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ORIGINAL PAPER

Myoepithelial and luminal breast cancer cells exhibit different responses to all-trans retinoic acid

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Abstract
Purpose Breast cancer is the leading cause of death among women worldwide. The exact role of luminal epithelial (LEP) and myoephitelial (MEP) cells in breast cancer development is as yet unclear, as also how retinoids may affect their behaviour. Here, we set out to evaluate whether retinoids may differentially regulate cell type-specific processes associated with breast cancer development using the bi-cellular LM38-LP murine mammary adenocarcinoma cell line as a model.
Materials and methods The bi-cellular LM38-LP murine mammary cell line was used as a model throughout all experiments. LEP and MEP subpopulations were separated using inmunobeads, and the expression of genes known to be involved in epithelial to mysenchymal transition (EMT) was assessed by qPCR after all-trans retinoic acid (ATRA) treatment. In vitro invasive capacities of LM38-LP cells were evaluated using 3D Matrigel cultures in conjunction with confocal microscopy. Also, in vitro proliferation, senescence and apoptosis characteristics were evaluated in the LEP and MEP subpopulations after ATRA treatment, as well as the effects of ATRA treatment on the clonogenic, adhesive and invasive capacities of these cells. Mammosphere assays were performed to detect stem cell subpopulations. Finally, the orthotopic growth and metastatic abilities of LM38-LP monolayer and mammosphere-derived cells were evaluated in vivo.
Results We found that ATRA treatment modulates a set of genes related to EMT, resulting in distinct gene expression signatures for the LEP or MEP subpopulations. We found that the MEP subpopulation responds to ATRA by increasing its adhesion to extracellular matrix (ECM) components and by reducing its invasive capacity. We also found that ATRA induces apoptosis in LEP cells, whereas the MEP compartment responded with senescence. In addition, we found that ATRA treatment results in smaller and more organized LM38-LP colonies in Matrigel. Finally, we identified a third subpopulation within the LM38-LP cell line with stem/progenitor cell characteristics, exhibiting a partial resistance to ATRA.
Conclusions Our results show that the luminal epithelial (LEP) and myoephitelial (MEP) mammary LM38-P subpopulations respond differently to ATRA, i.e., the LEP subpopulation responds with increased cell cycle arrest and apoptosis and the MEP subpopulation responds with increased senescence and adhesion, thereby decreasing its invasive capacity. Finally, we identified a third subpopulation with stem/progenitor cell characteristics within the LM38-LP mammary adenocarcinoma cell line, which appears to be non-responsive to ATRA.

Keywords Mammary cancer · Retinoic acid receptors · Luminal and myoepithelial cells · EMT

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1 Introduction

Breast cancer is associated with the highest incidence and death rates among woman in western countries [1–3]. Epidemiological data indicate that 1 in 8 women may suffer from this disease. In many breast cancers myoepithelial (MEP) cells co-exist with neoplasic luminal epithelial (LEP) cells, and both have been found to modulate the angiogenic, proliferative and invasive capacities of the neoplastic luminal component [4–7]. Furthermore, several authors have speculated that MEP cells may create a favourable micro-environment for tumour progression but, as yet, little is known about the exact interaction between both cell types [8–10]. It is relevant to note here that most in vitro breast cancer models encompass only one cell type which, thus may not be representative of the actual physiological condition within a neoplastic mammary gland [11]. Previously, we developed and characterized the LM38-LP cell line, which is derived from a spontaneous murine mammary adenocarcinoma and which contains both myoepithelial and neoplastic luminal components, similar to human triple negative mammary tumours for which it serves as a pre-clinical model [12, 13]. Basal subtype triple-negative tumors do not express estrogen or progesterone receptors (ER or PR, respectively) and lack HER2 expression and/or *HER2* gene amplification [14, 15]. These tumours are generally aggressive and exhibit a high probability of metastatic dissemination [16, 17]. Unlike other breast cancers, triple negative cancers are resistant to current therapies targeting HER2, including trastuzumab, hormone therapies based on tamoxifen, and therapies based on aromatase inhibitors.

Due to their role in growth regulation and differentiation, natural and synthetic retinoids are widely evaluated in clinical trials for cancer treatment and prevention [18–20]. Besides, since retinoic acid is known to act as a key regulator of embryonic stem cell behaviour, retinoids may not only induce apoptosis, but also differentiation of cancer stem cells. Due to their difference in origin, the susceptibility of LEP and MEP cells to drug treatment may differ. In the past, retinoid-based treatment of solid tumours turned out to be unsatisfactory, causing this approach to be abandoned [21], in spite of the fact that at that time little was known about the role played by the retinoid signalling pathway in solid tumour development [22–25]. Recent work has uncovered the importance of both LEPs and MEPs in breast cancer development, thereby opening avenues to revisit the potential of this retinoid-based therapeutic approach. We hypothesize that, although the solid tumour environment is complex and heterogeneous [26], changes in pathological conditions accomplished through differentiation induction may affect patients with cancer, i.e., differentiated cells may become more susceptible to conventional therapies, leading to better therapeutic responses. Retinoic acid is the active metabolite of vitamin A, playing a key role in controlling the development, proliferation and differentiation of diverse cell types [27], including embryonic and adult cells. The effects of retinoids are mainly mediated through retinoid receptors, members of the nuclear receptor super-family. These receptors include retinoic acid receptors (RARs) and rexinoid receptors (RXRs). Both receptor types constitute α, β and γ subtypes, which are encoded by distinct genes. Additionally, each subtype may be subject to alternative splicing, resulting in different isoforms with distinct regulatory and functional properties [28]. In response to ligand binding, retinoid receptors may form RAR/RXR heterodimers that can bind to regulatory regions within DNA, called retinoic acid responsive elements (RAREs). RAREs are present in a vast number of genes, including the *RARβ* (implicated in differentiation and apoptosis), C*ytochrome CYP26* (linked to ATRA metabolism), *CRBP-I, −II, −III and -IV* (retinol transporter proteins) and various *HOX* genes [29]. In the classic retinoid pathway, also known as the 'genomic pathway', retinoids bind to RXR-RARα1 heterodimers triggering co-repressor release and co-activator recruitment, which activates the expression of enzymes that can modify histones, thereby allowing chromatin conformational changes. This enables active RARα1 to induce the transcription of genes harbouring a RARE motif within their promoter [30]. RARs can also mediate rapid transcription-independent responses such as the modulation of phosphatidyl inositol-3-kinase (PI3K) or MAPK pathways [31]. Moreover, different retinoids can mediate signalling pathways distinct from those regulated by RARs. An example is the capacity of ATRA to modulate the cAMP-PKA signalling pathway which can lead to cellular differentiation independently of RAR activation [32].

The aim of the current study was to uncover the mechanisms involved in growth and metastatic dissemination inhibition exerted by retinoids on the MEP and LEP subpopulations of the LM38-LP mammary murine adenocarcinoma cell line. Our results show that both subpopulations respond differentially to retinoid (ATRA) treatment. The LEP subpopulation responds through activation of the 'genomic pathway' which leads to cell cycle arrest and apoptosis. In the MEP subpopulation a non-genomic pathway is activated that increases senescence and adhesion and, at the same time, decreases invasion. Finally, we identified a third subpopulation with stem/progenitor cell characteristics, which seems non-responsive to ATRA.

2 Materials and methods

2.1 Cell cultures

The previously established LM38-LP cell line (passage 15–25), derived from a BALB/c murine mammary papillary adenocarcinoma with tumourigenic and metastatic capacities [8], was used throughout this study. This cell line is composed of

two main subpopulations, antigenically characterized as luminal epithelial (LEP) and myoephitelial (MEP), which remain up to the 30th passage [12]. Cells were grown in DMEM/F12 medium with non-essential amino acids and 2 μM L-glutamine (Gibco, Life Technologies, Rockville, MD, USA), supplemented with 10 % fetal bovine serum (FBS) (Internegocios, Buenos Aires, Argentina) at 37 °C in plastic tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) in a humidified 5 % CO_2/air atmosphere. Serial passages were carried out through treatment of sub-confluent monolayers with 0.25 % trypsin and 0.02 % EDTA in Ca^{2+} and Mg^{2+}-free PBS (Gibco, Carlsbad, CA, USA).

2.2 Retinoids and doses

Except otherwise stated, the following compounds and doses were used: ATRA (Sigma–Aldrich, St. Louis, MO, USA) 1 μM, Rarα agonist AM580 (Biomol, Plymouth Meeting, PA, USA) 200 nM and Rarα antagonist Ro41-5253 (Biomol, Plymouth Meeting, PA, USA) 2 μM. All compounds were dissolved in DMSO at a 1000x final concentration.

