Ki67 density, and nuclear localization of β-catenin and Sox2 intensity. One-way analysis of variance (ANOVA) was used for statistical analysis of expression of OCT3/4, Sox2, CD49f and Bcl11b.Statistical analysis was performed using Statistical Package for Social Sciences for Windows (version 22.0, SPSS, Chicago, IL, USA).

III. RESULTS

Flow cytometry analysis of AM-1 cells

As previously described (Harada *et al.*, 1998), AM-1 cells had a heterogeneous morphology from small, round cells to large, flattened cells. The average cell size was 60.6 ± 32.4 μm^2, and the biggest cell was 12 times larger than the smallest one. As a result of flow cytometry analysis of AM-1 cells, it was largely divided into two populations (Population1-P1, Population2-P2) according to cell size (Fig. 3). The two populations showed relatively similar granularity, but were divided into a relatively large P1 group and a small P2 group according to cell size. Cytokeratin 14 and Sox2 were more expressed in the P2 group than in the P1 group in immunofluorescence staining (Fig 4 A, B). In addition, CD49 and Sox2 were hardly detected in P1, whereas 3 x 10^3 (CD49) and 6 x 10^2 (Sox2) were found in P2, respectively (Fig 5 A, B).

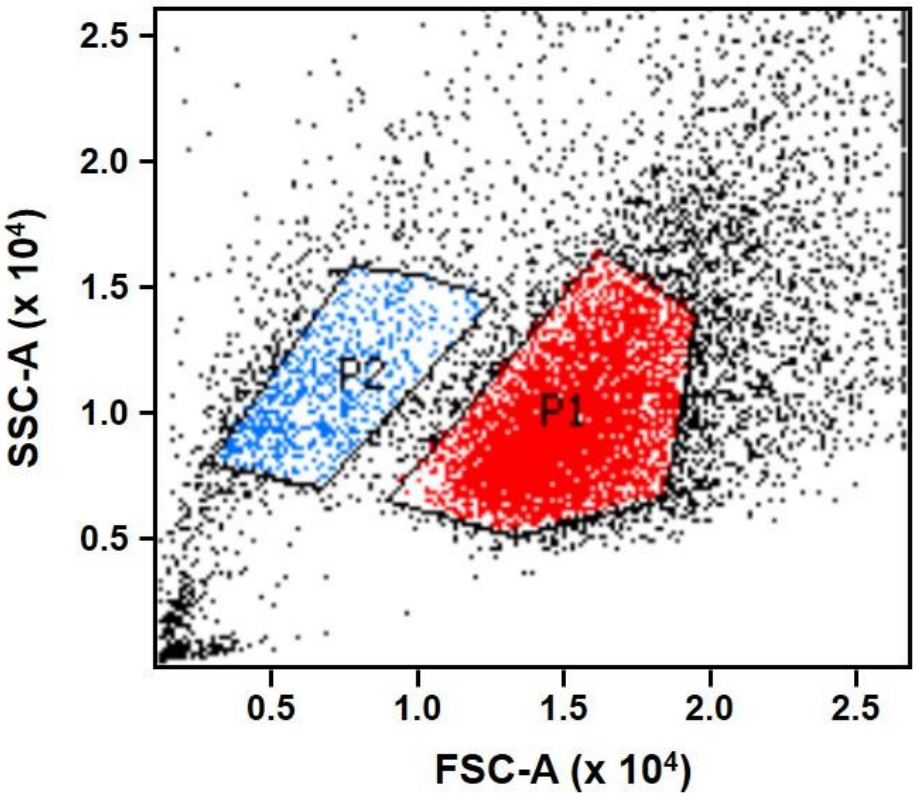
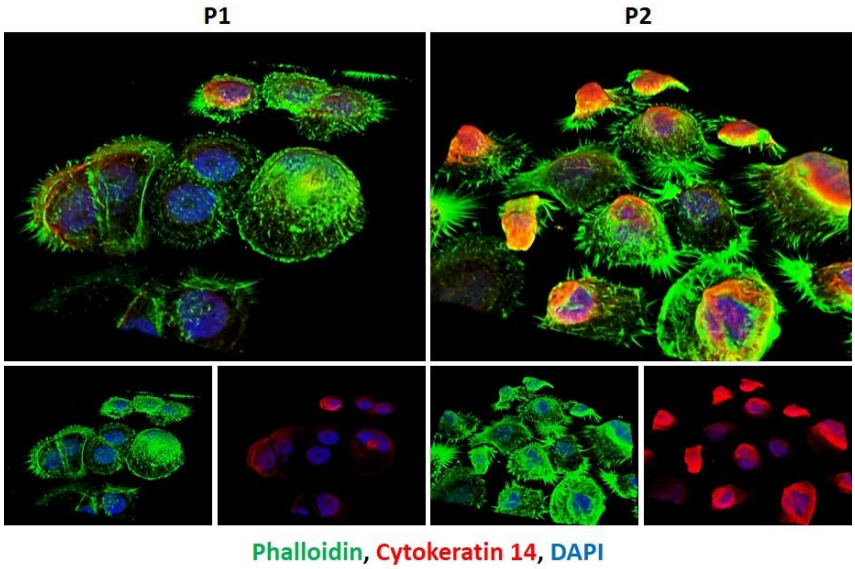


Figure 3. Histogram of flow cytometry analysis of AM-1 cells.
AM-1 cells divided two groups (Population1-P1, Population2-P2).

A



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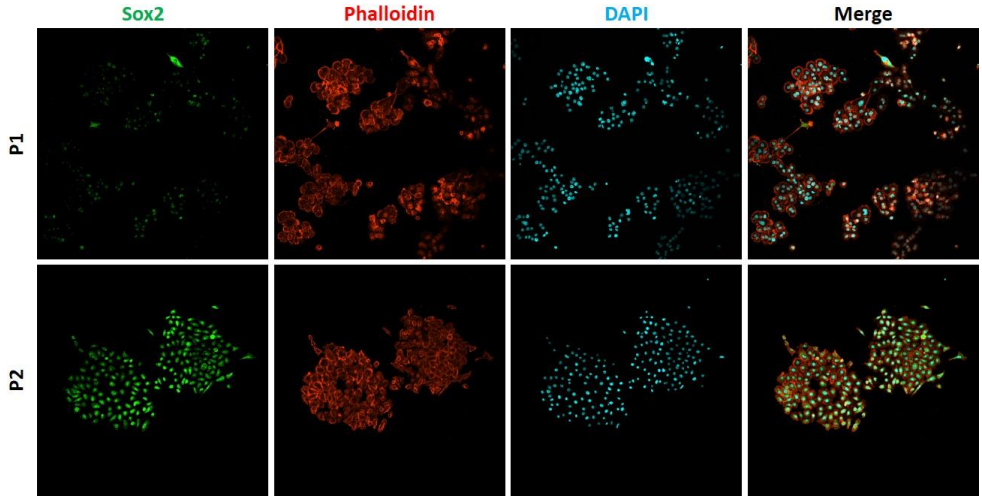


Figure 4. Immunostaining of P1 & P2 cells.
Cytokeratin 14 and Sox2 were more expressed in the P2 group than in the P1 group in immunofluorescence staining
A: Expression of Cytokeratin 14 in P1 & P2 cells.
B: Expression of Sox2 in P1 & P2 cells.

