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Mammary Gland Development in the Mouse

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We have examined the role of integrin–extracellular matrix interactions in the morphogenesis of ductal structures *in vivo* using the developing mouse mammary gland as a model. At puberty, ductal growth from terminal end buds results in an arborescent network that eventually fills the gland, whereupon the buds shrink in size and become mitotically inactive. End buds are surrounded by a basement membrane, which we show contains laminin-1 and collagen IV. To address the role of cell–matrix interactions in gland development, pellets containing function-perturbing anti-β1 integrin, anti-α6 integrin, and anti-laminin antibodies respectively were implanted into mammary glands at puberty. Blocking β1 integrins dramatically reduced both the number of end buds per gland and the extent of the mammary ductal network, compared with controls. These effects were specific to the end buds since the rest of the gland architecture remained intact. Reduced development was still apparent after 6 days, but end buds subsequently reappeared, indicating that the inhibition of β1 integrins was reversible. Similar results were obtained with anti-laminin antibodies. In contrast, no effect on morphogenesis *in vivo* was seen with anti-α6 integrin antibody, suggesting that α6 is not the important partner for β1 in this system. The studies with β1 integrin were confirmed in a culture model of ductal morphogenesis, where we show that hepatocyte growth factor (HGF)–induced tubulogenesis is dependent on functional β1 integrins. Thus integrins and HGF cooperate to regulate ductal morphogenesis. We propose that both laminin and β1 integrins are required to permit cellular traction through the stromal matrix and are therefore essential for maintaining end bud structure and function in normal pubertal mammary gland development.

Key Words: **integrin; mouse mammary gland; morphogenesis; hepatocyte growth factor; extracellular matrix; laminin.**

INTRODUCTION

Tissue morphogenesis relies on the dynamic interactions of cells with each other and with the extracellular matrix (ECM).[2] Key molecules involved in these interactions are the integrin family of cell surface ECM receptors. Most integrin functions have been defined using culture models and blocking antibodies, disintegrins, antisense constructs, or peptides corresponding to binding sites (Adler and Chen, 1992; Lallier *et al.*, 1992; Trikha *et al.*, 1994). Such interference with cell–ECM communication has been demonstrated to affect cell adhesion, migration, differentiation state, gene expression, proliferation, and survival (reviewed in Ashkenas *et al.*, 1996). However, it is still largely unresolved as to whether integrins are involved in similar mechanisms *in vivo*.

A number of studies have explored the effect of injecting integrin-blocking antibodies intravenously. These have concentrated on the subsequent behaviour of cells of the immune system (Abraham *et al.*, 1994; Gorczynski *et al.*, 1995; Weg *et al.*, 1993) or the degree of metastatic spread from primary tumours (Elliott *et al.*, 1994), which are essentially effects on single cells or small groups of cells. However, most cells *in vivo* do not exist singly but rather as

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[2] Abbreviations used: ECM, extracellular matrix; EHS, Engelbreth–Holm–Swarm; H&E, haematoxylin and eosin; HGF, hepatocyte growth factor; TGF, transforming growth factor; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

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a three-dimensional multicellular architecture, and few studies have explored how integrin function relates to the development and maintenance of tissue structure.

To investigate the importance of integrins in tissue morphogenesis, their role in developing systems *in vivo* needs to be examined and to this end, knockout mice have been created by homologous recombination. However, many homozygous integrin deletions are lethal at early stages of development (reviewed by Hynes, 1996, and Fassler *et al.*, 1996). Thus in order to study the function of integrins in late-developing organ systems such as the mammary gland, other approaches are required. The injection of function-blocking antibodies into specific sites of interest has been used in several developmental models. In nonmammalian systems, the presence of anti-β1 integrin antibody blocks interstitial cell migration of *Hydra* grafts *in vivo* (Agbas and Sarras, 1994), while injection of anti-β1 antibodies into the blastocoel of amphibian embryos disrupts gastrulation and mesodermal cell migration (Boucaut *et al.*, 1984; Howard *et al.*, 1992; Johnson *et al.*, 1993). In avian embryos, introduction of antibodies to β1 integrins into cranial neural crest cell migratory pathways results in serious perturbation of normal migration (Bronner Fraser, 1985). However, this approach has not previously been used to investigate integrin function in developing mammalian tissues *in vivo*.