2.3 Separation of LEP and MEP subpopulations

LM38-LP cells, pretreated or not with retinoids, were detached and separated into LEP and MEP subpopulations essentially as reported before [8]. Briefly, cells were incubated for 20 min with an anti-E-cadherin antibody (Santa Cruz, Dallas, TX, USA), thoroughly washed and, subsequently, incubated with magnetic immunobeads (Dynabeads M-280; Invitrogen, Carlsbad, CA, USA) for 20 min at 4 °C. Based on the fact that E-cadherin is exclusively expressed on LEP cells, cell populations were separated using a magnet [12]. It should be noted that in our experiments the LM38-LP cell subpopulations were first treated together to closely simulate a physiological situation, i.e., allowing them to interact, and subsequently separated to study the effects of treatment on each subpopulation.

2.4 Western blotting

Sub-confluent monolayers of LM38-LP cells were washed twice with ice-cold PBS, scraped off with a Teflon scraper and, finally, lysed with 1 % Triton X-100 in PBS. After protein concentration measurements, samples were denatured by boiling in sample buffer with 5 % β-mercaptoethanol and run in 10 % SDS-PAGE. 50 μg protein was loaded in each lane and, after electrophoresis, the gels were blotted to Hybond-P membranes. Next to incubation for 1 h in blocking buffer, containing 5 % skim milk and 0.1 % Tween-20 in PBS, the membranes were incubated for 18 h with primary anti-Erα (MC-20: sc-542, Santa Cruz, Dallas, TX, USA), anti-Pr (C262: sc-53943, Santa Cruz, Dallas, TX, USA) or anti-Her2 (44E7, Cell Signaling, Danvers, MA, USA) antibodies and, next, for 1 h with mouse or rabbit secondary antibodies coupled to horseradish peroxidase (Santa Cruz, Dallas, TX, USA). The final detection was performed by chemoluminiscence. Bands were digitalized using a Photo/Analyst Express System (Fotodyne Inc. Hartland, WI, USA) and the signal intensities were quantified using Image J software (NIH, USA).

2.5 RT-PCR assay

mRNA expression was determined by RT-PCR. Briefly, RNA from cells, treated or not with retinoids for 48 h, was extracted using a Gentra Purescript RNA isolation kit (Qiagen, Limburg, Netherlands) and cDNA was prepared using an iScript cDNA synthesis kit (Bio Rad, Richmond, CA, USA). PCR products were obtained using the human/mouse (h/m) primers *rar*α1 (sense: 5′-GCCAGGCGCTCTGACCACTC-3′, antisense: 5′-AGCCCTTGCAGCCCTCACAG-3′) and h/m *rar*α2 (sense: 5′-ACTCCGCTTTGGAATGGCTCAAAC-3′, antisense: 5′-AGCCCTTGCAGCCCTCACAG-3′) as reported before by Zelent [33], m*rar*γ1 (sense: 5′-TGGGGC CTGGATCTGGTTAC-3′, antisense: 5′-TTCACAGGAGCT GACCCCAT-3′), m*rar*γ2 (sense: 5′-GCCGGGTCGCGATG TACGAC-3′, antisense: 5′- TTCACAGGAGCTGACCCCAT -3′), h/m *rar*β2 (sense: 5′-ATGGAGTTCGTGGACTTTTC TGTG-3′, antisense: 5′-CTCGCAGGCACTGACGCCAT-3′) and *crbp*-1 (sense: 5′-TTGTGGCCAAACTGGCTCCA-3′, antisense: 5′-ACACTGGAGCTTGTCTCCGT-3′) as designed at Dr. Farias laboratory (Mount Sinai, School of Medicine, New York), *nanog* (sense: 5′-CCAGTGGAGTAT CCCAGCAT-3′, antisense: 5′- GAAGTTATGGAGCGGA GCAG-3′), *sox*2 (sense: 5′-AAGGGTTCTTGCTGGGTTTT -3′, antisense: 5′-AGACCACGAAAACGGTCTTG-3)′ and *gapdh* (sense: 5′-CGTAGACAAAATGGTGAAGG-3′, antisense: 5′-GACTCCACG ACATACTCAGC-3′).

2.6 qPCR assay

Cells were seeded in 100 mm culture dishes and treated with ATRA or (control) vehicle for 48 h. Then MEP and LEP cells were separated as described above and RNA was extracted using a purification RNeasy Mini Kit (Quiagen, Limburg, Netherlands). Subsequently, reverse transcription and qPCR were performed following the manufacturer's instructions (RT^2 First Strand Kit, catalog number 330401; RT^2 Profiler PCR Array Format D, PAMM-090A-2 EMT; Quiagen, Limburg, Netherlands). The results were analyzed using the PCR Array Data Analysis Software package (www.SABiosciencies.com/ pcrarraydataanalysis.php).

2.7 Matrigel colony assay

5×10^3 cells were re-suspended in 400 μl DMEM/F12 medium supplemented with 2 % FBS and 2 % Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The resulting monocellular suspensions were seeded in multi-well culture slides (Labteks, BD Biosciencies, Franklin Lakes, NJ, USA) containing 75 μl Matrigel. The respective retinoid treatments were initiated 3 days after seeding, when colonies were first detectable, and lasted for 48 or 120 h. Next, the cells were fixed with 2 % paraformaldehyde in PBS for 20 min at room temperature (RT) and subjected to immunofluorescence as outlined below, or for direct observation using an inverted phase contrast Eclipse E400 Nikon microscope (Tokyo, Japan).

2.8 Immunofluorescence and microscopy

LM38-LP cells were seeded (2×10^4/well) in multi-well culture slides (Labteks, BD Biosciences, Franklin Lakes, NJ, USA). 24 h after seeding, sub-confluent monolayers of cells were treated with retinoids for 48–120 h, and fixed with 3 % paraformaldehyde in PBS for 20 min at RT. Next, both monolayers and 3D Matrigel cultures (see above) were permeabilized with pre-cooled 0.5 % Triton X-100 for 10 min at 4 °C, rinsed with PBS-glycine, and incubated with blocking buffer (5 g BSA, 10 ml Triton X-100, 2 ml Tween 20 in 500 ml PBS+10 % serum). After this, the cells were incubated overnight at 4 °C with primary antibodies directed against smooth muscle actin (Santa Cruz, Dallas, TX, USA), phospho-Akt (Cell Signaling, Danvers, MA, USA), activated caspase-3 (Cell Signaling, Danvers, MA, USA) or p27 (Santa Cruz, Dallas, TX, USA) in blocking buffer (1:200). After washing, secondary antibodies conjugated with Alexa fluor 456 or 488 were added (Molecular Probes, Eugene, OR, USA) in blocking buffer (1:400) for 1 h at RT. Finally, the cells were incubated with DAPI (1:2000) for 10 min at RT, rinsed with PBS, and mounted in ProLong anti-fade solution (Molecular Probes, Eugene, OR, USA). Photographs were taken with a Leica TCS SP5 DM confocal microscope or with an Eclipse E400 Nikon epifluorescence microscope equipped with a Coolpix Nikon digital camera (Tokyo, Japan).

2.9 Proliferation assay

The proliferative potential of LM38-LP cells was determined by assessing the number of cells during the exponential growth phase of unsynchronized monolayer cultures under different treatment conditions. Briefly, 4×10^5 cells were seeded in 35 mm Petri dishes and cultured in normal media or in media with charcoal-stripped serum during 4 days. After this, cells from triplicate wells were washed twice with PBS, trypsinized, and counted using a hemocytometer in conjunction with trypan blue exclusion staining.

2.10 7-AAD cytometry assay

Cells treated with retinoids for 72 h were detached and rinsed with PBS. The cells were re-suspended in Staining Solution included in the 7-AAD Cell Viability Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) and incubated at RT for 10 min. Next, the cells were centrifuged at 400 g for 5 min and re-suspended in 0.5 ml Assay Buffer. Samples were analyzed using a BD FACS CANTO cytometer and the results obtained were analyzed using Diva and Flowjo software packages (BD Biosciences, Franklin Lakes, NJ, USA).

