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In vitro influence of the extracellular matrix in myoepithelial cells stimulated by malignant conditioned medium

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SUMMARY

In order to investigate the role of myoepithelial cell and tumor microenvironment in salivary gland neoplasma, we have performed a study towards the effect of different extracellular matrix proteins (basement membrane matrix, type I collagen and fibronectin) on morphology and differentiation of benign myoepithelial cells from pleomorphic adenoma cultured with malignant cell culture medium from squamous cell carcinoma. We have also analyzed the expression of α-smooth muscle actin (α-SMA) and FGF-2 by immunofluorescence and qPCR. Our immunofluorescence results, supported by qPCR analysis, demonstrated that α-SMA and FGF-2 were upregulated in the benign myoepithelial cells from pleomorphic adenoma in all studied conditions on fibronectin substratum. However, the myoepithelial cells on fibronectin substratum did not alter their morphology under malignant conditioned medium stimulation and exhibited a stellate morphology and, occasionally focal adhesions with the substratum. In summary, our data demonstrated that the extracellular matrix exerts an important role in the morphology of the benign myoepithelial cells by the presence of focal adhesions and also inducing increase FGF-2 and α-SMA expression by these cells, especially in the fibronectin substratum.

Introduction

The tumor microenvironment in the carcinogenesis process has been emphasized in many studies, especially in breast cancer in which is a determinant of its behavior. It is known that normal myoepithelial cells have an important role as a tumor suppressor, and so a defense against cancer progression.[1,2] Numerous in vitro and in vivo studies have demonstrated that the growth, survival, polarity, and invasive behavior of breast cancer cells can be modulated by myoepithelial cells and various stromal cells.[3]

The myoepithelial cell, which lies on the epithelial side of the basement membrane, is thought to contribute to both the synthesis and remodeling of the basal lamina. This anatomical relationship suggests that myoepithelial cells may exert important paracrine effects on normal glandular epithelium and may regulate the progression of ductal carcinoma in situ (DCIS) to invasive breast cancer. Thus, one important candidate for regulating the transition of DCIS to invasive cancer is the myoepithelial cell.[2] The tumor suppressor phenotype was identified based on the ability of myoepithelial cells to inhibit the growth and invasion of breast cancer cells in coculture assays in vitro and inhibit tumor growth by secretion of extracellular matrix proteins, protease inhibitors, and various growth factors.[1,4]

In a previous study of salivary gland, we have identified that some myoepithelial markers (CK14, α-smooth muscle actin, calponin, P63, CD10, and D2–40) as well as laminin and maspin, were stronger expressed in benign myoepithelial cells surrounding the malignant epithelial cells in carcinoma ex-pleomorphic adenoma (CXPA) in situ areas when compared with benign areas of pleomorphic adenoma (PA).[5] In order to better understand this fact, we have conceived an in vitro model attempting to simulate the cellular interactions of in situ structures of CXPA.[6] The results demonstrated that benign myoepithelial cells under the influence of conditioned medium underwent phenotypic alteration represented by an increased FGF-2 content.

Besides the myoepithelial cell influence on malignant cells, the extracellular matrix may also regulate cell proliferation and differentiation mediating the tumorigenesis process in vivo and may enable tumor cells to metastasize and grow in an inappropriate site of the body.[7] Thus, considering that the extracellular matrix is an important component of the tumor microenvironment, we have

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been currently investigating the effect of different extracellular matrix proteins, such as basement membrane matrix (Matrigel), type I collagen and fibronectin on benign myoepithelial cells from pleomorphic adenoma cultured with malignant cell culture medium from squamous cell carcinoma analyzing the cell morphology and the expression of α-smooth muscle actin (α-SMA) and FGF-2 .

Materials and methods

Cell culture

Benign myoepithelial cells were obtained from explants of pleomorphic adenoma (PA) tumors provided by surgery. In order to validate the analysis, cell culture replicates were obtained from three different donors according to the methodology described in our previous study.[6,8] This study was conducted following the approval of the Ethical Committee of São Leopoldo Mandic Institute and Research Center, Campinas, Brazil (Protocol # 09/0014).

The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented by 1% antimycotic–antibiotic solution (10,000 units of penicillin, 10 mg of streptomycin and 25 μg 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% CO_2) following the used protocol for this cell lineage culture.[8] After the cells had reached confluence, they were detached with 0.05% trypsin and subculture at a density of 110 cells/mm^2 on the top of different extracellular matrix proteins. Matrigel (BD Biosciences, California, USA) at 6 mg/ml, Human Collagen Type I (BD Biosciences) at 0.3 mg/ml and Fibronectin (Sigma) at 20 μg/ml were placed in the polystyrene plate or 13 mm coverslips for the following experiments. The benign myoepithelial cells from PA were cultured in DMEM for 24 h before cultured with conditioned malignant medium.

