Perturbation of $\beta 1$ -integrin function alters the development of murine mammary gland

Marisa M.Faraldo¹, Marie-Ange Deugnier, Matvey Lukashev², Jean Paul Thiery and Marina A.Glukhova¹

UMR 144, CNRS-Institut Curie, Section de Recherche, 26, rue d'Ulm, 75248, Paris, Cedex 05, France and ²Department of Anatomy and Cancer, University of California, 3rd and Parnassus Avenue, San Francisco, CA 94143-0750, USA

¹Corresponding authors
e-mail: Glukhova@curie.fr

The expression of a transgene coding for a chimeric molecule, containing the cytoplasmic and transmembrane domains of the $\beta 1$ -integrin chain and the extracellular domain of the T-cell differentiation antigen CD4, was targeted to the mouse mammary gland by the mouse mammary tumor virus (MMTV) promoter. The chimera does not interact with the extracellular ligands; however, its expression in cultured cells was shown to interfere with focal adhesion kinase (FAK) phosphorylation following ligation of endogenous $\beta 1$ -integrin. Therefore, expression of the transgenic protein on the cell surface should uncouple adhesion from intracellular events associated with the $\beta 1$ -cytoplasmic domain and thus perturb $\beta 1$ -integrin functions. Although most of the transgenic females were able to lactate, their mammary glands had a phenotype clearly distinct from that of wild-type mice. At mid-pregnancy and the beginning of lactation, transgenic glands were underdeveloped and the epithelial cell proliferation rates were decreased, while the apoptosis levels were higher than in wild-type glands. In lactation, the amounts of the whey acidic protein (WAP) and β -casein gene transcripts were diminished, and the basement membrane component, laminin and the $\beta 4$ -integrin chain accumulated at the lateral surface of luminal epithelial cells, revealing defects in polarization. Our observations prove that *in vivo*, $\beta 1$ -integrins are involved in control of proliferation, apoptosis, differentiation and maintenance of baso-apical polarity of mammary epithelial cells, and therefore are essential for normal mammary gland development and function.

Keywords: cytodifferentiation/growth regulation/integrins/mammary epithelium/transgenic mice

Introduction

Integrins are a family of transmembrane heterodimer receptors composed of non-covalently associated α - and β -subunits. So far, at least 15 different α - and eight different β -subunits have been identified. The integrin $\beta 1$ -subunit is known to associate with at least ten distinct α -subunits to form heterodimers with different ligand specificity. The receptor consists of an extracellular, a

transmembrane and a cytoplasmic domain. The large extracellular domain binds to various ligands, i.e. to the extracellular matrix (ECM) proteins or to other cell surface receptors. The cytoplasmic domain of the receptor interacts with the cytoskeleton. Integrins function as signaling receptors and have been shown to regulate the reorganization of the cytoskeleton, intracellular ion transport, kinase activation and gene expression (for review see Dedhar and Hannigan, 1996; Meredith *et al.*, 1996; Giancotti, 1997).

Inactivation of the $\beta 1$ -gene results in early embryonic lethality (Fässler and Meyer, 1995; Stephens *et al.*, 1995). This observation suggests that the integrins of the $\beta 1$ family are essential for normal development. However, recent studies performed with chimeric mouse embryos and adult mice containing $\beta 1$ -null cells revealed that the embryos with a $\beta 1$ -null cell content of up to 25% developed normally, whereas in adults, $\beta 1$ -null cells were found in most of the tissues and organs (Fässler and Meyer, 1995). For instance, the chimeric mouse epidermis containing $\beta 1$ -null keratinocytes appeared well-organized (Bagutti *et al.*, 1996).

In this work, we have used the mouse mammary gland as a model to study the involvement of $\beta 1$ -integrins in the cellular functions *in vivo*. In order to perturb the function of $\beta 1$ -integrins, we have targeted the expression of a transgene coding for a chimeric molecule containing the cytoplasmic and the transmembrane domains of the $\beta 1$ -integrin subunit and the extracellular domain of the T-cell differentiation antigen, CD4, to the mammary gland using mouse mammary tumor virus (MMTV) promoter. Such a chimera neither binds to α -integrin subunits, nor interacts with the ECM integrin ligands. However, *in vitro*, it was shown to be delivered to focal contacts of the adherent cells and to stimulate tyrosine phosphorylation of FAK if clustered by an anti-CD4 extracellular domain antibody. On the other hand, when expressed at high levels, the chimera induced cell detachment, prevented adhesion to ECM proteins and interfered with FAK phosphorylation following antibody-induced $\beta 1$ -integrin clustering (Lukashev *et al.*, 1994). Thus the chimeric molecule can uncouple adhesion from the intracellular integrin-associated events and acts as a dominant inhibitor of integrin function.

The mammary gland consists of secretory alveoli interconnected by a system of branching ducts. The entire structure is surrounded by a basement membrane and embedded in connective tissue (fat-pad stroma). Epithelial cells that compose the gland are arranged in two layers, a luminal epithelial and a basal layer which consists of myoepithelial and a small number of so-called basal (or precursor) cells. Mammary gland development occurs mainly after birth. Ductal development is initiated in the virgin animal at the onset of puberty and with the attainment of sexual maturity the mammary tree, consisting

of branching ducts, occupies the entire stromal fat pad. During pregnancy, numerous alveolar buds appear along the epithelial branches and give rise to the secretory tissue. Active cell proliferation is observed during ductal growth in the virgin, during alveolar development and further ductal growth in pregnancy, and after parturition. In the luminal epithelial cells of the alveoli, the expression of milk-specific proteins begins during pregnancy, and increases dramatically with the onset of lactation when the fully mature phenotype is acquired.

