Retinoids induce lumen morphogenesis in mammary epithelial cells

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Summary

Lumen formation is a fundamental step in the development of the structural and functional units of glandular organs, such as alveoli and ducts. In an attempt to elucidate the molecular signals that govern this morphogenetic event, we set up an in vitro system in which cloned mammary epithelial cells grown in collagen gels under serum-free conditions form solid, lumen-less colonies. Addition of as little as 0.1% donor calf serum (DCS) was sufficient to induce the formation of a central cavity. Among a number of serum constituents analyzed, retinol was found to mimic the effect of DCS in inducing lumen morphogenesis. Since the biological activities of retinol are largely dependent on its conversion to all-trans-retinoic acid (RA), we examined in more detail the effect of RA on lumen formation. RA induced the formation of lumen-containing colonies (cysts) in a concentration- and time-dependent manner, a half-maximal effect after 9 days of culture being observed with 100 pM RA. The pleiotropic effects of retinoids are mediated by nuclear retinoic acid receptors (RARs; α, β and γ) and retinoid X receptors (RXRs; α, β and γ). To identify the signaling pathway involved in RA-induced lumen formation, we used receptor-specific synthetic retinoids. TTNPB, a selective RAR agonist, promoted lumen morphogenesis, whereas RXR-selective ligands lacked this activity. Lumen formation was also induced at picomolar concentrations by Am-580, a synthetic retinoid that selectively binds the RARα receptor subtype. Moreover, co-addition of Ro 41-5253, an antagonist of RARα, abrogated the lumen-inducing activity of both RA and DCS, indicating that this biological response is mediated through an RARα-dependent signaling pathway. To gain insight into the mechanisms underlying RA-induced lumen formation, we assessed the potential role of matrix metalloproteinases (MMP). Using gelatin zymography, we observed a dose-dependent increase in latent and active forms of gelatinase B (MMP-9) upon RA treatment. In addition, lumen formation was abrogated by addition of the synthetic MMP inhibitor BB94, indicating that this morphogenetic process is likely to require MMP activity. Collectively, our results provide evidence that RA promotes lumen formation by mammary epithelial cells in vitro and suggest that it plays a similar role during mammary gland development in vivo.

Key words: Mammary gland, Morphogenesis, Epithelium, Retinoids, Lumen

Introduction

The development of most glandular organs begins with the invagination of an existing epithelial sheet into the underlying mesenchyme. The primary bud thus generated subsequently undergoes a series of morphogenetic events that culminate in the formation of branching tubes (excretory ducts) and hollow spheres (follicles, alveoli or acini). A crucial step in the development of glandular units is lumen formation, that is, the creation of a central cavity within an initially solid epithelial primordium (Hogg et al., 1983; Gumbiner, 1996; Coucouvanis and Martin, 1995; Hogan and Kolodziej, 2002). Over the past decade, it has become increasingly clear that the processes involved in the generation of epithelial architecture are orchestrated by diffusible cues. Thus, a number of studies have led to the identification of polypeptide growth factors that promote the elongation and branching of epithelial tubes (e.g. Montesano et al., 1991b; Vega et al., 1996; Bellusci et al., 1997; Sakurai et al., 2001). However, with the exception of a few recent papers (Soriano et al., 1995; Lipschutz et al., 2000; Hirai et al., 2001; O'Brien et al., 2002), there is a paucity of information about the molecular signals specifically responsible for lumen formation. This stems in part from the limited availability of in vitro systems suited for analyzing this morphogenetic process (Petersen et al., 1992; Soriano et al., 1995; Yap et al., 1995; Hirai et al., 1998; Blatchford et al., 1999).

The isolation of clonal murine mammary epithelial cell lines endowed with the ability to form tubulocystic structures in collagen gels (Montesano et al., 1998) has provided an additional convenient model to investigate the molecular mechanisms of lumen formation. However, the identification of morphogenetic factors in serum-supplemented cultures is potentially hampered by the presence of numerous undefined components, including polypeptide growth factors, hormones and other biologically active molecules, which may mask or modify the effects of exogenously added agents. To circumvent this drawback, and to define the minimal requirements for lumen formation, we have developed a serum-free culture system in which mammary epithelial cells form solid multicellular colonies in collagen gels (R.M., unpublished). The goal of the present study was to use this experimental system as a bioassay to identify the molecular signals involved in lumen morphogenesis.

Retinoic acid (RA) and its precursor retinol (vitamin A) are key regulators of vertebrate embryonic patterning and organogenesis (reviewed by Morriss-Kay et al., 1999; Ross et al., 2000) and are potent inducers of cell differentiation (Gudas et al., 1994; Nau and Blaner, 1999). The pleiotropic effects of retinoids are mediated by retinoid receptors, a subgroup of the nuclear receptor superfamily. The retinoid receptor family includes the retinoic acid receptors (RARs), which bind both all-trans and 9-cis RA stereoisomers, and the retinoid X receptors (RXRs), which bind 9-cis-RA only. Each family is composed of three receptor isoforms, designated α, β and γ. RARs and RXRs can form heterodimers and homodimers that act as ligand-dependent transcription factors by binding to retinoid response elements in the promoters of target genes (reviewed by Chambon et al., 1996).

Herein, we report that retinoids are potent inducers of lumen formation in serum-free collagen gel cultures of mammary epithelial cells and that this activity is mediated through an RARα-dependent signaling pathway.

Materials and Methods

Reagents

The natural retinoids all-trans-retinoic acid (RA) and all-trans retinol were purchased from Sigma (Sigma Chemical Co., St Louis, MO), and the synthetic retinoids TTNPB and AM-580 were from Biomol Research Laboratories (Plymouth, PA). The RARα-selective antagonist Ro 41-5253 and the RXR-selective ligand Ro 25-7386 were generous gifts from E. M. Gutknecht and P. Mohr (F. Hoffmann-LaRoche Ltd., Basel, Switzerland). The retinoids were dissolved in DMSO to obtain a stock solution (either 1 mM or 10 mM), aliquoted, stored at –20°C and protected from light exposure until used. The final concentration of DMSO in the culture medium did not exceed 0.1%. The synthetic MMP inhibitor BB94 and the related inactive isomer BB1268 were kindly provided by P. Brown (British Biotech Pharmaceuticals Ltd., Oxford, UK).

Cells

J3B1 cells (Montesano et al., 1998), a subpopulation of the non-tumorigenic murine EpH4 mammary epithelial cell line (Fialka et al., 1996; Oft et al., 1996), were recloned by limiting dilution to ensure homogeneity. Clone A (J3B1A cells) was used throughout this study. The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Basel, Switzerland) supplemented with 10% donor calf serum (DCS, GIBCO) and 2 mM L-glutamine and used between passages 7 and 17.

Lumen morphogenesis assay

J3B1A cells were harvested with trypsin/EDTA from confluent cultures, centrifuged and washed in serum-free DMEM/F12 medium (1:1). The cells were centrifuged once again and resuspended in a serum-free chemically defined medium consisting of DMEM/F12, 1 μg/ml insulin (Sigma), 1 μg/ml holo-transferrin (Sigma) and 200 pg/ml recombinant human epidermal growth factor (EGF, Boehringer Mannheim) (this medium will hereafter be referred to as 'defined medium'). J3B1A cells were mixed with a type I collagen solution prepared as described previously (Montesano et al., 1991a; Montesano et al., 1998) to obtain a concentration of 1×10^4 to 5×10^4 cells/ml. The cell suspension was then dispensed into multiwell plates (Falcon, Becton Dickinson and Co., Franklin Lakes, NJ, USA; or Nunc, Kampstrup, Roskilde, Denmark) or into 35 mm dishes (Nunc). After a 10 minute incubation at 37°C to allow collagen gelation, the cultures were grown in defined medium with or without the indicated agents. The medium and treatments were changed every 2-3 days. In experiments designed to assess the effect of RA on pre-formed cell aggregates, J3B1A cells (5×10^4 cells/ml) were grown for 3 days on a 0.5% agarose gel cast in 60 mm dishes to allow formation of floating cell clusters (Hirai et al., 2001; Simian et al., 2001). The clusters were then resuspended in a collagen gel (2 ml) cast in 35 mm dishes and incubated for a further 48 hours with or without various concentrations of RA (1 nM to 1 μM). In additional experiments, we used a 'collagen sandwich' morphogenesis assay (Chambard et al., 1981; Hall et al., 1982; Montesano et al., 1991a) to further analyze the ability of untreated and RA-treated J3B1A cells to form lumina. The sandwich assay was carried out by seeding the cells in defined medium onto the surface of a 1 ml collagen gel in a 35 mm dish (2×10^4 cells/dish) and allowing them to attach for approximately 2 hours. The cells were subsequently overlaid with a second collagen gel (1 ml) as described previously (Montesano et al., 1983) and incubated at 37°C in defined medium with or without RA for a further 48 hours.

Quantification of lumen formation

J3B1A cells were suspended in the collagen solution, and 1 ml aliquots of the suspension (3×10^4 cells/ml) were dispensed in the 22 mm wells of a multiwell plate (Falcon). After the collagen had gelled, 1 ml of defined medium with or without the indicated agents was added. Media and treatments were renewed every 2-3 days. At the indicated time points, five randomly selected fields (measuring 1 mm×1.4 mm) per experimental condition in each of at least four separate experiments were photographed under bright field illumination using the 10× objective of a Nikon Diaphot TMD inverted photomicroscope. Quantification of lumen formation was carried out on positive prints (125× final magnification) by determining the percentage of cell colonies exhibiting a cystic structure (colonies were arbitrarily classified as cysts when containing a translucent cavity whose minor axis exceeded 20 μm). Data were expressed as mean percentage of cysts±s.e.m., and statistical significance was determined using the Student's unpaired *t*-test.