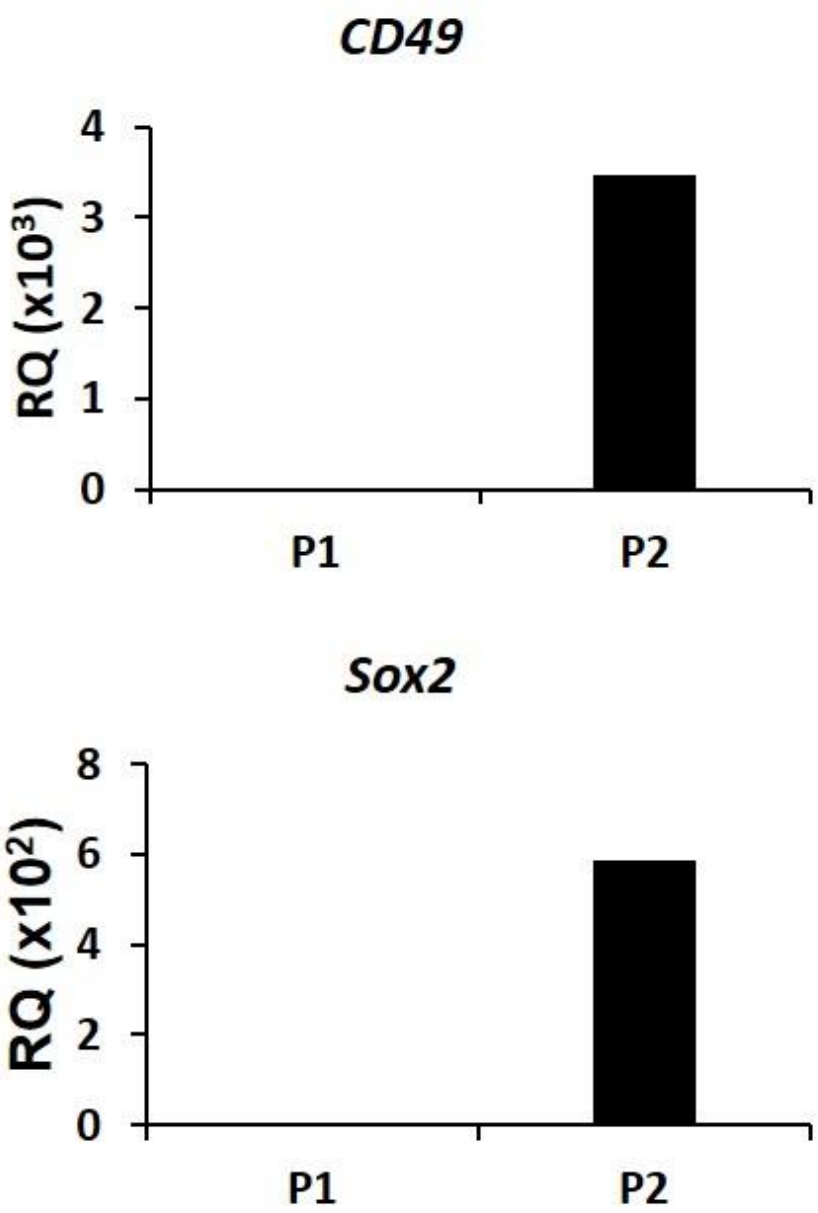
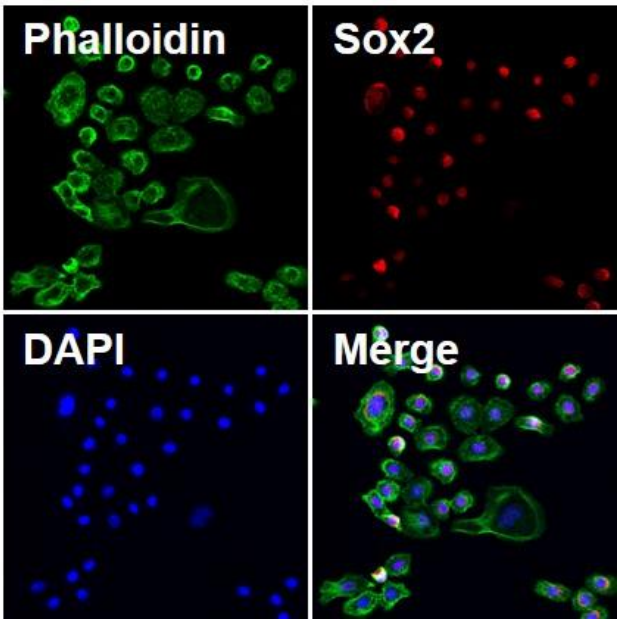


Figure 5. Graph of expression of CD49, Sox2 in P1 & P2 cells.
CD49 and Sox2 were hardly detected in P1, whereas 3 x 10^3 (CD49) and 6 x 10^2 (Sox2) were found in P2.

A putative CSCs population in AM-1 cells

To detect a possible CSCs population inside of these heterogeneous cells, Sox2, a marker of DESCs (Juuri *et al.*, 2013; Juuri *et al.*, 2012; Lee *et al.*, 2016), was red stained and visualized (Fig. 6A). Sox2 expression significantly differed among the cells and was especially strong in small round cells (Fig. 6A). Quantification of the results revealed a negative correlation between Sox2 expression and the cell size of AM-1 cells (Fig. 6B, Pearson's correlation coefficient $r = -0.616$, $p<0.05$). Expression of Ki67, a marker of cell proliferation, also showed a wide variation among the cells (Fig. 7A). Ki67 expression was also negatively correlated with cell size in AM-1 cells (Fig. 7B, $r = -0.691$, $p<0.05$). Wnt signaling, an essential signaling pathway for ameloblast differentiation, is known to be suppressed in DESCs and AM-1 cells. The staining results of β-catenin revealed that the effector molecule of Wnt signaling was localized differently in AM-1 cells; some cells had nuclear β-catenin, which indicates activation of Wnt signaling, but cytosolic β-catenin was observed in other cells (Fig. 8A). Quantitative analysis showed that the nuclear localization of β-catenin was negatively correlated with Sox2 expression (Fig. 8B, $r = -0.734$, $p<0.05$). Overall, small, round cells revealed high expression of proliferation and stem cell markers with low Wnt signaling activity, which identifies them as putative CSCs in AM-1 cells.

A



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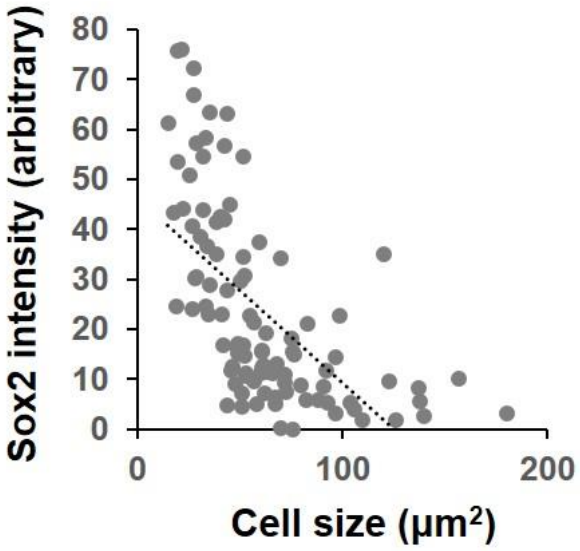
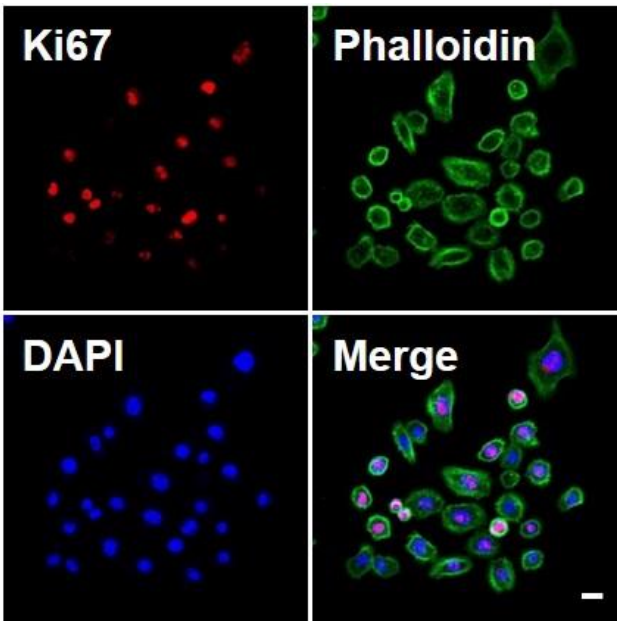


Figure 6. Expression of Sox2 in AM-1 cells.

A: Immunostaining of Sox2 in AM-1 cells.

B: Correlation of Sox2 and cell size (Pearson's correlation coefficient $r = -0.616$, $p<0.05$).

