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ORIGINAL ARTICLE

A clinically relevant bi-cellular murine mammary tumor model as a useful tool for evaluating the effect of retinoic acid signaling on tumor progression

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Abstract
Background The effect of retinoic acid (RA) on breast cancer progression is controversial. Our objective was to obtain information about breast cancer progression, taking advantage of the ER-negative murine mammary adenocarcinoma model LM38 (LM38-LP constituted by luminal (LEP) and myoepithelial-like cells (MEP), LM38-HP mainly composed of spindle-shaped epithelial cells, and LM38-D2 containing only large myoepithelial cells), and to validate the role of the retinoic acid receptors (RARs) in each cell-type compartment.
Materials and methods We studied the expression and functionality of the RARs in LM38 cell lines. We analyzed cell growth and cell cycle distribution, apoptosis, the activity of proteases, motility properties, and expression of the molecules involved in these pathways. We also evaluated tumor growth and dissemination in vivo under retinoid treatment.
Results LM38 cell lines expressed most retinoic receptor isotypes that were functional. However, only the bi-cellular LM38-LP cells responded to retinoids by increasing RARβ2 and CRBP1 expression. The growth of LM38 cell sublines was inhibited by retinoids, first by inducing arrest in MEP cells, then apoptosis in LEP cells. Retinoids induced inhibitory effects on motility, invasiveness, and activity of proteolytic enzymes, mainly in the LM38-LP cell line. In in-vivo assays with the LM38-LP cell line, RA treatment impaired both primary tumor growth and lung metastases dissemination.
Conclusion These in-vivo and in-vitro results show that to achieve maximum effects of RA on tumor progression both the LEP and MEP cell compartments have to be present, suggesting that the interaction between the LEP and MEP cells is crucial to full activation of the RARs.

Keywords Breast cancer · Luminal and myoepithelial cells · Retinoids · Metastasis

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Introduction

The normal ductal and lobular tree of the mammary gland is lined by two main cell types, the luminal (LEP) facing the luminal space and the myoepithelial (MEP) facing the adipose stroma. Although myoepithelial cells have been regarded as natural cancer suppressors, there has recently been growing awareness of their potential activity in cancer progression [1–3]. Analysis of gene-expression profiles of breast carcinomas associated estrogen receptor (ER)-positive status with a luminal phenotype and ER-negative status with a basal/myoepithelial phenotype and worse prognosis [4]. In addition to the possible contradictory

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action of both phenotypes, few models are currently available for investigating the effect of myoepithelial cells in the critical multistep process of invasion and metastasis in breast cancer. In our laboratory we have developed and characterized a spontaneous ER-negative murine mammary adenocarcinoma model (LM38) with papillary differentiation, capacity to metastasize to draining lymph nodes and lung, and containing both luminal and myoepithelial tumoral cell components, from which several tumor cell lines with different in-vitro and in-vivo phenotypes have been established [5].

There is scarce information in the literature about the expression and function of the retinoid acid (RA) system in mammary myoepithelial cells. A deeper insight into the function of myoepithelial cells in the physiology and pathology of mammary glands would enable better understanding of carcinogenesis mechanisms and possible application of specific therapy for breast cancer [6].

The vitamin A metabolite RA regulates several biological processes and is of crucial importance in embryonic development and in tissue remodeling in the adult. RA activity is mainly mediated by retinoic acid receptors (RARα, β, and γ), ligand-inducible transcription factors that are members of the superfamily of nuclear hormone receptors. RARs associate with the rexinoid receptor (RXR) to form heterodimers that bind to regulatory regions of specific target genes and modulate their transcription [7–9]. Transcriptional activation by RAR may trigger differentiation [10, 11], cell cycle arrest [12], and apoptosis [13, 14].

Much evidence supports the notion that RA can interfere with oncogenesis, impairing tumor development and tumor growth [15]. Kupumbati et al. [16] have demonstrated that inactivation of homozygous RAR isotypes affects the susceptibility of mice to oncogene-induced mammary carcinogenesis. In addition, retinoids, including natural or synthetic vitamin A analogues, can regulate cell growth, differentiation, and apoptosis in a variety of tumor cell types, including ER-breast cancer cells [17, 18]. Human acute promyelocytic leukemia (APL) cells can produce, as a result of chromosomal translocations, mutant forms of RARα that prevent appropriate deacetylase activity and result in deregulated gene activation, effects that can be reversed by all-*trans* retinoic acid (ATRA) at doses that induce APL cell differentiation. In fact APL can be effectively eradicated by retinoid signaling combined with chemotherapy [19]. Besides APL, retinoid-based therapy for other human cancers, including solid tumors, is undergoing clinical evaluation with quite variable results [7, 15, 20].

Retinoid resistance has been attributed to deficient RARα and β gene expression and/or to an impaired responsiveness to retinoids via RARβ, typical features of human breast cancer and other tumors [21, 22]. In addition, it has been demonstrated that CRBP1, a protein that regulates RA biosynthesis from its precursor retinol, is frequently downregulated in breast cancer and other human cancers, by an epigenetic mechanism [23].

Taking into account that most cancer patients die from their metastases rather than from primary tumor growth, and that the involvement of RA signaling in the molecular mechanisms of metastasis is still elusive, the studies presented here provide relevant information on the involvement of the RA signaling in mammary cancer progression, taking advantage of the ER-negative bi-cellular LM38 model, which offers a unique opportunity to address this involvement in both the luminal and myoepithelial tumoral cell compartments at the same time. Our in-vivo and in-vitro results show that the response to ATRA of the LM38 cell lines is not only restricted to cell growth inhibition but includes a set of complex mechanisms in the malignant progression cascade that ultimately convert breast cancer cells to a less malignant phenotype.

Materials and methods

Tumor model

From a BALB/c spontaneous transplantable mammary papillary differentiated adenocarcinoma (M38) able to metastasize to draining lymph nodes and lung, several continuous cell lines were established. Whereas the LM38-LP cell line was composed of two main cell subpopulations antigenically characterized as LEP and MEP cells up to the 30th subculture, the high-passage LM38-HP cell line was mainly composed of small homogeneous spindle-shaped epithelial cells, and the large spindle cell clone LM38-D2 contained only large MEP cells [5]. Although all three cell lines were tumorigenic when inoculated into syngeneic mice, their incidence, growth rate, and metastasizing capacity were remarkably different. In fact, the bi-cellular LM38-LP cell line developed more aggressive papillary adenocarcinomas of high tumorigenicity and growth rate that spread both to the lung and to regional lymph nodes [5].

Cell-culture conditions

LM38 cell lines were cultured in DMEM/F12 medium with non-essential amino acids and 2 μM L-glutamine (Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS) (Bioser, Buenos Aires, Argentina) at 37°C, in plastic flasks (Nunc, Roskilde, Denmark) in a humidified 5% CO_2/air atmosphere. Serial passages were made by treatment of confluent monolayers with 0.25% trypsin and 0.02% EDTA in Ca^{2+} and Mg^{2+}-free PBS.

Retinoids and doses

Except where otherwise stated, the following compounds and doses were used: ATRA (Sigma–Aldrich, St Louis, MO, USA) 1 μM; 9-*cis* RA (Sigma) 1 μM; retinol (Sigma) 1 μM; AM580 (Biomol, PA, USA) 50–200 nM; Ro41-5253 (Biomol) 2 μM. All compounds were resuspended in DMSO at ×1000 final concentration. Medium with freshly added retinoids was changed every day, with care taken to protect cells from light during the whole experiment.

RT-PCR

Transcription of RAR and RXR receptors at the mRNA level was analyzed by RT-PCR. Briefly, RNA from cells treated or not with retinoids for 48 h was prepared using the Gentra Purescript RNA isolation kit. (Qiagen, Valencia, CA, USA). cDNA was prepared with the iScript cDNA synthesis kit (Bio Rad, Richmond, CA, USA). PCR products were obtained by use of the primers h/m RARα1, h/m RARα2, mRARβ1/β3, mRARβ2, mRARγ1, mRARγ2, and mRXRα from Zelent [24]. h/m RARβ2 primer was designed at Mira y Lopez laboratory (RARβ2-S 5′ ATG GAGTTCGTGGACTTTTCTGTG 3′: RARβ2-AS 5′ CTC GCAGGCACTGACGCCAT).

