# Role of $Ca^{2+}$ signaling in collective migration and invasion of ameloblastoma

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# Role of $Ca^{2+}$ signaling in collective migration and invasion of ameloblastoma

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The Doctoral Dissertation
Submitted to the Department of Dentistry
And the Graduate School of Yonsei University
In partial fulfilment of the
Requirements for the degree of
Doctor Philosophy in Dental Science

Sung-Ho Park

June 2020

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June 2020

# Acknowledgements

본 학위 논문을 완성하기까지 많은 분들의 도움이 있었습니다.

우선, 많이 부족한 저를 오랜 기간 끊임없이 지도해주신 정영수 교수님께 한없는 존경과 감사의 마음을 드립니다. 구강악안면외과 임상가이면서도, 끝까지 학자의 길을 갈 수 있도록 이끌어 주셔서 진심으로 감사드립니다. 본 논문을 마칠 수 있는 역량에 도달하기까지 많은 지도를 해주신 정한성 교수님께도 진심으로 감사드리며, 존경을 표합니다. 교수님께서 주신 가르침을 마음속에 깊이 담고 배움의 자세를 간직하겠습니다. 연구기간 내내, 항상 따뜻하게 대해주시고 많이 신경 써주신 이종민 교수님께도 감사의 말씀을 드립니다. 임상가로서 한번 더 연구의 목표를 생각할 수 있도록 논문 작성 과정에서 부족한 점을 많이 지도해 주신 정휘동 교수님께 감사의 말씀을 드립니다.항상 너무 바쁘신 와중에도 이끌어주신 김현이 교수님께도 감사의 말씀을 드립니다.

조직학 교실에서 연구 기간 내내 저와 가장 많은 토론의 시간을 가져주시고, 조언을 해주신 이동준 선생님께 진심으로 감사의 마음을 드립니다. 연구가 잘 마칠 수 있도록 항상 많은 도움을 주신 이서진 선생님께도 진심으로 감사드립니다. 교실 생활을 하며 잘 적응할 수 있도록 도와주신, 김은정 박사님, Zhang Sushan 선생님, 오상빈 선생님, 이승두 선생님에게도 감사의 말씀을 드립니다.

구강악안면외과 박사과정을 마치는 지금까지, 전문의로서 역량

을 키워주신 김명래 교수님께 항상 가슴 깊이 존경심과 감사의 마음을 드립니다. 뛰어난 의술 외에도 항상 따뜻한 인품을 지니는 모습을 보여주시는 삼성서울병원 김창수 교수님께 존경과 감사의 마음을 드립니다. 저를 많이 챙겨주시고, 항상 열심히 노력하시는 모습을 몸소 보여주시는 이대서울병원 김선종 교수님께도 존경과 감사의 마음을 드립니다. 항상 같이 지식을 공유하며 의지가 되어 주는 의국원 선생님들과 직장 동료 선생님들에게도 감사 드립니다.

지금까지 제가 몸 건강히, 부족한 점 없이 임상 진료 및 학위에 매진 할 수 있도록 잘 키워주신 부모님께 감사의 말씀을 드리고, 평소에 하지 못한 사랑한다는 말씀을 드립니다. 제가 건강을 챙길 수 있도록 항상 신경 써주신 장인어른, 장모님께도 감사의 마음을 드립니다. 항상 형인 저를 잘 따라주고, 먼저 챙겨주는 동생 지호에게도 감사함을 표합니다.

마지막으로, 학위 졸업을 위해 소홀했던 육아 및 가정 생활을 홀로 묵묵히 보조해준 사랑하는 아내 강미나에게 깊은 사랑과 감사한 마음을 전합니다. 내 삶의 원동력, 사랑하는 아들 재연이에게도 감사함을 전하며 앞으로도 지금처럼만 건강하고 밝게 자라주길 바란다.

2020년 6월

박 성 호

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| | |
|---|---|
| CaSR | Calcium-sensing receptor |
| CRAC | $Ca^{2+}$ release-activated $Ca^{2+}$ channel |
| DMEM | Dulbecco's modified eagle's medium |
| ECM | Extracellular matrix |
| EMT | Epithelial–mesenchymal transition |
| ICC | Immunocytochemistry |
| KSFM | Keratinocyte serum free medium |
| NCX1 | $Na^{+}$/ $Ca^{2+}$ exchanger 1 |
| SCC | Squamous cell carcinoma |
| SOCE | Store-operated $Ca^{2+}$ entry |
| TRPC | Transient receptor potential channels |
| ZO-1 | Zonula occludens-1 |

**Abstract**

# Role of $Ca^{2+}$ signaling in collective migration and invasion of ameloblastoma

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Research on collective invasion is considered to be very important for conservative treatment and long-term prognosis of cancer. Particularly in malignant oral cancer and ameloblastoma, compared to breast cancer or lung cancer, the clear mechanism between calcium and collective invasion is unknown, and further studies are needed. Therefore, my objective is to investigate the role of $Ca^{2+}$ signaling in ameloblastoma during the collective invasion process. Human derived ameloblastoma cell line (AM-1) was used in this study. $CaCl_2$, SKF96365 and NPS2143 were added into the AM-1 culture medium to control the intracellular $Ca^{2+}$ concentration. Time-lapse imaging, tumor spheroid invasion collagen assay and immunofluorescence staining were used to observe the cell morphology, migration and invasion pattern of AM-1 under the control of $Ca^{2+}$ signaling. AM-1 cell showed a disparate cell morphology and migration pattern in different culture condition which is associated with $Ca^{2+}$ concentration. Furthermore, AM-1 cell attached to each other more tightly and migrated collectively in $Ca^{2+}$ dose-dependant manner. However, $Ca^{2+}$ induced cell-cell adhesion could be suppressed by SKF96365 not NPS2143. Moreover, $Ca^{2+}$ induced collective migration of AM-1 cell also can be suppressed by SKF96365. Finally, SKF96365 was sufficient to inhibit the cell-cell adhesion and collective invasion of AM-1 cell. Therefore, it can be concluded that calcium pump is expected to be a potential candidate target for inhibiting the collective invasion of ameloblastoma.

**Key words: Ameloblastoma; collective invasion; $Ca^{2+}$ signaling; SKF96365**

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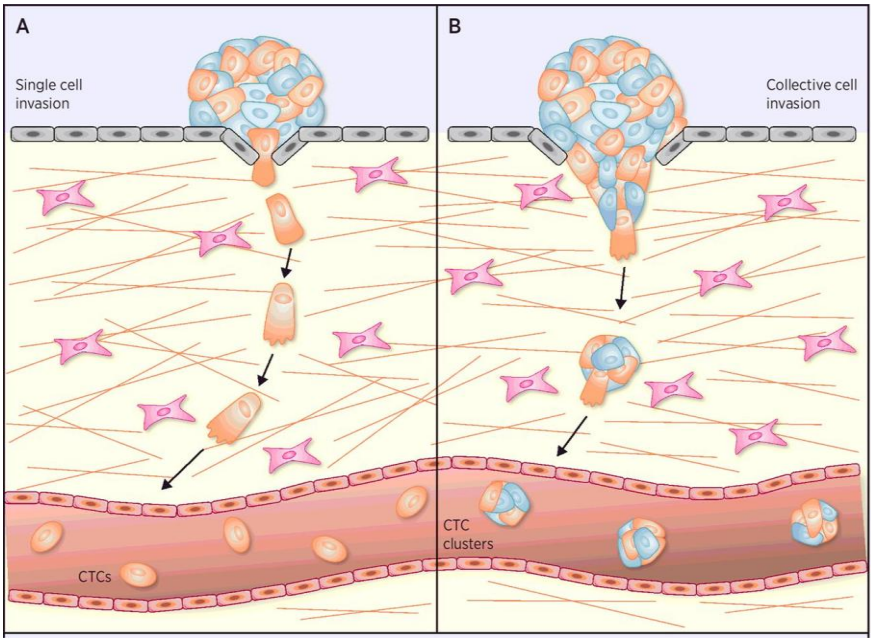
## I. INTRODUCTION

### 1. Cancer invasion

The ability of cancer cells to metastasize by invading adjacent tissues is often characteristic of cancer as metastasis to local or distant tissues. Traditionally, invasion in a histopathological sample is defined as the penetration of normal tissue by a single cell, a small group of cells, or an extended strand of linked tumor cells (Leighton *et al.*, 1960). For many types of cancer histologically, the most frequently observed cell type is the collective invasion type, which is

defined as malignant function (Friedl *et al.*, 1995; Friedl *et al.*, 2004; Christiansen and Rajasekaran, 2006; Vandervorst *et al.*, 2019) (Figure 1). In this group, cells maintain cohesion by expressing cell-cell junction molecules such as cadherins or adhesion of the immunoglobulin superfamily (Cavallaro and Christofori, 2004).




(Vandervorst *et al*., 2019)

**Figure 1. Carcinoma cell metastasis is mediated by multimodal cell migration**

(A) In the single cell invasion model, individual primary tumor cells undergo epithelial–mesenchymal transition (EMT) to acquire mesenchymal properties that enable them invade into the stroma, circulate as individual cells in blood vessels, and seed clonal metastatic lesions. (B) In the collective cell invasion model, groups of cohesive cells invade into the stroma, circulate in blood vessels as clusters of cells, and seed polyclonal metastatic lesions.

Indeed, histopathological analysis of specimens from patients with colorectal cancer (Yokoyama *et al*., 2013; Karagiannis *et al*., 2014), breast cancer (Yokoyama *et al*., 2013; Westcott *et al*., 2015; Yang *et al*., 2019), squamous cell carcinoma (Nakashima *et al*., 2013), lung cancer (Richardson *et al*., 2018), melanoma (Wang *et al*., 2018), head and neck squamous carcinoma (Gopal *et al*., 2017), thyroid cancer (Kim *et al*., 2017) and salivary adenoid cystic carcinoma (Gao *et al*., 2017; Wu *et al*., 2019) reveals collective invasion of cancer cells, and cells in the group characterized by sustained cell-to-cell adhesion and polarized invasions between them. In addition, it has been found that most metastatic cancer cells express E-cadherin, an epithelial cell marker protein involved in cell-cell adhesion, while few cells express mesenchymal cell marker proteins including ZEB1 and vimentin (Mizukoshi *et al*., 2020).

Similar to general cancer, oral cancer shows a similar pattern with collective invasion. In squamous cell carcinoma (SCC) among typical oral cancer, tissue invasion by invading cells collectively requires physical forces exerted by tumor cells on the surrounding extracellular matrix (ECM) (Grasset *et al*., 2018). Here we can see that the collective invasion of SCC is accelerated by matrix-dependent mechano-sensitization of EGF signaling. Calcium was a effective intracellular secondary messenger that induces actomyosin contractility.

## 2. Cancer cell invasion pattern

### 2.1 Solitary invasion

In the absence of cell-cell adhesion, tumor cells invade into a single cell form. Molecular and cellular systems resulting from solitary migration provide an important view in the spread of tumors that occur when cells migrate as single cells, such as leukemias and lymphomas, or are separated from aggregated cells through the epithelial to mesenchymal transition (EMT) (Thiery *et al*., 2009). However, cancer cell invasion is not limited to migrate as a single cell.