Cell-matrix interactions are important regulators of cell growth and programmed cell death. Recent studies have shown that adhesion mediated by specific integrins resulted in recruitment of an adaptor protein, Shc, and activation of the MAP-kinase pathway (Wary *et al.*, 1996). Adhesion to the ECM protects the cells from apoptosis (Frisch and Francis, 1994; Boudreau *et al.*, 1995; Frisch and Ruoslahti, 1997), whereas activation of FAK following adhesion appears to be an important factor in control of cell survival (Frisch *et al.*, 1996; Hungerford *et al.*, 1996). In addition to cell growth and survival, ECM can regulate the phenotype of various cell types, including the mammary epithelium. In particular, adhesion of mammary epithelial cells to laminin is believed to mediate β -casein gene expression (Streuli *et al.*, 1995b), whereas activity of STAT5 transcription factor, an essential regulator of milk gene expression, is also controlled by cell-ECM interactions (Streuli *et al.*, 1995a). *In vivo* studies have implicated matrix-degrading metalloproteinases in growth control and mammary gland morphogenesis (Talhouk *et al.*, 1992; Simpson *et al.*, 1994; Witty *et al.*, 1995; Alexander *et al.*, 1996; Lund *et al.*, 1996). Overexpression of the active stromelysin-1 in the mouse mammary gland resulted in hyperplasia of the virgin gland and apoptosis in the late pregnant gland. Further studies revealed that the balance between these proteolytic enzymes and their tissue inhibitor may be an important modulator of the functional cell-matrix interactions in the mammary gland (Alexander *et al.*, 1996).

To determine the effects of expression of the transgenic protein on mammary gland development, we have analyzed gland morphology, proliferation, apoptosis and differentiation of mammary epithelial cells in the transgenic animals at different stages of gland development. We found that in pregnancy and lactation, the transgenic glands were significantly underdeveloped due to decreased epithelial cell proliferation and increased apoptosis rates; in lactation, expression of milk-specific proteins, WAP and β -casein, was diminished and epithelial cell polarization was affected. The data prove that *in vivo*, β 1-integrins are involved in the control of mammary epithelium proliferation, apoptosis, differentiation and maintenance of baso-apical polarity.

Results

Transgene expression

We have constructed a plasmid containing MMTV promoter and a sequence coding for a chimeric molecule, consisting of the cytoplasmic and transmembrane domains of the mouse β 1-integrin chain and the extracellular domain of CD4 (MMTV-Ch1). Four founders (F₀) expressing the β 1-chimera under the control of MMTV

promoter were generated, and the corresponding transgenic lines were established. Southern blot analysis has revealed the presence of 10–20 transgene copies in all transgenic lines (data not shown). The females from three transgenic lines were able to feed litters of normal size, whereas 25% of the females of line 17 lost some or all the pups during the first and second days of lactation.

To estimate the transgene expression levels, total RNA was isolated from the mammary tissue of females from different transgenic lines and analyzed by Northern blot (Figure 1A). A probe for CD4 did not hybridize with the wild-type gland RNA and detected a band of 3.0 kb in the RNA from the transgenic gland. mRNA of the same size was revealed with a probe for the β 1-cytoplasmic domain, indicating that the 3.0 kb band corresponded to the transgene transcript. The β 1-probe also hybridized to a 3.8 kb band of the endogenous β 1-mRNA (Holers *et al.*, 1989). The expression of the transgene was high in line 17, moderate in line 42 and weak in lines 44 and 46.

The developmental profile of transgene expression is shown in Figure 1B. The transgene was already being expressed in 8-week-old virgin mice, expression was significantly up-regulated during pregnancy and further increased during lactation.

The MMTV promoter was previously reported to be expressed in organs other than the mammary gland (Leder *et al.*, 1986; Choi *et al.*, 1987). We found the transgene transcript in the kidney, lung, spleen and salivary gland (data not shown); however, the expression levels were much lower than those observed in the mammary gland during pregnancy and lactation.

Using anti-CD4 monoclonal antibody (mAb), we have detected the transgenic protein in the luminal epithelial cells in lactating transgenic mice (Figure 1C). In agreement with previous studies (Stöcklin *et al.*, 1993), the transgene expression driven by the MMTV promoter was not uniform throughout the gland: positive and negative cells could be found in the same alveoli. Expression of the transgenic protein was not revealed in the myoepithelial cells (Figure 1C and D).

β 1-chimera expression interferes with normal development of mammary gland in pregnancy and lactation

To study the effects of transgene expression on the mammary gland development, we compared the morphology of the wild-type and transgenic glands at different developmental stages. No obvious alteration in the size or branching pattern of the virgin transgenic mice glands was observed. At mid-pregnancy (days 10–12), the transgenic glands appeared smaller with a branching pattern less complex than that observed in normal glands (Figure 2A and B). By the end of pregnancy (days 18–19), the difference between wild-type and transgenic glands was less prominent (data not shown). Figure 2C and D shows sections through 2-day-lactating glands stained with hematoxylin–eosin. In wild-type glands, on the second day of lactation the fat pad was completely occupied by the secretory epithelium, and small fat-stroma islets were rarely seen between the lobules. On the contrary, in the transgenic glands, the alveoli appeared sparse and were surrounded by vast areas of fat stroma.

Consistent with the transgene expression levels, the morphological differences were drastic in females from

Fig. 1. Expression of the β1-integrin chimera in the transgenic mice. (A) Northern blot analysis of total mammary gland RNA (20 µg) from wild-type and transgenic 15-week-old virgin mice with cDNA probes for CD4 (CD4) and β1-cytoplasmic domain (β1-cyto). Wt, wild type; T17, T42, T44 and T46, transgenic lines, 17, 42, 44, and 46, respectively. Arrows, from top to bottom, show a 3.8 kb band corresponding to the endogenous β1-integrin chain mRNA and a 3 kb band corresponding to the transgene transcript. (B) Northern blot analysis of the transgene expression in line 17 at different developmental stages using the cDNA probe for β1 cytoplasmic domain. Wt, wild-type 5-day-lactating; V8, V13, P18 and L5, correspond to 8- and 13-week-old virgin, 18-day-pregnant and 5-day-lactating mice, respectively, all from the transgenic line 17. Twenty µg of total mammary RNA was loaded in each case. In (A) and (B) the ethidium bromide-stained 18S rRNA band is shown as a loading control. (C) Detection of the transgenic protein by indirect immunofluorescence in a 5-day-lactating transgenic mammary gland from line 17 using anti-CD4 mAb followed by Texas Red-conjugated secondary antibody. (D) The same section treated with FITC-conjugated anti-α-smooth muscle actin mAb to reveal the myoepithelial cells. Arrows in (C) and (D) show the alveoli containing expressing and non-expressing cells. The myoepithelial cells do not express the transgene. Bar, 50 µm.