In some experiments LM38-LP cells, pretreated or not with ATRA, were detached and separated into LEP and MEP subpopulations as follows. Briefly, cells were incubated for 20 min with anti-E-cadherin (Santa Cruz, CA, USA), thoroughly washed, and further incubated with magnetic immunobeads (Dynabeads M-280; Invitrogen, Oslo, Norway) for 20 min at 4°C. Cell components were separated with the aid of a magnet. E-cadherin is exclusively expressed by LEP cells [5]. Once separated, RNA and cDNA from each subpopulation and from the LM38-LP cell line were prepared as described above and assayed for RARβ2 mRNA expression.

Western blot

Semiconfluent monolayers pretreated or not for 48 h with retinoids were lysed with 1% Triton X-100 in PBS. Protein content was measured by use of Bradford's method (Bio-Rad protein assay). In some experiments monolayers were used to separate nuclear and cytoplasm fractions using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). Fifty micrograms of each total lysate or 20 μg from each nuclear fraction were denatured by boiling in sample buffer with 5% β-mercaptoethanol and 10% SDS-PAGE was performed. Gels were blotted to Hybond-P membranes (Bio Rad). After incubation for 1 h with PBS containing 5% skimmed milk plus 0.1% Tween-20, membranes were incubated overnight at 4°C with the first antibody, and then with a secondary antibody coupled to horseradish peroxidase: anti rabbit (Sigma) or anti goat (Zymed, Carlsbad, CA, USA). Detection was performed by ECL (Amersham). Bands were quantified by densitometry (Molecular Analyst software; Bio Rad, Hercules, CA, USA). Specific antibodies for RARβ1/β2, CRBP1, p27, cyclin D1, cyclin B1, and P-Erk/Erk (Santa Cruz) or for activated caspase 3 and P-Akt/Akt (Cell Signaling, Boston, MA, USA) were used.

RARs transcriptional activity

To quantify RARs transcriptional activity, a retinoic acid response element (RARE)-Luc transfection assay was performed using a plasmid construct that expresses firefly luciferase from a RARE-containing artificial promoter DR5. Cells of each line were resuspended in Optimem (Gibco Life Technologies) and transfected with DR5 reporter plasmid and SV-40 driven Renilla luciferase control plasmid with Fugene (Roche Applied Science, Indianapolis, IN, USA). Cells were plated and, 24 h after transfection, treated for 48 h with retinoids; the luciferase assay was conducted thereafter.

Cell-growth assays and reversibility of ATRA effect

Cells were resuspended in complete medium and seeded in 96 multiwell plates. After 24 h, cells received different treatments: 0.01 μM–10 μM ATRA, 1 μM retinol, 1 μM 9 *cis* RA, or vehicle for 2–6 days. Viability was assessed by reduction of the tetrazolium salt (MTS) to the formazan product in viable cells (Cell Titer 96 TM; Promega, Madison, WI, USA), as calculated by the 492/620 nm absorbance ratio, or by cell counting. In another experiment cells growing in 96-well plates were treated for 5 days either with ATRA or retinol alone or together with 2 μM Ro415253, RARα pharmacological antagonist. Cell viability was quantified with MTS as above.

To analyze the reversibility of the effect of ATRA, cells of each cell line were seeded in a 96-well plate. At 24 h cells were divided into 3 groups: medium + 10% FCS + vehicle for 7 days (control); continuous treatment with ATRA for 7 days (ATRA); and treated with ATRA for 4 days, washed out, and cultured for 3 days with control medium (ATRA + medium). The viability of the cell culture was measured by the MTS assay at 1, 4, and 7 days of treatment.

Quantification of mitosis and apoptosis

Cells of each line (4×10^3) were seeded on culture slides (BD Bioscience, San Jose, CA, USA). After 24 h cells received or not ATRA for 4 days. Cells were fixed in 4%

formalin in PBS and then stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma). The number of apoptotic and/or mitotic images/field (10 fields total) was recorded and indexes were calculated (i.e. Mitotic index = No. of cells in mitosis/Total no. of cells). Apoptosis was also quantified by use of Annexin-V + FITC (Molecular Probes, Carlsbad, CA, USA) staining as described by the manufacturer. Counting and photographic recording of apoptotic cells was conducted by use of the corresponding filter of a Nikon Eclipse E-400 fluorescence microscope.

Cell cytometry studies

Cell cycle distribution was analyzed in the bi-cellular LM38-LP cell line. Cell monolayers treated with ATRA, retinol, or ATRA plus Ro415253 for different times were detached and fixed in 70% ethanol. After staining with 100 μg/ml propidium iodide, samples were examined for DNA content by use of a FACS Canto cytometer and analyzed using Diva (Becton–Dickinson, San Jose, CA, USA) and FloJo (Ashland, OR, USA) software.

Adhesion assay

Cells were pretreated with/without ATRA for 5 days. Then monolayers were detached with trypsin and, after 2 h-incubation at room temperature to recover their surface, 3×10^4 cells/well of 24 multiwell plates were seeded in triplicate in medium containing 2% FCS with or without ATRA. After 60 min incubation at 37°C, non-adherent cells were removed, cells were washed twice with PBS and adherent cells were quantified by counting detached cells. Rate of adhesion was calculated as No. of adherent cells/Total no. of seeded cells. In some experiments wells were coated with fibronectin (2 μg/ml) before LM38-LP cell seeding.

Migration assay

Cell migration capacity was evaluated by use of a "wound healing assay" [25]. Wounds 400 μm wide were made in confluent monolayers of the different cell lines, pretreated or not with ATRA for 5 days, in 35 mm dishes. Wounded monolayers were then incubated in the presence of 2% FCS, with or without ATRA. Cells were allowed to migrate into the cell-free area for 10 h and fixed and stained with Giemsa. This short time was used to exclude cell proliferation as a confusing factor. Cell migration was quantified by calculating the percentage of the area occupied by the migratory cells in the original cell-free wounded area, by using densitometric analysis (Molecular Analyst software; Bio Rad, Hercules, CA, USA). Three independent experiments were performed, and each condition was assayed in triplicate.

Invasion assay

Transwell cell culture chambers (Corning, Union City, CA, USA) were used for invasion assay. The filters (8 μm membrane pores) were previously coated with 0.1% gelatin on the lower side and with a thin layer of reconstituted basement membrane Matrigel (BD) as described by the manufacturer. The lower chamber contained human cellular fibronectin (16 μg/ml) (Sigma) in 0.5 ml MEM, as chemoattractant. Cell lines were pretreated or not with ATRA for 5 days before the invasion assay, which was also conducted in the presence of ATRA and 10% FCS. After 24 h, cells on the upper side of membranes were thoroughly wiped off with a cotton swab. Membranes were fixed in Carnoy and stained with Hoechst 33258 (Sigma). Cells that invaded the Matrigel, passed through the pores, and became reattached to the lower surface of the filter were regarded as invasive cells and their nuclei were counted in ×400 fields under a fluorescence microscope (Eclipse E400, Nikon).

Quantification of proteases activity

Secreted urokinase-type plasminogen activator (uPA) and metalloproteinases (MMPs) activity were evaluated in conditioned media (CM). Semiconfluent monolayers growing in 24 multiwell plates treated or not with ATRA for 5 days were extensively washed with PBS to eliminate serum traces. Serum-free medium (500 μl) was added and the incubation was continued for 24 h. CM were harvested, the remaining monolayers were lysed with 1% Triton X-100 in PBS, and cell protein content was determined by use of the Bio-Rad Protein Assay. CM samples, aliquoted and stored at −20°C, were used only once after thawing. To quantify uPA activity, a radial caseinolytic method was used [26]. uPA activities were referenced to a standard urokinase curve ranging from 0.05 to 25 U/ml and normalized to the protein content of the original cell culture. Gelatinolytic MMP activity was determined on gelatine copolymerized gels as described elsewhere [27]. The clear lysed areas of the bands on the stained gels were measured with a GS-700 densitometer. Data were normalized to the protein content of the original cell culture.