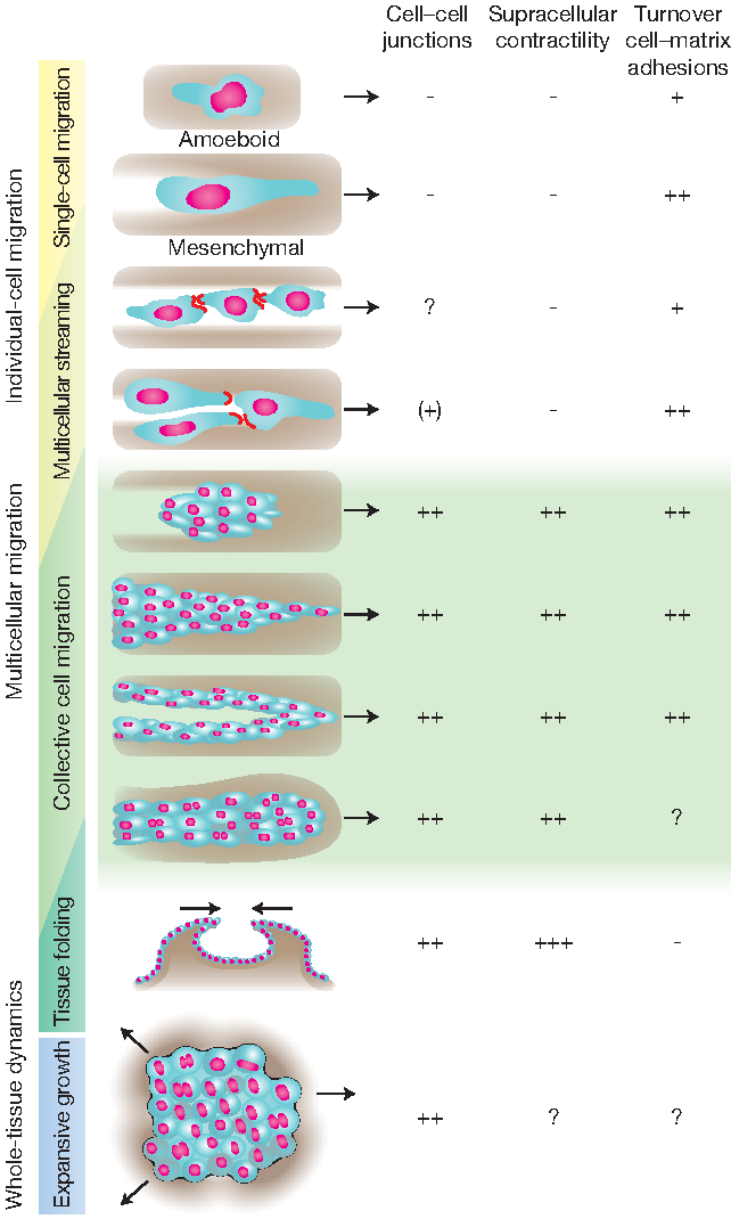
### 2.2 Collective cell invasion

During collective cell migration, cell-to-cell adhesion and functional interaction between the leader cells located at the end of the group and the follower cells that follow them plays an important role. It has been found that leader and follower cells have different cellular properties (cell behavior), such as cell polarity (Rorth, 2012; Konen *et al*., 2017; Mayor and Etienne-Manneville, 2016). Cell transition from non-migratory to migratory state is an important process for initiating collective or solitary cell migration (Nagai *et al*., 2020) .

Compared with solitary cell migration, it can be assumed that collective cell migration is more advantageous to overcome the extracellular matrix (ECM) barrier required for efficient sprouting of endothelial cells during angiogenesis, as well as invasion and metastasis of cancer cells. Like normal development and wound healing, EMT plays an important role in carcinoma cell initiation of collective migration and invasion. Although EMT is known to lose epithelial traits and promote transition to the mesenchymal state, collectively migrating cells do not lose their epithelial trait completely and rather require this for

metastasis (Cheung and Ewald, 2016; Padmanaban *et al*., 2019).

The morphological composition of cancer cells collectively invading can vary considerably (Friedl *et al*., 2012). Invading cell groups range in diameter from just one or two strands of cells to masses that do not come into contact with the ECM or even form large luminal structures (Ewald *et al*., 2008) (Figure 2). These are often seen in invasive carcinomas of the breast, prostate and pancreas (Christiansen and Rajasekaran, 2006). Certain combinations of cell-cell adhesion, cell-matrix adhesion and proteolysis probably contribute to determining the size and shape of the collective invasion structure.




(Friedl *et al*., 2012)

**Figure 2. Different types of cancer cell invasion**

Amoeboid or mesenchymal single-cell migration; multicellular streaming with or without weak junctional contacts; collective cell migration; tissue folding and expansive growth. Although each pattern can be described as a separate process, overlapping or shifting of cell morphology can result in mixed or unstable phenotypes. The strength range of the proposed property (from none to strong) is indicated by the plus and minus signs.

Cancer caused by this collective invasion is relatively more extensive in local tissue destruction than single cell invasion. This requires surgery with a wide range of resections, including major anatomical structures. Therefore, research on collective invasion is considered to be very important for conservative treatment of cancer and long-term prognosis. The therapeutic targeting of collective invasion aims to reduce the loss of biological tissue to prolong the patient's life and improve the quality of life, and may be meaningful in cases of local invasive and destructive diseases that cannot be resolved by surgical removal alone (Friedl *et al.*, 2012).

### 3. Role of $Ca^{2+}$ signaling in tumorigenesis

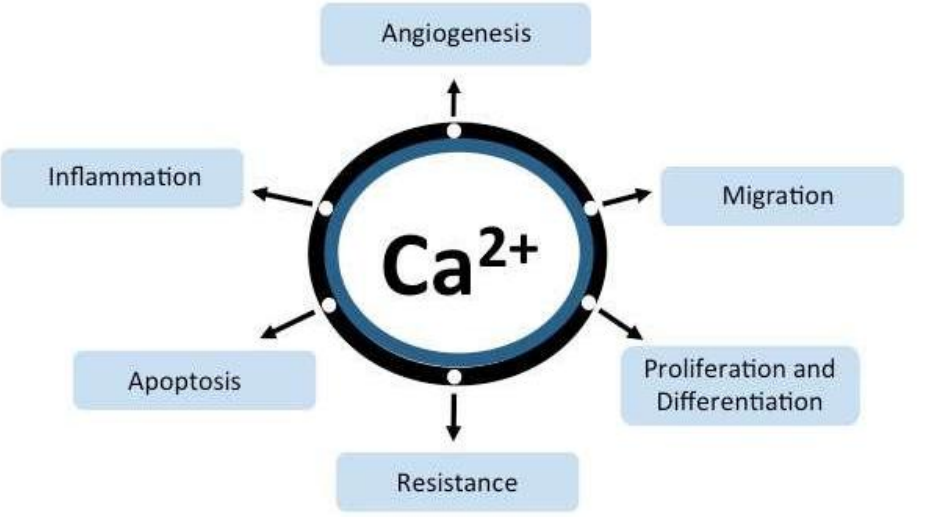
$Ca^{2+}$ is a ubiquitous secondary messenger that affects all aspects of cell fate and function. The $Ca^{2+}$ signaling pathway regulates the major processes from inflammation to apoptosis involved in tumor formation and resistance to chemotherapy in breast cancer.

In the resting state, $Ca^{2+}$ in the cytoplasm is maintained at a sudden low level (~ 50-100 nM) with a concentration difference up to 10000 compared to extracellular free $Ca^{2+}$ (1-2 mM). In non-excitable cells, P-type $Ca^{2+}$-ATPase is located in the endoplasmic reticulum, Golgi, secretory vesicles, plasma membrane, pump $Ca^{2+}$ out of the cytoplasm to maintain resting cytoplasmic $Ca^{2+}$ concentration. These responses with ligands, membrane voltage, mechanical forces, and volume changes in and out of the cell, causing temporary $Ca^{2+}$ channels opening to increase the $Ca^{2+}$ concentration in the cytoplasm, causing downstream effectors related to gene transcription, cell cycle control, differentiation, proliferation, cell migration, and apoptosis pathways (Makena and Rao, 2020) (Figure 3).

The prolonged elevation of $Ca^{2+}$ levels in the resting phase has the potential for

malignancy to gene expression, migration and proliferation to initiate and maintain tumor growth (Figure 4). Conversely, depletion of calcium storage and weakening of the calcium influx pathway lead to chemical resistance and apoptosis avoidance of tumor (Roderick and Cook, 2008; Marchi and Pinton, 2016). As both $Ca^{2+}$ level elevation and depletion can induce a malignant phenotype, the specific mechanisms that induce these changes need to be clearly understood.

In particular, in malignant oral cancer and ameloblastoma, compared to breast cancer or lung cancer, the clear mechanism between calcium and collective invasion is unknown, and further studies are needed.

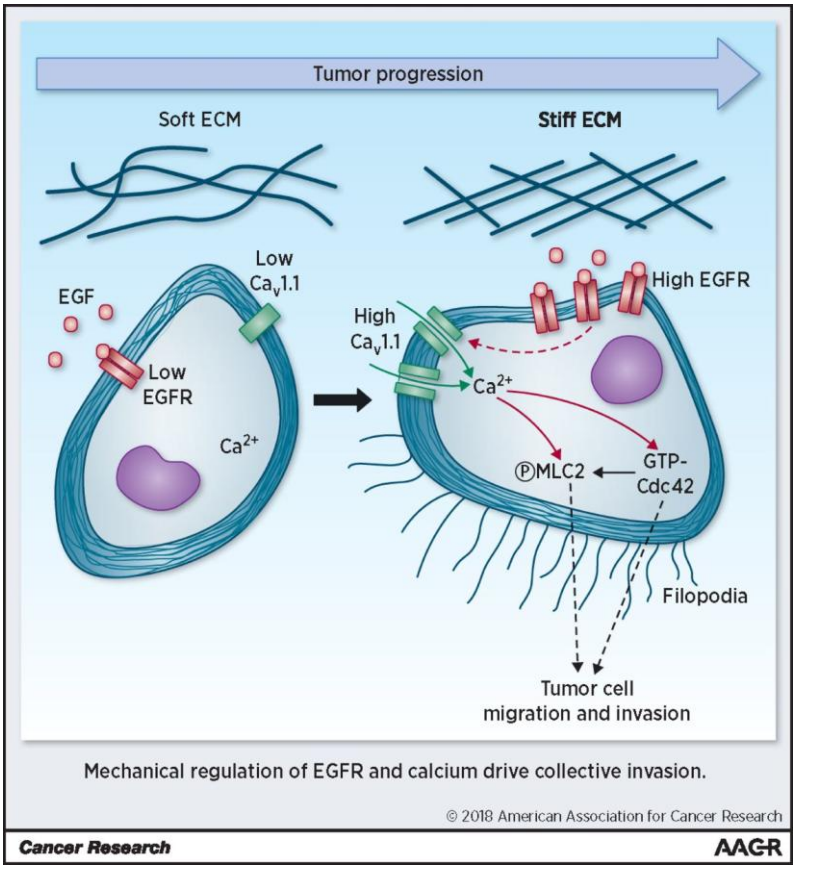



(Makena and Rao, 2020)

**Figure 3. Role of $Ca^{2+}$ signaling**

$Ca^{2+}$ is a ubiquitous secondary messenger that affects all factors of cell fate and function. The $Ca^{2+}$ signaling pathway regulates the major processes from inflammation to apoptosis involved in cancer formation and resistance to chemotherapy.

Increasing the $Ca^{2+}$ concentration in the cytoplasm causes downstream effectors related to gene transcription, cell cycle control, differentiation, proliferation, cell migration, and apoptosis pathways.