Processing for light and electron microscopy

After overnight fixation in situ with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4), collagen gels were gently removed from the dishes or wells and cut into 2×2 mm fragments. These were extensively rinsed in cacodylate buffer, post-fixed in 1% osmium tetroxide in Veronal acetate buffer for 45 minutes and further processed as described previously (Montesano et al., 1991a). Semi-thin (1 μm thick) and thin sections were cut with an LKB ultramicrotome (LKB Instruments, Gaithersburg, Maryland). Semi-thin sections were stained with 1% methylene blue and photographed under transmitted light using a Zeiss photomicroscope (Carl Zeiss, Orberkochen, Germany). Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Gelatinolytic and caseinolytic zymography

J3B1A cells were plated in 60 mm plastic dishes in defined medium, grown to confluence, and further incubated with or without different concentrations of RA. Conditioned media were collected after 24, 48 and 72 hours, supplemented with 0.5 mM PMSF and 15 mM HEPES, centrifuged for 5 minutes at 340 ***g***, and the resulting supernatants were stored at –20°C until used. Alternatively, cells were suspended in 3D collagen gels at 2×10^5 cells/ml and incubated with or without RA. Conditioned media contained within collagen gels were collected after 24, 48 and 72 hours by centrifuging the gels in Eppendorf tubes at 2000 ***g*** for 5 minutes and collecting the supernatant. 35 μl of conditioned media were electrophoresed under

non-reducing conditions in 7.5% SDS/polyacrylamide gels, co-polymerized with 1 mg/ml gelatin. After soaking in 2.5% Triton X-100 for 20 minutes to remove SDS, the gels were incubated in reaction buffer (50 mM Tris-HCl pH 8 containing 150 mM NaCl, 10 mM $CaCl_2$) at 37°C for 16 hours and then stained with ethanol:acetic-acid:water (30:10:60) containing 0.25% Coomassie Blue R250 for 4 hours. The conditioned media from MCF-7 and U937 cells lines, which are known to secrete MMP-2 and MMP-9 respectively, were used as positive controls. Gelatinolytic activity was detected as a clear band on a background of uniform blue staining. For the detection of plasminogen-dependent proteolytic activity, conditioned media from cells grown either in plastic dishes or in collagen gels were analyzed by casein zymography as previously described (Pepper et al., 1990).

Northern blot hybridization

Confluent monolayers of J3B1A cells in defined medium were incubated with or without different concentrations of RA. After 24 or 48 hours, the dishes were washed with ice-cold PBS and total cellular RNA was extracted with Trizol reagent (Life Technologies, Paisley, Scotland) according to manufacturer's instructions. RNA was denatured with glyoxal, electrophoresed in a 1% agarose gel (15 μg RNA per lane) and transferred overnight onto nylon membranes (Hybond-N, Amersham, Buckinghamshire, UK). RNAs were crosslinked by baking the filters at 80°C for 2 hours and stained with methylene blue to assess 18S and 28S ribosomal RNA integrity. Filters were hybridized for 16 hours at 65°C with 1.5×10^6 cpm/ml of ^{32}P-labeled cRNA probes generated from mouse MMP-9 or human MT1-MMP cDNAs (kindly provided by M. Pepper, Geneva, Switzerland). As an internal control for determining the amount of RNA loaded, the filters were simultaneously hybridized with a ^{32}P-labeled P0 ribosomal phosphoprotein cRNA probe. Post-hybridization washes were performed as previously described (Pepper et al., 1990). Filters were exposed to Kodak XAR-5 films at –70°C between intensifying screens.

Results

Lumen morphogenesis by J3B1A cells requires a factor present in serum

When grown in collagen gels in culture medium containing 10% DCS, J3B1A mammary epithelial cells formed irregularly shaped cysts and branching tubes enclosing a wide lumen, as previously described (Montesano et al., 1998). By contrast, when grown in collagen gels in serum-free, chemically-defined medium (DMEM/F12, 1 μg/ml insulin, 1 μg/ml holo-transferrin and 200 pg/ml EGF), they formed compact spheroidal or slightly elongated colonies, in which a clearly defined lumen could not be discerned by phase contrast microscopy (Fig. 1A). Addition of as little as 0.1% DCS to the defined medium was sufficient to induce the formation of a central cavity (Fig. 1B). These findings therefore suggested that DCS contains a factor(s) necessary for lumen morphogenesis by J3B1A cells.

In an attempt to identify the molecule(s) present in DCS that is/are responsible for lumen morphogenesis, we first

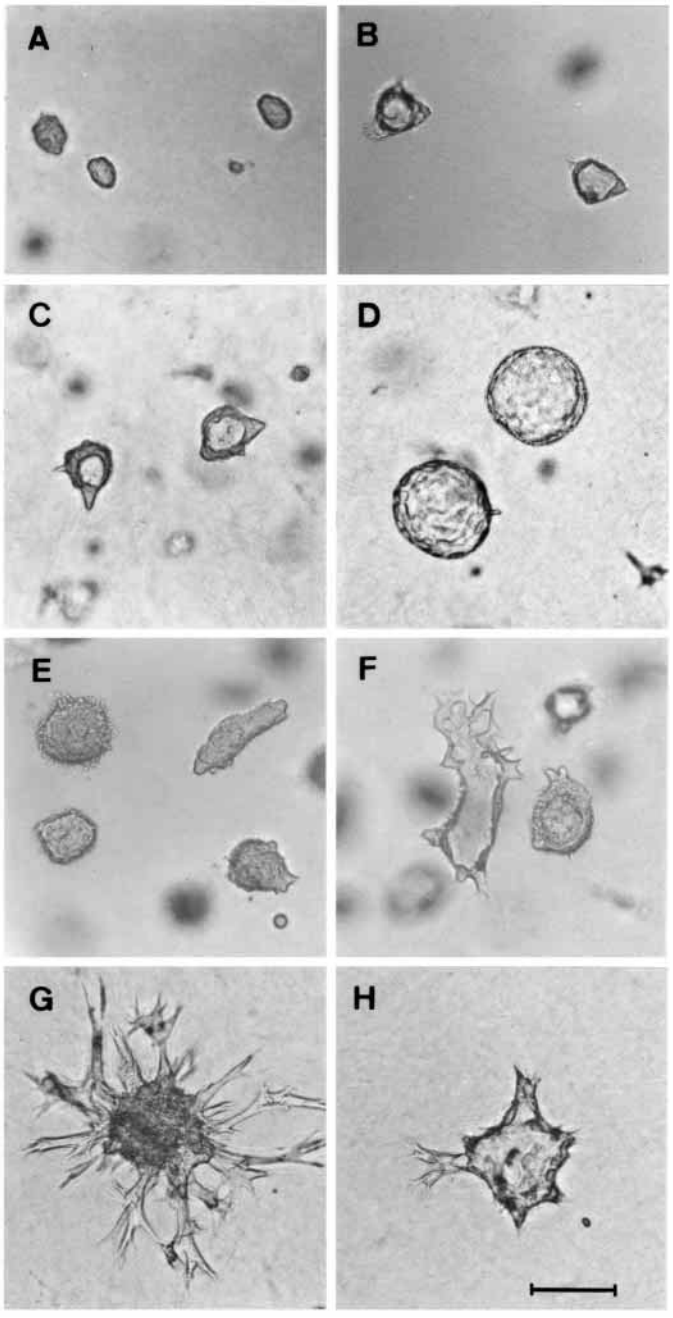

Fig. 1. Retinoids substitute for serum in inducing lumen formation by mammary epithelial cells. (A) J3B1A cells suspended in a collagen gel at a concentration of 3×10^4 cells/ml and grown in serum-free, chemically defined medium for 8 days form small solid colonies devoid of a discernible lumen. (B) J3B1A cells grown for 8 days in defined medium supplemented with 0.1% DCS form cystic structures containing a central cavity. (C) Addition of 1 nM RA to the defined medium mimics the lumen-inducing activity of DCS, resulting in the development of small, irregularly shaped cystic structures. (D) At higher (1 μM) concentration, RA elicits the formation of large spheroidal cysts. (E,F) J3B1A cells were grown for 7 days in defined medium to allow the formation of compact multicellular colonies and subsequently incubated in the presence or absence of 1 μM RA for a further 3 days. Whereas control colonies remain solid (E), RA treatment induces lumen formation (F). (G,H) Effect of RA on pre-clustered cells. J3B1A cells were grown in suspension on agarose for 3 days to obtain discrete cell aggregates, which were subsequently embedded in collagen gels. After 48 hours of incubation in the absence of RA, the aggregates extend branching cords in the collagen matrix but do not form cystic structures (G). Addition of RA (100 nM) at the time of embedding reduces the extent of branching and induces cavitation of the aggregates (H). Bar, 100 μm.

examined the potential effect of a number of polypeptide growth factors. Addition of hepatocyte growth factor (10 ng/ml), or increasing the concentration of EGF in the defined medium to 10 ng/ml, resulted in the formation of larger, moderately branched colonies, but did not induce lumen formation (data not shown). Likewise, addition of platelet-derived growth factor BB (50 ng/ml), basic fibroblast growth factor (30 ng/ml) or keratinocyte growth factor (20 ng/ml) resulted in a slight increase in colony size but failed to mimic the lumen-inducing activity of DCS (data not shown). In addition, preliminary physico-chemical characterization indicated that the lumen-inducing activity of DCS was resistant to boiling for 10 minutes, which raised the possibility that the 'factor' responsible for this activity was not a protein.

In light of the results described above, we next analyzed the effect of mammotrophic steroid hormones, including hydrocortisone [which we found previously stimulates lumen formation by a different mammary epithelial cell line (Soriano et al., 1995)], 17-β-estradiol and progesterone. None of these hormones elicited lumen formation by J3B1A cells (data not shown). We finally explored whether serum-borne bioactive lipids, such as lysophospholipids or retinoids, could substitute for DCS in inducing lumen formation. Lysophosphatidic acid (LPA), a lipid mediator responsible for many biological activities of serum (Moolenaar, 1999), stimulated the formation of branching cords by J3B1A cells at concentrations of 1 μM to 10 μM but did not induce lumen formation (Fig. 5C). Another serum-borne bioactive lipid, sphingosine-1-phosphate (1 μM) (Hla et al., 1999) had no manifest effect on the organization of J3B1A colonies (data not shown). Remarkably, however, addition of physiological concentrations (10 nM to 1 μM) of retinol (vitamin A) to the defined medium resulted in the formation of cystic structures comparable to those seen in DCS-supplemented cultures (data not shown). Because retinol exerts most of its biological activities by conversion to the active metabolite all-trans-retinoic acid (RA) (Gudas et al., 1994; Napoli, 1996; Gottesman et al., 2001), we conducted a series of experiments to analyze the effect of RA on the morphogenetic properties of J3B1A cells.

Retinoic acid can substitute for serum in the induction of lumen formation

A qualitative analysis of RA-treated cultures showed that cyst size and shape were dependent on RA concentration. Thus, at relatively low concentrations (100 pM to 100 nM), RA predominantly induced the formation of small, irregularly shaped cysts (Fig. 1C), whereas at higher (1-10 μM) concentrations it promoted the development of larger, spheroidal cysts (Fig. 1D). These findings were confirmed by a quantitative evaluation of lumen diameter (Fig. 2C).

