Members of the Fatty Acid Binding Protein Family Are Differentiation Factors for the Mammary Gland

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Abstract. Mammary gland development is controlled by systemic hormones and by growth factors that might complement or mediate hormonal action. Peptides that locally signal growth cessation and stimulate differentiation of the developing epithelium have not been described. Here, we report that recombinant and wild-type forms of mammary-derived growth inhibitor (MDGI) and heart–fatty acid binding protein (FABP), which belong to the FABP family, specifically inhibit growth of normal mouse mammary epithelial cells (MEC), while growth of stromal cells is not suppressed. In mammary gland organ culture, inhibition of ductal growth is associated with the appearance of bulbous alveolar end buds and formation of fully developed lobuloalveolar structures. In parallel, MDGI stimulates its own expression and promotes milk protein synthesis. Selective inhibition of endogenous MDGI expression in MEC by antisense phosphorothioate oligonucleotides suppresses appearance of alveolar end buds and lowers the β-casein level in organ cultures. Furthermore, MDGI suppresses the mitogenic effects of epidermal growth factor, and epidermal growth factor antagonizes the activities of MDGI. Finally, the regulatory properties of MDGI can be fully mimicked by an 11–amino acid sequence, represented in the COOH terminus of MDGI and a subfamily of structurally related FABPs. This peptide does not bind fatty acids. To our knowledge, this is the first report about a growth inhibitor promoting mammary gland differentiation.

GROWTH development, and differentiation of epithelial tissues are multistage processes that are driven by a combination of paracrine and autocrine signaling factors and interactions of a cell with its extracellular matrix (reviewed in 51, 60, 62). In the mammary gland, these complex interactions are regulated by various steroid and peptide hormones (29, 32, 46, 60). Development of the mouse mammary gland at puberty is characterized by sparsely branching ducts which invade the stroma, followed by the development of lobuloalveolar structures and functional differentiation, i.e., synthesis of milk constituents at pregnancy (1, 17, 32). By use of endocrine ablation (44), organ culture systems (1, 35, 54, 65) and mammary cells (MEC)[1] growing on a

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; CRABP, cellular retinoic acid binding protein; EGF, epidermal growth factor; FABP, fatty acid binding protein; H-FABP, heart FABP, 1-FABP, intestine FABP; L-FABP, liver FABP; LI, labeling index; MDGI, mammary-derived growth inhibitor; MEC, mammary epithelial cells; WAP, whey acidic protein.

basement membrane (39, 63), it has been documented that several steroid hormones in conjunction with prolactin and insulin regulate this process (reviewed in reference 66). Although the systemic importance of hormones such as estrogen, progesterone, prolactin, cortisol, and growth hormone in the development of the mammary gland has been well documented, these hormones virtually lack growth stimulatory or inhibitory activities in vitro (32, 46). This has prompted a search for polypeptide growth factors that might locally mediate or complement action of systemic hormones. A number of growth factors that either stimulate or inhibit epithelial DNA synthesis in the murine mammary gland have been described (32). For example, mitogenic effects on MEC have been described for epidermal growth factor (EGF) (16, 27, 65, 70) and TGF α (55, 59), demonstrating the importance of these growth factors for ductal growth and lobuloalveolar development. TGF-β1 has been shown to inhibit ductal outgrowth in the mammary gland of virgin mice (47, 53). In addition, the same molecule can inhibit functional differentiation in vitro (41, 50), and it impairs differentiation and milk protein synthesis in mice (34).

In contrast to these reports, there are no available data that

describe growth inhibitory and differentiation stimulatory growth factor activities during functional differentiation of the mammary gland.

When we screened for epithelial growth inhibitors, we have detected and purified from lactating bovine mammary gland (7) and from milk fat globule membranes (12) the mammary-derived growth inhibitor (MDGI) and a COOH-terminally truncated form of MDGI (13). The 14.5-kD protein was sequenced (7), and its cDNA was cloned (38). Originally, MDGI has been discovered according to its growth inhibitory activities on breast cancer cells in vitro (8). Our studies on MDGI mRNA and protein expression in the developing bovine and mouse mammary gland revealed a strong correlation between expression of MDGI in MEC and differentiation (6, 38, 43). MDGI expression was found to be hormonally regulated (6), suggesting a local role of the growth inhibitor in mediating or complementing hormonal action during differentiation (23, 24).

MDGI belongs to a multigene family of conservative proteins, referred to as fatty acid binding proteins (FABP), known to bind long-chain fatty acids, retinoids, and eicosanoids (reviewed in references 21, 40, 68, 69). A striking homology was evident between MDGI and the heart fatty acid binding protein (FABP) (H-FABP), which differ only in 10 positions of the amino acid sequence (7), and most likely represent isoforms that are encoded by the same gene (67). Other closely related members include the FABPs found in the brain and the cellular retinoic acid binding protein (CRABP) (52, 14, 56, 68). The function of these proteins is not yet defined, although a role in fatty acid transport, sequestration, or metabolism has been widely discussed (21, 69). Evidence for the existence of physiologically interesting FABP ligands such as selenium (4), E-type prostaglandins (18), or hydroxyeicosatetraenoic acid (48), as well as mitosis-associated expression of liver FABP (36), gave rise to speculations of some role of FABP in growth control.

To address directly the question of a role for MDGI and H-FABP as growth inhibitors and differentiation factors of MEC, we have cultured primary mouse mammary epithelial cells and whole mouse mammary glands in presence of wild-type, recombinant as well as mutated MDGI and H-FABP forms. We show that MDGI and H-FABP inhibit epithelial DNA synthesis, promote morphological differentiation, and stimulate milk protein synthesis. Selective inhibition of MDGI expression in organ cultures suppresses alveolar budding and impairs β-casein synthesis. The MDGI and H-FABP properties do not require fatty acid binding, and can be fully mimicked by a peptide that comprises COOH-terminal amino acids conservatively maintained in a subgroup of MDGI-related FABPs. This first report about a mammary epithelial differentiation factor suggests that other members of the FABP family might similarly to MDGI regulate growth and differentiation.

Materials and Methods

Cell and Organ Culture

Primary Cultures from Mammary Glands of Adult Virgin Mice. Mammary cells were collected after collagenase digestion of mammary glands excised from 8- to 13-wk-old mice. Approximately 10^5 cells were plated onto plastic culture dishes (60 mm in diameter) and maintained in DME supplemented with 10% fetal calf serum, penicillin, streptomycin, 4 μg/ml of insulin, and 10 ng/ml EGF.

Mammary Gland Organ Culture. Whole second thoracic mammary glands of hormonally primed virgin mice were cultured in serum-free medium according to Banarjee et al. (2). 4-wk-old BALB/c mice were injected subcutaneously for nine consecutive days with 1 mg progesterone and 1 μg estradiol (Jenapharm, Jena, Germany). The second pair of thoracic glands was excised and cultivated for 5 d as whole organ on perforated cyclopore membranes (No. 3090; Falcon Plastics, Cockeysville, MD) inserted into wells of culture plates (No. 3046; Falcon Plastics) in 2 ml medium 199 with Hanks' salts (Gibco BRL, Gaithersburg, MD) and 50 μg/ml gentamycin in an atmosphere of 50% O_2/5% CO_2, with medium changes every 2 d. The medium was supplemented with aldosterone (No. 12050; Serva, Sigma, Germany), ovine-prolactin (31 IU/mg, No. L-6520; Germany), insulin (Berlin-Chemie, Berlin, Germany) (each at 5 μg/ml), and 1 μg/ml hydrocortisone (No. 24608; Merck, Darmstadt, Germany), and will be referred to in this paper as the APIH medium. This medium has been used in all experiments described below. EGF was prepared as described before (58). Generally, three pairs of contralateral glands representing control and test groups were examined.

Histology and Morphometry

Whole-mount hematoxylin stains of mammary glands were obtained as described (49). For histology, formalin-fixed glands were embedded in paraffin. 5-μm sections were cut, deparaffinized, stained with hematoxylin and eosin, and analyzed as described before (6). Histologic criteria used were as follows (6): for ductules, a linear coat of cubic epithelial cells without adjacent mesenchyme and signs of secretion; for alveoli, presence of secretory cells with vacuoles and luminal secretion. Only structures exhibiting lumina were counted. At least three pairs of contralateral glands were examined. Fat droplet index was defined as the quotient of the number of alveoli consisting of >50% of epithelial cells with intraepithelial fat droplets to the total number of alveoli. Alveolar secretion was defined as the quotient of the number of alveoli with lumina containing secretory products to the total number of alveoli. An experimental group comprised of ~500 different structures for the morphometric analysis.

DNA Synthesis and Immunocytochemistry

Primary Cultures. To estimate DNA synthesis, cells were seeded at a density of 2 × 10^6 cells in 60-mm dishes in DME containing 10% FBS. 12 h later, the peptide to be tested was added. Cultures were maintained for 48 h before 5-bromo-2'-deoxyuridine (BrdU) at 10 μM was added according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). For BrdU staining, cultures were washed three times with PBS and fixed in 70% ethanol/glycine (50 mM, pH 2.0) for 30 min at −20°C. Cells were first incubated with 1:10 diluted anti BrdU antiserum, followed by an incubation with a secondary anti–mouse IgG conjugated to alkaline phosphatase. Bromo chloro indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) was used as substrate. Colonies >1,000 cells were counted. On the average, 10–20 colonies were analyzed for BrdU immunostaining. Results (expressed as percent of the control, which received the appropriate buffer) were obtained from triplicate experiments (SD = ±20%).

Mammary Gland Organ Culture. Growth inhibition was determined by labeling cells with BrdU (100 μM) for 4 h before cultures were terminated. Then organ cultures were fixed in 4% formalin/0.5% trichloroacetic acid before embedding. Deparaffinized sections were washed in PBS and analyzed as described above with the anti-BrdU antiserum kit (Boehringer Mannheim GmbH). Sections were counterstained with fast nuclear red and the labeling index (LI) estimated as described before (6). At least 1,000 nuclei were counted for each experimental group and histologic structure. Percent inhibition = 100-LI treated/LI controls × 100. Immunohistochemical analysis for expression of MDGI and β-casein in tissue sections of a mammary gland was performed as described before (38, 43). Deparaffinized sections were analyzed with a rabbit anti–mouse β-casein antiserum or an affinity-purified IgG fraction of a rabbit antiserum against bovine MDGI (43). For staining, sections were washed with PBS and treated with the streptavidin-biotinylated peroxidase complex (1:200) (Amersham International, Amersham, IL) using diaminobenzidine as substrate. Sections were counterstained with hematoxylin.

