The Interplay of Hypoxia and Autophagy in Epithelial-Derived Ameloblastoma Cell Survival: A Pilot Study

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Abstract
Ameloblastoma is the most clinically-significant benign odontogenic jaw tumor with a locally-aggressive growth pattern and high malignant transformation rate. Epithelial-derived ameloblastoma cells (EPAMCs) demonstrate enhanced basal autophagy but the etiopathogenesis of ameloblastoma and the roles of hypoxia and autophagy in EPAMCs survival and recurrence are still unknown. The goals of this study were to assess expression of ameloblastoma-specific markers and the roles of hypoxia and autophagy on EPAMC survival. Primary and recurrent ameloblastoma tissues from two patients were immunostained with pan-cytokeratin, vimentin and SQSTM1/p62. Additionally, EPAMCs were subjected to severe hypoxia (0.1% O₂) to define responsiveness to hypoxia based on expression of hypoxic and autophagic markers. Human odontoma-derived cells (HODCs) served as control. Both primary and recurrent tissue samples stained positive for pan-cytokeratin. Vimentin and SQSTM1/p62 were undetectable but the connective tissue stained positive for vimentin. Phosphorylated-40S ribosomal protein S6 (pS6) levels were decreased in EPAMC in both hypoxia and post-hypoxia. There were no significant changes among the remainder markers or between the EPAMC and HODCs. While the small sample size of this pilot study limited the statistical power several interesting trends were observed. In EPAMCs, canonical autophagy tended to be active at baseline, hypoxia, and re-oxygenation but did not increase when cells were subjected to hypoxia. Cells displayed reduced levels of pS6 and elevated levels of LC3ABII/LC3ABI and p62 24 hours following hypoxia. The vimentin expression and pan-cytokeratin pattern are consistent with an epithelial origin of ameloblastoma. Our data also suggests EPAMCs are using autophagy to survive severe hypoxia.

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**Introduction**

Ameloblastoma is a benign epithelial odontogenic tumor of the jaws, with the majority (80%) occurring in the mandible (McClary et al. 2016). It is locally invasive in growth, has a high recurrence rate, and represents the most clinically significant odontogenic tumor (Boeddinghaus and Whyte 2008). It compromises 11-18% of all odontogenic tumors, thus making it the second most common odontogenic tumor after odontomas (Boeddinghaus and Whyte 2008; Kreppel and Zoller 2018; Lee and Kim 2013; Siar and Ng 2014). According to the World Health Organization (WHO) classification of Head and Neck tumors, ameloblastomas can be categorized into different clinicohistologic types that include ameloblastoma (formerly solid/multicystic), unicystic, extraosseous/peripheral, or metastasizing (malignant) types of ameloblastoma (Speight and Takata 2018). A high malignant transformation rate of about 70% has been identified, out of which 2% metastasize usually to the lungs (Effiom et al. 2018). Ameloblastomas are usually treated with surgical approaches. Surgery can include a conservative approach, where the tumor is enucleated or curetted, or a more radical approach where the tumor undergoes wide local excision, including 1-2cm normal bone margins, followed by reconstruction of bone and maxillofacial prosthetic therapy (Effiom et al. 2018; McClary et al. 2016). Ameloblastoma has a high recurrence rate, especially after conservative therapy (McClary et al. 2016).

The etiology of ameloblastoma pathogenesis remains unknown. Most tumors display mutations in the mitogen-activated protein kinase (MAPK) signaling pathway, which is responsible for cell proliferation, as well as mutations in the sonic hedgehog (SHH) pathway (McClary et al. 2016; Sweeney et al. 2014). Sweeney et al. reported that 39%
of ameloblastomas exhibited mutations in Smoothened (SMO), a critical component of the SHH pathway, while 46% displayed BRAF (the gene encoding serine/threonine-protein kinase B-Raf protein) mutations (Sweeney et al. 2014). BRAF is a serine-threonine kinase within the MAPK pathway. It is constitutively activated in a BRAF V600E mutation, where valine is substituted for glutamic acid at position 600, promoting cell proliferation and malignant transformation. The BRAF V600E mutation was implicated as a marker of recurrence (Brown et al. 2014; do Canto et al. 2019).

Epithelial-mesenchyme transition (EMT) is a process that occurs during embryogenesis and is important for the development of multicellular organisms (Siar and Ng 2014). Dysregulation of this process has been implicated in cancer progression (Siar and Ng 2014). E-cadherin is a major regulator while repressors of EMT that act by down-regulating E-cadherin include Snail, Slug, SIP1, and Twist (Siar and Ng 2014). Snail has been linked to induction of EMT in ameloblastomas (Siar and Ng 2014). Epithelial cells of follicular ameloblastomas were found to contain epithelial-mesenchymal cells that express EMT markers and this is further evidence supporting the role of EMT in ameloblastoma formation (Jiang et al. 2017). Ameloblastoma-derived mesenchymal stromal cells have also been found to produce a significant levels of interleukin (IL) 6 which, in turn, promoted epithelial-derived ameloblastoma cells (EPAMC) to undergo EMT (Jiang et al. 2017).

Autophagy is the process by which cells degrade and recycle old and/or damaged organelles and proteins to sustain homeostasis (Kim and Overholtzer 2013). Canonical autophagy involves the formation of a double membrane autophagosome, the formation of which is regulated by various autophagy-related (Atg) genes and proteins (Florey and
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Overholtzer 2012). The Atg1 homolog, Ulk1 is a kinase responsible for the activation of autophagy in mammalian cells. This kinase is inhibited by mammalian target of rapamycin (mTor) when the cells are in a nutrition-abundant environment, which effectively inhibits autophagy (Florey and Overholtzer 2012). There are two ubiquitin-like conjugation systems downstream of Ulk1 that also play a role in autophagosome formation. One system involves various Atg proteins, while the other system involves the cleavage of pro-LC3 by Atg4, which forms LC3-I. LC3-I is then conjugated to phosphatidylethanolamine (PE), a phospholipid, by two different Atg proteins (Atg3 and Atg7), thus forming LC3-II. This initiates autophagosome formation (Florey and Overholtzer 2012). The formation of an autophagosome is important for lysosomal degradation of intracellular material (Florey and Overholtzer 2012). Extra-cellular material is transported to lysosomes by single membrane vesicles called phagosomes. The lysosomes then degrade or recycle the contents of the phagosome through the macroendocytic pathway (named as such due to the size of the vesicle) (Florey and Overholtzer 2012). It is now known that the intracellular and extracellular degradations pathways are not entirely distinct. Canonical autophagy proteins play a role in the macroendocytic pathway by allowing the fusion of the lysosome to the single membrane vesicle.