(Grasset *et al*., 2018)

**Figure 4. Mechanical regulation of EGFR and calcium drive collective invasion**

Tumor-derived matrix stiffness and EGFR signaling induced increased intracellular $Ca^{2+}$ through $Ca_V1.1$ expression in SCC cells. Blocking L-type calcium channel expression or activity using $Ca^{2+}$ channel blockers as verapamil and diltiazem suppressed SCC cell collective invasion both *in vitro* and *in vivo*.

### 4. Ameloblastoma

Ameloblastoma is histologically benign and slow to grow, but because it grows infiltratively, recurrence is well developed and grows to a considerable size without any particular symptom, causing swelling or destruction of the cortical bone, leading to aesthetic defects of the facial and functional failure of chewing (Gardner, 1984). Because of these clinical characteristics, ameloblastoma can be treated with conservative surgical methods such as marsupialization, enucleation, curettage. Otherwise, it is treated using radical surgical methods such as en bloc or marginal resection, segmental resection and unilateral hemisection (Nakamura *et al*., 2002). The choice of treatment is determined by considering the radiological findings, histologic findings, site, size, age, general condition, aesthetics, socioeconomic conditions, prognosis, and ability of the surgeon. Conservative surgery can be selected to preserve a large amount of bone remaining in the patient, and the amount of bone regeneration has aesthetic and functional advantages (Hinds *et al*., 1954). Nevertheless radical surgery may be preferred over conventional surgery because of high recurrence rate. However, radical resection of the mandible has problem such as swallowing, pronunciation, and aesthetic morphology, conservative treatments are needed to improve the patient's quality of life.

Like breast and lung cancer, ameloblastoma is thought to be able to regulate cell migration by calcium concentration. The extracellular and intracellular calcium concentration can be controlled by a calcium pump inhibitor and a calcium sensing receptor inhibitor. Each inhibitor can be used to analyze the cell migration morphology by regulating the calcium concentration in the cytoplasm of the ameloblastoma tumor cell.

### 5. AM-1 cell line

However, to the best of our knowledge, there are no reports associated with cellular factors that may influence the different patterns of invasion in ameloblastoma. This may be due to the limited lifespan of ameloblastoma cells from primary cultures, it is not available to researchers to do such studies without a reproducible *in vitro* model until 1998.

Immortalized ameloblastoma cell line (AM-1) using human papillomavirus type-16 was established (Harada *et al*., 1998).

### 6. Invasion pattern of ameloblastoma

As stated, pathologic classification is a benign tumor in that metastasis rarely occurs, but there are recent attempts to classify it as a carcinoma because of its aggressive invasion pattern (Slootweg and Muller, 1984).

Thus, until now, there are no alternative surgical options for the surgeon other than expanded resection, even if it is an imperfect therapeutic strategy that may burden the patients' life. Interestingly, various pathological images of all types of ameloblastoma demonstrate that the cells invade collectively into surrounding tissues without losing the cell-cell adhesions and maintains the epithelial cell characteristic (Hao *et al*., 2018).

In this study, we cultured immortalized AM-1 in two different concentrations of calcium and detect the different cell-cell adhesion and migration pattern. We hypotheses that the Calcium signaling may involve in the collective migration and invasion of AM-1 cell. To determined this hypothesis we examined the expression of cell-cell junction protein on AM-1 cell. We also observed the alternation of AM-1 cells migration and invasion pattern after calcium pump inhibitor treatment. While several studies have observed the association between collective invasion pattern and $Ca^{2+}$ signaling in ameloblastoma,

however, little is known on their details of the invasive mechanism, which has substantially hindered clinical decision making. Thus, clarification of these biological mechanisms will provide a foundation to develop new treatment strategies for this disease.

## II. MATERIALS AND METHODS

### 1. Cell culture

The human ameloblastoma cell line AM-1 was kindly provided by Prof. Harada H. (Iwate Medical University, Japan). AM-1 was cultured in Keratinocyte serum free medium (Keratinocyte-SFM; 10724-011, Gibco, USA) supplemented with 2.5 g EGF Human Recombinant (10450-013; Gibco, USA), 25 mg Bovine Pituitary Extract (13028-014; Gibco, USA) and Dulbecco's modified eagle's medium (DMEM; 11995-065, Gibco, USA) supplemented with 10% fetal bovine serum (FBS; C0235, Gibco, USA) and 10,000 U/mL Penicillin-Streptomycin (15140163, Gibco, USA) respectively at 37℃ in a humidified atmosphere with 5% carbon dioxide ($CO_2$).

### 2. Reagents

The following reagents were used: Calcium chloride (Sigma-aldrich, USA), SKF 96365 hydrochloride (Tocris Bioscience, USA), NPS 2143 hydrochloride (Tocris Bioscience, USA), E-cadherin antibody (BD Bioscience, USA), ZO-1 antibody (Invitrogen, USA), Alexa Fluor™ 488 Phalloidin (Thermo Fisher Scientific, USA), CellTracker™ Green CMFDA (Thermo Fisher Scientific, USA), Collagen Type I (Corning, USA).

### 3. Instruments

The following instruments were used: Inverted Laser Confocal Microscope (DMi8, Leica, Germany), Confocal Quantitative Image Cytometer (CQ-1, YOKOGAWA, Japan), Inverted Microscope (CKX41SF, Olympus, Japan), Hera cell $CO_2$ Incubator (Thermo Fisher Scientific, USA).

**4. Time-Lapse imaging of AM-1 cell**

The AM-1 cell suspensions with a density of $3x10^5$ were seeded on a 35 mm glass bottom confocal dish for at least 24hr. Mix 25mM CellTracker™ Green CMFDA with the medium and incubated in the 37℃ incubator for 30 min. These staining methods with fluorescent probe enable us to visualize the AM-1 cell movement clearly and no effect on cell viability. After washing with PBS 3 times, confocal dishes with AM-1 cells in 2 ml of conditional medium were placed into the microscope stage adaptor, and z-stacks of confocal images were acquired using a spinning disk confocal imaging system based on a CQ-1 inverted fluorescence microscope. Then 145 serial two-dimensional confocal images through 517 nm channels were recorded in the environmental chamber, which ensured a constant temperature (37℃), humidity and 5% $CO_2$ atmosphere throughout the duration of imaging. Intervals between image acquisition were 10 minutes. All image acquisition settings were identical for experimental variants in each experiment. Image J software package was use for graphical analysis. Image J plugins “FiloQuant” was used to count the number of filopodia on the AM-1 cells. Image J plugins “Tracking” was used to record the movement trace of AM-1 cells. Migration distance and average velocity were calculated by Particle analysis.

**5. Immunofluorescence**

AM-1 cell were grown on the cover-slips with a density of $2.5x10^5$ for at least 24hr and subsequently washed, fixed, permeabilized, blocked, and incubated overnight with E-cadherin and ZO-1 primary antibodies. Subsequently, the cover-slips were washed with PBT, incubated with Alexa Fluor™ 488 Phalloidin and secondary antibodies in PBT for 1hr at room temperature, washed again with PBT, incubated with DAPI in PBT for 15 min.

### 6. Spheroids invasion collagen assay

AM-1 spheroids were fabricated in Ultra-low attachment plates (Prime Surface 3D Culture Spheroid 96U plates, Sbio, Japan) using $2.5x10^4$ cells per spheroid. 24 hours post-incubation, spheroids were mixed with collagen (Rat tail collagen type I, Corning, NY) in pH7, and incubated at 37 °C for 30 minutes. After the gel has set and stabilized, in order to ensure proper media supply, spheroids were detached using a tip, and 300 μl of proper media was added per well. Pictures were taken every 12 hours using an Inverted Polarized Light Microscope.

### 7. Statistical methods

All data are expressed as the mean value ± standard deviation. GraphPad Prism 7 software package was used for statistical analysis, and the One-way ANOVA were used for comparisons between 3 samples. $P < 0.05$ was considered statistically significant.

## III.RESULTS

### 1. Disparate cell morphology and the migration pattern of AM-1 were observed in a different culture condition.

Calcium is a fundamental intracellular signaling that regulates a variety of cellular functions. One of the most important functions is to control the cell movement and many previous research data supported this theory. Inhibition of $Ca^{2+}$ transients prevents adhesion disassembly, retraction, and cell movement. Human adherent neutrophils generate calcium spikes during migration while no calcium surges are observed when cells are stationary. A non-homogeneous $Ca^{2+}$ distribution caused flattening, polarization and crawling of eosinophils and accompanied neutrophil polarization. To explore the effects of different $Ca^{2+}$ concentrations on AM-1 cell, cell morphology and migration pattern in AM-1 cell that cultured in Keratinocyte serum free medium (KSFM) and Dulbecco's modified eagle's medium (DMEM) were detected. Under the KSFM culture condition, AM-1 cell had a small polygonal morphology with numerous thin filopodia protruding from the cell surface (Figure 5A). Under the DMEM culture condition, AM-1 cell shows a large flatted morphology with many lamellipodia that were found at the leading edge, and well-developed actin filament was formed numerous actin bundles (Figure 5B). To understand the relationship between $Ca^{2+}$ signaling and cell mobility, time-lapse images of the AM-1 cell were performed, which collected every 15 min for 8 hr by using confocal time-lapse imaging equipment after labeling with fluorescence dye. After cultured in 2 different media which contains a different concentration of $Ca^{2+}$, AM-1 shows different migration patterns. Under the KSFM condition, AM-1 cell lose their cell-cell adhesion and migrated individually, however, under the DMEM condition, it maintains the cell-cell adhesion and shows a

collective migration pattern (Figure 5C, D). Furthermore, each cell of the time-lapse images was tracked, and the total distances and average velocities of cells were calculated using an image analyzing software. The moving distance of AM-1 cell which was cultured in KSFM was significantly higher than the DMEM culture condition group (Figure 5E). Similarly, the average velocity of AM-1 cell which was cultured in KSFM was higher than the DMEM culture condition group (Figure 5F). Altogether, this result demonstrates that AM-1 cell shows disparate morphology and migration pattern in different concentration of $Ca^{2+}$ condition and it the $Ca^{2+}$ signaling may involve in the AM-1 cell migration and invasion process.

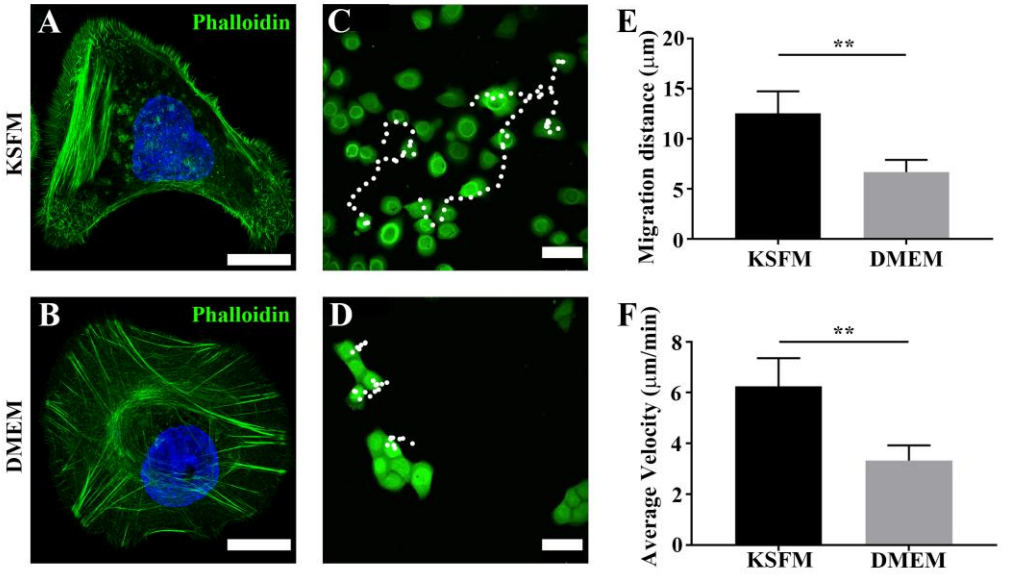



**Figure 5. Disparate cell morphology and migration pattern of AM-1 in different culture condition**

(A) Under the KSFM culture condition, AM-1 cells show a small polygonal morphology with numerous thin Filopodia protruding from the cell surface. (B) Under the DMEM culture condition, AM-1 cells show a large flatted morphology with many lamellipodia that were found at the leading edge, and well-developed actin filament was formed numerous actin bundle. (C-D) Time-lapse images of the AM-1 cells were collected every 15 min for 8 hr and the white dots line shows the trajectory of single-cell migration. AM-1 cells grown in KSFM migrate individually, and the range of movement is wider than DMEM culture condition. (E-F) Quantification of AM-1 cells moving distance and maximum velocity index. Scale bars; A-B, 20μm; C-D 50μm. $^{**}p < 0.01$.

