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ORIGINAL ARTICLE

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Microvesicles derived from squamous cell carcinoma induce cell death, autophagy, and invasion of benign myoepithelial cells

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Abstract

Background: There has been great interest recently in the mechanisms of cell-to-cell communication through microvesicles (MV). These structures are produced by many different cell types and can modulate cellular activity by induction of epigenetic alterations. These vesicles may promote tumor mass increase either by stimulating cell proliferation *via* growth factors or by inhibiting apoptosis, which reinforces the role of such vesicles as important modulators of tumor progression.

Methods: The present in vitro study aimed to characterize MV derived from malignant neoplastic epithelial cell cultures (EP) and their effect on the expression of apoptosis/autophagy and invasion related genes of benign myoepithelial (Myo) cell cultures.

Results: The results revealed round structures with a mean size of 153.6 (±0.2) nm, with typical MV morphology. CD63 quantification indicated that EP cell culture at 70%-80% confluence secreted 3.088 × 10^8 MV/mL. Overall, Myo exposed to MVs derived from EP showed both up- and downregulation of tumorigenesis promoting genes. MVs from EP cells promoted cell death of Myo cells and positively modulate *BAX*, *SURVIVIN*, *LC3B*, *MMP-2*, and *MMP-9* expression. Furthermore, an increasing of MMP-2 and MMP-9 secretion by Myo was observed after MV exposure.

Conclusions: These findings suggest that MVs from EP modulate autophagy of Myo cells, which may, in part, explain the disappearance of these cells in in situ areas of invasive carcinoma ex-pleomorphic adenoma. Additionally, the overexpression of MMPs contributes to the development of an invasive phenotype of Myo cells, which could favor the dissolution of the basement membrane during tumorigenesis process.

KEYWORDS

cell biology, cell death, cell-derived microvesicles, salivary gland neoplasms, tumor microenvironment

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1 | INTRODUCTION

Cellular communication is essential for normal development of multicellular organisms. The classic mechanisms of cellular communication involve the participation of cellular junctions as well as adhesion molecules and soluble factors secreted by cells, which can act in an autocrine, paracrine, and endocrine manner.[1]

In carcinogenesis, communication among cells within the tumor microenvironment is fundamental for the development and progression of neoplastic tissues. Additionally, studies have been supporting the role of cellular interactions as important modulators of aggressiveness in neoplastic cells.[2] In this context, with the purpose of evaluating the interactions between cells, Martinez et al[3] developed an in vitro model that mimics an in situ tumor condition. In this model, myoepithelial (Myo) cells derived from pleomorphic adenoma are co-cultivated with malignant neoplastic epithelial cells, thus permitting specific analysis on how reciprocal inductions can affect cell phenotype. Indeed, Silva et al[4] observed an induction of senescence and autophagy processes in Myo cells mediated by malignant epithelial cells, which could explain the reduction in the former cell population during the progression of carcinomas ex-pleomorphic adenoma.[5,6]

Recent studies have demonstrated that cell-cell communication may be mediated by vesicles known as microvesicles (MVs) or exosomes.[7] These vesicles are derived from the endocytic-lysosomal system and are surrounded by a bilipid membrane associated with surface proteins, such as CD63 tetraspanins and major histocompatibility factors I and II.[8] MV may vary in size from 100 to 1000 nm and are produced by a variety of cells, both in physiological and pathological conditions.[9]

MVs are capable of affecting cellular activity by inducing epigenetic changes, which occur due to the influence of proteins, mRNA, and micro RNA (miRNA).[10] In malignant neoplasia, MV positively modulate different stages of tumorigenesis.[11] In fact, studies demonstrate that these vesicles promote an increase in tumor mass either by stimulating cell proliferation *via* growth factors or by inhibiting apoptosis process,[12] which reinforces the important role played by such vesicles on modulating tumor progression. Moreover, MVs from various cell types contain metalloproteinases (MMP), such as MMP-2 and MMP-9, that are involved in local invasion by extracellular matrix (ECM) degradation.[13]

Considering the importance of MV in tumor biology and cellular communications and the lack of studies in salivary gland neoplasia, the present in vitro study aims to characterize MV derived from malignant epithelial cell cultures and to assess their effects on apoptosis/autophagy gene expression, as well as molecules involved in tumor progression, such as MMPs and tissue inhibitor of metalloproteinases (TIMP), of Myo cell cultures.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

Benign neoplastic Myo cells derived from three primary pleomorphic adenomas were established, characterized, and frozen according to the methodology described by Miguita et al.[14] Briefly, the cells were thawed and transferred to centrifuge tubes containing 10 mL of Dulbecco's modified Eagle's medium (DMEM, Invitrogen/Life Technologies, CA, USA) and centrifuged at 336 g for 3 min. The supernatant was discarded, and the cells grew in culture flasks of 75 cm^2 (Corning Costar, Corning, NY) containing DMEM supplemented with 10% fetal bovine serum (Invitrogen/Life Technologies), 50 μg/mL of vancomycin (Acros Organics, Geel, Antwerp, Belgium), and 50 μg/mL gentamicin (Invitrogen/Life Technologies). The culture medium was changed every 2 or 3 days. Throughout the culture time, the cells were maintained at 37°C in humidified atmosphere containing 5% CO_2 and 95% atmospheric air. All procedures were approved by the Research Ethics Committee of the São Leopoldo Mandic Institute and Research Center, Campinas, Brazil (Registration 2011/0401). The cells were cultured up to the seventh passage maximum.