We next performed a detailed dose-response and kinetic analysis of the effect of RA on the percentage of cystic (i.e. lumen containing) colonies. At the earliest time point analyzed (3 days), the percentage of cystic colonies was proportional to RA concentration (P<0.05 for values of 1 nM RA versus 100 pM RA; P<0.0025 for 10 nM RA versus 100 pM RA; P<0.025

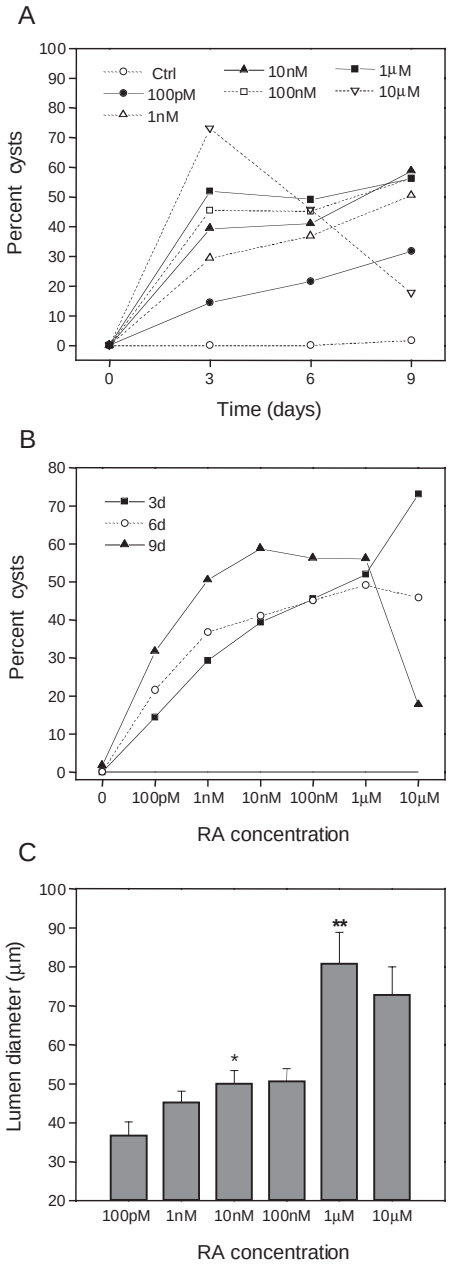

Fig. 2. Time course and dose-dependence of retinoic acid effect on lumen formation. J3B1A cells were suspended in collagen gels at 3×10^4 cells/ml and incubated with either 0.1% DMSO (control) or a wide range of RA concentrations. (A,B) Lumen formation was quantified after 3, 6 and 9 days as described in Materials and Methods. Results are expressed as a mean percentage of cysts (i.e. colonies containing a central cavity) in 25 randomly selected photographic fields (five fields from each of five separate experiments) per experimental condition. In A, values are plotted against time of treatment, whereas in B, the same values are plotted against RA concentration. P=0.01 for values of 100 pM RA at 3 days compared with the control; P<0.025 for values of 100 pM RA at 9 days compared with 3 days; P<0.01 for values of 1 nM and 10 nM RA at 9 days compared with 3 days; P<0.0025 for values of 10 μM RA at 6 days compared with 3 days and at 9 days compared with 6 days. (C) Lumen diameter was quantified on the micrographs taken at day 9 by measuring the minor axis of the central cavity in all cystic colonies. Data are expressed as mean±s.e.m. from five separate experiments. * P<0.01 compared with 100 pM RA; ** P<0.0005 compared with 100 nM RA.

for 100 nM RA versus 1 nM RA; $P<0.05$ for 1 μM RA versus 10 nM RA; $P<0.0005$ for 10 μM RA versus 1 μM RA) with a maximum 73% value observed at 10 μM (Fig. 2A,B). Upon continued treatment with relatively low (100 pM to 10 nM) RA concentrations, the percentage of cysts formed by J3B1A cells further increased between days 3 and 9 ($P<0.025$ for values of 100 pM RA at 9 days compared with 3 days; $P<0.01$ for values of 1 nM and 10 nM RA at 9 days compared with 3 days). By contrast, in cultures treated with 1 μM RA, the cyst percentage did not increase significantly between days 3 and 9, and with the highest RA concentration (10 μM), it even steadily decreased ($P<0.0025$ for values of 10 μM RA at 6 days compared with 3 days, and at 9 days compared with 6 days) (Fig. 2A,B). In a separate set of experiments, we quantitatively analyzed cyst formation after long term treatment (up to 28 days) with RA. A significant increase in the percentage of cysts between 7 and 21 days was observed after treatment with RA at 10 nM (73% cysts at 21 days versus 50% at 7 days, $P<0.005$) as well as with RA at 100 nM (76% cysts at 21 days versus 49% at 7 days, $P<0.0025$); no further significant increase being observed between 21 and 28 days (data not shown). By contrast, the percentage of cysts did not change significantly between 7 and 28 days in response to 100 pM, 1 nM or 1 μM RA. Thus, in long-term culture (21-28 days), the optimal effect on lumen formation was obtained with 10 nM and 100 nM RA (data not shown). In summary, our qualitative and quantitative findings indicate that relatively low concentrations of RA (10 to 100 nM) elicit a sustained induction of lumen formation in cultures of J3B1A cells. By contrast, at the highest concentrations tested (10 μM), RA induces rapid formation of cystic structures, which, however, partially regress during protracted culture, possibly owing to the dose-dependent growth-inhibitory activity of RA on J3B1A cells (R.M., unpublished).

The findings described above raised the question of why in short term (6-9 day) cultures only approximately 50% of the cells formed cysts in response to RA. A potential explanation was provided by the observation that lumen-less colonies were generally quite small when compared with cystic structures (data not shown), which suggested that lumen formation was hindered below a threshold colony size. To explore this possibility, and to determine at the same time whether RA induces lumen formation by modulating cell proliferation, we analyzed the effect of RA on pre-formed cell aggregates. For this purpose, J3B1A cells were cultured in a collagen gel for 7 days in the absence of RA to obtain the formation of compact multicellular colonies, at which time RA (1 μM) was added for a further 3 days. This induced the formation of patent lumina within the initially solid epithelial colonies (Fig. 1E,F). These results were corroborated using an alternative approach, in which cells were pre-clustered in suspension and subsequently embedded in collagen gels (Hirai et al., 2001; Simian et al., 2001). In this experimental setting as well, addition of RA (1 nM to 10 μM) to the collagen gels induced the cavitation of the clusters within 48 hours (Fig. 1G,H). Notably, under these conditions, lumen formation occurred in virtually 100% of pre-formed clusters, in contrast to the maximum 50-70% cysts induced by RA in short-term cultures of singly suspended cells. These findings therefore support the hypothesis formulated above, that is, that a 'critical mass' of cells may be required for optimal lumenogenesis in response to RA. They also indicate that modulation of J3B1A cell proliferation is unlikely to be responsible for RA-induced lumen formation.

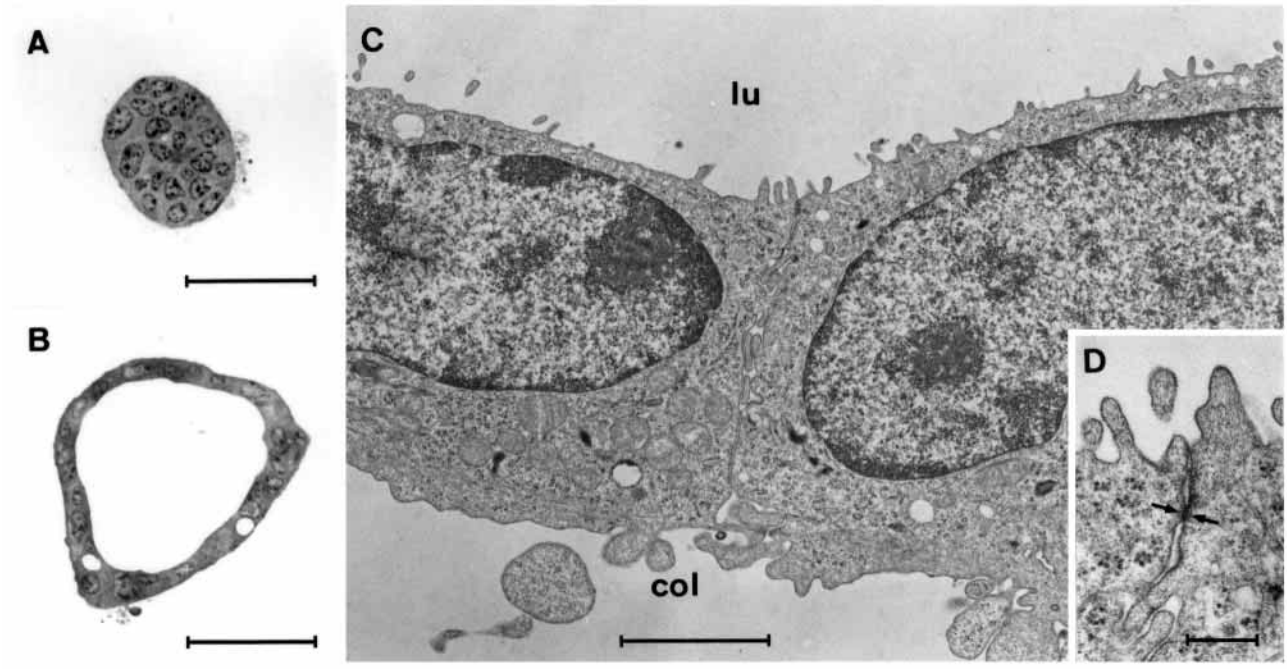

Fig. 3. Structural organization of RA-induced cysts. (A) Semi-thin sections of a 12-day-old collagen gel culture incubated with solvent (DMSO) alone show that the colonies of J3B1A cells consist of compact cell aggregates. (B) Semi-thin sections of cultures treated with 10 nM RA show cystic structures enclosing a wide lumen. (C) Thin sections of the wall of a cyst. The cells have a clearly defined polarity, with a microvilli-bearing surface facing the lumen (lu) and a basal surface in contact with the collagen matrix (col). (D) Detail of an apically located junctional complex (arrows). (A,B) Bar, 50 μm. (C) Bar, 2 μm. (D) Bar, 0.5 μm.

To analyze the organization of the colonies formed by J3B1A cells, collagen gel cultures were processed for light and electron microscopy. In semi-thin sections of cultures incubated with solvent (DMSO) alone, J3B1A colonies appeared to consist of solid cell aggregates (Fig. 3A), within which small lumen-like spaces were only occasionally observed. Electron microscopy confirmed that the aggregates were composed of closely packed cells lacking obvious signs of structural polarization (data not shown). In sharp contrast, semi-thin sections of RA-treated cultures showed that J3B1A colonies consisted of a palisade of epithelial cells circumscribing a central lumen (Fig. 3B). Thin-section electron microscopy revealed that the cells forming the wall of the cysts had a clearly defined polarity, with a microvilli-bearing luminal surface oriented towards the cyst cavity, a lateral surface provided with an apically located junctional complex and a basal surface in contact with the collagen matrix (Fig. 3C,D).