For the in vitro induction with conditioned medium, squamous cell carcinoma cells (CAL27) obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were used in the same density (110 cells/mm^2) and medium (DMEM) described for myoepithelial cells. The medium of these cells was removed 48 h after plating and then benign myoepithelial cells from PA cultures in DMEM for 24 h were incubated with the non-filtered and filtered in 0.22 μm sterile syringe filter (Corning Inc., Germany) malignant conditioned medium for 4 days. These two different conditions were used because our previous study showed that in non-filtered malignant conditioned medium there are non-adherent viable malignant epithelial cells from squamous cell carcinoma capable of seeding in the plate where the benign myoepithelial cells are cultured. As control, the analysis was carried out without malignant conditioned medium (i.e. DMEM).

Immunofluorescence

Cell growth on coverslips in different substrata were fixed in methanol for 6 min at 20 °C, rinsed in PBS followed by blocking with 1% bovine albumin in phosphate buffer saline (PBS) for 30 min at room temperature. The primary polyclonal antibodies used were FGF-2 (1:50, anti-rabbit, Sta. Cruz) and α-Smooth Muscle Actin (1:50, anti-mouse, Dako Corp., Carpenteria, CA, USA). Control staining reaction was performed using PBS in substitution to the primary antibody. The secondary antibody used was biotinylated anti-rabbit IgG or anti-mouse IgG (Vector Laboratories Inc, Burlingame, CA, USA). Following, fluorescein–streptavidin conjugated (Vector) was used. After washing, preparations were mounted using Vectashield® DAPI-associated (4′-6-diamidino-2-phenylindole) (Vector) and observed on a Zeiss Axioskop 2 conventional fluorescence microscope (Carl Zeiss MicroImaging GmbH, Germany) equipped with 63× Plan Apochromatic 1.4NA and 100× Plan Apochromatic 1.4NA objectives in standard conditions (Carl Zeiss, Oberköchen, Germany). To verify the morphological changes of benign myoepithelial cells from pleomorphic adenoma cultured with malignant cell culture medium in different substrata, the cells were also immunostained with vimentin (1:400, anti-mouse, Dako). The immunofluorescence experiments were repeated three times for each benign myoepithelial cell donor.

Transmission electron microscopy (TEM)

For further investigation of the myoepithelial cell interactions with the studied substrata, after 4 days of culture in DMEM medium, the main morphological aspects were analyzed by TEM. The cells were fixed in 0.1% glutaraldehyde and 4% formaldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. Then, the cells were post-fixed with 1% osmium tetroxide for 1 h, dehydrated in a graded series of ethanol and embedded in Spurr resine. Representative areas were selected for 80-nm-thick ultrathin sections by using a Leica (Leica Instruments GmbH, Nussloch, Germany) Ultracut R ultramicrotome with diamond knife and examined with a JEM 1010 electron microscope (Jeol USA Inc., Peabody, MA) operating at 80 kV.

Real Time quantitative (q) PCR

Total RNA was isolated from myoepithelial cells from PA cultured in different substrata using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), submitted to electrophoresis on 1.2% agarose/6% formaldehyde gels to check quality/integrity, and quantified with a NanoVue spectrophotometer (GE Healthcare, Bjorkgatan, Sweden). Aliquots of 1 μg total RNA from each sample were treated with DNase I (Invitrogen) and used for duplicate reverse transcription reactions with the Superscript III First Strand cDNA Synthesis kit (Invitrogen), according to the manufacturer's instructions. The primer sets were as follows: 5′-GTGCTAACCGTTACCTGGCTAT-3′ and 5′-CCAATCGTTCAAAAAAGAAACAC-3′ for *FGF-2*; 5′-ATGCTCCCAGGGCTGTTTT-3′ and 5′-GCTTCGTCACCCACGTAGCT-3′ for α-*SMA* (α-smooth muscle actin); 5′-AGGCCAACCGCGAGAAG-3′ and 5′-ACAGCCTGGATAGCAACGTACA-3′ for *ACTB* (beta actin), used as internal gene reference. Quantitative real time PCR was performed using a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green as detection dye. Cycling conditions were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. No template and no reverse transcription controls were used to test possible contaminations. Dissociation curve analysis was performed after each completed PCR for primer specificity and primer–dimer absence determinations. The quantification data were analyzed with the SDS System Software (Applied Biosystems) and the relative expression levels were calculated according to the Comparative Ct method, as $2^{-\Delta\Delta Ct}$. From each donor, mRNA was obtained from the cell culture performed in duplicate which was done in 3 different sets. The values obtained from the donors in each experiment were merged to perform the statistical analysis. Important to note is that the inter-individual variation was very low (data not show).

Statistics

Results are expressed as the mean ± standard deviation. In order to compare the results among the different substrata and medium conditions, the two-way analysis of variance (ANOVA) with post hoc Tukey test was applied, with a significance level of 0.05.

