Cellular senescence and autophagy of myoepithelial cells are involved in the progression of in situ areas of carcinoma ex-pleomorphic adenoma to invasive carcinoma. An in vitro model

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Abstract During tumor invasion, benign myoepithelial cells of carcinoma ex-pleomorphic adenoma (CXPA) surround malignant epithelial cells and disappear. The mechanisms involved in the death and disappearance of these myoepithelial cells were investigated via analysis of the expression of regulatory proteins for apoptosis, autophagy and cellular senescence in an in situ in vitro model. Protein expression relating to apoptosis (Bax, Bcl-2, Survivin), autophagy (Beclin-1, LC3B) and cellular senescence (p21, p16) was evaluated using indirect immunofluorescence. β -galactosidase expression was assessed via histochemistry. Biopsies of CXPA (ex vivo) allowed immunohistochemical evaluation of p21 and p16, whilst LC3B, p21 and p16 protein expression was analyzed by western blotting. In the in vitro model, the myoepithelial cells were positive for LC3B (cytoplasm) and p21 (nucleus), whilst in vivo positivity for p21 and p16 was observed. In vitro, β -galactosidase activity increased in the myoepithelial cells over time. Western blotting analysis revealed an increased LC3B, p16 and p21 expression in the myoepithelial cells with previous contact with the malignant cells when compared with those without contact. The investigation of behavior of benign myoepithelial cells in ductal areas of CXAP revealed that the

myoepithelial cells are involved in the autophagy-senescence phenotype that subsequently leads to their disappearance.

Keywords Autophagy · Cellular Senescence · Myoepithelial Cells · Tumor Microenvironment

Abbreviations

AE1/AE3	Pan cytokeratin
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonistic killer
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma-2 protein
Bcl-xl	B cell lymphoma-extra long
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
cIAP	Baculoviral IAP repeat containing
CK7	Cytokeratin 7
CXAP	Carcinoma ex-pleomorphic adenoma
DAPI	4'-6-diamidino-2phenylindole
DMEM	Dulbecco's modified Eagle medium
DCIS	Ductal carcinoma in situ
EDTA	Ethylenediaminetetraacetic acid
IAP	Apoptosis inhibitors proteins
LC3	Microtubule-associated protein 1 light chain 3
Mcl-1	Myeloid cell leukemia 1 protein
NAIP	NLR family, apoptosis inhibitory proteins
PA	Pleomorphic adenoma
PBS	Phosphate buffer saline
RIPA	Radio immuno precipitation assay buffer
TBST	Tris-buffered saline and tween 20
XIAP	X-linked inhibitor of apoptosis protein

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Introduction

Carcinoma in situ is a precursor lesion that can give rise to invasive cancer. Breast is the most studied carcinoma in situ, with research in this field primarily focusing on prognostic and predictive biomarkers (Bartlett et al. 2014), as well as the tumor stroma, which has been implicated in the invasion process (Metwaly et al. 2012). Despite the great bulk of studies dealing with this tumor, there is still little understanding of the events involved in the progression of in situ to invasive carcinoma.

Although in situ carcinoma in salivary gland is a rare event, it can be observed in areas of carcinoma ex-pleomorphic adenoma (CXPA), where in situ areas are characterized by the presence of benign myoepithelial cells surrounding malignant epithelial cells, both originating from pleomorphic adenoma (PA).

In studies of CXPA using immunohistochemistry, myoepithelial cells in direct contact with malignant epithelial cells exhibited differentiation in in situ areas, observed by the presence of all of the normal myoepithelial cell immunomarkers, which is a rarity in PA (Altemani et al. 2005; Araújo et al. 2006). Various reports, mainly in breast cancer, consider that myoepithelial cells act as a tumor suppressor, since they present a low matrix degrading enzyme expression, yet produce high levels of proteinase inhibitors, (Sternlicht and Barsky 1997; Sternlicht et al. 1997) which makes the invasion process and angiogenesis more difficult (Nguyen et al. 2000; Jones et al. 2003; Barsky and Karlin 2005; Silva et al. 2012). Myoepithelial cells have also been reported to exert an anti-proliferative effect on the tumor cells (Shao et al. 1998). In CXPA, however, their role as a tumor suppressor fails and they can no longer survive, evident by the presence of both in situ and invasive areas in this tumor. The absence of myoepithelial cells could be attributed to cell death, whose mechanisms, including apoptosis, autophagy and senescence, have been widely studied in tumorigenesis.