RNA Preparation and Northern Blot Analysis

All methods for analyzing mRNA expression in cultures from mammary

glands were used exactly as described before (6). Total RNA was extracted, denatured, and applied to 1% agarose gels for transfer to Hybond C-extra membranes (Amersham International) by capillary blotting. Levels for mRNAs were estimated from Northern blots hybridized with ^{32}P-labeled cDNA probes for MDGI, β-casein, and whey acidic protein (WAP), and were normalized to 28S ribosomal RNA, as described earlier (6). The 0.4-kD cDNA probe in pBR327 for WAP was provided by Dr. N. Hynes (Friedrich Miescher Institute, Basel, Switzerland).

Synthesis and Application of Oligonucleotides

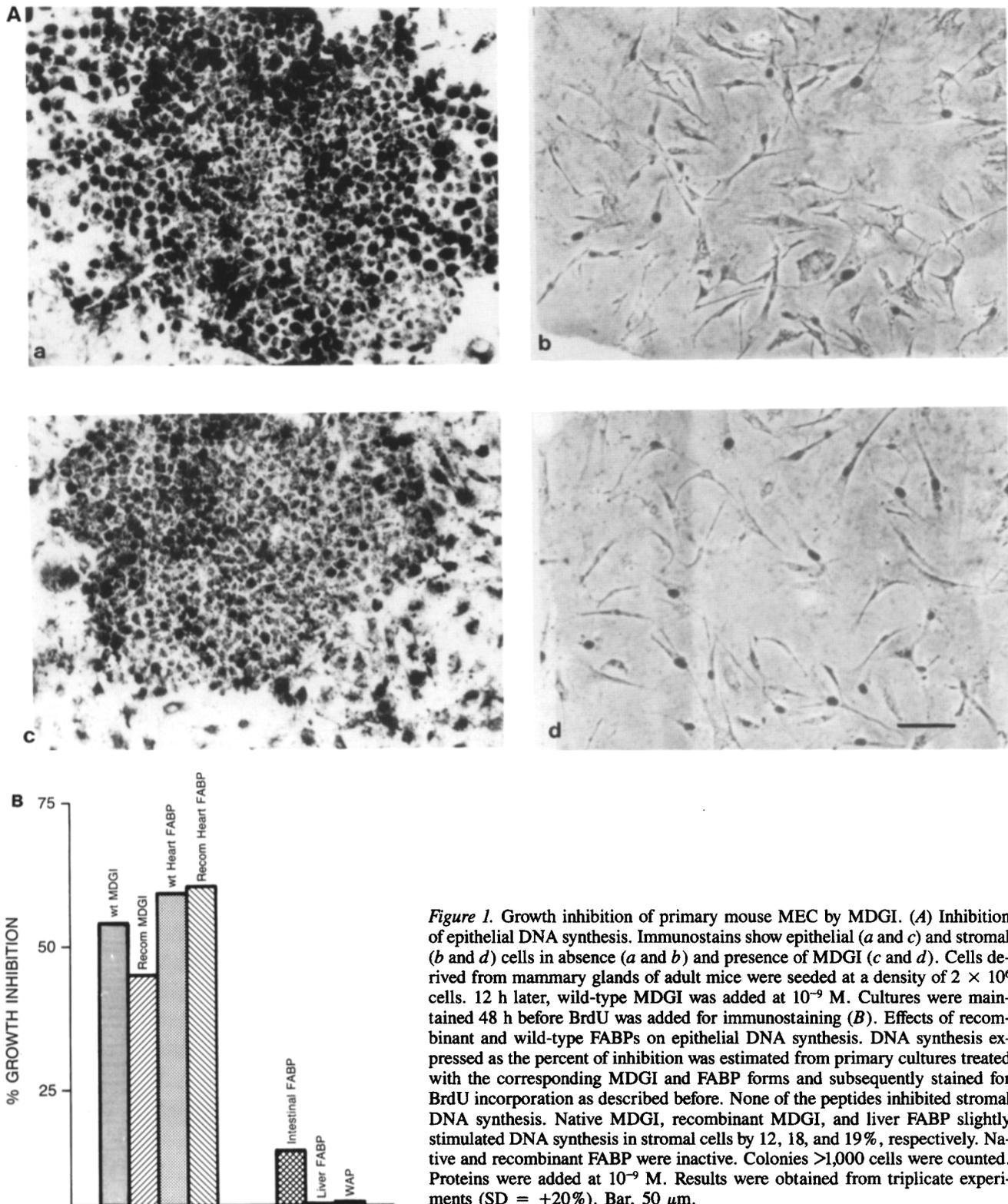The 17-mer phosphothioate oligonucleotides were synthesized and purified

Figure 1. Growth inhibition of primary mouse MEC by MDGI. (A) Inhibition of epithelial DNA synthesis. Immunostains show epithelial (a and c) and stromal (b and d) cells in absence (a and b) and presence of MDGI (c and d). Cells derived from mammary glands of adult mice were seeded at a density of 2 × 10^6 cells. 12 h later, wild-type MDGI was added at 10^{-9} M. Cultures were maintained 48 h before BrdU was added for immunostaining (B). Effects of recombinant and wild-type FABPs on epithelial DNA synthesis. DNA synthesis expressed as the percent of inhibition was estimated from primary cultures treated with the corresponding MDGI and FABP forms and subsequently stained for BrdU incorporation as described before. None of the peptides inhibited stromal DNA synthesis. Native MDGI, recombinant MDGI, and liver FABP slightly stimulated DNA synthesis in stromal cells by 12, 18, and 19%, respectively. Native and recombinant FABP were inactive. Colonies >1,000 cells were counted. Proteins were added at 10^{-9} M. Results were obtained from triplicate experiments (SD = ±20%). Bar, 50 μm.
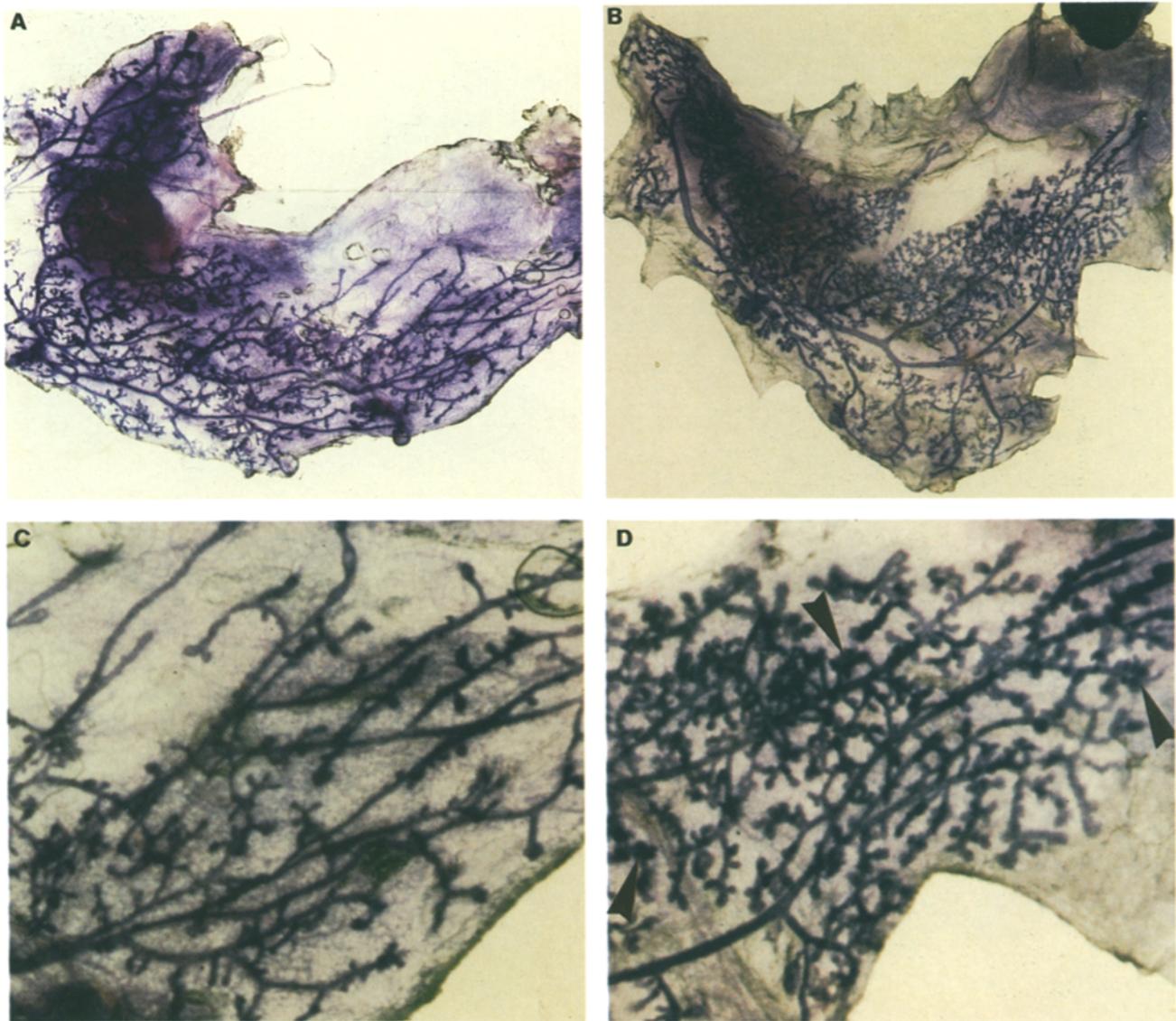
Figure 2. Effects of MDGI on mammary gland morphology and differentiation. Whole mouse mammary glands were cultured for 5 d in the APIH medium in the presence or absence of MDGI. Morphology: (*A* and *C*) Control contralateral gland. (*B* and *D*) Mammary glands treated with 10⁻⁹ M of wild-type MDGI (15 ng/ml). Same typical result was obtained with recombinant MDGI, and with recombinant or wild-type H-FABP. Arrows indicate alveolar buds formed in presence of MDGI. Histology: (*E*) Control. (*F*) MDGI treated. Sections of whole mammary glands stained with hematoxylin/eosin show upon MDGI treatment a secretory active and monolayered epithelium surrounding enlarged lumina. Note fat droplets accumulation in alveolar epithelial cells. Bar, 50 μm. β-casein immunostaining: (*G*) Control. (*H*) MDGI treated. MDGI immunostaining: (*I*) Control. (*J*) MDGI treated. Immunodetection was performed with a rabbit anti–mouse β-casein or anti-MDGI antibody visualized with a biotin/streptavidin detection system using diaminobenzidine as peroxidase substrate. Note marked β-casein expression in ductal and alveolar MEC, and strong luminal β-casein secretion if compared to MDGI. Bar, 100 μm.

by high-performance liquid chromatography (BioTez Berlin Buch GmbH). Sequences correspond to the mouse MDGI cDNA (6) with the translation start as first codon of the sense or the complementary antisense strand. The base composition of the random oligonucleotide was made identically to the antisense probe. The modified oligonucleotides were directly added to the medium on day 1 of the culture. Cultures received the oligonucleotides with every medium change.