**2. Cell-Cell adhesion was enhanced with the increase of $Ca^{2+}$ concentration.**

$CaCl_2$ is an inorganic compound, that clinically applicable for hypocalcemic tetany (abnormally low levels of calcium in the body that cause muscle spasm). To confirm the effect of $Ca^{2+}$ on cell-cell adhesion and migration pattern of AM-1 cells, $CaCl_2$ was added into the KSFM as a supplement of $Ca^{2+}$ ion. Then the AM-1 cell was cultured in the KSFM with or without $CaCl_2$ and time-lapse imaging was performed again on AM-1 cells, which collected the image every 15 min for 12 hr. The representative images of the individual migrating and collective migrating AM-1 cell were observed at several time points (Figure 6A-C). As a control group, the cell grown in KSFM which is in the low $Ca^{2+}$ condition, the locations of AM-1 cells in the same view were varied greatly, and individual cell migration was frequently observed during 12 hours of imaging. However, in the 0.6 mM and 1.2 mM calcium chloride treatment group, the locations of AM-1 cells in the same view were almost constant and collective migration was frequently observed. Quantification of moving distance and average velocity index also shows that the AM-1 cells which grown in KSFM move more broadly and quickly than the cell after calcium chloride treatment, however, there is no significant difference between the 0.6 mM and 1.2 mM calcium chloride treatment group (Figure 7A, B). Furthermore, cytoskeletons of the AM-1 cells were stained using phalloidin conjugated with fluorescence dye.

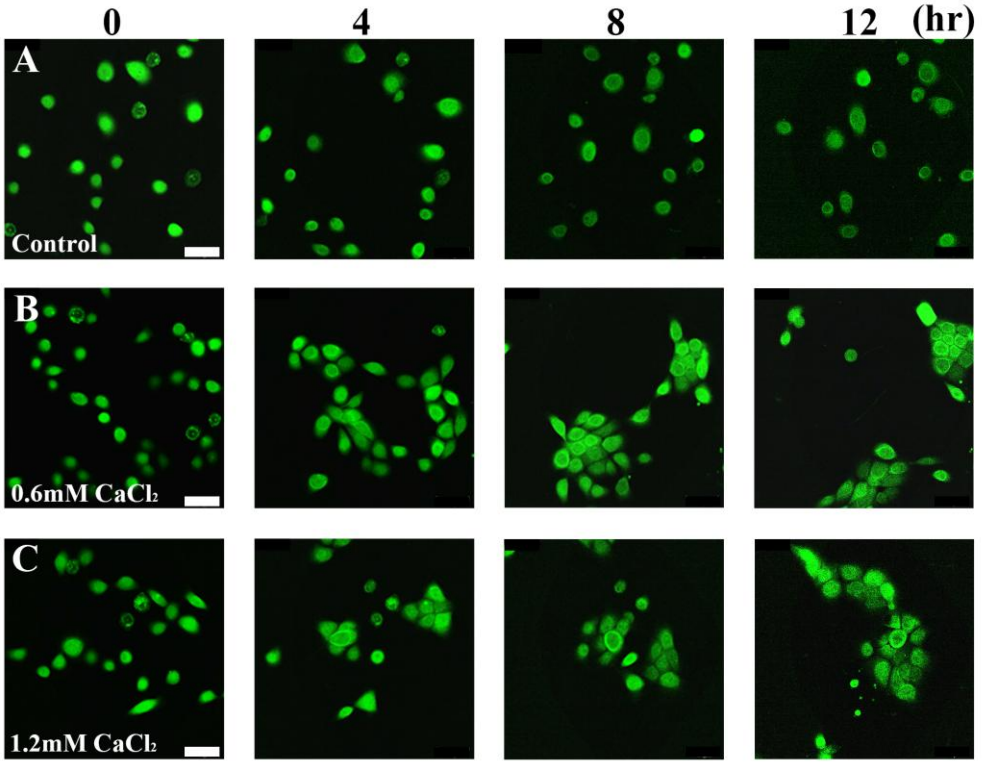



**Figure 6. The collective migration and motility of AM-1 enhanced with increasing of $Ca^{2+}$ concentration**

The representative time-lapse sequences of AM-1 cell cultured in 3 mediums with increasing $Ca^{2+}$ concentration. (A) The cells grown in KSFM migrate individually, and the locations of AM-1 cells in the same view were varied greatly. (B, C) In the 0.6 mM and 1.2 mM Calcium chloride treatment group, AM-1 cells migrate collectively as the Calcium chloride treatment time goes on, and the location of AM-1 cells in the same view is constant. Scale bars; A-C, 50μm.

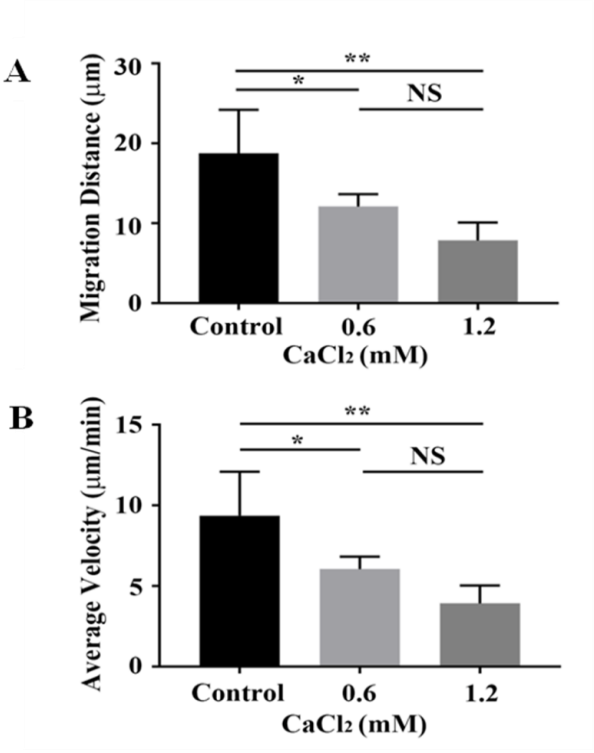



**Figure 7. Migration distance and average velocity of AM-1 depending on $Ca^{2+}$ concentration**

Time-lapse images of each cell were tracked every 15 min for 12 hr, and the migration distance (A) and average velocity (B) of cells were calculated by using an image analyzing software.

### 3. Collective migration was enhanced with the increase of $Ca^{2+}$ concentration.

Increasing level of cell-cell adhesion in the calcium chloride treatment group was observed (Figure 8A-C). Therefore, results suggested that $Ca^{2+}$ concentration was associated with AM-1 cell mobility and migration and cell-cell adhesion was enhanced by the increasing $Ca^{2+}$ concentration.

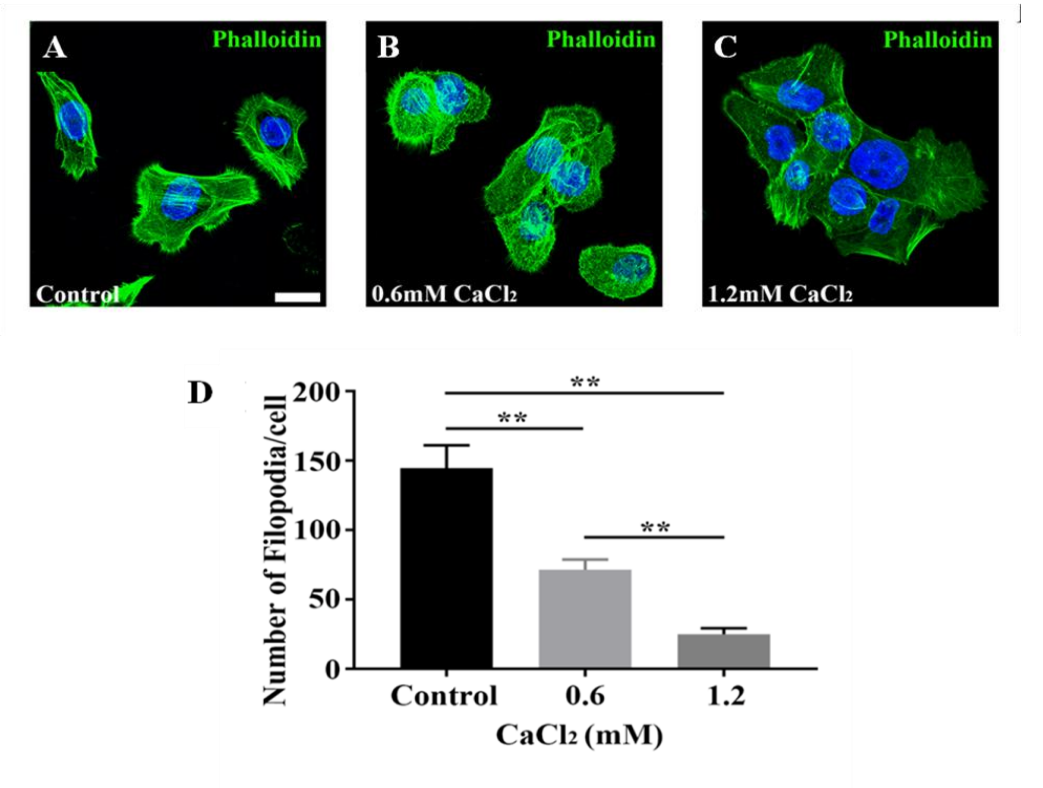



**Figure 8. Actin filament and filopodia formation depending on the $Ca^{2+}$ concentration**

(A-C) Cytoskeletons of the cells were stained using Phalloidin conjugated with fluorescence dye (green). Nuclei are counter stained with DAPI (blue). (D) Quantification of the number of filopodia per cells was calculated by image analyzing software. Scale bars; A-C, 50μm; *$p < 0.05$, **$p < 0.01$.

