Author’s Accepted Manuscript

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PII: S0014-2999(15)30199-0
DOI: http://dx.doi.org/10.1016/j.ejphar.2015.08.023
Reference: EJP70171

To appear in: European Journal of Pharmacology

Received date: 4 May 2015
Revised date: 12 August 2015
Accepted date: 17 August 2015

Cite this article as: Baharak Farhanji, Mostafa Latifpour, Ali Mohammad Alizadeh, Hamid Khodayari, Saeed Khodayari, Mahmood Khaniki and Sarieh Ghasempour, Tumor suppression effects of myoepithelial cells on mice breast Cancer, European Journal of Pharmacology, http://dx.doi.org/10.1016/j.ejphar.2015.08.023

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Tumor suppression effects of myoepithelial cells on mice breast cancer

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Abstract

Several studies have assumed that myoepithelial cells (MECs) loss may contribute to epithelial tumor induction and/or progression. We adopted an in vitro assay and a syngeneic mice breast cancer model with histological and molecular characteristics resembling human lesions to evaluate tumor suppression effects of MECs. Flow cytometric, cell viability, blood chemistry, transmission electron microscope, immunohistochemistry and qRT-PCR assays were performed at the end of the study. We demonstrated that MECs could significantly suppress the viability of cancer cells at different time points ($P < 0.05$). At the end of the fourth and fifth weeks, treated mice had smaller tumor volume compared with control animals. Average tumor volume was significantly less in treated groups than control group at days 21 ($0.38 \pm 0.19$ vs. $1.99 \pm 0.13$ cm$^3$), 28 ($0.57 \pm 0.3$ vs. $2.5 \pm 0.37$ cm$^3$) and 35 ($0.7 \pm 0.35$ vs. $2.65 \pm 0.4$ cm$^3$) after tumor cell injection ($P < 0.05$). No hematological, hepatocellular, and renal toxicities were seen in MECs treated groups. Ultrastructural features revealed severe relationship between adjacent tumoral cells and loose interconnections of neoplastic cells in treated group. Immunohistochemical examinations of breast tumors showed high p63 and low alpha-smooth muscle actin protein expression in treated mice compared to control ($P < 0.05$). mRNA expressions of TNF-$\alpha$, smooth muscle–myosin heavy chain, connexin 43, and maspin were significantly up-regulated in breast tumor tissues in treated group compared to control ($P < 0.05$). VEGF and alpha-smooth muscle actin mRNA expression were reduced in treated animals ($P < 0.05$). The present study highlighted the potential tumor suppression effects of MECs on breast cancer in a typical animal model.

Key words: Myoepithelial cell, breast cancer, mice
1. Introduction

Cell therapy facilitates therapeutic modalities at various clinical clues in multiple organs based on some main principles. Several cell lines have the capacity to release soluble factors such as cytokines and growth factors to improve organ self-healing. Surprisingly, the importance of myoepithelial cells (MECs) in breast cancer has always been underestimated. These cells appear to have dual functions as tumor suppressor and promoter. MECs are a specialized combination of muscular and epithelial cells capable of contracting mammary ducts to push milk and a system of secretory apparatus of the mammary gland (Gage, 1998; Pandey et al., 2010). They considerably contribute to basement membrane creation, moreover, their myogenic differentiation is responsible for contractile phenotype mediated by various substances such as oxytocin (Alizadeh and Mirzabeglo, 2013; Imanieh et al., 2014; Murrell, 1995). Several studies have assumed that intact MECs are essential determinant of normal breast differentiation, and loss of their functions may contribute to induction and/or progression of epithelial tumors (Pechoux et al., 1999; Slade et al., 1999). While epithelial cells are susceptible targets for transforming events leading to cancer, MECs are still resistant. Although evidence proposes MECs suppressive effects on tumor growth, invasion and angiogenesis, their role remains a main puzzle in breast cancer biology (Alizadeh et al., 2014). Indeed, a number of myoepithelial-specific proteins called tumor-suppressive proteins such as alpha-smooth muscle actin (SMA), smooth muscle myosin heavy chain (SM-MHC) (Okamoto-Inoue et al., 1999), calponin, caveolin-1 (Lee et al., 1998), connexin 43 (Hirschi et al., 1996), maspin(Zou et al., 1994), and activin (Liu et al., 1996) have shown to inhibit epithelial tumor formation.