2.11 Annexin-V staining and epifluorescence microscopy

Cells were seeded in multi-chamber slides (Labteks, BD Biosciences, Franklin Lakes, NJ, USA) and treated with retinoids for 72 h. Next, they were rinsed with PBS after which 100 μl Annexin-binding buffer, 1 μl Annexin-V conjugated to Alexa-Fluor 488 (Invitrogen, Carlsbad, CA, USA) and 2 μl propidium iodine (PI) were added to each well. Subsequently, the cells were incubated for 15 min at RT and the wells were rinsed with Annexin-binding buffer. Finally, labtek slides were mounted using ProLong anti-fade solution (Molecular Probes, Eugene, OR, USA) and photographs were taken using an Eclipse E400 Nikon microscope equipped with a digital Coolpix Nikon camera, as mentioned above (Tokyo, Japan).

2.12 Senescence assay

A Senescence Detection Kit (Biovision, Milpitas, CA, USA) was used following the manufacturer's instructions. Briefly, 5×10^3 cells were seeded in 12 well-plates. Cells were treated with retinoids for 96 h, rinsed with PBS and fixed with 0.5 ml Fixation Solution (Biovision, Milpitas, CA, USA) for 15 min at RT. Next, the cells were washed twice with PBS, and 470 μl staining solution, 5 μl supplement for staining and 25 μl of a 20 mg/ml X-gal solution in DMSO were added to each well. After overnight incubation at 37 °C, the cells were rinsed again with PBS and mounted in PBS-Glycerol (1:1). Photographs were taken using a phase contrast Eclipse E400 Nikon microscope equipped with a digital Coolpix Nikon camera (Tokyo, Japan).

2.13 Transwell invasion assay

Transwell cell culture chambers (Corning, Union City, CA, USA) were used for the invasion assay. The filters (8 μm membrane pores) were first coated with a thin layer of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The lower chamber was

supplemented with human cellular fibronectin (16 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) in 0.5 ml MEM, as chemo-attractant. $1x10^5$ LM38-LP cells or its LEP/MEP components pre-treated or not with ATRA for 5 days, were seeded into the chambers in triplicate. After 24 h, the cells on the upper side of the membranes were thoroughly wiped off with a cotton swab, after which the membranes were fixed with formalin and stained with DAPI. The cells that had invaded the Matrigel, passed through the pores and re-attached to the lower surface of the filter were regarded as invasive, and their nuclei were counted in 400x fields under a fluorescence microscope (Eclipse E400, Nikon, Tokyo, Japan).

2.14 Adhesion assay

LM38-LP cells were treated with or without ATRA for 5 days. When appropriate, pre-treated cells were separated as outlined above. Pre-treated monolayers were detached with trypsin and, after 2 h incubation at RT to recover their surface, 3×10^4 cells/well were seeded in triplicate in 24 multi-well plates containing medium supplemented with 2 % FBS, with or without ATRA. The wells were first coated with 0.1 % fibronectin (Sigma–Aldrich, St. Louis, MO, USA) or collagen I (Labtek with Collagen I, BD Biosciences, Franklin Lakes, NJ, USA). After 60 min incubation at 37 °C, non-adherent cells were removed by washing twice with PBS and adherent cells were detached by trypsinization and quantified by direct counting (see above). The rate of adhesion was calculated as number of adherent cells/total number of seeded cells.

2.15 Clonogenicity assay

1×10^5 cells were seeded in 35 mm Petri dishes and treated for 5 days with retinoid or (control) vehicle. Next, these pre-treated cells were detached with trypsin and 1000 cells of each group were seeded in quadruplicate in 35 mm Petri dishes. Half of these dishes continued receiving the retinoid treatment for an additional 5 days. After this, colonies were fixed with neutral formalin and stained with violet crystal. Colonies larger than 20 cells were counted using a StereoStar Stereoscopic Zoom Microscope model 568 (American Optical, Las Vegas, NV, USA).

2.16 ALDH activity assay

An Aldefluor kit (Stem Cell Technologies, Vancouver, Canada) was used to determine de percentage of aldehyde dehydrogenase (ALDH) positive LM38-LP cells according to the manufacturer's instructions. Green fluorescence, which is produced as a result of ALDH activity, was assessed using a BD FACS CANTO cytometer (BD Biosciences).

2.17 Mammosphere formation assay

A 1×10^4 mono-cellular suspension of LM38-LP cells was seeded in low attachment plates with mammosphere serum-free medium: DMEM-F12, 10 ng/ml FGF, 20 ng/ml EGF, B27 without vitamin A (Invitrogen, Carlsbad, CA, USA) [34]. After 10–12 days, mammospheres were treated or not with ATRA for 96 h. Next, mammospheres were photographed using a digital Coolpix Nikon camera coupled to a phase contrast Eclipse E400 Nikon microscope. Subsequently, mammospheres were disaggregated and the cells were cultured under normal conditions for adherent growth up 12 days to evaluate their capacity to reconstitute LM38-LP mixed populations.

2.18 Orthotopic tumour growth and metastasis assays

Randomized inbred 2 to 4 month-old virgin female BALB/c mice, obtained from our Animal Care Facility, were used for in vivo assays. Food and water were administered ad libitum. All animal studies were conducted in accordance with the NIH Guide for the Care and the Use of Laboratory Animals. Mice were inoculated orthotopically into the fat pad of the 4th mammary gland with 2×10^5 LM38-LP cells or 1000 cells from mammospheres treated or not with ATRA for 96 h. When tumours became palpable (approximately 5 days post inoculation for LM38-LP and 14 days for cells from mammospheres), tumour diameters were measured twice a week with a caliper and tumour volumes were calculated (D x d2/2) for the assessment of growth rates. Four weeks after the tumours became palpable, mice were sacrificed and necropsied. The tumours were used for histopatological assessments and, to delineate the presence of spontaneous metastases, lungs were removed and the numbers of surface lung nodules were recorded.

2.19 Statistical analyses

All experiments were performed in triplicate and each experiment was repeated at least twice. The significance of differences between groups was calculated using Student's t or ANOVA tests, as indicated. The nonparametric Mann–Whitney U test was used to analyze differences in metastatic ability. A value of $p<0.05$ was considered to be statistically significant.

3 Results

3.1 LM38-LP mammary tumour cells grow in a hormone-independent manner

In order to define the LM38-LP mammary tumour cell model, we assessed the expression of the estrogen receptor alpha

(ERα), the progesterone receptor (PR) and the tyrosine-protein kinase receptor ErbB2 (HER2). Whereas we could not detect any expression of ERα or HER2, we found low levels of expression of PR-B (Fig. 1a). This result led us to test whether LM38-LP cells can grow in a hormone-independent manner. To this end, we performed an in vitro growth assay in normal media and in media containing charcoal-stripped serum. As shown in Fig. 1b, LM38-LP cells could equally well grow in normal media and media containing charcoal-stripped serum for at least 120 h, indicating that LM38-LP cells can indeed grow in a hormone-independent manner.