Synthesis of Recombinant Proteins

Including microheterogeneities found upon protein sequencing in five positions (7), MDGI differs from the H-FABP sequence deduced from cDNA cloning or protein sequencing (5, 7, 38) in 10 positions of the amino acid sequence: 12S in MDGI/D in H-FABP, 14E/K, 40L/T, 36A/T, 43S/E,

85N/T, 88E/G, 93Q/H, 127V/T, and an additional 132A in H-FABP. Respective codons were successively exchanged in the H-FABP cDNA using the thiophosphate method to obtain the recombinant MDGI form. Double-stranded M13mpl8DNA was cut by KpnI and HindIII. The vector pET-BHFABP, which is a construct of the pET-3d (61) and of a NcoI/HindIII fragment of the H-FABP-cDNA, was used for cloning the MDGI cDNA into an expression vector. To this end, an NcoI/HindIII fragment of the unmutated H-FABP cDNA (derived from pET-BHFABP) was replaced by the respective MDGI fragment. The resulting expression vector was named pET-BHMDGI. To express proteins, the pRT-3d vector in BL21 (DE3) cells was used (61). Soluble recombinant proteins were harvested from supernatants and isolated by CM Sephadex C50 and Sephacryl S-100 HR, as described (5). All amino acid substitutions were confirmed by protein sequencing as described (5, 7). Recombinant forms for bovine liver FABP

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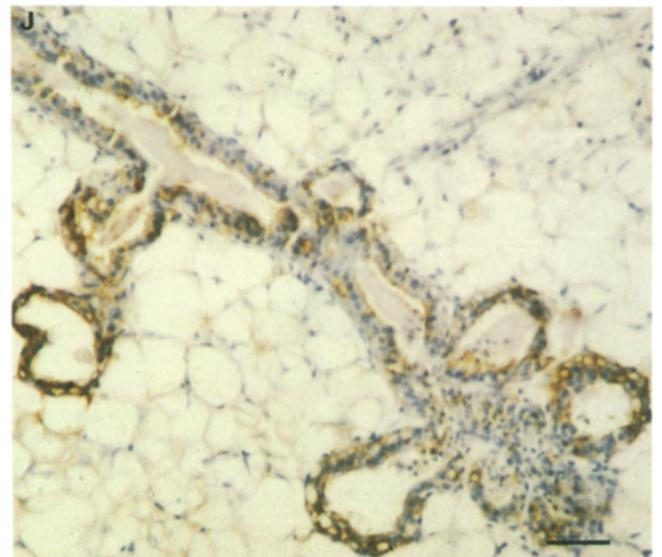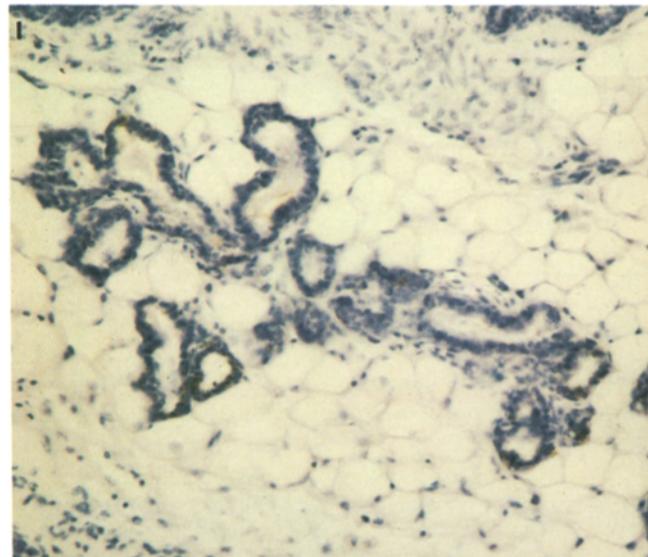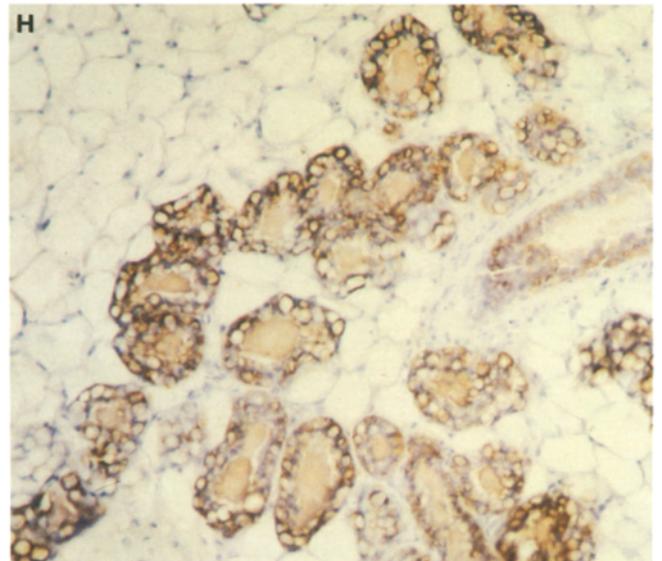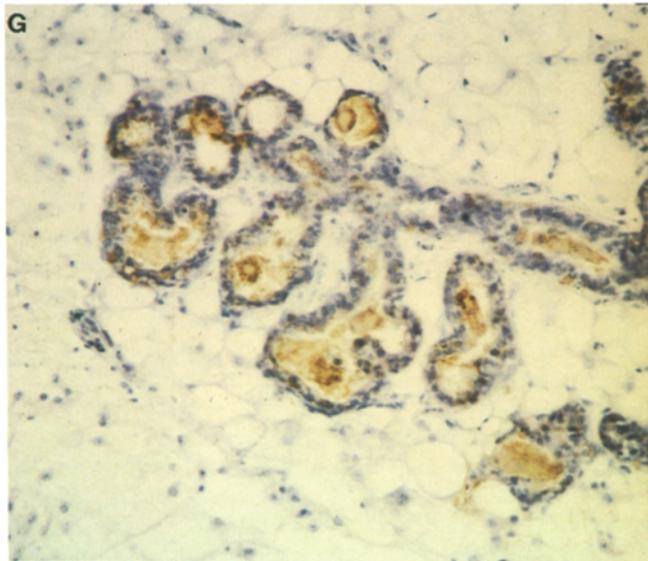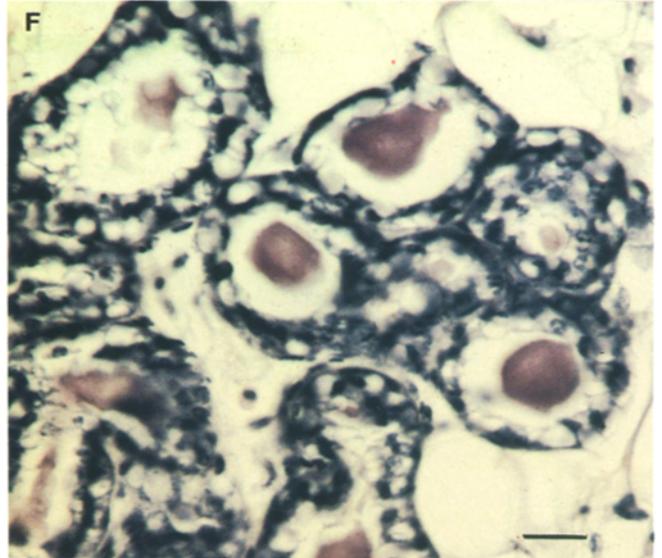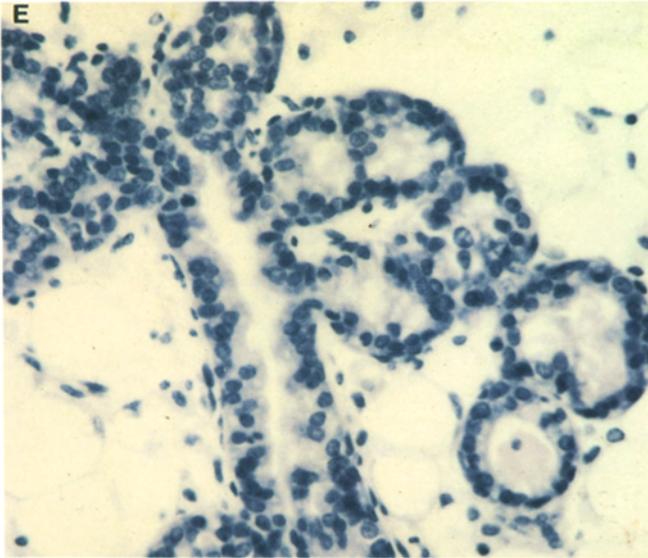
(L-FABP) and rat intestine FABP (I-FABP) were produced according to their sequences (68). The mutant human H-FABP carrying 106 Thr for Arg was prepared by site-directed mutagenesis using the uracil selection method (37).

Fatty Acid Binding

Fatty acid binding was measured at 2 μM oleic acid and 10 μg protein according to the lipidex method (22) as described by us earlier (9).