Loss of function of autophagy genes has been implicated in disease occurrence. Autophagy associated degradation in the form of LC3 Associated Phagocytosis (LAP) has been speculated to affect the growth potential of tumors and their response to treatment (Kim and Overholtzer 2013). Loss of critical autophagy proteins, Beclin1, ATG5, and ATG7 causes pro-inflammatory cytokines to be released by macrophages which changes the tumor microenvironment (Kim and Overholtzer 2013).
Cancer cells utilize autophagy in order to survive (Goulielmaki et al. 2016). The involvement of autophagy in the oncogenesis of odontogenic epithelium is supported by data showing Beclin1 and ATG5 expression in ameloblastic tumors (Okada et al. 2014). Autophagy promotes tumor survival in a hypoxic environment and deprivation of nutrients and this can be detected using LC3 as a marker (Okada et al. 2014). An immunohistochemical study displayed increased expression of ATG7, LC3, and sequestosome-1 (SQSTM 1)/p62 (a substrate for LC3, hereafter referred as p62) in ameloblastomas and a lower expression of LC3 and p62 in recurrent ameloblastomas when compared to the primary form (Okada et al. 2014). An increase in p62 expression causes tumors to progress and occurs when autophagy is dysregulated while a decrease of p62 signifies an increase in basal autophagy (Okada et al. 2014). Autophagy has also been proposed to play a role in preventing tumorigenesis as Beclin1 disruption has been implicated in certain tumor formation, including liver carcinoma, in mice (Okada et al. 2014). Several studies confirmed the presence of a BRAF mutation in ameloblastomas (Brown et al. 2014; Brunner et al. 2015; do Canto et al. 2019; Kurppa et al. 2014; Sweeney et al. 2014). Tumors with a BRAF mutation require degradation of mitochondria, a process that may be facilitated by autophagy, specifically mitophagy (Goulielmaki et al. 2016). A BRAFV600E mutation was found to induce LC3 in colorectal cancer cells by activating the MEK/ERK pathway (Goulielmaki et al. 2016). The role of EMT in ameloblastoma formation, as aforementioned, has been confirmed (Jiang et al. 2017). There is evidence that EMT demonstrates increase autophagic activity, as determined in breast cancer cells; human breast cancer tissues that expressed SNAI2, an EMT marker, also expressed ATG5 (Akalay et al. 2013). It has also been suggested that autophagy in breast
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cancer cells inhibited EMT by degrading key inducers (Snail and Twist) which lead to metastasis inhibition (Lv et al. 2012). Hypoxia has been found to activate autophagy in tumors which promotes cell survival (Pursiheimo et al. 2009). The clearance of p62 and an increase of the RAS/ERK signaling pathway by autophagy under hypoxic conditions protects tumor cells against the oxidative stress imposed by hypoxia (Pursiheimo et al. 2009). Hypoxia-inducible factor (HIF) was found to be involved in hypoxia-activated autophagy in mouse embryo fibroblasts (Zhang et al. 2008). These authors suggest that when hypoxia is acute (<24 hours), p62 clearance by autophagy occurs independent of HIF, while chronic hypoxia (>24 hours) would promote a more prevalent autophagic process that would rely on HIF (Pursiheimo et al. 2009). Tumors may also employ autophagy in a HIF1 independent manner as there is a hypoxia-reoxygenation cycle of cancer cells that would require protein levels to be regulated in a quicker fashion (Pursiheimo et al. 2009). This occurs through either 5'AMP-activated protein kinase (AMPK) or the unfold protein response (UPR) pathway (Bassam Janji 2013). In a nutrient depleted environment autophagy is activated by AMPK (Bassam Janji 2013). Clearly the role of autophagy in cell survival and tumorigenesis is complex and likely tumor specific.

Little is known as to what contributes to the recurrence of ameloblastomas. Rates have been reported to be 0-15% (Kreppel and Zoller 2018). One study reports a 60% recurrence rate after conservative therapies (Hammarfjord et al. 2013). Due to this high rate of recurrence, especially with conservative approaches, the treatment of choice for primary ameloblastoma is usually the radical approach which includes resection of the jaws with wide margins; the morbidity associated with this is significant (Heikinheiro et
al. 2015). Recurrent ameloblastomas have a 2% chance of malignant transformation and metastasis (Kreppel and Zoller 2018). They are also best treated with radical resection (Effiom et al. 2018; Karathanasi et al. 2013). Understanding the mechanism underlying ameloblastoma formation and recurrence can provide a means of significantly reducing, or preventing, rates of recurrence. Conservative nonsurgical therapy, such as drug therapy, will have a minimal impact on quality of life compared to the more radical approach that usually results in disfigurement.

Drugs that inhibit BRAF mutations, Vemurafenib, and dabrafenib, have been developed and approved for metastatic melanomas that display BRAF mutations (Heikinheimo et al. 2015). Experiments in vitro reveal ameloblastoma cells with BRAFV600 mutations also respond to vemurafenib (Brown et al. 2014; Sweeney et al. 2014). An approved drug for basal cell carcinoma, Vismodegib, inhibits SMO (Heikinheimo et al. 2015). Because autophagy is implicated in both tumor suppression and progression, the role of chemical agents that target both have been investigated in cancer therapy. Chloroquine (or hydroxychloroquine), an inhibitor of autophagy, is currently investigated in clinical trials for the treatment of solid oncological and hematological tumors (Rubinsztein et al. 2012). Tamoxifen, vitamin D and metformin all induce autophagy but have not been investigated in clinical trials (Rubinsztein et al. 2012). Other drugs that induce autophagy include rapamycin, resveratrol, and spermidine (Rubinsztein et al. 2012). None of those drugs, however, have been approved for ameloblastoma.

