Enhanced Basal Autophagy Supports Ameloblastoma-Derived Cell Survival and Reactivation

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Abstract
Objectives: Ameloblastoma is an aggressive odontogenic jaw neoplasm. Its unlimited growth confers high potential for malignant transformation and recurrence. It is unclear why ameloblastoma is highly recurrent despite surgical resection with a wide margin of normal tissue. While canonical autophagy can be used to degrade and eliminate damaged cellular components, it is also a protective mechanism that provides energy and vital metabolites for cell survival. We used ameloblastoma-derived cells to test the hypothesis that autophagic processes play a role in survival and reactivation of ameloblastoma. Methods: Primary epithelial (EP-AMCs) and mesenchymal (MS-AMCs) ameloblastoma-derived cells were established from tissue samples of solid multicystic ameloblastoma. Clonogenic capacity and basal autophagic capacity were assessed in ameloblastoma-derived cells relative to human odontoma-derived cells (HODCs) and maxilla-mesenchymal stem cells (MX-MSCs). Ability of ameloblastoma-derived cells to survive and form new ameloblastoma was assessed in mouse tumor xenografts. Results: EP-AMCs were highly clonogenic (p < 0.0001) and demonstrated enhanced basal levels of autophagic proteins microtubule-associated protein 1-light chain 3 (LC3) (p < 0.01), p62 (Sequestosome 1, SQSTM1) (p < 0.01), and the LC3-adapter, melanoregulin (MREG) (p < 0.05) relative to controls. EP-AMCs xenografts regenerated solid ameloblastoma-like tumor with histological features of columnar ameloblast-like cells, loose stellate reticulum-like cells and regions of cystic degeneration characteristic of follicular variant of solid multicystic ameloblastoma. The xenografts also displayed stromal epithelial invaginations strongly reactive to LC3 and p62 suggestive of epithelial-mesenchymal transition and neoplastic odontogenic epithelium. Conclusions: EP-AMCs exhibit altered autophagic processes that can support survival and recurrence of post-surgical ameloblastoma cells. © 2018 Elsevier Ltd

Keywords
Ameloblastoma; Autophagy; Recurrence; Survival

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Enhanced basal autophagy supports ameloblastoma-derived cell survival and reactivation

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ABSTRACT

Objectives: Ameloblastoma is an aggressive odontogenic jaw neoplasm. Its unlimited growth confers high potential for malignant transformation and recurrence. It is unclear why ameloblastoma is highly recurrent despite surgical resection with a wide margin of normal tissue. While canonical autophagy can be used to degrade and eliminate damaged cellular components, it is also a protective mechanism that provides energy and vital metabolites for cell survival. We used ameloblastoma-derived cells to test the hypothesis that autophagic processes play a role in survival and reactivation of ameloblastoma.

Methods: Primary epithelial (EP-AMCs) and mesenchymal (MS-AMCs) ameloblastoma-derived cells were established from tissue samples of solid multicystic ameloblastoma. Clonogenic capacity and basal autophagic capacity were assessed in ameloblastoma-derived cells relative to human odontoma-derived cells (HODCs) and maxilla-mesenchymal stem cells (MX-MSCs). Ability of ameloblastoma-derived cells to survive and form new ameloblastoma was assessed in mouse tumor xenografts.

Results: EP-AMCs were highly clonogenic (p < 0.0001) and demonstrated enhanced basal levels of autophagic proteins microtubule-associated protein 1-light chain 3 (LC3) (p < 0.01), p62 (Sequestosome 1, SQSTM1) (p < 0.01), and the LC3-adapter, melanoregulin (MREG) (p < 0.05) relative to controls. EP-AMCs xenografts regenerated solid ameloblastoma-like tumor with histological features of columnar ameloblast-like cells, loose stellate reticulum-like cells and regions of cystic degeneration characteristic of follicular variant of solid multicystic ameloblastoma. The xenografts also displayed stromal epithelial invaginations strongly reactive to LC3 and p62 suggestive of epithelial-mesenchymal transition and neoplastic odontogenic epithelium.

Conclusions: EP-AMCs exhibit altered autophagic processes that can support survival and recurrence of postsurgical ameloblastoma cells.