Peptide Synthesis

Synthesis of peptides with a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA), purification, and identification of purified peptides, has been described elsewhere (72).

Results

MDGI and Heart FABP Specifically Inhibit Epithelial DNA Synthesis

Protein sequencing showed coexistence in the bovine mammary gland of H-FABP and of MDGI, which differ from each other in 10 amino acids (7). To test whether MDGI and H-FABP both possess growth inhibitory activity and whether growth inhibition is specific for epithelial cells, the respective recombinant and wild-type proteins were produced, and were compared in activity in primary cultures of MEC (Figs. 1, A and B). As shown, wild-type MDGI at 10^{-9} M specifically inhibited DNA synthesis in MEC (Fig. 1 A c) if compared to the control (Fig. 1 A a), whereas growth of mammary stromal cells was unaffected (Fig. 1 A, b and d). Wild-type and recombinant MDGI- or H-FABP inhibited DNA synthesis of MEC by ~50-60% (Fig. 1 B). Half-maximal inhibition was obtained with ~10^{-10} M of the MDGI or H-FABP forms (not shown). Recombinant L- and I-FABP, which represent more distant members of the FABP family and share an overall sequence homology of 44-55% with MDGI (7), had no significant growth inhibitory effect (Fig. 1 B). Therefore, growth inhibition seems to be specific for MEC and for MDGI and the heart type of FABP.

MDGI Inhibits Ductal Growth and Promotes Functional Differentiation

To ascertain whether MDGI might regulate differentiation, whole-organ mammary glands that were derived from virgin mice were cultured for 5 d in a serum-free medium containing aldosterone, prolactin, insulin, and hydrocortisone (APIH medium) (Fig. 2). This hormone milieu results in complete lobuloalveolar development of the gland and terminates in functional differentiation. Under the same conditions, organ cultures were treated for 5 d with MDGI at 10^{-9} M. The presence of MDGI resulted in appearance of smaller ducts and ductules with numerous side branches (Fig. 2 B) when compared with the control (Fig. 2 A). H-FABP produced essentially the same morphological changes (not shown). Accordingly, when labeling indices were estimated in alveoli, ducts and ductules of MDGI- or H-FABP-treated mammary glands, strongest inhibition of DNA synthesis (~60% if compared to the untreated gland) was found in ductular MEC (Table I). In treated cultures, ductules were terminated by bulbous alveolar end buds (Fig. 2 D) when compared with the control gland (Fig. 2 C). The

Table I. Inhibition of Ductal DNA Synthesis by MDGI and Bovine H-FABP

Protein	Inhibition of DNA Synthesis (%)			
	Alveoli	Ducts	Ductules	Overall
wt. MDGI	9 ± 2	34 ± 4	61 ± 6	45 ± 5
wt. H-FABP	13 ± 2	33 ± 4	57 ± 4	46 ± 4

Overall epithelial cell inhibition of DNA synthesis induced by the recombinant forms of MDGI, human H-FABP, and human mutated H-FABP (106Thr for 106Arg) amounted to 49 ± 5, 40 ± 4, 46 ± 4, and 53 ± 5%, respectively. Oleic acid binding activities for wild-type and mutant human H-FABP amounted to 25.7 and 2.0 nmol/mg protein, respectively. For estimation of DNA synthesis, mammary glands were cultured in the APIH medium for 3 d. Epithelial cell inhibition of DNA synthesis was determined by labeling cells with BrdU for 4 h before cultures were terminated. In controls, ~16-20% of epithelial cells were BrdU positive. Histologic criteria were as follows: ducts, an adjacent layer of connective tissue; ductules, a linear coat of cubic epithelial cells without adjacent mesenchyme; alveoli, presence of secretory cells with vacuoles and luminal secretion.

alveolar end buds represent a developmental pathway that eventually leads to secretory alveoli at functional differentiation. Indeed, histological examination of MDGI-treated glands revealed the appearance of mono-layered secretory active alveoli with enlarged luminal spaces (Fig. 2, E and F). In accord with these changes, which are characteristic for the differentiated phenotype, functional differentiation evidenced by β-casein expression was stimulated (Fig. 2, G and H). Intracellular and luminal β-casein levels, which can be considered to indicate an earlier period of functional differentiation, were elevated (Fig. 2 H). MDGI treatment produced an autostimulation of its mRNA expression (Table II). Correspondingly, a clear increase in endogenous MDGI production was detected in MEC and, to a much less extent, in the luminal spaces (Figs. 2, I and J). The striking differences between the pattern of the β-casein and MDGI expression exactly reflect the situation we have described for the developing mammary gland (6). Substantiation that MDGI, as well as the human and bovine forms of H-FABP, might function as differentiation factors in the mammary gland was assessed by quantifying "early" and "late" markers of functional differentiation such as β-casein and WAP expression, fat droplet accumulation, and secretory activity (Table II). As shown, MDGI and H-FABP elevated the levels of markers for functional differentiation up to threefold.

Inhibition of MDGI Expression Suppresses Alveolar Differentiation and β-Casein Expression

In mammary glands of primed virgin mice, the MDGI gene is not expressed; however, if these glands are taken in culture in presence of APIH, MDGI expression can be first detected after 3 d (6). Hormonally induced MDGI expression, which reaches some maximum after 8 d (6), follows closely the pattern that has been found during pregnancy (6, 38). This finding argues in favour of a local role for endogenous MDGI during growth and differentiation, and we have therefore tested whether MDGI expression is a prerequisite for normal development. To this end, antisense phosphothioate oligonucleotides complementary to position 1-17 (relative to the translation initiation side) of the murine MDGI coding region (6) (as-oligonucleotide) were added on day 1 to the

Table II. Stimulation of Functional Differentiation by MDGI and H-FABP

Protein	mRNA Levels			Fat droplet index	Alveolar secretion
	β-casein	WAP	MDGI		
		(%)		(%)	(%)
wt. MDGI	295 ± 12	275 ± 10	295 ± 10	179 ± 21	153 ± 18
wt. bovine H-FABP	280 ± 10	287 ± 15	267 ± 12	195 ± 19	157 ± 17
Rec. human H-FABP	241 ± 4	251 ± 9	255 ± 10	218 ± 13	271 ± 22
Rec. human mut. H-FABP	251 ± 6	260 ± 7	282 ± 9	210 ± 10	248 ± 13

Mammary glands were cultured for 5 d in the APIH medium. Levels are expressed as percent of the RNA level in the control representing the contralateral gland. Three pairs of contralateral glands were examined. For definition of morphometric parameters, see Materials and Methods.

APIH-maintained whole organ cultures. Controls included the sense (s) and one random (r) oligonucleotide. As shown in Fig. 3 *B*, a 5-d treatment with the as-oligonucleotide almost completely blocked MDGI expression in MEC if compared with the untreated control gland (Fig. 3 *A*). Whole-mount stains of two pairs of contralateral glands (Fig. 3, *C* and *D*) demonstrate the typical morphological effect observed upon as-oligonucleotide treatment. Glands placed on the right side of Fig. 3, *C* and *D*, received as-oligonucleotide, whereas left glands represent the respective control glands. As can be seen, inhibition of MDGI expression leads to prevention of alveolar end bud formation and to the appearance of glands that seem to be retarded in development. The two other control glands, which received the s-oligonucleotide (Fig. 3 *E*, *right gland*) or r-oligonucleotide (Fig. 3 *F*, *right side*) show the same normal development as the corresponding untreated glands, depicted on the left side in Figs. 3, *E* and *F*. As could be expected, the appearance of less developed glands was accompanied by a fourfold increase in the labeling index of ductal and ductular epithelial cells (not shown). In accord with impaired alveolar growth shown in Fig. 3, *C* and *D*, we found profoundly decreased β-casein levels in as-oligonucleotide–treated glands (Fig. 3 *H*), if compared to the normal control (Fig. 3 *G*). This was particularly evident for the luminal species of alveoli, compared to the intracellular β-casein level, which was somewhat less affected.

MDGI and EGF Are Functional Antagonists

We showed recently that EGF strongly suppresses MDGI mRNA expression and prevents differentiation in explant cultures derived from virgin or pregnant mouse mammary glands (6, 59). It was, therefore, obvious to ascertain whether EGF and MDGI might functionally interact during growth and differentiation. To this end, organ cultures were treated for 5 d with both factors in a concentration range of 1–3 nM (Fig. 4). As shown, EGF exerts mitogenic activities and suppresses the mRNA levels for WAP, β-casein, and MDGI. The effects of EGF on growth and differentiation were suppressed with increasing MDGI concentrations. MDGI at 3 nM completely prevented activities of 1 nM EGF. On the other side, stimulation of functional differentiation by MDGI could be strongly reduced in presence of 3 nM EGF. In conclusion, increasing the EGF or the MDGI concentrations was mutually antagonistic.