Immunofluorescence and confocal microscopy

Cells were seeded (4×10^3/well) on culture slides (BD Bioscience). Twenty-four hours after seeding, cells were treated with retinoids for 5 days, as described above, and then fixed with 3% paraformaldehyde in PBS for 20 min at

RT, permeabilized with precooled 0.5% Triton X-100, 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). Cells were incubated overnight at 4°C with the primary antibodies for phospho-pRb (Cell Signaling, Danvers, MA, USA) and p27 (Santa Cruz) in blocking buffer (1:200). After washing, the secondary antibodies conjugated with Alexa fluor were added (Molecular Probes) in blocking buffer (1:200) for 45 min at RT. Then cells were incubated with DAPI (1:2000) for 10 min at RT, rinsed with PBS, and mounted with Prolong anti-fade (Molecular Probes). Photographs were taken with a Leica TCS SP5 DM confocal microscope.

In-vivo assays

Animals

Randomized inbred 2 to 4-month-old virgin female BALB/c mice, obtained from our Animal Care Area, 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.

Experimental lung metastasis assay

This assay evaluates the capacity to colonize the lung independently of the growth of the primary tumor. Subconfluent monolayers, pretreated or not with ATRA for 5 days, were detached by trypsinization and 3×10^5 LM38-LP cells were injected into the tail vein of syngeneic mice ($n = 10$ for each group). Mice were monitored daily and killed 21 days later. Lungs were removed and the number and size of superficial lung colonies were determined under a stereoscopic microscope.

Orthotopic tumor growth and spontaneous metastatic ability

Mice were inoculated orthotopically into the fat pad of the 4th mammary gland with 2×10^5 LM38-LP cells. When tumors became palpable approximately 7 days post-inoculation, animals began their oral treatment with ATRA (2 mg/animal) diluted in olive oil, through a gastric gavage. Treated animals received a total of five doses given twice a week for 3 weeks, whereas controls received only the vehicle. Mice were monitored daily. Twice a week tumor diameters were measured with a caliper and tumor volume was calculated ($D \times d^2/2$) for assessment of growth rate. Forty-five days post-inoculation of tumor cells mice were sacrificed and necropsied. Tumors were used for histopathological studies. To investigate the presence of spontaneous metastases, lungs were removed and the number of surface lung nodules were recorded, as indicated above, or analyzed by histological examination. In another protocol, mice received a subcutaneous pellet containing ATRA (10 mg/animal).

As a better approach to mimic the clinical situation of an oncological patient, in other experiments LM38-LP primary tumors, grown subcutaneously, were surgically removed 20 days after inoculation and then mice were treated orally with ATRA or vehicle. Ten days after ending the treatment the mice were sacrificed, the lungs were removed, and the number of lung surface nodules was recorded.

Angiogenesis assay

Briefly, syngeneic BALB/c mice were inoculated i.d. in both flanks with 2×10^5 LM38-LP cells, pretreated or not with ATRA in 0.1 ml DMEM/F-12 without FBS, and with trypan blue (Sigma) to mark the inoculation site. After 5 days, mice were sacrificed; the skin was carefully separated from the underlying tissues and the vascular response was measured. The inoculated sites were photographed with a digital camera coupled to a dissecting microscope at 6.4× magnification. The number of vessels was quantified in the digital images, using a reticular screen to count number of vessels/mm^2 skin. Angiogenesis was expressed as vessel density (δ):

$$\delta = \frac{\Sigma \text{ Number of vessels per square}}{\text{Number of total squares}}$$

Statistical analysis

All experiments were performed in triplicate and each experiment was repeated at least twice. The significance of differences between groups was calculated by use of Student's 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.

Results

LM38 mammary tumor cell lines express functional retinoid receptors and CRBP1

By using RT-PCR it was observed that LM38-LP cells express mRNAs of the different RARα, β, and γ isotypes (Fig. 1a, b) and RXRα mRNA (data not shown). LM38-HP and LM38-D2 cell lines had a similar pattern of expression (data not shown). Treatment of LM38-LP cells with different retinoids for 2 days did not modify the level of

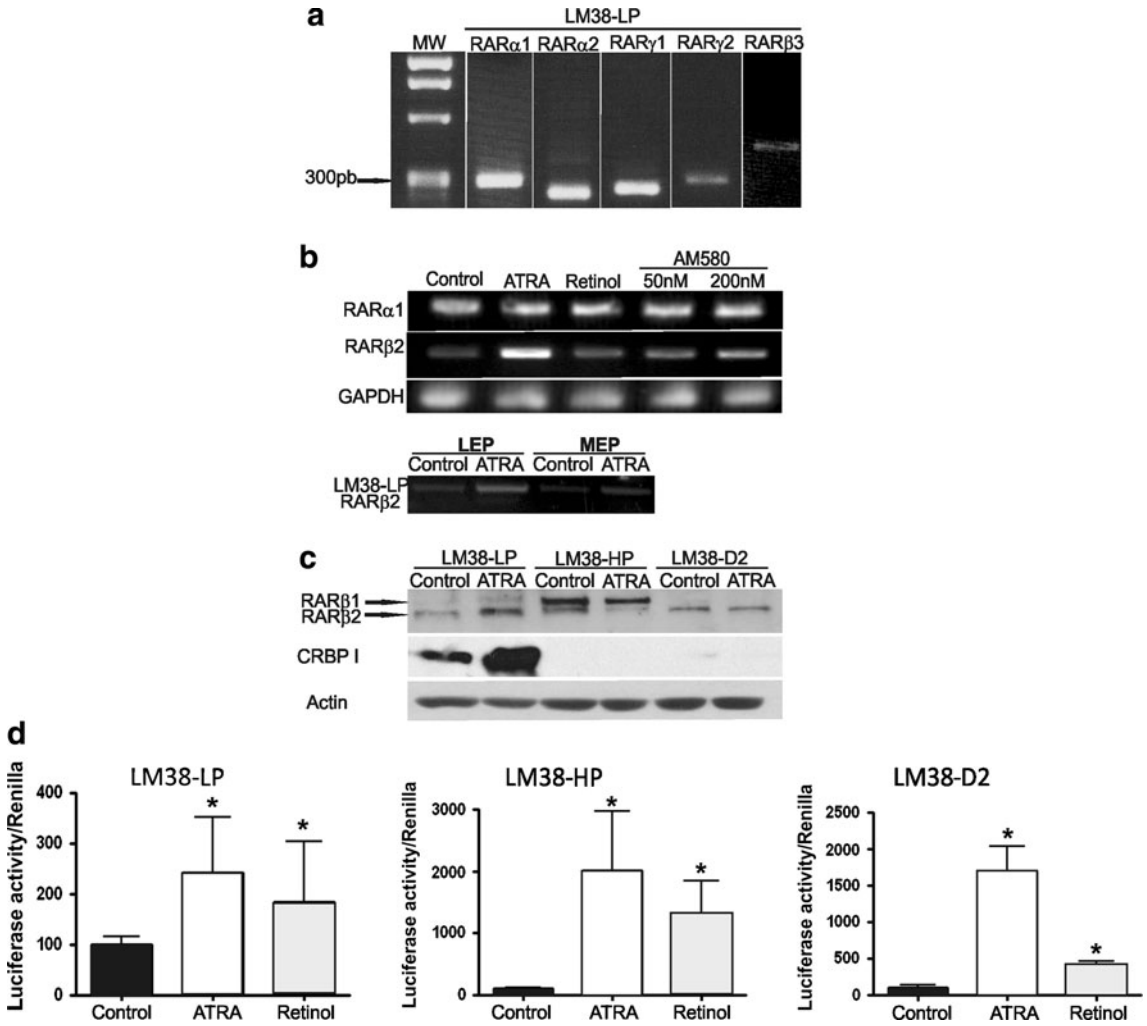

Fig. 1 Expression of functional RARs and CRBP1 by mammary tumor LM38 cell lines. **a** RNA from LM38-LP cells was isolated and expression of different RAR isotypes was analyzed by RT-PCR. **b** RT-PCR for RARα1 and RARβ2 expression in LM38-LP cell line and in separated LEP/MEP components, treated or not with retinoids. **c** Nuclear and cytoplasmic fractions of LM38 cell lines. Western blot was performed for RARβ2 (nuclear fraction) and CRBP1 (cytosolic fraction). **d** To quantify RARs functionality in response to retinoids, a retinoic acid response element (RARE)-Luc transfection assay was performed. $*p < 0.05$ (ANOVA). All figures are representative of three independent experiments