1. Introduction

Ameloblastoma is the most common odontogenic tumor in sub-Saharan Africa (Ogundana, Effiom, & Odukoya, 2017) while odontoma formerly regarded as a dental hamartomatous tissue (El-Naggar, 2017) is the most common odontogenic tumor among Caucasians. However, ameloblastoma is the most aggressive of all odontogenic tumors (Bassey, Ounde, & Anyanechi, 2014). Ameloblastoma arises from odontogenic epithelium within a mature fibrous stroma that is devoid of odontogenic ectomesenchyme (Sciubba, Eversole, & S lootweg, 2005). The unlimited growth of ameloblastoma confers a high potential for malignant transformation, metastasis and recurrence (DeVilliers, Suggs, Simmons, Murrah, & Wright, 2011; McClary et al., 2016). Management of ameloblastoma is surgical resection with a wide margin of surrounding normal tissue. Radical surgery causes significant morbidity, disfigurement and tumor recurrence that can range from 50 to 70% (Dandriyal, Gupta, Pant, & Baweja, 2011; Ghandhi et al., 2006; Laborde, Nicot, Wojcik, Ferri, & Raoul, 2017). Unfortunately, nonsurgical conservative therapy is associated with a higher recurrence of 90% (Laborde et al., 2017). It is unclear why ameloblastomas are highly recurrent despite surgical resection that includes a wide margin of surrounding normal tissue. It is possible that invasive ameloblastoma...
cells at the surgical margin predispose to surgical ‘tumor cell seeding’ that eventually reactivates to induce recurrence (Arotiba et al., 2012). Most ameloblastomas display genetic mutations of *BRAF* that encodes the serine/threonine protein kinase B-Raf, an activator of MAPK/ERK-signaling pathway as well as mutations of *SMO* (Smoothened) a component of the hedgehog signaling pathway (Effiom, Ogundana, Akinshipo, & Akintoye, 2017; Sweeney et al., 2014). Additionally, ameloblastoma neoplastic cells express high levels of autophagic markers LC3 (microtubule associated protein 1 light chain 3), p62 (sequestosome 1 (SQSTM1)), and ATG7 (autophagy related 7) which suggests that autophagic process may play a role in the neoplastic microenvironmental processes within ameloblastoma (Okada et al., 2014). As *BRAF* oncogenes induce the expression of key autophagic markers that include LC3, p62 and BECN1 (Becn1), and ameloblastomas demonstrate a high frequency of the oncogenic *BRAF* V600E mutation similar to colorectal cancer cells (Goulillemaki et al., 2016), it is possible that recurrence of ameloblastoma is activated by autophagic cell survival mechanisms of residual post-surgical marginal tumor cells. Since ameloblastoma and odontoma are the most common odontogenic tumors and ameloblastoma displays locally-aggressive growth unlike odontoma, we tested the hypothesis that autophagic process is disproportionately activated in ameloblastoma-associated odontogenic cells to support recurrence relative to the non-aggressive odontoma-associated odontogenic cells. Our goal is to further understand the mechanistic cues that induce ameloblastoma recurrence.

2. Methods

2.1. Tissue processing

Fresh ameloblastoma tissues were collected from a gross surgical sample of ameloblastoma. This study was approved by Lagos University Teaching Hospital Health Research Ethics committee protocol # 2205 and University of Pennsylvania Office of Regulatory Affairs Institutional Review Board (IRB) protocol # 829,501. A representative portion of the fresh surgical tissue sample was fixed in buffered formalin and 5 μm sections were stained with hematoxylin-eosin for microscopic analysis. Two board-certified oral pathologists confirmed a diagnosis of solid multicystic ameloblastoma-follicular pattern (SMA-f) based on current World Health Organization histological classification of odontogenic tumors (Effiom et al., 2017; Wright, Odell, Speight, & Takata, 2014). Another representative portion of the ameloblastoma surgical sample was processed for culture and establishment of primary ameloblastoma-derived cells.