The Activities of MDGI Do Not Depend on Fatty Acid Binding and Can Be Mimicked by an 11–Amino Acid Sequence Represented in the COOH terminus of MDGI and Related FABPs

The physiological role of retinoids, eicosanoids, or long-chain fatty acids endogenously bound to FABPs is unclear. We, therefore, tested whether bound fatty acids could be directly involved in the biological activities. The first approach was to produce a recombinant FABP form that cannot bind fatty acid. Replacement of 106 Arg by Thr in human H-FABP abolished the capacity of H-FABP to bind oleic acid, however, did not affect its growth inhibitory (Table I) or differentiation stimulating (Table II) activities. This finding supports a mechanism not involving bound fatty acids. Direct evidence was obtained for a functional domain in MDGI that does not require lipid binding by testing various synthetic peptides of the COOH-terminal MDGI sequence. By comparing FABP sequences encoded by exon 4, a striking homology was detected for a COOH-terminal stretch of 11 amino acids (designated as P108) (Fig. 5 *A*). Notably, we have shown earlier that P108 does not bind fatty acids (72). P108 at 10^{-10} M inhibited DNA synthesis of primary MEC by 55% (not shown), and was therefore further tested in the organ culture system under conditions as described before for MDGI (Fig. 5 *B*, *a–d*). Analogous to MDGI, treatment of APIH-cultured mammary glands for 5 d with 10^{-9} M P108 affected ductal branching and caused appearance of more bulbous alveolar end buds (Fig. 5 *B*, *a* and *b*). In accordance with the morphological changes that suggest suppression of ductal growth by P108, we found that after 3 d, DNA synthesis in MEC was inhibited by ∼60% (Fig. 5 *C*). Prolonged treatment for 5 d of the mammary glands with 10^{-9} M P108 resulted in the appearance of differentiated epithelium characterized by monolayered, secretory active alveoli (Fig. 5 *B*, *c* and *d*). In parallel, and similarly to MDGI β-casein synthesis was clearly stimulated by P108 (not shown). The growth inhibitory and differentiation stimulating activities of P108 are summarized in Fig. 5 *C*. As shown, the 11 COOH-terminal residues of MDGI are sufficient to fully mimic the activities of MDGI, including stimulation of β-casein and WAP expression, as well as autostimulation of endogenous MDGI gene expression. Single replacements of 130Lys by Asn or of 128Tyr by Ala abolished the activities of P108. Two other peptides including a shorter sequence of P108 (residues 126-130), representing a functional domain of growth hormone (42), were inactive.
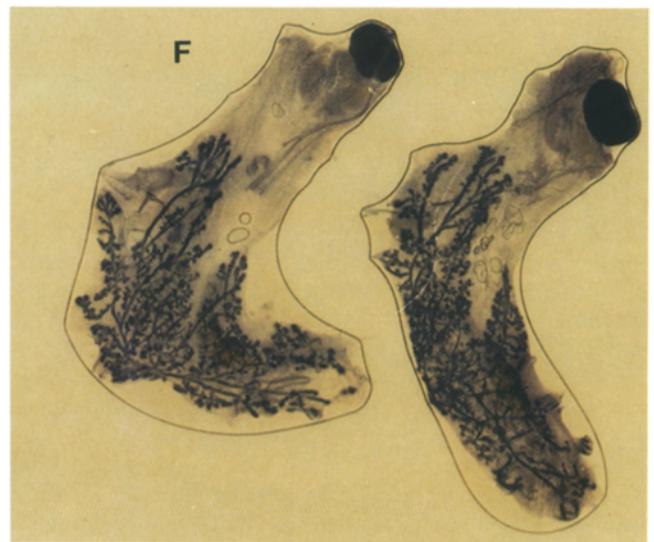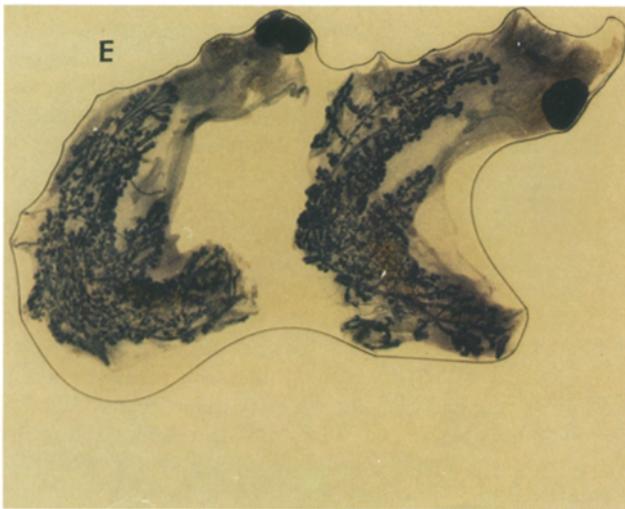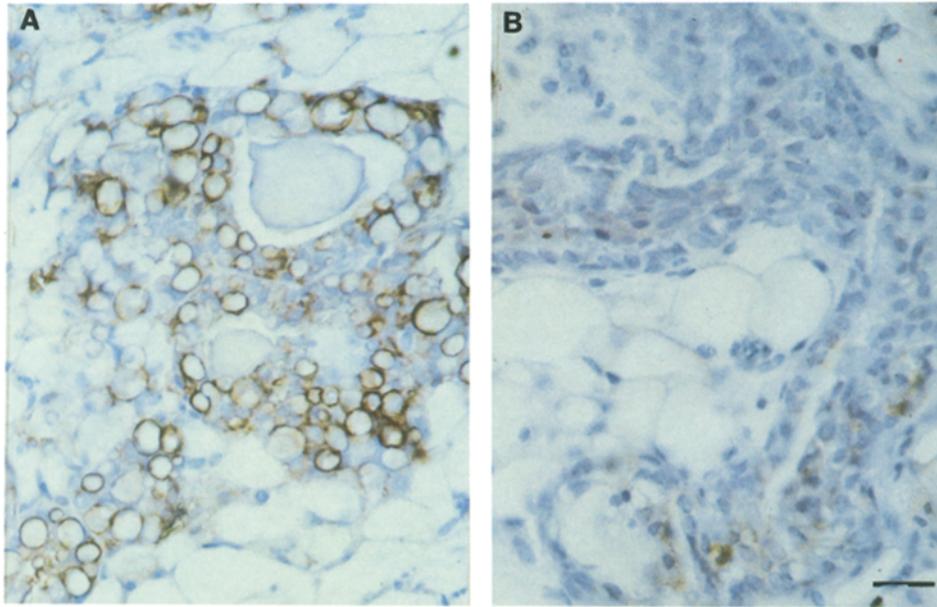
Figure 3. Effect of inhibition of MDGI expression on differentiation. Whole organ cultures of mammary glands maintained for 5 d in the APIH medium received 10 μM of a modified antisense oligonucleotide complementary to position 1-17 of the MDGI encoding region. Immunostains for MDGI show inhibition of MDGI expression (*B*) if compared with the control contralateral gland (*A*). Bar, 50 μm. Whole mount stains of mammary glands treated with the antisense (as) oligonucleotide (glands shown on the right side of *C* and *D*) show the
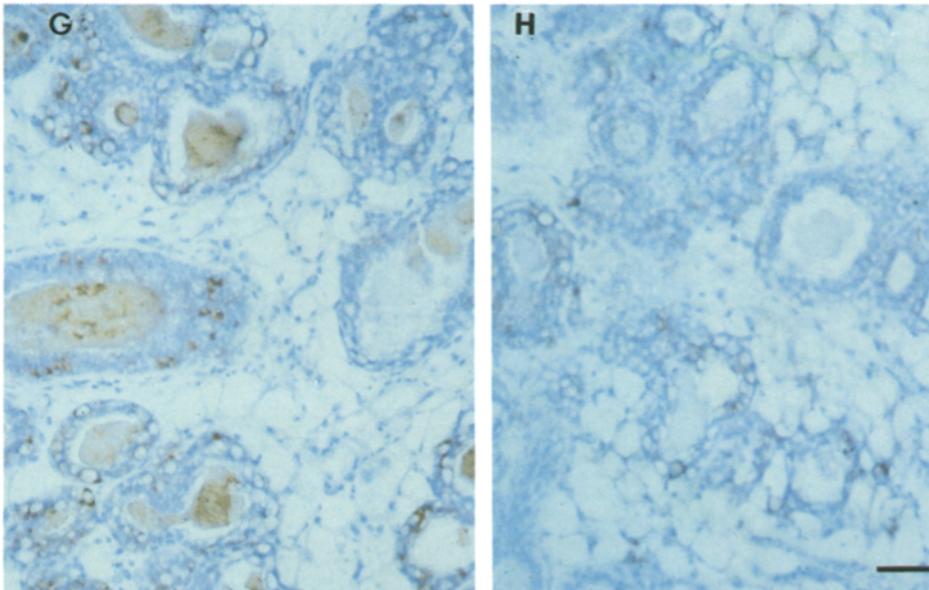
Figure 3.

Discussion

We demonstrate here for the first time that a polypeptide growth inhibitor promotes morphological and functional differentiation in the developing mammary gland. The in vitro model we have been using ensures to follow ductal growth and branching, lobuloalveolar development, and functional differentiation, i.e., developmental stages normally occurring during pregnancy. We were, therefore, able to test for both growth and differentiation activities of MDGI. It turned out that MDGI, which is expressed in the mammary gland in a developmentally and hormonally regulated manner (6, 38, 43), specifically inhibits epithelial DNA

synthesis, promotes formation of alveolar buds, supports development of differentiated lobuloalveoli, and stimulates milk protein synthesis and fat droplet accumulation. This means that MDGI stimulates both "early" and "late" phases (more characterized by fat droplet accumulation) of functional differentiation.

These are all the properties that distinguish MDGI from TGF-β1, which strongly inhibits ductal outgrowth in virgin mice; however, it does not affect epithelial growth during later stages of development like pregnancy. Moreover, contrary to MDGI, TGF-β1 does impair functional differentiation in vitro (41, 50) and in vivo (34). Therefore, MDGI can
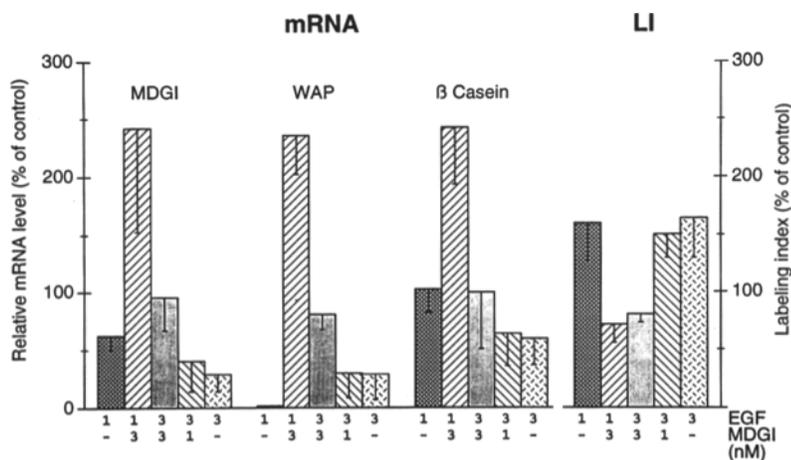

Figure 4. Functional interaction between MDGI and EGF. Mammary glands were cultured for 5 d in the presence of APIH. MDGI and EGF were additionally present at the indicated concentrations for 5 d. Overall epithelial DNA synthesis expressed as labeling index (*LI*) was determined by labeling cells with BrdU for 4 h before cultures were terminated, and mRNAs levels were estimated from Northern blots hybridized with ^{32}P-labeled probes as outlined before. Values are expressed as percent of the control representing the contralateral gland. Three pairs of contralateral glands were examined.