There is little knowledge regarding the role of autophagy in ameloblastoma activation and recurrence. In efforts to understand ameloblastoma pathogenicity, the objective of this
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The study was to characterize ameloblastoma and assess whether the hypoxic tumor microenvironment induces oxidative stress and activates autophagy in epithelial-derived ameloblastoma cells (EPAMCs) favoring survival of EPAMCS and possibly causing tumors to recur. Exploring the pathogenesis of ameloblastoma recurrence can impact treatment choice and ultimately lead to a favorable clinical outcome and patient care.
Materials and methods

Immunohistochemical analysis

An immunohistochemical study of human primary and recurrent ameloblastoma tissue specimen was performed. The tissue sections were deparaffinized as follows: Xylene 2 x 5 minutes each, 100% ethanol - 2 x 5 min each, 95% ethanol - 2 x 5 min each, 80% ethanol - 2 x 5 min each, 70% ethanol – 2 x 5 min each, 50% ethanol – 2x 5 min each. Tissues were then rinsed with phosphate-buffered saline (PBS) (pH 7.4) 2 x 5 min each. Next tissue sections were isolated using a ‘Pap’ pen. Tissues were blocked with endogenous peroxidase in 3% hydrogen peroxide (made with 1 part 30% H₂O₂ + 9 parts absolute methanol) then incubated in a humidified chamber for 20 minutes at room temperature. Tissues were then rinsed with PBS (pH 7.4) 2 x 5 min each. Endogenous peroxidase activity was removed by incubating tissues with BLOXALL™ Blocking Solution (SP-6000) for 10 minutes then rinsing them with PBS 1 x 5 min. Tissue sections were then incubated with 2.5% normal goat blocking serum (Vector Laboratories: ImmPRESS™ HRP IgG (Peroxidase) Polymer Detection Kit) for 40 minutes at room temperature. Antibody dilutions (Table 1) were prepared, and tissue sections were incubated with the primary antibody for 2 hours in a humidified chamber at 4°C. Blocking serum (same used above) was used as negative control. Tissues were then rinsed with PBS 2 x 5 min each then incubated with appropriate Vector ImmPRESS HRP Reagent for the host of antibody for 30 minutes at room temperature. Tissue sections were then washed with PBS 3 x 5 min each. Tissues were stained with 3,3’-Diaminobenzidine (DAB) for 10 minutes, washed 2 x 5 min each with deionized water, counterstained with hematoxylin for 3 minutes, and washed with tap water. PBS was applied to the slide until
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tissue sections were blue (approximately 30 seconds). Mounting media was applied to the slides and coverslip placed. Slides were labeled with antibody and dilution, including controls. Imaging with a microscope was done, and pictures were taken with a microscope camera.

*Cell samples*

All cell samples used in this study were previously isolated, characterized and preserved for long-term-storage. The most common form of ameloblastoma is the conventional type that accounts for 91% of all cases. (Effiom et al. 2018) Epithelial cells from conventional ameloblastoma (follicular variant) surgical samples (EPAMCs) previously isolated were evaluated in this study. Control cells included human odontoma-derived mesenchymal cells (HODCs). HODCs represent undifferentiated post-natal stem cells that are derived from odontomas and can differentiate into any tooth structure (i.e., enamel, dentin, pulp, or cementum) (Song et al. 2009).

*Culturing cells under normoxic and 0.1% oxygen (O₂) (severe hypoxic) conditions*

All cells were cultured under normoxic and severe hypoxic conditions (0.1% O₂) with a recovery period in a Billups-Rothenburg hypoxic chamber (Billups-Rothenberg; Figure 1, Table 2) as described briefly (Wu and Yotnda 2011). Cells were cultured in six wells (9.5x10⁴ cells per well) using α-Minimum Essential Media (MEM) growth medium. Once cell confluence reached 80% confluence, a subset of wells were kept at 37°C in a humidified incubator containing 21% O₂ and 5% carbon dioxide in air for normoxia. Another subset was transferred to a modular incubator chamber-MIC-101 (Billups-Rothenberg, Del Mar CA) (Figure 1) to induce severe hypoxia at 0.1% O₂ per the
manufacturer’s directions. The hypoxic chamber was flushed for 20 minutes with 0.1% $O_2$ + 5% carbon dioxide + nitrogen. Cells were then kept in a humidified incubator at 37°C.

**Collection of cell lysates**

Culture media of the cell samples was removed and cells were washed with PBS. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer made with 50mM Tris-Cl pH7.4, 150mM sodium chloride (NaCl), hydrochloride acid (HCL) for pH adjustment, Triton-x-100, 10% (w/v) Na-deoxycholate, 10%(w/v) SDS. 10 µL protease inhibitor and 20 µL phosphatase inhibitors per 1 ml were added to RIPA buffer. Cells were scraped and lysate was collected. Lysates were centrifuged at 15,000 rpm for 15 minutes at 4°C temperature. Supernatant was separated from the pellet, transferred to a tube, and stored.

**Assessment of autophagic and survival markers by western blotting**

Western Blot Protocol:

The basal and hypoxic level of autophagic and survival markers (Table 3), were measured in EPAMCs and HODCs by the following western blot protocol: The protein amount was determined using Bicinchoninic Acid (BCA) Assay so that equal protein amounts were loaded per lane. Water was calculated using the following: 30µl – (x µl sample + 7.5 µl loading buffer + 3µ reducing agent). Running buffer was prepared as follows:40ml NuPAGE MOPS Running Buffer (20x) + 760ml distilled water. NuPAGE 12% Bis-Tris Gel was assembled in a basin and snapped in place. Protein was transferred from gel to membrane as follows: Transfer buffer was prepared: 50ml NuPAGE Transfer Buffer (20x) + 100ml methanol + 850ml distilled water. Two pieces of filter paper were soaked in transfer buffer. 5% nonfat dry milk was prepared as follows: 2.5g nonfat dry milk +50ml
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tris-buffered saline + tween (TBST). Once the transfer was completed, membranes were placed in a container with the 5% milk preparation and placed on a shaker at room temperature for 1 hour.