A



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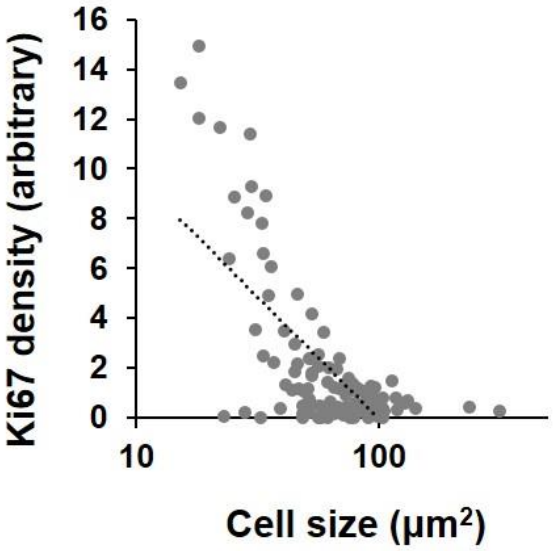
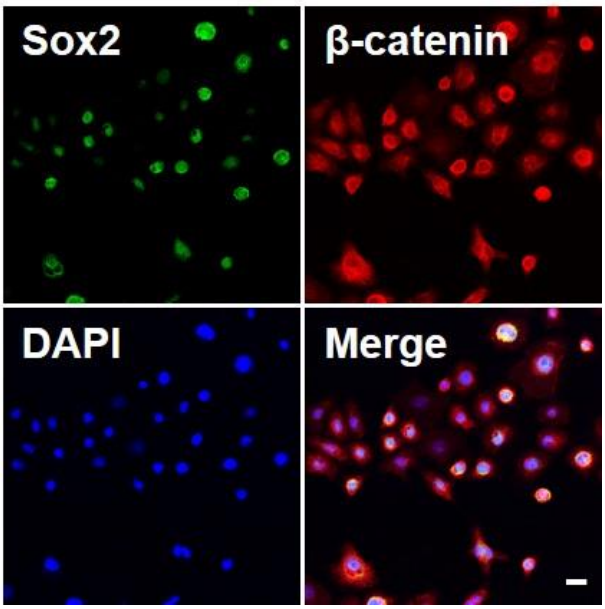


Figure 7. Expression of Ki67 in AM-1 cells.

A: Immunostaining of Ki67 in AM-1 cells.

B: Correlation of Ki67 and cell size (Pearson's correlation coefficient $r = -0.691$, $p<0.05$).

A



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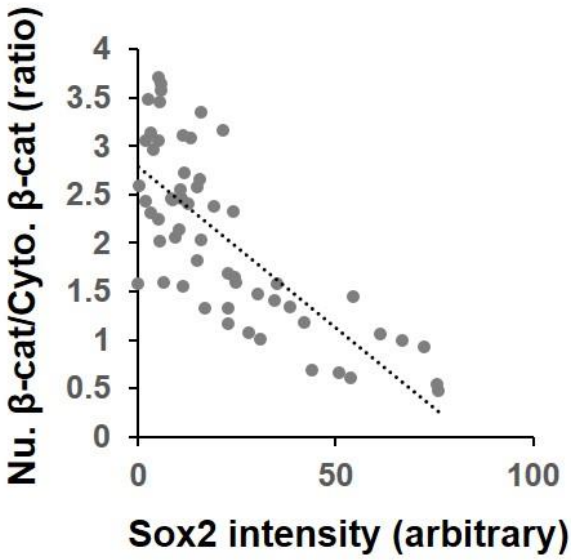

Figure 8. Expression of β-catenin in AM-1 cells.

A: Immunostaining of β-catenin in AM-1 cells.

B: Correlation of nuclear localization of β-catenin and Sox2 intensity (Pearson's correlation coefficient $r = -0.734$, $p<0.05$).

In vitro spheroid-forming capacity of AM-1 cells

The *in vitro* spheroid-forming assay is a well-established method of demonstrating the self-renewal capacity of stem cells from various organs (Chang *et al.*, 2013). To optimize spheroid formation conditions, various numbers of AM-1 cells were plated on low attachment surface cell culture plates with different culture media (Fig. 9). No spheroid formation was observed when the seeded number of cells was 2×10^5 per a well in 6-well plate with Keratinocyte growth medium (Fig. 9). However, the same number of cells grown in DMEM formed spheroids (Fig. 9). Interestingly, the dissected spheroids showed a similar structure to that of ameloblastoma; the hyperchromatic outer shell was reminiscent of peripheral palisading cells at the basal layer of ameloblastoma, and the eosinophilic spots inside the spheroids were similar to keratin pearls, a typical structure found in acanthomatous ameloblastoma (Fig. 10). Real-time PCR showed that the expression of stem cell markers (OCT3/4, Sox2, and CD49f) and an anti-apoptotic marker (Bcl11b) increased in cells that were 3-dimensionally cultured as spheroids compared with those cultured in conventional cell culture system (Fig. 11).

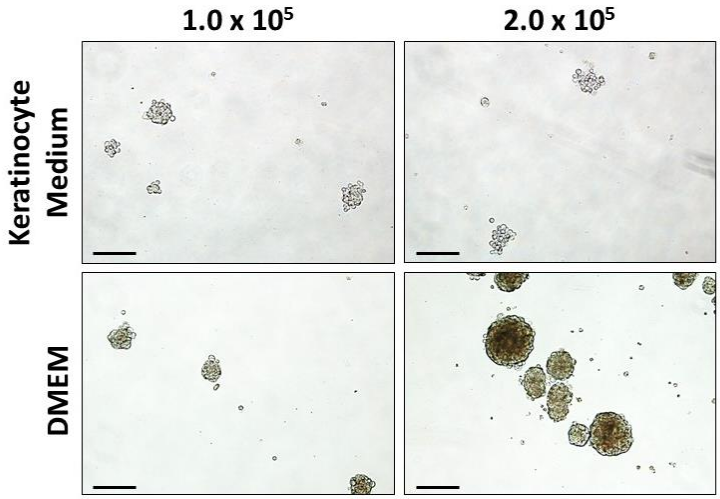

Figure 9. AM-1 shows spheroid forming activity.
Indicated numbers of AM-1 cells were plated on a well of low attachment surface 6-well cell culture plates and cultured for 7 days in Keratinocyte growth medium or DMEM. Bright field images of a spheroids are displayed. Scale bar = 100 μm

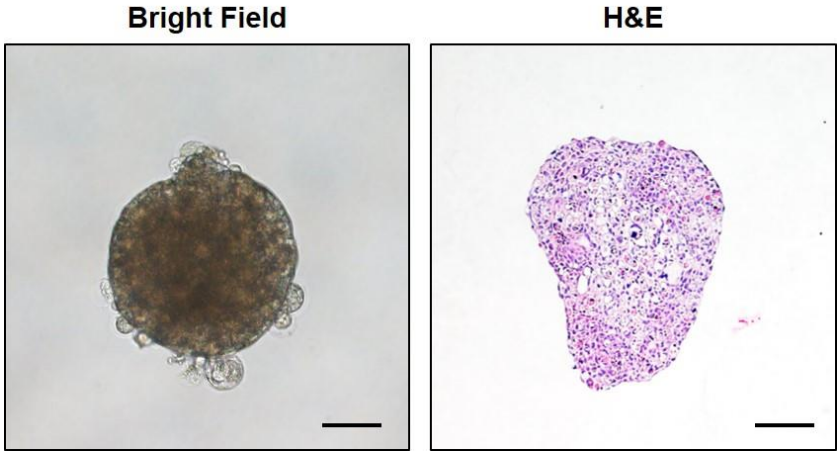

Figure 10. Bright Field and H&E staining of AM-1 spheroid.
The dissected spheroids showed a similar structure to that of ameloblastoma; the hyperchromatic outer shell was reminiscent of peripheral palisading cells at the basal layer of ameloblastoma, and the eosinophilic spots inside the spheroids were similar to keratin pearls, a typical structure found in acanthomatous ameloblastoma.