Malignant epithelial cells (CAL27, ATCC, Manassas, VA; EP) were thawed and transferred to centrifuge tubes containing 10 mL DMEM/Ham's F12 (Invitrogen/Life Technologies) and centrifuged at 336 g for 3 min. The supernatant was discarded, and the cells were cultivated in 75 cm^2 culture flasks (Corning) containing DMEM/Ham's F12 supplemented with 10% fetal bovine serum (Invitrogen/Life Technologies), 50 μg/mL of vancomycin (Acros Organics), 50 μg/mL gentamicin (Invitrogen/Life Technologies), and 0.4 μg/mL hydrocortisone (Sigma Aldrich, St. Louis, MO). The cultures were maintained at the same conditions as described above.

2.1.1 | MV isolation, identification, and quantification

Once reached 70%-80% of cell confluence in 75 cm^2 flasks, the medium of EP cell cultures was discarded and a serum-free medium was added. The cell cultures were maintained at 37°C for 18 hours. The medium was then supplemented with 4-aminophenylmercuric acetate (Sigma Aldrich) for 6 hours.[15] Subsequently, the medium was collected and centrifuged for 30 minutes at 2000 g. The supernatant was collected and transferred to a 50 mL tube, which *Total Kit exosome Isolation Reagent* (Invitrogen/Life Technologies) solution was added. Incubation was overnight at 4°C. Afterward, the solution was centrifuged for 1 hour at 10 000 g. The supernatant was discarded, and the pellet was resuspended in phosphate buffer.

2.2 | Scanning electron microscope

MV were identified by scanning electron microscopy as previously described by Sokolova et al.[16] Briefly, MV were fixed in phosphate-buffered solution of glutaraldehyde at 3.7% for 15 minutes. They were then dehydrated in increasing concentrations of ethanol solution (40%, 60%, 80%, and 100%), deposited on glass coverslips, and left at room temperature for 24 hours. Subsequently, samples were sputter coated with gold and examined under scanning electron microscope (SEM).

2.3 | Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to determine the number of MV secreted by EP cell cultures in a LM20 instrument (NanoSight, Amesbury, UK) equipped with a 532 nm laser. As described by Filipe et al,[17] the technique is based on light scattering and the individual track of Brownian motion of nanoparticles and is the only real-time approach to establish MV concentration (particle/mL). The solution containing the MV was injected into the sample chamber with sterile syringes until the liquid filled the tip of the syringe. All measurements were performed at room temperature.

2.4 | MV quantification by ELISA

Quantification of MV was performed using CD63 ExoELISA Kit (System Biosciences, Mountain View, CA), according to the manufacturer's instructions.

2.5 | MV treatment on Myo cells

To assess the effects of MV, benign Myo cells were culture in serum-free media containing ≅ 2-3 × 10^7 MV/10^3 cells, for 48 h. As negative control, benign Myo cells were cultures in the absence of MV.

2.6 | PCR Array

The expression of genes involved with tumorigenesis in Myo cells exposed to MVs from malignant epithelial cells after 48 h of cell culture, under the experimental conditions described above, was evaluated by means of the RT^2 Profiler™ PCR Array System (Human Cancer Pathway Finder, Cat # PAHS-033ZC, SuperArray Bioscience Corporation, Frederick, MD). First strand synthesis was performed with 1 μg of total RNA using the RT2 PCR Array First Strand Kit (SuperArray Bioscience Corporation). Five housekeeping genes (*Beta-2-microglobulin*, *Hypoxanthine phosphoribosyltransferase 1*, *Ribosomal protein L13a*, *Glyceraldehyde-3-phosphate dehydrogenase*, and *Beta-actin*) were used for the normalization. Amplification and detection were performed using 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA).The results were calibrated according to Myo cells that grown alone and analyzed by RT^2 Profiler™ PCR Array Data Analysis Template v3.5. The data were analyzed using the $2^{-\Delta\Delta CT}$ method.