In this study we have used the mammary gland as a model system to examine the role of integrin–matrix interactions in ductal morphogenesis. At birth the murine mammary gland is a simple rudiment consisting of a few ducts that extend from the nipple a short distance into the subcutaneous fat pad. Glandular growth then arrests until the onset of puberty at around 3 weeks of age when cells in bulbous structures at the ductal tips, known as end buds, begin to proliferate, resulting in lengthening and branching of the epithelial ductal network. This growth results in an arborescent network of interconnecting tubes, which eventually fills the subcutaneous fatty stroma of the gland. In areas where there is no room for further ductal expansion, the end buds regress to become quiescent terminal structures. By contrast, active terminal end buds are highly mitotic, multilayered epithelial structures found only during ductal growth. They consist of a teardrop-shaped group of cells containing 6–10 layers of body or luminal cells. This is surrounded by an outer layer of cap cells at the tip of the end bud and myoepithelial cells at its neck and extending along the length of the duct. These lie on a specialised form of ECM, the basement membrane. The duct in turn is ensheathed in a collagenous stroma that extends along its length up to the neck of the end bud. The fibrous stroma may constrict the direction of growth by only allowing ductal elongation from the tip of the end bud (Williams and Daniel, 1983); side branches form in areas of breaks in the sheath. The mammary ductal network is in turn surrounded by the adipose cells of the fat pad.

A number of factors have been shown to affect end bud formation and ductal elongation including growth factors such as transforming growth factor (TGF) α and β, epidermal growth factor, and hepatocyte growth factor (HGF), hormones such as oestrogen and growth hormone, and cell adhesion molecules including E- and P-cadherin (Coleman *et al.*, 1988; Daniel *et al.*, 1995; Kleinberg, 1997; Silberstein and Daniel, 1987; Snedeker *et al.*, 1991; Yang *et al.*, 1995). We now present a study examining the role of integrin–matrix interactions in the developing virgin mammary gland *in vivo*. Earlier work using culture models of primary mammary epithelial cells isolated from pregnant mice has demonstrated a role for integrin–ECM interactions in the suppression of apoptosis and also in milk production at a later stage of mammary gland development (Pullan *et al.*, 1996; Streuli *et al.*, 1991), and β1 integrin has been shown to be required for full pregnancy–associated development *in vivo* (Faraldo *et al.*, 1998). However, it has not previously been determined whether integrin receptors have an additional role in the control of ductal elongation during development in the nonpregnant pubertal virgin animal.

To address this question, we examined the effect of function-blocking anti-β1 and anti-α6 integrin antibodies on ductal morphogenesis and report here for the first time that blocking β1 but not α6 integrin dramatically reduces the number of end buds in the gland *in vivo* compared with controls. Furthermore, we show that the anti-β1 integrin antibody disrupts morphogenesis in a culture model of mammary tubulogenesis. Similar results were also obtained *in vivo* with a function-blocking anti-laminin antibody. These effects were not due to simple disruption of all cell–matrix interactions since the mammary gland retained its histoarchitecture; the effects observed were specific to the developing buds. We therefore propose that contact with the extracellular matrix, and with laminin in particular, via β1 integrins is a key factor in maintaining end bud structure and function, and thus that normal development and ductal morphogenesis of the mammary gland depend on functional β1 integrins. We also demonstrate that the key partner for these interactions is not the α6 integrin subunit.