After 1 hour, primary antibodies* in 5% milk were added to membranes and left overnight on a shaker at 4 °C. Next day: membrane was washed with TBST three times by adding TBST to membranes and placing on a shaker at room temperature (5-10 min between each wash). After the third wash, secondary antibodies** in 5% milk were added to membranes and membranes were left on a shaker at room temperature for 1 hour. After 1 hour, membranes were washed again with TBST three times. Membranes were then imaged and results were quantified.

*Primary antibodies: For hypoxia– anti-HIF-1α. Survival markers – anti- total ERK 1/2 (ERK), anti-p-ERK 1/2 (p-ERK). Autophagy- anti-Beclin1, anti-Phospho-beclin1 (p-Beclin), anti-phosphorylated 40S ribosomal protein S6 (pS6), anti-p62, anti-LC3A/B. Control -anti-Actin; source and dilutions as specified in Table 3.

**Secondary antibodies: Goat anti-rabbit / goat anti-mouse; dilution 1:2,500.

Statistics

All experiments were performed in at least triplicates. Data from the western blotting were analyzed as fold-change relative to normoxia. Highly divergent points were excluded from analysis. Data was then expressed as mean +/- standard deviation. A paired t-test comparison of means was done to compare the expression of autophagy proteins when cells were under normoxia to protein expression under hypoxia and recovery conditions; a Welch’s two-sample t-test was used to compare fold-changes from
normoxia in EPAMC and HODCs cells to each other. p-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure.

**Results**

*Comparative expression of ameloblastoma/autophagy markers in primary and recurrent ameloblastoma samples*

Ameloblastoma tissue samples from 2 patients were available for immunohistochemical assessment, of which only one patient had comparative primary and recurrent tissue samples. Pan-cytokeratin was positive in the cytoplasm of both primary (Figure 2a) and recurrent (Figure 2b) ameloblastoma tissue samples of one patient. In the second patient, pan-cytokeratin was weakly positive (Figure 2c), however the recurrent tissue sample was not available.

Vimentin and p62 were not reactive in the ameloblastoma cells in any of the patient samples, however the connective tissue stroma of all samples were positive for vimentin ( vimentin Figures 3a-c; p62 Figures 4a-c).

*Expression of HIF-1α and p-ERK 1/2 by EPAMC under and post- hypoxia*

HIF-1α is activated in order to regulate the cells’ response to hypoxia and allows them to adapt to hypoxic conditions (Pursiheimo et al. 2009). When measuring HIF-1α by western blotting (Figure 5), the HIF-1α response of EPAMCs was increased 1.5 fold relative to normoxia (p=0.22), in contrast to the HODSC response at 2.5 fold (p=0.16) (Figure 6). However, the expression of p-ERK/ERK 1/2 ratio detected by western blotting (Figure 5) did not change for either cell type (EPAMC p=0.92 HODCs p=0.83) (Figure 6). When the cells were left to recover for 24 hours, the HIF-1α level gradually increased to about 5 fold of normoxia (p=0.19) compared to HODCs that remained stable and unchanged at about
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2 fold (p=0.19) (Figure 7). The p-ERK/ERK 1/2 level relative to normoxia for the 2 cell types were inconclusive (EPAMC p=0.80, HODCs p=0.27) (Figure 7).

**Basal autophagy of EPAMCs under hypoxia and recovery**

Beclin1 was examined to evaluate autophagy activity because Beclin1 is required for the formation of the autophagosome (Koukourakis et al. 2010). Under 0.1% O₂, the phosphorylated-Beclin1 (p-beclin)/Beclin ratio of EPAMCs remained unchanged compared to basal levels, at 1.13 fold (p=0.85) (Figure 9), when measured by western blotting (Figure 8). The HODCs under hypoxia similarly exhibited levels close to normoxia at 0.87 fold change (p=0.58) (Figure 9). This did not change upon reoxygenation where the ratio of EPAMCs was 1.24 fold (p=0.65), in comparison HODCs levels remained close to normoxia at 1.53 fold (p=0.19) (Figure 10). To determine if autophagy was down regulated, we analyzed the levels of pS6 in our samples. PS6 in EPAMCs were decreased under hypoxia with a 0.50 fold change compared to normoxia (p=0.048)(Figure 9), suggesting active autophagy. No change in pS6 levels were observed when cells were allowed to recover (0.55 fold change (p=0.005) (Figure 10). In contrast in the HODCs, pS6 was similar to baseline levels when cells were hypoxic with a 0.9 fold change (p=0.83) (Figure 9) and this was reduced when cells were reoxygenated at 0.48 fold change (p=0.30) (Figure 10).

p62/SQSTM1 binds directly to LC3 via a specific sequence motif, p62 accumulates when autophagy is inhibited, and decreased levels are observed when autophagy is induced. Under 0.1% O₂, the p62 levels of EPAMCs were close to baseline with a fold change of 0.81 , when compared to normoxia (p=0.68) (Figure 9), and this increased to 1.72 fold when the cells were allowed to recover (p=0.10)(Figure 10), suggesting autophagy
inhibition as the cell recovers. In HODSCs, p62 similarly did not change under hypoxia when compared to normoxia, at 1.02 fold change (p=0.83) (Figure 9), however in contrast to EPAMCs, p62 was reduced to 0.57 fold change with recovery (p=0.08) (Figure 10). One interesting observation is the significant difference of p62 levels upon recovery between EPAMC and HODCs (p=0.04).