Apoptosis is a highly regulated form of cell death, in which, the organism self-maintains homeostasis and growth control, which are important for both physiological and pathological conditions (Townson et al. 2003; Wong 2011). This process is characterized by specific morphological and biochemical changes in the dying cells (Ouyang et al. 2012). Among the central regulators of apoptosis are the Bcl-2 family, which includes both pro- (Bax, Bak, Bad) and anti-apoptotic regulators (Bcl-2, Bcl-xl, Mcl-1) (Placzek et al. 2010), as well as inhibitors of apoptosis (IAPs), including Survivin, NIAP, XIAP and c-IAP (Plati et al. 2011; Ulukaya et al. 2011; Cheung et al. 2011).

Autophagy, a cellular degradation and recycling process highly conserved in eukaryotes, was originally identified as a mechanism for survival under conditions of stress, such as in nutrient or energy starvation (Ouyang et al. 2012; Kondo et al.

2005). Despite autophagy being a primarily cytoprotective mechanism, excessive self-digestion can also be detrimental (Cao and Klionsky 2007; Pattingre et al. 2008). The most significant genes to have been studied to date are BECLIN1 and LC3B (Chen and Karantza-Wadsworth 2009; Miracco et al. 2010), with many studies having demonstrated the influence of deregulation in their expression during tumorigenesis (Levine 2007; Roy and Debnath 2010).

Senescence is a cellular program that leads to an irreversible arrest of cell growth, associated with dramatic changes in cell morphology (large flat cells), metabolism, gene expression and secretion patterns (senescence-associated secretory phenotype or SASP) (Shay and Roninson 2004; Evan and Fagagna 2009; Dulic 2013). This irreversible cell cycle arrest is established and maintained by the p53-p21 and p16-pRB tumor suppressor pathways, via inactivation of Cyclin-dependent Kinase (CDK) and key cell cycle regulators, in response to myriad senescence-inducing stimuli (Dimri 2005; Campisi et al. 2011; Larsson 2011).

Therefore, excited by these facts and based on the in vitro model previously described by Martinez et al. (2012), the aim of this study was to clarify the consequence of cross-talking between malignant epithelial and benign myoepithelial cells, by the evaluation of protein expression for apoptosis, autophagy and cellular senescence, using an in vitro model, which mimics the in situ situation.

Material and methods

Cell culture

This study was conducted following the approval of the Ethical Committee of São Leopoldo Mandic Institute and Research Center, Campinas, Brazil (Number 2011/0401).

Benign myoepithelial cells were obtained from explants of salivary gland Pleomorphic Adenoma (PA) provided by surgery from three different donors and characterized using α -smooth actin, vimentin, CK7 and calponin, as described by Miguita et al. (2010). The oral squamous cell carcinoma cells (CAL 27) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 1 % antimycotic-antibiotic solution (10,000 units of penicillin, 10 mg of streptomycin and 25 μ g of amphotericin B per ml in 0.9 % sodium chloride; Sigma), containing 10 % of donor calf serum (DCS; GIBCO, Buffalo, NY), plated in 60-mm diameter plastic culture dishes and incubated under standard cell culture conditions (37°C, 100 % humidity, 95 % air, and 5 % CO₂). Once confluence was reached, they were detached with 0.05 % trypsin and sub-cultured for the subsequent experiments.

The in vitro model

The in vitro model that mimics an in situ malignant neoplasia was performed according Martinez et al. (2012). For this, 13 mm coverslips were coated with fibronectin (Sigma) at 20 µg/ml and incubated at room temperature for 1 h. Myoepithelial cells were plated on the top of fibronectin. Twenty-four hours after, the culture medium of myoepithelial cells was removed and placed by the non-filtered malignant conditioned medium from squamous cell carcinoma cells, which was prepared 48 h before its utilization. To certify the formation of in situ-like neoplastic areas, the cells were examined by phase contrast microscopy, in each studied period (4, 7, 10 and 14 days) and also immunostained with vimentin and AE1/AE3, markers for tumoral benign myoepithelial cells and squamous cell carcinoma lineage, respectively (Fig. 1). As control, the malignant (CAL27) and the myoepithelial cells were cultured without previous contact of malignant cells.