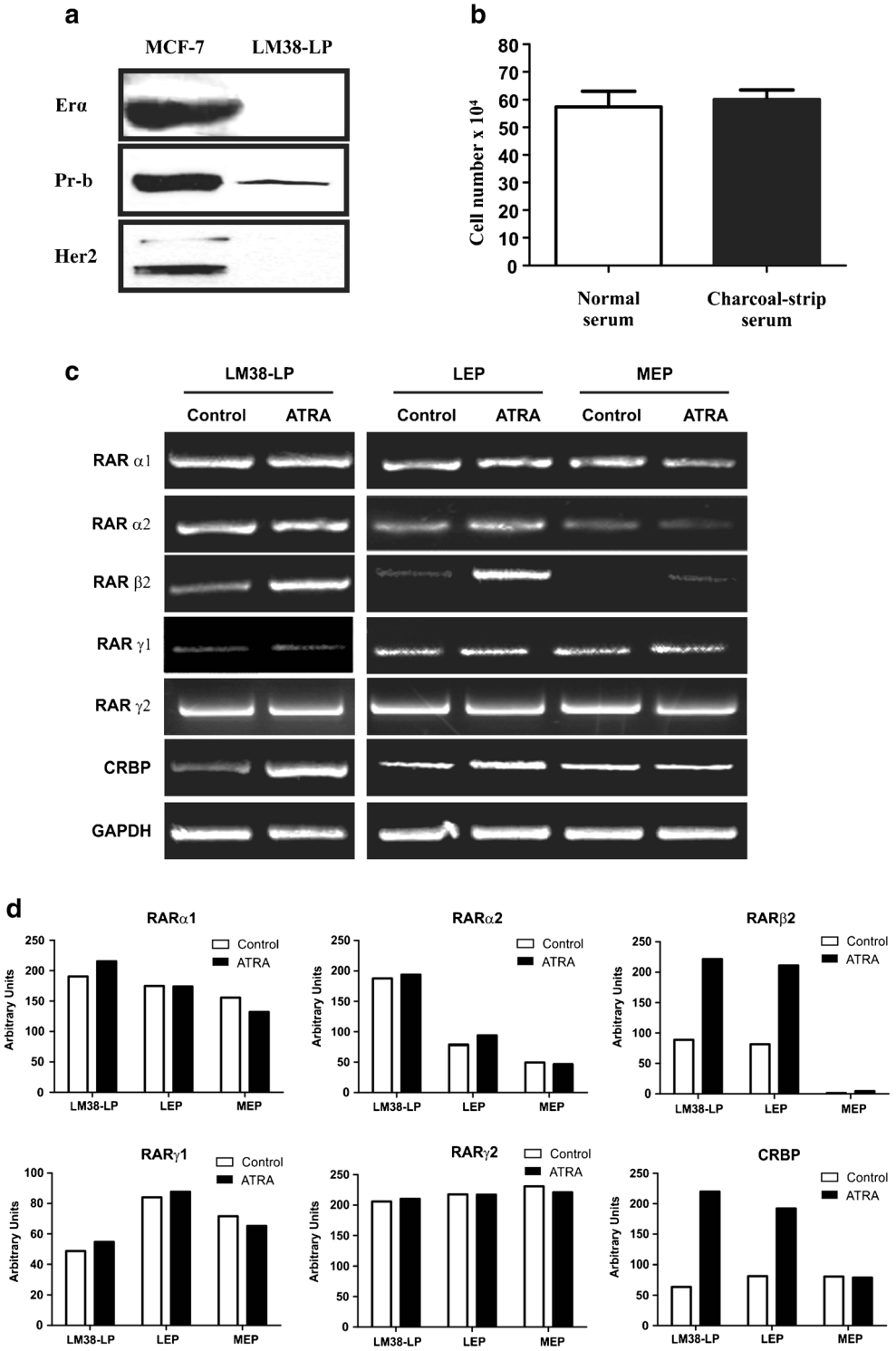


Fig. 1 **a** Whole cell lysates prepared from LM38-LP and MCF-7 (positive control) cells were subjected to Western blotting using antibodies directed against Erα, Pr and Erb2 (Her2). Similar results were obtained in three independent experiments. **b** LM38-LP cell numbers assessed 96 h after culture in medium with 2 % charcoal-stripped serum. At least 3 independent experiments were performed with similar results. **c** RT-PCR analysis of *rar* isoforms and *crbp*-1 in LM38-LP cells and its derived LEP/MEP components. Results are representative of two independent experiments. **d** Densitometric representation of each RT-PCR experiment

3.2 Retinoic acid signalling pathway-associated genes are differentially expressed in LEP and MEP cells

Since our previous work indicated that LM38-LP cells express all RAR and RXR receptors and, concomitantly, respond to ATRA treatment [13], we set out to determine whether ATRA may differentially affect its luminal epithelial (LEP) or myoephitelial (MEP) subpopulations. To this end, LEP and MEP cells from ATRA treated (48 h) LM38-LP cells were separated by magnetic immunobeads (see materials and methods), and analysed by RT-PCR for the expression of several retinoic acid signalling pathway-associated genes. By doing so, we found that LM38-LP and LEP cells exhibited an increased expression of *rar*β2 and *crbp*-1, a known Rarα1 target, upon ATRA treatment (Fig. 1c and d), whereas no changes in the *rar*α1, *rar*α2, *rar*γ1 and *rar*γ2 isoforms were observed, neither in the LM38-LP cell line nor in its LEP and MEP subpopulations.

3.3 LEP and MEP cells differentially regulate EMT genes in response to ATRA treatment

Since epithelial to mesenchymal transition (EMT) has been implicated in tumour progression and metastatic dissemination [35], we decided to assess whether ATRA may affect the expression of genes involved in EMT, and whether LEP and MEP cells may respond differentially to this treatment. To this end, LM38-LP cells were incubated or not with ATRA for 48 h. Next, both subpopulations were separated and real time PCR (qPCR) analyses were performed for 84 genes known to be involved in EMT. The results, depicted in Table 1, show that 27/84 genes were modulated by more than 2.5-fold in at least one of the two subpopulations. The LEP subpopulation was found to respond to ATRA by negatively regulating 22 of these 27 genes, most of them known to be involved in proliferation and survival, including *fzd7*, *mst1r*, *tmem132a*, *tcan* and *wnt11*, as well as genes known to be involved in migration, invasion and metastatic dissemination, including *jag1*, *mmp9* and *wnt11* or *wnt5A* (Table 1). On the other hand, MEP cells were found to respond to ATRA treatment in a different way than LEP cells, i.e., only 2/27 genes showed a significantly decreased expression, i.e., *mmp2* and *mmp9*, both known to be implicated in migration and invasion. MEP cells also showed an increased expression of 5 genes known to be implicated in differentiation and migration, including *bmp1* and *itgb1*, which were not modulated by ATRA treatment in LEP cells (Table 1).

3.4 ATRA inhibits growth through different mechanisms in LEP and MEP cells

Since ATRA treatment was found to differentially modulate the expression of EMT genes involved in differentiation, proliferation and survival of LEP and MEP cells, we set out to assess whether these differences are associated with different biological responses of these cells to ATRA. Regarding proliferation and survival, we previously found [13] that exposure to ATRA reduces the phosphorylation levels of Erk and Akt in LM38-LP cells. Given the fact that these molecules play important roles in cellular proliferation and survival, we questioned which cellular component of the LM38-LP cell line is implicated in the ATRA-induced decrease in phosphorylated Akt (pAkt). To this end, monolayers of LM38-LP cells were treated with retinoids for 48 h and then analyzed for the expression of pAkt and, concomitantly, smooth muscle actin (i.e., a myoepithelial cell marker) by immunofluorescence in conjunction with confocal microscopy (Fig. 2a). Before treatment, some LEP cells and a few MEP cells were found to be positive for pAkt immunostaining. Subsequent treatment of the cells with ATRA resulted in a notable decrease in pAkt expression, whereas treatment with the RARα antagonist Ro41-5253 resulted in a reversion of this effect. However, a few cells located at the outer limit of LEP islets and in contact with MEP cells continued to express pAkt, suggesting that they were unresponsive to ATRA (Fig. 2a).

Next we investigated the effect of retinoids on the capacity of LM38-LP cells to form 3D structures in Matrigel, which represents a more physiological condition than cells growing in monolayer. We found that LM38-LP cells have the ability to form large and rather disorganized 3D epithelial colonies. These colonies became smaller and more differentiated glandular-like with an evident lumen after 96 h of ATRA treatment (Fig. 2b). We also found that after 48 h of ATRA treatment the inner cells of these luminal colonies showed an increase in nuclear p27 immunostaining. This observation suggests that growth arrest may be a mechanism implicated in the differentiation effect of ATRA on the luminal compartment (Fig. 2**c**). In order to assess whether apoptosis, a mechanism that includes growth arrest, is differentially regulated by retinoids in LM38-LP cells, we performed a 7-ADD assay using flow cytometry on monolayer cells treated with ATRA for 72 h. By doing so, we indeed found that this treatment causes an increase in the number of apoptotic cells (Fig. 2d). To subsequently evaluate which subpopulation was affected, monolayer cultures were treated with ATRA for different time periods, stained with Annexin-V and propidium iodine (PI) and evaluated by fluorescence microscopy. After 72 h, ATRA treatment induced apoptosis mainly in LEP cells (Fig. 2e), whereas a 120 h treatment period was required to induce apoptosis in some of the MEP cells (data not shown). Concordantly, we found that in 3D Matrigel colonies caspase-3, a robust apoptosis marker, was activated in the centres after 5 days of ATRA treatment (Fig. 2f).