Table I. BrdU incorporation in normal and transgenic mammary glands

Stage	BrdU-positive nuclei (%)	
	Wild-type	β-1 transgenic
12-day-pregnant	19.91 ± 1.47 (3)	12.10 ± 2.35 (4)
2-day-lactating	7.96 ± 0.64 (4)	3.21 ± 2.08 (4)

Values are presented as mean ± SD; in all cases, 1500–2000 nuclei per specimen were counted. The females with litters of 7–8 pups were selected; number of animals analyzed is indicated in parentheses. Statistically significant differences were observed for comparisons between non-transgenic and transgenic glands ($P < 0.004$ and < 0.02 for 12-day-pregnant and 2-day-lactating mice, respectively).

line 17, much less pronounced in line 42 and hardly detectable in lines 44 and 46.

β1-chimera expression inhibits proliferation and induces apoptosis in glands of pregnant and lactating mice

The mammary glands of the transgenic mice might be underdeveloped due to decreased proliferation and/or increased apoptosis rates. To compare the mammary

epithelium proliferation levels in the transgenic and wild-type animals, BrdU was injected 2 h prior to sacrifice and its incorporation in DNA was estimated by immunohistochemical technique in the mammary gland paraffin sections. In normal mice, proliferation rates are high at mid-pregnancy, drop at the end of the gestation period and temporarily increase again post-partum with the onset of lactation (Traurig, 1967a,b). Proliferation was significantly diminished in transgenic glands at mid-pregnancy ($12.10 \pm 2.35\%$ compared with $19.91 \pm 1.47\%$ in wild-type) as well as at the second day of lactation ($3.21 \pm 2.08\%$ compared with $7.96 \pm 0.64\%$ in wild-type) (Table I).

We have observed rather high apoptosis levels in wild-type glands at mid-pregnancy (Table II). The cells with apoptotic nuclei comprised $1.11 \pm 0.11\%$ of total epithelial cell number in glands of 12-day-pregnant mice. By the end of pregnancy, the level dropped to $0.42 \pm 0.10\%$ and remained low during lactation ($0.28 \pm 0.10\%$ in 2-day-lactating and $< 0.1\%$ in 4- and 10-day-lactating glands). In transgenic glands, the number of apoptotic nuclei varied significantly between animals. However, a statistically significant increase in apoptosis was observed in glands of pregnant and 2-day-lactating transgenic mice (Table II). Later in lactation, similar to wild-type glands, apoptosis rates were low and did not exceed 0.1%.

Fig. 2. Morphology of the transgenic and wild-type mammary glands. Whole-mount staining of the glands from 12-day-pregnant mice, (A) wild-type, and (B) transgenic, line 17. Hematoxylin–eosin stained sections from 2-day-lactating mice, (C) wild-type and (D) transgenic. Bars, 2 mm in (A) and (B); 100 μ m in (C) and (D).

Table II. Apoptosis in mammary glands of wild-type and transgenic pregnant and lactating animals

Stages	Apoptotic nuclei (%)	
	Wild-type	β -1 transgenic
12-day-pregnant	$^{a}1.11 \pm 0.11$ (4)	$^{a}2.46 \pm 1.19$ (5)
18-day-pregnant	$^{a}0.42 \pm 0.10$ (4)	$^{a}0.90 \pm 0.18$ (4)
2-day-lactating	$^{a}0.28 \pm 0.10$ (3)	$^{a}1.15 \pm 0.41$ (3)
4-day-lactating	<0.1	<0.1
10-day-lactating	<0.1	<0.1

^aValues are presented as mean $\pm$ SD; 1500–2000 nuclei per specimen were counted. The females with litters of 7–8 pups were selected; number of animals analyzed is indicated in parentheses. Statistically significant differences were observed for comparisons between nontransgenic and transgenic glands ($P < 0.05$).

β 1-chimera expression affects differentiation of secretory epithelium during lactation

During the second half of pregnancy, mammary epithelium undergoes a differentiation process when luminal cells begin expressing the milk protein genes. The fully mature phenotype is reached at lactation, with the onset of milk secretion occurring under suckling stimuli. To determine whether the transgene expression could affect the differentiation of mammary secretory epithelium, we isolated total RNA from 2-day-lactating normal and transgenic mammary glands and probed it for expression of WAP and β -casein genes (Figure 3). mRNA for both proteins was reduced in transgenic glands; however, the expression levels varied in different animals. Figure 3 shows four representative cases. We observed a 40–80% reduction of WAP mRNA and a 20–60% reduction of β -casein mRNA.

Fig. 3. Expression of milk-protein genes in normal and transgenic lactating mammary glands. Total RNA (20 μ g) from 2-day-lactating mammary glands from two normal and four transgenic females (all with 7–8 pups) was analyzed by Northern blot for the expression of WAP and β -casein milk proteins and cytokeratin 8. Expression of GAPDH was used as a loading control. The blot was exposed to Amersham film after hybridization with the probe: WAP, 90 min; β -casein, 30 min; cytokeratin 8, 5 days; GAPDH, 15 h. For quantitative analysis, the values obtained for WAP and β -casein were normalized to GAPDH.

mRNA coding for the cytokeratin 8, a marker of luminal cells, was more abundant in the majority of the transgenic glands compared with wild-type.

β 1-chimera expression in luminal epithelial cells affects polarity

Polarity is an important property of all organized epithelia and its establishment is mediated by the interaction of the

Fig. 4. Double immunofluorescence staining of sections through lactating transgenic (A and B; E and F) and wild-type (C and D) mammary glands with: (A) anti-CD4 and (B) anti-laminin; (C) anti-β4-integrin and (D) anti-laminin; (E) anti-β4-integrin and (F) anti-laminin. Note that in lactating transgenic mammary glands, in addition to basal distribution, laminin accumulates at lateral cell surfaces at the sites of transgene expression, whereas β4-integrin chain co-localizes with laminin. In wild-type animals, both laminin and β4-integrin chains are restricted to the basal cell surface. Bar, 20 μm (A, B), 10 μm (C–F).

cells with their basement membrane. The overall mammary epithelium bilayer organization in the transgenic animals appeared normal, and we did not reveal any difference in the distribution of actin, vinculin and E-cadherin (data not shown). At the light microscopy level in transgenic glands, the basement membrane around alveoli appeared continuous as revealed by staining with an anti-laminin antibody. However, surprisingly, in addition to basal localization, at the sites of transgene expression, laminin was accumulated at the lateral surface of luminal epithelial cells (Figure 4A and B).