MATERIALS AND METHODS

Antibody Preparation

The detailed method for the preparation of the anti-β1 integrin antibody is published elsewhere (Edwards and Streuli, 1999). In brief, rat monoclonal antibody PS/2 (anti-α4 integrin) was used to purify $\alpha4\beta1$ integrin from E14.5–16.5 mouse embryo homogenate. After prebleeding, two New Zealand White rabbits were immunized with 50 μg purified $\alpha4\beta1$ integrin and boosted after 12 weeks. Rabbit IgG was purified by protein A chromatography, but not further purified on an integrin column because we wished to retain as much of the integrin function-perturbing component of the antiserum as possible. An antibody to the COOH-terminal domain of the mouse β1 integrin subunit was developed in rabbits and affinity-purified on an integrin peptide column as described previously (Delcommenne and Streuli, 1995). Rabbit polyclonal antibodies against purified Engelbreth–Holm–Swarm (EHS) tumour

laminin-1 and mouse laminin E3 fragment specific for the laminin α1 chain (kind gifts of P. Yurchenko) were also raised in this laboratory. Purified IgG fractions were subsequently affinity-purified on EHS–Sepharose columns. Rat anti-α6 integrin monoclonal antibody (GoH3) was obtained from Serotec (Oxford, UK), as was the appropriate rat IgG2a control antibody.

Pellet Preparation

Elvax pellets were prepared according to the methods of Silberstein and Daniel (1982). Briefly, 10 mg of anti-β1 integrin, anti-laminin, anti-E3, or control rabbit IgG or 1 mg of anti-α6 integrin or control rat IgG2a was added to 20 mg bovine serum albumin (Sigma) and lyophilised. The lyophilate was mixed with 250 μl of a 10% (w/v) Elvax (Dupont, gift of G. Silberstein) solution in dichloromethane and immediately frozen. The resulting pellet was left to dry overnight at −20°C and then vacuum desiccated. Antibody content was calculated as weight of incorporated material (i.e., 10 or 1 mg antibody respectively) per total weight of pellet. The large original pellet was cut into smaller pieces (~1 mm^3) for implantation. The typical amount of antibody implanted in these experiments was 300 μg/pellet piece for rabbit polyclonal or 30 μg/pellet piece for rat monoclonal antibodies.

Implantation of Pellets

Age-matched 5-week-old virgin ICR or MF1 outbred mice were anaesthetised and the ventral skin was retracted to expose both abdominal No. 4 mammary glands. A small pocket was made in the fat pad of one gland proximal to the lymph node (i.e., just in front of the advancing ducts), and an antibody pellet was inserted. The contralateral gland 4 either had no implant or had a control IgG pellet. The skin was then sutured closed. The mice were administered analgesia and left to recover on a heated pad.

BrdU Injection and Detection

To assess proliferation rates in the mammary gland, 10 μl BrdU labelling reagent (Amersham International Plc, Little Chalfont, UK) per gram of mouse body weight was injected intraperitoneally 2 h before the glands were removed from the mouse and processed for whole-mounting, cryoembedding, or paraffin embedding. Detection of BrdU incorporation in paraffin sectioned tissue was performed using an anti-BrdU primary antibody and an alkaline phosphatase-conjugated secondary antibody to cause a colour reaction with DAB-Ni (Amersham). Sections were counterstained with 0.5% methyl green in 0.1 M sodium acetate, pH 4.0, for 15 min. Proliferation indices were calculated as the proportion of labelled cells in an end bud.

Mammary Gland Whole-Mount

Mice were killed by cervical dislocation. The No. 4 glands were removed and spread flat on microscope slides, fixed in 70% ethanol, 5% formalin, 5% glacial acetic acid, and defatted in acetone. Cell nuclei were stained with haematoxylin. The tissue was then dehydrated sequentially in ethanol and cleared in 100% methyl salicylate. Photomicrographs were taken on Kodak Ektachrome 160T film using an Olympus OM4 camera mounted on a Leica dissecting microscope.

Paraffin Embedding

Number 4 glands were removed, cut into pieces, fixed in 4% paraformaldehyde (1 h at 4°C), and embedded in paraffin using standard techniques. Alternatively, for tissue that had already been whole-mounted, pieces of gland were cut from slides and washed in two changes of xylene before being embedded in wax. Five-micrometer sections were cut on a rotary microtome and mounted on uncoated glass slides.

Haematoxylin and Eosin Staining (H&E Staining)

Paraffin sections were dewaxed in xylene and rehydrated through alcohols. After a wash in distilled water, slides were stained for 1 min in Harris's haematoxylin, "blued" in tap water, and stained for 1 min in eosin. Tissue that had been whole-mounted and stored in methyl salicylate before sectioning required longer staining in eosin and less time in haematoxylin.