2.7 | Expression of apoptosis, autophagy, and invasion genes

After 48 hours of exposure to MVs derived from EP cells, total RNA from Myo cell cultures was collected and the expression of *BAX*, *BCL-2*, *SURVIVIN*, *BECLIN-1*, *LC3B*, *MMP-2*, *MMP-9*, *TIMP-1*, and *TIMP-2* was evaluated by qPCR. The primer sets (Invitrogen; Thermo Fisher Scientific, Inc) were as follows: F5'- TGTTTTCTGACGGGCAACTTCA-3' and R5'-CAGTTCCGGCACCTTGGT-3' for *BAX*; F5'-AACTTGACAGAGGATCATGCTGTACT-3' and R5'-GATCTTTATTTCATGAGGCACGTTATT-3' for *BCL-2*; F5'- AAGGACCACCGCATCTCTACA-3' and R5'-CCAAGTCTGGCTCGTTCTCAGT-3' for *SURVIVIN*; F5'-TGGCAGAAAATCTCGAGAAGGT-3' and R5'-GCTGCTGTCGTTTAAATTCACTGAT-3' for *BECLIN-1*; F5'-CCATGCCGTCGGAGAAGA-3' and R5'-CTGCTCTCGAATAAGTCGGACAT-3' for *LC3B*; F5'-AGCGAGTGGATGCCGCCTTTAA-3' and R5'-CATTCCAGGCATCTGCGATGAG-3' for *MMP-2*; F5'-GCCACTACTGTGCCTTTGAGTC-3' and R5'- CCCTCAGAGAATCGCCAGTACT-3' for *MMP-9*; F5′-TTCGTGGGGACACCAGAAGTCAAC-3′ and R5′-TGGACACTGTGCAGGCTTCAGTTC-3′ for *TIMP-1*; F5′-AAGCGGTCAGTGAGAAGGAGTGG-3′ and R5′-CCTTGGAGGCTTTTTTGCAGTTG-3′ for *TIMP-2* and F5'- GCACCGTCAAGGCTGAGAAC-3' and R5'-CCACTTGATTTTGGAGGGATCT-3' for *GAPDH*, used as internal gene reference.

2.7.1 | RNA isolation

For total RNA isolation from the MV-exposed Myo cell cultures, TRIzol reagent (Invitrogen/Life Technologies) was used according to the manufacturer's instructions. Briefly, cells grown in 10 cm^2 plates were homogenized with 1 mL of TRIzol. Then, 0.2 mL of chloroform was added, and the samples were centrifuged at 12 000 g for 15 minutes at 4°C. Total RNA was precipitated from the aqueous phase with 0.5 mL of isopropanol. Then, the samples were centrifuged at 16 000 g for 10 min and the pellet was rinsed in 75% ethanol. The samples were centrifuged again, and the pellet was resuspended in nuclease-free water. RNA samples were obtained in duplicate for each condition studied. The integrity of the samples was verified by 1.2% agarose gel electrophoresis. Integral samples exhibit two ribosomal subunits: 18S and 28S. After electrophoresis, RNA samples were stored at − 80°C until use.

2.7.2 | Synthesis of complementary DNA (cDNA)

Total RNA was quantified using a spectrophotometer (NanoVue, GE Healthcare Life Sciences, Piscataway, NJ). Complementary DNA (cDNA) was synthesized using 1 μg of total RNA and a reverse transcription reaction kit (Superscript III RTTM kit, Invitrogen/Life Technologies). The reaction occurred for 50 minutes at 50°C, followed by 5 minutes at 85°C.

2.7.3 | qPCR

qPCR was performed using SyberGreen as the detection system. The reactions were carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). For each reaction, a total of 40 ng cDNA were used. Relative gene expression was calculated using

the comparative $2^{-\Delta\Delta Ct}$ method. PCR conditions were performed at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All samples were run in triplicates, normalized to internal GAPDH, and calibrated to the Control group levels (Myo cells not exposed to MVs).

2.8 | The effect of MV on apoptosis rate

Myo cells were exposed to MVs derived from EP cells. After 48 hours, the cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS, pH 7.4). After centrifugation, the cells were suspended in a 100 µL containing annexin V fluorescein isothiocyanate (Annexin V-FITC, MACS Miltenyi Biotec, Bergisch Gladbach, GE) and propidium iodide (PI, MACS Miltenyi Biotec). After 15 minutes under dark conditions, the cells were resuspended with 400 µL binding buffer (MACS Miltenyi Biotec). The percentage of apoptotic or dead cells was established by flow cytometer using Gallios flow cytometer (Beckman Coulter, USA).

2.9 | ELISA

After 48 h of MVs exposure, the supernatants from Myo cell cultures were harvested and centrifuged at 5000 g for 15 minutes at 4°C. Aliquots of each sample were assayed using enzymatic immunosorbent assay (ELISA) to determine the MMP-2, MMP-9, TIMP-1, and TIMP-2 levels, according to the manufacturer's recommendations (R&D Systems, USA). Briefly, 100 µL of detection antibody was added to each well, with the exception of the blank well, mixed gently, and incubated overnight (16-24 hours) at 4°C. The wells were then rinsed three times prior to adding the standard and supernatant in duplicate. After incubation, the plates were again washed and incubated with 200 µL of conjugate at room temperature for 60 minuts. The plates were then rinsed three times before 200 µL of substrate was added and subsequently incubated for 15 minutes in the dark at room temperature. The reaction was stopped by adding 50 µL of stop solution, and the color was measured in an automated microplate spectrophotometer (Epoch, Biotek, Winooski, VT, USA). Total MMPs and TIMPs were quantitated in nanograms per ml (ng/mL). Results were calculated using the standard curves created in each assay. The ELISA assays were performed in a blind fashion in triplicate.

2.10 | Statistical analysis

All experiments were performed in biological triplicates. The numerical data were subjected to the Mann-Whitney test using SigmaStat software, version 3.5 (Systat Software Inc, San Jose, CA). PCR array data were analyzed by Student's t test followed by Benjamini-Hochberg multiple test correction. The cutoff applied for the fold change in gene expression was 2.0. For all data, the significance level was set at 5%.

3 | RESULTS

3.1 | Scanning electron microscopy

The representative images of isolated MVs from EP cell cultures are demonstrated in Figure 1. The images revealed round structures with size ranging from 100 to 180 nm.