Previously, we showed that in LM38-LP monolayer cultures, under normal conditions, many MEP cells were already arrested, and that ATRA increased the number of MEP cells positive for nuclear p27 staining [13]. Taking into account that in addition to apoptosis, senescence may be an outcome of

Table 1 Genes involved in EMT that are regulated by ATRA. Negative values indicate times down-regulation compared to the untreated control, and positive values indicate times up-regulation compared to the untreated control

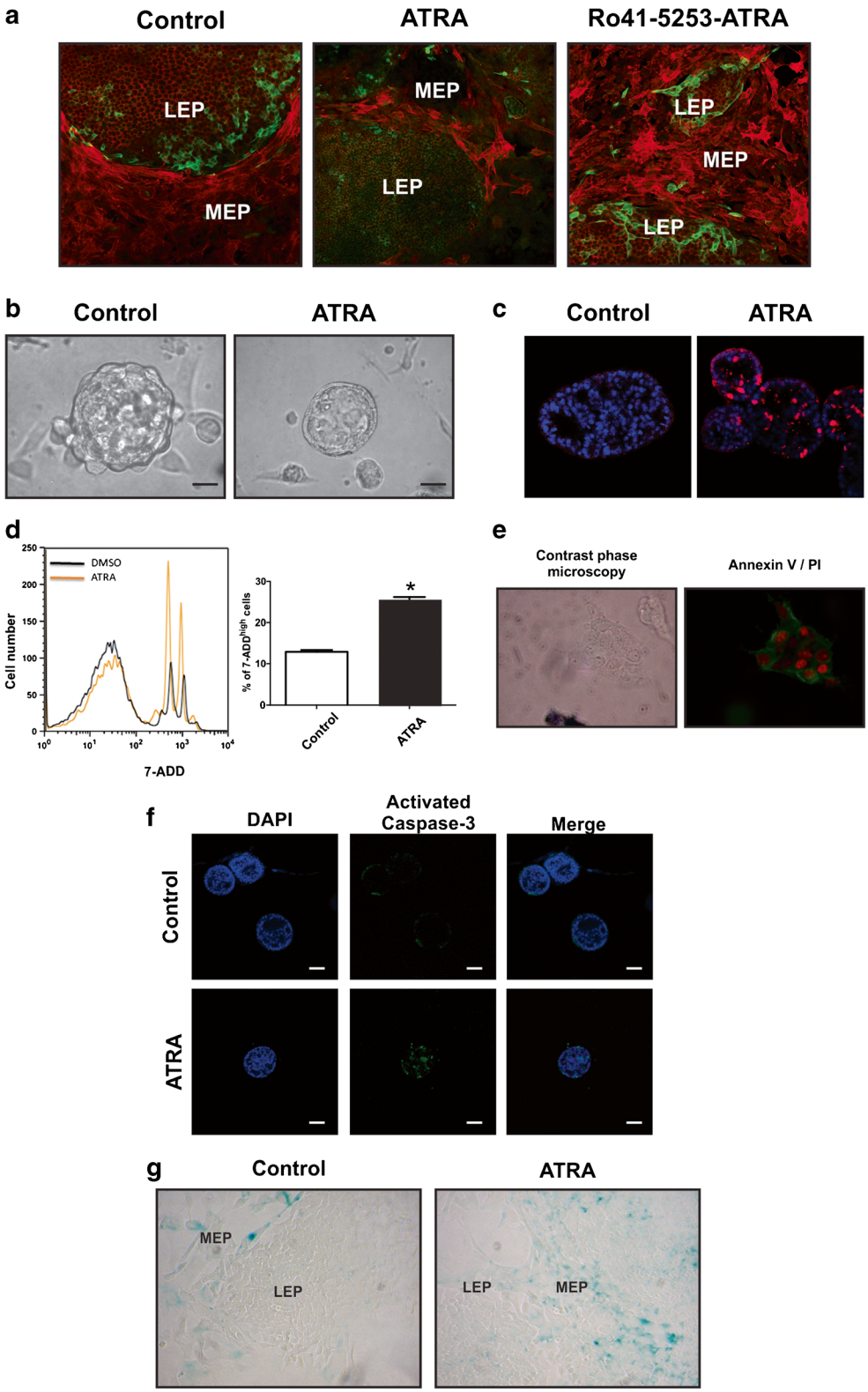
Genes	LEP	MEP	Most relevant fuction	Reference
ahnak	———	+3.6	Role in cellular adhesion. Regulates actin cytoskeleton in plasmatic membrane.	[36]
akt1	−9.9	———	Cellular survival	[37]
bmp1	———	+3.1	Cellular differentiation	[38]
col1a2	———	+4.7	Cellular adhesion and cytoskeleton organization	[39]
egfr	−37.0	———	Growth and cellular proliferation	[40]
foxc2	−19.2	———	Transcription factor involved in EMT activation. It is correlated to the capability of forming metastasis	[41]
fzd7	−127.7	———	Decreased expression diminishes survival, invasion and metastatic capability of cancer colon cells	[42]
ilk	−3.6	———	Promotes tumorigenesis in neurobastoma cells. Role in EMT induction	[43, 44]
itga5	−64.5	———	Promotes migration in Breast cancer cells. Modulates migration and invasion.	[45, 46]
itgb1	———	+3.9	Modulates cytoskeleton components affecting migration, invasion and cellular adhesion	[47]
jag1	−131.2	+3.3	Protein associated to metastatic capacity of breast cancer cells. Its overexpression is associated with bad prognostic in breast cancer	[48, 49]
mmp2	−88.3	−3.2	Alters extracellular matrix and basal membranes organization. It is augmented in numerous tumors	[40]
mmp3	−3.7	———	Alters extracellular matrix and basal membranes organization. It is augmented in numerous tumors	[40]
mmp9	−318.4	−7.7	Alters extracellular matrix and basal membranes organization. It is augmented in numerous tumors	[40]
mst1r	−192.9	———	Its overexpression promotes tumor formation	[50]
notch1	−41.9	———	Critical role in balance maintenance between cellular proliferation, differentiation and apoptosis	[50]
ptk2	−15.1	———	Related to tumoral invasion and survival. It is overexpressed in highly metastatic colon and breast cancer	[51]
smad2	−25.1	———	Mediates TGF-beta growth factor signal regulating proliferation, apoptosis and differentiation	[52]
stat3	−83.4	———	Controls various cellular processes including proliferation, oncogenesis y metastasis	[53, 54]
steap1	−11.3	———	Overexpressed in breast cancer and is negatively regulated by17-β-estradiol in MCF-7 cells	[55]
tgfb1	−18.2	———	Regulates proliferation, adhesion, migration and others cellular functions	[40]
tmem132a	−569.2	———	Important survival factor	[56]
twist1	−21.3	———	Important function in EMT regulation. When it is negatively regulated, migration and invasion capacity decrease in some tumor cells.	[57, 58]
vcan	−232.5	———	Predictive relapse marker and it has negative impact in survival rate.	[59]
vim	+4.1	———	Related to cell migratory capacity	[60]
wnt11	−294.6	———	Implicated in multiple signalization pathways and can affect proliferation and cellular migration	[61–63]
wnt5a	−46.2	———	Implicated in multiple signalization pathways and can affect proliferation and cellular migration	[64, 65]

retinoid-induced cellular arrest, and since senescence is usually associated with pre-malignant stages and prevention of tumour progression, we set out to assess whether retinoid treatment may induce senescence in LM38-LP cells and, if so, which subpopulation is affected. We used SA-β-galactosidase activity as a well-established senescence marker. Under normal (control) conditions, only some MEP cells showed SA-β-galactosidase activity (i.e., were senescent). After 96 h treatment with ATRA or the RARα agonist AM580, an increase in SA-β-galactosidase activity was mainly found in MEP cells, while only a few LEP cells were positive for this marker (Fig. 2g).