The major integrin chains expressed by mammary epithelial cells, α2, α3, α6, β1 and β4, can potentially form the dimers, α2β1, α3β1, α6β1 and α6β4. In wild-type glands, the β1-, α3- and α6-integrin chains were found at the basal and lateral surfaces of the epithelial cells (data not shown), whereas β4 chains were restricted to the basal cell side (Figure 4C). In transgenic glands, β4 was revealed at the basal cell surface and, in addition, co-localized with laminin at the lateral surface of luminal epithelial cells (Figure 4E and F). We did not reveal any obvious alteration in the distribution of β1-, α3- and α6-integrin chains (data not shown).

In order to estimate the ability of the mammary epithelial

Fig. 5. Formation of lumen in the aggregates of mammary epithelial cells in Matrigel. Mammary epithelial cells were isolated by collagenase digestion and seeded in Matrigel, and after 3 days (A) cysts and (B) aggregates without lumen were counted. Where indicated, 1 μM of dexamethasone was added to induce transgene expression. (C) The results of representative experiments performed with the mammary cells isolated from transgenic animals and their wild-type littermates of lines 17 and 42. Compare the cells from transgenic and wild-type animals grown with dexamethasone. In each case, >300 aggregates were counted. In (A) and (B), bar represents 100 μm.

cells expressing the transgene to establish polarity when interacting with the basement membrane components *in vitro*, we isolated epithelial cells from mammary glands of wild-type and transgenic mice and cultured them in Matrigel. To induce the transgene expression, dexamethasone was added to the growth medium. It was previously demonstrated by Soriano *et al.* (1995) that glucocorticoid hormones enhanced cell polarization and lumen formation in the aggregates of the mammary epithelial cells growing in collagen gel. In agreement with these data, the cells isolated from wild-type animals and cultured in Matrigel without dexamethasone formed aggregates without lumens (Figure 5), whereas in dexamethasone-containing cultures, numerous cysts (aggregates containing cells organized in a monolayer with a clearly distinguished lumen; Figure 5A) were detected. The cells obtained from the transgenic glands failed to form cysts either in the presence or absence of dexamethasone and remained in aggregates without lumen.

Discussion

We demonstrate here that perturbation of the $\beta 1$ -integrin function impairs development of mammary gland and differentiation of secretory epithelium.

To interfere with the function of $\beta 1$ -integrin *in vivo* we used the MMTV promoter to target the expression of a chimeric protein to the mammary gland. The chimeric protein contained the $\beta 1$ -integrin cytoplasmic and transmembrane domains and the extracellular domain of a molecule not relevant to integrins, the T-differentiation antigen CD4. Several *in vitro* studies have shown a dominant inhibitory effect on the $\beta 1$ -integrin function of chimeras containing the $\beta 1$ -cytoplasmic and extracellular domains of CD4 or interleukin 2 receptor, expressed on the cell surface as single chains (Akiyama *et al.*, 1994; LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994; Smilenov *et al.*, 1994). Such chimeric molecules do not have any role in adhesion; however, their expression interferes with FAK phosphorylation following ligation of endogenous $\beta 1$ -integrin. Thus, expression of the transgenic protein should uncouple adhesion from associated intracellular events and perturb integrin-mediated outside-inside signaling.

It should be taken into consideration that endogenous $\beta 1$ -integrin is present in the mammary gland of the transgenic animals and, therefore, to interfere with its function, very high transgene expression levels are required. We have obtained four transgenic mouse lines expressing different levels of the $\beta 1$ -chimera and, as expected, the most severe phenotype was observed in line 17, which had the highest expression level. The majority of transgenic females were able to feed normal size litters even though their glands were clearly underdeveloped, whereas in line 17, some of the females lost pups during the first two days of lactation.

An exhaustive study of mammary epithelial cell integrin repertoire has yet to be carried out; however, luminal epithelial cells were shown to express $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6$ chains that can be associated with $\beta 1$ or $\beta 4$ chains. *In vitro* data implicated $\alpha 2\beta 1$ in the branching morphogenesis of mammary epithelial cells in collagen gels (Berdichevsky *et al.*, 1994); however, the *in vivo* relevance of such data has not been confirmed to date, since the inactivation of the $\alpha 2$ gene has not yet been achieved. Knock-outs of $\alpha 3$ -, $\alpha 6$ - and $\beta 4$ -integrin genes resulted in perinatal lethality, whereas ablation of the $\beta 1$ gene produced early embryonic lethality (reviewed in Hynes, 1996). In $\alpha 6$ - and in $\beta 4$ -null mice, the most apparent defects were found in the dermal-epidermal junctions, where lack of hemidesmosomes led to detachment of the epidermis (Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996). In $\alpha 3$ -null mice, medullary collecting ducts in the kidneys and bronchi in the lungs displayed decreased branching (Kreidberg *et al.*, 1996). Probably due to the fact that the mammary gland is still poorly developed at birth, no data are so far reported regarding the mammary epithelium organization in the integrin-deficient mice.

Perturbation of $\beta 1$ -function leads to decreased proliferation and increased apoptosis

Interactions of mammary epithelial cells with the basement membrane are essential for cell survival as their disruption

triggers signals for apoptosis and cell-cycle arrest by induction of interleukin-1 β -converting enzyme (Boudreau *et al.*, 1995, 1996). Integrins, being the major ECM receptors, play an important role in growth control. In transgenic mice expressing $\beta 1$ -chimera, at mid-pregnancy and during lactation, mammary glands were clearly less developed than in normal animals. We have found that the gland was underdeveloped due to decreased proliferation, as well as increased apoptosis rates. It was recently demonstrated that ligation of a subset of $\beta 1$ -integrins leads to recruitment of adaptor protein Shc, activation of the MAP-kinase pathway and suppression of the apoptosis signal (Wary *et al.*, 1996). Similar to $\beta 1$ -integrins, ligation of $\alpha 6\beta 4$ results in Shc recruitment and therefore the roles of these integrins in growth control and other cellular functions may be overlapping. Since transgene expression perturbs $\beta 1$ -integrins directly, rather than $\alpha 6\beta 4$, our data suggest that perturbation of $\beta 1$ -integrin function cannot be entirely compensated for by adhesion via $\alpha 6\beta 4$. However, the possibility that $\alpha 6\beta 4$ function is indirectly impaired by transgene expression should not be excluded.