Therefore, MECs may have an important role in paracrine regulation of normal and tumor cells by influencing the epithelial and luminal compartments, and ultimately altering breast tissue microenvironment.
To better characterize the MECs role in tumor progression, we adopted an *in vitro* and a syngeneic mice breast cancer model with histological and molecular characteristics resembling human lesions in a typical animal model. In fact, investigators have revealed that the myoepithelium in mice can be targeted with special molecules using mammary tumor models. Development of new therapies of human breast cancer requires suitable animal models. Orthotopic models of cell therapy have additional advantages including retention of differentiated structures within the tumor, vascular growth differences, realistic tissue pharmacokinetics at the tumor site and metastatic spread (McConville et al., 2007). Further studies to understand the exact molecular mechanisms of MECs suppressive tumor function may lead to attain a novel therapeutic target for breast cancer.

2. Materials and methods

2.1. Materials

Mouse mammary adenocarcinoma cell line (MC4-L2) was a gift from Buenos Aires University, Argentina (Lanari et al., 2001). Methylthiazol tetrazolium (MTT), phosphate-buffered saline (PBS) solution, Ketamine and Xylazine were purchased from Sigma Aldrich Co. (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were from Invitrogen.

2.2. Mouse MECs isolation

Inbred female BALB/c mice (7-9 weeks old) were placed on their back on a corkboard after euthanasia, then pinned in place through the feet, and swabbed with 70% ethanol. A ventral midline incision through the skin was made to expose mammary glands (Rasmussen et al., 2000). The mammary glands were dissected from the skin using a scalpel, starting from the proximal area close to the nipple till to the distal end of the gland, and carefully separated from the peritoneum with a blunt-edged instrument. Dissected tissues were kept in tissue dishes filled with PBS and diluted antibiotics floating on ice cubes. Immediately after
delivery, sections of the mammary tissues were transported to the laboratory in sterile Hank’s balanced salt solution contained 40 µg/ml of gentamicin at room temperature. The pieces were then rinsed several times with sterile phosphate buffered saline containing 2 µg/ml amphotericin B, 100 U/ml penicillin, and 100 µg/ml streptomycin.

2.3. Mammary gland digestion and MECs recovery

Mammary gland pieces were transferred to a 10-cm sterile petri dish. Tissues were mechanically minced with two scalpels inside the petri dish under sterile conditions. They were then transferred to a small flask containing PBS (10 ml/g tissue) and antibiotics. Appropriate volume of 1% collagenase was added, and the tissues were stirred at 37 °C for 30 to 90 min. Tissue digestion was checked starting at 30 min by aseptically removing small aliquots under the low-power microscopic examination. The desired endpoint was an epithelial preparation with no visible tissue pieces and more than 80% of epithelial organoids free of adhering stromal tissue. Then, 20 ml of F12 plus 5% FBS were added to samples digest, and allowed the clumps to be settled for 2 min. The supernatant centrifuged at 1500 g for 5 min to prepare mammary epithelial cells and washed three times with F12 plus 5% FBS. Cells were kept in a humidified incubator setting at 37 °C in a medium refreshing three times a week. MECs were meticulously separated after organoids administration in primary culture.

2.4. Cell labeling

To track proliferation, MECs were labeled with vital dye CellTracker CM-DiI, according to the manufacturer’s protocol. To test whether fluorescent nuclear track detector interferes with standard fixation and staining procedures, hybrid detector cell layer was labeled with a series of dyes. Cells were labeled with a fluorescent dye, Cell-tracker CM-DiI Molecular Probe (Cat. No. C700) at a concentration of 1.5 µg in Dulbecco’s phosphate buffered saline (DPBS, 1 ml) for 8 min at humidified atmosphere first, and then for additional 15 min at room temperature. After labeling, cells gently washed twice with DPBS.
2.5. Flow cytometric analysis

Cells were incubated at \(10^6/ml\) in L15/10% FCS with anti-CD10-fluorescein isothiocyanate (clone M1/69, BD Biosciences, Oxford, UK, 0.5 µg/ml) for 45 min at 4 °C, washed in L15/10% FCS and resuspended in L15/10% FCS/0.01% 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). Analysis was carried out on a BD FACSVantageSE DiVa (BD Biosciences) equipped with two coherent 90 C-4 argon ion lasers (Coherent, Santa Clara, CA, USA) set at 488 nm and 333.6 to 333.8 nm. Samples were gated on the basis of forward-and side-scatter. Doublets and high order clumps were excluded using a time-of-flight approach, where forward-scatter-height was plotted against forward-scatter area. Routine examination of sorted cells revealed >99% single cellularity.