3.5 LEP and MEP cells respond differently upon retinoid treatment in metastatic dissemination

From our real time PCR results we deduced that ATRA may exert different effects on the expression of genes involved in adhesion, migration and invasion in either LEP or MEP cells. Since invasion and metastasis are key events related to tumour progression, we assessed whether ATRA may indeed have

Fig. 2 **a** Effect of 48 h ATRA treatment on pAkt expression. LM38-LP monolayer cells were subjected to immunofluorescence and confocal microscopy to assess the expression of pAkt (*green*) and smooth muscle actin (*red*) (400x). **b** Effect of ATRA treatment on colony formation of LM38-LP cells in Matrigel. Phase contrast microscopy (200x). **c** Effect of ATRA treatment on p27 (*red*) nuclear translocation within LM38-LP cells in Matrigel culture. Nuclei were counter-stained with DAPI (*blue*) (400x). **d** Annexin V flow cytometry of LM38-LP cells treated for 72 h with ATRA. *$p<0.05$, *t*-test, $n=4$. (**e**) ATRA effect on LM38-LP monolayer cells. Luminal apoptotic cells show double staining with IP (*red*) and Annexin (*green*) (400x). **f** Activated caspase-3 detection by immunofluorescence and confocal microscopy of LM38-LP Matrigel colonies treated or not (control) with ATRA (400x). **g** SA-β-galactosidase activity assay of LM38-LP monolayer cells treated or not (control) with ATRA. Blue staining indicates senescent cells (200x). Figures are representative of three independent experiments. Scale bar=100 μM



differential effects on the in vitro adhesive and invasive capacities of LEP and MEP cells. To this end, we evaluated invasion using Tanswell culture chambers and ATRA treated or non-treated LM38-LP cells, or their separated LEP/MEP subpopulations. By doing so, we found that LM38-LP cells and its MEP subpopulation responded to ATRA treatment by reducing their invasive capacities, while the LEP compartment was not affected. However, we must take into account

here that the LEP subpopulation is virtually non-invasive in the control situation (Fig. 3a). As shown in Fig. 3b, ATRA treatment also increased the adhesion of LM38-LP cells to fibronectin, an extracellular matrix (ECM) component. The same result was obtained with MEP cells, whereas the adhesive capacity of LEP cells was not modified by this treatment. Conversely, we found that ATRA treatment increased the adhesion to collagen I in both cell populations studied, but its effect was more pronounced in the MEP subpopulation.

3.6 The LM38-LP cell line harbours a third subpopulation with stem/progenitor cell characteristics

Given the fact that a low number of cells located in the outer limits of the luminal colonies expressed both pRb [13] and pAkt (Fig. 2a) in the presence of ATRA, we next set out to assess whether ATRA may affect the clonogenic capacity of LM38-LP cells. For this, LM38-LP monolayers were treated with vehicle (control) or ATRA for 5 days after which the cells were seeded at a very low density and cultured for 5 more days with or without ATRA treatment. We found that approximately 4 % of the LM38-LP cells were able to form colonies (Fig. 4a). This result suggests that the LM38-LP cell line contains a minor clonogenic subpopulation which is not inhibited by ATRA.

Based on this finding, we set out to investigate whether this clonogenic subpopulation represents, at least in part, mammary cancer stem/progenitor cells (CSC). To this end, we assessed the presence of ALDH activity, which is a well-known CSC marker, and found that the LM38-LP cell line contains 4.73 % ALDH positive cells (Fig. 4b), which closely matches the above observed clonogenic capacity. In addition, we assessed whether the LM38-LP cell line has the capacity to form mammospheres. We found that the LM38-LP cell line can indeed form mammospheres after 10–12 days in suspension culture (Fig. 4c). In addition, we found that these mammospheres express relatively high levels of pluripotent marker genes, such as *nanog* and *sox2*, compared to LM38-LP monolayer cells, thus corroborating stem/progenitor cell enrichment (Fig. 4d). To further support the presence of this third subpopulation with CSC characteristics within the LM38-LP cell line, mammospheres were disaggregated and the cells were propagated at low densities under standard monolayer culture conditions. We found that each mammosphere cell can regenerate the LM38-LP cell line, including its main two cellular compartments (LEP and MEP; Fig. 4e). This result further confirms the existence of a subpopulation of bi-potent mammary stem/progenitor cells within this adenocarcinoma cell line.

To analyse whether this mammosphere forming subpopulation can generate in vivo tumours with the same characteristics as LM38-LP monolayer cells, BALB/c mice were orthotopically inoculated into the 4th mammary gland with 1×10^3 cells from disaggregated mammospheres or with 2×10^5 LM38-LP cells. As shown in Fig. 5a, we found that cells derived from mammospheres were able to form mammary tumours similar to the parental LM38-LP cells, with a similar growth rate but with a longer latency period. Regarding the formation of spontaneous lung metastases, no differences were found between both groups (Fig. 5b). Furthermore,

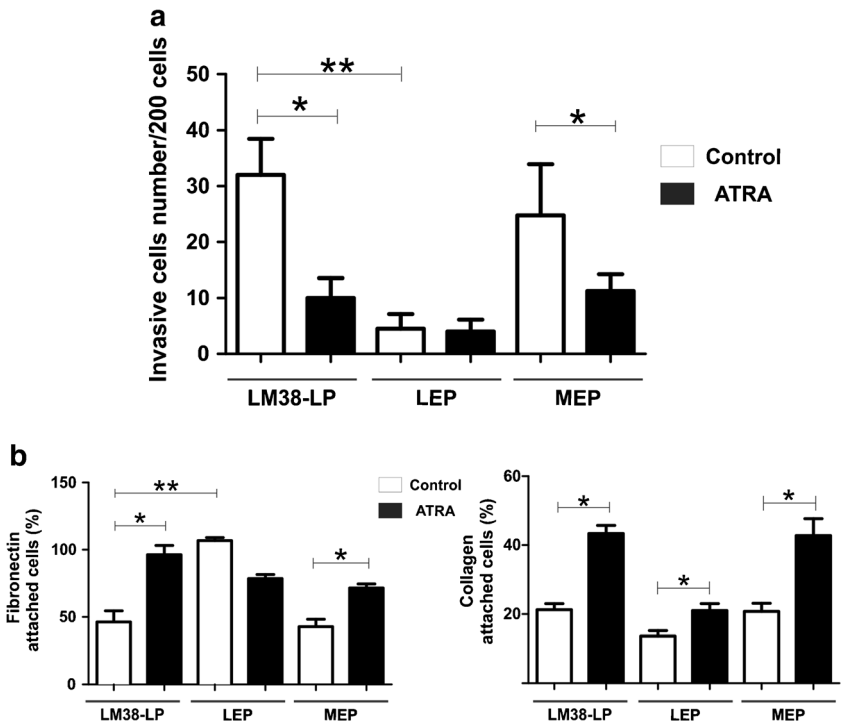


Fig. 3 **a** Invasion of LM38-LP cells and its derived subpopulations LEP and MEP, treated or not (control) with ATRA, as determined in Transwell culture chambers. **b** Adhesion of LM38-LP cells and its derived subpopulations LEP and MEP, treated or not (control) with ATRA, to fibronectin and collagen I. *$p<0.05$ control versus ATRA, **$p<0.05$ LM38-LP cells versus LEP cells; ANOVA