3.2 | Nanoparticle tracking analysis

MVs size distribution and concentration are depicted in Figure 2A. MVs derived from EP cell cultures exhibited a mean size of 153.6 (± 0.2) nm. Screenshot of NTA from EP MVs is demonstrated in Figure 2B.

3.3 | Microvesicles quantification

CD63 quantification by ELISA indicated that EP cell cultures at 70%-80% confluence secreted 3.088×10^8 MVs/mL.

3.4 | PCR Array

Myo cells cultured with MVs from EP cells overexpressed genes involved to growth factors and tumorigenesis (Table 1).

3.5 | Expression of apoptosis, autophagy, MMP-2, MMP-9, TIMP-1, and TIMP-2 genes

The effect of MVs from EP cells on the expression of apoptosis and autophagy genes in Myo cell cultures is represented in Figure 3. The results indicated that MVs positively modulate *BAX*, *LC3B*, and *SURVIVIN*

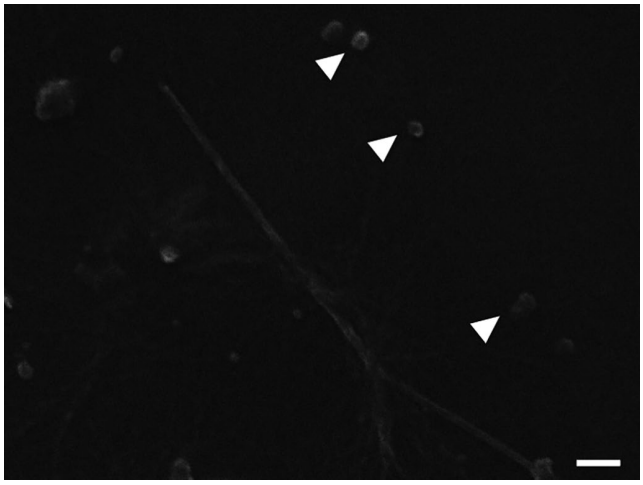

FIGURE 1 Morphological characterization of microvesicles (head arrow) isolated from EP cell cultures by SEM. Scale bar = 350 nm

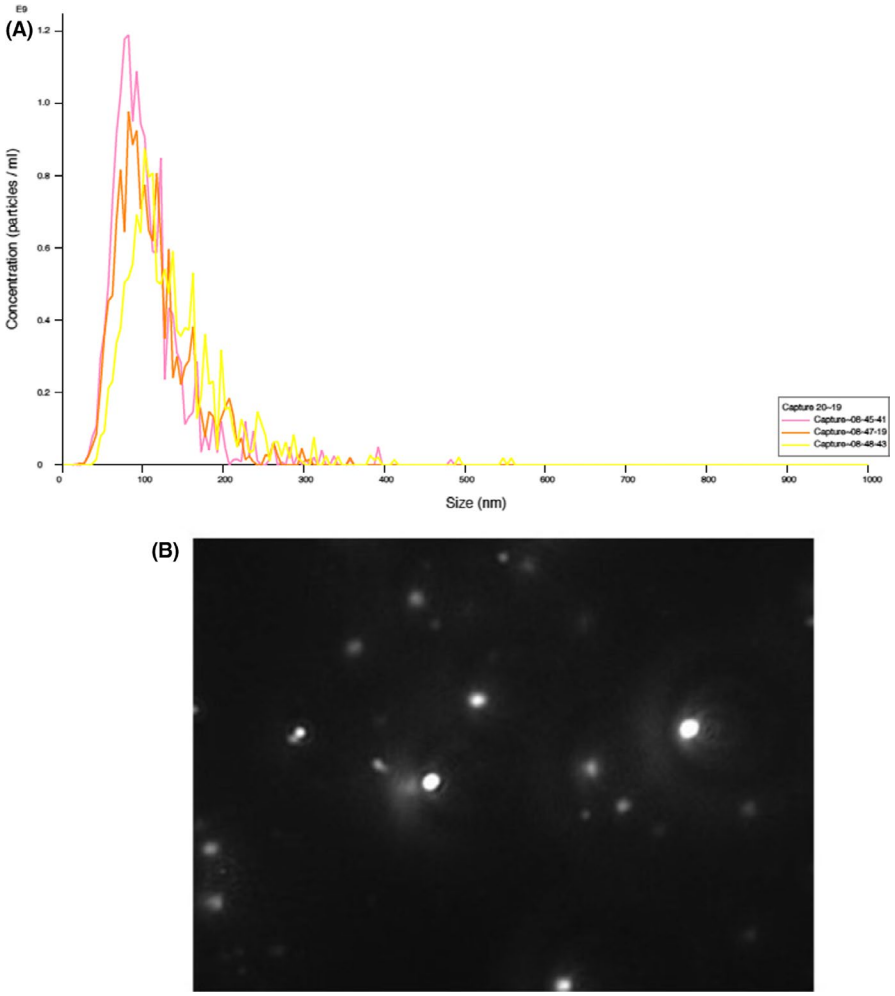

FIGURE 2 NTA analysis of MV derived from EP cell cultures. (A) Size distribution and concentration of MV. (B) Screenshot of NTA from EP MV

expression and negatively modulate *BCL-2* expression ($P < .05$). *BECLIN-1* was not affected ($P > .05$). In contrast, there was an increase in *MMP-2*, *MMP-9*, *TIMP-1*, and *TIMP-2* gene expression in Myo cells exposed to MVs from EP cells when compared to control group ($P < .05$).