Results

Myoepithelial cell morphology did not alter under malignant conditioned medium on fibronectin and type I collagen substrata

To investigate whether the morphology of myoepithelial cells was affected by the presence of different substrata under malignant conditioned medium, the cells were examined by phase contrast microscopy and immunostained with vimentin which highlights the morphology of these cells in immunofluorescence (Fig. 1). No morphological alteration was observed in cells cultured in human type I collagen (Fig. 1g, h and i) or fibronectin (Fig. 1j, k and l) substrata under malignant conditioned medium stimulation. In these substrata, the cells assumed a polyhedral and stellate morphology in all studied conditions (DMEM, filtered conditioned malignant medium, and non-filtered conditioned malignant medium). On the other hand, the cells cultured with conditioned malignant medium (filtered and non-filtered) in polystyrene (Fig. 1b and c) and Matrigel (Fig. 1e and f) presented a spindle-shaped morphology differently from the control condition (DMEM) (Fig. 1a and d). The ultrastructural morphological examination revealed cells containing well-developed rough endoplasmic reticulum and Golgi apparatus, some of them exhibiting a peripheral layer of myofilaments (Fig. 2). These cells also displayed a notched (indented) nucleus with granular chromatin. The presence of focal adhesions was visualized in type I collagen and fibronectin substrata (Fig. 2c and d). No site of adhesion was visualized in polystyrene and matrigel substrata (Fig. 2a and b).