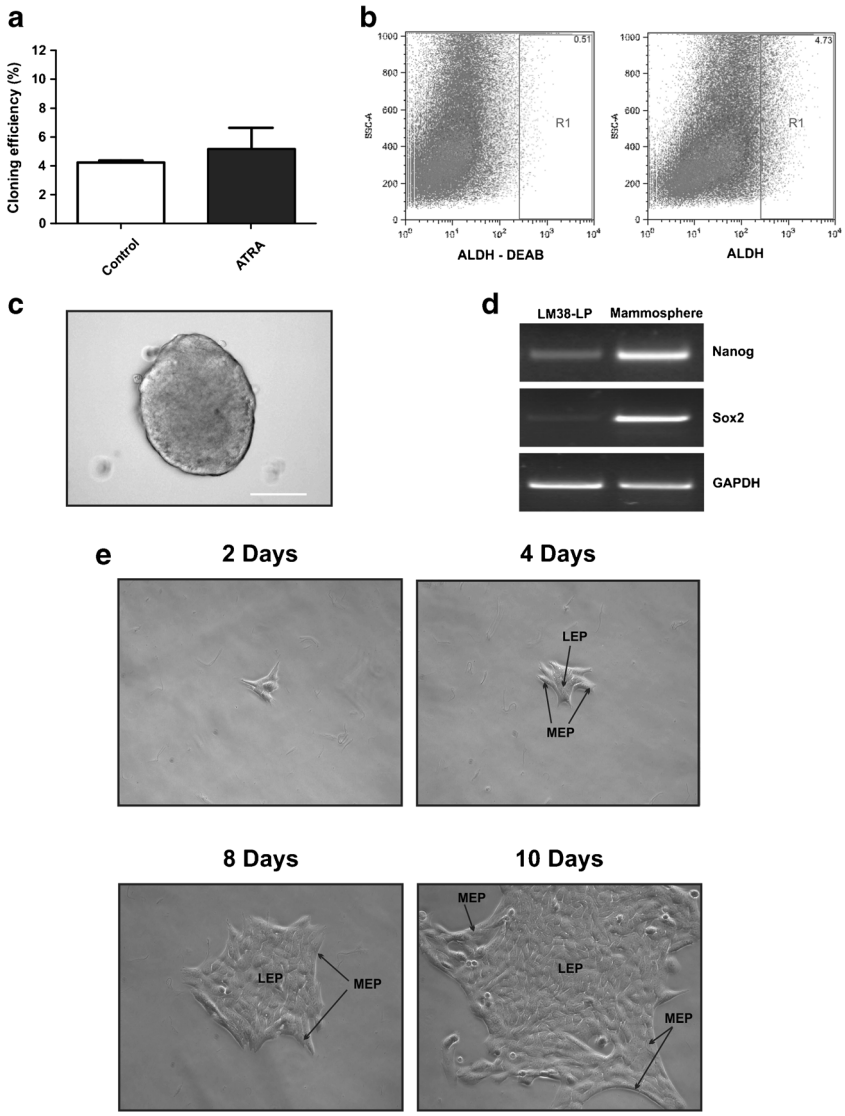


Fig. 4 **a** Clonogenicity assay of LM38-LP cells treated with ATRA, during the whole experiment or after pre-treatment for 5 days (control). **b** ALDH assay of LM38-LP cells to assess the presence of stem cells. Representative analysis of flow cytometries of cells treated (left) or not (right) with the ALDH inhibitor DEAB. Region 1(R1) indicates cells that are considered positive in this assay. **c** A typical mammosphere of the LM38-LP cell line (Scale bar = 100 μM. **d** nanog and sox2 RT-PCR in LM38-LP cells and mammosphere derived cells. The result shown is representative of two independent experiments. **e** Phase contrast microscopy of cells derived from LM38-LP mammospheres in monolayer culture at different time points (2–10 days; 400x). The results are representative of three independent experiments. *$p<0.05$ control versus ATRA; ANOVA

histopathological analyses revealed that both tumours exhibited the same morphological characteristics (Fig. 5c).

Finally, we treated mammospheres with ATRA or not for 96 h in vitro. We found that ATRA treatment reduced the diameter of the mammospheres (Fig. 5d), but when the ATRA treated mammospheres were inoculated orthotopically in mice, no differences in tumour volumes were observed (Fig. 5e), suggesting that only part of their cellular components was sensitive to the ATRA inhibitory effect.

4 Discussion

The mammary gland is composed of basically two cell types, i.e., luminal epithelial (LEP) and myoepithelial (MEP) cells. In the past, several investigators have speculated that MEP cells may create a microenvironment favourable for LEP-based mammary tumour progression but, as yet, little is known about the exact interaction between both cellular components [12, 66, 67]. Considering the distinct spatial organization of both subpopulations and the potential genetic modifications acquired by transformed cells, it is expected that each subpopulation will respond differently to similar retinoid exposures. These different responses may also explain why some mammary tumours are more resistant or less sensitive to retinoid treatment than others, as probably also to other therapies. Previously, we found that retinoids can inhibit the growth and metastatic dissemination of breast cancer cells [13]. Here, we aimed at understanding the mechanisms underlying these processes, including the responses of each cellular component to retinoid treatment. To this end, we used the LM38-LP cell line which represents an excellent breast cancer model harbouring both LEP and MEP components, as well as a putative stem/progenitor cell compartment [12, 13, 68].

In order to determine whether this model shares molecular similarities with human triple negative tumours, we first

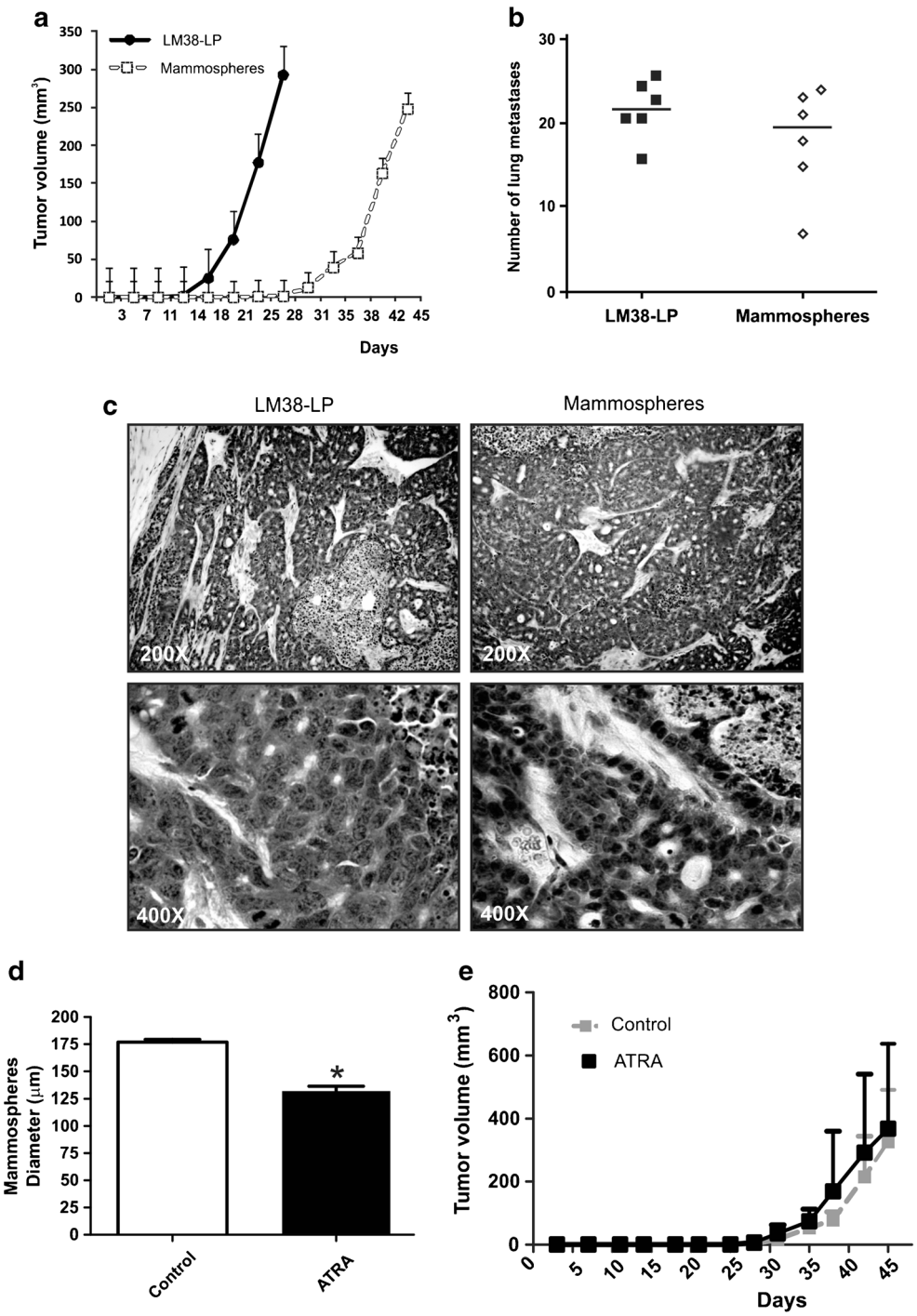


Fig. 5 In vivo LM38-LP tumour growth. **a** Tumour volume and **b** number of lung metastases in BALB/c mice inoculated with LM38-LP cells or with cells derived from mammospheres **c** Histologies of tumors grown from LM38-LP cells or from cells derived from mammospheres. **d** Diameters of mammospheres treated or not with ATRA for 96 h. *$p<0.05$ control versus ATRA; ANOVA. **e** Tumour volumes in mice inoculated with mammospheres pretreated or not with ATRA

assessed the expression of Erα, Pr and Her2 receptors. Surprisingly, we could detect low levels of Pr-b expression. To exclude the possibility that LM38-LP derived tumors may exhibit hormone dependence, we assessed whether these cells can grow in the absence of hormones. As we found that LM38-LP cells can grow in both normal media and in media containing charcoal-stripped serum, a hormone dependence of these cells could be excluded, and lack of Pr functionality could be inferred. These results, together with its aggressive in vivo behaviour [12] suggest that this cell line may be considered as an interesting model for human triple negative tumours.