3.6 | Cell death and apoptosis

The flow cytometer analysis for annexin V assay revealed no difference on Myo cells either exposed or not to MVs from EP cell cultures (Figure 4A). However, MVs derived from EP cell cultures promoted cell death of Myo cell cultures, as demonstrated by propidium iodide staining, with 6.2%- PI positive Myo cells (Figure 4B).

3.7 | ELISA

MMP-2, MMP-9, TIMP-1, and TIMP-2 levels are represented in Figure 5. MMP-2 and MMP-9 levels were increased in Myo cells exposed to MVs from EP cells when compared to Myo control group. There was a significant reduction in TIMP-2 levels when Myo cells were exposed to MVs from EP ($P < .05$). TIMP-1 levels showed no statistical difference.

4 | DISCUSSION

The results of the present study indicate that MVs from EP cells positively modulate the expression of genes associated with proliferation, differentiation, and regulation of cell death processes in benign Myo cells. These findings suggest that MV produced by malignant epithelial cells may play an important role in the transition from in situ to invasive carcinoma in cases of carcinomas ex-pleomorphic adenoma (CXAP). During the progression of this neoplasia, Myo cells disappear,[5,6] and recent evidence has suggested that these cells may undergo autophagy-senescence.[4]

In this context, interactions between tumor cells and stroma are crucial to tumor progression. MVs are considered a novel class of intercellular signal mediators secreted by different cell types and may drive cell signaling in both physiological and pathological processes.[18] Accordingly, mRNAs and microRNAs present inside such MV can be transferred between cells, modulating gene expression, and, consequently, promoting tumor progression.[9,12] Thus, this study aimed to characterize MV derived from EP cell cultures and to assess their participation on possible biological mechanisms, for instance, apoptosis and autophagy, on Myo cell disappearance during CXPA progression.

TABLE 1 Tumorigenesis gene expression of Myo cell cultures isolated or exposed to microvesicles derived from EP cell cultures (Myo + MV EP)

Function/symbol/description	Relative expression Myo + MV CAL 27
Angiogenic growth factor	
ERAP1 *(Endoplasmic reticulum aminopeptidase 1)*	–
EREG *(Epiregulin)*	3.98
FGF1 *(Fibroblast growth factor 1)*	2.26
FGF2 *(BFGF – Fibroblast growth factor 2)*	–
FIGF *(VEGFD – Vascular endothelial growth factor D)*	−7.46
IL18 *(Interleukin 18)*	–
JAG1 *(Jagged canonical Notch ligand 1)*	−2.28
PGF *(Placental growth factor)*	–
TNNT1 *(Troponin T1, slow skeletal type)*	−3.88
TYMP *(Thymidine phosphorylase)*	4.52
VEGFA *(Vascular endothelial growth factor A)*	–
Apoptosis regulation	
GDNF *(Glial cell-derived neurotrophic factor)*	2.27
IL1A *(Interleukin 1 alpha)*	6.01
IL1B *(Interleukin 1 beta)*	11.91
IL2 *(Interleukin 2)*	−6.46
SPP1 *(Secreted phosphoprotein 1)*	3.49
VEGFA *(Vascular endothelial growth factor A)*	–
Cell differentiation	
BMP2 *(Bone morphogenetic protein 2)*	–
BMP3 *(Bone morphogenetic protein 3)*	–
BMP4 *(Bone morphogenetic protein 4)*	−2.39
BMP5 *(Bone morphogenetic protein 5)*	
BMP6 *(Bone morphogenetic protein 6)*	2.44
BMP8B *(Bone morphogenetic protein 8b)*	–
CSF1 *(Colony stimulating factor 1)*	2.88
CSPG5 *(Chondroitin sulfate proteoglycan 5)*	3.57
ERAP1 *(Endoplasmic reticulum aminopeptidase 1)*	–
EREG *(Epiregulin)*	3.98
FGF1 *(Fibroblast growth factor 1)*	2.26
FGF2 *(BFGF – Fibroblast growth factor 2)*	–
FGF22 *(Fibroblast growth factor 22)*	–
FGF9 *(Fibroblast growth factor 9)*	−6.35
FIGF *(VEGFD – Vascular endothelial growth factor D)*	−7.46
IL11 *(Interleukin 11)*	–
IL12B *(Interleukin 12B)*	−32.91
IL2 *(Interleukin 2)*	−6.46
INHA *(Inhibin subunit alpha)*	−3.61
INHBA *(Inhibin subunit beta A)*	–
JAG1 *(Jagged canonical Notch ligand 1)*	−2.28
JAG2 *(Jagged canonical Notch ligand 2)*	–
LTBP4 *(Latent transforming growth factor beta binding protein 4)*	–
MDK *(Midkine)*	−2.76
NRG1 *(HGL – Neuregulin 1)*	–
OSGIN1*(Oxidative stress induced growth inhibitor 1)*	5.12
PGF *(Placental growth factor)*	–
SLCO1A2 *(Solute carrier organic anion transporter family member 1A2)*	−2.66
SPP1 *(Secreted phosphoprotein 1)*	3.49
TNNT1 *(Troponin T1, slow skeletal type)*	−3.88
Developmental regulators	
AMH *(Anti-Mullerian hormone)*	4.80
BDNF *(Brain-derived neurotrophic factor)*	−2.27
BMP2 *(Bone morphogenetic protein 2)*	–
BMP3 *(Bone morphogenetic protein 3)*	–
BMP4 *(Bone morphogenetic protein 4)*	−2.39
BMP6 *(Bone morphogenetic protein 6)*	2.44
BMP8B *(Bone morphogenetic protein 8b)*	–
CECR1 *(ADA2 – Adenosine deaminase 2)*	–
CSF2 *(Colony stimulating factor 2)*	6.20
CSF3 *(Colony stimulating factor 3)*	129.35
CSPG5 *(Chondroitin sulfate proteoglycan 5)*	3.57
CXCL1 *(C-X-C motif chemokine ligand 1)*	536.50
DKK1 *(Dickkopf WNT signaling pathway inhibitor 1)*	–
FGF11 *(Fibroblast growth factor 11)*	–
FGF13 *(Fibroblast growth factor 13)*	−2.07
FGF14 *(Fibroblast growth factor 14)*	−2.01
FGF17 *(Fibroblast growth factor 17)*	–
FGF2 *(Fibroblast growth factor 2)*	–
FGF5 *(Fibroblast growth factor 5)*	–
FGF7 *(Fibroblast growth factor 7)*	–
GDF10 *(Growth differentiation factor 10)*	−3.40
GDNF *(Glial cell-derived neurotrophic factor)*	2.27
GPI *(Glucose-6-phosphate isomerase)*	2.25
HBEGF *(Heparin binding EGF like growth factor)*	10.89
IGF1 *(Insulin like growth factor 1)*	−4.16
IGF2 *(Insulin like growth factor 2)*	−2.34
INHA *(Inhibin subunit alpha)*	−3.61
INHBA *(inhibin subunit beta A)*	–
JAG1 *(Jagged canonical Notch ligand 1)*	−2.28
LEFTY1 *(Left-right determination factor 1)*	−2.34
LEFTY2 *(Left-right determination factor 2)*	–