The effects of RA were further studied using a collagen gel sandwich assay of lumen morphogenesis in which epithelial cells are allowed to attach onto the surface of a collagen gel and are subsequently covered with a second layer of collagen (Chambard et al., 1981; Hall et al., 1982; Montesano et al., 1991a). In control cultures, collagen overlay resulted after 48 hours in the formation of a network of flattened cell cords containing only a few small lumen-like spaces (Fig. 4A,C). Under the same conditions, incubation with RA (10 nM to 1 μM) induced the formation of numerous large lumina (Fig. 4B,D).

Taken together, the results described above indicated that RA induces lumen morphogenesis by J3B1A cells, resulting in the generation of cystic structures that are reminiscent of mammary gland alveoli. Since lumen formation is also necessary for the development of the mammary gland ductal tree, we next wished to determine whether, under appropriate conditions, RA mediates the formation of hollow tubular structures as well. To address this issue, we applied two experimental strategies. In the first approach, we took advantage of the finding that serum-free conditioned medium from confluent monolayer cultures of J3B1A cells induce the formation of branching cords by J3B1A cells suspended in collagen gels in defined medium (R.M., unpublished). J3B1A cells were first incubated with 50% (v/v) J3B1A conditioned medium for 3 days to initiate cord formation and subsequently grown for a further 7 days in the presence or absence of RA. Although cells maintained in conditioned medium alone formed branching cords devoid of central lumen (Fig. 5A), addition of RA induced the formation of wide lumina within the initially solid epithelial cords, thereby resulting in the generation of hollow tubes (Fig. 5B). The alternative approach consisted of inducing cord formation by treatment with LPA. As previously alluded to, incubation with LPA alone stimulated branching morphogenesis but did not promote the formation of

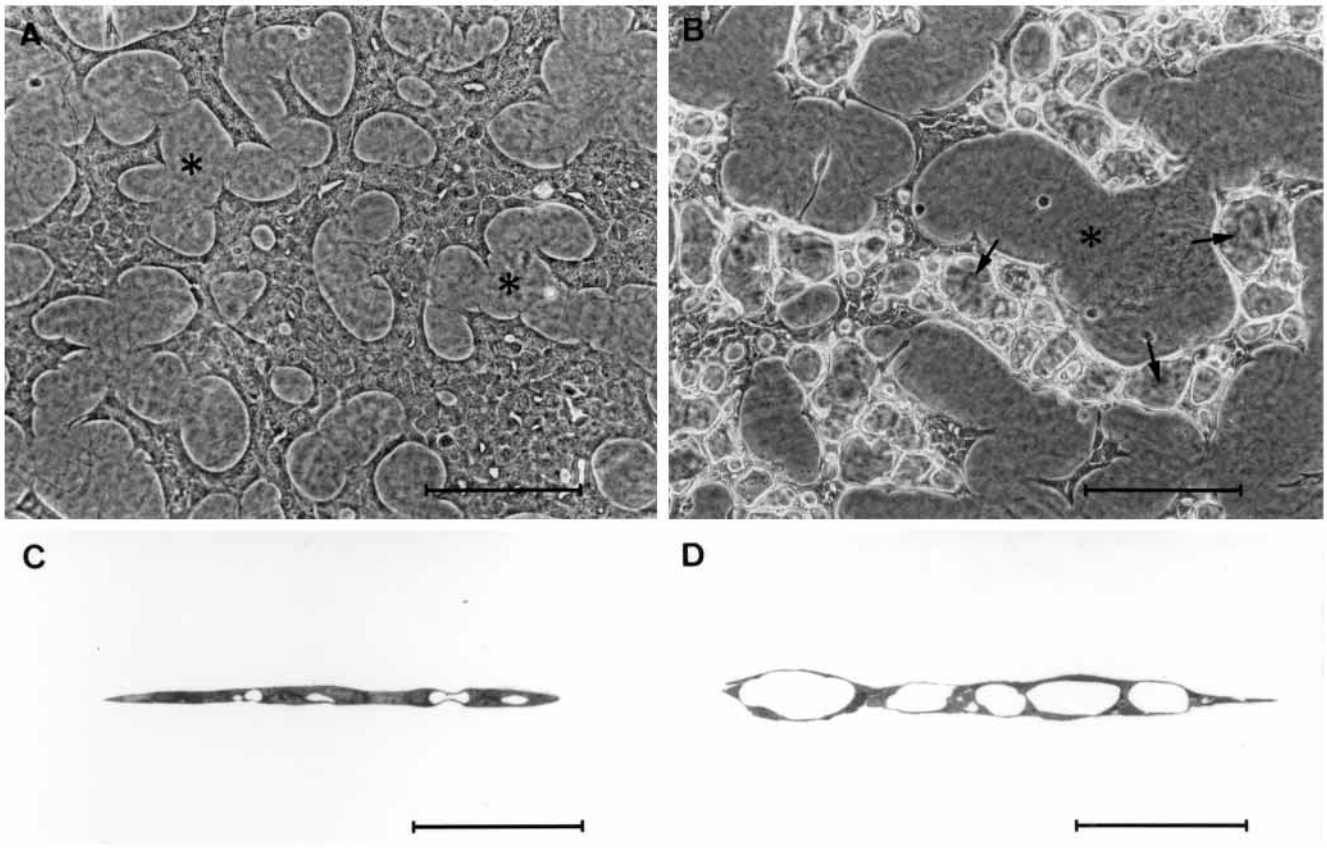

Fig. 4. Retinoic-acid-induced lumen morphogenesis in a collagen gel sandwich assay. (A,B) Phase contrast micrographs (asterisks indicate cell-free gel areas). (C,D) Semi-thin (1 μm thick) sections perpendicular to the culture plane. When sandwiched between two collagen layers (see Materials and Methods) for 48 hours, untreated J3B1A cells form flattened cell cords containing only a few small lumina (A,C). By contrast, in the presence of 1 μM RA (B,D), J3B1A cells form large lumina (arrows in B). (A,B) Bar, 200 μm. (C,D) Bar, 100 μm.

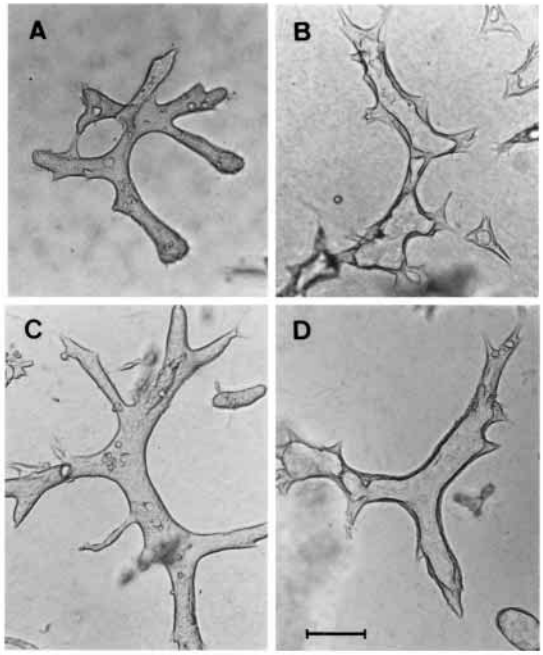

Fig. 5. When added together with factors that promote branching morphogenesis, RA induces the formation of hollow tubular structures. (A) Serum-free conditioned medium from confluent monolayer cultures of J3B1A cells induces the formation of solid branching cords by J3B1A cells grown in collagen gels (10 day culture). (B) When incubated with conditioned medium for 3 days to allow initial cord formation and subsequently treated with 1 μM RA for a further 7 days, J3B1A cells form branching tubes enclosing a widely patent lumen. (C) Addition of 10 μM LPA stimulates colony branching without inducing lumen formation. (D) Concomitant treatment with 10 μM LPA and 10 nM RA results in the formation of arborized tubular structures that recapitulate the 3D organization of mammary gland ducts. Bar, 100 μM.

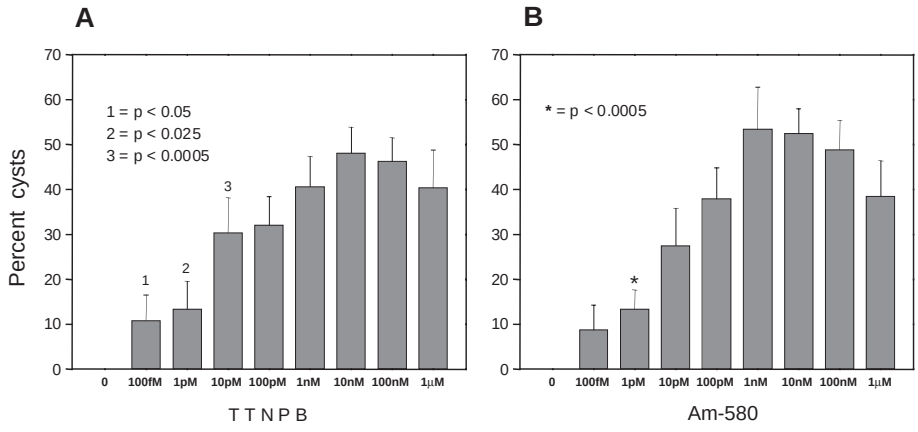

Fig. 6. Induction of lumen formation by RAR-pan-reactive and RARα-selective synthetic retinoids. (A) J3B1A cells were suspended in collagen gels and incubated either with 0.1% DMSO (control) or with a wide range of concentrations of the RAR-selective agonist TTNPB. (B) J3B1A cells were suspended in collagen gels and incubated either with 0.1% DMSO (control) or with different concentrations of Am-580, a selective RARα ligand. Lumen formation was quantified after 6 days as described in Materials and Methods. Results are expressed as mean percentage of cysts±s.e.m. in 20 randomly selected photographic fields (five fields from each of four separate experiments) per experimental condition. In A, 1=P<0.05; 2=P<0.025; 3=P<0.0005 (compared with control values); in B, *=P<0.0005 (compared with control values).

detectable lumina (Fig. 5C). Strikingly, however, simultaneous addition of LPA and RA elicited the formation of arborized tube-like structures that closely mimic the organization of mammary gland ducts (Fig. 5D).