2.6. Co-culture of MC4-L2 and MECs to assay cell viability

To evaluate MECs effects on MC4-L2, co-cultures of MC4-L2 and MECs were performed using 6-well Transwell plates. To prevent direct cell-cell contact, MECs were seeded at a total of \(5 \times 10^5\) cells on a 0.4 µm pore size Transwell filter (SPL Life Sciences Co., Ltd). MC4-L2 was also seeded at a total of \(6 \times 10^5\) cells in 6-well flat-bottom tissue culture plate. Cells were cultured in Gibco® high glucose Dulbecco’s Modified Eagle Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). All cells were grown at 37 °C in a humidified atmosphere of 5% carbon dioxide. In the first step, cells were seeded in 6 wells and allowed to be attached overnight. Seeded MECs were then transferred to the top of six wells containing MC4-L2 except one as the control and incubated. MTT assay was done to determine cell viability of triplicate wells at days 1, 3 and 5, and represented as the viability percentage using an enzyme-linked immunosorbent assay method at 570 nm.
2.7. Animals

Female inbred BALB/c mice aged 6-8 weeks old, purchased from Iran Pasteur Institute and maintained in large group houses under 12-h dark and light cycles with free access to food and water. Animals were handled according to relevant national and international guidelines of the Weatherall report, and Institutional Animal Care and Use Committee (IACUC) of Tehran University of Medical Sciences.

2.8. Tumorigenicity

MC4-L2 was trypsinized and re-suspended in 10-fold excess culture medium. After centrifugation, the cells were re-suspended in a serum-free medium. Prepared cells (1 × 10^6 / 0.1 ml) were subcutaneously injected in the right inguinal flank of the female mice under ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg, i.p) anesthesia (Farhangi et al., 2015). Two weeks post MC4-L2 injection, twelve mice were taken and equally divided into control and treated groups. MECs (1 × 10^6) were intratumorally injected once a week for three consecutive weeks using a 21-gauge needle under ketamine and xylazine anesthesia in treated group, while in control animals, saline solution was administered. Animals were euthanatized at day 35 post MC4-L2 injection.

2.9. MECs tracing

Cells were stained with CellTracker™ CM-DiI Dye 72 h before cell injection, according to the manufacturing protocol (Life technologies, USA). CellTracker™ CM-DiI is a red fluorescent dye well suited for monitoring cell movement or location. Almost 1 × 10^6 viable cells were re-suspended in 50 µl PBS, and then infected at the periphery of the tumor arena.

2.10. Body weight and tumor size measurement

Animals were weekly weighed and regularly monitored for abnormal sequels. Tumor volume was measured by a digital vernier caliper (Mitutoyo, Japan) on a weekly basis and reported as cm^3 using the following formula (Mohsenikia et al., 2013):
V = 1 / 6 (\pi \text{LWD}), \text{where} \ L = \text{length}, \ W = \text{width} \text{and} \ D = \text{depth.}

2.11. Hematology and blood chemistry tests

At the end of the present study, animals were anesthetized first and then 1.5 ml of blood was intracardially withdrawn. Blood samples were taken and added into the ethylene-diamine-tetra-acetic-acid (EDTA)-coated tubes for hematology and heparin-coated tubes for clinical chemistry tests. Differentiated leukocyte count (WBC), erythrocyte count (RBC), platelets count (Plt), hemoglobin (Hgb) and hematocrit (Hct) levels were measured using an animal blood counter (Celltac; Nihon Kohden, Tokyo, Japan). Plasma urea nitrogen (BUN) and creatine (Cr) were also determined with CCX System (CCX WB; Nova Biomedical, USA). Plasma alkaline phosphatase (ALP), albumin (ALB), total bilirubin (T.Bil), direct bilirubin (D.Bil), gamma-glutamyl transpeptidase (GGT), alanine transaminase (ALT) and aspartate transaminase (AST) were all measured (Autoanalyser Model Biotecnica, BT 3500, Rome, Italy).