Indirect immunofluorescence

Cells grown on coverslips were fixed in methanol for 6 min at −20 °C, rinsed in phosphate buffer saline (PBS) and then blocked using 1 % bovine albumin in PBS at room temperature for 30 min. The primary antibodies are described in Table 1. The control staining reaction was performed using PBS as a substitute for the primary antibody. The secondary

Table 1 Primary antibodies

Antibody	Dilution	Clone	Host	Sources
Calponin	1:20	CALP	Mouse	Dako*
α-smooth actin	1:50	1A4	Mouse	Dako*
Vimentin	1:300	V9	Mouse	Dako*
CK7	1:50	OV-TL 12/30	Mouse	Dako*
AE1/AE3	1:75	Polyclonal	Mouse	Dako*
Bcl-2	1:100	Polyclonal	Rabbit	Abcam**
Bax	1:20	6A7	Mouse	Abcam**
Survivin	1:100	Polyclonal	Rabbit	Abcam**
Beclin-1	1:50	EPR1733Y	Rabbit	Abcam**
LC3B	1:500	Polyclonal	Rabbit	Abcam**
p21	1:500	Polyclonal	Rabbit	Abcam**
p16	1:200	4C11	Mouse	Sigma***

*Dako Cytomation, Glostrup, Copenhagen, Denmark

**Abcam, Cambridge, MA, USA

***Sigma-Aldrich, Saint Louis, MO, USA

antibody used was either biotinylated anti-rabbit IgG or anti-mouse IgG (Vector Laboratories Inc, Burlingame, CA, USA). Conjugated fluorescein–streptavidin (Vector) was then used. After washing, the preparations were mounted using Vectashield with DAPI (4′-6-diamidino-2-phenylindole) (Vector) and observed under a conventional Zeiss Axioskop 2 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Germany) equipped with a 63x Plan Apochromatic 1.4NA

Fig. 1 Immunostaining for Vimentin (*green*) in myoepithelial cells (MC) and for AE1/AE3 (*red*) in carcinoma cells (CAL27) on a fibronectin substrata after 4, 7, 10 and 14 days. At 4 days (**a**), malignant epithelial cells surrounded by benign myoepithelial cells assuming a cluster formation are observed. At 7 and 10 days (**b** and **c**) the number of carcinoma epithelial cells is more abundant. After 14 days of cell culture (**d**), malignancy predominates with the disappearance of myoepithelial cells. Bar: A, B=100 µm; C, D=50 µm

and 100x Plan Apochromatic 1.4NA lenses under standard conditions (Carl Zeiss, Oberkochen, Germany). Immunofluorescence was repeated three times for each benign myoepithelial cell donor.

β -galactosidase staining assay

β -galactosidase activity was detected using a senescence β -galactosidase staining kit (Cell Signaling Technology, USA). The cells were washed with PBS and fixed for 15min at room temperature with 2 % formaldehyde and 0.2 % glutaraldehyde in PBS after 4, 7 and 11 days of establishment of the in vitro model. They were then washed twice with PBS and incubated overnight at 37 °C in a dry

incubator with the β -galactosidase staining solution. The cells were observed under a phase contrast microscope (Nikon TS100, Japan).

Immunohistochemistry

One case of PA and 3 cases of CXPA were retrieved from the files of the Departments of Pathology of the São Leopoldo Mandic Institute and Research Center, Campinas, Brazil and the State University of Campinas (UNICAMP). Cellular senescence, using the markers p21 and p16, was evaluated. Three-micrometer-thick serial sections were obtained from paraffin-embedded samples. Once dewaxed, the sections were processed for antigen retrieval.

Fig. 2 Immunostaining for Bax, Bcl-2 and Survivin in benign myoepithelial cells (*) and CAL27 cells (**). All proteins were strongly positive in malignant cells and negative in myoepithelial cells, at all studied time periods. Nuclei stained with DAPI appear in *blue*. Bars: **a, b, c, f, g, h, k, l, m**=100 μ m; **d, e, i, j, n, o**=50 μ m

Endogenous peroxidase was blocked by incubation with 3 % hydrogen peroxide and methanol (1:1). After washing, the sections were incubated with the primary antibodies p21 (1:100, anti-rabbit, polyclonal, Abcam) and p16 (CINtec kit, Roche). Signal detection was performed using DAKO

EnVision Peroxidase (DakoCytomation, Carpinteria, CA, USA), followed by a diaminobenzidine chromogen solution and then counterstained with Mayer's hematoxylin. The reactions were performed using Dako Autostainer Plus (DakoCytomation).