(Continues)

(Continues)

TABLE 1 (Continued)

Function/symbol/description	Relative expression Myo + MV CAL 27
LIF *(LIF interleukin 6 family cytokine)*	5.41
LTBP4 *(Latent transforming growth factor beta binding protein 4)*	–
MDK *(Midkine)*	−2.76
MSTN *(Myostatin)*	−4.41
NDP *(Norrin cystine knot growth factor NDP)*	−10.00
NGF *(Nerve growth factor)*	–
NODAL *(Nodal growth differentiation factor)*	−3.75
NRG1 *(Neuregulin 1)*	–
NRTN *(Neurturin)*	–
PSPN *(Persephin)*	–
PTN *(Pleiotrophin)*	–
TNNT1 *(Troponin T1, slow skeletal type)*	−3.88
VEGFA *(Vascular endothelial growth factor A)*	–
THPO *(Thrombopoietin)*	−5.70

Note: Data are expressed as the fold-regulation relative to Myo grown alone. All depicted values are statistically significant at 5%. "–" indicates a lack of effect relative to Myo.

The results revealed that EP cells secreted spherical structures, with an average size of 153.6 (± 0.2) nm, compatible with MV dimensions described in literature. These structures are derived from the endosomal system measuring approximately 100 to 1000 nm in diameter, different from exosomes, which vary in size from 40 to 100 nm.[19] Morphological distinction between both types of extracellular vesicles is noticed as well as the molecular content within such structures, which can be highly heterogeneous[20] and may vary even further depending on cell type.[21] The term MV is more appropriate, since in vitro and in vivo studies have demonstrated that isolated vesicles represent a diverse population of either exosomes or shedding vesicles ranging from 40 to 1000 nm in diameter.[19] Notwithstanding that both exosomes and MV contain a myriad of cellular components capable of affecting cellular communication on local and distant cells.

MV are highly enriched in a protein superfamily named tetraspanin,[22] especially the tetraspanins CD9, CD63, CD37, CD81, or CD82, which are commonly expressed in the MV membrane and often used as biomarkers. Among them, CD63 and CD81 are considered the most commonly identified in MV.[23] In EP cells, CD63 has been frequently used as a label for released MV.[24] Indeed, the findings presented herein supported by NTA morphology assay showed that the EP cell culture at 70%-80% confluence secreted approximately 3.088×10^8 MVs/mL.

The results obtained in the proposed in vitro model showed the participation of MV from EP in the modulation of genes involved with proliferation, differentiation, and in general, in regulatory events of angiogenesis and hypoxia, as well as related to the tumor immune response by Myo cells. Although the expression of genes involved with a possible Myo cell suppressing activity was observed, the most significant effects were observed with the expression of genes encoding proteins responsible for proliferation, differentiation, and consequent, tumor progression and invasion. Among these genes, overexpression of CSF (*Colony-stimulating factor*), CXCL1 (*chemokine ligand 1*), EREG (*Epiregulin*), IL11 (*Interleukin 11*), IL1 beta (*Interleukin 1 beta*), LIF (*Leukemia inhibitory factor*), and TYMP (*Thymidine phosphorylase*) and decreased expression of CECR1 (*Cat eye syndrome chromosome region, candidate 1*), FIGF (*C-fos induced growth factor*), and TNNT1 (*Troponin T type 1*) were notably observed.