The effects of retinoic acid are mediated through the RARα signaling pathway

Two biologically active stereoisomers of RA have been identified, all-trans-RA (RA) and 9-cis-RA. Although 9-cis-RA is an activating ligand for both RARs and RXRs, RA binds with high affinity to RARs only. However, when added at supraphysiological concentrations, RA has been reported to activate RXRs as well, possibly as a result of limited isomerization to 9-cis-RA (Van Heusden et al., 1998). To identify the receptors involved in the regulation of lumen formation by J3B1A cells, we therefore utilized synthetic retinoids that selectively bind to either RARs or RXRs. TTNPB, a RAR agonist that activates RARα, RARβ and RARγ but does not affect RXRs (LeMotte et al., 1996) exhibited potent lumen-inducing activity, a highly significant (P<0.0005) effect (30% cysts) being observed at concentrations as low as 10 pM and a maximal effect at 10 nM (Fig. 6A). By contrast, RXR-selective ligands, including the synthetic retinoid Ro 25-7386 (10 nM to 1 μM) (Toma et al., 1998) as well as the naturally occurring compounds phytanic acid (50 μM) (Kitareevan et al., 1996) and docosahexaenoic acid [5 to 20 μM (Mata de Urquiza et al., 2000)] were unable to induce lumen formation (data not shown), suggesting that RXR activation does not mediate lumen formation in J3B1A cells.

The RAR subfamily of retinoid receptors consists of three members: RARα, RARβ and RARγ. RARα is the most ubiquitously expressed, whereas RARβ and RARγ display more restrictive patterns of distribution (reviewed by Chambon, 1996). We first explored the potential role of RARα in lumen formation by incubating collagen gel cultures of J3B1A cells with the synthetic retinoid Am-580. This RARα-selective agonist (Delescluse et al., 1991) induced lumen formation in a dose-dependent manner, a highly significant (P<0.0005) effect (27.5% cysts) being observed at concentrations as low as 1 pM and a maximal effect (53% cysts) at 1 nM (Fig. 6B). To determine whether the lumen-inducing activity of RA was mediated by RARα, we next used Ro 41-5253, a RARα-selective antagonist. Ro 41-5253 binds RARα without inducing transcriptional activation, thereby counteracting RARα-mediated effects of retinoids (Apfel et al., 1992). Ro 41-5253 alone (100 nM to 1 μM) did not induce lumen formation (data not shown). However, when co-added with RA, R0 41-5253 abrogated lumen formation in a dose-dependent manner (Fig. 7A), indicating an involvement of RARα in this process. Finally, we wished to ascertain whether RARα also

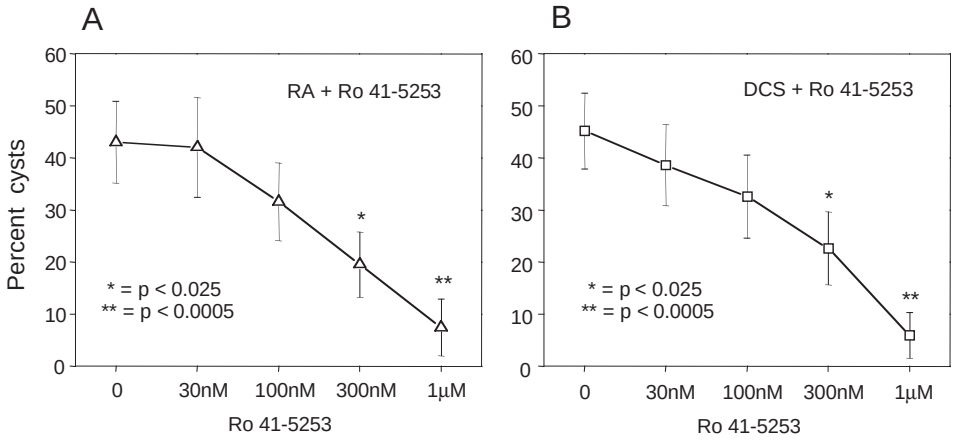

Fig. 7. An RARα-selective antagonist inhibits both RA- and DCS-induced lumen formation. (A) J3B1A cells suspended in collagen gels were either treated with RA alone (1 nM) or co-treated with RA and different concentrations of Ro 41-5253, a selective RARα antagonist. (B) J3B1A cells were suspended in collagen gels and incubated either with 0.1% DCS alone or with 0.1% DCS plus different concentrations of Ro 41-5252. Lumen formation was quantified after 6 days. **P*<0.025 and ***P*<0.0005 compared with control values.

mediated the lumen-promoting activity of DCS. As seen in Fig. 7B, Ro 41-5253 inhibited the lumen-inducing activity of DCS in a dose-dependent manner, resulting in the formation of solid multicellular structures. Collectively, these finding strongly suggest that retinoids are responsible for the ability of DCS to promote lumen formation by J3B1A cells and that activation of RARα is sufficient to mediate this biological response.

Lumen formation requires matrix metalloproteinase activity

In light of our previous finding that generation of cystic structures by other cell types is dependent on extracellular proteolysis (Montesano et al., 1990), we wished to determine whether cyst formation by J3B1A cells also requires pericellular matrix degradation. Extracellular matrix remodeling is mediated primarily by matrix metalloproteinases (MMP), a family of secreted and membrane-associated zinc-dependent endopeptidases (reviewed by Vu and Werb, 2000). To assess the potential role of MMP in RA-induced lumen formation, we first examined the effect of RA on the production of MMP-2 (72 kDa gelatinase, gelatinase A) and MMP-9 (92 kDa gelatinase, gelatinase B). Zymographies of conditioned media from untreated J3B1A cells grown in plastic dishes contained bands of gelatin lysis corresponding to the reported molecular weight of the latent form of MMP-9. Treatment with RA (10 nM to 1 μM) resulted in a marked time- and dose-dependent increase in this activity. In addition, the lower molecular weight activated species of MMP-9, which was barely visible in control cultures, became clearly detectable after 72 hours of RA treatment (Fig. 8A). A similar dose-dependent (albeit less robust) induction of the 92 kDa gelatinase was observed in conditioned media from J3B1A cells suspended in 3D collagen gels in the presence of RA (data not shown). By contrast, proteolytic activity corresponding to the molecular weight of MMP-2 (72 kDa) was not detected in gelatin zymographies of J3B1A cells grow either on plastic or in collagen gels. To assess whether MMP-9 mRNA is upregulated by RA, total cellular RNA was analyzed by

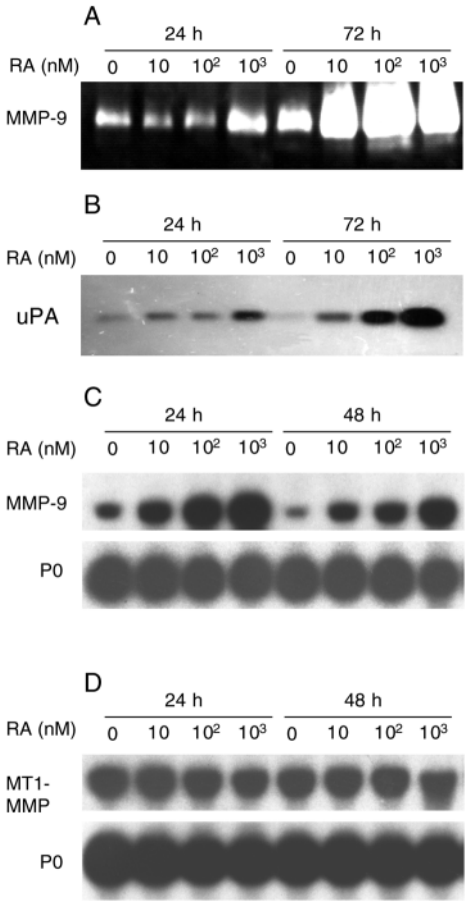

Fig. 8. RA induces the production of MMP-9 and uPA. (A) Effect of RA on gelatinase activity. Gelatin zymography of conditioned medium from monolayer cultures of J3B1A cells incubated with 10,100 and 1000 nM RA. The larger band of gelatin lysis corresponds to the reported molecular weight of latent MMP-9, whereas the smaller lower molecular weight band corresponds to the activated MMP-9 species. The zymogram is representative of five distinct experiments. (B) RA induces a dose- and time-dependent increase in uPA activity, as detected by casein zymography of conditioned media. (C,D) RA induces a dose-dependent increase in MMP-9 mRNA but does not modulate MT1-MMP mRNA. Confluent J3B1A cell monolayers were incubated with or without various concentrations of RA for 24 or 48 hours, after which RNA samples (5-10 μg per lane) were prepared and hybridized with either MMP-9 or MT1-MMP cRNA probes, as described in Materials and Methods. A bovine P0 ribosomal phosphoprotein cRNA probe was used to assess uniformity of loading.

northern blot hybridization. This showed a clear dose-dependent induction of MMP-9 mRNA by RA (10 nM to 1 μM) (Fig. 8C). As it is known that MMP-9 is activated extracellularly by other proteinases such as plasmin (Baramova et al., 1997), we next investigated whether RA modulates plasminogen-dependent extracellular proteolysis in J3B1A cells. By casein zymography of cell supernatants of J3B1A cells grown either on plastic (Fig. 8B) or in collagen gels (data not shown), we observed a clear dose-dependent induction of the urokinase-type plasminogen activator (uPA). These findings provide a potential mechanism for activation of MMP-9. Recently, it has been shown that membrane type-1 MMP (MT1-MMP, MMP-14) not only participates in the activation of progelatinase A but also directly cleaves fibrillar collagen (Ohuchi et al., 1997). By northern blot analysis, we found that MT1-MMP mRNA is constitutively expressed by J3B1A cells but is not detectably modulated by RA (Fig. 8D). Collectively, these results suggest that the cooperative activities of MT1-MMP and MMP-9 contribute to the process of lumen morphogenesis in J3B1A cells.

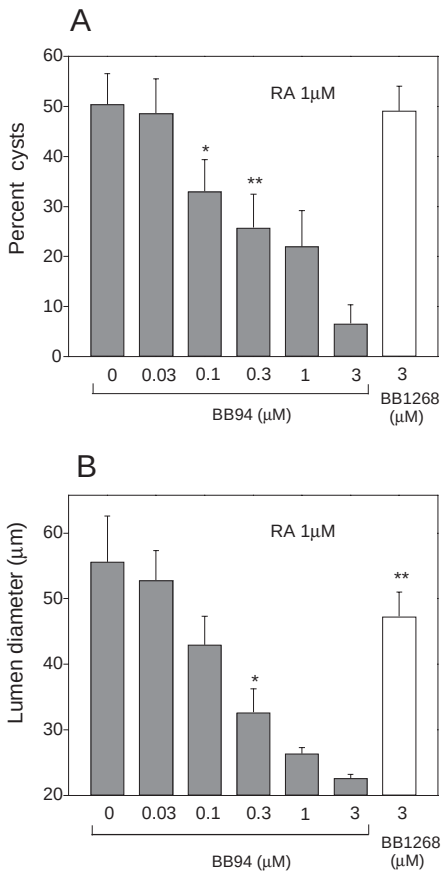

Fig. 9. RA-induced lumen formation requires metalloproteinase activity. (A,B) Effect of the synthetic MMP inhibitor BB94 on RA-induced lumen formation. J3B1A cells suspended in collagen gels were treated with RA alone (1 μM), co-treated with RA (1 μM) and BB94 (30 nM to 3 μM) or co-treated with RA (1 μM) and the inactive isomer BB1268 (3 μM). In A, cyst formation was quantified after 6 days as described in Materials and Methods and results are expressed as mean percentage of cysts±s.e.m. in 20 randomly selected photographic fields (five fields from each of four separate experiments) per experimental condition. Cyst formation is inhibited in a dose-dependent manner by BB94 but is not affected by the inactive isomer BB1268 (*P<0.05 and **P<0.01 compared to cultures incubated with RA alone). In B, lumen diameter was quantified after 6 days by measuring the minor axis of the central cavity in all cystic colonies. Lumen diameter is reduced in a dose-dependent manner by BB94, but is not significantly decreased by BB1268 (*P<0.01 compared with cultures incubated with RA alone; **not significantly different from cultures incubated with RA alone).