2.12. Histological and immunohistochemical examinations

Breast tumor samples were fixed and preserved in 4% buffered formaldehyde for at least 24 h, passaged and embedded in paraffin. Paraffin blocks were then sectioned by 3-5 \mu m thickness for hematoxylin and eosin (H & E) staining (Mohsenikia et al., 2013). Slides were studied under OLYMPUS-BX51 microscope. The breast tumors were stained for immunohistochemistry using avidin-biotin immunoperoxidase method (Mohsenikia et al., 2013). Sections were stained with polyclonal mouse antiRat/Rabbit p63 and a-SMA antibodies (DAKO Corporation, USA) according to the manufacturer’s instructions. Briefly, paraffin sections deparaffinized with xylene and rehydrated through a series of descending graded ethanol solutions. Slides kept into TBS-EDTA buffer and put into a microwave oven for 15 min at 90 °C. Endogenous peroxidase activity blocked by 0.3% H2O2 buffer incubation for 15 min. Biotinylated secondary antibody and avidin-biotin complex with horseradish
peroxidase were applied followed by chromogen 3, 3’-diaminobenzidine (Sigma Chemical) addition. The criteria used for p63 and a-SMA markers evaluation was based on the estimated proportion of positive cells and average staining intensity of positive cells for these markers. The semi-quantitative score was adopted as total score of the staining intensity and population of the positive cells which ranged from 0 to 6 points. Each of scoring system for intensity and population is as follows (Mohsenikia et al., 2013):

No staining and/or no positive cells: 0
Faint/barely staining and/or faint/barely positive cells up to 25% of cells: 1
Moderate staining and/or moderate positive cells in 25% to 50% of cells: 2
Strong staining and/or strong positive cells in more than 50% of cells: 3

2.13. Transmission electron microscopy

Breast tumors were fixed in 2.5% glutaraldehyde (Proscitech, Thuringowa, Australia) in PBS and processed for transmission electron microscopy (TEM) (Alizadeh et al., 2012). Samples were post fixed in 1% OsO₄ (TAAB; Berkshire, UK) dehydrated through an ethanol series, equilibrated in propylene oxide, and embedded in araldite (Proscitech). Thin sections were stained with uranyl acetate and lead citrate. Specimens were observed under a LEO 906 (Zeiss, Germany) transmission electron microscope and recorded with a camera (Zeiss, Germany) for subsequent assessments.

2.14. RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from tumor tissues using TRIzol® reagent (Life Technologies) followed by DNase I digestion (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized by PrimeScript™ RT reagent kit (Fermentas, Germany). The list of primers for specific genes and housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are indicated in table 1. Real-time PCR was performed
using the SYBR® Premix Ex Taq™ II (Takara). Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$ (Farhangi et al., 2015).

2.15. Statistical Analysis

Statistics were presented in Prism® 6.1 software (GraphPad Software, Inc, La Jolla, CA, USA) and analyzed using one-way analysis of variance followed by Newman–Keuls multiple comparison test or Student’s t-test. Differences among groups were stated to be statistically significant when $P < 0.05$.

3. Results

3.1. Cell morphology

Cells of the primary cultures showed a multipolar shape except that a few of spindle form. Cells in primary cultures were passaged on day 2. Immediately after passage light microscopic examination showed elongated and spindle shaped cells with retractile and highly migratory properties together with pale cytoplasm and nuclei (myoid or fibroblast like) (Fig. 1).

3.2. Flow cytometric analysis

Flow cytometric analysis showed cells with myoepithelial marker including CD10 (2.5%) expression against myoepithelial cancerous cells. CD10 positive cells consistently revealed three distinct populations: CD10$^{\text{High}}$ ($69.9 \pm 5.5\%$), CD10$^{\text{Low}}$ ($22.2 \pm 5.6\%$), and CD10$^{\text{Negative}}$ ($6.3 \pm 1.5\%$) (Result from 15 independent sorts).

3.3. Cell viability assay of MC4-L2 co-cultured with MECs

Cell-cell interaction was analyzed in the heterotypic co-culture system. Transwell and MTT studies revealed MECs inhibitory effects on MC4-L2 proliferation at different times ($P < 0.05$) (Fig. 2).
3.4. **Immunohistochemical staining to trace MECs**

Thirty-five days post transplantation, CellTracker™ CM-DiI Dye incorporated cells were observed in the transplanted area (Fig. 3). Fluorescent microscopy using DIL-labeled cells showed viable proliferated MECs evidenced with red colored regions. Interestingly, there were some areas filled with contacted transplant cells furnishing series of quasi-spherical structures such as normal breast acini.