Assessment of Apoptotic Indices

Apoptosis was quantified by examining H&E-stained paraffin sections under an Olympus microscope. Apoptotic cells were defined as those that had dark, condensed, and lobular nuclei and whose cytoplasm was eosinophilic. In our experience this is a much more reliable and reproducible indicator of apoptosis than TUNEL labelling for this tissue. End buds were identified and the number of apoptotic cells as a proportion of the total number of cells in each end bud section was calculated.

Cryoembedding

Number 4 glands were removed, placed in OCT mounting medium (Tissue-tek), and frozen. Sections (7 or 30 μm) were placed on glass slides, fixed in precooled methanol:acetone 1:1 at −20°C (10 min), and stored at −20°C until required.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Streuli and Bissell, 1990). Anti-β1 integrin antibody was used at 10 μg/ml. Anti-laminin-1 and laminin E3 fragment antibodies were used at 1 and 3 μg/ml, respectively. Rabbit polyclonal serum raised in this laboratory against collagen IV was used at 1:100 dilution. Donkey anti-rabbit FITC-conjugated secondary antibody was used at a concentration of 1:100 (Jackson ImmunoResearch Laboratories, Inc.). Nuclei were counterstained with 4 μg/ml Hoechst 33258 and mounted in 1 mg/ml *p*-phenylene diamine in 10% PBS, 90% glycerol, pH 8.0. Stained sections were examined on a Zeiss epifluorescence microscope and photographed using Tmax 400 film or on a Leica confocal microscope.

Metabolic Labelling and Immunoprecipitation of Integrins

This was performed as previously described (Delcommenne and Streuli, 1995). Briefly, K-1735 M2 melanoma, CID-9, or primary mammary epithelial cells were radiolabelled overnight with [^{35}S]methionine (NEN) and lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 1 mM $CaCl_2$, 1 mM $MgCl_2$ containing 0.45 TIU/ml aprotinin, 10 μM leupeptin, 0.5 μM PMSF, and 1.5 μM

pepstatin A (Sigma). Aliquots of lysates containing equal TCA-precipitable counts were reacted with 10 μg antibody and immune complexes precipitated with protein A–Sepharose (Zymed Laboratories Inc., South San Francisco, CA). The bound complexes were washed and analysed by SDS–PAGE under nonreducing conditions on 6.25% gels. Gels were dried and autoradiographed with Kodak XAR-5 film at −70°C.

Immunoblotting

Purified laminin-1 (0.2 μg) or its E3 component, EHS matrix, and purified collagen IV (Becton Dickinson Oxford, UK) were separated under reducing conditions on 4.0–7.5% SDS–polyacrylamide gradient gels, transferred to Immobilon-P membrane (Millipore LTD., Oxford, UK), incubated with 1 μg/ml affinity-purified anti-laminin or anti-E3 polyclonal antibodies and detected by enhanced chemiluminescence using an ECL kit (Amersham).

Adhesion Assays

First-passage primary mouse mammary epithelial cells, CID-9 or FSK-7 mammary epithelial cell lines (Pullan and Streuli, 1996; 4×10^4 cells/well), were incubated for 60 min in the presence of 0–400 μg/ml anti-β1 integrin, anti-laminin-1, or anti-E3 IgG or 400 μg/ml control rabbit IgG in 96-well dishes (Maxisorb, Nunc, Kampstrup Roskilde, Denmark) coated with physiological substrata (12 μg/ml fibronectin, 2 μg/ml collagen IV, 12 μg/ml laminin-1, and 3 μg/ml vitronectin (all Sigma), or 16 μg/ml collagen I and 1 μg/ml EHS matrix; Pullan and Streuli, 1996). After unattached cells were washed away, adhered cells were fixed, stained with crystal violet, and solubilised in 2 M guanidine hydrochloride before the absorbance was read at 595 nm.