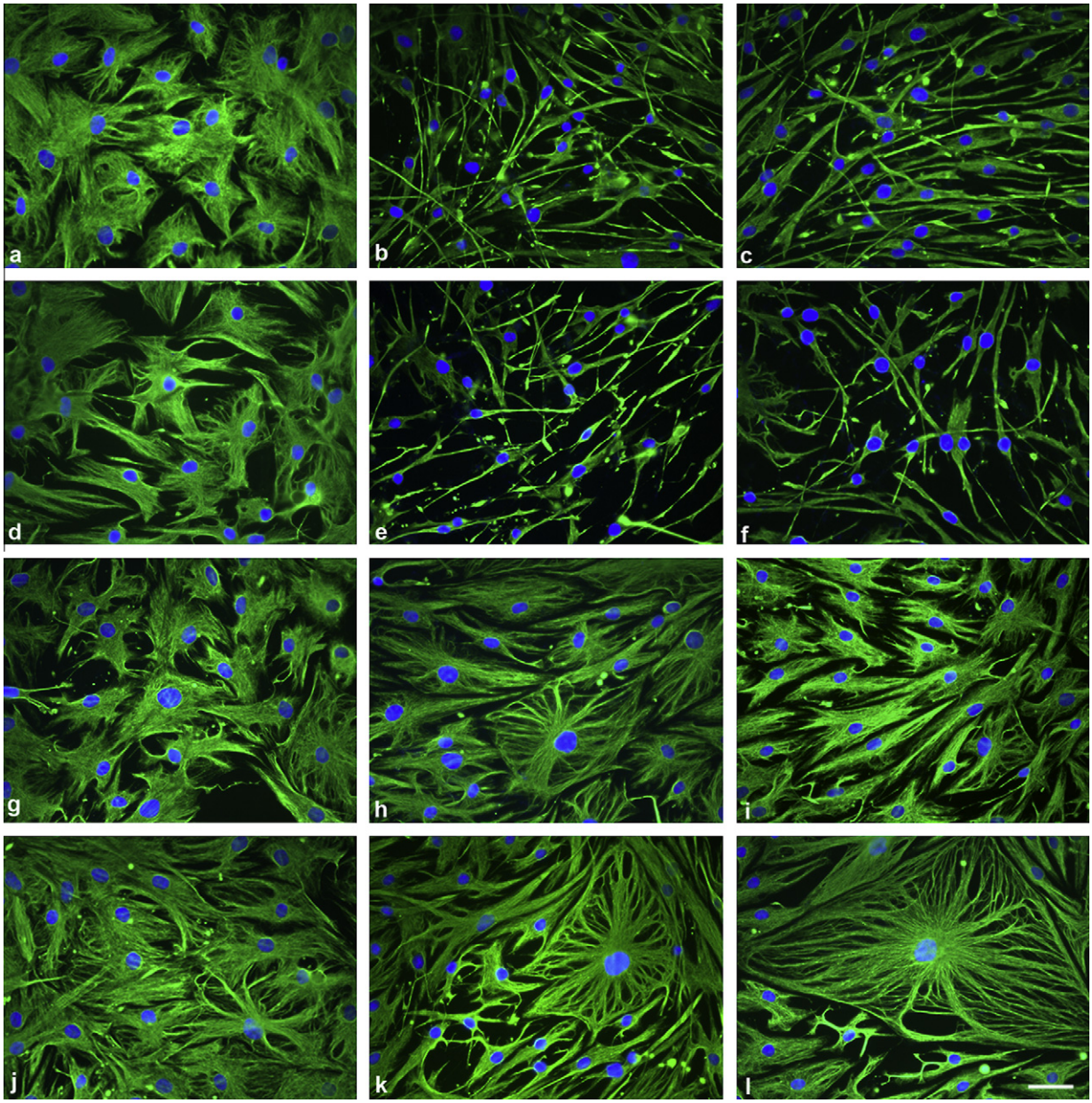

Figure 1 Myoepithelial cells morphology on polystyrene (a, b, c), matrigel (d, e, f), type I collagen (g, h, i) and fibronectin (j, k, l) substrata. Immunostaining for vimentin in myoepithelial cells from PA. The cells maintained the stellate morphology when cultured in type I collagen (g, h, i) or fibronectin (j, k, l) substrata independently of the malignant medium stimulation. A spindle-shaped morphology is observed on polystyrene (b, c) and Matrigel (e, f) when the myoepithelial cells were cultured with conditioned malignant medium. a, d, g, j: DMEM; b, e, h, k: filtered malignant conditioned medium; c, f, i, l: non-filtered malignant conditioned medium. Nuclei stained with DAPI appear in blue. Bar: 100 μm.

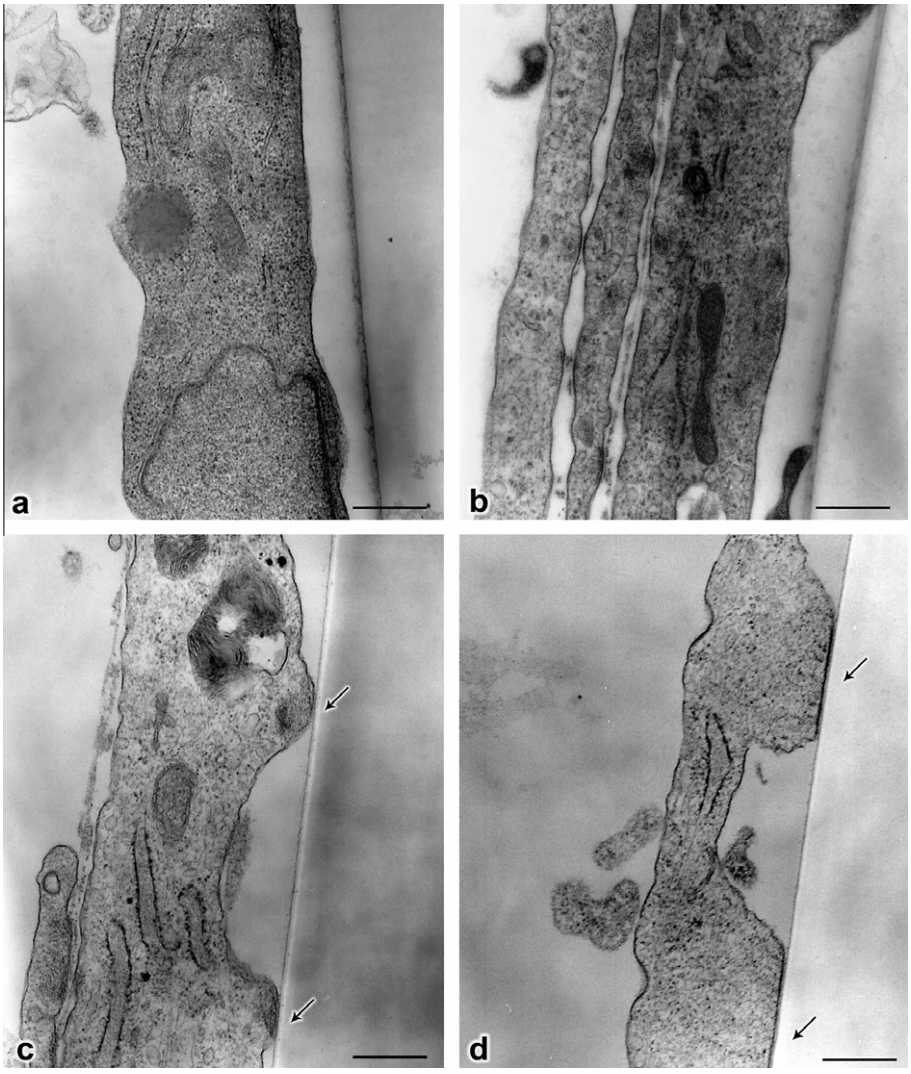

Figure 2 Transmission electron micrographs in myoepithelial cells on polystyrene (a), matrigel (b), type I collagen (c) and fibronectin (d) substrata in DMEM condition. Some cells exhibited a peripheral layer of myofilaments and also displayed a notched (indented) nucleus with granular chromatin. No site of adhesion was visualized in polystyrene and matrigel substrata (a, b). The presence of focal adhesions (arrows) was visualized in type I collagen and fibronectin substrata (c, d). Bars: a, b, c: 500 nm; d: 1 μm.