Fig. 3 Immunostaining for Beclin1 and LC3B in benign myoepithelial cells (*) and CAL27 cells (**). All proteins were positive in malignant cells, yet only LC3B was observed in myoepithelial cells, at all studied time periods. Nuclei stained with DAPI appear in *blue*. Bars: **a, b, c, f, g, h**=100 μ m; **d, e, i, j**=50 μ m

Magnetic cell separation

The oral squamous cell carcinoma cells (CAL27) and myoepithelial cells, which were co-cultivated in the in vitro model, were isolated via MACS micro-bead system using miniMACS columns (Miltenyi Biotec GmbH), in order to allow evaluation of autophagy and cellular senescence protein expression for each cell type by western blotting.

After 4 and 10 days, the cells were detached from the petri dishes by 0.05 % trypsin and counted using a Neubauer Chamber. A specific carcinoma cell surface marker was applied, the CD326EpCAM microbead (Miltenyi Biotec GmbH), for positive selection of the oral squamous cell

carcinoma cells. After incubation at 4 °C for 30 min, the cells were washed in 8 ml wash buffer (PBS, pH 7.2, 0.5 % bovine serum albumin (BSA), and 2 mM EDTA) and centrifuged at 300 g for 10 min. The cells were then suspended in 1 ml of wash buffer. For magnetic separation, the cell suspension was poured into a pre-rinsed MS column in the magnetic field of the MACS magnet, which had been previously washed with wash buffer. The magnetic labeled carcinoma cells (CD326EpCAM+) were bound to the column, whilst the non-labeled myoepithelial cells were collected in a falcon tube. The column was removed and the magnetic labeled carcinoma cells released from magnetic field via washing the column out with 1 ml of wash buffer.

Fig. 4 Immunostaining for p21 in benign myoepithelial cells (*) and CAL27 cells (**). p21 was positive in the cytoplasm of malignant cells and the nuclei of myoepithelial cells, at all studied time periods. Nuclei stained with DAPI appear in *blue*. Bars: **a, b, c, f, g, h, k, l, m**=100 μ m; **d, e, i, j, n, o**=50 μ m

Western blotting

After magnetic separation, proteins were extracted from the benign myoepithelial cells with RIPA buffer and were subsequently quantified using a BCA Protein Assay (Thermo Scientific). Protein extracts were separated on a 15 % SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membranes (Hybond; Amersham Biosciences, Piscataway, NJ, USA), and probed for 1 h with the primary antibodies anti-LC3B, anti-p21, anti-p16, (1:1000) diluted in TBST + 5 % skimmed milk. The GAPDH primary antibody was used as an endogenous control (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with either the mouse or rabbit secondary monoclonal antibody (1:2500), the reaction was revealed using Bio-Rad Laboratories (Hercules, CA, USA) Western blotting chemiluminescent detection reagents (Opti-4CN) onto radiographic films (GE Healthcare, Fairfield, CT, USA). OD measurements were performed with NIH Image 1.37 (National Institutes of Health, Bethesda, MD) for scanned membranes. All experiments were performed in 3 separate sets. OD values were calculated as a percentage density normalized to that of the control (myoepithelial cells without contact with malignant cells), taken as 100 %.

Results

Indirect immunofluorescence

Apoptosis markers

Positivity for the anti-Bax, anti-Bcl2 and anti-Survivin regulatory apoptosis proteins was evaluated to confirm the presence of the apoptosis mechanism in the *in vitro* model, as well as in the isolated cell population from the myoepithelial and squamous cell carcinoma cells. The results demonstrated that only squamous cell carcinoma cells exhibited immunostaining for anti-Bax, anti-Bcl2 and anti-Survivin in the isolated conditions, as in the *in situ* model. In all studied conditions, the myoepithelial cells were negative for the aforementioned markers (Fig. 2).

Autophagy markers

The anti-Beclin1 and anti-LC3B autophagy markers are shown in Fig. 3. In both conditions studied, anti-Beclin1 positivity was only seen in for squamous cell carcinoma cells, whilst, anti-LC3B was positive in both squamous cell carcinoma and myoepithelial cells.