**4. Character of AM-1 cells was regulated by $Ca^{2+}$ pump inhibitor, not $Ca^{2+}$ sensing receptor inhibitor.**

Cell shape and cell locomotion have been shown to change as a result of chemoattractant-induced $Ca^{2+}$ release from internal stores (Gilbert *et al*., 1994). It was known to be regulated by different calcium ion channels which are expressed in the cell membrane. There are two kinds of regulators on the cell membrane to mediate intracellular calcium homeostasis: Calcium pump and Calcium sensing receptor. Calcium pumps are a family of ion transporters that are responsible for the active transport of calcium , pumping out of the cell for the maintenance of the steep $Ca^{2+}$ electrochemical gradient across the cell membrane. The calcium-sensing receptor (CaSR) is a Class C G-protein coupled receptor that senses extracellular levels of calcium ion and controls calcium homeostasis by regulating the release of parathyroid hormone. Two intracellular calcium regulatory inhibitors were selected: SKF96365 which is originally identified as a inhibitor of receptor-mediated calcium entry is widely used diagnostically, as a inhibitor of the transient receptor (Singh *et al*., 2010) and NPS2143 is a catalytic drug which acts as an antagonist at the Calcium-sensing receptor (CaSR) and consequently stimulates the release of parathyroid hormone. To determined which intracellular calcium regulator associated with the AM-1 cell-cell adhesion, SKF96365 and NPS2143 were added respectively into the DMEM and cytoskeleton was stained with phalloidin again. Compare with the control group (KSFM), cell-cell adhesion level was changed after SKF96365 treatment, however, NPS2143 seemingly has no significant effect on cell-cell adhesion of AM-1 cells (Figure 9A-D).

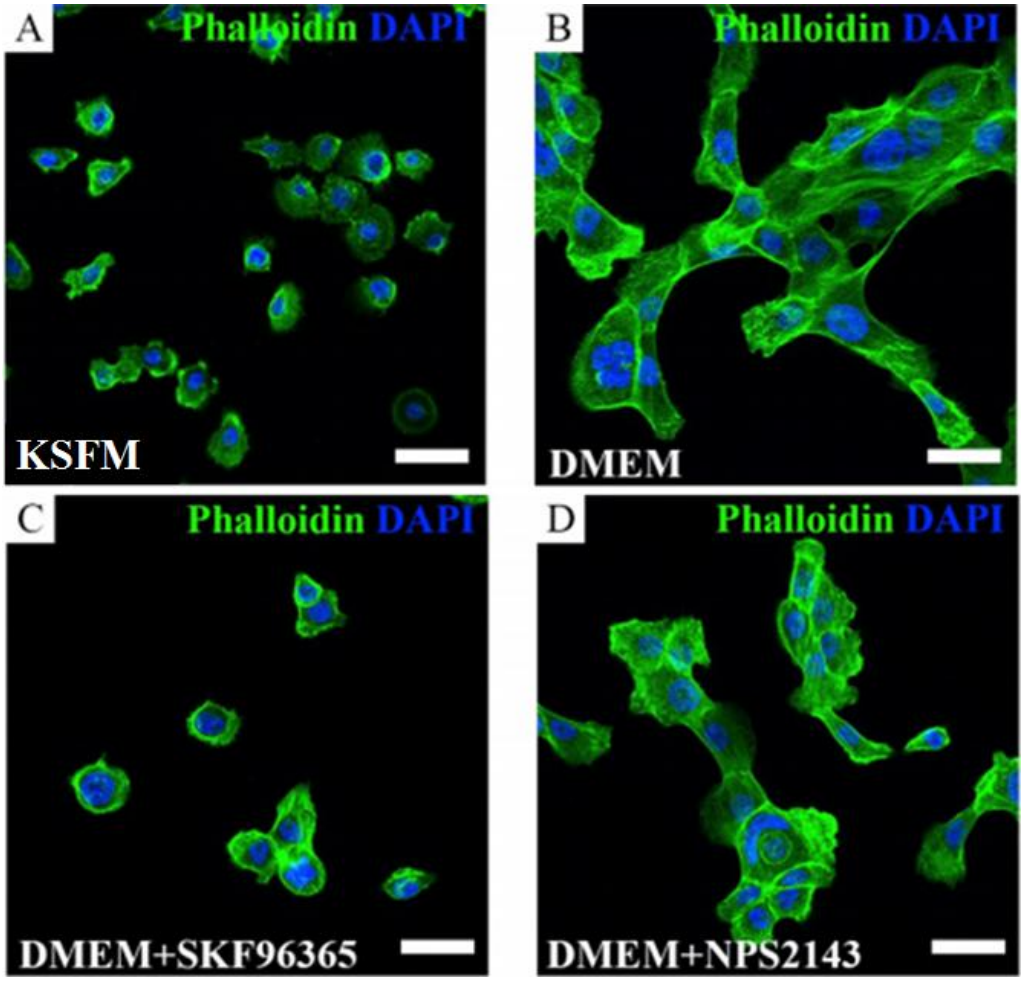



**Figure 9. Growth pattern of AM-1 regulated by $Ca^{2+}$ pump inhibitor or $Ca^{2+}$ sensing receptor inhibitor**

Cytoskeletons of AM-1 cells were stained with phalloidin conjugated with fluorescence dye (green). Nuclei are counterstained with DAPI (blue). (A) Numerous thin filopodia on the surface of the AM-1 cell membrane with small

polygonal morphology that grown separately from each other in KSFM condition. (B) AM-1 cells that grown in DMEM condition showed a large flatted morphology with lamellipodia and attached with each other. (C) After SKF96365 treatment on AM-1 cell that grown in DMEM conditions, the cell size was getting smaller, and the cell detached with other cells. (D) NPS2143 treatment on AM-1 cell that grown in DMEM condition, showed a little effect to the cell size and cell-cell adhesion. Scale bars; A-D, 50μm.

**5. Cell-Cell adhesion of AM-1 cell is suppressed by $Ca^{2+}$ pump inhibitor, not $Ca^{2+}$ sensing receptor inhibitor.**

Immunocytochemistry (ICC) was performed to confirm the expression level of cell-cell adhesion molecule E-cadherin and tight junction protein Zonula occludens-1 (ZO-1) in AM-1 cells after the intracellular regulatory inhibitor treatment. As a control group, in the KSFM culture condition, the E-cadherin and ZO-1 were rarely expressed in AM-1 cell even it almost contact with each other (Figure 10A). However, in the DMEM culture condition, AM-1 cells grown adherently to each other and E-cadherin and ZO-1 were expressed strongly at the junctional membrane (Figure 10B). After SKF96365 treatment, the expression levels of E-cadherin and ZO-1 were extremely decreased even in DMEM condition (Figure 10C). However, in the NPS2143 treatment group, E-cadherin and ZO-1 still could be detected in AM-1 cells membrane (Figure 10D). In summary, result suggested that the calcium pump plays a crucial role in the cell-cell adhesion of AM-1 cells, and the intracellular calcium regulatory inhibitor SKF96365 can efficiently inhibit the regulation of calcium pump on AM-1 cell-cell adhesion, while calcium-sensing receptor NPS2143 does not affect the regulation of AM-1 cell-cell adhesion.

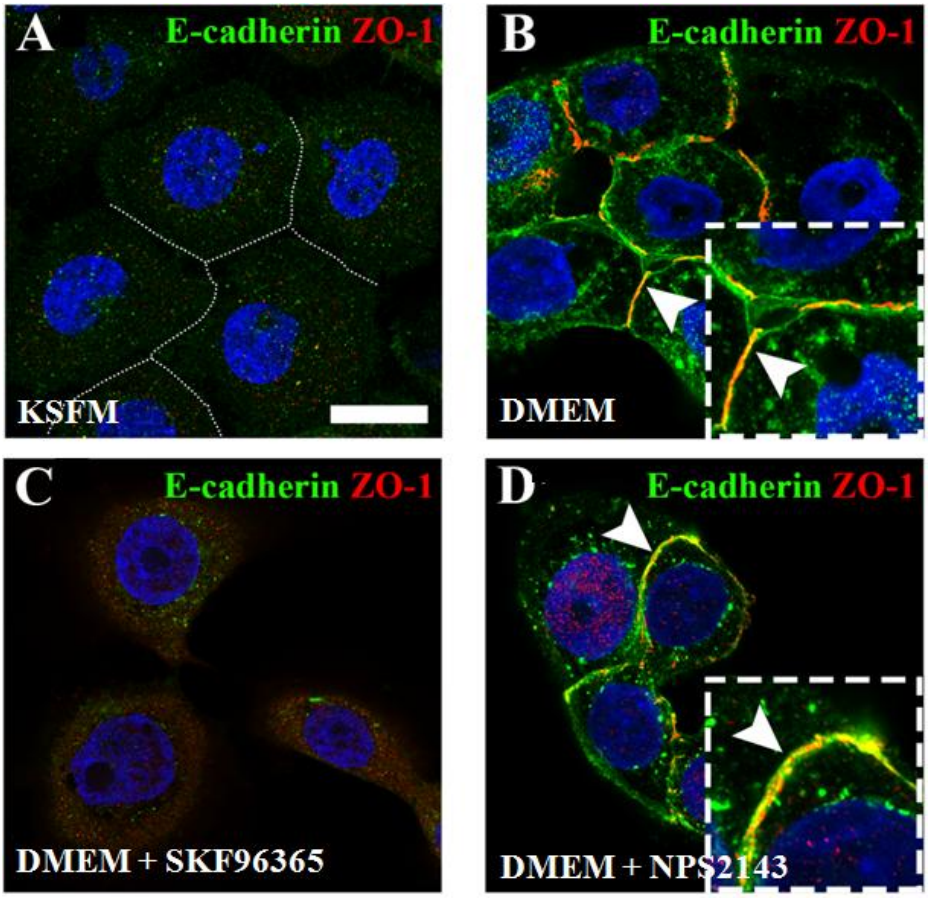



**Figure 10. Cell-Cell adhesion of AM-1 cell with $Ca^{2+}$ pump inhibitor or $Ca^{2+}$ sensing receptor inhibitor**

AM-1 cells were grown 4 different culture conditions respectively, which are KSFM, DMEM, DMEM added 25 mM $Ca^{2+}$ pump blocker (SKF96365) and DMEM added 25 mM $Ca^{2+}$ sensing receptor inhibitor (NPS2143) (A-D). AM-1 cells were subjected to ICC with anti E-cadherin antibody (green) and anti ZO-1 antibody (red). (A) E-cadherin and ZO-1 were not detected in the cell

membrane in KSFM condition group. (B) In DMEM group, E-cadherin and ZO-1 were strongly expressed in junctional region of cell membrane. (C) After SKF96365 treatment, the expressions of E-cadherin and ZO-1 were significantly reduced. (D) The expressions of E-cadherin and ZO-1 were not reduced after NPS2143 treatment. Scale bars; A-D, 10μm.

### 6. Aspect of collective migration under $Ca^{2+}$ and/or $Ca^{2+}$ pump inhibitor.

It already confirmed that cell-cell adhesion and collective migration of AM-1 cell were induced by $Ca^{2+}$ concentration. And it also illustrated that cell-cell adhesion could be suppressed by intracellular calcium regulatory inhibitor SKF96365 which inhibit the calcium pump which on the AM-1 cell membrane. Next, to demonstrate the relationship between $Ca^{2+}$ induced collective migration and $Ca^{2+}$ pump inhibitor SKF96365, 12.5 M SKF96365 was added to 0.6 mM calcium chloride treated AM-1 cell, and time-lapse imaging was performed again. The representative images of several time points suggest that in the control group (Figure 11A), the locations of AM-1 cells in the same view were varied greatly, and individual cell migration was frequently observed during 12 hours of imaging. However, in the 0.6 mM calcium chloride treatment group, the locations of AM-1 cells in the same view were almost constant and collective migration was frequently observed (Figure 11B). Interestingly, when after added SKF96365 to calcium chloride treated group, the collective migration was disassembled and some of AM-1 cells detached with other cells and migrated individually (Figure 11C). Quantification of moving distance and average velocity index also shows that the AM-1 cell which grown in KSFM move more broadly and quickly than the cells in calcium chloride treatment group (Figure 12A, B). However, in SKF96365 treated group, the moving distance and average velocity of AM-1 cells were almost equal to control group (KSFM).