3.5. **Body weight and tumor volume measurements**

Body weight in control and treated groups slightly increased and steadily rose to reach a plateau few weeks later but slightly fell in control group as tumors developed (Fig. 4). Treated mice had smaller tumor volume (cm$^3$) at the end of the fourth and fifth weeks compared with control animals (Fig. 5). Average tumor volume was significantly less in treated group than control at days 21 (0.38 ± 0.19 vs. 1.99 ± 0.13 cm$^3$), 28 (0.57 ± 0.3 vs. 2.5 ± 0.37 cm$^3$) and 35 (0.7 ± 0.35 vs. 2.65 ± 0.4 cm$^3$) post tumor cell injections ($P <0.05$). Final mean tumor volume reached to 2.65 ± 0.4 and 0.7 ± 0.35 cm$^3$ in control and treated groups, respectively (Fig. 5).

3.6. **Hematology, blood chemistry, and histopathological tests**

Hematological studies were done via complete blood count analysis. No inflammatory responses were doubted, since total leukocyte counts remained within normal range (Table 2). MECs injection did not cause hematological, hepatocellular, and renal toxicities based on kidney and liver functions evaluation (Table 2).

3.7. **Transmission electron microscopy**

Ultrastructural features revealed neoplastic tissues made up of clusters of epithelial like cells with high N/C ratio, large nuclei and nucleoli. MECs were spindle shaped and retractile with abundant mitochondria, pale cytoplasm and nuclei. Neoplastic cells which were surrounded by MECs had large nuclei and nucleoli within a relatively collagenous stroma.
They had loose interconnections with large mitochondria in some cells (Fig. 6A). MECs (left) with numerous large mitochondria had tight junction with tumor cells (center and top) (6B and 6C). They also interrelated with adjacent tumoral cells (6D).

3.8. Immunohistochemical examinations

Immunohistochemistry examinations of breast tumors showed high p63 protein expression in treated group compared with control ($P < 0.05$). Moreover, α-SMA protein expression was conceivably lowered in treated mice compared to control ($P < 0.05$) (Fig. 7).

3.9. Analysis of gene expression by real-time PCR

MRNA expression of TNF-α (2.39-fold change), SM-MHC (5.75-fold change), connexin 43 (2.34-fold change), and maspin (1.33-fold change) were significantly up-regulated in breast tumor tissues in the treated group compared to control ($P < 0.05$). Moreover, calponin mRNA expression was also up-regulated in breast tumor of the treated group but not in a significant way (1.86-fold change) ($P > 0.3$) (Fig. 8).

MRNA expression of VEGF (0.03-fold change) and SMA (0.16-fold change) was reduced in mice treated compared to control ($P < 0.05$) (Fig.8).

4. Discussion

The main purpose of this study was to develop novel therapeutic strategies for breast cancer. Our results confirmed in vitro inhibitory effects of MECs on cell viability of cancerous cells. Tumor size and weight were significantly declined in the treated group compared to control in mice breast tumor. Mrna expression of TNF-α, SM-MHC, connexin 43, and maspin was significantly up-regulated in breast tumor tissues of the treated group compared to control. VEGF and SMA mRNA expressions were reduced in treated mice. These data can prove tumor suppressive effects of MECs on mice breast cancer.

The exact mechanisms of concentration-dependent inhibitory effects of MECs on cell migration are not fully understood and needed to be worked. This may elucidate MECs role...
in mammary gland morphogenesis and malignant transformation and can also provide better insights into breast cancer biology, diagnosis and therapy. Board of evidence suggests MECs role in suppressing mammary oncogenesis via secreting suppressor proteins that limit cancer cell growth and invasiveness (Adriance et al., 2005; Hu et al., 2008). It seems that several paracrine factors such as protease inhibitors, various growth factors, and angiogenesis factors secreted by MECs exert their effects on tumor growth (Gudjonsson et al., 2002). These factors have a broad range of functions in tumor inhibitory such as growth and neoangiogenesity suppression, apoptosis induction, and cancer cells spread inhibition. Moreover, MECs express high amounts of proteinase inhibitors including maspin, connexins, and neogenin, which in turn can interfere with motile and invasive behavior of tumor cells, block angiogenesis and basement membrane degradation (Liu et al., 1996; Zhang et al., 2000). It seems that functional defects in cancer derived MEC lines may provide an explanation for the above observations of in vivo part of the present study.