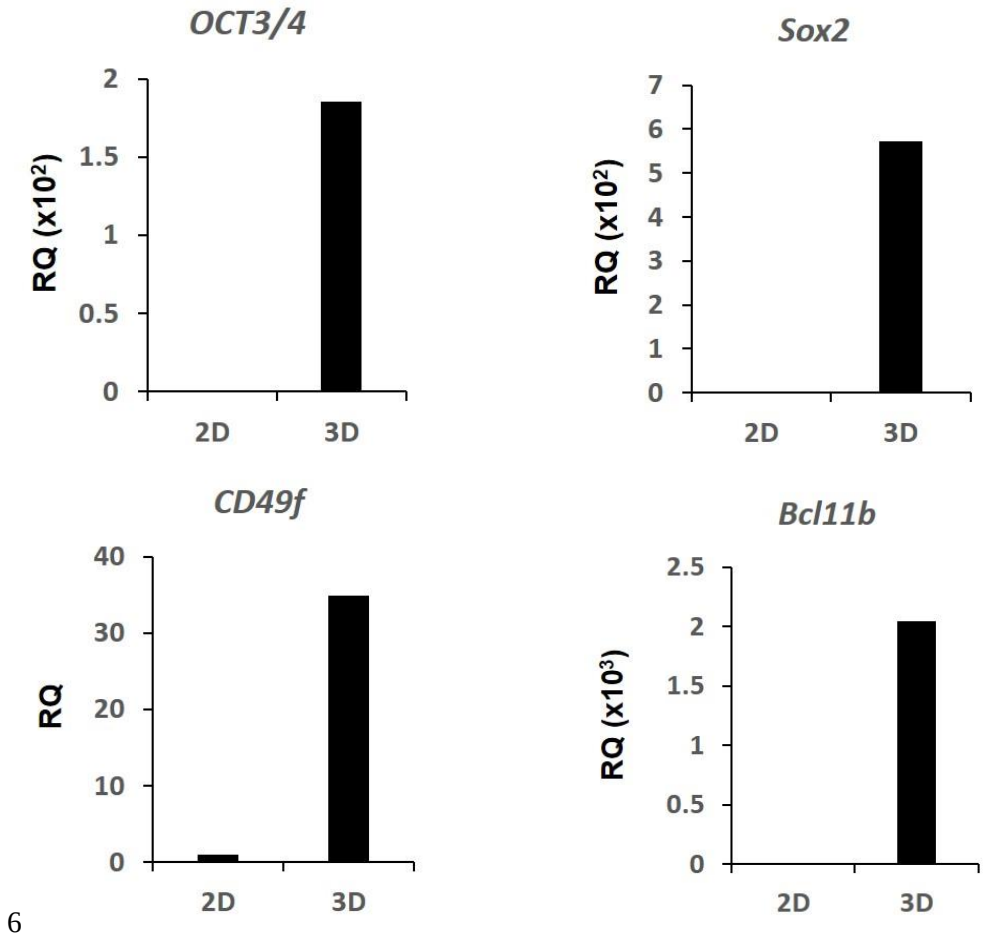

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Figure 11. Graph of expression of OCT3/4, Sox2, and CD49f, Bcl11b in 2D cultured cells and 3D cultured spheroid.
The expression of stem cell markers (OCT3/4, Sox2, and CD49f) and an anti-apoptotic marker (Bcl11b) increased in cells that were 3-dimensionally cultured as spheroids compared with those cultured in conventional cell culture system

In vivo tumor-forming capacity of AM-1 cells

The *in vivo* tumor-forming capacity of AM-1 cells was assessed by orthotopic grafts of ameloblastoma cells (Fig. 12). An AM-1 cell sheet lump was implanted into a hole prepared at the extraction site of the maxillary first molar of 8-week-old BALB/c nude mice (Fig. 12). After 1 week, complete closure of the extraction site was observed (Fig. 11, the area surrounded by the dotted line). Histological analysis of the maxillary tissue revealed the extraction of first molar and formation of an abnormal cell mass in the extraction site (Fig. 13A). Immunostaining for a human-specific antigen, human leukocyte antigen (HLA), showed the existence of exogenous cells in the mass (Fig. 13B). The exogenous cells formed eosinophilic structures that were reminiscent of those observed inside AM-1 spheroids (Fig. 13C, arrows).

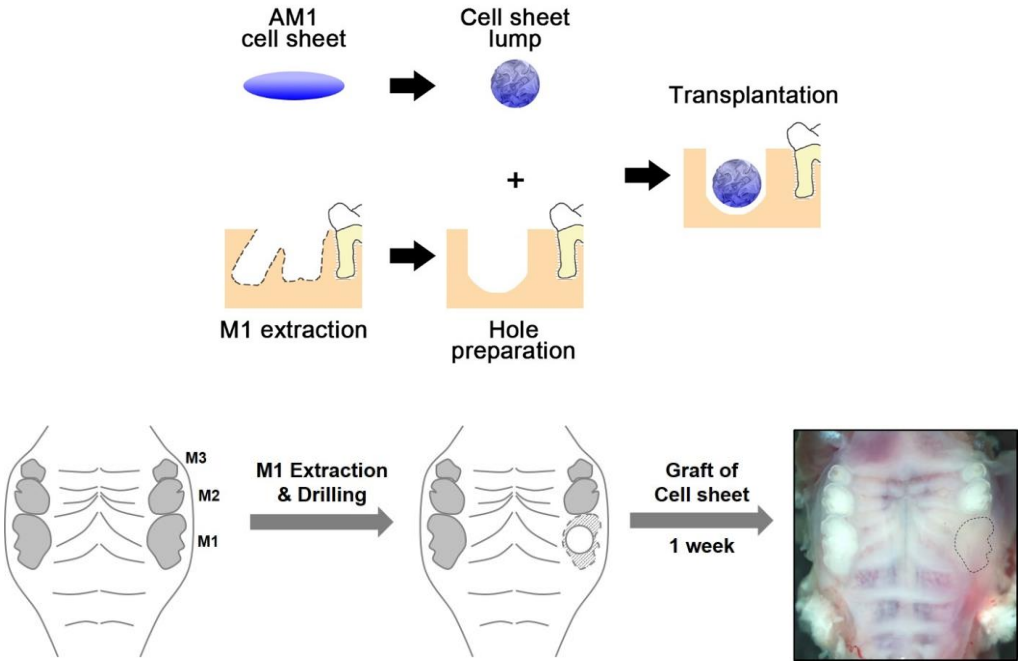


Figure 12. Schematic diagram of the orthotopic grafts of the AM-1 cell sheet into the maxillary of mouse.
An AM-1 cell sheet lump was implanted into a hole prepared at the extraction site of the maxillary first molar of 8-week-old BALB/c nude mice.

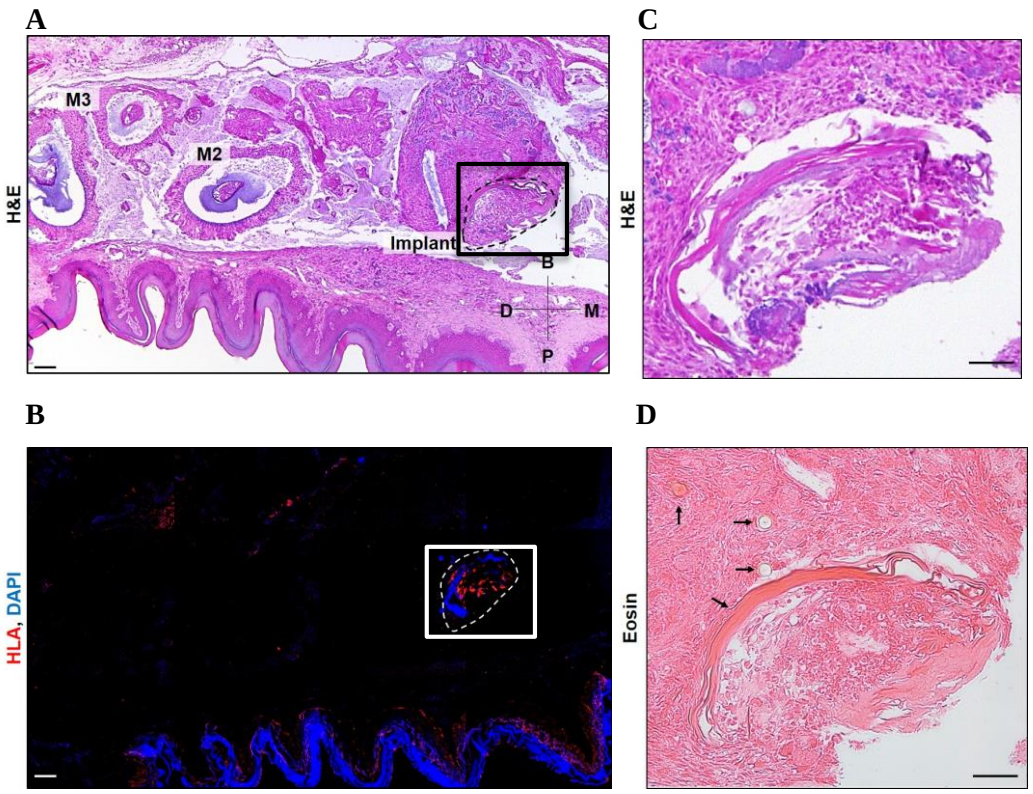


Figure 13. Histological analysis of the maxillary tissue.

A: H&E staining of maxillary tissue. An abnormal cell mass in the extraction site.
B: Immunostaining, Human leukocyte antigen (HLA), of maxillary tissue.
C: Enlargement of the implant site of H&E stained maxillary tissue.
D: The exogenous cells formed eosinophilic structures (arrows).