Fibronectin enhances expression of α-SMA and FGF2

The immunofluorescence assay to α-SMA and FGF-2 proteins in all substrata and conditions are represented in Figures 3 and 4, respectively. Myoepithelial cell cultured on fibronectin substratum exhibited an enhanced immunoexpression of α-SMA independently of the studied condition (DMEM, filtered conditioned and non-filtered conditioned medium) (Fig. 3j, k and l). On polystyrene (Fig. 3a, b and c), matrigel (Fig. 3d, e and f) and type I collagen (Fig. 3g, h and i) substrata, α-SMA was heterogeneously immunoexpressed in myoepithelial cells in all studied conditions. This could be identified through their nuclei labeled with DAPI, without cytoplasmic immunoreactivity for α-SMA. In addition, even under malignant conditioned medium (filtered conditioned and non-filtered conditioned medium) the myoepithelial cells exhibited the same pattern of immunoexpression of α-SMA when compared to the control medium condition (DMEM).

FGF-2 was immunoexpressed in the myoepithelial cell cultures in all substrata, independently of conditioned malignant medium stimulation (filtered or non-filtered), and was detected as punctuate deposits throughout the cytoplasm (Fig. 4). On fibronectin substratum, myoepithelial cells exhibited an increase in the expression of FGF-2 in all studied conditions (Fig. 4j, k and l) when compared to polystyrene substratum (Fig. 4a, b and c). Myoepithelial cells cultured in conditioned malignant medium seemed to present a stronger FGF-2 staining in polystyrene (Fig. 4b and c) and matrigel (Fig. 4e and f) substrata when compared to the control (DMEM) (Fig. 4a and d). On the other hand, no alteration in FGF-2 immunoexpression was observed in type I collagen (Fig. 4g, h and i).

FGF-2 and α-SMA mRNA was upregulated in fibronectin substratum

In order to quantify the results obtained by immunofluorescence, we further assessed the potential influence of the substrata in the expression of αsmooth muscle-actin and FGF-2 in benign myoepithelial cells induced or not with the conditioned malignant medium by qPCR (Fig. 5a and b).

The expression of α-smooth muscle actin (α-SMA) and FGF-2 mRNA was statistically significant upregulated in benign myoepithelial cells cultured in fibronectin substratum in all medium conditions (DMEM, filtered conditioned medium, and non-filtered conditioned medium). In addition, FGF-2 mRNA expression, in polystyrene and matrigel, was only statistically significant