In agreement with previous studies (Stöcklin *et al.*, 1993), we have observed that the MMTV promoter is not evenly expressed in the mammary epithelium and both expressing and non-expressing cells can be found in the same alveoli. Therefore, transgenic protein levels may vary from cell to cell. In transgenic glands, individual cell fate may conceivably depend on its transgene expression level, i.e. a particularly high transgenic protein level might be required to induce apoptosis, whereas moderate levels could retard entrance into the cell cycle.

In 4- and 10-day-lactating glands, practically no proliferation was observed and apoptosis rates were low in both wild-type and transgenic animals. This observation is in apparent contradiction to the fact that transgene expression is up-regulated as lactation proceeds. However, even though the amount of transgenic protein is higher late in lactation, in the absence of growth when basement membrane is well-organized and hemidesmosomes are well established, the transgenic protein may be less efficient in perturbing cell-matrix interactions and inducing apoptosis.

We did not observe any persistent alteration in virgin glands; however, at this developmental stage the transgene expression levels might still be too low to compete efficiently with the endogenous $\beta 1$ -integrin.

Perturbation of $\beta 1$ -integrin function impairs differentiation of secretory epithelium

Northern blot analysis has revealed that β -casein and, in particular, WAP transcript levels were decreased in transgenic glands. These data are in keeping with the results of the *in vitro* studies suggesting that expression of the β -casein gene in mammary epithelial cells required interaction with laminin mediated by $\beta 1$ -integrin (Streuli *et al.*, 1995b), and that DNA-binding activity of a transcription factor STAT5, a regulator of milk-protein gene expression, depended on adhesion to basement membrane (Streuli *et al.*, 1995a). Interestingly, unlike wild-type animals, in transgenic females milk gene expression varied significantly between individual animals. Such differences may be explained by a natural asynchrony of differentiation marker expression observed in mammary epithelial cells

(Robinson *et al.*, 1995) together with mosaic MMTV-driven transgene expression pattern (Stöcklin *et al.*, 1993).

We have also observed a relative increase of the cytokeratin 8 transcript levels. These data suggest that in transgenic animals, differentiation of secretory epithelium is impaired, and cells continue to produce cytokeratin 8, a structural protein, rather than switch completely to secretion.

Cellular mechanisms of the transgene action

In vitro expression of β 1-CD4 chimera resulted in cell rounding and detachment (Lukashev *et al.*, 1994). In the transgenic glands, at the light microscopy level, overall tissue organization was not altered and basement membrane formed a continuous sheath around the ducts and alveoli. However, in addition to basal localization, laminin was found at lateral surfaces of the luminal epithelial cells in correlation with transgene expression. In mammary epithelium α 3-, α 6- and β 1-chains (thus, α 3 β 1- and α 6 β 1-integrin dimers) are more abundant at the basal cell surface, but also localize at sites of cell-cell contact between luminal epithelial cells while β 4-chains (i.e. α 6 β 4 dimers) are found only at the basal side of luminal epithelial cells. Using immunofluorescence methods, we did not reveal any redistribution of α 3, α 6 and β 1 in transgenic glands compared with wild-type. Supposedly, the major integrin receptors for laminin in mammary epithelium are α 3 β 1 and α 6 β 4. Similar to laminin, distribution of the β 4-integrin chain was altered in transgenic glands, as it was co-localized with laminin at lateral cell surfaces. These observations reveal defects in cell polarization and suggest malformation of cell-cell junctions. In agreement with these findings, cells isolated from the glands of transgenic animals and cultured in Matrigel failed to establish polarity and to organize into cysts. Similarly, polarization and organization of normal mammary epithelial cells into cysts in collagen gels as well as in Matrigel were reported to require function of β 1-integrins (Howlett *et al.*, 1995), whereas treatment of the mammary tumor cells with function-blocking anti- β 1 antibody resulted in establishment of polarity and organization into acinus-like structures, probably by mimicking normal interactions with the ECM (Weaver *et al.*, 1997).

The intracellular events accompanying the transgene expression are not known precisely. It has been suggested that single β 1-cytoplasmic domain may block integrin dimer activation by titration of intracellular proteins essential for this process (Chen *et al.*, 1994; Fenczik *et al.*, 1997). On the other hand, transgene expression might affect integrin-mediated signaling. Phosphorylation/activation of FAK is usually linked to the formation of integrin-mediated sites of contact between cell and ECM (focal adhesions). We did not observe any statistically significant difference in FAK phosphorylation levels between transgenic and wild-type glands (data not shown). However, it should be taken into consideration that FAK may be phosphorylated in response to stimulation by growth factors or biologically active peptides, rather than exclusively by adhesion (Zachary and Rozengurt, 1992). Further studies are required to clarify the mechanism of the transgenic protein action and its effects on bidirectional transmembrane signaling mediated by β 1-integrins. The

cells isolated from transgenic glands and cultured *in vitro* should provide a good model system for such studies.

One of the important consequences of the single β 1-cytoplasmic domain expression may be perturbed ECM assembly (LaFlamme *et al.*, 1994), which in turn, may cause alterations in stromal-epithelial interactions and affect the critical balance in the production of extracellular matrix proteinases and their inhibitors. Such changes could lead to reactive stroma formation and promote tumor development (Sympson *et al.*, 1994, 1995; Lund *et al.*, 1996). On the other hand, numerous studies have shown that expression of integrins is down-regulated in many tumor types, whereas complementary experiments have revealed that expression of certain integrins and integrin-associated molecules in the transformed cells can suppress the transformed phenotype (for recent review see Ben-Ze'ev, 1997; Meredith *et al.*, 1996; Schwartz, 1997). Thus, perturbation of β 1-integrin function may lead to selection of a cell or a cell population that would be anchorage-independent, that is, able to proliferate and avoid apoptosis in the absence of adhesion. In agreement with this suggestion, we observe hyperplasia in the mammary glands of 1.5-year-old parous females; however, more data are required to prove that these anomalies result from the transgene expression.