Effect of Wnt signaling activators on the putative CSCs population in AM-1 cells

To further investigate the role of Wnt signaling in the putative CSCs, Wnt signaling was activated in AM-1 cells by using the Wnt signaling activators lithium chloride (LiCl) and valproic acid (VPA) and observed cellular and molecular changes (Fig. 14, Fig. 15 and Fig. 16). Upon treatment with LiCl, the number of large, flattened cells without Ki67 expression increased, while the expression of Ki67 was weak (Fig. 14 A-C). Sox2 also showed a dose dependent decease after the LiCl treatment (Fig. 15A). AM-1 cells classified into 4 types (Fig. 15B) according to size (small or large), shape (round or flat), Sox2 expression ($Sox2^{high}$ or $Sox2^{low}$), and nuclear accumulation of β-catenin (nuclear β-cat.^{high} or nuclear β-cat.^{low}). Upon treatment with LiCl, type I (small, round, $Sox2^{high}$, and nuclear β-cat.^{low}) and type II (small, dented, round, $Sox2^{high}$, and nuclear β-cat.^{low}) cells decreased, while type III (small, flat, $Sox2^{low}$, and nuclear β-cat.^{low}) and type IV cells (large, flat, $Sox2^{low}$, and nuclear β-cat.^{high}) increased (Fig. 15C).

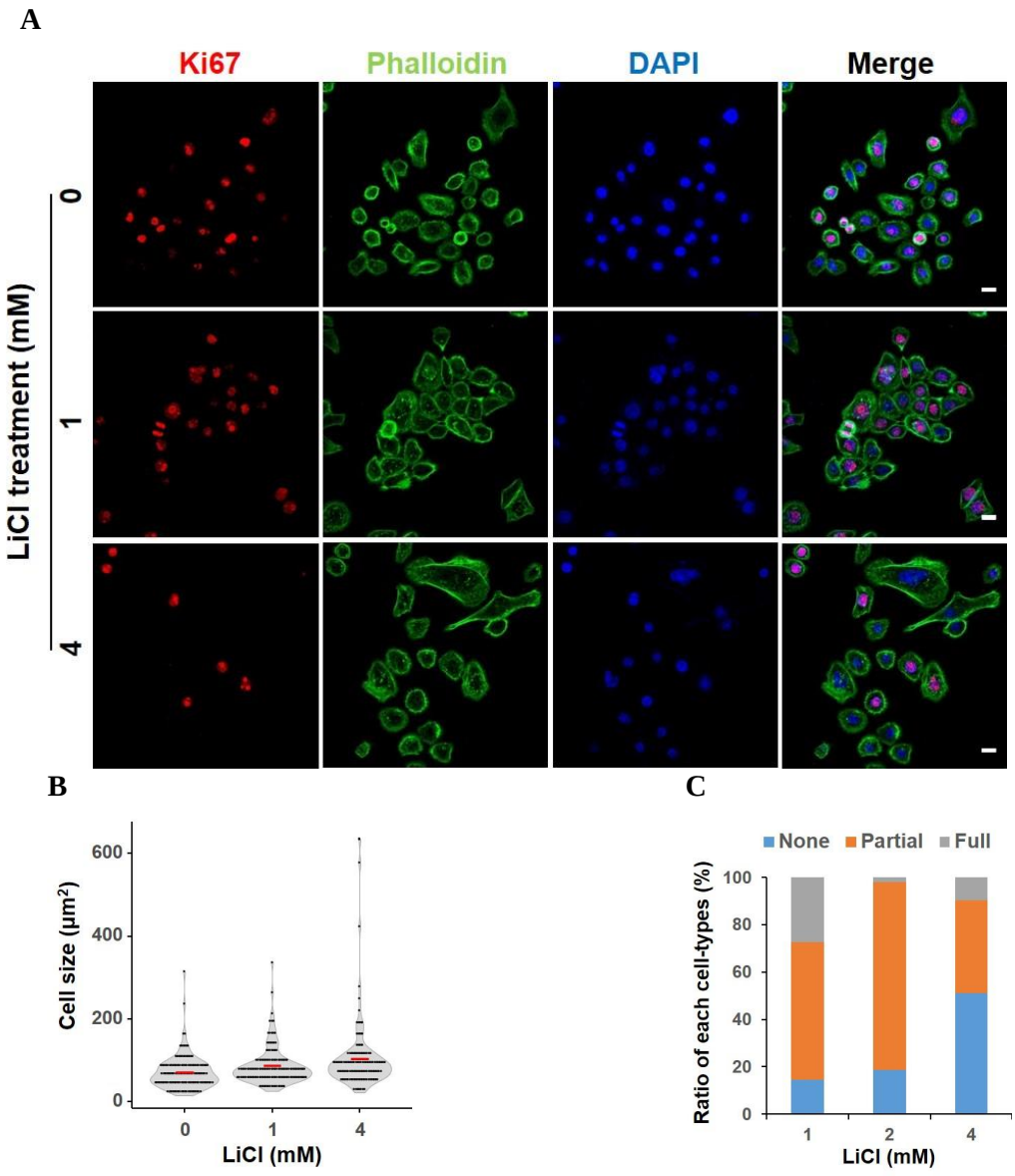


Figure 14. Expression of Ki67 in LiCl treated AM-1 cells.

A: Immunostaining of LiCl treated AM-1 cells.

B: Graph of LiCl and cell size.

C: Graph of LiCl and Ratio of each cell-types.

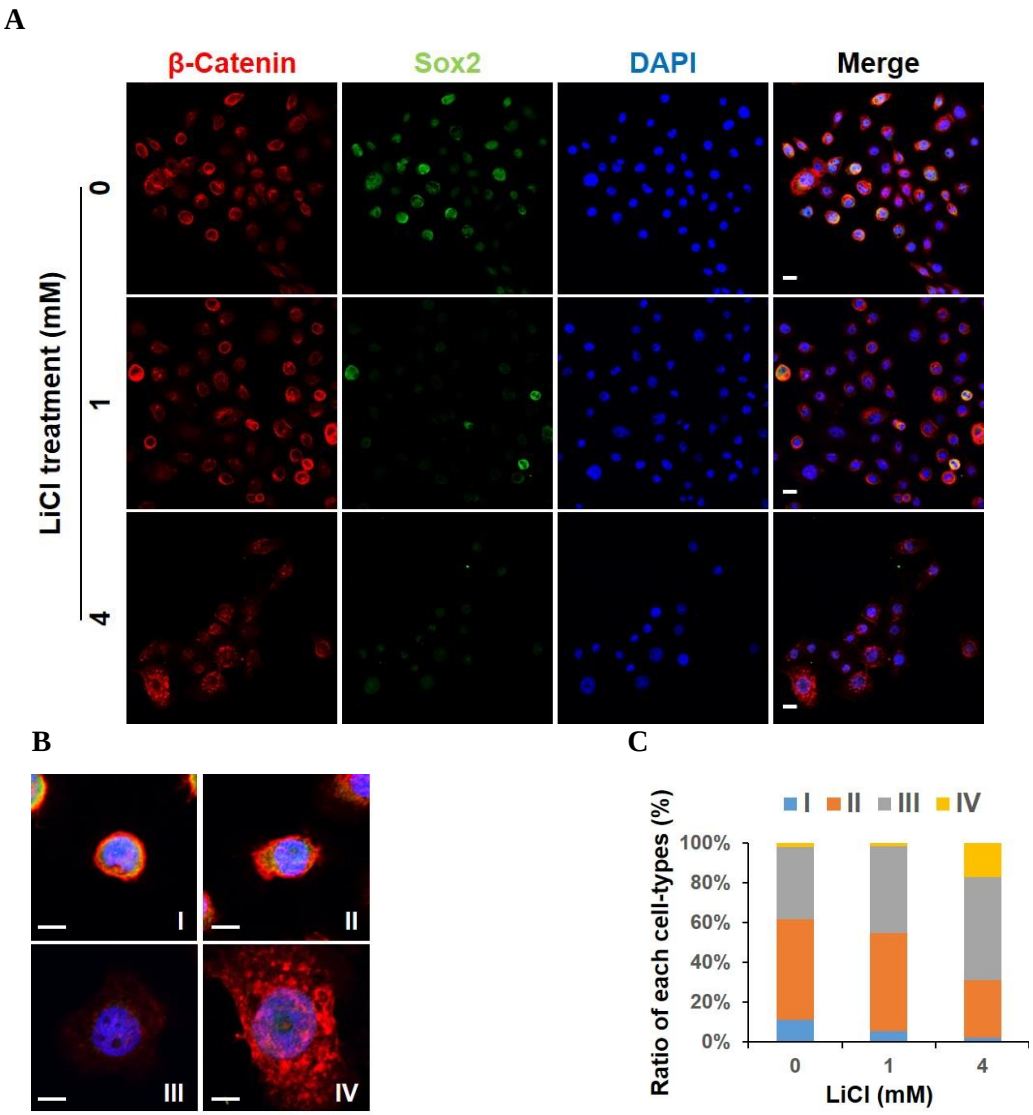


Figure 15. Expression of Sox2 in LiCl treated AM-1 cells.