effect on lobuloalveolar development in comparison to the untreated control gland, placed on the left side. Shown are two typical pairs of mammary glands. Note almost complete regression of alveolar end buds in glands treated with the as-oligonucleotide. *E* and *F* represent control glands treated for 5 d with the sense probe (*E, gland on right*) or with the random oligonucleotide (*F, right side*), while the respective untreated contralateral glands are shown on the left side. (*G* and *H*) Sections immunostained for β-casein expression derived either from the gland treated with the as-oligonucleotide (*H*) or from the untreated contralateral gland (*G*). Note preferential reduction of luminal immunostaining. Bar, 100 μm.
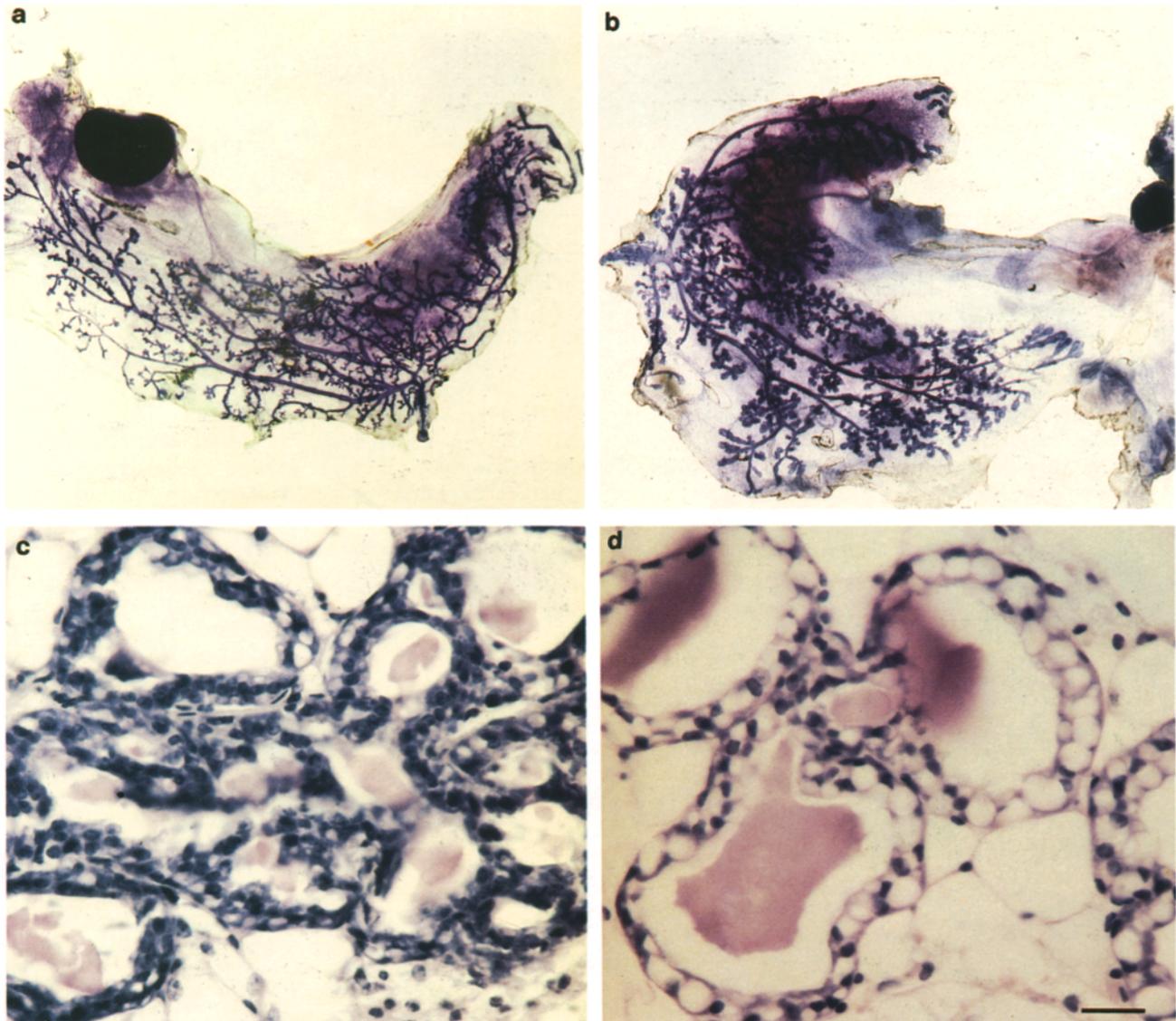
Figure 5. Effects of P108 on growth and differentiation of the mammary gland in organ culture. (*A*) Comparison of the COOH-terminal sequence of bovine MDGI (7) with sequences of bovine brain FABP (52), bovine heart FABP (5), bovine myelin P2 (68), mouse P422 (68), and bovine CRABP (68). Areas containing identical or conservatively exchanged residues are shadowed. Residues 126-131 are identical to residues 108-112 of bovine growth hormone (42), and they are bordered by two highly conserved positively charged amino acids. Biologically active P108 comprises residues 121-131 in which cysteine has been replaced by serine to prevent dimerization and inactivation (72). Numbering is according to the MDGI sequence in (7). For comparison, sequences of inactive bovine L-FABP and rat I-FABP are included. (*B*) Effects of P108 on morphology and histology. Glands were cultured for 5 d in APIH medium in the absence (*a* and *c*) or
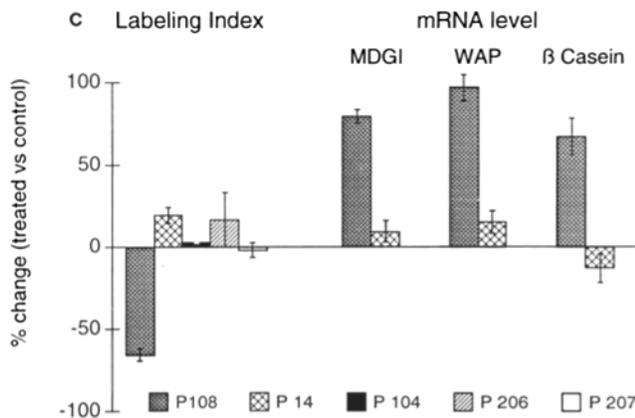
Figure 5.

be considered as a unique and novel differentiation factor for the mammary gland.

Functionally, MDGI and the bovine and human forms of H-FABP can not be distinguished. Considering that at least one other member of the family, the brain FABP, has been described to be identical with MDGI (52), one can assume a role for these proteins in tissue differentiation. This assumption concurs with data from an earlier study (73), where we have shown that teratocarcinomas formed in syngenic mice after injection of pluripotent embryonic stem cells treated with MDGI or P108 contain more differentiated neural tissue.

Interestingly, an immunohistochemical analysis of MDGI and H-FABP expression shows immunoreactivity exclusively in postmitotic, terminally differentiated cells of the myocard, skeletal muscle, and surface epithelial cells of intestinal and respiratory tracts (Zschiesche, W., A. Kleine, J. H. Veerkamp, and J. Glatz, manuscript submitted for publication). The developmental, cell and tissue specific expression of various other FABPs (14, 15, 28, 30) indeed seems to support a more general role for these proteins in growth and differentiation similarly to that described here for MDGI.

MDGI expression can be hormonally induced in organ culture and its synthesis is further stimulated when MDGI or P108 were added. To test for a relationship between endogenous MDGI expression and a function of MDGI during development, we have inhibited MDGI expression in MEC. Our findings about a suppression of alveolar growth and β-casein synthesis might indicate an intracellular mode of action and again indicate to MDGI as a differentiation factor. This is in correspondence with the specific pattern of gene expression during pregnancy showing MDGI transcripts preferentially in alveolar cells adjacent to the surrounding stroma (38). In contrast to the situation in organ cultures, MDGI can not be induced in MEC grown as monolayers, in-

cluding responsive to lactogenic hormones murine cell lines (unpublished data). Single MEC provided with an exogenous basement membrane that can be induced to express β-casein also do not express MDGI unless they form multicellular structures with luminal spaces (unpublished data). These data and our present findings strongly argue in favor of a role for MDGI at a distinct state of development that is characterized by a decline of ductal growth and is associated with lobuloalveolar morphogenesis and functional differentiation.

It has been recently reported about a structural identity between MDGI and a cysteine protease inhibitor specific for papain, cathepsin B and cathepsin L (74). It is tempting to speculate that the high level of MDGI expression found in the lactating mammary gland and in milk (12), suggests a different function which might rather be associated with lipid transport and metabolism, tissue remodeling, or with preserving milk protein products. Our findings about a dominant presence of MDGI in cells with high steroid metabolism such as testicular Leydig' cells, adrenal cells, or cells of the corpus luteum, might indicate future directions of work (Zschiesche, W., A. Kleine, J. H. Veerkamp, and J. Glatz, manuscript submitted for publication):

In breast cancer cells, the antiproliferative activity of MDGI can be antagonized by EGF in a dose-dependent manner (10, 23). EGF and TGF-α strongly suppress MDGI mRNA expression in explant and organ cultures of pregnant or virgin mice (6, 59). On the other hand, several lines of indirect evidence indicate that EGF might have a role in differentiation. The levels of EGF in plasma (46) and its receptor in the mammary gland (20) increase during pregnancy. Both EGF and TGF-α have been detected in the mammary epithelium of pregnant mice (55, 59). However, conflicting reports about EGF or TGF-α activities demonstrated both differentiation-inhibiting (31, 64, 71) and -stimulating activities (71). According to our data, EGF is able to produce a growth stimulatory effect on MEC under conditions of functional differentiation, provided that MDGI was absent or added at low concentrations. The data show that increasing the EGF or MDGI concentration was mutually antagonsitic. We have shown earlier that EGF suppresses MDGI mRNA expression (6), suggesting that under physiological conditions, concentration of both factors might be critical for maintaining balanced growth and differentiation. In early pregnancy, when EGF and TGF-α are present, they could suppress MDGI overexpression, thus preventing impaired growth and differentiation of the mammary gland. Likewise, MDGI could regulate EGF activities to maintain normal differentiation.