RARα1 mRNA, but markedly increased RARβ2 levels (Fig. 1b). In addition, protein levels of RARβ2 and CRBP1 (both products of RARE-regulated genes) were also studied in the nuclear and cytosolic fractions, respectively, of the three cell lines (Fig. 1c). Although both LM38-LP and LM38-D2 cells expressed RARβ2, only the bi-cellular cell line responded to ATRA with an increase in its protein level. In contrast, LM38-HP cells expressed RARβ1 and low levels of RARβ2 but were not regulated by ATRA (Fig. 1c). Interestingly, when the two cellular subpopulations that compose the LM38-LP cell line were separated after ATRA treatment, by use of E-cadherin-immunobeads, increased RARβ2 mRNA expression was observed for LEP cells only (Fig. 1b). CRBP1, a key mediator in the synthesis of RA from its precursor retinol, was mainly expressed by LM38-LP cells, which responded to ATRA with a marked increase in CRBP1 expression (Fig. 1c). The transient expression of a RARE-Luc reporter was used to determine the transcriptional activity of the RARs, and showed that the three LM38 cell lines responded to ATRA and retinol treatment, as luciferase activity indicated (Fig. 1d).

LM38 mammary tumor cell lines response to growth inhibition by retinoids

To analyze the sensitivity of LM38 cell lines to cell growth inhibition by ATRA, cells were treated for 5 days with different concentrations of the compound and evaluated with the MTS assay. The growth of the three LM38 cell lines was significantly inhibited by 1 μM ATRA (Fig. 2a). Similar effects were obtained with 9-*cis* RA and retinol

(data not shown). Four-day treatment was necessary to detect significant differences. Similar results were obtained with LM38-HP and LM38-D2 cell lines (data not shown). To evaluate whether RARα mediated the growth inhibitory effect of retinoids on these cell lines, the RARα antagonist Ro415253 was used. We observed that Ro415253 partially interfered the inhibitory effect of ATRA and retinol (Fig. 2b), supporting the idea that RARα, in particular, is implicated in the growth-inhibitory effect. Interestingly, 4-day exposure to ATRA followed by extensive washing and a further 3-day culture without treatment, resulted in irreversible growth inhibition of LM38-HP and LM38-D2 cells, but the bi-cellular LM38-LP recovered its growth capacity three days after ATRA removal (Fig. 2c).

Cell growth arrest and apoptosis are involved in retinoids-induced growth inhibition of LM38 cell lines

To evaluate mechanisms involved in ATRA-induced growth inhibition, we analyzed the number of mitotic and apoptotic cells at different times after ATRA exposure. For LM38-LP cells ATRA induced a significant reduction in the percentage of mitosis after 96 h of exposure and an increase in the number of apoptotic cells after 72 h of treatment (Fig. 3a). LM38-HP cells became arrested with a significant decrease in the percentage of mitosis after 72 h of treatment, and a significant increase in the apoptotic index was detected only after treatment for 5 days (data not shown). Finally, ATRA did not modulate the mitotic index of the myoepithelial LM38-D2 cells but induced a significant increase in cell death after 72 h of treatment. Apoptosis induction by ATRA was confirmed by annexin V staining (Fig. 3a, inset). The effect of retinoids on cell cycle distribution of the bi-cellular LM38-LP cell line was analyzed by PI cytometry. Whereas near-diploid LEP cells accumulated in the G1 phase after 4 days of ATRA or retinol treatment, growth of the aneuploid MEP subpopulation was not arrested in any specific phase of cell cycle (data not shown). G1 phase arrest of LEP cells was prevented when LM38-LP cells were co-treated with ATRA and the RARα antagonist Ro415253, confirming RARα involvement in this effect (Fig. 3b).

We also analyzed expression of some cell-cycle regulatory markers by use of immunofluorescence and confocal microscopy. In control, untreated cultures approximately half of MEP cells had p27 immunopositivity whereas LEP islets expressed phosphorylated pRB (PpRB, proliferation marker). After 3-day treatment with ATRA we observed an increase in the number of p27-stained MEP cells at the nuclear level, whereas LEP cells in the islets acquired p27 expression and became negative for PpRB, except for a positive single-cell layer around the islets, suggesting that they kept cycling under ATRA treatment. Similar results were found by exposing the cells to the RARα agonist, AM-580 (Fig. 3c).

In WB analysis, LM38-LP cells treated with ATRA for 48 h showed a decrease of cyclin B1 and D1 and an increase of p27 levels expression, in agreement with the inhibition of cell growth observed (Fig. 3d). ATRA also induced an increase of activated Caspase 3 and a significant reduction of pAkt and pErk levels (Fig. 3d), in line with the pro-apoptotic effect observed. Taken together these results strongly suggest that the LM38-LP cells are sensitive to the anti-proliferative and pro-apoptotic known effects of ATRA.

Effects of ATRA treatment on in-vitro properties associated with tumor progression and metastasis dissemination of LM38 cell lines

The ability of tumor cells to disseminate to distant sites is a complex process of sequential steps dependent on both tumor and host properties. Taking into account the different metastatic potential of LM38 cell lines [5], and hypothesizing that ATRA, besides inhibiting cell proliferation and inducing apoptosis, could also affect their capacity to disseminate, we analyzed in vitro the effect of ATRA on cell adhesion, migration, proteases secretion, and invasion, all of which are critical mechanisms of the metastasis process.

As shown in Fig. 4a, 5-day treatment with ATRA increased the adhesive capacity of LM38-LP and LM38-D2 cells to the plastic surface. LM38-LP cells were also assayed for an effect of ATRA on their adhesion to the extracellular matrix component fibronectin, and an evident increase of this capacity was induced by the retinoid (Fig. 4a). The migratory ability of LM38-HP and LM38-D2 cells was not modulated by the same treatment with ATRA. Conversely, ATRA induced 64% inhibition in the ability of the bi-cellular LM38-LP cell line to migrate (Fig. 4a). By use of transwell culture chambers we observed that LM38-D2 cells had the highest invasive capacity. Treatment with ATRA for 5 days significantly inhibited the invasive capacity of the bi-cellular LM38-LP and the myoepithelial LM38-D2 cell lines (Fig. 4a). Regarding secretion of proteases, we found a correlation with invasive ability, with LM38-D2 cells having the highest proteolytic activity. We found that the three mammary tumor cell lines responded to 5-day ATRA treatment with significant inhibition of MMP-9 activity secreted to the culture medium (Fig. 4b). Secreted uPA activity was significantly reduced by ATRA only in the invasive LM38-LP and LM38-D2 cell lines (Fig. 4b), with no effect on the non-invasive LM38-HP cells (data not shown).