A: Immunostaining of LiCl treated AM-1 cells.

B: Classification of AM-1 cell types (I: small, round, $Sox2^{high}$, and nuclear β-cat.^{low}, II: small, dented, round, $Sox2^{high}$, and nuclear β-cat.^{low}, III: small, flat, $Sox2^{low}$, and nuclear β-cat.^{low}, IV: large, flat, $Sox2^{low}$, and nuclear β-cat.^{high}.

C: Graph of LiCl and Ratio of each cell-types.

Treatment with VPA showed a similar result to that with LiCl (Fig. 16A). The number of large, flat cells (Fig. 16A) and expression of β-catenin in the cells increased (Fig. 16A and B). Expression of Bcl11b and Sox2 were decreased upon VPA treatment (Fig. 16C). The opposite effect was observed in cells treated with fibroblast growth factor (Fgf), a mitogenic factor that stimulates ameloblastoma proliferation (Nakao *et al.*, 2013); the number of small, round, $Sox2^{high}$ cells increased upon basic Fgf (bFgf) treatment (Fig. 17). These results show that small, round, $Sox2^{high}$ cells respond to their micro-environment, which is one of the basic feature of CSCs (Carnero *et al.*, 2016).

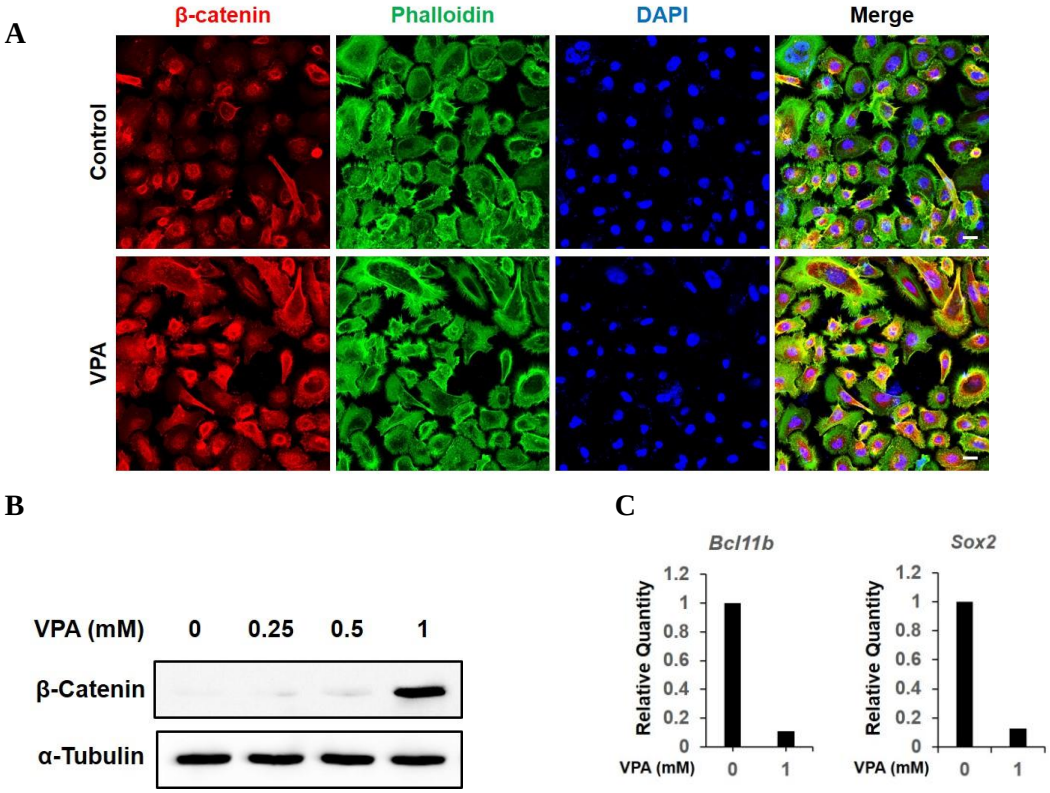


Figure 16. Expression of β-catenin in valproic acid (VPA) treated AM-1 cells.
A: Immunostaining of VPA treated AM-1 cells.

B: Dose dependent elevation of β-catenin.

C: Graph of VPA and Bcl11b, Sox2.

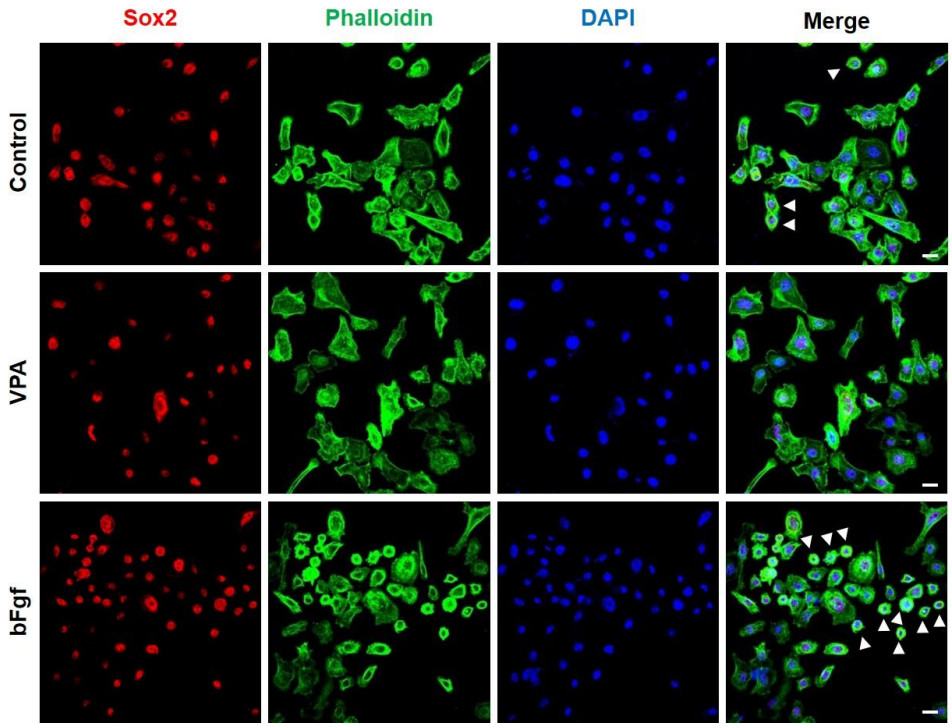


Figure 17. Immunostaining of VPA and bFgf treated AM-1 cells.
The number of small, round, Sox2^{high} cells increased upon bFgf treatment at the merged image (arrowheads).

Effect of Wnt signaling activator on AM-1 spheroid

To investigate the effect of Wnt signaling on spheroid formation, suspended AM-1 cells in DMEM were cultured with or without VPA (Fig. 18A). Both groups produced a similar number of spheroids. However, larger sized spheroids were observed in the VPA-treated group (Fig. 18B). The effect of VPA on AM-1 spheroids was further investigated by histological and immunohistochemical analyses (Fig. 19A and B). VPA-treated spheroids showed larger and more eosinophilic inner structures compared with non-treated ones (Fig. 19A). β-catenin staining visualized both an increase and nuclear accumulation of β-catenin in VPA-treated spheroids (Fig. 19B). VPA treatment of the spheroids decreased Sox2 and Bcl11b expression in a dose-dependent manner (Fig. 20).