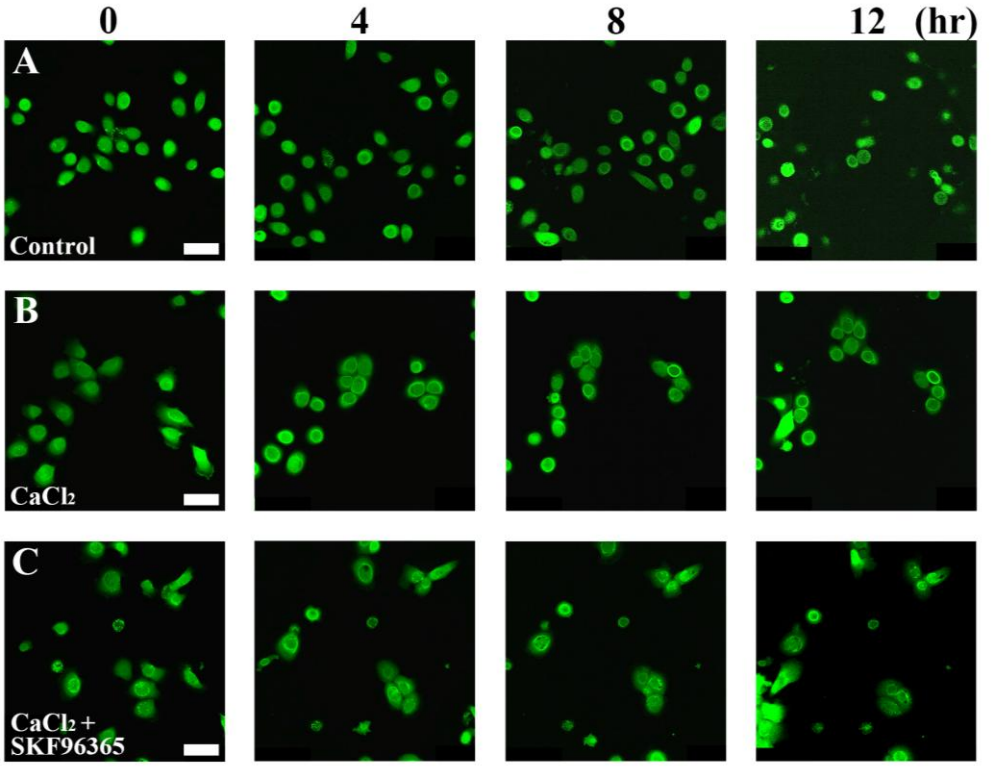



**Figure 11. $Ca^{2+}$ induced collective migration and Cell-Cell adhesion could be suppressed by $Ca^{2+}$ pump inhibitor**

Time-lapse imaging were performed on fluorescence dye (green) labeled cells for 12 hr every 10 min. (A) The AM-1 cell grown in KSFM, showed a individual migration pattern. (B) After added exogenous $Ca^{2+}$ into the KSFM, the cells migrated collectively. (C) The cells added exogenous $Ca^{2+}$ that treat with SKF96365 showed the collective migration was disassembled. Scale bars; A-C, 50μm.

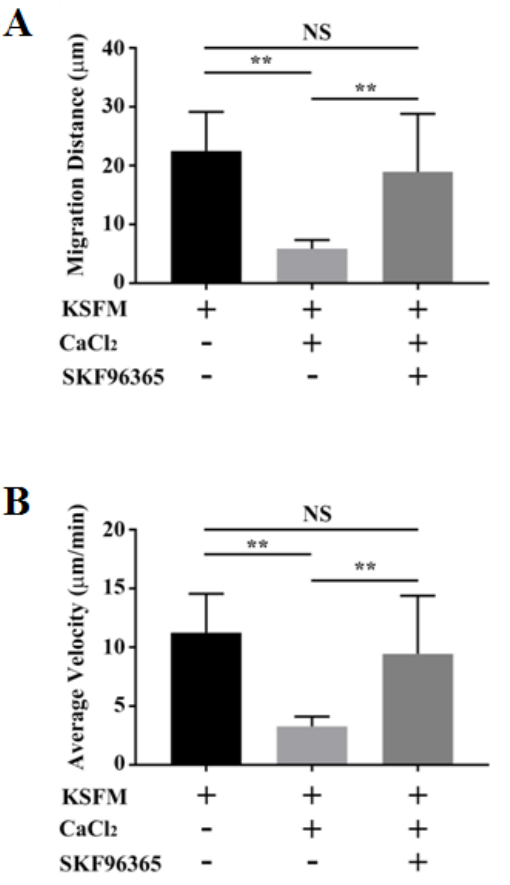



**Figure 12. Migration distance and average velocity of AM-1 with $Ca^{2+}$ and/or $Ca^{2+}$ pump inhibitor**

Time-lapse images of each cell the were tracked every 10 min for 12 hr, and the distance (A) and average velocity (B) of cells were calculated using an image analyzing software. Migration distance and velocity of AM-1 cells were decreased with $Ca^{2+}$ concentration. With $Ca^{2+}$ pump inhibitor, migration pattern of AM-1 cells was rescued.

### 7. Cell-Cell adhesion with $Ca^{2+}$ and/or $Ca^{2+}$ pump inhibitor.

Immunocytochemistry result also shows that to compare with control group (Figure 13A), the E-cadherin and ZO-1 expressed strongly at the edge of junctional membrane of AM-1 cells in calcium chloride treatment group (Figure 13B). However, after added SKF96365, the E-cadherin and ZO-1 were rarely expressed in AM-1 cells (Figure 13C). Altogether, the collective migration and cell-cell adhesion level could be induced by $Ca^{2+}$ signaling, however, the calcium pump inhibitor SKF96365 is sufficient to suppress the $Ca^{2+}$ signaling to dismissed the cell-cell adhesion and collective migration of the AM-1 cells.

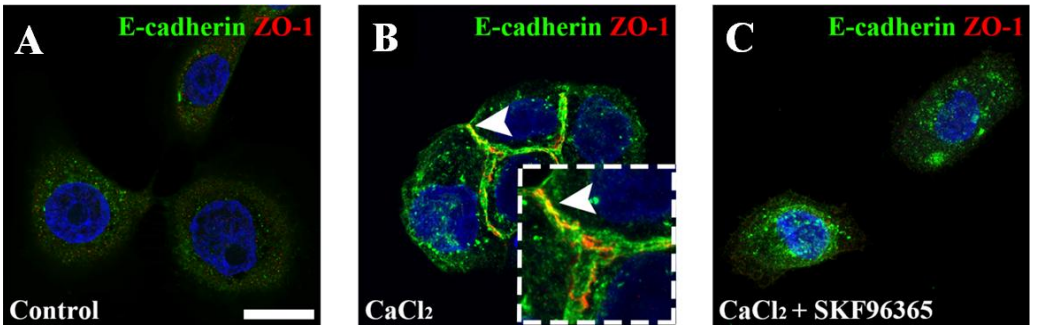



**Figure 13. Cell-Cell adhesion could be regulated by $Ca^{2+}$ or $Ca^{2+}$ pump inhibitor**

AM-1 cells were subjected to ICC with anti E-cadherin antibody (green) and anti ZO-1 antibody (red). (A) No valid signal was detected in KSFM condition. (B) E-cadherin and ZO-1 were expressed in the junctional region of the AM-1 cell membrane at the condition of exogenous $Ca^{2+}$ added in the KSFM. Arrowhead indicates the co-localization region of E-cadherin and ZO-1 protein which was shown clearly in the high magnification image of the white dot line box. (C) After SKF96365 treatment, the E-cadherin and ZO-1 expressions were significantly reduced. Scale bars; A-C, 10μm.

### 8. $Ca^{2+}$ signaling induced collective invasion of AM-1 cells in a three-dimensional culture system.

To investigate the role of $Ca^{2+}$ signaling in AM-1 invasion pattern, spheroid invasion assay was performed in a three-dimensional culture system. AM-1 cells were seeded on the Ultra-low attachment 96 well and incubated for 48 hr. After spheroid made formation, it was embedded into 4mg/ml collagen type I gel on the 2 mg/ml PolyHema coated 35mm dish and solidified in 37 ℃ incubator for 1 hr. Time-lapse images of AM-1 spheroid that labeled with red fluorescence dye were collected every 15 min for 72 hr in the 3 different culture condition. In the control group, the spheroid shows very low level of expansive growth (Figure 14A, A'). The $CaCl_2$ treatment group shows an aggressive collective invasion pattern (Figure 14B, B'). However, the AM-1 spheroid treated $CaCl_2$ with SKF96365 group shows a low levels of expansive growth similar with control group (Figure 14C, C').

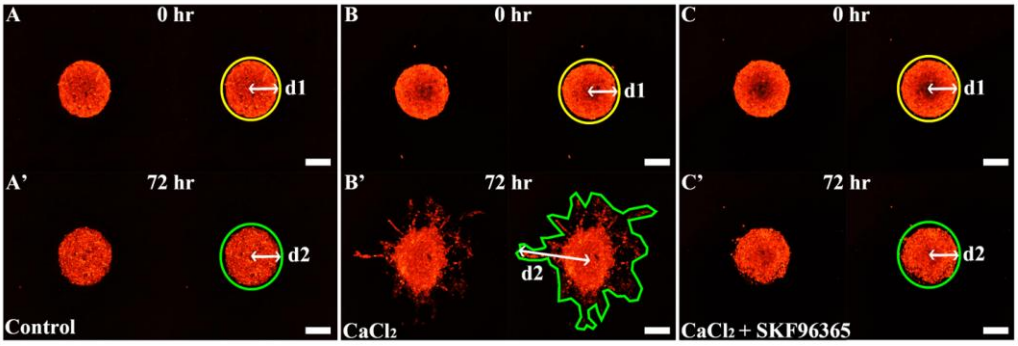



**Figure 14. $Ca^{2+}$ signaling induced collective invasion of AM-1 cells in a three-dimensional culture system**

AM-1 spheroids were embedded into the collagen I matrix and cultured in 3 different culture conditions respectively, which are KSFM, KSFM added 1.2 mM $CaCl_2$, KSFM added 1.2 mM $CaCl_2$ with 25 mM $Ca^{2+}$ pump blocker (SKF96365). Representative images of initial AM-1 spheroids (A-C) and expanded spheroids (A'-C'), along with their corresponding binarized images which were labeled with fluorescence dye (red) were collected every 15 min for 72 hr by using confocal time-lapse imaging equipment. Decomposition of the binarized image was performed into two components: spheroid core (yellow circle) and edging cells (green circle). Illustration of the parameters used for global measurements : total distance of cell invasion starting from the spheroid center (d1) or border (d2). Comparison of global measurements at t = 0 and t = 72 hr. Scale bars: 100 μm.

### 9. The invading distance and invasion area of AM-1 spheroids.

The invading distance and invasion area of AM-1 spheroids cultured in $CaCl_2$ treatment group were dominantly higher than other group (Figure 15A, B). Altogether, this result indicated that $Ca^{2+}$ signaling was sufficient to induce collective invasion of AM-1 spheroid and it can be suppressed by $Ca^{2+}$ pump inhibitor SKF96365.