Cellular senescence markers

The results demonstrated that for the squamous cell carcinoma cells, anti-p21 and anti-p16 proteins were present in the

cytoplasm as punctuate deposits, in both studied conditions. Anti-p21 was found infrequently, only in the nucleus of myoepithelial cells in the isolated condition, yet immunostaining increased in the *in situ* condition. However, anti-p16 was negative for the myoepithelial cells in all conditions studied (Figs. 4 and 5).

Fig. 5 Staining for p16 in benign myoepithelial cells (*) and CAL27 cells (**). p16 was positive only in the cytoplasm of malignant cells at all studied time periods. Nuclei stained with DAPI appear in blue. Bars: a, b, c=100 μ m; d, e=50 μ m

β -galactosidase staining assay

The expression of the β -galactosidase lysosomal enzyme was evaluated using a colorimetric assay, after 4, 7 and 11 days of cell culture. In both studied conditions, the enzyme was present in myoepithelial cells only. However, in the in situ condition, an increase in staining was observed, principally after the later time intervals. There was no staining for β -galactosidase lysosomal enzyme in the squamous cell carcinoma cells (Fig. 6).

Immunohistochemistry

In order to confirm the in vitro results obtained, immunohistochemistry for cellular senescence proteins in the ex-vivo PA specimens and CXPA in situ areas (Fig. 7) was performed. p21 was observed in the epithelial cells of pleomorphic adenoma ductal structures, whilst in the in situ areas of CXPA, it was present in the nucleus and cytoplasm of the myoepithelial (arrows) and malignant epithelial cells, respectively. However, p16 was only present in the myoepithelial cell nucleus (arrows) of the in situ areas of CXPA.

Western blotting

The expression of the LC3B, p21 and p16 proteins was evaluated in myoepithelial cells by western blotting analysis at 4 and 10 days, to confirm the results obtained by immunofluorescence and immunohistochemical analyses.

After 10 days, LC3B, p21 and p16 protein expression was statistically increased ($P < 0.05$) in the myoepithelial cells with previous contact with the malignant cells when compared with those without contact (Fig. 8) (LC3B – 56.44 %; p21 – 92.27 %; p16 – 89.23 %).

Discussion

Epithelial-mesenchymal transition is a process involving loss of intercellular adhesion, acquisition of increased migration and

invasion potential, all of which are required for tumor invasion and dissemination. This process has been widely studied with regard to the search for genetic alterations in the carcinoma cells, in addition to the role of the tumor microenvironment in progression from in situ to invasive carcinoma (Cowell et al. 2013). Studies have involved both surface and glandular epithelial carcinoma, including ductal carcinoma in situ (DCIS) of the breast, where they are characterized by the presence of intercalated myoepithelial cells between carcinomatous epithelial cells and the basement membrane. The capacity for tumor suppression has been attributed to myoepithelial cells, namely via the formation of a physical barrier, which is lost once invasion occurs. In CXPA of salivary gland, in situ areas are often observed together with invasive areas, alongside contained myoepithelial cells separating the malignant epithelial cells from the basement membrane and extracellular matrix. In CXPA, the myoepithelial cells originate from the PA, and hence are considered benign neoplastic cells as opposed to normal myoepithelial cells, as observed in breast cancer. It is important to highlight that these cells disappear during invasion, despite little attention having been given to this fact. In order to investigate myoepithelial cell death, the model created by Martinez et al. (2012) was used, in which benign myoepithelial cells surround carcinoma cells, simulating an in situ condition.

Primarily, myoepithelial cell apoptosis was studied, with negativity for Bax, Bcl-2 and Survivin observed. These proteins were only detected in squamous cell carcinoma cells in the in vitro model and in cells cultured without previous contact. The deregulated expression of these proteins has been considered a prominent hallmark of cancer (Plati et al. 2011; Roberg et al. 2007), and seemingly it is not the absolute quantity but rather the ratio of these pro- and anti-apoptotic proteins that plays an important role in the regulation of cell death (Wong 2011; Roberg et al. 2007).

Since the expression of the proteins involved in apoptosis was not detected in myoepithelial cells, therefore excluding the possibility that this process could be responsible for their disappearance, the potential involvement of autophagy was investigated using the same model.