To determine the functional relevance of MMP in lumen formation, we next examined the organization of the colonies formed by J3B1A cells in the presence of RA and different concentrations of the synthetic MMP inhibitor BB94 (Brown, 1998). A quantitative analysis demonstrated that BB94 reduced in a dose-dependent manner both the percentage of cystic colonies (Fig. 9A) and lumen diameter in residual cysts (Fig. 9B). By contrast, the related inactive isomer BB1268 did not inhibit cyst formation (Fig. 9A,B). These findings support a role for MMP in RA-induced lumen morphogenesis.

Discussion

To begin elucidating the factors that regulate the process of lumen formation in glandular organs, we have developed an experimental system in which J3B1A mammary epithelial cells grown in collagen gels under serum-free conditions form solid, lumen-less colonies. Addition of low concentrations of bovine serum to the defined medium is sufficient to elicit the formation of a central cavity. The effect of serum cannot be reproduced by a number of polypeptide growth factors or hormones but is accurately recapitulated by the addition of retinol (vitamin A) in a range of concentrations that are normally found in plasma. All-trans-retinoic acid (RA), the active metabolite of retinol, also induces lumen formation in a dose- and time-dependent manner. These findings therefore identify a novel biological activity of retinoids that is likely to play an important role in the development and maintenance of glandular epithelial structures.

Several lines of evidence indicate that induction of lumen formation in cultures of J3B1A cells is mediated by a specific retinoid signaling pathway that involves the RARα receptor. First, the RAR-selective ligand TTNPB, a synthetic retinoid that binds specifically to RAR but not to RXR receptors (LeMotte et al., 1996), is a potent inducer of lumen formation. By contrast, RXR-selective ligands, including the synthetic retinoid Ro 25-7386 (Toma et al., 1998) as well as the naturally occurring compounds phytanic acid (Kitareevan et al., 1996) and docosahexaenoic acid (Mata de Urquiza et al., 2000), are unable to induce lumen formation. These results suggest that activation of RARs rather than RXRs is a requirement for induction of lumen formation by J3B1A mammary epithelial cells. Second, lumen formation is also potently induced by the synthetic retinoid Am-580, which is a selective agonist of the RARα isoform (Delescluse et al., 1991). Third, lumen formation induced by either RA or serum is suppressed by Ro 41-5253, a synthetic agonist that competes with RA for binding to RARα (Apfel et al., 1992). Collectively, these findings indicate that activation of RARα is sufficient to mediate lumen formation, although the potential contribution of RARβ- and γ-selective pathways cannot be definitively ruled out.

Information concerning retinoid effects on lumen morphogenesis is presently scant. Using 2D cultures of kidney epithelial cells, Humes and Cieslinski showed that RA, added

in combination with transforming growth factor-β1 and EGF, induced the retraction of a confluent monolayer resulting in the formation of cord-like structures containing a central lumen (Humes and Cieslinski, 1992). Lee et al. observed that in primary cultures of rat mammary epithelial cells grown in a basement membrane gel, retinoids increase the number of alveolar-like lobular colonies (Lee et al., 1995). In light of the findings reported here, this effect might have resulted in part from the ability of retinoids to promote lumen formation. Seewaldt et al. applied a dominant-negative strategy to explore the effect of inhibiting RA receptor function in normal human mammary epithelial cells (Seewaldt et al., 1997). They found that mammary epithelial cells expressing a truncated RARα are unable to form polarized lumen-containing structures when grown in a 3D extracellular matrix environment. Thus, using two different but complementary approaches, the work of Seewaldt et al. and our own study strongly suggest that RA signaling plays an important role in the process of lumen formation in epithelial tissues. This conclusion is further corroborated by a recent analysis of the effects of retinoids on ovarian carcinoma cells maintained in organotypic culture (Guruswamy et al., 2001). The authors of this study reported that retinoid treatment promoted a more orderly organization of the cells, which were sometimes arranged around a central space to form gland-like structures.

When added alone to serum-free collagen gel cultures of J3B1A cells, RA induces the formation of alveolar-like cysts that only occasionally present short branching outgrowths. It therefore appears that, in the absence of additional morphogens, RA is unable to promote the generation of arborized tubular structures. Nonetheless, evidence obtained in this study indicates that when acting in concert with other factors, RA plays a critical role in the process of tubulogenesis by virtue of its lumen-inducing activity. Thus, under experimental conditions that induce the formation of branching cords devoid of a central lumen (e.g. incubation with serum-free conditioned medium from monolayer cultures of J3B1A cells or exposure to the bioactive lipid LPA), the concomitant addition of RA results in the formation of duct-like structures enclosing a widely patent lumen. These results indicate that induction of branching is not by itself sufficient to generate hollow tubes and that lumen formation requires additional signals, which in our experimental system are provided by retinoids. The finding that simultaneous treatment with LPA and RA fully recapitulates the tubulogenic program of J3B1A cells also indicates a wider role for lipid messengers in epithelial morphogenesis than has previously been considered.

The cellular and molecular mechanisms responsible for lumen formation in glandular tissues are still largely conjectural. To facilitate their analysis, it is useful to divide the process of lumenogenesis into two phases: initial lumen formation and subsequent lumen expansion (for details, see Yap et al., 1994). Initial lumen formation is thought to be the direct consequence of cell polarization. Specifically, it has been proposed that when epithelial cells within a 3D aggregate begin to polarize, intracellular vacuoles containing apical membrane proteins fuse with the prospective apical pole, thereby forming a nascent lumen (Wang et al., 1994; Yap et al., 1995). The subsequent enlargement of the primitive cavitary structure may be due to proteolysis of the surrounding pericellular matrix (Montesano et al., 1990; Kadono et al., 1998; Obermüller et al., 2001) or to fluid accumulation resulting from vectorial transport of water and solutes by the epithelial cells lining the cavity (Grantham et al., 1989; Macias et al., 1992; Yap et al., 1994). To begin to address the mechanisms responsible for RA-induced lumen formation, we have examined in this study the potential role of matrix-remodeling proteases. By gelatin zymography, we found that RA increases in a dose-dependent manner the expression of latent and active forms of MMP-9 (gelatinase B) by J3B1A cells. Gelatinases, by acting sequentially after the initial cleavage of the triple helix by constitutively expressed interstitial collagenase (MMP-1) or MT1-MMP, are thought to constitute a critical and possibly rate-limiting step in collagen degradation (Creemers et al., 1998). The findings that RA induces a parallel dose-dependent increase in: (1) the production of MMP-9 (but not of MMP-2); (2) the percentage of cystic colonies and (3) cyst lumen diameter, suggest that MMP-9-mediated pericellular matrix remodeling is involved in the process of lumen formation by J3B1A cells. Importantly, the functional relevance of MMP in our system is supported by the finding that lumen formation is abrogated by the synthetic MMP inhibitor BB94 but not by the related inactive isomer BB1268. It should be noted, however, that BB94 inhibits not only MMP but also members of the 'adamalysin' family of metalloproteinases (Moss et al., 1997), which are thought to play important roles in developmental processes by promoting membrane protein ectodomain shedding (Peschon et al., 1998). We cannot therefore exclude the possibility that inhibition of metalloproteinases other than MMP contributes to the ability of BB94 to abrogate lumen formation. With this caveat in mind, the findings reported in this study lend significant support to the notion (Sympson et al., 1994; Witty et al., 1995; Simian et al., 2001; Lee et al., 2001) that the balance of MMP and MMP inhibitors is crucial for mammary gland development.

Although our results point to an important role for MMP-mediated matrix remodeling in RA-induced lumen morphogenesis, it is conceivable that additional mechanisms participate in this process. Thus, it has been reported that retinoids increase the expression of MUC1, an anti-adhesive apical membrane glycoprotein, in epithelial cells (Guruswamy et al., 2001). Interestingly, MUC1 has been proposed to facilitate the formation and maintenance of lumen in glandular tissues by preventing interaction between the prospective apical domains of contiguous epithelial cells (Hilkens et al., 1992). RA has also been shown to stimulate the deposition of basement membrane components, such as laminin (Gudas et al., 1994), which have the ability to induce cell polarization and lumen formation (Klein et al., 1988; Wang et al., 1990; O'Brien et al., 2001; Gudjonsson et al., 2002). If operative in our system, these mechanisms would probably contribute to initial lumen formation. Subsequent MMP-dependent cyst enlargement may be compounded by positive intraluminal hydrostatic pressure resulting from active fluid secretion (i.e. inward transport of solutes and water), decreased fluid reabsorption and/or enhanced sealing capacity of tight junctions. Finally, recent studies suggest the existence of a functional link between retinoids and epimorphin, a membrane-bound morphoregulatory protein. Epimorphin has been shown to promote lumen formation by mammary epithelial cells (Hirai et al., 1998) by increasing the expression

of the transcription factor C/EBPβ (Hirai et al., 2001) and of MMP (Simian et a., 2001). Intriguingly, RA has been reported to enhance the expression of epimorphin (Plateroti et al., 1998), of C/EBPβ (Menéndez-Hurtado et al., 1997; Lee et al., 2002) and of MMP (this study) in epithelial cells. These findings therefore raise the possibility that the lumen-inducing activity of retinoids is mediated in part through the induction of epimorphin. Further studies will be necessary to establish whether the potential mechanisms alluded to above actually contribute to lumen morphogenesis under our experimental conditions.