In conclusion, we demonstrate here that perturbation of β 1-integrin function by targeted expression of β 1-cytoplasmic domain in mammary epithelium leads to decreased proliferation and increased apoptosis, and affects expression of milk-protein genes. The data prove that β 1-integrins are involved in growth control and differentiation of mammary epithelium and are essential for normal mammary gland development and function.

Materials and methods

Generation of transgenic mice

Construction of the plasmid pBS-Ch1 was as previously described (Lukashev *et al.*, 1994). Briefly, the N-terminal fragment of the mouse β 1-integrin cDNA, coding for the cytoplasmic and transmembrane domains, was fused in-frame with the C-terminal sequence of mouse CD4 cDNA coding for the extracellular domain. The MMTV-Ch1 construct used in this work was created by inserting the 1.5 kb *Xba*I-*Xho*I fragment of pBS-Ch1 into a psp72-derived plasmid, containing the MMTV promoter, and intronic and polyadenylation sequences of simian virus 40 (provided by Dr B.Groner; Stöcklin *et al.*, 1993). To generate transgenic mice, a 4.5 kb *Sal*I-*Sal*I fragment corresponding to the insert was purified by electroelution from the agarose gel band followed by Elutip-d column (Schleicher and Schuell, Dassel, Germany) and microinjected in the pronucleus of B6/D2 F₁ fertilized eggs. The injected embryos were transferred to C57BL/6 pseudopregnant females and the offspring genotype was tested for the integration of transgene by PCR analysis of genomic DNA. The following oligonucleotides, complementary to the exons 2 and 3 of the gene coding for CD4, were used for amplification: 5'-GATTCCCAACCAACAAGAGC-3' and 5'-GCAGGGCAGTTCTGCTGATT-3'. Transgenic lines were generated by mating founder animals to C57BL/6 mice. Heterozygous transgenic mice were used for analysis of phenotype. Negative littermates served as a wild-type control.

Mammary tissue preparation for morphological analysis

The fourth (inguinal) glands were used for all morphological analysis, cell proliferation and apoptosis assays. For whole-mount staining, mammary glands were flattened on microscopic slides, fixed overnight in Carnoy's solution (75% ethanol, 25% acetic acid) and stained with carmine alum as described by Sympson *et al.* (1994). For histological analysis, mammary glands were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in ethanol, cleared in

chloroform and embedded in paraffin, or alternatively, fixed in Methacarn solution (60% methanol, 30% chloroform, 10% acetic acid), dehydrated in methanol, cleared in xylene and embedded in paraffin. Sections of 5 µm were deparaffinized and stained with hematoxylin and eosin.

For immunofluorescence staining, dissected mammary glands were immediately embedded in Tissue-Tek (Miles Diagnostic Division, Elkhart, IN) and frozen in isopentane cooled by liquid nitrogen. Prior to staining, 5 µm cryosections were fixed for 10 min in acetone at -20°C and incubated with 10% fetal calf serum for 20 min at room temperature. The sections were then incubated with primary antibodies for 1 h at 37°C, washed with PBS, and treated with FITC- or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, Pennsylvania) diluted 1:100, for 30 min at 37°C. mAb against α-smooth muscle actin conjugated to FITC (Sigma) was diluted 1:50; rat anti-mouse CD4 (Pharmingen) and rat anti-β4-integrin chain (Pharmingen) mAbs were used at 10 µg/ml; rat anti α6-integrin Mab GoH3 (provided by A.Sonnenberg; Sonnenberg *et al.*, 1986) was used as undiluted culture supernatant; rabbit anti-β1-integrin cytoplasmic domain (provided by R.Hynes; Marcantonio and Hynes, 1988) and rabbit anti-α3-integrin cytoplasmic domain (provided by F.Watt, ICRF, London) antibodies were used at 5–10 µg/ml. A polyclonal antibody against mouse laminin was kindly provided by Dr H.Feracci (Institut Curie, Paris, France) and used at a dilution of 1:250. Nuclei were stained with 1 µg/ml DAPI (4',6-diamidino-2-phenylindole; Sigma) solution for 5 min at room temperature. In all cases, mice with litters of 7–8 pups were analyzed. Negative female littermates served as wild-type controls.

Cell proliferation assay

For the detection of cell proliferation, mice were intraperitoneally injected with 250 µg/g body weight of BrdU (Sigma) 2 h prior to sacrifice. Sections of mammary glands, pre-fixed in Methacarn, were deparaffinized and rehydrated according to standard protocols. Endogenous peroxidase was blocked by incubation in 3% H₂O₂ in methanol at room temperature for 20 min. Sections were then treated with 2 N HCl at 37°C for 20 min and with 0.1 M Na-borate buffer, pH 8.5, for 10 min. Incorporated BrdU was detected using the Amersham Cell Proliferation Kit following the manufacturer's instructions. A weak hematoxylin counterstaining was performed, and 1500–2000 nuclei per sample were counted.

Apoptosis analysis

To detect apoptotic nuclei, paraformaldehyde pre-fixed paraffin sections were analyzed by the TdT digoxigenin nick end-labeling with Apoptag Plus (Oncor) following the manufacturer's instructions. After counterstaining with methyl green, 1500–2000 nuclei per sample were counted.

RNA isolation and analysis

The third (thoracic) gland was used for RNA extraction. Total RNA was isolated using RNA-plus reagent (Bioprobe Systems) following the manufacturer's instructions. Twenty µg of total RNA was separated on 1% agarose/formaldehyde gels, transferred to nylon membranes (Hybond-N, Amersham) and hybridized with [³²P]dCTP random-primed labeled cDNA probes. Two different probes were used to detect transgene expression, a 250 bp *Bam*HI–*Eco*RV fragment from MMTV–Ch1 plasmid (containing 200 bp of β1-integrin and 50 bp of CD4) and a 1.2 kb *Bam*HI–*Hind*III fragment corresponding to CD4. cDNA probes: for mouse WAP (Campbell *et al.*, 1984) was kindly provided by Dr R.Jaggi (Laboratory for Clinical and Experimental Research, Bern, Switzerland); for mouse β-casein (Gupta *et al.*, 1982), by Dr J.Rosen (Baylor College of Medicine, Baylor, TX, USA); and for mouse cytokeratin 8 (Sèmat *et al.*, 1984), by Dr J.L.Jorcano (CIEMAT, Madrid, Spain). The blots were exposed to the high performance autoradiography film (Amersham). Quantitative analysis of the results was performed by PhosphorImager (Molecular Dynamics).