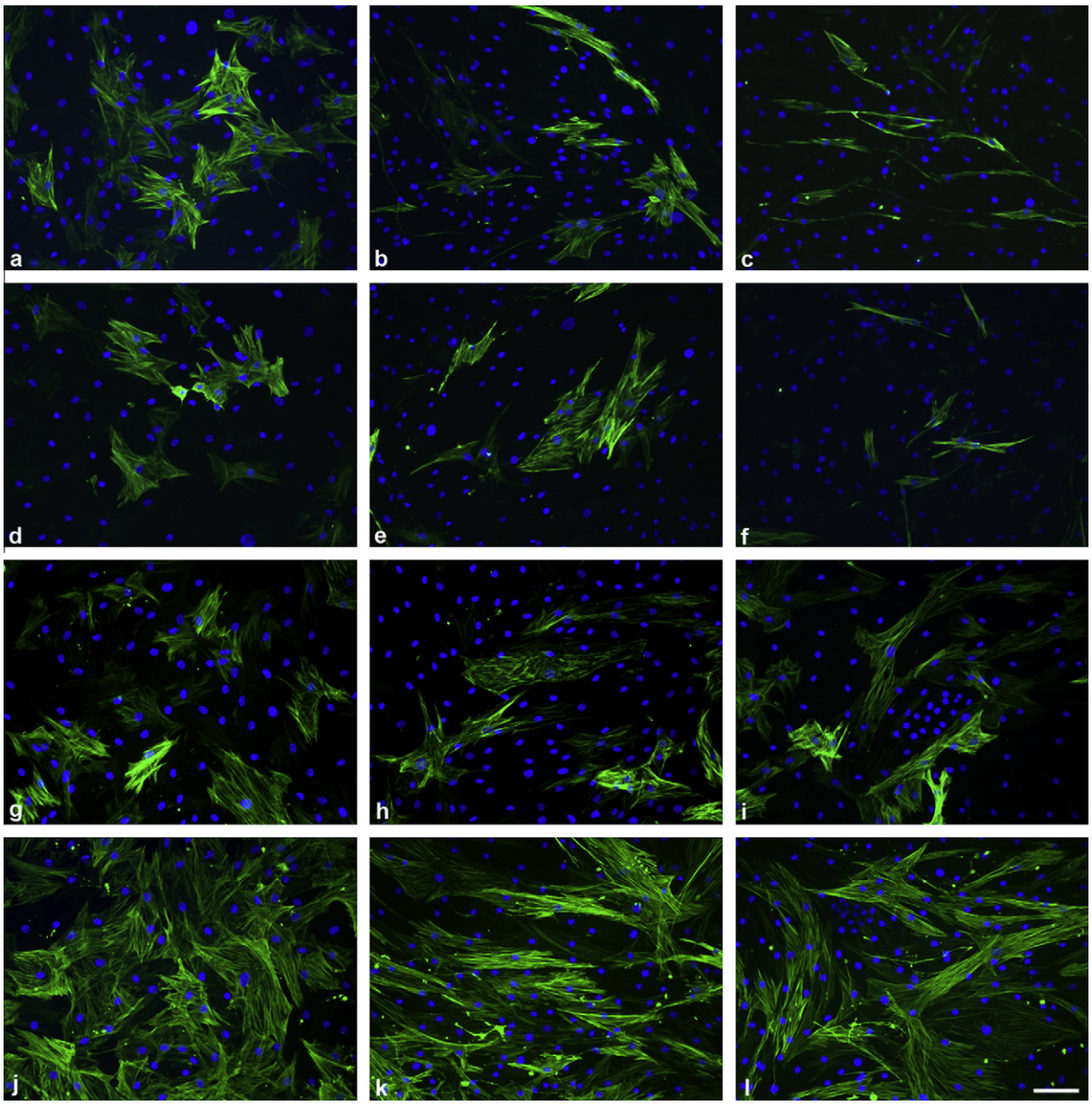

Figure 3 Immunostaining for α-SMA in myoepithelial cells on polystyrene (a, b, c), matrigel (d, e, f), type I collagen (g, h, i) and fibronectin (j, k, l) substrata. α-SMA was heterogeneously immunoexpressed in myoepithelial cells in all studied conditions, on polystyrene (a, b, c), matrigel (d, e, f) and type I collagen (g, h, i) substrata. However, on fibronectin substratum the myoepithelial cells exhibited an enhanced immunoexpression of α-SMA independently of the studied condition (j, k, l). a, d, g, j: DMEM; b, e, h, k: filtered malignant conditioned medium; c, f, i, l: non-filtered malignant conditioned medium. Nuclei stained with DAPI appear in blue. Bar: 50 μm.

upregulated in myoepithelial cells under stimulation of malignant conditioned medium filtered and non-filtered (Fig. 5b).

Discussion

The present results show the importance of the extracellular matrix in salivary gland tumor morphology and immunophenotype. Fibronectin substratum regulates in vitro the immunoexpression of FGF-2 and α-SMA in myoepithelial cells when compared with type I collagen and Matrigel. This differential role in cell organization and gene expression was suggested in fetal hepatocytes primary culture indicating the importance of this extracellular matrix during normal development.[9] Moreover, cancer cells are influenced by paracrine regulators from the host microenvironment. Such host regulation may be important to determine tumor cell behavior in vivo.[10,11]

FGF-2 and α-SMA mRNA expression were also upregulated in benign myoepithelial cells on fibronectin substratum when compared with type I collagen and Matrigel in all studied conditions. Previously, we have shown that FGF-2 was increased in benign myoepithelial cells under stimulation by conditioned medium obtained from malignant cells on polystyrene. This fact suggests that the myoepithelial cells may receive divergent signals or a change in the balance among the signals may occur, with excessive release of FGF-2, eventually favouring the growth of malignant cells.[6] In addition, FGF-2 triggers the expression of α5β1 integrin on endothelium of tumors which regulates angiogenesis favoring the