Next, we found that the LM38-LP cell line expresses the main retinoic acid receptors. Interestingly, we found that its cellular subpopulations express these factors in a different fashion. Thus, while both MEP and LEP cells were found to express the *rarα1* gene, the all-trans retinoic acid (ATRA) inducible genes *rarβ2* and *crbp-1* were only expressed by

the LEP cells. These results indicate that only LEP cells may respond to ATRA through the 'genomic pathway'. Previously we have shown [9] that ATRA can modulate the metastatic dissemination of LM38-LP cells. Based on this observation, we here assessed the effect of ATRA on the process of epithelial to mesenchymal transition (EMT), a process known to be involved in tumour progression and metastasis [40]. We determined the transcript levels of several EMT related genes in the presence or absence of ATRA, and whether LEP and MEP cells display similar or different EMT gene expression profiles. We found that both cellular compartments, in response to ATRA treatment, shared a decreased expression of only the metalloprotease genes *mmp2* and *mmp9*. These data are in agreement with our previous results, indicating that the LM38-LP cell line responds to a 5-day ATRA treatment with a significant inhibition of its migrative capacity and, concomitant, its Mmp-9 protein activity [13]. In addition, we found that ATRA-treated LEP as well as MEP cells exhibited a down-regulation of genes involved in migration and invasion, which is in agreement with other reports in the literature indicating that retinoic acid has an anti-invasive effect on breast cancer cells [69–71]. We must, however, take into account here that the LEP cells were virtually non-invasive under control conditions, whereas the MEP cells showed a strong decrease in its invasiveness after ATRA treatment.

It has been reported that retinoic acid may induce the expression of factors involved in cell adhesion [72, 73]. Here, we found that each cellular compartment differed in its ability to adhere to extracellular matrix components. While LEP cells showed an increased adhesion to fibronectin, MEP cells only reached these levels under retinoid treatment conditions. On the other hand, we found that both subpopulations increased their adhesion to collagen I, being more evident in MEP cells. Concomitant with this increment, ATRA treatment induced *itgb1* and *col1* gene expression, both known to be involved in EMT. Taken together, we conclude that LM38-LP derived MEP cells constitute the subpopulation that responds to ATRA, thereby increasing its adhesion to extracellular matrix components and reducing its invasive capacity.

Apoptosis may represent an important mechanism by which retinoids can inhibit tumour cell growth [74–76]. We found that the ATRA growth inhibitory effect on LM38-LP cells was associated with a lower expression of phosphorylated Erk (pErk) and phosphorylated Akt (pAkt), suggesting the inactivation of two main regulatory pathways related to cellular proliferation and survival [13]. We next questioned whether ATRA induced growth inhibition may occur through different mechanisms in the two main LM38-LP cell compartments (LEP and MEP). We found that both ATRA and AM580, a Rarα agonist, decreased the number of pAkt positive cells in LEP islets, whereas only a few MEP cells were positive. In other words, we found that under control conditions MEP cells were arrested whereas LEP cells were proliferative, and that after retinoid treatment the number of proliferating LEP cells decreased. We also found that LEP cells turned apoptotic after a 72 h ATRA treatment period, whereas MEP cells only turned apoptotic after a 5-day treatment period. In agreement with this, it has been reported by others that retinoids can promote apoptosis in breast cancer cells through Rarβ2 induction [77]. Our data indicate that ATRA can induce apoptosis in LEP cells, which is the subpopulation that shows an increase in Rarβ2 expression in response to ATRA treatment. Therefore, disruption of retinoid receptor activity and/or its expression may suppress apoptosis, resulting in the accumulation of aberrant cells and, thus, tumour development.

Senescence is considered a tumour suppressive mechanism [78, 79] and it has been reported that accumulation of p27 is the main inducer of senescence in different tumour cell models [80, 81]. Previously, we found that several MEP cells are positive for nuclear p27 staining under control conditions, and that the number of p27 positive cells increased after ATRA treatment [13]. Here, we found that only the MEP

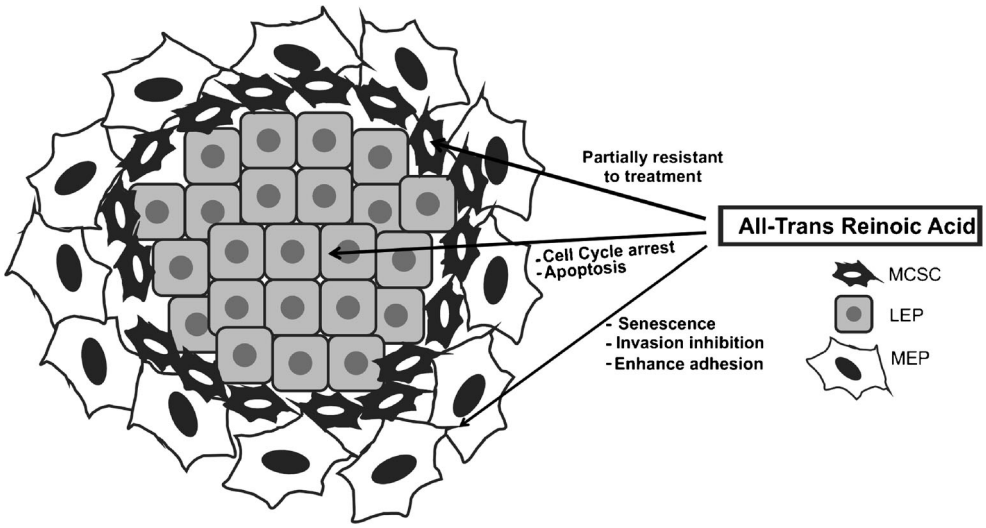


Fig. 6 Hypothetical responsiveness model to retinoids for each LM38-LP subpopulation. In this model, the final effect of all-trans retinoic acid (ATRA) treatment depends on the summation of the responses of the LEP and MEP compartment, and the behaviour of a partially resistant group of cells located between both compartments with stem/progenitor characteristics

compartment responded to retinoids with senescence before cell death, suggesting a differential mechanism of growth inhibition. It is important to highlight here that many of the senescence related genes do not harbour RARE sites in their promoters [82, 83]. Therefore, we suggest that some of these genes may be regulated by retinoids in an alternative, indirect manner.

We found that after a 96 h ATRA treatment period, LM38-LP cells growing in Matrigel formed primarily large disorganized colonies with a scarce lumen. Under retinoid treatment, the colonies were found to be smaller, more organized and to present with an apparent lumen. Moreover, retinoid treatment was found to induce caspase-3 activity within the colony centres. These data confirm the presence of apoptosis, and suggest the occurrence of differentiation events induced by retinoids. Although the effect of retinoids on cellular differentiation has amply been documented in embryonic development and tumour models [84, 85]. more studies are required to confirm the differentiating effects of retinoids in the current breast cancer model.

It is a widely accepted concept that many tumours possess cancer stem cells that are responsible for tumour progression, metastasis, recurrence and resistance to treatment, since they are capable of self-renewal and differentiation in distinct lineages [86]. According to this concept, we suspected the existence of a subpopulation with progenitor capacity within the LM38-LP cell line, since luminal colonies always gave rise to both LEP and MEP cells upon propagation [12]. We found that ~4 % of the LM38-LP cells did not respond to retinoid treatment, and that pAkt and pRb [13] positive cells were present in the outer limits of the luminal islets, even under retinoid treatment conditions. In addition, we found that 4.73 % of the cells showed ALDH activity, which is a well-known marker for cancer stem cells (CSC). From these results we conclude that the LM38-LP cell line indeed harbours a third stem/progenitor cell subpopulation that is capable of forming mammospheres. In addition, we found that mammosphere-derived cells exhibit high expression levels of pluripotency genes, such as *nanog* and *sox2*. The presence of stem cell properties was further confirmed by the observation that single cells derived from mammospheres were capable of completely regenerating the LM38-LP cell line. Besides, we found that orthotropic inoculation of mammosphere cells in BALB/c mice resulted in tumours with a similar histology and metastatic capacity to those resulting from LM38-LP inoculated cells. Since Gudas and Wagner [87] reported that retinoid-induced differentiation in stem cells may serve as a therapeutic approach, the presence of cancer stem cells within the LM38-LP cell line turns this model into an interesting tool to design new therapeutic strategies targeting the stem/progenitor cell niche. Regarding the response of this stem/progenitor cell population to ATRA treatment, it appears to be resistant when considering Akt phosphorylation, the percentage of clonogenic cells and the in vivo growth capacity. However, when mammospheres were treated with ATRA, we observed a decrease in their diameter. Possibly, the mammary cancer stem/progenitor cell population is heterogeneous in nature, and only some of these cells die or undergo cell cycle arrest when treated with ATRA [88]. In Fig. 6 we postulate a hypothetical responsiveness model to retinoids (ATRA) for each LM38-LP subpopulation. Our data suggest that previous controversial results of clinical retinoid use, particularly for breast cancer, may at least partly be explained by differential responses of the main cellular components that constitute these tumors.

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

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