Apoptosis and autophagy are described as types of programmed cell death that play a crucial role on tissue homeostasis, controlling cell proliferation and death under physiological and pathological conditions. During tumorigenesis, a deregulation of these biological processes occurs, which may contribute to tumor progression.[25] Loss of the complex balance between the Bcl-2 family of pro (*BAX*) and anti-apoptotic (*BCL-2*) proteins can provide cell resistance to apoptotic stimulation and contribute to the survival of cancer cells.[26] Similarly to apoptosis, autophagy is a mechanism that usually occurs at basal levels in cells, though it can be strongly induced in certain cellular stress conditions, for example, cancer.[27]

The results presented herein indicate that the MV from EP cell cultures upregulate *BAX* and downregulate *BCL-2*, which suggests

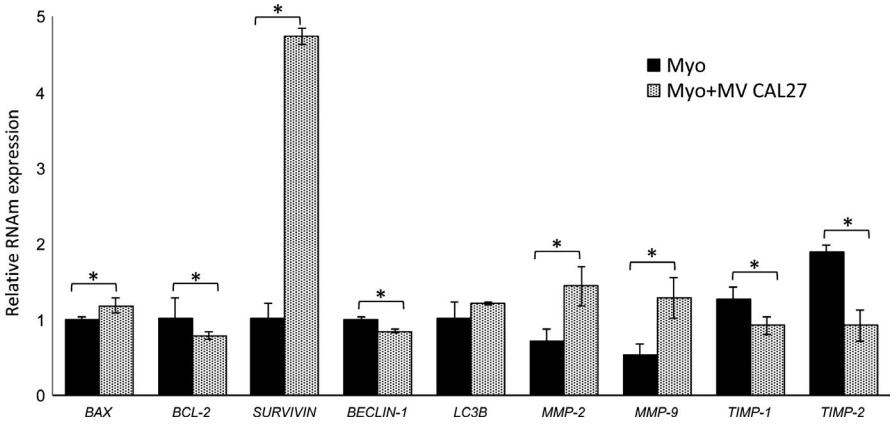

FIGURE 3 Relative BAX, BCL-2, SURVIVIN, BECLIN-1, LC3B, MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression in Myo cell cultures exposed to MVs derived from EP cell cultures (Myo + MV EP). *indicates statistical difference between the studied conditions. The values are expressed in means (± SD)