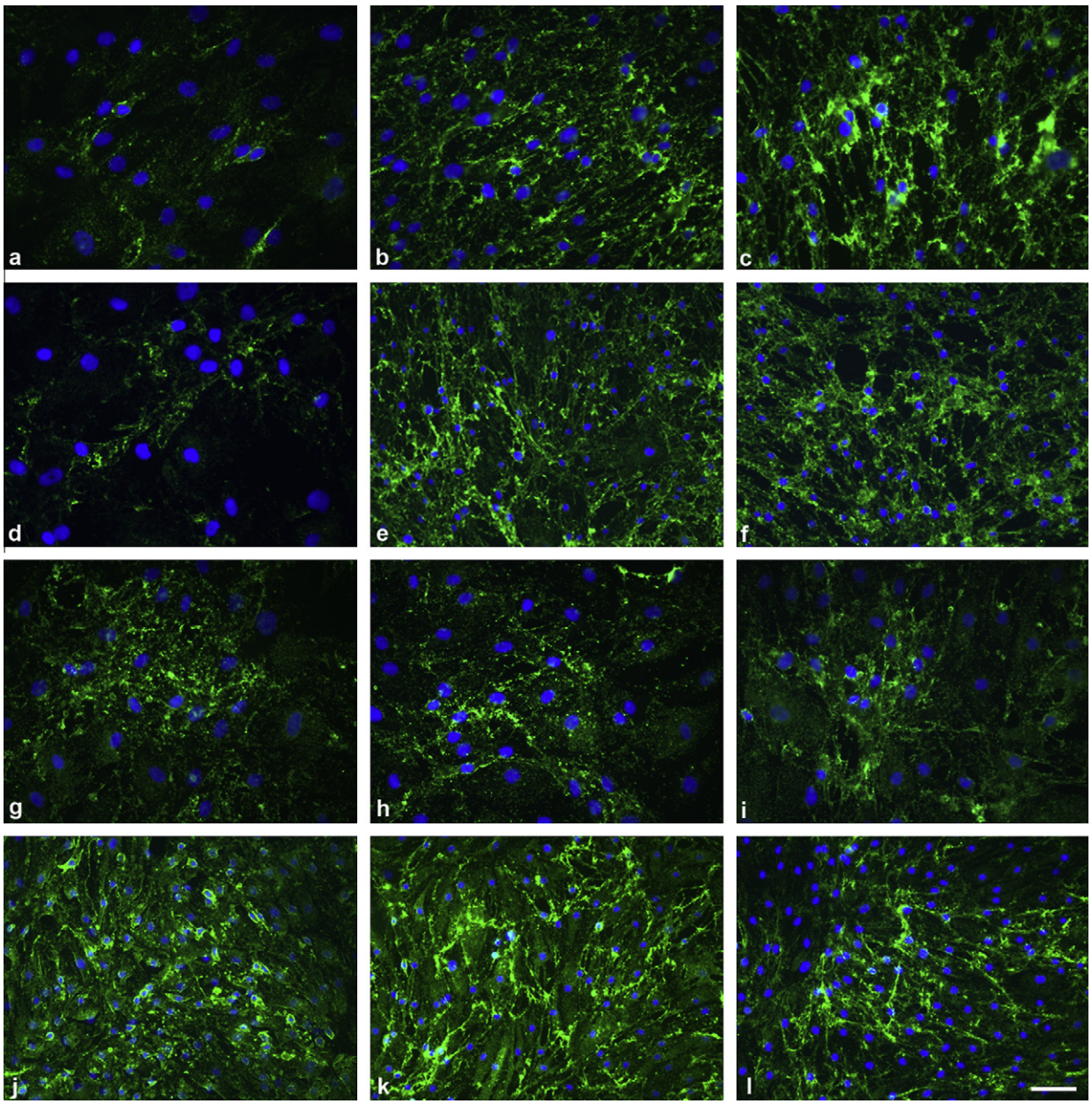

Figure 4 Immunostaining FGF-2 in myoepithelial cells on polystyrene (a, b, c), matrigel (d, e, f), type I collagen (g, h, i) and fibronectin (j, k, l) substrata**.** FGF-2 was immunoexpressed in the myoepithelial cell cultures in all substrata, independently of conditioned malignant medium stimulation (filtered or non-filtered), and appeared as punctuate deposits throughout the cytoplasm. On fibronectin substratum, myoepithelial cells exhibited an increase in the expression of FGF-2 in all studied conditions (j, k, l), on polystyrene (b, c) and matrigel (e, f) substrata cultured in conditioned malignant medium. No alteration in FGF-2 immunoexpression was observed in type I collagen (g, h, i). a, d, g, j: DMEM; b, e, h, k: filtered malignant conditioned medium; c, f, i, l: non-filtered malignant conditioned medium. Nuclei stained with DAPI appear in blue. Bars: a, b, c, d, g, h, i: 100 μm; e, f, j, k, l: 50 μm.

microenvironment vascularization swift which then becomes prone to the invasion of malignant epithelial cells in in situ areas.[12,13] However, in line with our findings, FGF-2 overexpression on fibronectin substratum may regulate myoepithelial cell differentiation which is highlighted by the increase of α-SMA expression.

Furthermore, matrix adhered cells are more responsible to factors present in the environment which then control cell proliferation and gene expression.[14] This could be observed by the upregulation of α-SMA and FGF-2 in benign myoepithelial cells cultured in fibronectin substratum in all medium conditions. Indeed, combined integrin aggregation and ligand occupancy promotes the formation of a large complex of cytoskeletal molecules that interact with actin cytoskeleton as well as transient coaggregation of growth factor receptors.[15] Thus, Fibronectin substratum increases the expression of α-SMA and FGF-2 independently of the influence of malignant conditioned medium.