Primary cell culture

Primary mammary epithelial cell cultures were obtained as previously described (Smith, 1996). Both inguinal glands were removed from 15-week-old virgin mice and minced with scalpel blades to fragments of ~1 mm³ in RPMI medium containing 10% fetal calf serum, 5 µg/ml insulin and 10 ng/ml EFG (complete medium). After an overnight incubation in 1 mg/ml collagenase (Worthington Biochemical) in complete medium at 37°C, the fragments were washed and plated in tissue culture flasks at a density of ~2.5×10⁵ fragments per T-75 flask. Fibroblasts were removed by gentle trypsin digestion and the epithelial cells were seeded in Matrigel (Becton Dickinson Labware) and on glass

coverslips in complete medium. To induce transgene expression, 1 µM dexamethasone (Sigma) was added to the growth medium. Induction of the transgene expression was analyzed by indirect immunofluorescence method using anti-CD4 mAb. After 2–3 days of the dexamethasone treatment, ~90% of the cells were CD4-positive.

Acknowledgements

We are especially grateful to Dr I.Cerutti, and to the personnel of the Service d'Expérimentation Animale et de Transgénèse, Villejuif; particularly to R.Duchâteau, A.Loeuillet and P.Médina for taking care of transgenic mice. We greatly appreciate the advice of Dr C.Daniel on the phenotype analysis. We express our gratitude to Dr Z.Werb for helpful advice, to Dr A.M.Vallés for critical comments on the manuscript, and to V.Petit and C.Marchaland for help with densitometric and statistical analysis of the data, respectively. We appreciate the generous gifts of reagents by Drs H.Feracci, B.Groner, R.Hynes, R.Jaggi, J.L.Jorcano, J.Rosen, A.Sonnenberg, F.Watt and Z.Werb. This work was supported by the Association pour la Recherche contre le Cancer (ARC 1161). M.M.F. was supported by a Human Frontier Science fellowship and by a fellowship from the Ligue Contre le Cancer. M.-A.D. is Chargé de Recherche and M.A.G. is Directeur de Recherche at the Institut de la Santé et de la Recherche Médicale.

References

- Akiyama,S.K., Yamada,S.S., Yamada,K.M. and LaFlamme,S.E. (1994) Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. *J. Biol. Chem.*, **269**, 15961–15964.
- Alexander,C.M., Howard,E.W., Bissell,M.J. and Werb,Z. (1996) Rescue of mammary epithelial cell apoptosis and ectactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. *J. Cell Biol.*, **135**, 1669–1677.
- Bagutti,C., Wobus,A.M., Fässler,R. and Watt,F.M. (1996) Differentiation of embryonal stem cells into keratinocytes: comparison of wild-type and β1 integrin-deficient cells. *Dev. Biol.*, **179**, 184–196.
- Ben-Ze'ev,A. (1997) Cytoskeletal and adhesion proteins as tumor suppressors. *Curr. Opin. Cell Biol.*, **9**, 99–108.
- Berdichevsky,F., Alford,D., D'Souza,B. and Taylor-Papadimitriou,J. (1994) Branching morphogenesis of human mammary epithelial cells in collagen gels. *J. Cell Sci.*, **107**, 3557–3566.
- Boudreau,N., Sympson,C.J., Werb,Z. and Bissell,M.J. (1995) Suppression of ICE and apoptosis in mammary epithelial cells by ECM. *Science*, **267**, 891–893.
- Boudreau,N., Werb,Z. and Bissell,M.J. (1996) Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. *Proc. Natl Acad. Sci. USA*, **93**, 3509–3513.
- Campbell,S.M., Rosen,J.M. and Hennighausen,L.G. (1984) Comparison of the whey acidic protein genes of the rat and mouse. *Nucleic Acids Res.*, **12**, 8685–8697.
- Chen,Y.P., O'Toole,T.E., Shipley,T., Forsyth,J., LaFlamme,S., Yamada,K., Shattil,S. and Ginsberg,M. (1994) 'Inside-out' signal transduction inhibited by isolated integrin cytoplasmic domains. *J. Biol. Chem.*, **269**, 18307–18310.
- Choi,Y., Henrard,D., Lee,I. and Ross,S. (1987) The mouse mammary tumor virus long terminal repeat directs expression in epithelial and lymphoid cells of different tissues in transgenic mice. *J. Virol.*, **61**, 3013–3019.
- Dehdar,S. and Hannigan,G.E. (1996) Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr. Opin. Cell Biol.*, **8**, 657–669.
- Fässler,R. and Meyer,M. (1995) Consequences of lack of β1 integrin gene expression in mice. *Genes Dev.*, **9**, 1896–1908.
- Fenczik,C.A., Sethi,T., Ramos,J.W., Huges,P.E. and Ginsberg,M. (1997) Complementation of dominant suppression implicates CD98 in integrin activation. *Nature*, **390**, 81–85.
- Frisch,S.M. and Francis,H. (1994) Disruption of epithelial cell–matrix interactions induces apoptosis. *J. Cell Biol.*, **124**, 619–626.
- Frisch,S.M. and Ruoslahti,E. (1997) Integrins and anoikis. *Curr. Opin. Cell Biol.*, **9**, 701–706.
- Frisch,S.M., Vuori,K., Ruoslahti,E. and Chan-Hui,P.-Y. (1996) Control of adhesion-dependent cell survival by focal adhesion kinase. *J. Cell Biol.*, **134**, 793–799.