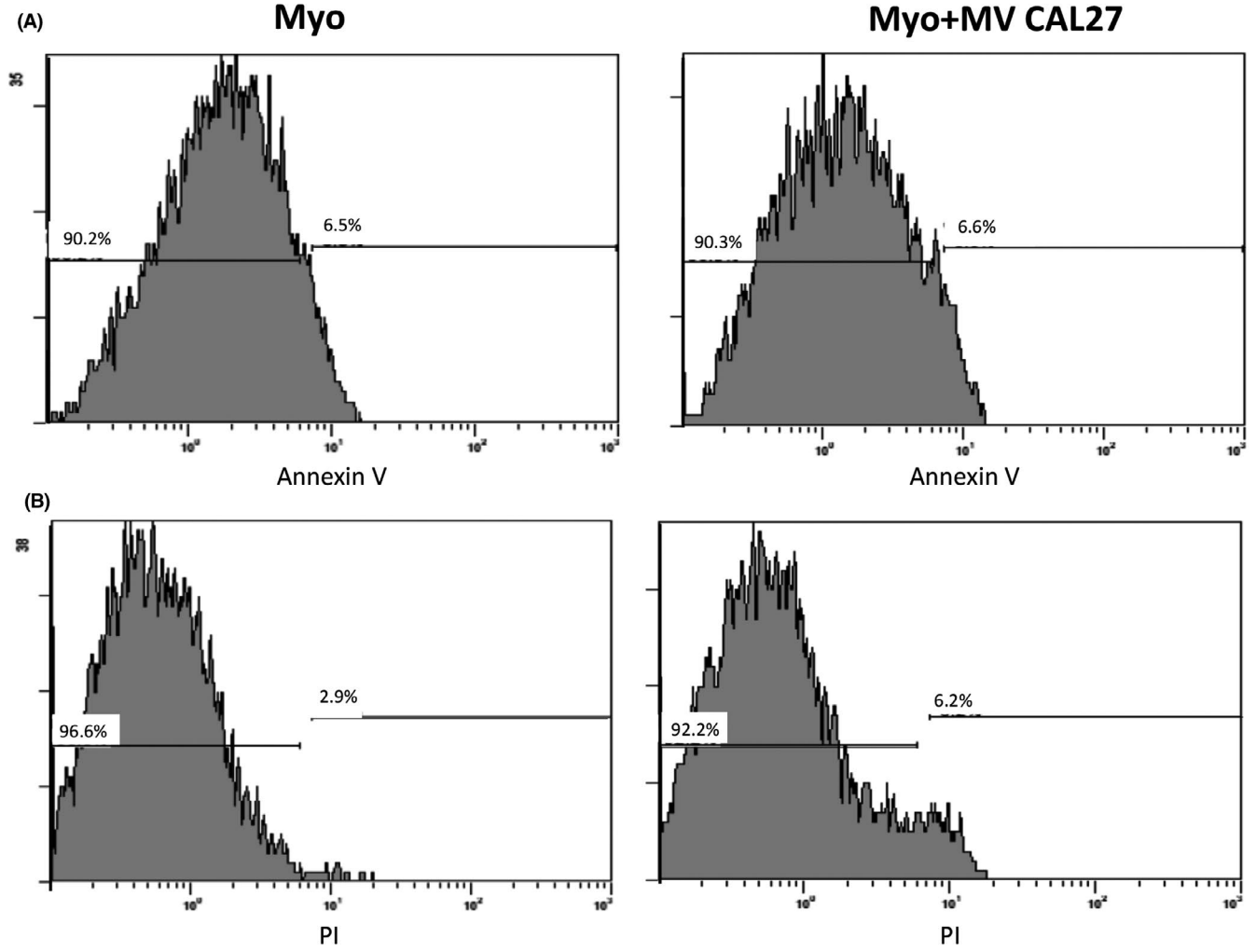

FIGURE 4 Myo cell cultures isolated or exposed to microvesicles derived from EP cell cultures (Myo + MV EP). (A) Apoptosis assay by annexin V. (B) Cell death by propidium iodide. Charts are representative of three experiments

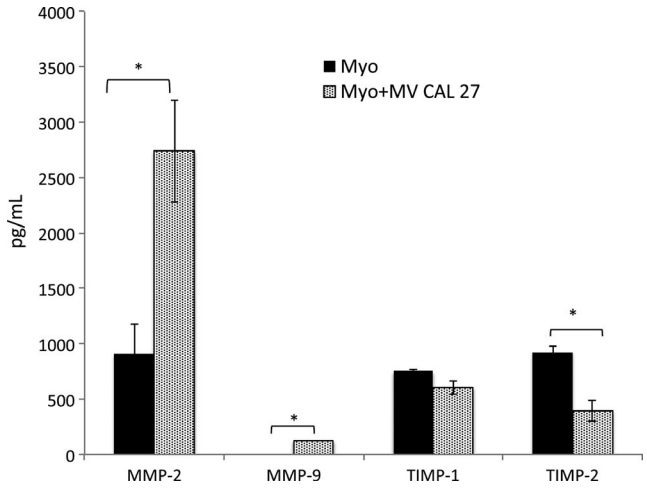

FIGURE 5 MMP-2, MMP-9, TIMP-1, and TIMP-2 (pg/mL) quantification in Myo cell cultures isolated or exposed to microvesicles derived from EP cell cultures (Myo + MV EP). The error bars represent the standard deviations. The asterisk symbol indicates statistical difference among the conditions

an increase in apoptosis. Nevertheless, an upregulation of *SURVIVIN* was observed, which could contradict an increase in apoptosis. Flow cytometer analysis showed an increase in cell death and not apoptosis when Myo cells were exposed to MVs from EP. On the other hand, LC3B was found to be upregulated when MVs were added to Myo cells. This protein plays a crucial role on autophagy[28] and has been associated with the progression of different types of cancers,[29] which reinforces the role of MV modulating the autophagic and non-apoptotic process presented in this model.

These findings corroborate a previous study by Silva et al,[4] who demonstrated that Myo cell disappearance from areas of invasive carcinoma in situ was not associated with apoptosis but with an induction of senescence and autophagy when Myo cells were co-cultured with malignant epithelial cells. This could explain the loss observed in Myo cell population during the progression of ex-pleomorphic adenoma carcinoma.[5,6] These findings could be related, at least in part, to the effect of MV produced by malignant epithelial cells on invasive areas of CXPA in situ.

Additionally, the results of the present study suggest a possible role of MVs in molecules involved in degradation of almost all

components of the basement membrane, contributing to invasion. qPCR and ELISA showed and increased expression of MMP-2 and MMP-9, whereas TIMP-2 levels were decreased when Myo cells were exposed to MVs from EP cells. In this context, we emphasize the role of Myo cell in matrix degradation, optimizing the tumor microenvironment for cell migration, although its role in tumor suppressor activity is described in the literature.

Despite these interesting preliminary findings concerning the role of MV derived from EP cell cultures on cell death, autophagy and MMPs and its inhibitors related genes in benign Myo cell cultures, further investigation is necessary to evaluate such biological processes at the protein level as well as their phenotypic features under MV stimulation.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

Elizabeth Ferreira Martinez: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology. **VC Araujo:** Conceptualization; Data curation; Formal analysis. **Natalia Festugatto Navarini:** Data curation; Methodology. **Isabela Fernandes Souza:** Data curation; Methodology. **Gabriel Bernardo Rena:** Data curation; Methodology. **Ana Paula Demasi:** Data curation; Formal analysis. **Eneida de Paula:** Data curation; Formal analysis. **Lucas Novaes Teixeira:** Conceptualization; Data curation; Formal analysis; Methodology.

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