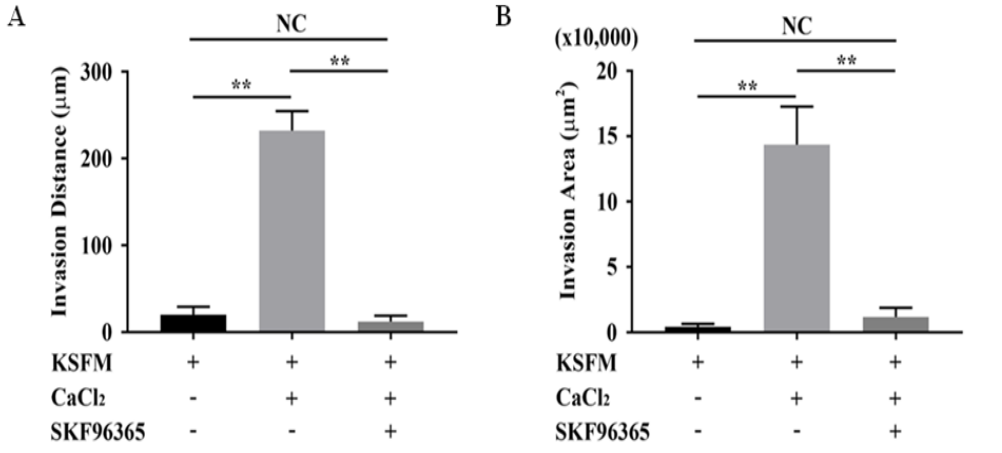



**Figure 15. $Ca^{2+}$ signaling induced invasion distance and areas of AM-1 cells in a three-dimensional culture system**

AM-1 spheroids were embedded into the collagen I matrix and cultured in 3 different culture conditions respectively, which are KSFM, KSFM added 1.2 mM $CaCl_2$, KSFM added 1.2 mM $CaCl_2$ with 25 mM $Ca^{2+}$ pump blocker (SKF96365). Graph representing the AM-1 cells invading distance (A) and invasion areas (B). $^{**}p < 0.01$.

## IV. DISCUSSION

Ameloblastoma was classified into benign odontogenic tumors of epithelial origin by the World Health Organization (El-Naggar, 2017), however, its distinct invasive growth pattern is more similar to the malignant tumor. In previous study, various pathological images of all types of ameloblastoma demonstrate that the cells invade collectively into surrounding tissues without losing the cell-cell adhesions and maintains the epithelial cell characteristic (Hao *et al*., 2018). EMT-related proteins, such as E-cadherin, β-catenin, and Vimentin are involved in the occurrence and development of ameloblastoma, so they can serve as biomarkers for tumor invasion. Expression of Vimentin occurred in the epithelium with the recurrence and malignant transition, accompanied by reduced E-cadherin. Down-regulated expression or dysfunction of E-cadherin in tumor cells can cause destruction of the epithelium and acquire the ability to migrate from the primary site, which can lead to invasiveness and metastasis of tumor cells. Nevertheless, expression of E-cadherin in epithelial hyperplasia and benign tumor is similar to that in normal tissues, and this results suggest the preservation of epithelial adhesion. Results of marker expression at the tissue and cell level confirmed the presence of EMT in ameloblastoma, which may play an important role in the invasive growth of ameloblastoma. Other previous attempts to clarify the mechanism of ameloblastoma cell migration and invasion that controll invasive signaling pathway, receptor-ligand interaction and protease-substrate interaction have largely failed (Florescu *et al*., 2012; Anne *et al*., 2014). An important genetics study on the ameloblastoma deepened understanding of its etiopathogenesis (Kurppa *et al*., 2014), however, it is not enough to clarify that showing an aggressive growth pattern as a benign neoplasm.

For this study of ameloblastoma, an immortalized cell line is needed to replace the primary cell. Primary cells have a limited lifespan because of the shortening of chromosomal telomeres accompanied with each cell division. After about 20 to 60 times doubling, telomeres become too short to withstand another cell cycle, resulting in termination of cell division. This phenomenon is called Hayflick limit (Hayflick, 1965). To have an infinite doubling capacity of cell line, it must be immortalized through transformation using viral or chemical induction methods. Viral genetic engineering introduces viral genes (e.g. SV-40, E6, E7) that efficiently promote proliferation and allow cells to undergo infinite cell division. Likewise, chemical induction methods depend on carcinogens (e.g., ionizing radiation, nickel chloride, benzopyrene) to alter the genetic makeup of primary cells to favor unlimited proliferation.

Immortalized cell lines are often used for research instead of primary cells (Kaur and Dufour, 2012). They are cost-effective, easy to use, offer unlimited supplies of materials and offer a number of benefits including bypassing ethical issues related to the use of animal and human tissues. Immortalized cell lines also provide a pure cell population, which is important because it provides consistent samples and reproducible results. Immortalized cell lines should display and maintain functional characteristics as close to the primary cell as possible. But, cell lines are genetically engineered, they can change phenotype, intrinsic function, and responsiveness to stimuli. Serial passage of cell lines can cause genotypic and phenotypic variation over an extended period of time and genetic drift can also lead to heterogeneity in culture. Thus, cell lines may not properly represent primary cells and may give different results (Table 1).

Each ameloblastoma cell lines also show different invasion patterns depending on the culture environment. Without fibroblasts, AM-1 cells are sharp and form

an invasive process similar to plexiform, while AM-3 cells form a series of blunt processes often observed during collective migration (Fuchigami *et al*., 2017). In comparison, under co-culture with HFF-2 fibroblasts, AM-3 cells formed a tuft-like invasive process and invaded collectively into outer layer more than observed in AM-1 cells. In addition, HFF-2 fibroblasts were localized to the tip of the invasive tumor process. These results suggest that tumor-associated cells assist invasion of tumor cells. However, comparative studies on AM cell lines are needed in the future.

| **Cell characteristics** | **Primary cells** | **Spontaneously immortalized cells** | **Artificially immortalized cells** |
|---|---|---|---|
| **Immortalization** | None | Occurs only after multiple passages | Possible at low passage number |
| **Genetic background** | Close to *in vivo* situation | Artificial (severe changes can occur upon immortalization) | Artificial (severe changes can occur upon immortalization) |
| **Proliferative capacity** | Mostly low | High | High |
| **Life span** | Limited, finite | Not limited, infinite | Not limited, infinite |
| **Biological properties** | Close to *in vivo* situation | Dedifferentiation, more distant from *in vivo* situation | Dedifferentiation depends on immortalization methods |
| | | Selection of distinct cell types difficult | Selection of distinct cell types possible, conditional immortalization strategy feasible |
| **Preferred culture medium** | Specialized medium (expensive) | Standard medium | Standard medium |
| **Properties of cell population** | Close to *in vivo* cell types | Loss of distinct cell types during subcultivation | Selection of distinct cell types possible |
| **Standardization and reproducibility** | Limited cell number, standardization only possible for some cell types | Standardization possible due to unlimited amount of cells | Standardization possible due to unlimited amount of cells |

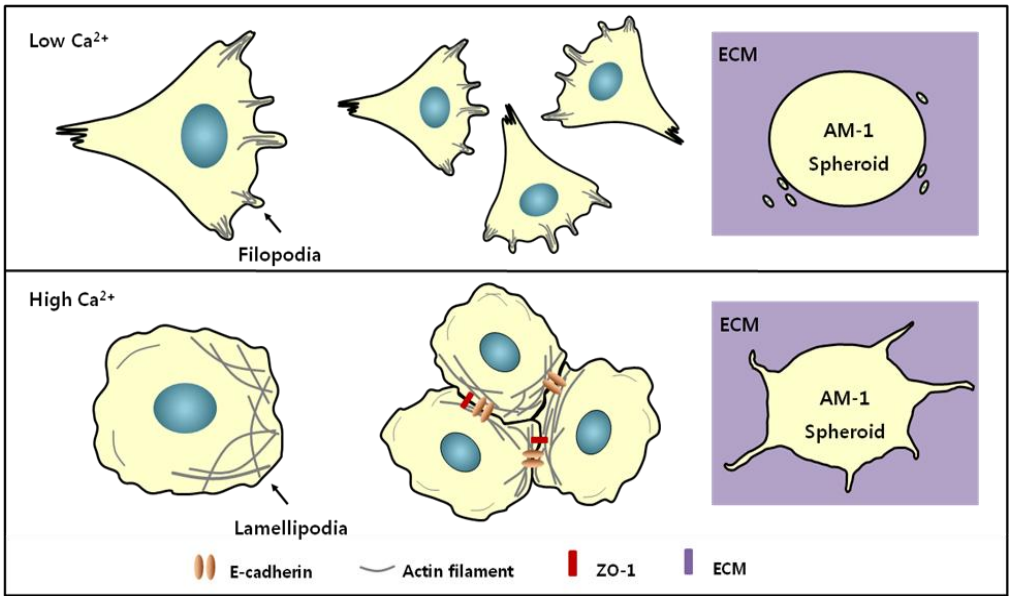
(Eckerle *et al.*, 2014)

**Table 1. Advantages and disadvantages of primary vs. spontaneously vs. artificially immortalized cell culture**

Immortalized cell lines are cost-effective, easy to use, offer unlimited supplies of materials and offer a number of benefits including bypassing ethical issues related to the use of animal and human tissues. Immortalized cell lines also provide consistent samples and reproducible results *in vitro*.

Ameloblastoma cell line 1 (AM-1) shows a disparate morphology and migration pattern in different culture media which are Keratinocyte serum-free medium and Dulbecco's modified eagle's medium. The biggest difference between these two mediums is $Ca^{2+}$ concentration. As the most abundant second messenger in the human body, intracellular calcium ions have a substantial diversity of roles in fundamental cellular physiology, including gene expression, cell cycle control, cell motility, autophagy, and apoptosis (Cui *et al*., 2017). Based on result, hypothesis is that $Ca^{2+}$ signaling may be responsible for collective migration of AM-1 cell.