ATRA inhibits in-vivo tumor growth, local invasion, and experimental and spontaneous metastatic dissemination of the LM38-LP cell line

All in-vivo assays were performed with the bi-cellular LM38-LP cell line, because this is the only one with high tumorigenicity, local invasion of the skin, and metastatic dissemination to the lungs [5]. Because several important determinants of metastatic potential, for example adhesion, migration, invasion, proteases secretion, and cell growth, were significantly modulated after 5-day exposure of LM38-LP cells to 1 μM ATRA, next we studied whether these features had in-vivo correlation. First we performed an experimental metastasis assay, because this design

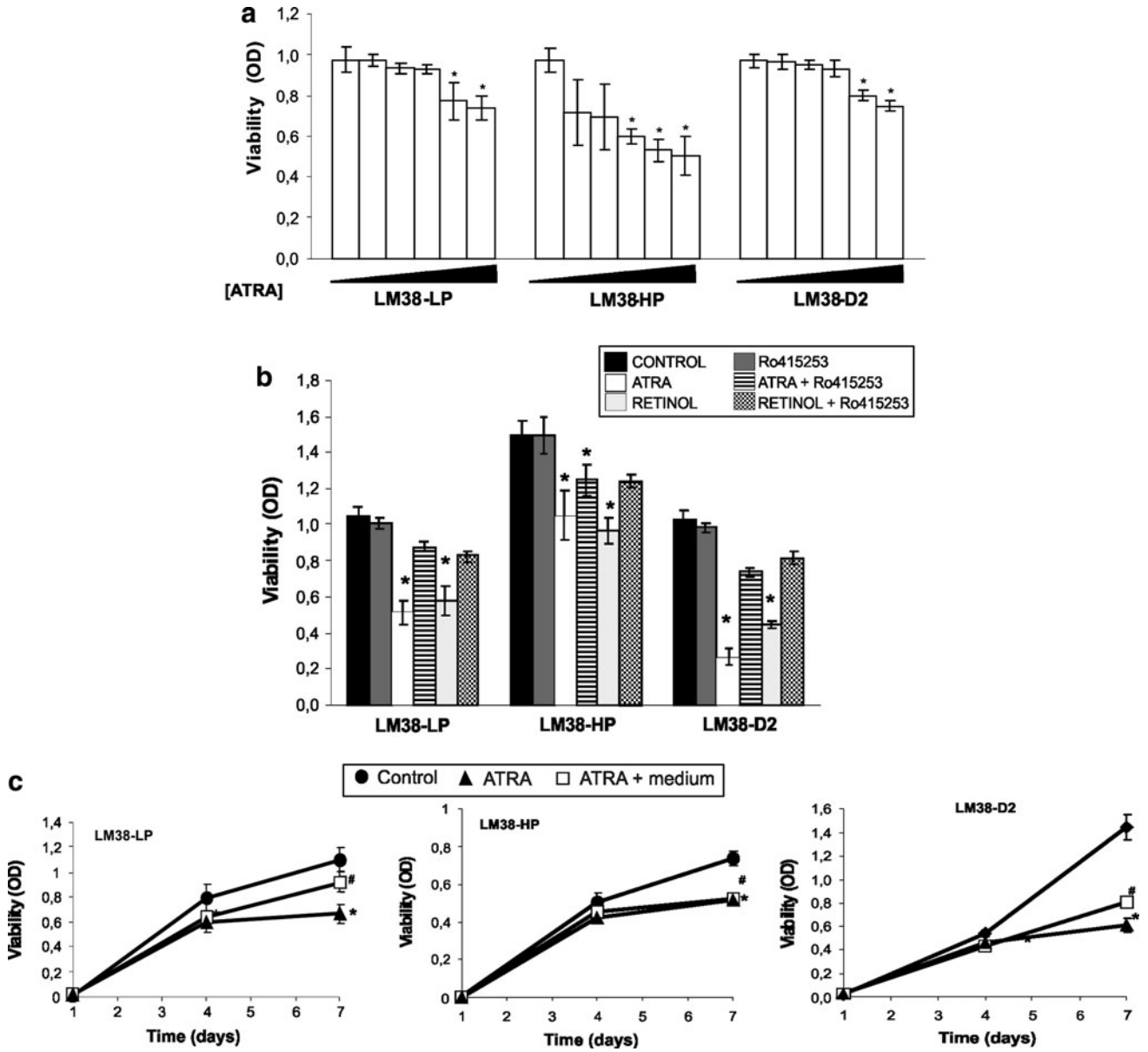

Fig. 2 Effect of retinoids on the in-vitro cell growth of LM38 cell lines. **a** LM38 cells were exposed for 5 days to different doses of ATRA: 0.0, 0.001, 0.01, 0.1, 1, and 10 μM. $^{*}p < 0.05$ (ANOVA). Determinations were performed in quintuplicate by MTS assay. Data are representative of three independent experiments. **b** RARα participation in retinoid growth inhibitory effect. $^{*}p < 0.05$ versus control (ANOVA). Determinations were performed in quintuplicate by MTS assay. Data are representative of three independent experiments. **c** To analyze the reversibility of the effect of ATRA, cells of each cell line were divided into 3 groups: medium + 10% FCS + vehicle for 7 days (control); continuous treatment with ATRA for 7 days (ATRA); and treated with ATRA for 4 days, washed out, and cultured for 3 days with control medium (ATRA + medium). The figures shown correspond to one experiment, of two independent experiments, for each cell line. $^{*}p < 0.05$ versus control, $^{\#}p < 0.05$ between ATRA and ATRA + medium (ANOVA). Determinations were performed in quintuplicate by MTS assay