In a number of in vivo and in vitro systems, lumen formation has been shown to result from selective apoptosis of centrally located cells (Coucouvanis and Martin, 1995; Humphreys et al., 1996; Blatchford et al., 1999; Murray and Edgar, 2000). However, apoptosis is unlikely to represent the primary mechanism of cavitation in our experimental setting for two reasons. First, in sections of cystic colonies, lumina appeared essentially free of cell debris or apoptotic bodies. Second, the percentage of nuclei with condensed chromatin (as visualized by staining with the DNA-binding dye DAPI) or containing fragmented DNA (as visualized by a TUNEL assay) was virtually identical (approximately 1%) in J3B1A colonies formed in control and RA-treated cultures (R.M. and P.S., unpublished).

Retinoids are essential for embryonic patterning and organogenesis (Chytil, 1996; Niederreither et al., 1999; Morriss-Kay et al., 1999; Ross et al., 2000; Malpel et al., 2000), yet virtually nothing is known concerning their potential effects on mammary gland development. Although caution needs to be exercised when extrapolating information gained from in vitro systems to the intact organism, the results of this study raise the possibility that retinoids regulate lumen formation during embryonic and/or postnatal mammary gland morphogenesis. How could retinoids control this process? Metabolic studies have shown that blood levels of retinol are maintained within a narrow range (Napoli, 1996; Gottesman et al., 2001). Consequently, stage-specific retinoid effects on mammary epithelium, rather than being determined by variations in plasma retinol concentration, may be mediated by spatially and temporally restricted changes in either retinoid receptor expression or intracellular retinol metabolism. With respect to the latter possibility, it is well documented that retinol undergoes a series of oxidative reactions that first generate retinaldehyde and subsequently RA, which eventually binds to and activates retinoid nuclear receptors (Gudas et al., 1994; Napoli, 1996). Among the enzymes involved in this pathway, retinaldehyde dehydrogenase-2 (Raldh-2) has been shown to be developmentally regulated and to play an essential role in embryogenesis by controlling RA availability to target cells (Niederreither et al., 1999; Ulven et al., 2000; Gottesman et al., 2001). RA catabolism by inactivating enzymes (e.g., CYP26) provides an additional mechanism for the fine-tuning of intracellular RA concentration. Interestingly, chronological and/or regional variations in RA availability, largely determined by a balance between RA-synthesizing and RA-catabolizing enzymes, have been reported to occur during embryogenesis (Abu-Abed et al., 2001; Sakai et al., 2001) and to be crucial for lung development (Malpel et al., 2000; Cardoso, 2001). In light of the notions outlined above, it will be important in future studies to establish whether spatiotemporal changes in retinoid metabolism or signaling contribute to the embryonic and/or postnatal organogenesis of the mammary gland, with particular respect to the process of lumen formation.

Vitamin A deficiency in experimental animals has long been associated with a higher incidence of spontaneous and carcinogen-induced tumors. Further, epidemiological studies have indicated that individuals with a lower dietary vitamin A intake are at higher risk of developing cancer. Conversely, retinoid administration has been shown to prevent tumor development in a variety of animal models as well as in patient studies (reviewed by Lotan, 1996). More recently, lack of expression of specific RARs (reviewed by Yang et al., 1999) or inability to synthesize RA from retinol (Mira-y-Lopez et al., 2000) have been observed in a number of mammary carcinoma cell lines, indicating that defects in the retinoid signaling pathway likely contribute to breast cancer development. The mechanism by which retinoids exert their chemopreventive activity, however, is not fully understood. We have shown here that mammary epithelial cells grown in the presence of retinoids generate polarized, lumen-containing structures that mimic the organization of mammary gland alveoli and ducts, whereas in the absence of retinoids they form solid colonies devoid of a central lumen. Given these findings, and considering that early stages of breast cancer are characterized by loss of tissue-specific organization, we propose that in addition to their morphogenetic function during embryogenesis, retinoids act post-natally as stabilizers of epithelial tissue architecture, thereby preventing tumorigenesis.

In conclusion, by growing clonal mammary epithelial cells in a 3D extracellular matrix environment under defined serum-free conditions, we have uncovered a biological activity of retinoids that would have not been detected using conventional, 2D cultures. The experimental system we have developed provides a basis for further dissecting the mechanisms responsible for the lumen-inducing activity of retinoids and may also serve as a suitable bioassay to identify additional regulators of epithelial morphogenesis.

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References

Abu-Abed, S., Dollé, P., Metger, D., Beckett, B., Chambon, P. and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Devl*. **15**, 226-240.

Apfel, C., Bauer, F., Crettaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W. and Klaus, M. (1992). A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. *Proc. Natl. Acad. Sci. USA* **89**, 7129-7133.

Baramova, E. N., Bajou, K., Remacle, A., L'Hoir, C., Krell, H. W., Weidle, U. H., Noel, A. and Foidart, J. M. (1997). Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. *FEBS Lett*. **405**, 157-162.

Bellusci, S., Grindley, J., Emoto, H., Itah, N. and Hogan, B. L. (1997). Fibroblast growth factor 10 (FGF 10) and branching morphogenesis in the embryonic mouse lung. *Development* **124**, 4867-4878.

Blatchford, D. R., Quarrie, L. H., Tonner, E., McCarthy, C., Flint, D. J. and Wilde, C. J. (1999). Influence of microenvironment on mammary epithelial cell survival in primary culture. *J. Cell. Physiol.* **181**, 304-311.

Brown, P. D. (1998). Synthetic inhibitors of matrix metalloproteinases. In: *Matrix Metalloproteinases* (ed. W. C. Parks and R. P. Mecham), pp 243-261. St. Louis, Missouri: Academic Press.

Cardoso, W. (2001). Molecular regulation of lung development. *Annu. Rev. Physiol*. **63**, 471-494.

Chambard, M., Gabrion, J. and Mauchamp, J. (1981). Influence of collagen gel on the orientation of epithelial cell polarity: follicle formation from isolated thyroid cells and from preformed monolayers. *J. Cell Biol*. **91**, 157-166.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J*. **10**, 940-954.

Chytil, F. (1996). Retinoids in lung development. *FASEB J*. **10**, 986-992.

Coucouvanis, E. and Martin, G. L. (1995). Signals for death and survival: a two step mechanism for cavitation in the vertebrate embryo. *Cell* **83**, 279-287.

Creemers, L. B., Jansen, I. D. C., Docherty, A. J. P., Reynolds, J. J., Beersten, W. and Everts, V. (1998). Gelatinase A (MMP-2) and cysteine proteinases are essential for the degradation of collagen in soft connective tissue. *Matrix Biol.* **17**, 35-46.

Delescluse, C., Cavey, M. T., Martin, B., Bernard, B. A., Reichert, U., Maignan, J., Darmont, M. and Shroot, B. (1991). Selective high affinity retinoic acid receptor α or β-γ ligands. *Mol. Pharmacol.* **40**, 556-562.

Fialka, I., Schwarz, H., Reichmann, E., Oft, M., Busslinger, M. and Beug, H. (1996). The estrogen-dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. *J. Cell Biol*. **132**, 1115-1132.

Gottesman, M. E., Quadro, L. and Blaner, W. S. (2001). Studies of vitamin A metabolism in mouse model systems. *BioEssays* **23**, 409-419.

Grantham, J. J., Uchic, M., Cragoe, E. J., Jr, Kornhaus, J., Grantham, J. A., Donoso, V., Mangoo-Karim, R., Evan, A. and McAteer, J. (1989). Chemical modification of cell proliferation and fluid secretion in renal cysts. *Kidney Int.* **35**, 1379-1389.

Gudas, L., Sporn, M. B. and Roberts, A. B. (1994). Cellular biology and biochemistry of the retinoids. In *The Retinoids: Biology, Chemistry and Medicine*, 2nd edn (ed. M. B. Sporn, A. B. Roberts and D. S. Goodman), pp. 443-520. New York: Raven Press.

Gudjonsson, T., Rønnov-Jessen, L., Villadsen, R., Rank, F., Bissell, M. J. and Petersen, O. W. (2002). Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. *J. Cell Sci.* **115**, 39-50.

Gumbiner, B. M. (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-357.

Guruswamy, S., Lightfoot, S., Gold, M. A., Hassan, R., Berlin, K. D., Ivey, R. T. and Benbrook, D. M. (2001). Effects of retinoids on cancer phenotype and apoptosis in organotypic cultures of ovarian carcinoma. *J. Natl. Cancer Inst*. **93**, 516-524.

Hall, H. G., Farson, D. A. and Bissell, M. J. (1982). Lumen formation by epithelial cell lines in response to collagen overlay: a morphogenetic model in culture. *Proc. Natl. Acad. Sci. USA* **79**, 4672-4676.

Hilkens, J., Ligtenberg, M. J. L., Vos, H. L. and Litvinov, S. V. (1992). Cell-membrane-associated mucins and their adhesion-modulating properties. *Trends Biochem. Sci.* **17**, 359-363.

Hirai, Y., Lochter, A., Galosy, S., Koshida, S., Niwa, S. and Bissell, M. J. (1998). Epimorphin functions as a key morphoregulator for mammary epithelial cells. *J. Cell Biol.* **140**, 159-169.

Hirai, Y., Radisky, D., Boudreau, R., Simian, M., Stevens, M. E., Oka, Y., Takebe, K., Niwa, S. and Bissell, M. J. (2001). Epimorphin mediates mammary luminal morphogenesis through control of C/EBPβ. *J. Cell Biol*. **153**, 785-794.

Hla, T., Lee, M.-J., Ancellin, N., Liu, C. H., Thangada, S., Thompson, B. D. and Kluk, M. (1999). Sphingosine-1-phosphate: extracellular mediator or intracellular second messenger? *Biochem. Pharmacol*. **58**, 201-207.

Hogan, B. L. M. and Kolodziej, P. A. (2002). Molecular mechanisms of tubulogenesis. *Nat. Rev. Genet.* **3**, 513-523.

Hogg, N. A. S., Harrison, C. J. and Tickle, C. (1983). Lumen formation in the developing mouse mammary gland. *J. Embryol. Exp. Morphol*. **73**, 39-57.

Humes, D. H. and Cieslinski, D. A. (1992). Interactions between growth factors and retinoic acid in the induction of kidney tubulogenesis in tissue culture. *Exp. Cell Res*. **201**, 8-15.

Humphreys, R. C., Krajewska, M., Krnacik, S., Jaeger, R., Weiher, H., Krajewski, S., Reed, J. C. and Rosen, J. M. (1996). Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. *Development* **122**, 4013-4022.

Kadono, Y., Shibahara, K., Namiki, M., Watanabe, Y., Seiki, M. and Sato, H. (1998). Membrane type 1-matrix metalloproteinase is involved in the formation of hepatocyte growth factor/scatter factor-induced branching tubules in Madin-Darby canine kidney epithelial cells. *Biochem. Biophys. Res. Commun*. **251**, 681-687.