**LC3ABII/LC3ABI levels in EPAMC following hypoxia and recovery**

To investigate which form of autophagy was activated, LC3 levels were measured. Basal levels of LC3ABII/LC3ABI in both EPAMCs and HODSc were unchanged when subjected to hypoxia (1.07 p=0.67, and 1.45 fold p=0.16, respectively) (Figure 9) but doubled in EPAMCs following reoxygenation to a 2.28 fold increase (p=0.80) (Figure 10). In HODSCs, the LC3ABII/LC3ABI ratio levels remained similar to baseline levels after reoxygenation at 1.24 fold change (p=0.58) (Figure 10).
Discussion

The expression of pan-cytokeratin, vimentin, and p62 were assessed in both primary and recurrent ameloblastoma to characterize ameloblastoma and further understand behavioral differences. Ameloblastoma and non-pathologic odontogenic tissue have been reported to exhibit similar cytokeratin expression (Hunter and Speight 2014). Depending on the type of ameloblastoma, cytokeratin is expressed in different locations (Hunter and Speight 2014). The expression pattern can change in odontogenic neoplasms (Sherlin et al. 2013). The antibody used in this study targeted the AE1/AE3 cytokeratin. We found both the primary and recurrent ameloblastoma of the patients similarly exhibited positive staining that was localized to the cytoplasm of the ameloblastoma islands. A prior study similarly found positivity of AE1/AE3 cytokeratin in all of their follicular and plexiform ameloblastomas (Wato et al. 2006). The primary ameloblastoma of the second patient in this study exhibited weak, inconsistent staining which coincides with a previous study that found a similar staining pattern of the AE1/AE3 cytokeratin in desmoplastic and unicystic ameloblastomas (Sherlin et al. 2013). Our data is in line with previously reported ameloblastoma behavior previously described in the literature. Vimentin is also a filament but it is expressed mostly in fibroblasts (Sherlin et al. 2013). The primary and recurrent ameloblastoma of the first patient stained negative for ameloblastoma islands but positive in the connective tissues stroma due to the presence of fibroblasts in the connective tissue. In the primary ameloblastoma of the second patient, the staining was weak; this is because vimentin is typically positive in neoplasms of mesenchymal origin (Wei et al. 2017). Based on vimentin expression in ameloblastoma, it has been proposed that both epithelium and connective tissue play a
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role in the formation of hard tissue in ameloblastoma, with the connective tissue playing a bigger role in the EMT (Sherlin et al. 2013). Our observation of vimentin expression alongside the pan-cytokeratin pattern are consistent with an epithelial origin of ameloblastoma. Because it is difficult to speculate the role of p62 in autophagy based only on measuring its levels by immunoblotting (Mizushima and Yoshimori 2007), an immunohistochemical analysis was performed to further understand its role in autophagy and ameloblastoma recurrence. p62 was found to be negative in ameloblastoma samples of both patients. This coincides with the results of one study that found two of their primary ameloblastomas negative for p62, although the rest of their samples positively stained (Okada et al. 2014). When they looked at primary and recurrent ameloblastoma from the same patient, as we did, they also did not encounter strong staining with p62 (Okada et al. 2014). Typically, hypoxia induces oxidative stress which results in the presence/accumulation of unfolded proteins that need to be degraded and these proteins trigger p62 expression (Pursiheimo et al. 2009). The staining varies with different histological subtypes of ameloblastoma examined. The absence of staining could indicate p62 degradation by autophagy which could suggest the tumor cells are utilizing autophagy to survive. Because the western blot cell lysates in this study were not extracted from the same samples used in the immunohistochemical study, it is difficult to make a direct comparison between both. The negative p62 staining, however, could provide further insight into the role of autophagy in ameloblastoma. It is difficult to make any conclusions based solely on the tissue specimen of two patients; one way to further advance our understanding of ameloblastoma behavior is to examine its cells. In a previous study, ameloblastoma cells have been shown to have enhanced basal
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Autophagy (Sharp et al. 2019). The ameloblastic microenvironment is composed of both surviving and dying cells due to the nature of cell turnover in tumors (Sharp et al. 2019). The rapid cell proliferation rate requires high amounts of ATP and this is the main source of metabolic stress in tumor cells (Bassam Janji 2013). Ameloblastomas have been shown to display several autophagy markers including Atg7, p62, and LC3 (Sharp et al. 2019). More specifically, EPAMCs were found to have an increase of LC3B and p62 expression when compared to HODCs cells (Sharp et al. 2019). P62, Beclin1, and LC3 are induced by a BRAF mutation, which ameloblastomas exhibit. The role of autophagy in cancer progression depends on a multitude of factors such as the type and stage of tumor. Autophagy can either promote or suppress tumor formation and metastasis (Bassam Janji 2013). It is activated under hypoxic conditions and it allows the cells to adapt to limited oxygen availability by decreasing oxygen consumption (Pursiheimo et al. 2009). Autophagy was found to be activated in non-small cell lung carcinoma in response to hypoxia and this protected the tumor against lysis by T lymphocytes (Bassam Janji 2013). On the other hand, it has been speculated that autophagy is inhibited due to metabolic stress and this activates apoptosis and thus prohibits further cancer cell growth (Bassam Janji 2013). Evidence also shows metastatic cancer cells are resistant to apoptosis and that autophagy activation will thus favor metastasis (Bassam Janji 2013). Autophagy is stimulated by hypoxia in order to sustain ATP levels and preserve cell vitality (Pursiheimo et al. 2009). The small sample size of this pilot study limited the statistical power but does allow the observation of several interesting trends. To simulate the hypoxic environment characteristic of some tumors, the cells in this study were subjected to 0.1% O₂ to represent severe hypoxic conditions. The cellular response to hypoxia is