In relation to morphology, interesting observations were found. It is already established that myoepithelial cells in cell culture exhibit a polyhedral and stellate morphology.[6,8] This morphology was maintained in myoepithelial cells cultured on polystyrene and Matrigel substrata with no conditioned medium (DMEM). On the other hand, on the same substrata, the benign myoepithelial cells under the influence of conditioned medium (filtered and non-filtered) underwent morphological alteration characterized by spindle-shaped morphology which is similar to normal myoepithelial cells and the benign myoepithelial cells that surrounded the malignant epithelial cells in in situ like structures of CXPA.[2] It is important to highlight that the myoepithelial cell needs to present this differentiated morphology pattern to exert its tumor

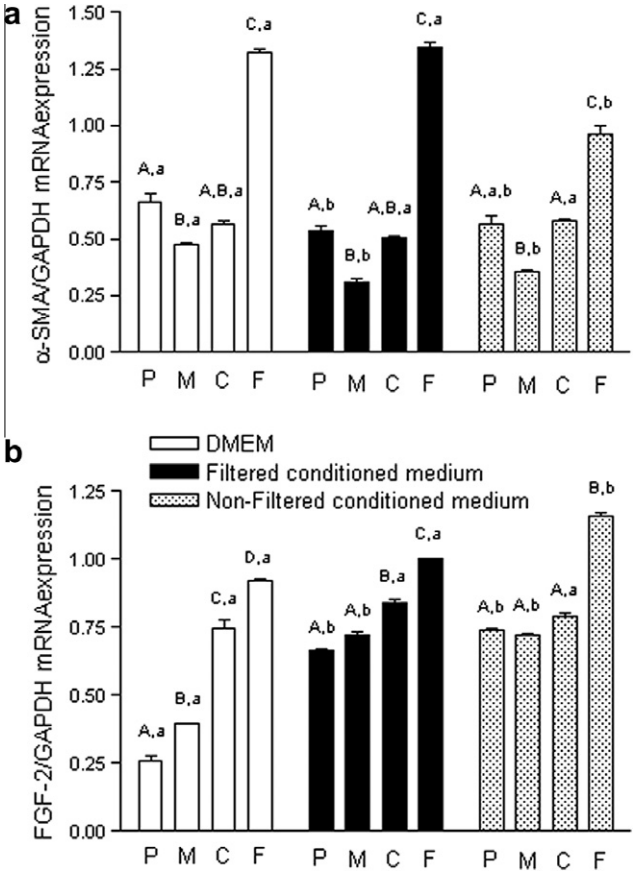

Figure 5 Relative α-*SMA* (a) and *FGF-2* (b) mRNA expression. On fibronectin substratum, the expression of α-*SMA* and *FGF-2* was statistically upregulated in all studied conditions (DMEM, filtered conditioned medium, non-filtered conditioned medium). Indeed, the *FGF-2* mRNA expression was up-regulated in myoepithelial cells stimulated by malignant conditioned medium on polystyrene and matrigel substrata (b). P: polystyrene, M: matrigel, C: type I collagen, F: fibronectin. Capital letter indicates statistical difference among the substrata. Lowercase letter indicates statistical difference among the conditions.

suppressive ability.[16] Nevertheless, the myoepithelial cells on fibronectin and type I collagen substrata, in all studied conditions, did not alter their morphology exhibiting a polyhedral and stellate morphology. Moreover, on the ultra structural morphological analysis, the myoepithelial cells have interacted with fibronectin and type I collagen substrata promoting sites of adhesion (focal contacts). Therefore, in vitro, these cell-binding sites could promote a close relationship between myoepithelial cells and substrata, establishing a high tensile strength preventing any morphological alteration.

Cell adhesion, signaling and morphogenesis of salivary glands depend on the interaction between the extracellular matrix and the integrin adhesion molecules.[15,17,18] It is worth noting that these findings are in accordance with the literature data which indicates that neither fibronectin nor non-fibrillar collagen are present in the tumor front of invasion.[19,20]

In malignant invasion, the ECM exerts extraordinary control on the behavior of cells dictating whether they will proliferate or undergo growth arrest, migrate or remain stationary and thrive or undergo apoptotic death. The effects of the ECM on cells are mainly mediated by the integrins, which organize the cytoskeleton and activate intracellular signaling pathways.[21] It is known that cellular cohesion plays a critical role not only establishing compartments and boundaries between tissues but also providing influence in malignant invasion.[22] These findings then demonstrate that the presence of growth factors in the malignant conditioned medium is not sufficient to alter the myoepithelial cell morphology on fibronectin and type I collagen substrata.

In conclusion, this in vitro study showed that type I collagen and especially fibronectin may modify the action of malignant cells, inducting the increase of FGF-2 by the benign myoepithelial cells favouring the malignant cells proliferation. In addition, fibronectin also holds the myoepithelial cells in the matrix avoiding any morphologic alteration. Thus, all together probably contributes to the tumor growth and impairs the function of myoepithelial cells as a tumor suppressor. This issue must be taken into account when the invasion process in in situ carcinoma both in breast as in salivary gland cancer is analyzed.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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