A

B

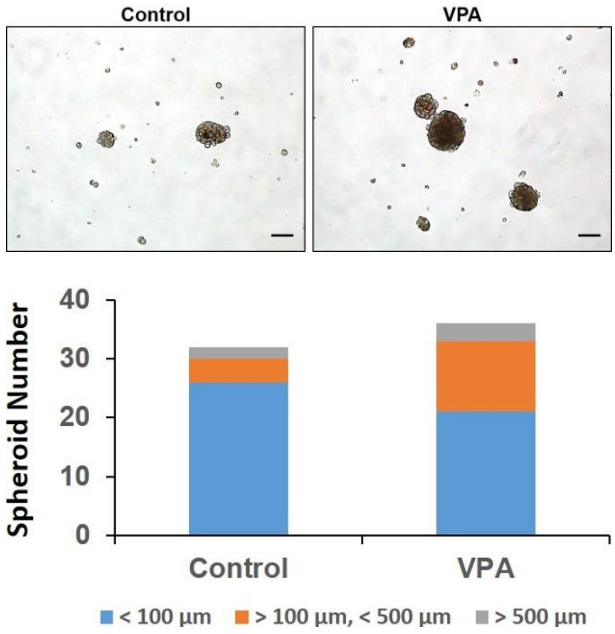
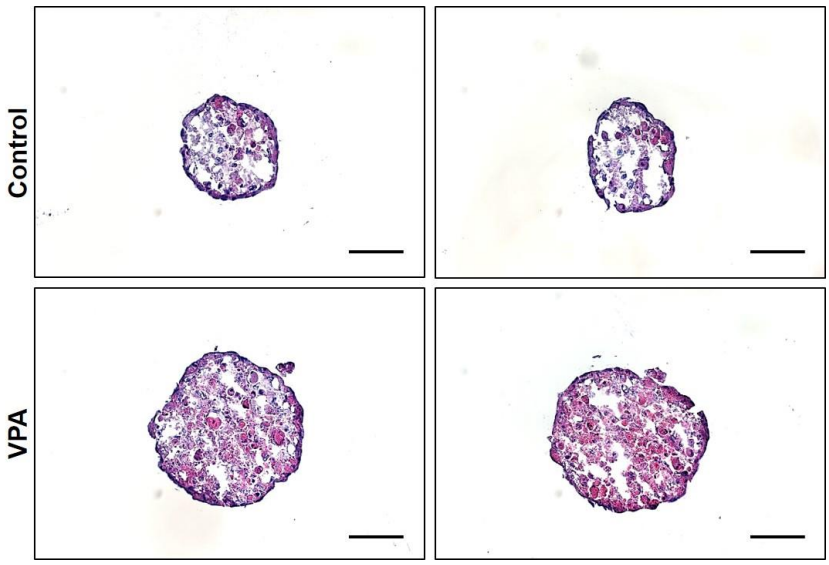


Figure 18. Effect of VPA on AM-1 spheroid formation.
Both groups produced a similar number of spheroids. However, larger sized spheroids were observed in the VPA-treated group
A: AM-1 spheroid in DMEM with and without VPA.
B: Graph of Spheroid number and control, VPA.

A



B

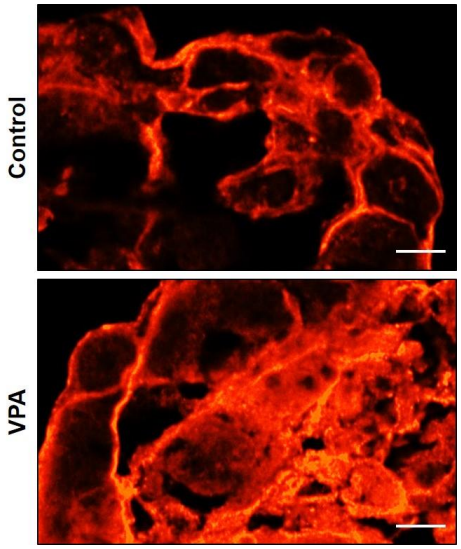


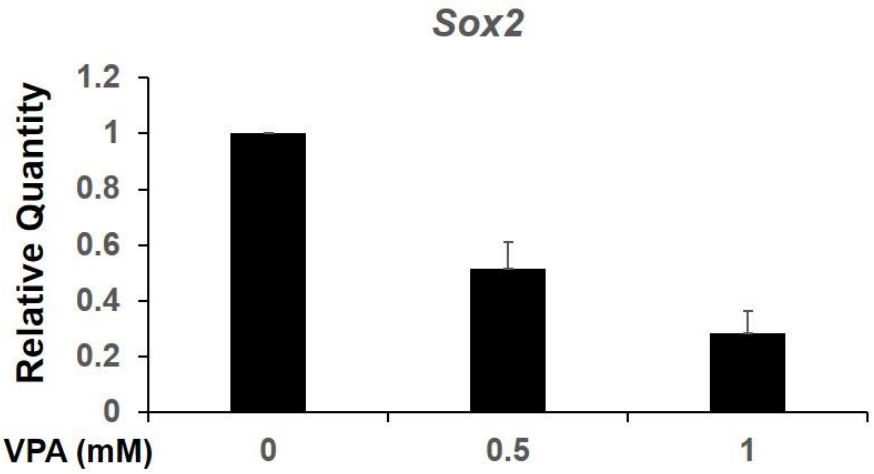
Figure 19. Histological and immunohistochemical analyses of VPA treated AM-1 spheroid.

VPA-treated spheroids showed larger and more eosinophilic inner structures compared with non-treated ones. β-catenin staining visualized both an increase and nuclear accumulation of β-catenin in VPA-treated spheroids

A: H&E staining of control and VPA treated AM-1 spheroid.

B: β-catenin staining of control and VPA treated AM-1 spheroid.

A



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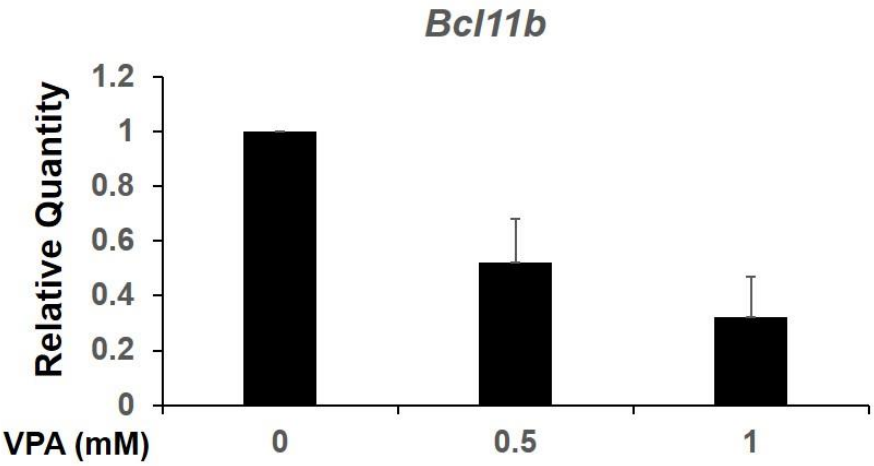

Figure 20. Decrease of Sox2 and Bcl11b expression according to the dose of VPA in AM-1 spheroid.
VPA treatment of the spheroids decreased Sox2 and Bcl11b expression in a dose-dependent manner.
A: Graph of VPA dose and Bcl11b.
B: Graph of VPA dose and Sox2.

IV. DISCUSSION

Treatment of ameloblastoma depends on the surgeon's treatment object. Some surgeons perform surgery as conservative as possible, even if they are prepared for recurrence of ameloblastoma. But to prevent recurrence of ameloblastoma, radical surgery is the current mainstay of ameloblastoma treatment and includes an en bloc resection with 1–2 cm adjacent bone, which usually requires immediate bone reconstruction for speech and swallowing (Mendenhall *et al.*, 2007). This reconstruction is unnecessary for a conservative surgery, such as simple enucleation or curettage of the bone margin. However, the rate of recurrence after these operations can be as high as 60–90% (McClary *et al*., 2016). With the high recurrence rate, the characteristics of ameloblastoma, such as slow and long-term proliferation, resistance to radiation and chemotherapy and complex histological structures inside tumors (Laborde *et al*., 2017), strongly imply the possible existence of CSCs in ameloblastoma. However, only several pathological studies have reported stem cell marker staining in tumor tissue (Chang *et al.*, 2013; Kumamoto and Ohki, 2010), and the characteristics and roles in tumorigenesis of CSCs remains to be elucidated.

In this study, a population of putative CSCs was characterized from AM-1 cells, a well-established human ameloblastoma cell line. The small, round cell population of AM-1 cells were proliferative and expressed a stem cell marker. It has been found that AM-1 cells harbor spheroid-forming capacity, which is an indicator for the stemness of cells. In addition, the orthotopic graft of AM-1 cells formed a mass with keratinization. These findings can pave the way to study CSCs populations of ameloblastoma *in vitro*.

In this study, two populations (P1, P2) according to the cell size of AM-1 were found using flow cytometry. It was confirmed that there was a difference in expression of Sox2 and CD49 in these two groups. Among the two groups divided according to cell size, cells in the P2 group with relatively small cell size and overexpressed Sox2 and CD49 are considered to be cells closer to CSCs than the P1 group with large cell size. On the other hand, P1 group cells are considered to be more differentiated mature cells than P2 cells. In

addition, cell mobility was more active in P2 cells than in P1 cells. This suggests that the role of the P2 cell may be larger in epithelial-mesenchymal transition (EMT) process of the CSCs, and this also seems to be evidence that the P2 cell group is a cell close to the CSCs. However, since no specific marker of ameloblastoma CSCs has been reported, it seems to require further study.