Our findings about growth inhibitory and differentiation promoting activities of human and bovine H-FABP provide first direct evidence for a regulatory function of members of this conservative multigene family. Based on expression data, it has been suggested that some FABPs might have

in the presence (*b* and *d*) of 10^{-9} M of the synthetic peptide P108. Whole mount stain of P108 treated gland shows bulbous alveolar end buds (*b*). Histology shows monolayered secretory active epithelium upon peptide treatment (*d*). Bar, 50 μm. (*C*) Peptide P108 fully mimics growth and differentiation properties of MDGI. Whole mammary glands were cultured for 3 d to determine the labeling index in MEC, or for 5 d (to estimate the mRNA levels for MDGI, WAP and β-casein) in the APIH medium in presence of 1 nM of the different peptides. To verify specificity of P108, LIs were determined with modified peptides: 130Lys→130Asn, designated as P206; 128Tyr→128Ala, designated as P207. In addition, amino acids 69-78 and 126-130 of the MDGI sequence are represented by P14 and P104, respectively.

some function in cellular growth, differentiation, and signal transduction (21, 69). For the I-FABP, endogenous expression in cryptal cells of the intestinum is strictly associated with inhibition of DNA synthesis (15). However, a dominant expression in more differentiated cells described for the I-FABP (15), the adipocyte FABP, (also referred to as ap2 or P422 [57]), or for the CRABP (14) has not been directly related to growth inhibitory activity. Unexpectedly, transfection of liver cDNA into hepatoma cells (36) or fibroblasts (33) cause these cells to grow in response to linoleic acid and to reach higher cell densities, respectively. Overexpressing CRABP-1 in F9 teratocarcinoma cells resulted in reduction of differentiation (11). However, there are no data available to show the consequences of overexpression of FABPs into normal cells, which can be induced to undergo differentiation in vitro. We report here that P108, which represents the COOH terminus of MDGI, fully mimics the MDGI properties. This peptide has a strong homology with the COOH terminus of other FABPs, including myelin P2, which is expressed during differentiation of Schwann's cells (28), with CRABP and brain FABP. P108 does not bind fatty acids (72) rendering involvement of endogenously bound ligands in the activities we described here very unlikely. Rather, our data argue in favor of a mechanism implying interaction of P108 with cellular targets related to growth signaling pathways. For the CRABP, a function in signaling pathways has been suggested (19).

Although the molecular mechanism(s) by which MDGI or P108 inhibit growth and promote differentiation are not yet known, preliminary data indicate steps likely to be involved. We have shown that P108 over a concentration range of 10^{-10} to 10^{-12} M prevents arachidonic acid or 15S-hydroxyeicosatetraenoic acid–induced hypersensitivity of neonatal rat heart cells to β_2-adrenergic stimulation (72). From these studies, we concluded that a mechanism of interference of MDGI or P108 subsequent to generation of 15S-hydroxyeicosatetraenoic acid is involved. Among the different peptides tested, only P108 turned out to be active in MEC and heart myocytes. Therefore, it seems possible that the growth modulatory effects of MDGI and of P108 may result from an interference with some pathway subsequent to the generation of lipoxygenase products. For TNF (25), IFN-α (26), and TGF-β (45), involvement of the arachidonic acid pathway has been documented.

References

1. Banarjee, M. R., and M. Antoniou. 1985. Steroid and polypeptide hormone interaction in milk protein gene expression. *In* Biochemical Actions of Hormones. G. Litwack, editor. Academic Press, New York. pp. 237–289.
2. Banarjee, M., B. Wood, and D. Kinder. 1973. Whole mammary gland organ culture: selection of appropriate gland. *In Vitro Cell. Dev. Biol.* 9:129–133.
3. Bandyopadyay, G., S. Hwang, W. Imagawa, and S. Nandi. 1993. Role of polyunsaturated fatty acids as signal transducers. Amplification of signals from growth factor receptors by fatty acids in mammary epithelial cells. *Prostaglandins Leukotrienes Essent. Fatty Acids.* 48:71–78.
4. Bansal, M. P., R. G. Cook, K. G. Danielson, and D. Medina. 1989. A 14-kilodalton selenium binding protein in mouse liver is a fatty acid binding

protein. *J. Biol. Chem.* 264:13780–13784.
5. Billich, S., T. Wissel, H. Kratzin, U. Hahn, B. Hagendorff, A. Lezius, and F. Spener. 1988. Cloning of a full-length complementary DNA for fatty acid binding protein from bovine heart. *Eur. J. Biochem.* 175:549–556.
6. Binas, B., E. Spitzer, W. Zschiesche, B. Erdmann, A. Kurtz, T. Müller, C. Nieman, W. Blenau, and R. Grosse. 1992. Hormonal induction of functional differentiation and mammary derived growth inhibitor expression in cultured mouse mammary gland explants. *In Vitro Cell. Dev. Biol.* 28A:625–634.
7. Böhmer, F.-D., R. Kraft, A. Otto, C. Wernstedt, A. Kurtz, T. Müller, K. Rohde, G. Etzold, P. Langen, C.-H. Heldin, and R. Grosse. 1987. Identification of a polypeptide growth inhibitor from bovine mammary gland. MDGI sequence homology to fatty acid and retinoid binding proteins. *J. Biol. Chem.* 262:15137–15143.
8. Böhmer, F.-D., W. Lehmann, H. Schmidt, P. Langen, and R. Grosse. 1984. Purification of a growth inhibitor for Ehrlich ascites mammary carcinoma cells from bovine mammary gland. *Exp. Cell Res.* 150:466–476.
9. Böhmer, F.-D., M. Mieth, G. Reichmann, C. Taube, R. Grosse, and M. D. Hollenberg. 1988. A polypeptide growth inhibitor isolated from lactating bovine mammary gland (MDGI) is a lipid carrying protein. *J. Cell Biochem.* 38:199–204.
10. Böhmer, F.-D., W. Lehmann, F. Noll, R. Samtleben, P. Langen, and R. Grosse. 1985. Specific neutralizing antiserum against a polypeptide growth inhibitor for mammary cells purified from bovine mammary gland. *Biochim. Biophys. Acta.* 846:145–154.
11. Boylan, J. F., and L. J. Gudas. 1991. Overexpression of the cellular retinoic acid binding protein-I (CRABP-I) results in a reduction in differentiation-specific gene expression in F9 teratocarcinoma cells. *J. Cell Biol.* 112:965–979.
12. Brandt, R., M. Pepperle, A. Otto, R. Kraft, F.-D. Böhmer, and R. Grosse. 1988. A 13-kD protein purified from milk fat globule membranes is closely related to mammary derived growth inhibitor. *Biochemistry.* 27:1420–1425.
13. Brandt, R., and R. Grosse. 1992. Purification of a mammary-derived growth inhibitor related polypeptide expressed during pregnancy. *Biochem. Biophys. Res. Commun.* 189:406–413.
14. Busch, C., P. Sakena, K. Funa, H. Nordlinder, and U. Erikson. 1990. Tissue distribution of cellular retinol-binding protein and cellular retinoic acid-binding protein: use of monospecific antibodies for immunohistochemistry and cRNA for in situ localization of mRNA. *Methods Enzymol.* 189:315–324.
15. Cohn, S., T. Simon, K. Roth, E. Birkenmeier, and J. Gordon. 1992. Use of transgenic mice to map cis-acting elements in the intestinal fatty acid binding protein gene (FABPi) that controls its cell lineage-specific and regional patterns of expression along the duodenal-colonic ad crypt-villus axes of the gut epithelium. *J. Cell Biol.* 119:27–44.
16. Coleman, S., G. Silberstein, and C. Daniel. 1988. Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor. *Dev. Biol.* 127:304–315.
17. Daniel, C., and G. Silberstein. 1987. Postnatal development of the rodent mammary gland. *In* The Mammary Gland 1: Development, Regulation and Function. M. Neville and C. Daniel, editors. Plenum Publishing Corp., New York. pp. 3–36.
18. Dutta-Roy, A., N. Gopalswamy, and D. Trulzsch. 1987. Prostaglandin E1 binds to Z protein of rat liver. *Eur. J. Biochem.* 162:615–619.
19. Durand, B., M. Sanders, P. Lervy, M. Leid, and P. Chabon. 1992. All-trans and 9-cis retinoic acid induction of CRABP II transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. *Cell.* 71:73–85.
20. Edery, M., K. Pang, L. Larson, T. Colosi, and S. Nandi. 1985. Epidermal growth factor receptor levels in mouse mammary glands in various physiological states. *Endocrinology.* 117:405–411.
21. Glatz, J. F. C., M. Vork, D. Cistola, and G. van der Vusse. 1993. Cytolplasmic fatty acid binding proteins: significance for intracellular transport of fatty acids and putative role on signal transduction pathways. *Prostaglandins Leukotrienes Essent. Fatty Acids.* 48:33–41.
22. Glatz, J. F. C., C. Baerwaldt, J. H. Veerkamp, and H. J. Kempen. 1984. Diurnal variation of cytosolic fatty-acid binding protein content and of palmitate oxidation in rat liver and heart. *J. Biol. Chem.* 259:4295–4300.
23. Grosse, R., and P. Langen. 1990. Mammary derived growth inhibitor. *In* Handbook of Experimental Pharmacology 95/II. M. Sporn and A. Roberts, editors. Springer Verlag, Heidelberg. pp. 249–265.
24. Grosse, R., F.-D. Böhmer, B. Binas, A. Kurtz, E. Spitzer, T. Müller, and W. Zschiesche. 1992. Mammary derived growth inhibitor. *In* Cancer Treatment and Research. Genes Oncogenes and Hormones. R. B. Dickson and M. Lippman, editors. Kluwer Academic Press, Boston. pp. 69–97.
25. Haliday, E., C. Rameshda, and G. Ringold. 1991. TNF induces c-fos via a novel pathway requiring conversion of arachidonic acid to a lipoxygenase metabolite. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:109–115.
26. Hannigan, G., and B. Williams. 1991. Signal transduction by interferon alpha through arachidonic acid metabolism. *Science (Wash. DC).* 205:204–206.
27. Haslam, S., L. Counterman, and K. Nummy. 1993. Effects of epidermal growth factor, estrogen, and progestin on DNA synthesis in mammary

cells in vivo are determined by the developmental state of the gland. *J. Cell. Physiol.* 155:72–78.