Maspin, an important tumor suppressor secreted by MECs, can inhibit tumorigenesis, tumor cell migration and metastasis (Hopkins and Whisstock, 1994). Additionally, it acts as an angiogenesis and locomotion inhibitor (Czerwenka et al., 2000; Pemberton et al., 1995). These properties may explain the anti-angiogenic and anti-invasive effects of MECs on tumor and precancerous cells. Maspin which is synthesized by mammary glands MECs has been shown to be reduced. This finding coincides with the transition of in situ cancers to invasive form (Karnoub et al., 2007; Maass et al., 2001; Zou et al., 1994). In addition, maspin expression is down-regulated in breast, prostate, gastric and melanoma cancers but over-expressed in pancreatic, gallbladder, colorectal, and thyroid cancers. This may suggest maspin different activities in different cell types. It is also expressed in the normal breast epithelium but its expression or its mRNA level is often reduced in breast cancer cells (Maass et al., 2001). In the present study, maspin mRNA expression was significantly up-
regulated in breast tumor tissues in the treated group compared to control, which can confirm the tumor suppression effects of MECs on breast cancer.

Connexins (Cx), which take part in gap junctional intercellular communication (GJIC), play an important role in cell growth and cell death, and can act as tumor suppressors. They play a critical role in tissue development and differentiation. In the normal mammary gland there was an intercellular and punctuate staining pattern, mainly between MECs. Down-regulation of Cx expression is often observed in tumors, and is believed to contribute to loss of cell growth control (McLachlan et al., 2006). Xu et al. (2008) reported that connexin-43 may induce E-cadherin expression, and inhibit cell proliferation and progression of lung cancer (Xu et al., 2008). Studies have shown that connexin-43 was down-regulated in human breast cancer cell lines. In addition, its expression was found to be reduced in all stages of human breast cancer (Carystinos et al., 2001). In the present study, connexin-43 mRNA expression was significantly up-regulated in breast tumor tissues in the treated group compared to control. This may focus the inhibitory effects of MECs in breast cancer tumor.

Activin, which belongs to TGF-beta superfamily, is expressed by MECs as well. Activin has been reported to inhibit growth of breast cancer cells by activating Smad proteins, and blocking p38 mitogen-activated protein kinase pathway (Burdette et al., 2005). In mouse mammary glands, mammary basal epithelial cells are positive for SMA and calponin. SMA is a robust myoepithelial marker. It has been shown to be positive even in suboptimally fixed or infarcted tissues. In addition, SM-MHC is more specific than SMA with less staining of myofibroblasts (Barsky and Karlin, 2006; Pandey et al., 2010). In this study we showed SM-MHC up-regulation against SMA down-regulation on the treated breast tumor with MECs. Based on ultrastructural features of the present study, and other reports, it seems that MECs form a semi-continuous protective sheet separating the human breast epithelium and the surrounding stroma. This cell layer disruption may stimulate growth and angiogenic factors
release, and induce reactive oxygen species which altogether can alter at cell microenvironment (Pandey et al., 2010). In the present study, proteins made by MECs including p63 and SMA, are used to determine intact or broken myoepithelium. This MECs ammunition layer against cancer creates a defensive barrier and prevents cancer cells from escaping into the surrounding breast tissue.

In summary, MECs potential role in tumor progression has always been neglected. It is now believed that they have an important regulatory role in breast cancer pathology via influencing epithelial and luminal compartments, and the tissue microenvironment. Further studies are needed to understand the exact molecular mechanisms of tumor suppressive effects of MECs followed by finding a novel therapeutic target for breast cancer. Research on this area may eventually lead to the development of novel approaches for the prevention and treatment of breast cancer.

Declaration of interest

The author(s) report no conflicts of interest and are responsible for the content of the paper.

Acknowledgments

This study was co-founded by Tehran University of Medical Sciences (Grant Number: 16128) and Fasa University of Medical Sciences. We would like to thank Professor Claudia Lanari to assist in providing cell lines.