2.2. Establishment of ameloblastoma-derived cells

Ameloblastoma-derived cells were isolated using an established protocol for isolation of human orofacial mesenchymal stem cells (OMMSCs) (Akintoye et al., 2006). Described briefly, fresh ameloblastoma tissue collected in cold α-modified Minimum Essential Medium (α-MEM, Catalogue # 11095072, Thermo-Fisher Invitrogen, Waltham MA) was processed under aseptic conditions within 48 h of collection. Loose soft tissues on the external surface of ameloblastoma sample were carefully cleaned with sterile surgical blade and washed three times in phosphate-buffered saline (PBS). The sample was cut in tiny fragments with sharp surgical scissors in a reaction vial containing ice cold α-MEM followed by digestion with 1:1 collagenase-type I (3 mg/ml) and dispase (4 mg/ml) for 1 h at 37 °C as previously reported (Jiang et al., 2017). The cell suspension was passed through a 70 μm nylon cell strainer (Catalogue # 352350, BD Biosciences Discovery Labware, San Jose, CA) to release single cells, split into two equal volumes and then washed twice in PBS plus centrifugation at 1500 rpm for 5 min. Both epithelial and mesenchymal-derived primary cell lines were cultured. The primary epithelial ameloblastoma-derived cells (EP-AMCs) were established by seeding one half of the single cell suspension in a T-75 flask of Keratinocyte Growth Medium KGM-Gold™ (Catalogue #:192151, Allendale NJ), 2 mM glutamine, 100/μl penicillin, 100 μg/ml streptomycin sulfate, 100 μM L-ascorbate-2-phosphate and 10% fetal bovine serum. The remaining single cell suspension was seeded in T-75 flask containing α-MEM, 2 mM glutamine, 100/μl penicillin, 100 μg/ml streptomycin sulfate, 100 μM L-ascorbate-2-phosphate and 10% fetal bovine serum to establish mesenchymal ameloblastoma-derived cells (MS-AMCs). Both cell types were maintained in a humidified incubator at 37 °C and 5% CO2. The culture media were changed every three days. At 75% confluence, primary cells were detached with 0.25% trypsin/EDTA (Thermo-Fisher Invitrogen, Waltham MA) and sub-cultured so that both primary and passage 1 cells were stored in liquid nitrogen. For all experiments, early passages 3–5 cells were tested. Previously described human odontoma-derived cells (HODCs) (Song, Stefanik, Damek-Poprawa, Alawi, & Akintoye, 2009) and ameloblast-lineage cells (ALCs) (courtesy of Drs. Caroline Gibson and Sugiyama) served as odontogenic controls while maxillary mesenchymal stem cells (MX-MSCs) (Akintoye et al., 2006) served as non-odontogenic control.

2.3. Colony forming efficiency

Primary EP-AMCs, MS-AMCs and MX-MSCs were sub-cultured at 102, 103, 104 and 105 cells per flask in triplicate T-25 flasks using growth medium of KGM and α-MEM respectively as described above. At 14 days, cells were fixed with 100% methanol, stained with methyl violet solution and colonies of 50 or more cell aggregates were counted to calculate colony forming efficiency (CFE).

2.4. Immunostaining for autophagic markers

The different cell populations (EP-AMCs, MS-AMCs, HODCs, ALCs and MX-MSCs) were cultured at low density in collagen-coated 35 mm dishes (No. 1.5 Coverslip, 10 mm Glass Diameter; catalogue # P35GCOL-1.5-10-C, MatTek, Ashland, MA). At 60–70% confluence, cells were fixed with 4% paraformaldehyde for 10 min followed by immunostaining with rabbit anti-LC3B (1:100, #2775S, Cell Signaling Technology, Danvers MA), and guinea pig anti-p62 / SQSTM1 (1:50, GP62-C, Progen) as previously described (Reyes-Reveles et al., 2017). Briefly, the samples were blocked in blocking solution containing 5% BSA and 0.2% Triton X-100 in PBS (PBST) at 37 °C for 1 h, incubated with primary antibody diluted in blocking solution at 4 °C overnight, washed and incubated with appropriate secondary antibody (goat anti-rabbit Alexa Fluor 594 or goat anti-guinea pig Alexa Fluor 488 conjugates (Invitrogen). For negative control cells, the primary antibodies were substituted with non-immune sera. Images were acquired with the same laser settings using Nikon A1R laser scanning confocal microscope with a PLAN APO VC 60x water (NA 1.2) objective at 18 °C and Nikon Elements AR 4.30.01 software.