Fig. 6 β -Galactosidase expression in the in vitro model. Malignant cells (CAL27) did not exhibit activity for this enzyme. There was an increase in β -Galactosidase expression in the myoepithelial cells at 7 and 11 days. Bars: a, b=50 μ m; c=25 μ m

Fig. 7 Immunohistochemistry for p21 and p16 in PA and CXPA. In PA, myoepithelial cells were negative for both markers (**a** and **c**) whilst in CXPA, the myoepithelial cells were positive for p21 (**b**, arrows) and p16 (**d**, arrows). Bar=250 μ m

In the present study, under both conditions studied, myoepithelial cells were only positive for LC3B, whilst both Beclin1 and LC3B were observed in squamous cell carcinoma cells. This suggests that, in this *in vitro* model, autophagy presents different functions and consequences depending on cell type and tumor compartment, since at the end of the 14-day period oral squamous cell carcinoma cells proliferated whilst myoepithelial cells disappeared. Western-blotting confirmed the induction of autophagy in the benign myoepithelial cells, since an increase in LC3B was detected for myoepithelial cells isolated from the *in situ* model as compared to cells cultured alone. By providing essential nutrients and mitochondrial fuels (L-lactate, ketones, glutamine and free fatty acids) in a paracrine fashion, the induction of autophagy in the stromal cells could “energize” the anabolic

growth of cancer cells. This suggestion corroborates the study by Capparelli et al. (2012b), which proposed an “autophagic tumor stroma model of cancer metabolism”, emphasizing that autophagy may be both compartment- and cell-type specific, playing an important role in tumorigenesis.

Autophagy has been proposed as being sufficient to induce cellular senescence (Chen and Goligorsky 2006; Chen et al. 2012), with autophagy and senescence possibly being part of the same biological phenomenon, termed the “autophagy-senescence transition (AST)” (Capparelli et al. 2012b, c; Narita et al. 2009). Based on these facts, the present study aimed to investigate, *in vitro*, senescence in myoepithelial cells, analyzing the expression of the senescence protein markers p16 and p21. Western blotting analysis revealed an increased expression of p16 and p21 in the myoepithelial cells isolated from the *in vitro* model when compared with cells cultured without previous contact with malignant cells. These results showed that the contact between the malignant and benign myoepithelial cells may lead to the induction of senescence. Immunohistochemistry for senescence proteins in three *ex vivo* specimens of CXAP was also performed. p21 and p16 were observed in the nuclei of the myoepithelial cells in the *in situ* areas of CXAP, whilst p21 was also detected in the myoepithelial cell nuclei in the *in vitro* model. Moreover, *in vitro* activity of the β -galactosidase lysosomal enzyme, which is pronounced in senescent cells, was only observed in the myoepithelial cells, principally at the later studied time intervals. Altogether, these results indicate that myoepithelial cells could be undergoing a differentiation and/or senescence

Fig. 8 LC3B, p21 and p16 protein expression in myoepithelial cells. Representative immunoblot of LC3B, p21 and p16 in lysates of myoepithelial cells alone (**a**) or isolated from the *in vitro* model (**b**), after 4 and 10 days of cell culture. GAPDH was used as loading control

processes. Therefore, the benign myoepithelial cells in this in vitro in situ model became autophagic and senescent.

Various studies have suggested that senescent microenvironment cells may present tumor promoting activities, which could be attributed to the secretion of numerous cytokines, growth factors and proteases that facilitate cancer progression, known as senescence-associated secretory phenotype (SASP) (Campisi et al. 2011; Martinez et al. 2010, 2013; Campisi 2011; Campisi and Fagagna 2007). Furthermore, the autophagic-senescent phenotype of stromal cells result in the production of high-energy mitochondrial fuels transferred to epithelial cancer cells to satisfy their high-energy demands, driving anabolic tumor growth (Capparelli et al. 2012a, b).

Considering the cross-talking between malignant epithelial and benign myoepithelial cells, the results of this study suggest that the autophagy-senescence phenomenon may explain why benign myoepithelial cells of CXPA are more differentiated than myoepithelial cells of ductal structures of PA, as demonstrated by Araújo et al. (2006).

In conclusion, the investigation of the behavior of benign myoepithelial cells in ductal areas of carcinoma ex pleomorphic adenoma revealed that the myoepithelial cells are involved in the autophagy-senescence phenotype that leads to their disappearance. This mechanism liberates the first invasion barrier for the malignant cells, explaining one of the early events involved in the progression of in situ to invasive carcinoma.

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Conflict of Interest The authors declare that they have no conflict of interest.

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