28. Hayasaka, K., K. Nano, M. Tahara, and W. Sato. 1991. Isolation and sequence determination of cDNA encoding P2 protein of human peripheral myelin. *Biochem. Biophys. Res. Commun.* 181:204–207.

29. Howlett, A., and M. Bissel. 1993. The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. *Epithel. Cell Biol.* 2:79–89.

30. Hunt, C.R., J. Ro, D. Dobson, H. Min, and H. Spiegelman. 1986. Adipocyte P2 gene: developmental expression and homology of 5′-flanking sequences among fat cell-specific genes. *Proc. Natl. Acad. Sci. USA.* 83:3786–3790.

31. Hynes, N., D. Taverna, I. Harweth, F. Ciardiello, D. Salomon, T. Yamamoto, B. Groner. 1990. Epidermal growth factor receptor, but not c-erbB-2, activation prevents lactogenic induction of the β-casein gene in mouse mammary epithelial cells. *Mol. Cell. Biol.* 10:4427–4434.

32. Imagawa, W., G. Bandyopadhyay, and S. Nandi. 1990. Regulation of mammary epithelial cell growth in mice and rats. *Endocrinol. Rev.* 11:494–523.

33. Jefferson, J., J. Slotte, G. Nemecz, A. Pastuszyn, T. Scallen, and F. Schroeder. 1991. Intracellular sterol distribution in transfected mouse L cell fibroblasts expressing rat liver fatty acid binding protein. *J. Biol. Chem.* 266:5486–5496.

34. Jhappan, C., A. Geiser, E. Kordon, D. Bagheri, L. Henninghausen, A. Roberts, G. Smith, and G. Merlino. 1993. Targeting of a transforming growth factor beta1 transgene to the pregnant mammary gland inhibits alveolar development. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1835–1845.

35. Juergens, W., F. Stockdale, and Y. Topper. 1965. Hormone dependent differentiation of mammary gland in vitro. *Proc. Natl. Acad. Sci. USA.* 54:629–634.

36. Keler, T., C. Barker, and S. Sorof. 1992. Specific growth stimulation by linoleic acid in hepatoma cell lines transfected with the target protein of a liver carcinogen. *Proc. Natl. Acad. Sci. USA.* 89:4830–4834.

37. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzym.* 154:367–382.

38. Kurtz, A., F. Vogel, K. Funa, C.-H. Heldin, and R. Grosse. 1990. Developmental regulation of mammary derived growth inhibitor expression in bovine mammary tissue. *J. Cell Biol.* 110:1779–1789.

39. Li, M., D. Aggeler, D. Farson, C. Hatier, and M. Bissel. 1987. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci. USA.* 84:136–140.

40. Matarese, V., R. Stone, D. Waggoner, and D. Bernlohr. 1989. Intracellular fatty acid trafficking and the role of cytosolic lipid binding proteins. *Prog. Lipid Res.* 28:245–272.

41. Mieth, M., F. Böhmer, R. Ball, B. Groner, and R. Grosse. 1990. Transforming growth factor beta inhibits lactogenic hormone induction of beta-casein expression in HC 11 mouse mammary epithelial cells. *Growth Factors.* 4:9–15.

42. Miller, W., J.A. Martial, and J. Baxter. 1980. Molecular cloning of DNA complementary to bovine growth hormone. *J. Biol. Chem.* 255:7512–7524.

43. Mueller, T., A. Kurtz, F. Vogel, H. Breter, F. Schneider, U. Engstroem, M. Mieth, D.-D. Boehmer, and R. Grosse. 1989. A mammary derived growth inhibitor (MDGI) related 70-kD antigen identified in nuclei of mammary epithelial cells. *J. Cell. Physiol.* 138:415–423.

44. Nandi, S. 1958. Endocrine control of mammary gland development and function in the C3 11/HE Crgl mouse. *J. Natl. Cancer Inst.* 21:1039–1062.

45. Newman, M. 1990. Inhibition of carcinoma and melanoma cell growth by type 1 transforming growth factor is dependent on the presence of polyunsaturated fatty acids. *Proc. Natl. Acad. Sci. USA.* 87:5543–5547.

46. Oka, T., M. Yoshimura, S. Lavandero, K. Wada, and Y. Ohba. 1990. Control of growth and differentiation of the mammary gland by growth factors. *J. Dairy Sci.* 74:2788–2800.

47. Pierce Jr., D. F., M. Johnson, Y. Matsui, S. Robinson, L. Gold, A. Purchio, C. Daniel, B. Hogan, and H. Moses. 1993. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β1. *Genes & Dev.* 7:2308–2317.

48. Raza, H., J. Pongubala, and S. Sorof. 1989. Specific high affinity binding of lipoxygenase metabolites of arachidonic acid by liver fatty acid binding protein. *Biochem. Biophys. Res. Commun.* 161:448–455.

49. Rivera, E. M. 1971. Mammary gland culture. *Methods Mammal. Embryol.* 31:442–471.

50. Robinson, F. D., A. Roberts, and C. Daniel. 1993. TGFβ suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. *J. Cell Biol.* 120:245–251.

51. Sakakura, T. 1991. New aspects of stroma-parenchyma relations in mammary gland differentiation. *Intern. Rev. Cytol.* 125:165–202.

52. Schöntgen, F., G. Pignede, L. Bonnano, and P. Jolles. 1989. Amino acid sequence and some ligand binding properties of fatty acid binding protein from bovine brain. *Eur. J. Biochem.* 185:35–40.

53. Silberstein, G., and C. Daniel. 1987. Reversible inhibition of mammary ductal growth by transforming growth factor beta. *Science (Wash. DC).* 37:291–293.

54. Smith, G. 1987. Functional differentiation of virgin mouse mammary epithelium in explant culture is dependent upon extracellular proline. *J. Cell. Physiol.* 131:190–199.

55. Snedeker, S., C. Brown, and R. DiAugustine. 1991. Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary ductal morphogenesis. *Proc. Natl. Acad. Sci. USA.* 88:276–280.

56. Spener, F., C. Unterberg, T. Börchers, and R. Grosse. 1990. Characteristics of fatty acid binding proteins and their relation to mammary derived growth inhibitor. *Mol. Cell. Biochem.* 98:57–68.

57. Spiegelmann, B., M. Frank, and H. Green. 1983. Molecular cloning of mRNA from 3T3 adipocytes. Regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation dependent proteins during adipocyte development. *J. Biol. Chem.* 258:10083–10089.

58. Spitzer, E., R. Grosse, D. Kunde, and H. Schmidt. 1987. Growth of mammary epithelial cells in breast cancer biopsies correlates with EGF binding. *Int. J. Cancer.* 39:279–282.

59. Spitzer, E., W. Zschiesche, B. Binas, R. Grosse, and B. Erdmann. 1994. EGF and TGF-alpha modulate structural and functional differentiation of the mammary gland from pregnant mice. *J. Cell. Biochem.* In press.

60. Stoker, A., C. Streuli, M. Martins-Green, and M. Bissel. 1990. Designer microenvironments for the analysis of cell and tissue function. *Curr. Opin. Cell Biol.* 2:864–874.

61. Studier, F., A. Rosenberg, J. Dunn, and J. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60–89.

62. Streuli, C., and M. Bissel. 1991. Mammary epithelial cells, extracellular matrix and gene expression. *In* Regulatory Mechanisms in Breast Cancer. M. Lippman and R. Dickson, editors. Kluwer Academic Publishers, Norwell, MA. pp. 365–381.

63. Streuli, C., and M. Bissel. 1990. Expression of extracellular matrix components is regulated by substratum. *J. Cell Biol.* 110:1405–1415.

64. Taketani, Y., and T. Oka. 1983. Tumor promoter 12-O-tetradecanoylphorbol 13-acetate, like epidermal growth factor, stimulates cell proliferation and inhibits differentiation of mouse mammary epithelial cells in culture. *Proc. Natl. Acad. Sci. USA.* 80:1646–1649.

65. Tonelli, Q., and S. Sorof. 1980. Epidermal growth factor requirement for development of cultured mammary gland. *Nature (Lond.).* 285:250–252.

66. Topper, Y., and C. Freeman. 1980. Multiple hormone interactions in the development biology of the mammary gland. *Physiol. Rev.* 60:1049–1106.

67. Treuner, M., C. Kozak, D. Gallhan, E. Spitzer, R. Grosse, and T. Mueller. 1994. Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor/heart-fatty acid binding protein. *Gene (Amst.).* In press.

68. Veerkamp, J., R. Peeters, and R. Maatman. 1991. Structural and functional features of different types of cytoplasmic fatty acid binding proteins. *Biochem. Biophys. Acta.* 1081:1–24.

69. Veerkamp, J., T. van Kuppevelt, R. Maatman, and C. Prinsen. 1993. Structural and functional aspects of cytosolic fatty acid binding proteins. *Prostaglandins Leukotrienes Essent. Fatty Acids.* 49:887–906.

70. Vonderhaar, B. 1987. Local effects of EGF, TGF alpha, and EGF like growth factors on lobuloalveolar development of the mouse mammary gland in vivo. *J. Cell. Physiol.* 132:581–584.

71. Vonderhaar, B., and H. Nakhashi. 1986. Bifunctional activity of epidermal growth factor on alpha and kappa-casein gene expression in rodent mammary glands in vitro. *Endocrinology.* 119:1178–1184.

72. Wallukat, G., F.-D. Böhmer, U. Engstroem, P. Langen, M. Hollenberg, J. Behlke, H. Kuehn, and R. Grosse. 1991. Modulation of the beta-adrenergic response in cultured rat heart cells. II. Dissociation from lipid-binding activity of MDGI. *Mol. Cell. Biochem.* 102:49–60.

73. Wobus, A., W. Zschiesche, and R. Grosse. 1990. Differentiation-promoting effects of mammary-derived growth inhibitor (MDGI) on pluripotent mouse embryonic stem cells. *Virchows Arch. B Cell Pathol.* 59:339–342.

74. Zabari, M., M. Berri, P. Rouchon, F. Zamora, C. Tassy, B. Ribadeau-Dumas, and A. Quali. 1993. Purification and characterization of a polymorphic low molecular weight bovine muscle cysteine proteinase inhibitor: structural identity with fatty acid binding proteins. *Biochemie.* 75:937–945.