- Georges-Labouesse, E., Messaddeq, N., Yehia, G., Cadalbert, L., Dierich, A. and LeMeur, M. (1996) Absence of the α -6 integrin leads to epidermolysis bullosa and neonatal death in mice. *Nature Genet.*, **13**, 370–373.
- Giancotti, F.G. (1997) Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr. Opin. Cell Biol.*, **9**, 691–700.
- Gupta, P., Rosen, J.M., D'Eustachio, P. and Ruddle, F.H. (1982) Localization of the casein gene family to a single mouse chromosome. *J. Cell Biol.*, **93**, 199–204.
- Howlett, A.F., Bailey, N., Damsky, C., Petersen, O.W. and Bissell, M.J. (1995) Cellular growth and survival are mediated by β 1 integrins in normal human breast epithelium but not in breast carcinoma. *J. Cell Sci.*, **108**, 1945–1957.
- Holers, V.M., Ruff, T.G., Parks, D.L., McDonald, J.A., Ballard, L.L. and Brown, E.J. (1989) Molecular cloning of a murine fibronectin receptor and its expression during inflammation. *J. Exp. Med.*, **169**, 1589–1605.
- Hungerford, J.E., Compton, M.T., Matter, M.L., Hoffstrom, B.G. and Otey, C.A. (1996) Inhibition of p125FAK in cultured fibroblasts results in apoptosis. *J. Cell Biol.*, **135**, 1383–1390.
- Hynes, R.O. (1996) Targeted mutations in cell adhesion genes: what have we learned from them? *Dev. Biol.*, **180**, 402–412.
- Kreidberg, J.A., Donovan, M.J., Goldstein, S.L., Rennke, H., Shepherd, K., Jones, R.C. and Jaenisch, R. (1996) α 3 β 1 integrin has a crucial role in kidney and lung organogenesis. *Development*, **122**, 3537–3547.
- LaFlamme, S.E., Thomas, L.T., Yamada, S.S. and Yamada, K.M. (1994) Single subunit chimeric integrins as mimics and inhibitors of endogenous integrin functions in receptor localization, cell spreading and migration, and matrix assembly. *J. Cell Biol.*, **126**, 1287–1298.
- Leder, A., Pattengale, P.K., Kuo, A., Stewart, T.A. and Leder, P. (1986) Consequences of widespread deregulation of the *c-myc* gene in transgenic mice: multiple neoplasms and normal development. *Cell*, **45**, 485–495.
- Lukashev, M.E., Sheppard, D. and Pytela, R. (1994) Disruption of integrin function and induction of tyrosine phosphorylation by the autonomously expressed β 1 integrin cytoplasmic domain. *J. Biol. Chem.*, **269**, 18311–18314.
- Lund, L.R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M.J., Dano, K. and Werb, Z. (1996) Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development*, **122**, 181–193.
- Marcantonio, E.E. and Hynes, R.O. (1988) Antibodies to the conserved cytoplasmic domain of the integrin β 1 subunit react with proteins in vertebrates, invertebrates and fungi. *J. Cell Biol.*, **106**, 1765–1772.
- Meredith, J.E., Winitz, S., McArthur, Lewis, J., Hess, S., Ren, X.-D., Renshaw, M.W. and Schwartz, M.A. (1996) The regulation of growth and intracellular signaling by integrins. *Endocrinol. Rev.*, **17**, 207–220.
- Robinson, G.W., McKnight, R.A., Smith, G.H. and Hennighausen, L. (1995) Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development*, **121**, 2079–2090.
- Schwartz, M.A. (1997) Integrins, oncogenes, and anchorage independence. *J. Cell Biol.*, **139**, 575–578.
- Sémat, A., Vasseur, M., Maillat, L., Brulet, P. and Darmon, M. (1984) Sequence analysis of murine cytokeratin endo A (No. 8) cDNA. Evidence for mRNA species initiated upstream of the normal 5' end in PCC4 cells. *Differentiation*, **37**, 40–46.
- Smilenov, L., Briesewitz, R. and Marcantonio, E.E. (1994) Integrin β 1 cytoplasmic domain dominant negative effects revealed by lysophosphatidic acid treatment. *Mol. Biol. Cell*, **5**, 1215–1223.
- Smith, G.H. (1996) Experimental mammary epithelial morphogenesis in an *in vivo* model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. *Breast Cancer Res. Treat.*, **39**, 21–31.
- Sonnenberg, A., Daams, H., van der Valk, M.A., Hilken, J. and Hilgers, G. (1986) Development of mouse mammary gland: identification of stages of differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. *J. Histochem. Cytochem.*, **139**, 787–799.
- 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.
- Stephens, L.E., Sutherland, A.E., Klimanskaya, I.V., Andrieux, A., Meneses, J., Pedersen, R.A. and Damsky, C.H. (1995) Deletion of β 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev.*, **9**, 1883–1895.
- Stöcklin, E., Botteri, F. and Groner, B. (1993) An activated allele of the *c-erbB-2* oncogene impairs kidney and lung function and causes early death of transgenic mice. *J. Cell Biol.*, **122**, 199–208.
- Streuli, C.H., Edwards, G.M., Delcommenne, M., Whitelaw, B.A., Burdon, T.G., Schindler, C. and Watson, C. (1995a) Stat5 as a target for regulation by ECM. *J. Biol. Chem.*, **270**, 21639–21644.
- Streuli, C.H., Schmidhauser, C., Bailey, N., Yurchenko, P., Skubitz, A.P.N., Rockley, C. and Bissell, M.J. (1995b) Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.*, **129**, 591–603.
- 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.
- Sympson, C.J., Bissell, M.J. and Werb, Z. (1995) Mammary gland tumor formation in transgenic mice overexpressing stromelysin-1. *Semin. Cancer Biol.*, **6**, 159–163.
- Talhouk, R.S., Bissell, M.J. and Werb, Z. (1992) Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *J. Cell Biol.*, **118**, 1271–1282.
- Taurig, H.H. (1967a) Cell proliferation in the mammary gland during late pregnancy and lactation. *Anat. Rec.*, **157**, 489–504.
- Taurig, H.H. (1967b) A radioautographic study of cell proliferation in the mammary gland of the pregnant mouse. *Anat. Rec.*, **159**, 239–248.
- van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C.M. and Sonnenberg, A. (1996) Epithelial detachment due to absence of hemidesmosomes in integrin β 4 null mice. *Nature Genet.*, **13**, 366–369.
- Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E. and Giancotti, F.G. (1996) The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell*, **87**, 733–743.
- Weaver, V., Petersen, O., Wang, F., Larabel, C., Briand, P., Damsky, C. and Bissell, M.J. (1997) Reversion of the malignant phenotype of human breast cells in three-dimensional culture and *in vivo* by integrin blocking antibodies. *J. Cell Biol.*, **137**, 231–245.
- 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.
- Zachary, I. and Rozengurt, E. (1992) Focal adhesion kinase (p125FAK): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell*, **71**, 891–894.

Received November 28, 1997; revised February 10, 1998;
accepted February 18, 1998