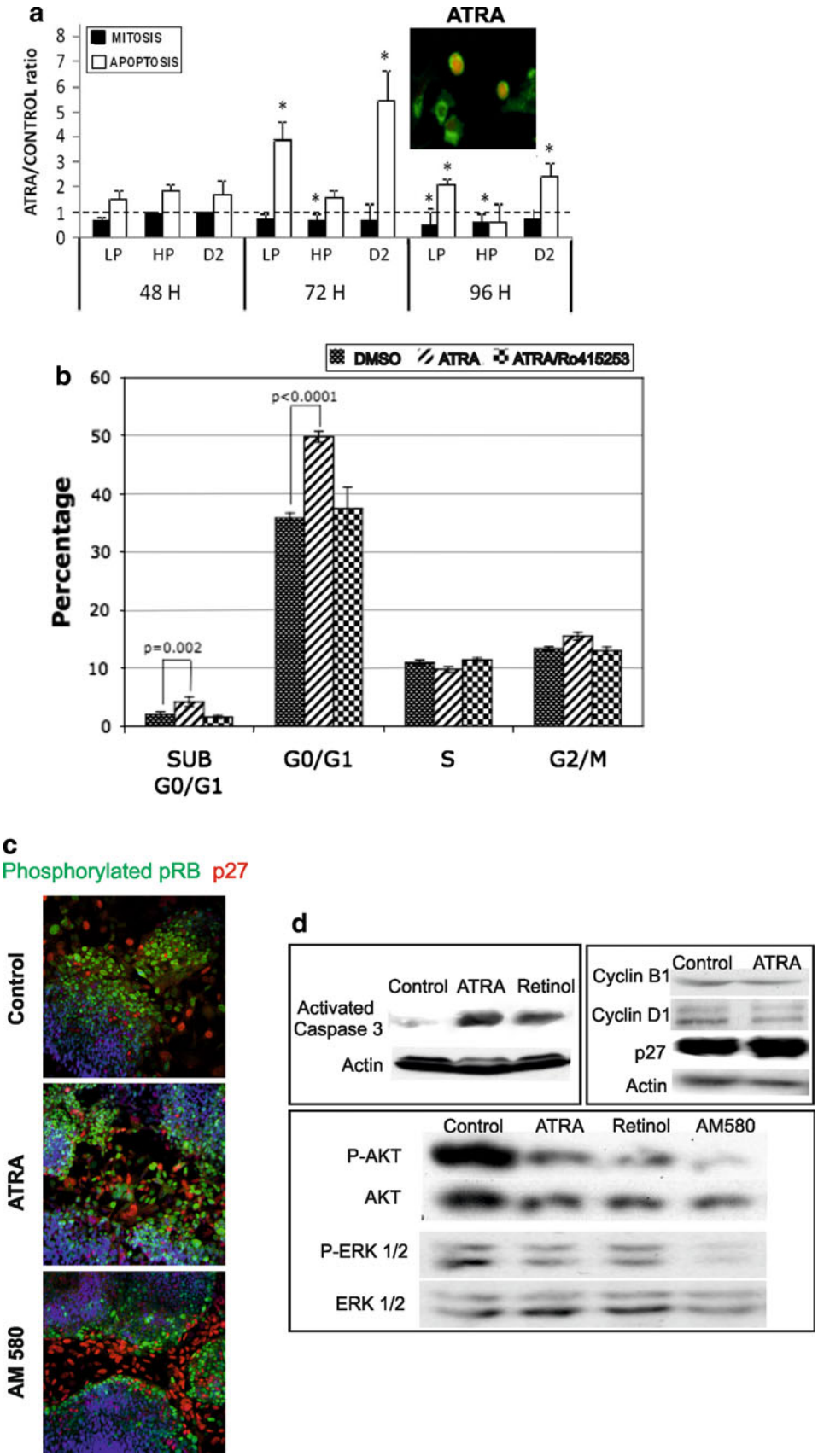

Fig. 3 Some mechanisms involved in the effect of retinoids on in-vitro cell growth of LM38 cell lines. **a** Indexes of apoptosis and mitosis were determined by counting the number of apoptotic and/or mitotic images/field. Data were expressed as ATRA/control ratio. $*p < 0.05$ (t test). *Inset* Annexin V-FITC staining of ATRA-treated cells. **b** Effect of the RARα antagonist Ro415253 on LM38-LP cell cycle distribution. After staining with propidium iodide, samples were examined for DNA content in a FACS Canto cytometer and analyzed using Diva and FloJo software. $*p < 0.01$ (t test). $X \pm$ SD of three experiments. **c** Immunofluorescence and confocal microscopy of LM38-LP cell line under different treatments with retinoids for 5 days, for expression of p27 and phosphorylated pRB. Photographs were taken with a Leica TCS SP5 DM confocal microscope (×400). **d** Cell lysates from monolayers pre-treated or not for 48 h with retinoids were used to study by WB the expression of proteins related to the regulation of cell cycle and apoptosis/survival. All figures are representative of three independent experiments

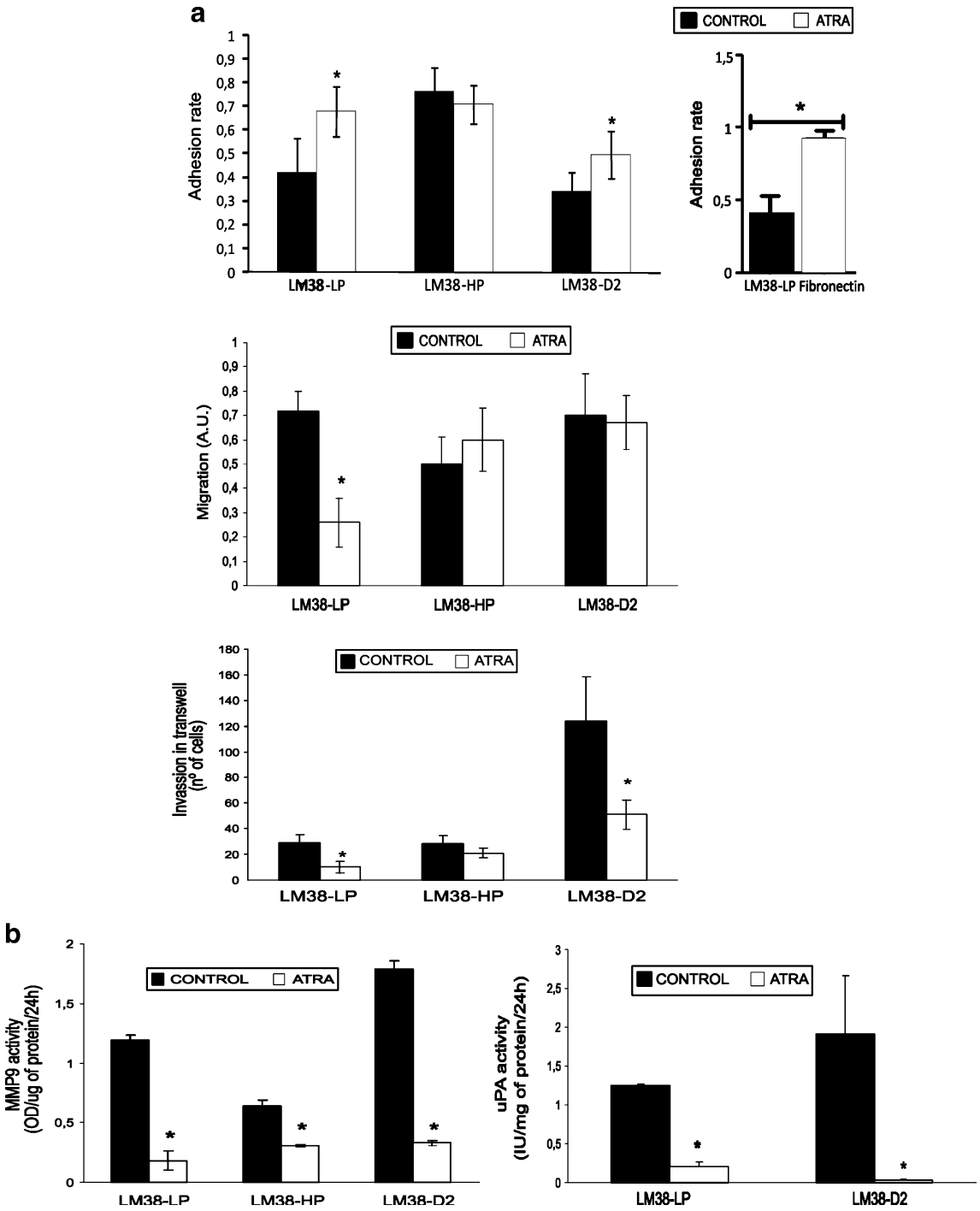

Fig. 4 Effects of ATRA treatment on in-vitro properties associated with tumor progression in LM38 cell lines. **a** To evaluate adhesion, migration, and invasion cells were pretreated with/without ATRA for 5 days. For the adhesion assay, either coated or not with FN, non-adherent cells were washed out and adherent cells were detached and counted 60 min after seeding. Cell migration capacity was evaluated by use of a "wound healing assay". For the invasion assay, nuclei on the lower side of Transwell cell-culture chambers were counted in ×400 fields under a fluorescence microscope. Determinations, expressed as $X \pm SD$, were made in triplicate. $^*p < 0.05$ versus control (t test). Data are representative of three independent experiments. **b** Secreted MMP-9 and uPA activity were evaluated by zymograms or radial caseinolysis, respectively. Determinations were performed in duplicate. $^*p < 0.05$ versus control (t test). Figures are representative of three independent experiments

enables detection of the ability to colonize the target organ independently of the growth of the primary tumor. As shown in Fig. 5a, ATRA-treated LM38-LP cells were much less able to colonize the lungs than untreated control cells. Next, we studied the effect of ATRA oral treatment on LM38-LP tumor growth and dissemination. As depicted