Wnt signaling is osteogenic signaling, which promotes bone generation by activating osteogenesis and inhibiting osteoclastogenesis (Monroe *et al.*, 2012). In tooth development, Wnt signaling is known to be activated in the late stage of development and facilitate ameloblast differentiation and movement (Guan *et al.*, 2016). Expression of Sfrp, an antagonist of Wnt signaling, is observed in Sox2-positive DESCs (Juuri *et al.*, 2012). Interestingly, AM-1 also expresses the Wnt antagonist Sfrp2 (Sathi *et al*., 2009), and osteogenic genes related to Wnt signaling are suppressed in this cell-line (Sathi *et al*., 2012). It has been revealed that the Sox2-positive population in AM-1 showed low Wnt signaling activity. Exogenous activation of Wnt signaling by treatment of Wnt activators reduced the number of Sox2-positive cells and spheroid forming activity of AM-1. These results suggest that Wnt activators suppress CSCs populations of ameloblastoma.

The ability to form spheroids as an anchorage independent growth is a typical characteristic of stem cells or CSCs. According to a study by Liu *et al.*, A study of CSCs using spheroid-forming cells among gastric cancers confirmed that spheroid body cells showed characteristics of CSCs, such as resistance to chemotherapy (Liu *et al*., 2013). AM-1 showed anchorage-independent growth, which is a property of transformed cells (Ju *et al*., 2014). Proliferation of AM-1 was observed in suspended cultures with both keratinocyte growth medium, which has low calcium concentration (Pillai *et al.*, 1988), and DMEM. However, spheroid formation was only observed in suspended cultures of AM-1 with DMEM. Suspended AM-1 cells formed compact and round spheroids that were approximately 200 μm in diameter. Meanwhile, small and loosely connected cell masses were observed with keratinocyte growth medium. Calcium is known to be an important co-factor of cell adhesion molecules that affects cell differentiation and migration

(Tharmalingam and Hampson, 2016). Further study is required to reveal the role of calcium on the spheroid formation activity of AM-1. In addition, even though the spheroids revealed some histological properties reminiscent of ameloblastoma, the culture conditions should be further optimized to represent more characteristics, such as reverse polarization of cells from the basement membrane of ameloblastoma.

In the area of clinical oral and maxillofacial surgery, ameloblastoma was a very difficult disease to treat. Treatment has been established for decades with the principle of surgical removal, and it has been difficult to apply other treatments such as medication. However, recent efforts have been made to target therapies such as neoadjuvant BRAF inhibitor treatment (Kaye *et al.*, 2015). In this study, it has been found that activation of Wnt signaling can be effective in the treatment of ameloblastoma. Through this, drugs such as rosozumab, which has recently been developed and used as an osteoporosis drug, as well as VPA, a Wnt activator currently used in humans, can provide clues for the treatment of ameloblastoma. If the osteogenic activity of Wnt signaling activation is utilized, it will be able to help with the recovery of the jaw while simultaneously removing the lesion (Gaur *et al.*, 2005). It is thought that further study of CSCs of ameloblastoma will be needed for future treatment of ameloblastoma. In this case, the possibility of using AM-1 as an *in vitro* and *ex vivo* model of CSCs study of ameloblastoma was confirmed.

V. CONCLUSION

Experiments were performed using AM-1 cells to identify the presence of CSCs in ameloblastoma and to verify the role of the Wnt signal activator in these CSCs. Through the above experiments process, the following results were obtained.

1. AM-1 cells exhibited significant heterogeneity in cell morphology. The largest cell was 12 times larger than the smallest cell, and various shapes of cells, from round to amorphous, were observed.
2. Immunocytochemistry analysis showed that small-sized and round-shaped cells were proliferative and expressed a marker of dental epithelial stem cells (DESCs), SRY related HMG box 2 (Sox2).
3. Sox2 expression in cells was negatively correlated with activation of Wnt signaling.
4. I*n vitro* spheroid-forming assays and the orthotopic graft of the first molar extraction of nude mice confirmed the stem cell-like characteristics of AM-1 cells.
5. Exogenous activation of Wnt signaling using lithium chloride (LiCl) and valproic acid (VPA) increased the cell size and decreased proliferation and expression of Sox2.

This study confirmed the putative CSCs population in AM-1. This confirmed the possibility of using AM-1 as an *in vitro* and *ex vivo* model for the study of CSCs in ameloblastoma. And reduction of CSCs cell population and reduction of spheroid in AM-1 were confirmed through activation of Wnt signaling. Through this, the possibility of activating Wnt signaling as an agent for treatment of ameloblastoma and suppression of recurrence was confirmed.

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ABSTRACT (in Korean)

Wnt 신호 전달의 활성화가 법랑모세포종의 암 줄기세포의 감소에 미치는 영향

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박 진 후

암 줄기세포(Cancer stem cells, CSCs)는 비대칭 분열에 의해 종양의 이질성(heterogeneity)에 기여하는, 무제한 자가 재생능력(self-renewing)을 가진 종양 세포의 아집단(subpopulation)이다. 임상적으로, CSCs는 방사선 치료 및 항암 화학 치료에 대한 내성을 보이며, 종양의 재발에 원인이 되는 것으로 알려져있다. 치성 상피성 종양인 법랑모세포종(ameloblastoma)은 성장 속도가 비교적 느리고, 전이 되지 않는 특성으로 인해 양성 종양으로 분류된다. 그러나, 법랑모세포종은 국소적 재발이 아주 흔하고, 약 1%에서 악성 변화 및 전이가 관찰된다. 아직까지 법랑모세포종은 방사선 요법이나 약물 요법으로 치료가 어려운 것으로 알려져 있다. 이 때문에 법랑모세포종의 치료는 병소 주변부를 포함하는 광범위한 외과적 절제술을 요하며, 환자의 악골에 큰 결손을 남긴다. 본 연구의 목적은 AM-1 세포를 사용하여 법랑모세포종의 CSCs를 확인하고, 이 CSCs에 Wnt 신호 전달 활성화제의 효과를 규명하고자 함이다. 먼저 잘 알려진 인간 법랑모세포종 세포주(cell line)인 AM-1 세포를

유세포 분석법(flow cytometry)을 사용하여 분석 하였다. 이때 획득 된 세포 중에서 CSCs로 의심되는 세포를 확인하기 위해 면역 염색을 하였다. 면역 염색에 의해 CSCs로 식별 된 세포에 대한 Wnt 신호 전달 활성화제를 적용하여 효과를 규명하 였다. 상기의 연구과정을 통하여 아래와 같은 결과를 얻었다.

1. AM-1 세포는 세포 형태에서 상당한 이질성을 나타내었다. 가장 큰 세포는 가장 작은 세포보다 12 배 더 크며, 원형에서 비정질에 이르는 다양한 형태의 세포가 관찰되었다.
2. 면역 세포 화학 분석 결과 소형 및 원형 세포가 증식하였으며, 이들 세포가 dental epithelial stem cells (DESCs), SRY related HMG-box-2 (Sox2)의 마커를 발현시켰다.
3. 세포에서의 Sox2 발현은 Wnt 신호 전달의 활성화와 음의 상관 관계가 있었다.
4. 현탁 배양에서 회전타원체(spheroid)의 형성과, 누드 마우스의 제 1 대구치 추출에 orthotopic graft를 통해 AM-1 세포의 줄기 세포와 같은 특성을 확인하였다.
5. 리튬 클로라이드 (LiCl) 및 발프로산 (valproic acid, VPA)을 사용한 Wnt 신호의 외인성 활성화는 세포 크기를 증가시키고 세포 증식 및 Sox2의 표현을 감소시켰다.

이번 연구를 통해 AM-1에서 CSCs로 추정되는 세포군을 확인할 수 있었다. 이는 법랑모세포종의 CSCs연구의 체외(*in vitro*) 및 생체외(*ex vivo*) 모델로 AM-1을 사용할 수 있는 가능성을 보여주었다. 그리고 Wnt 신호 전달의 활성화를 통해 AM-1에서 CSCs 세포군의 감소와 회전타원체의 감소를

확인할 수 있었다. 이를 통해 Wnt 신호 전달의 활성제의 법랑모세포종 치료 및 재발 억제를 위한 약물로서의 가능성을 확인하였다.

핵심 되는 말: 암 줄기세포, 법랑모세포종, Wnt 신호전달, AM-1