References


Fig. 1. Light microscopic features of MECs

Myoepithelial cells are seen as the elongated and spindle shaped cells with retractile and highly migratory properties together with pale cytoplasm and nuclei. Slides studied by OLYMPUS-BX51 microscope (H & E, 40×). MECs = myoepithelial cells

Fig. 2. Cell viability of MC4-L2 co-cultured with MECs

To prevent direct cell-cell contact, MECs were seeded at a total of $5 \times 10^5$ cells on a 0.4 μm pore size Transwell filter. MC4-L2 was also seeded at a total of $6 \times 10^5$ cells in 6-well flat-bottom tissue culture plate. Cells were cultured in Gibco® high glucose Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. In the first step, cells were seeded in 6 wells and allowed to be attached

Legends
overnight. Seeded MECs were then transferred to the top of six wells containing MC4-L2 except one as the control and incubated. MTT assay was done to determine the cell viability of triplicate wells at days 1, 3 and 5, and represented as the viability percentage using an enzyme-linked immunosorbent assay method at 570 nm. Data reported are mean ± S.D; * $P < 0.05$ compared to curcumin; DNC = dendrosomal curcumin; DEN = dendrosome; DOX= doxorubicin. MECs = myoepithelial cells

**Fig. 3.** A feature of tracing MECs injected mice breast tumor

MECs were stained with CellTracker™ CM-DiI Dye 72 h before cell injection. Almost $1 \times 10^6$ viable cells were re-suspended in 50 µl PBS, and then injected at the periphery of the tumor arena. Thirty-five days post transplantation, the incorporated cells were observed in the transplanted area. Fluorescent microscopy using DIL-labeled cells showed viable proliferated MECs evidenced with red colored regions. There were some areas filled with contacted transplant cells furnishing series of quasi-spherical structures such as normal breast acini.

MECs = myoepithelial cells

**Fig. 4.** A plot of MECs effects on mice weight

Animals were weekly weighed and regularly monitored for abnormal sequels. Data reported are mean ± S.D; * $P < 0.05$ compared to control; MECs = myoepithelial cells

**Fig. 5.** MECs effects on tumor size (cm$^3$) in an animal model of breast cancer

Animals were regularly monitored for abnormal sequels. Tumor volume weekly measured by a digital vernier caliper and by using the following formula: $V=\frac{1}{6}(\pi LWD)$, where $L=$ length, $W=$ width and $D=$ depth. The average tumor volume was significantly less than control group in the second and third weeks after MECs injection. Data reported are mean ± S.D; * $P < 0.05$ compared to control MECs = myoepithelial cells

**Fig. 6.** Transmission electron microscopic photomicrographs from MECs injected to mammary tumor
Neoplastic cells surrounded by MECs had large nuclei and nucleoli within a relatively collagenous stroma. They had loose interconnections with large mitochondria in some cells (A, 7000×). MECs (left) with numerous large mitochondria had tight junction with tumor cells (center and top) (B and C, 7000×). They also interrelated with adjacent tumoral cells (D, 7000×). MECs = myoepithelial cells

**Fig. 7.** MECs effects on expression of α-SMA and p63 proteins in tumor tissue of mammary tumor-bearing mice

Sections were stained with polyclonal mouse antiRat/Rabbit p63 and α-SMA antibodies according to the manufacturer’s instructions. The criteria used for p63 and α-SMA markers evaluation was based on the estimated proportion of positive cells and average staining intensity of positive cells for these markers. The semi-quantitative score was adopted as total score of the staining intensity and population of the positive cells. It is ranged from 0 to 6 points. Each of scoring system for intensity and population is as follows (17):

No staining and/or no positive cells: 0
Faint/barely staining and/or faint/barely positive cells up to 25% of cells: 1
Moderate staining and/or moderate positive cells in 25% to 50% of cells: 2
Strong staining and/or strong positive cells in more than 50% of cells: 3

Data reported are mean ± S.D; * P < 0.05 compared to control MECs = myoepithelial cells

**Fig. 8.** MECs effects on expression of NF-kB, TNF-α, α-SMA, SMA-MHC, Cnx 43, Maspin and Calponin in an animal model of breast cancer