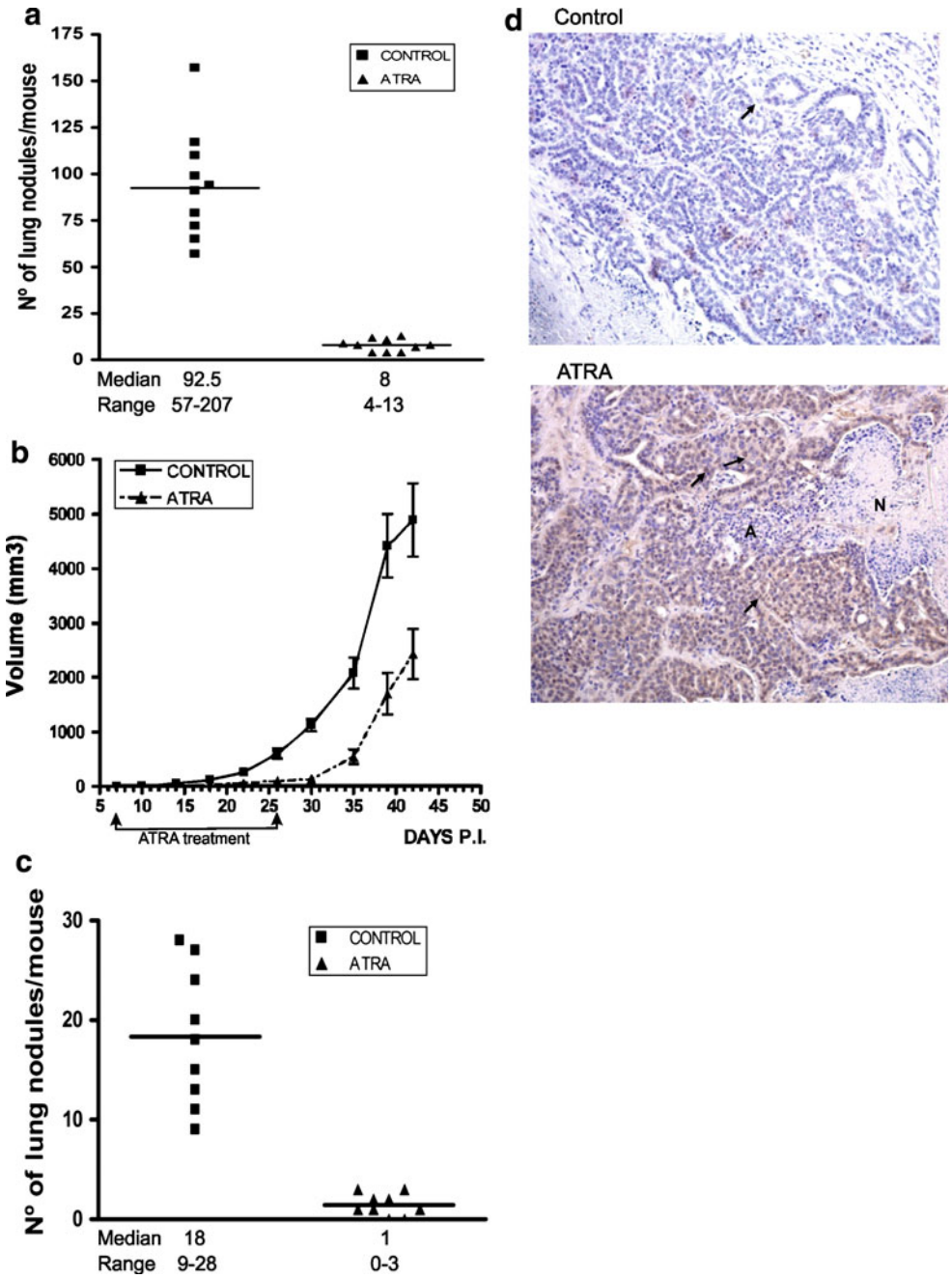

Fig. 5 Effect of ATRA treatment on in-vivo tumor growth and metastatic dissemination of LM38-LP cells. **a** Experimental metastasis assay. Female BALB/c mice were inoculated intravenously with LM38-LP cells pretreated in vitro for 5 days with 1 μM ATRA. Twenty-one days post-inoculation superficial lung colonies were counted under a stereoscopic microscope. $^*p < 0.001$ versus control (Mann–Whitney test). The figure is representative of two independent experiments. **b** Evaluation of LM38-LP orthotopic tumor growth. Oral ATRA treatment was initiated when tumors were palpable. Twice a week tumor diameters were measured and tumor volume was calculated for growth rate assessment. $^*p < 0.01$ versus control (ANOVA). The figure is representative of two independent experiments. **c** Evaluation of LM38-LP spontaneous metastatic ability. Six weeks after inoculation of tumor cells the number of spontaneous lung metastases was recorded as indicated above. $^*p < 0.001$ versus control (Mann–Whitney test). The figure is representative of two independent experiments. **d** Immunohistochemistry for p27 in sc LM38-LP tumors (×200). *Arrows* indicate examples of p27 stained nuclei. *A* apoptotic cells, *N* necrotic area

in Fig. 5b, the volume of the orthotopic tumors were significantly lower when mice were treated with ATRA (either orally or with a pellet). However, 10 days after interrupting the treatment, partial recovery of tumor growth rate was recorded (Fig. 5b). ATRA oral treatment also reduced local invasiveness of the mammary subcutaneous LM38-LP tumor (15/19 (78.9%) invasive tumors in control mice vs. 9/19 (47.4%) in ATRA-treated, $p < 0.05$), confirmed by histological analysis. Necropsy performed 6 weeks after tumor inoculation revealed a significant reduction in the number of spontaneous lung metastases (Fig. 5c). Interestingly, similar inhibition of spontaneous metastatic capacity was observed when oral administration of ATRA was initiated after surgical removal of the subcutaneous tumor (20 days post-inoculation), mimicking the clinical situation of an adjuvant therapy (data not shown). Microscopic examination did not reveal any effect of ATRA treatment on the histopathological features of LM38-LP tumors, which maintained their papillary differentiated phenotype. A remarkable increase of p27 immunoreactivity in the nuclear compartment of the malignant cells was evident for in-vivo tumor cell arrest induced by ATRA. In addition large areas of apoptotic and necrotic cells were also evident (Fig. 5d).

To further elucidate whether tumor angiogenesis was affected by retinoids we used an in vivo angiogenesis assay. Our results did not reveal any modulation of LM38-LP cells angiogenic ability in response to 5-day in-vitro ATRA pretreatment.

Discussion

Breast cancer development and progression to metastatic disease are determined by both the intrinsic properties of the breast cancer cells and the host microenvironment, together with their interactions. These processes are associated with deregulation of cell growth and cell death and with the transient or stable expression of properties associated with cell motility and invasion [28, 29]. Mouse models of breast cancer can serve as a valuable tool not only for understanding mammary cancer biology but also for development and validation of new tumor intervention strategies. Among the different models available, spontaneous transplantable breast tumors in mice similar in morphology, histopathology, and molecular characteristics to human adenocarcinomas are not frequent; they usually have limited vascularization and very few metastasize.

Although RA or its synthetic retinoid derivatives inhibit in-vitro cell growth of a variety of murine and human tumor cell lines by several mechanisms, for example apoptosis, cell cycle arrest, or cell differentiation [30], use of retinoid therapy for solid tumors progression in the clinic is still controversial. In this work we demonstrate that retinoids inhibited in-vitro cellular functions relevant to tumor progression, for example cellular proliferation, survival, motility, and invasiveness, and in-vivo tumor growth and metastasis, in a murine breast cancer model constituted by luminal and myoepithelial tumor cells [5].

Whereas alteration of the expression of RAR isotypes has been described in cancer cells, expression and functional studies have revealed that the bi-cellular LM38-LP cell line, and the derived LM38-HP and D2 cell lines, expressed the main RAR isotypes in a functional state. In particular we have detected expression of the RARβ2 isoform, which is reduced or silenced in many cancers, allowing its nomination as a tumor suppressor, and RARγ isoforms, which have also been found altered in several advanced tumors [22, 31–33]. There are few literature reports of expression and activity of RARs in mammary MEP cells. Contrary to our findings, Ariga et al. [34], who examined normal human breast tissues, pre-cancerous lesions, and/or ductal carcinomas in situ, found that immunoreactivity for RARβ was detected exclusively in myoepithelial cells but not in ductal luminal epithelia.