Kitareevan, S., Burka, L. T., Tomer, K. B., Parker, C. E., Deterding, L. J., Stevens, R. D., Forman, B. M., Mais, D. E., Heyman, R. A., McMorris, T. and Weinberger, C. (1996). Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. *Mol. Biol. Cell* **7**, 1153-1166.

Klein, G., Langegger, M., Timpl, R. and Ekblom, P. (1988). Role of laminin A chain in the development of epithelial cell polarity. *Cell* **55,** 331-341.

Lee, I.-H., Lee, J.-H., Lee, M.-J., Lee, S.-Y. and Kim, I.-S. (2002). Involvement of CCAAT/enhancer-binding protein-α in haptoglobin gene expression by all-trans-retinoic acid. *Biochem. Biophys. Res. Commun.* **294**, 956-961.

Lee, P.-P. H., Lee, M.-T., Darcy, K. M., Shudo, K. and Ip, M. M. (1995). Modulation of normal mammary epithelial cell proliferation, morphogenesis, and functional differentiation by retinoids: a comparison of the retinobenzoic acid derivative RE80 with retinoic acid. *Endocrinology* **136**, 1707-1717.

Lee, P.-P. H., Hwang, J.-J., Mead, L. and Ip, M. M. (2001). Functional role of matrix metalloproteinases (MMPs) in mammary epithelial cell development. *J. Cell. Physiol.* **188**, 75-88.

LeMotte, P. K., Keidel, S. and Apfel, C. M. (1996). Characterization of synthetic retinoids with selectivity for retinoic acid or retinoid X receptors. *Biochim. Biophys. Acta* **1289**, 298-304.

Lipschutz, J. H., Guo, W., O'Brien, L. E., Nguyen, Y. H., Novik, P. and Mostov, K. (2000). Exocyst is involved in cystogenesis and tubulogenesis and acts by modulating synthesis and delivery of basolateral plasma membrane and secretory proteins. *Mol. Biol. Cell* **11**, 4259-4275.

Lotan, R. (1996). Retinoids in cancer chemoprevention. *FASEB J.* **10**, 1031-1039.

Macias, W. L., McAteer, J. A., Tanner, J. A., Fritz, A. L. and Armstrong, W. M. (1992). NaCl transport by Madin-Darby canine kidney cyst epithelial cells. *Kidney Int.* **42**, 308-319.

Malpel, S., Mendelsohn, C. and Cardoso, W. V. (2000). Regulation of retinoic acid signaling during lung morphogenesis. *Development* **127**, 3057-3067.

Mata de Urquiza, A., Liu, S., Sjöberg, M., Zetterström, R. H., Griffiths, W., Siövall, J. and Perlmann, T. (2000). Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* **290**, 2140-2144.

Menéndez-Hurtado, A., Vega-Núñez, E., Santos, A. and Perez-Castillo, A. (1997). Regulation by thyroid hormone and retinoic acid of the CCAAT/enhancer binding Protein α and β genes during liver development. *Biochem. Biophys. Res. Commun*. **234**, 605-610.

Mira-y-Lopez, R., Zheng, W. L., Kuppumbatti, Y. S., Rexer, B., Jing, Y. and Ong, D. E. (2000). Retinol conversion to retinoic acid is impaired in breast cancer cell lines relative to normal cells. *J. Cell. Physiol*. **185**, 302-309.

Montesano, R., Mouron, P., Amherdt, M. and Orci, L. (1983). Collagen matrix promotes reorganization of pancreatic endocrine cell monolayers into islet-like organoids. *J. Cell Biol.* **97**, 935-939.

Montesano, R., Soriano, J. V., Fialka, I. and Orci, L. (1998). Isolation of EpH4 mammary epithelial cell subpopulations which differ in their morphogenetic properties. *In Vitro Cell. Dev. Biol.* **34**, 468-477.

Montesano, R., Pepper, M. S., Möhle-Steinlein, U., Risau, W., Wagner, E. F. and Orci, L. (1990). Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing middle T oncogene. *Cell* **62**, 435-445.

Montesano, R., Schaller, G. and Orci, L. (1991a). Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. *Cell* **67**, 697-711.

Montesano, R., Matsumoto, K., Nakamura, T. and Orci, L. (1991b). Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67**, 901-908.

Moolenaar, W. H. (1999). Bioactive lysophospholipids and their G protein-coupled receptors. *Exp. Cell Res*. **253**, 230-238.

Morriss-Kay, G. M. and Ward, S. J. (1999). Retinoids and mammalian development. *Int. Rev. Cytol.* **188**, 73-131.

Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Carter, H. L., Chen, W.-J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R. et al. (1997). Cloning of a disintegrin metalloproteinase that processes tumour-necrosis factor-α. *Nature* **385**, 733-736.

Murray, P. and Edgar, D. (2000). Regulation of programmed cell death by basement membrane in embryonic development. *J. Cell Biol.* **150**, 1215-1221.

Napoli, J. L. (1996). Retinoic acid biosynthesis and metabolism. *FASEB J.* **10**, 993-1001.

Nau, H. and Blaner, W. S. (eds.) (1999). *Retinoids: the biochemical and molecular basis of vitamin A and retinoid action. Handb. Exptl. Pharmacol.*, Vol. 139. Berlin: Springer-Verlag.

Niederreither, K., Subbarayan, V. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.

Obermüller, N., Morente, N., Kränzlin, B., Gretz, N. and Witzgall, R. (2001). A possible role for metalloproteinases in renal cyst development. *Am. J. Physiol.* **280**, F540-F550.

O'Brien, L. E., Jou, T.-S., Pollack, A. L., Zhang, Q., Hansen, S. H., Yurchenco, P. and Mostov, K. E. (2001). Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat. Cell Biol.* **3**, 831-838.

O'Brien, L. E., Zegers, M. M. P. and Mostov, K. E. (2002). Building epithelial architecture: insights from three-dimensional culture models. *Nat. Rev. Mol. Cell Biol.* **3**, 531-537.

Oft, M., Peli, J., Rudaz, C., Schwartz, H., Beug, H. and Reichmann, E. (1996). TGF-β1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev.* **10**, 2462-2477.

Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1997). Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.* **272**, 2446-2451.

Pepper, M. S., Belin, D., Montesano, R., Orci, L. and Vassalli, J. D. (1990). Transforming growth factor beta-1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. *J. Cell Biol.* **111,** 743-755.

Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N. et al. (1998). An essential role for ectodomain shedding in mammalian development. *Science* **282**, 1281-1284.

Petersen, O. W., Ronnov-Jessen, L., Howlett, A. R. and Bissell, M. J. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial. *Proc. Natl. Acad. Sci. USA* **89**, 9064-9068.

Plateroti, M., Rubin, D. C., Duluc, I., Singh, R., Foltzer-Jourdainne, C., Jean-Noël Freund, J.-N. and Kedinger, M. (1998). Subepithelial fibroblast cell lines from different levels of gut axis display regional characteristics. *Am. J. Physiol. Gastrointest. Liver Physiol.* **274**, G945-G954.

Ross, S. A., McCaffery, P. Y., Drager, U. C. and de Luca, L. M. (2000). Retinoids in embryonal development. *Physiol. Rev.* **80**, 1021-1054.

Sakai, Y., Meno, C., Fujii, H., Nishino, J., Shiratori, H., Saijoh, Y., Rossant, J. and Hamada, H. (2001). The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. *Genes Dev.* **15**, 213-225.

Sakurai, H., Bush, K. T. and Nigam, S. K. (2001). Identification of pleiotrophin as a mesenchymal factor involved in ureteric bud branching morphogenesis. *Development* **128**, 3283-3293.

Seewaldt, V. L., Caldwell, L. E., Johnson, B. S., Swisshelm, K., Collins, S. J. and Tsai, S. (1997). Inhibition of retinoic acid receptor function in normal human mammary epithelial cells results in increased cell proliferation and inhibits the formation of a polarized epithelium in vitro. *Exp. Cell Res.* **236**, 16-28.

Simian, M., Hirai, Y., Navre, M., Werb, Z., Lochter, A. and Bissell, M. J. (2001). The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development* **128**, 3117-3131.

Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L. and Montesano, R. (1995). Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *J. Cell Sci.* **108**, 413-430.

Sympson, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J. and Werb, Z. (1994). Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J. Cell Biol.* **125**, 681-693.

Toma, S., Isnardi, L., Riccardi, L. and Bollag, W. (1998). Induction of apoptosis in MCF-7 breast carcinoma cell line by RAR and RXR selective retinoids. *Anticancer Res.* **18**, 935-942.

Ulven, S. M., Gundersen, T. E., Weedon, M. S, Landaas, V. O., Sakhi, A. K., Fromm, S. H., Geronimo, B. A, Moskaug, G. O. and Blomhoff, R. (2000). Identification of endogenous retinoids, enzymes, binding proteins, and receptors during early postimplantation development in mouse: important role of retinal dehydrogenase type 2 in synthesis of all-*trans*-retinoic acid. *Dev. Biol.* **220**, 379-391.

Van Heusden, J., Wouters, W., Ramaekers, F. C. S., Krekels, M. D. W. G., Dillen, L., Borgers, M. and Smets, G. (1998). All-*trans*-retinoic acid metabolites significantly inhibit the proliferation of MCF-7 human breast cancer cells in vitro. *Br. J. Cancer* **77**, 26-32.

Vega, Q. C., Worby, C. A., Lechner, M. S., Dixon, J. E. and Dressler, G. R. (1996). Glial cell line-derived neurotrophic factor activates RET and promotes kidney morphogenesis. *Proc. Natl. Acad. Sci. USA* **93**, 10657-10661.

Vu, T. H. and Werb, Z. (2000). Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* **14**, 2123-2133.

Wang, A. Z., Wang, J. C., Ojakian, C. K. and Nelson, W. G. (1994). Determinants of apical membrane formation in multicellular epithelial MDCK cysts. *Am. J. Physiol.* **267**, C473-C481.

Witty, J. P., Wright, J. H. and Matrisian, L. M. (1995). Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol. Biol. Cell* **6**, 1287-1303.

Yang, L. M., Tin-U, C., Wu, K. and Brown, P. (1999). Role of retinoid receptors in the prevention and treatment of breast cancer. *J. Mam. Gland Biol. Neoplasia* **4**, 377-388.

Yap, A. S., Stevenson, B. R., Armstrong, J. W., Keast, J. R. and Manley, S. W. (1994). Thyroid epithelial morphogenesis in vitro: a role for bumetanide-sensitive Cl^- secretion during follicular lumen development. *Exp. Cell Res.* **213**, 319-326.

Yap, A. S., Stevenson, B. R., Keast, J. R. and Manley, S. W. (1995). Cadherin-mediated adhesion and apical membrane assembly define distinct steps during thyroid epithelial polarization and lumen formation. *Endocrinology* **136**, 4672-4680.