2.5. Assessment of basal level autophagic proteins by western blotting

Lysates of EP-AMCs, MS-AMCs, HODCs, MX-MSCs and ALC were prepared in RIPA buffer (Cat # R0278; MilliporeSigma, St Louis MO) supplemented with 1% protease inhibitor cocktail (Cat # P8340; MilliporeSigma, St Louis MO) and 2% phosphatase inhibitor cocktail 2 (Cat # P5726; MilliporeSigma, St Louis MO). Total protein amount was determined using bichinchonic acid protein assay (Cat # 23,225; Thermo Scientific Waltham MA) and 15 μg protein per sample was separated under reducing condition in a 12% Bis-Tris polyacrylamide gel electrophoresis (Cat # NP 0341; Invitrogen-Thermo Scientific, Waltham MA). The protein bands were transferred to a PVDF membrane (Cat # IPVH00010, Millipore, Billerica, MA) blocked with 5% milk buffer (5% milk in PBS, 0.1% Tween-20) for 1 h at room temperature and incubated with the following primary antibodies: anti-p62 (1:1000; Cat # PM045, MBL International, Woburn MA), anti-LC3B (1:1500; Cat #
2.6. Mouse xenograft model of ameloblastoma

The animal protocol (# 806,165) was approved by the University of Pennsylvania Institutional Animal Use and Care Committee. After ex vivo expansion of EP-AMCs, MS-AMCs, HODCs, MX-MSCs and ALCs, 2 × 10⁶ cells of each type were attached to 40 mg of spheroidal hydroxyapatite/tricalcium phosphate (HA/TCP, particle size 0.5–1.0 mm, Zimmer, Warsaw, IN) before transplantation into separate subcutaneous pockets of 4-week-old immunocompromised nude female mice (Charles River Laboratories, Wilmington, MA). Each mouse received four grafts of randomly selected cell type so that every mouse harbored graft from at least three different cell types. The grafts were harvested by week 4, fixed in 4% paraformaldehyde in PBS (pH 7.4), decalcified in 10% EDTA (pH 8.0) and embedded in paraffin. Four different 5 µm sections were stained with hematoxylin/eosin (H&E) for histological evaluation.

2.7. Immunohistochemical assessment of ameloblastoma xenograft

Xenograft tissue sections were deparaffinized, rehydrated, blocked with 3% hydrogen peroxide followed by 1-hour steaming in citrate buffer for antigen retrieval. Tissue sections were rinsed in water, blocked for 40 min in 2.5% normal horse serum (Cat # S-2012, Vector Laboratories, Burlingame CA) and immunostained with primary antibodies to p62 (1:1000; Cat # PM045, MBL International, Woburn MA) and LC3 (1:1500; Cat #3868S, Cell Signaling Technology, Danvers MA) overnight at 4 °C. Tissue sections were rinsed in PBS before incubating for 30 min in ImmPRESS™ HRP goat anti-rabbit IgG (peroxidase) polymer detection reagent (Cat # MP-7451, Vector Laboratories, Burlingame CA), stained with DAB peroxidase substrate solution (Cat # SK 4105, Vector Laboratories, Burlingame CA), and counterstained with hematoxylin. Images were evaluated microscopically and captured with Nikon Eclipse 80i (Nikon Instruments, Melville, NJ) equipped with SPOT Flex digital camera (Diagnostic Instruments, Sterling Heights, MI).

2.8. Statistical analysis

The ameloblast-derived cells were seeded in triplicate flasks with appropriate parallel sets of odontogenic and non-odontogenic controls. All animal transplants were performed using triplicate animals and each animal received 3–4 grafts to minimize individual animal variability. All data were expressed as mean ± standard deviation. Differences between ameloblast-derived cells and controls were assessed with one-way analysis of variance (ANOVA) followed by post hoc comparisons with Turkey–Kramer test. Statistical significance was set at P < 0.05.
3.3. EP-AMCs regenerated ameloblastoma-like odontogenic tissues in mouse xenograft model

Among the di
differ
tent cell types transplanted in vivo, only EP-AMCs xenografts doubled in size within 4 weeks. Unlike other xenografts, histological analysis of EP-AMCs tumor xenograft displayed amelo-
blastoma-like tumor (Fig. 4) with similar columnar ameloblast-like cells, loose stellate reticulum-like cells and regions of cystic degenera-
tion characteristic of SMA-f (Fig. 4).