Several authors have reported that ER-positive breast cancer cells are sensitive to the growth-inhibitory effects of ATRA because of correlation with the presence of RARα positivity, whereas most ER-negative cells are resistant [18, 35, 36]. In contrast with other models, ER-negative LM38 cells, both LEP and MEP, express RARα; this probably determines the growth inhibition and apoptosis response to ATRA and 9-*cis* RA. Moreover, evidence also suggests that induction of RARβ2 is involved in mediating the growth-inhibitory effects of retinoids, regulating genes that induce cellular arrest [37, 38]. In our mammary cancer model we found that only the LM38-LP cell line clearly induced RARβ2 at the mRNA and protein levels in response to ATRA, suggesting that LEP/MEP interaction may be necessary for RARβ2 induction. Further studies revealed that only LEP cells of the bi-cellular LM38-LP increased RARβ2 expression in response to ATRA.

We also determined that the bi-cellular LM38-LP cell line expressed CRBP1, a protein involved in intracellular RA biosynthesis from retinol. In addition we observed that expression of this RA target gene was enhanced about sevenfold on exposure to ATRA for 48 h, as reported by others [23]. This result, and the growth inhibition by retinol in all three cell lines, suggest that the pathway for metabolism of vitamin A into its active metabolites is intact, although expression of CRBP1 in LM38-HP and LM38-D2 is low, almost undetectable by WB. However, we must bear in mind this effect could be mediated by other CRBP isoforms, for example CRBPIII, not studied here [39, 40].

We demonstrated that retinol, ATRA, and its isomer 9-*cis* RA were similarly effective at inhibiting the growth of the three LM38 cell lines. On ATRA removal, however, reversibility of the inhibitory effect was observed for the bi-cellular LM38-LP, suggesting the importance of the continuous presence of retinoids to achieve a good and permanent response. In addition, LM38-LP cell line reversibility could be explained by the probable existence of a quiescent and/or resistant cellular subpopulation (stem cell?) able to give rise to both LEP and MEP compartments and to repopulate the culture after ATRA withdrawal. Our finding of a small group of cells surrounding the epithelial islets that kept cycling in the presence of retinoids supports this hypothesis.

The main mechanism described by which ATRA inhibits the proliferation of breast cancer cells is by inducing G1 cell cycle arrest [18, 41]. Interestingly, we have found remarkable differences between LEP and MEP cells in this aspect. Whereas ATRA-treated LEP cells were arrested at G1 before death, MEP cells were found distributed along the cell cycle. Alterations in the expression and activity of cell cycle regulators have been associated with the antiproliferative effects of ATRA in breast cancer cells, for example reduced expression of cyclin D1 [42], reduced phosphorylation of pRb [43], and increased levels and/or activation of Cdk inhibitors [44, 45]. In this sense

we have found that after 5-day treatment with retinoids, LEP islets became negative for phosphorylated pRB, acquiring nuclear p27 expression. In addition, approximately 50% of MEP cells already expressed p27 under basal conditions, which increased to 100% after treatment, strongly suggesting that cell cycle arrest has a crucial function in impairment of tumor progression in LM38-LP cells. Moreover, RARα involvement in this antiproliferative activity is supported by the fact that the same effect was observed when monolayers were treated with the specific RARα agonist AM580, and by prevention of the growth-inhibitory effect when cells, either epithelial or myoepithelial, were co-treated with the specific RARα antagonist Ro415253. Decrease of cyclin D1 is also evidence of the impairment of the G1-S transition induced by ATRA, at least in the luminal compartment of the LM38-LP cell line. As a mechanism associated with its apoptotic effect, ATRA treatment induced activation of Caspase 3, in agreement with results reported for different tumoral cell lines [46, 47]. Whereas other authors reported that approximately 6 days continuous exposure to ATRA was necessary to induce apoptosis in breast cancer cells [48], we found that 3 days of ATRA treatment induced apoptosis in the bi-cellular LM38-LP and D2 cell lines. In contrast, treatment for at least 5 days was required to induce apoptosis in epithelioid LM38-HP cells.

It has been reported that ATRA can exert rapid, non-genomic effects, mediated by different signaling pathways [49, 50]. Here we have found that ATRA inhibited the activity of MEK/Erk and PI3K/Akt, two of the pathways known to be involved in the regulation of cell proliferation and cell survival. It is possible that these non genomic pathways participate in ATRA growth inhibitory and apoptotic effects on LM38 cell lines.

Tumor invasion and metastatic dissemination occur by a sequence of steps including specific cell–stroma interactions, proteolytic degradation of the extracellular matrix (ECM), and migration and invasion of tumoral cells [28, 29]. Because information about the potential role of ATRA signaling on different events related with tumor progression, and the mechanisms involved, is rather scarce, we initiated studies to analyze retinoid effects on breast cancer progression in the LM38 mammary cancer model. First we determined that 5-day pretreatment with ATRA induced higher adhesion, both to the tissue culture surface and to fibronectin, only in LM38 cell lines with MEP component. This enhancing effect could be related with the fact that ATRA increased the expression of some ECM components, for example fibronectin and laminin 1, in these cells (our unpublished results), as shown by other authors using different models [51, 52].

Regarding the effect of retinoids on cell motility we found that only the migratory ability of the bi-cellular LM38-LP cell line was specifically inhibited by ATRA. In addition we found that ATRA markedly impaired the invasiveness of LM38-LP and LM38-D2 cells, both highly invasive in vivo [5] and in vitro, whereas the poorly invasive epithelioid LM38-HP cell line was not modulated.

Tumor invasion and metastasis entail production of proteolytic enzymes by most cells within a tumor. Important proteases in this process include uPA and MMPs [53]. We observed that ATRA induced significant inhibition of MMP-9 secreted activity in the three LM38 cell lines whereas uPA secreted activity was only impaired in the invasive LM38-LP and D2 cell lines. Some reports have revealed that RA inhibits MMP-9 or matrilysin expression/activity and the metastatic phenotype [54, 55], and other authors have shown that RA up-regulates uPA production in mammary tumors [56].

To summarize, we found that ATRA induced remarkable in-vitro modulation of several mechanisms associated with breast cancer progression that together with the deleterious effects of retinoids on cell growth and survival could result in impairing of LM38-LP in-vivo malignant behavior. Thus we investigated whether in-vitro effects of retinoid treatment had an in-vivo correlate. First we demonstrated that ex-vivo ATRA treatment induced a significant reduction in the number of experimental lung metastases, confirming that a direct effect of RA on the tumor cells was impairing the last steps of the metastatic cascade. This effect could not be associated with a decrease of the angiogenic capacity of the mammary cancer cells. Administration of ATRA to tumor-bearing mice affected both primary tumor growth and spontaneous metastatic dissemination, because we observed a significant reduction of tumor growth up to 10 days after the last dose. The LM38-LP tumor then recovered its growing capacity in part, as also found when the same cell line received in-vitro treatment. Similar to us, other authors have reported that ATRA has a reversible cytostatic effect on another carcinoma cell line [57]. Independently of its effect on primary tumor growth, it is important to note that ATRA reduced the local invasiveness of LM38-LP tumor and that its anti-metastatic activity was still evident even 20 days after suspension of the treatment, suggesting that ATRA was affecting dissemination of tumor cells from the primary tumor. Moreover, this anti-metastatic effect was also evident when ATRA treatment was initiated after the surgical removal of the local tumor, thus mimicking an adjuvant treatment in an approach closer to the clinical situation. Other reports have also studied the potential use of ATRA or synthetic retinoids on the growth and dissemination of solid tumors in vivo, using rat rhabdomyosarcoma cells [58], a head and neck human cell line [59], a model of gastric cancer [60], and others [61].

Our results in this ER-negative LM38 breast cancer model, constituted by LEP and MEP malignant cells, are consistent with a putative role for retinoids in the regulation of tumor invasion and metastasis. Whereas ATRA induced arrest and apoptosis of LEP cells, the MEP compartment, highly invasive and basally arrested, responded to retinoids with impairment of proteases secretion and of their invasion capacity, and with an irreversible non-proliferative status and cell death. The strong antimetastatic effect of ATRA suggests that the development of mechanism-based combinations of retinoids and other agents may result in a very effective and lasting effect preventing breast cancer progression. Therefore, to develop rational retinoid-based therapy for breast cancer it becomes important to have a better understanding of LEP–MEP cross-talk.

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