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typically modified by the HIF pathway, although there is evidence that hypoxia can trigger autophagy independent of HIF (Bassam Janji 2013; Pursiheimo et al. 2009). HIF1 is an enzyme that cells utilize to detect oxygen level variations and to regulate the RAS/ERK signaling pathway to promote cell survival (Pursiheimo et al. 2009). HIF1 is comprised of the HIF-1α and HIF-1β subunits, which heterodimerize and form a complex (Daskalaki et al. 2018). HIF-1β is expressed under normoxic conditions while HIF-1α expression is induced by hypoxia (Masoud and Li 2015). HIF-1β normally translocates to the nucleus by binding to aryl hydrocarbon receptor (AhR) (Masoud and Li 2015). Under normoxic conditions, HIF-1α is hydroxylated which leads to its degradation by the von Hippel-Lindau tumor suppressor protein (Pursiheimo et al. 2009). HIF-1α and autophagy orchestrate a series of events that results in the activation of transcription-3 (STAT-3), a signal transducer, which promotes cell survival (Bassam Janji 2013). In this study, both HODCs and EPAMCs responded to severe hypoxia by enhanced activation of HIF-1α, however the response was higher in HODCs than in EPAMC. The hypoxic response continued to be sustained over a 24 hour time period suggesting the cells did not fully recover, even upon reoxygenation. Hypoxia does not appear toxic to the cells as they are responding by activating HIF-1α. HIF-1α activates autophagy under hypoxic conditions by promoting the expression of BH3-only protein Bcl-2/adenovirus E1B 19kDa-interacting protein 3 (BNIP3) and BNIP3L (Pursiheimo et al. 2009). Under normoxic conditions the Beclin1-Bcl-2 complex inhibits autophagy (Daskalaki et al. 2018), however when hypoxia is sustained, the Beclin1-Bcl-2 complex is cleaved by BNIP3 and BNIP3L and the released Beclin1 activates autophagy (Bassam Janji 2013). Beclin1 is a yeast homolog that is required for the formation of the autophagosome (Koukourakis et al. 2010). It forms a complex with
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PI3KC3/Vps34 and regulates autophagy through its control of different autophagic proteins (Kang et al. 2011). Beclin1 is commonly expressed in different carcinomas and has been found in the cytoplasm and nuclei of colorectal adenocarcinoma (Koukourakis et al. 2010). It is either excessively expressed or lost in tumors, depending on the type of cancer (Koukourakis et al. 2010). Beclin1 has been shown to prevent tumor growth by interacting with the BCL-2 protein (Cao and Klionsky 2007). In addition, one study linked the loss of Beclin 1 to recurrence of hepatocellular carcinoma (Ding et al. 2008). On the other hand, it is suggested that tumor progression is related to an increase of Beclin1 expression and this is potentiated in a hypoxic environment (Koukourakis et al. 2010). Hypoxia activates an anti-apoptotic role in Beclin1 in order to promote cell survival, the exact mechanism is unknown (Bassam Janji 2013). Our data showed in HODCs, the p-beclin/Beclin1 ratio decreased under 6 hour hypoxia and increased with recovery while the ratio in EPAMCs is slightly elevated and remains so with recovery, suggesting that as hypoxia increases, residual cells are not fully recovered which is consistent with our finding of persistently elevated HIF-1α levels upon reoxygenation. In addition, hypoxia did not appear to increase Beclin1 activity. In a previous study of tongue oral squamous cell carcinoma, Beclin1 expression was found to be decreased and Beclin1 did not affect autophagy levels (determined in that study by measuring p62 and LC3 levels) (Hu et al. 2016). Another study also found decreased levels of both Beclin1 and LC3-II in hypopharyngeal squamous cell carcinoma (Wang et al. 2013). In EPAMCs, the basal levels of Beclin1 activity (as determined by measured p-beclin/beclin ratios) did not seem to increase with hypoxia. To determine the effects of hypoxia on cell survival, the p-ERK / ERK levels were measured. The RAS/ERK signaling pathway is usually activated by
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hypoxia (Pursiheimo et al. 2009). Our data show that both cell lines seem to survive under 6 hour hypoxia, which is evaluated by measuring phosphorylated ERK/total ERK 1/2 ratio levels. This is consistent with previous findings where the authors suggest that the decrease of p62 under hypoxic conditions plays a role in the increase of phosphorylated ERK-1/2 levels (Pursiheimo et al. 2009). It is speculated that the decrease in p62 and increase in phosphorylated ERK, and thus increase in RAS/ERK signaling pathway, under hypoxia promotes cell survival of cancer cells (Pursiheimo et al. 2009). The decreased p-ERK/ERK levels upon recovery suggests cells might not be completely recovered. P62 is a marker of autophagy and plays a role in autophagosome formation alongside LC3-II (Tan et al. 2016). LC3 can be bound to p62 and so measuring p62 levels could detect autophagic activity; but p62 levels can also fluctuate regardless of autophagic activity and thus measuring it independently might not be a conclusive indicator of autophagy (Mizushima and Yoshimori 2007). P62 is typically decreased when canonical autophagy is activated and this indicates that the autophagosome is fusing to the lysosome and is subsequently degraded properly (Sharp et al. 2019). When autophagy is inhibited, autophagosome fusion to lysosome is impaired and this leads to accumulation of p62 (Tan et al. 2016). Hypoxia induces autophagy which degrades p62; when autophagy is inhibited – degradation of p62 is inhibited (Pursiheimo et al. 2009). P62 was greatly reduced in one study when different cells (keratinocytes, embryonic kidney cells, osteosarcoma cells, and head and neck squamous carcinoma cells) were placed under hypoxic conditions (1% O₂) for 24 and 48 hours (Pursiheimo et al. 2009). Following hypoxia, cells were re-oxygenated and allowed to recover for 2,4,6,8 hours. P62 recovered to baseline (similar to normoxic conditions) between 4-6 hours post re-
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oxygenation (Pursiheimo et al. 2009). Interestingly, in our study the HODCs’ p62 levels at hypoxia were observed to be similar at baseline and tended to decrease with recovery, suggesting hypoxia might not be affecting them at 6 hours but rather the cells are establishing a new baseline at hypoxia. The p62 levels of the EPAMCs decreased with hypoxia then increased with recovery. One possible explanation is that the cells are recovering to a higher baseline. The role of p62 in cancer is not fully understood. One study evaluated adenocarcinomas and found cells survive partly because Ras promotes p62 expression, but when cells are placed under hypoxia, the low oxygen availability may promote p62 clearance in order to alter the cellular energy metabolism (Duran et al. 2008). The increase of p62 with recovery seen in our EPAMCs might not portray an accurate representation of autophagic activity. P62 levels have been found to be unchanged when autophagy is activated in certain cells (Mizushima and Yoshimori 2007). In addition, the upregulation of P62 when autophagy is activated has been demonstrated in AML cells (Trocoli et al. 2011). P62 upregulation also occurs when the RAF1/Raf-MAP2K/MEK-MAPK/ERK signaling pathways are activated(Kim et al. 2014) or when cells are starved(Sahani et al. 2014), in an effort to recover p62. In addition, the gene responsible for the encoding of p62 has been found to be induced in autophagic conditions preventing the degradation of p62 in some situations (Mizushima and Yoshimori 2007). This makes it important to consider p62 levels in the overall context. In this study, the elevated p62 levels observed during recovery of EPAMCs coincided with an elevated LC3ABII/LC3ABI which could indicate a new baseline is established at hypoxia, with an increase in autophagy 24 hours post-hypoxia as the cells are attempting to recover. To further evaluate autophagy, the levels of pS6 were measured. PS6, a ribosomal protein, plays a