Total RNA was extracted from tumor tissues using TRIzol® reagent followed by DNase I digestion. Complementary DNA was synthesized by PrimeScript™ RT reagent kit. Real-time PCR was performed using the SYBR® Premix Ex Taq™ II (Takara). Relative gene expression was calculated as 2^{ΔΔCt}. mRNA expression of TNF-α, SM-MHC, connexin 43, and maspin were significantly up-regulated in breast tumor tissues in the treated group
compared to control. Moreover, mRNA expression of VEGF and SMA was reduced in mice treated compared to control. Data reported are mean ± S.D; *P < 0.05 compared to control;

MECs = myoepithelial cells

Table 1. Nucleotide sequences of the primers used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CAGCAAGGACACTGAGCAAG</td>
<td>TGATGGTATTCAAGAGAGTAGGG</td>
<td>141</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GTGTTATCCATTCTACCA</td>
<td>TCCCAGCATCTTGTTTCT</td>
<td>177</td>
</tr>
<tr>
<td>α-SMA</td>
<td>TCAGGGGATATGGTTGGAATG</td>
<td>GTTGGTGATGATGCGGTGTT</td>
<td>115</td>
</tr>
<tr>
<td>SMA-MHC</td>
<td>CACATCTTCTACTACCTGCTCG</td>
<td>CCTGGAACATTCATCATCTTG</td>
<td>139</td>
</tr>
<tr>
<td>Cnx 43</td>
<td>TATGTGATGAGAACAGGAAGAG</td>
<td>AAGAGGATGCTGATGATGATGG</td>
<td>191</td>
</tr>
<tr>
<td>Maspin</td>
<td>CAGTGGAGAAGAGAGGAGGAT</td>
<td>TGGCAAGAAATGGAAGAAAGA</td>
<td>256</td>
</tr>
<tr>
<td>Calp0nin</td>
<td>CAACAAGTTTGCCAGTCAGC</td>
<td>GCGAGGAAGCCCATACAC</td>
<td>286</td>
</tr>
<tr>
<td>VEGF</td>
<td>AGGCTGCTGTAACGATGAG</td>
<td>GTGCTGGCATTGTGGAGG</td>
<td>97</td>
</tr>
</tbody>
</table>
Table 2. Hematological and blood chemical indices in MECs treated and control mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Treated</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC (Millin/mm³)</td>
<td>8.5 ± 0.8</td>
<td>8.9 ± 0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>38.9 ± 1.7</td>
<td>40.3 ± 2.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Hgb (g/dl)</td>
<td>12.8 ± 1.2</td>
<td>13.3 ± 1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Plt (1000/mm³)</td>
<td>567 ± 50</td>
<td>590 ± 91</td>
<td>0.2</td>
</tr>
<tr>
<td>WBC (1000/mm³)</td>
<td>6.7 ± 0.8</td>
<td>7.6 ± 2</td>
<td>0.09</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>53.5 ± 5</td>
<td>55.6 ± 6</td>
<td>0.1</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>2.1 ± 3</td>
<td>2.4 ± 0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>43.5 ± 6.4</td>
<td>39.4 ± 7</td>
<td>0.09</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>43.2 ± 4.6</td>
<td>48.3 ± 6</td>
<td>0.08</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.48 ± 0.08</td>
<td>0.5 ± 0.07</td>
<td>0.6</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>455 ± 45</td>
<td>518 ± 85</td>
<td>0.08</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>67 ± 8.8</td>
<td>73.6 ± 9.7</td>
<td>0.1</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>763 ± 50</td>
<td>790 ± 70</td>
<td>0.5</td>
</tr>
<tr>
<td>GGT (U/l)</td>
<td>5.1 ± 0.5</td>
<td>5.3 ± 0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>ALB (mg/dl)</td>
<td>3 ± 0.2</td>
<td>3.3 ± 0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>T.BIL (mg/dl)</td>
<td>6.1 ± 0.4</td>
<td>6.5 ± 0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>D.BIL (mg/dl)</td>
<td>0.40 ± 0.06</td>
<td>0.35 ± 0.05</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Values are means ± S.D. *P < 0.05 compared to control. RBC= Red blood cell, HCT= hematocrit, WBC= White blood cells, Plt = Platelets, Cr = Creatinine, AST = Aspartate transaminase, ALT = Alanine transaminase, ALP = Alkaline phosphatase, GGT = Gamma-glutamyl transpeptidase, ALB = Albumin, T.BIL = Total bilirubin and D.BIL = Direct Bilirubin.
Figure 5

Tumor volume (Cm$^3$)

Days (after cells injection)

Control
Treated

14
21
28
35

*