Next, to confirm hypothesis, adding exogenous $Ca^{2+}$ into the culture medium shows result that exogenous $Ca^{2+}$ could induce the collective migration in the AM-1 cells. Moreover, exogenous $Ca^{2+}$ also reduces the number of filopodia which are actin-rich finger-like protrusions that extend from the plasma membrane and have been implicated in cell migration and invasion both *in vitro* and *in vivo*. In breast cancer, L-type $Ca^{2+}$ channels promote filopodia stability through the spatially restricted regulation of calcium entry (Jacquemet *et al*., 2016). This result indicates that mobility of the AM-1 cell was declined in high $Ca^{2+}$ concentration and the migration pattern was changed to collectively (Figure 16).




| **Collective invasion of ameloblastoma ($Ca^{2+}$↑)** | |
|---|---|
| **Morphology** | Large, Flat, Filopodia ↓ |
| **Migration pattern** | Collectively, Slowly |
| **Adhesion** | E-cadherin ↑, ZO-1 ↑ |

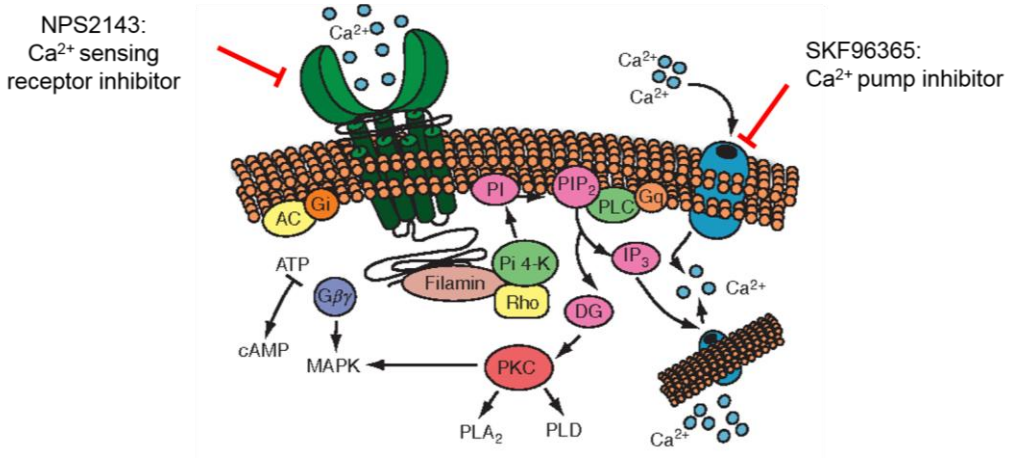
**Figure 16. Schematic representation of the $Ca^{2+}$ signal induced alternation of AM-1 cell morphologies, cell-cell adhesion, and invasion pattern**

At low $Ca^{2+}$ concentration condition, AM-1 cell shows a polygonal morphology with numerous thin filopodia protruding from the plasma membrane. The junction protein was rarely expressed and cells detached with each other. The spheroid of AM-1 cell shows very low levels of expansive

growth in the extracellular matrix. At high $Ca^{2+}$ concentration condition, AM-1 cell shows a flatted morphology with many lamellipodia found at the leading edge, and well-developed actin filament was aggregated to numerous actin bundles. The junction protein (E-cadherin, ZO-1) was strongly expressed in the plasma membrane, and cells adherent to each other. The spheroid of AM-1 cells became more aggressive and started high collective strand invasion in the extracellular matrix.

SKF96365, originally identified as a inhibitor of receptor-mediated calcium entry, is widely used diagnostically as a inhibitor of transient receptor potential canonical type (TRPC) channels (Singh *et al*., 2010). $Ca^{2+}$ influx in human uterine epithelial cells triggers adhesiveness for trophoblast-like cells, however, it could be suppressed by SKF96365 (Tinel *et al*., 2000). Here, $Ca^{2+}$ pump inhibitor SKF96365 and $Ca^{2+}$ sensing receptor inhibitor NPS2143 are used to check the effect on cell-cell adhesion level (Figure 17). The result shows that $Ca^{2+}$ pump inhibitor SKF96365 could suppress $Ca^{2+}$ induced cell-cell adhesion. E-cadherin is the representative cell-cell junctional molecular and ZO-1 is a tight junction protein that expressed in the plasma membrane. It further can be demonstrated that SKF96365 is effective on down-regulation of the expression of E-cadherin and ZO-1 in AM-1 cells. The result indicated that $Ca^{2+}$ signaling may regulate the AM-1 cell-cell adhesion *via* $Ca^{2+}$ pump, not $Ca^{2+}$ sensing receptor.

Moreover, it can be demonstrated that $Ca^{2+}$ induced collective migration also can be suppressed by SKF96365. Store-operated $Ca^{2+}$ entry regulates glioma cell migration and invasion, however, it could be suppressed by SKF96365 (Zhu *et al*., 2014). After adding exogenous $Ca^{2+}$, the AM-1 cell shows a collective migration pattern and strong expression of E-cadherin and ZO-1 compare with the control group. However, SKF96365 could effectively change the migration pattern and the expression of junctional molecular. The result indicated that the $Ca^{2+}$ induced collective migration and cell-cell adhesion could be suppressed effectively by SKF96365.




(Hendy *et al*., 2018)

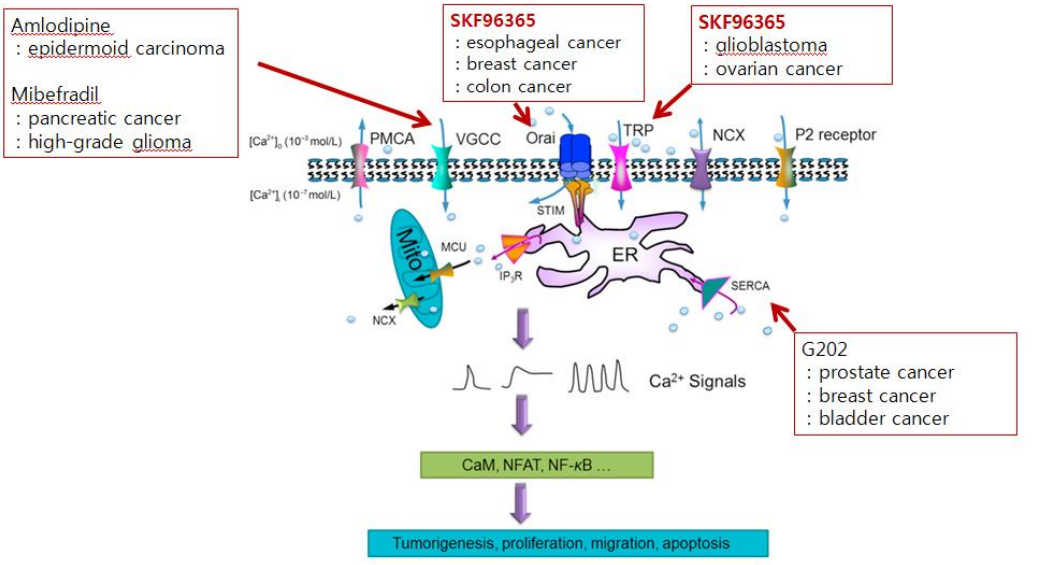
**Figure 17. $Ca^{2+}$ pump inhibitor SKF96365 and $Ca^{2+}$ sensing receptor inhibitor NPS2143**

Both the calcium signaling and calcium ion influx were inhibited in this study. NPS2143 inhibits $Ca^{2+}$ sensing receptor of cell membrane. NPS2143 blocks the following signal transduction of $Ca^{2+}$ sensing receptor. SKF96365 blocks the $Ca^{2+}$ pump of the cell membrane. Through SKF96365, the influx of calcium ion is blocked and concentration of calcium ion decreases in the cytosol.

It's becoming clear that $Ca^{2+}$ channels, $Ca^{2+}$ transporters and $Ca^{2+}$ pumps are involved in a wide range of tumorigenesis (Cui *et al*., 2017). This relatively new field has already made an important contribution to the identification of possible chemotherapeutic agents for a certain number of cancers, some of which have been transferred to clinical trials.

The imidazole compounds SKF-96365 and related antifungal compounds can inhibit $Ca^{2+}$ release-activated $Ca^{2+}$ channel (CRAC) and some transient receptor potential channels (TRPC). SKF-96365 was initially described as blocking receptor-mediated $Ca^{2+}$ entry in human platelets, neutrophils and endothelial cells, but was later used to suppress ovarian cancer cell growth and tumor formation by reducing the activity of different subtypes of TRPCs. Treatment of SKF-96365 has been reported to improve radiation sensitization in glioblastoma containing high expression levels of TRPC6 channels. SKF-96365 also enhances the reverse mode of $Na^{+}/Ca^{2+}$ exchanger 1 (NCX1) and regardless of TRPC, which can induce cell cycle arrest at the S and G2 stages of glioblastoma cells. Moreover, as an inhibitor of Orai1 channel, SKF-96365 can inhibit breast cancer cell migration *in vitro* and reduce tumor growth and metastasis *in vivo* (Yang *et al*., 2009). SKF-96365 inhibited Orai1-mediated store-operated $Ca^{2+}$ entry (SOCE) and intracellular $Ca^{2+}$ oscillations in esophageal cancer cells and resulted in significant reduced tumor growth in nude mice (Cui *et al*., 2017). In addition, voltage calcium channel blocks such as amlodipine and mibefradil are used as anticancer drugs (Figure 18).

Based on this trend, if a therapeutic candidate is established as a target for calcium signaling in ameloblastoma in the future, it can be very helpful for the conservative treatment of ameloblastoma.




(Cui *et al*., 2017)

**Figure 18. Schematic representation of targeting calcium signaling in cancer therapy**

Calcium channel inhibitors are already used as targeted therapeutics for different calcium channels in many cancers. SKF96365 is also used as a therapeutic agent in esophageal cancer, breast cancer, colon cancer, glioblastoma and ovarian cancer targeting ORI channels and TRP channels.

## V. CONCLUSION

This study demonstrated the differential migration pattern and morphology of AM-1 according to different culture condition. Intercellular adhesion and collective migration was enhanced with the increase of $Ca^{2+}$ concentration. On the other hand, intercellular adhesion and collective migration could be suppressed by $Ca^{2+}$ pump inhibitor.

Therefore, calcium pump may be a potential therapeutic candidate target for reducing collective invasion of ameloblastoma. However, it can not be clarified the mechanism of $Ca^{2+}$ pump inhibitor in the collective migration and invasion process of ameloblastoma. As such, it is necessary to identify the downstream molecular that influence the collective migration and invasion of ameloblastoma particularly with respect to $Ca^{2+}$ pump associated signaling pathway in order to develop effective therapies to treat this disease in the future.

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**ABSTRACT** (In Korean)

# 법랑모세포종의 collective migration and invasion 에서 칼슘 이온의 역할

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Collective invasion 에 대한 연구는 암의 보존적 치료와 장기 예후에 매우 중요하다. 특히, 악성 구강암 및 법랑모세포종에서는 유방암 또는 폐암 등의 다른 암과 비교하여 칼슘과 collective invasion 사이의 명확한 기전이 알려져 있지 않아 추가 연구가 필요하다. 따라서, 본 연구의 목표는 법랑모세포종에서 collective invasion 과정에 관여하는 $Ca^{2+}$ 신호의 역할을 연구하는 것이다.

본 연구에서는 인간에서 유래된 Immortalized ameloblastoma cell line (AM-1)을 사용 하였다. $CaCl_2$, Calcium pump inhibitor (SKF96365) 및 Calcium sensing receptor inhibitor (NPS2143)을 AM-1 배양 배지에 첨가하여 세포 내 $Ca^{2+}$ 농도를 제어 하였다. $Ca^{2+}$ 신호의 제어 하에 AM-1 세포의 세포 형태, 이동 및 침습 패턴을 분석하기 위해 Time-lapse imaging, Tumor spheroid invasion collagen assay, Immunofluorescence staining 을 사용 하였다.

AM-1 세포는 $Ca^{2+}$ 농도와 관련된 각기 다른 배양 조건에서 상이한 세포 형태 및 이동 패턴을 보여 주었다. 그리고 AM-1 세포는 $Ca^{2+}$의 농도가 증가함에 따라 서로 더 밀접하게 부착되고 collective migration 의 패턴을 보였다. 그러나 $Ca^{2+}$ 농도를 제어 했을 때, NPS2143 이 아닌 SKF96365 에 의해 $Ca^{2+}$ 유도 cell-cell adhesion 이 억제되었다. 또한, SKF96365 에 의해 AM-1 세포의 $Ca^{2+}$ 유도 collective invasion 도 억제되었다. 최종적으로, Calcium pump inhibitor 는 AM-1 세포의 cell-cell adhesion 과 collective invasion 을 억제하는 역할을 하였다. 따라서, 세포막의 칼슘 펌프는 법랑모세포종의 collective invasion 을 억제하기 위한 잠재적인 치료 표적이 될 것으로 결론 지을 수 있다.

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핵심되는 말: 법랑모세포종, collective invasion, $Ca^{2+}$ signaling, SKF96365