role in protein synthesis and autophagy regulation, among other functions, and has been implicated in tumorigenesis (Meyuhas 2015). The phosphorylation of S6 triggers autophagosomal formation and thus autophagic activity (Heijnen et al. 2014). In basal autophagy, S6 can activate autophagy in 2 ways; indirectly by inhibiting the PI3K/Akt pathway, a signaling pathway that is upstream of target of rapamycin (Tor), blocking mammalian Tor (mTor) (which inhibits autophagy) (Klionsky et al. 2005). An overactivity of phosphorylated S6 would thus activate autophagy, and this is important in maintaining homeostasis under normal conditions. PS6 directly activates autophagy, independent of the PI3K/Akt pathway, by interacting with Atg (Klionsky et al. 2005). There are studies that suggest a nutrient deplete environment would cause the inhibition of Tor and this leads to the decrease of pS6 and a highly active immediate autophagic response that will then return to baseline levels (Meyuhas 2015). Hypoxia has also been shown to inhibit mTOR and trigger phosphorylation of S6 (Meyuhas 2015). It appears the role of S6 has yet to be fully understood (Klionsky et al. 2005). Our data showed the pS6 in HODCs remains at baseline under hypoxia while it decreased in EPMACs. PS6 in both cell lines, however, did not recover with reoxygenation. In line with the aforementioned data, this further supports the possibility that the cells are establishing some form of new baseline at hypoxia and do not recover fully upon re-oxygenation. It has been proposed that the inhibition of Tor activates autophagy and when starvation (in a nutrition deplete condition such as hypoxia) persists, there is a decrease of p6 which would activate the PI3K/Akt pathway and thus Tor; this would inhibit and prevent autophagy from causing excess damage to the cell (Klionsky et al. 2005). This further supports the speculation of the cells’ incomplete recovery upon reoxygenation. Taking the Beclin1 data into consideration
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(there was no dramatic increase in p-Beclin/Beclin levels with hypoxia), the hypoxia and various autophagy marker data, this could suggest the ameloblastic cells are surviving using some form of autophagy. To further evaluate autophagy, LC3 levels were measured. Atg proteins that lipidate LC3-1 to PE and are important in the formation of the double membrane autophagosome (in canonical autophagy) have also been found to target phagosomes of the macroendocytic pathway and allow the fusion of single membrane phagosomes to the lysosome (independent of their role in autophagy). LC3-II (or the lipidation of LC3) occurs on membranes that are not involved in autophagy, which suggests that LC3 is not exclusively involved in the formation of the autophagosome and this is called LC3-associated phagocytosis (LAP) or non canonical autophagy (Florey and Overholtzer 2012). Unlike canonical autophagy, LAP does not rely on activation from the Atg1 homolog Ulk1 kinase complex and occurs even in a nutrition-abundant environment where canonical autophagy is typically inhibited (Florey and Overholtzer 2012). Atg5 and the conjugation of LC3 to PE is required in both canonical and non-canonical autophagy (Martinez et al. 2015). While LC3A/B was activated under 6 hour hypoxia in both cell lines (more so in HODSC), this was similar to basal levels. There was an increase of the LC3II/LC3I ratios in EPAMCs, compared to HODSCs, during recovery, which could indicate that the utilization of non-canonical autophagy to survive is the major difference between the two cell types. There has been recent evidence implicating LC3B in a non-canonical role of anoikis in ovarian cancer cells (Satyavarapu et al. 2018). In addition, Resveratrol (a polyphenol that affects tumorigenesis) has been shown to inhibit tumor formation by inducing non canonical autophagy in breast cancer cells (Scarlatti et al. 2008). Non-canonical autophagy has also been activated by luteolin (a flavonoid) in lung
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cancer cells as a mechanism to inhibit tumor progression (Park et al. 2013). Non canonical autophagy appears to inhibit tumorigenesis in certain cancer cell lines but not enough evidence is available to evaluate its role in ameloblastoma pathogenesis. When considering the data presented, it appears that EPAMCs have increased autophagy (likely non-canonical) during hypoxia and recovery, suggesting the establishment of a new baseline at hypoxia and a delayed response as the ameloblastic cells were unable to fully recover. The remaining data pertaining to hypoxia, survival, and autophagy, however, are inconclusive due to the limited sample size of this pilot study. Investigating the role of autophagy in ameloblastic cells has implications in therapy. Autophagy can contribute to cancer therapy resistance by allowing tumor cells to survive. However, inhibiting autophagy can result in either tumor progression or inhibition; it can aid in the recovery of cells damaged by chemotherapy or, on the other hand, can provide nutrients to cancer cells. The need to establish the specific role of autophagy in each cancer type before targeting autophagy is therefore of upmost importance (Bassam Janji 2013). It is difficult to definitively conclude the role of autophagy in ameloblastoma due to the nature of the study design and limited sample size but several observations have been made. The EPAMCs in this study did not completely recover upon re-oxygenation and utilized some form of autophagy, we speculate non-canonical, to protect the cells against oxidative stress. This suggests autophagy could be potentially be a target of chemotherapy but this needs to be evaluated by a larger scale study. There are several factors to consider when implementing this study on a larger scale. The ameloblastoma tissue samples in this study came from only two patients, one of whom did not have a recurrent sample. It would be ideal to compare both primary and recurrent tissue samples of a higher number of
patients. In addition, the EPAMCs evaluated were only from a primary ameloblastoma. Evaluating recurrent ameloblastoma cells as well for hypoxia, survival, and autophagy markers and comparing them to their primary counterpart would prove beneficial to determine the role those markers play in ameloblastoma cell survival. To confirm the role of LAP in autophagy, functional studies activating and inhibiting LAP in both primary and recurrent ameloblastoma samples and then evaluating the cells for autophagy markers would provide a more accurate representation of autophagy activity in those cells. The limited sample size in this pilot study provides insight to the possible behavior of EPAMC when subjected to hypoxia but a larger sample size study is needed for more definitive conclusions. Examining both primary and recurrent ameloblastomas will provide a better understanding of ameloblastoma pathogenicity and the role of hypoxia-induced autophagy in EPAMC survival.
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References:


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Tables.

Table 1. Ameloblastoma and autophagy markers detected by immunohistochemical staining.

<table>
<thead>
<tr>
<th>Marker + antibody source</th>
<th>Antibody concentration</th>
<th>Antibody validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan-cytokeratin AE1/AE3</td>
<td>1:1,000</td>
<td>(Dikina et al. 2018)</td>
</tr>
<tr>
<td>(Santa Cruz Biotechnology #sc-81714)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin (Santa Cruz Biotechnology #sc-6260)</td>
<td>1:1,000</td>
<td>(Kopecki et al. 2018)</td>
</tr>
<tr>
<td>p62 (Santa Cruz Biotechnology #sc-48402)</td>
<td>1:1,000</td>
<td>(Sharma et al. 2018)</td>
</tr>
</tbody>
</table>

Table 2. Cell culture conditions.

| 6 hours | Normoxia | Hypoxia | Hypoxia followed by 24 hour recovery |

Table 3. Hypoxia, survival, and autophagy markers detected by immunoblotting.

<table>
<thead>
<tr>
<th>Marker + antibody source</th>
<th>Antibody concentration</th>
<th>Antibody validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1-α (Abcam #ab2185)</td>
<td>1:1,000</td>
<td>(Zepeda-Orozco et al. 2017)</td>
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<tr>
<td>Total ERK 1/2 (Cell Signaling #9107)</td>
<td>1:1,000</td>
<td>(Strub et al. 2018)</td>
</tr>
<tr>
<td>p-ERK 1/2 (Cell Signaling #9101)</td>
<td>1:1,000</td>
<td>(Zhou et al. 2018)</td>
</tr>
<tr>
<td>Beclin1 (Cell Signaling #3738)</td>
<td>1:1,000</td>
<td>(Zhao et al. 2017)</td>
</tr>
<tr>
<td>Phospho-beclin (Novus #NBP2-29654)</td>
<td>1:1,000</td>
<td>(Muniz-Feliciano et al. 2017)</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Antibody/Control</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6 (Cell Signaling #2211)</td>
<td>1:1,000</td>
<td>(Ogawa et al. 2017)</td>
</tr>
<tr>
<td>p62 (MBL #PM045)</td>
<td>1:1,000</td>
<td>(Velikkakath et al. 2012)</td>
</tr>
<tr>
<td>LC3A/B (Cell Signaling #12741t)</td>
<td>1:1,000</td>
<td>(Ito et al. 2018)</td>
</tr>
<tr>
<td>Actin (control) (Sigma #A2228)</td>
<td>1:2,500</td>
<td>(Kanakkanthara et al. 2011)</td>
</tr>
</tbody>
</table>
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Figures

Figure 1. Modular Incubator Chamber-MIC-101.
Figure 2a. Immunohistochemical staining primary ameloblastoma first patient. Pan- 
cytokeratin 1:1,000. Magnification 10x.
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Figure 2b. Immunohistochemical staining recurrent ameloblastoma first patient. Pan-cytokeratin 1:1,000. Magnification 10x.
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Figure 2c. Immunohistochemical staining primary ameloblastoma second patient. Pan-cytokeratin 1:1,000. Magnification 10x.
Figure 3a. Immunohistochemical staining primary ameloblastoma first patient. Vimentin 1:1,000. Magnification 10x.
Figure 3b. Immunohistochemical staining recurrent ameloblastoma first patient. Vimentin 1:1,000. Magnification 10x.
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Figure 3c. Immunohistochemical staining primary ameloblastoma second patient. Vimentin 1:1,000. Magnification 10x.
Figure 4a. Immunohistochemical staining primary ameloblastoma first patient. p62 1:1,000. Magnification 10x.
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Figure 4b. Immunohistochemical staining recurrent ameloblastoma first patient. p62 1:1,000. Magnification 10x.
Figure 4c. Immunohistochemical staining primary ameloblastoma second patient. p62 1:1,000. Magnification 10x.
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Figure 5. HIF-1α and p-ERK 1/2 western Blotting images
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Figure 6. HIF-1α and p-ERK 1/2 hypoxia graph.

Figure 7. HIF-1α and p-ERK 1/2 post-hypoxia graph.
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Figure 8. Autophagy western blotting images.
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**Figure 9. Autophagy hypoxia graph.**

![Autophagy hypoxia graph](image)

**Figure 10. Autophagy post- hypoxia graph.**

![Autophagy post-hypoxia graph](image)