3.4. EP-AMCs induced epithelial-mesenchymal transition and recreated neoplastic odontogenic epithelium in vivo

Next, we probed the ameloblastoma-like tumor sections with anti-
bodies to LC3 and p62. The epithelial-mesenchymal invaginations of the odontogenic epithelium (Fig. 5A) were strongly reactive to LC3 and p62 (Fig. 5B–C) suggestive of autophagy-related survival of EP-AMCs and ameloblastoma-like tumor.

4. Discussion

Solid multicystic ameloblastoma (SMA) is the most common type of ameloblastoma. Histologically, it displays the follicular, plexiform or mixed follicular/plexiform arrangement of proliferating odontogenic epithelium that extends into the stroma (Effiom et al., 2017). We suc
cessfully isolated EP-AMCs and MS-AMCs from follicular SMA (SMA-f) and found EP-AMCs to be the significantly clonogenic sub-population. Abundance of cancer-stem cells have been identified in several cancers including head and neck cancers (Almeida, Guimaraes, Squarize, & Castilho, 2016). Specifically, an enriched amount of stem cell-related genes has been demonstrated in ameloblastoma stroma (Jiang et al., 2017). Taken together, these suggest that putative sub-population of stem cells reside in ameloblastoma that possibly support odontogenic epithelial proliferations characteristic of ameloblastoma (Effiom et al., 2017). Since stem cell-like increase in clonogenic index is a common feature of tumor growth and recurrence it can be conceived that re-
sidual ameloblastoma cells acquire this survival mechanism to adapt to their microenvironment and reactivate later (Almeida et al., 2016).

Tumor growth, progression and recurrence are orchestrated by mechanistic processes that favor survival in an unfavorable environ-
ment. Cytoprotective autophagy is associated with resistance to therapy of several oral-epithelial tumors (Huang & Liu, 2016; Lu & Xie, 2016). Additionally, EP-AMCs compared to HODCs, ALCs and MX-MSCs displayed increased levels of both LC3II and MREG, an LC3 binding partner required in a hybrid degradation process known as LC3-asso-
ciated phagocytosis (LAP). Interestingly, stromal cells in ameloblastoma (MS-AMCs) displayed much lower basal levels of autophagic proteins relative to the epithelial-derived cells (EP-AMCs) (Fig. 3).

Fig. 2. Enhanced autophagy in EP-AMCs. Confocal immunofluorescent images of EP-AMCs (A–C) show strong expression of LC3 and p62 (A, red, Alexa Fluor 594-labeled-anti-LC3B; B, green, Alexa Fluor 488-labelled anti-p62 and C, merge). Relatively lower reactivity was displayed in merged images of positive control odontogenic cells (D, ALCs; E, HODCs) and negative control non-odontogenic cells (F, MX-MSCs). Nuclei stained blue with Hoechst. [EP-AMCs = epithelial ame-
lloblastoma cells; ALCs = mouse ameloblast lineage cells; HODCs = human odontoma-derived cells; MX-MSCs = maxillary mesenchymal stem cells].
edge that further supports a possible unique role for EP-AMC autophagy-associated pathways in ameloblastoma recurrence. Through canonical autophagy, intracellular substrates are enwrapped as cargo by double membrane structures known as autophagosomes formed by the actions of LC3. This allows for bulk turnover of cytoplasmic components, enabling among other functions, the survival of nutrient-deprived cells (Florey & Overholtzer, 2012). The autophagic protein p62 strongly expressed by EP-AMCs is a major signaling hub involved in multiple signaling pathways and its high expression is associated with poor prognosis of several epithelial cancers such as ovarian cancer (Iwadate et al., 2014). Most ameloblastomas display genetic mutations of BRAF that encode the serine/threonine protein kinase B-Raf, an activator of MAPK/ERK-signaling pathway (Efimov et al., 2017); and ameloblastomas are associated with elevated levels of autophagic

![Fig. 3. Accumulation of autophagic proteins in EP-AMCs.](image)

Western blot immunoreactive bands (A) and quantitative analysis (panel B) of autophagic proteins p62, MREG, LC3-I and LC3II demonstrate strong immunoreactivity by EP-AMCs relative to control cells [positive controls = ALCs and HODCs; negative control = MX-MSCs]. Accumulation of autophagic proteins was higher in EP-AMCs versus MS-AMCs isolated from ameloblastoma stroma and control cells. LC3II (p < 0.01), p62 (p < 0.01) and MREG (p < 0.05) were significantly expressed in EP-AMCs relative to HODCs, another odontogenic cell population.

![Fig. 4. In vivo recreation of ameloblastoma-like odontogenic tumor by EP-AMCs.](image)

Histological pattern of ameloblastoma (A) was recreated in a mouse tumor xenograft model of subcutaneous transplant of EP-AMCs (B, C). The EP-AMCs tumor xenograft displayed similar columnar ameloblast-like cells (black arrow heads), loose stellate reticum-like cells (♦) and regions of cystic degeneration (green arrows) characteristic of SMA-f.
markers, LC3, BECN1, and p62 (Okada et al., 2014). Additionally, BRAF signaling and autophagy induce resistance to therapy in other tumors such as melanoma, epithelial ovarian cancers and pancreatic cancers (Iwadate et al., 2014; Yang et al., 2011).

Since high rates of cell death correlate with high mitotic index and tumor aggressiveness, the tumor microenvironment at various stages contains a population of dying cells (Lipponen, Aaltomaa, Kosma, & Syrjanen, 1994; Liu, Edgerton, Moore, & Thor, 2001). These dying cells serve as substrates for other phagocytic cells in a process known as entosis. During entosis, cells are engulfed alive and mediate their own internalization. Entosis may serve as a tumor suppressor (Florey, Kim, Sandoval, Haynes, & Overholtzer, 2011) or may promote tumor progression in the long term (Krajcovic et al., 2011). In these studies, we observe increased p62 levels, that suggests a decrease in canonical autophagy due to defective autophagolysosomal degradative capacity. Additionally, there was also an increase in both LC3B and its adapter protein, MREG required in LAP. Ability of LAP to facilitate destruction of cells corpses or cells stimulates anti-inflammatory mediators, hence therapeutically, the Inhibition of LAP dependent processes could shift the tumor microenvironment to one that is more pro-inflammatory or tumorigenic. As diagrammatically summarized in Fig. 6, it is possible that either decreased autophagolysosome capacity or increased LAP could promote ameloblastoma recurrence.

Ameloblastoma recurrence is independent of histologic type although tumor microenvironment plays a determinant role in accelerating tumor growth and cancer reoccurrence after surgical resection or chemotherapy (Hanahan & Coussens, 2012). One report has shown that epithelial cells isolated from SMA-f can be induced by stromal-derived IL-6 to promote expression of stem cell related genes and epithelial-mesenchymal transition (Jiang et al., 2017). The tumor microenvironment is usually hypoxic, and hypoxia-activated autophagy has been associated with degradation of p62 in some cells, so interplay of hypoxia and autophagy but not necessarily histologic variant possibly plays a role in ameloblastoma survival. Phosphatidylserine-positive (PS+) dying tumor or normal cells induce autophagy mediated degradation, often through LAP (Florey & Overholtzer, 2012). In LAP, LC3 is directly conjugated to a single membrane phagosome in a nutrient-independent manner (Martinez et al., 2011; Sanjuan et al., 2007). Two components critical for LAP-mediated degradation of ingested PS+ components by epithelial cells are LC3 and its binding partner MREG (Frost et al., 2015) both of which were upregulated in EP-AMCs. LAP dependent degradation of ingested PS+ structures by epithelial cells provides fuel for oxidative metabolism (Reyes-Reveles et al., 2017). It is possible that residual ameloblastoma cells predisposed to LAP mediated cargo degradation provide recycled bioenergetic components for survival and proliferation of residual ameloblastoma cells (Fig. 6). Therefore, like other head and neck cancers (Almeida et al., 2016), ameloblastoma possibly adapts dynamically to its microenvironment using putative clonogenically active stem-like cells, hypoxia and autophagic process. Additional studies are underway to delineate how EP-AMCs utilize autophagy associated process to promote ameloblastoma recurrence.
Conflicts of interest

The authors declare no conflict of interests.

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