Culture Assay for Mammary Ductal Morphogenesis

TAC-2.1 cells (Soriano *et al.*, 1996), a clonally derived subpopulation of the TAC-2 cell line (Soriano *et al.*, 1995), were cultured in tissue culture flasks (Falcon, Becton Dickinson and Co., San José, CA) coated with collagen I in DME (Gibco, Basel, Switzerland) supplemented with 10% FCS (Gibco), penicillin (110 iu/ml), and streptomycin (110 μg/ml). Recombinant human HGF was a generous gift of Dr. R. Schwall (Genentech Inc., South San Francisco, CA). TAC-2.1 cells were suspended at 6×10^3 cells/ml in collagen gels (500 μl) in 16-mm wells of four-well plates (Nunc) and incubated in 500 μl medium with or without HGF and anti-β1 integrin antibody. Medium and treatments were renewed every 2–3 days. After 9 days, the cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and at least three randomly selected fields (measuring 2.2 mm × 3.4 mm) per experimental condition in each of at least three separate experiments were photographed under bright-field illumination with a Nikon Diaphot TMD inverted photomicroscope. The total length of cords in each individual colony was measured with a Qmet 500 image analyser (Leyca Cambridge Ltd., Cambridge, UK). Cord length was considered as 0 in (a) spherical colonies and (b) slightly elongated structures in which the length to diameter ratio was less than 2. Values for cord length obtained from the largest colonies are an underestimate, since in these colonies a considerable proportion of the cords were out of focus and therefore could not be measured. Values are expressed as mean cord length per photographic field (Soriano *et al.*, 1996). The mean values for each experimental condition were compared to controls using Student's unpaired *T* test.

Analysis of c-Met Phosphorylation

TAC-2.1 cells were cultured on collagen I to subconfluency, serum starved overnight, and treated with 20 μg/ml HGF (R&D Systems, Abingdon, UK) and/or 50 μg/ml anti-β1 integrin antibody or control IgG. Cells were washed in ice-cold PBS containing 1 mM Na_3VO_4, 1 mM NaF (Sigma) and their contents were extracted with lysis buffer (1% Triton X-100, 1 mM PMSF, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 10 mM leupeptin and aprotinin). After the detergent insoluble proteins were cleared by centrifugation, equal amounts of protein (as estimated by Coomassie-stained gel electophoresis) were immunoprecipitated with anti-c-Met antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or control rabbit IgG followed by protein A–Sepharose (Zymed) before separation by 7.5% SDS–PAGE. After transfer to Immobilon-P membrane (Millipore), phosphorylated proteins were revealed with the anti–phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) followed by enhanced chemiluminescence using an ECL kit (Amersham). Blots were stripped according to the Amersham protocol and reprobed with precipitating antibody.

RESULTS

Distribution of Extracellular Matrix Components and α6 and β1 Integrins within Virgin Mammary Gland

The end buds and ducts of normal virgin mammary glands are surrounded by a sheath of ECM containing both laminin and collagen IV (Figs. 1A–1D). Laminin-1 was adjacent to the end buds as it was recognised both by the anti-laminin-1 antibody and by the anti-laminin α1 chain antibody raised against its E3 domain. Collagen IV was present at the end bud tips, although it was thinner in this region. The only previous study carefully examining the distribution of ECM proteins around end buds, rather than other types of mammary epithelial structures, did not detect basement membrane components around the distal end bud region (Sonnenberg *et al.*, 1986), but our investigations using confocal microscopy indicate that the proteins are indeed present.

Staining for β1 integrins was seen throughout the end bud, particularly strongly at the basal surface of both the end bud epithelium and the subtending ducts (Figs. 1E–1G). This corresponds to the cap cell layer in end buds and the myoepithelial cell layer of ducts (Williams and Daniel, 1983). In addition, β1 integrins were present at sites of cell–cell interaction within the luminal epithelium, and occasional cells within end buds were strongly stained (Fig. 1G). There was weak immunoreactivity with stromal cells. β1 integrins have not previously been examined in mouse mammary gland, but their localisation is similar to that observed in human breast lobules (Zutter *et al.*, 1990). α6 integrin was present around all epithelial cells of the mammary gland and in the stroma (data not shown), as

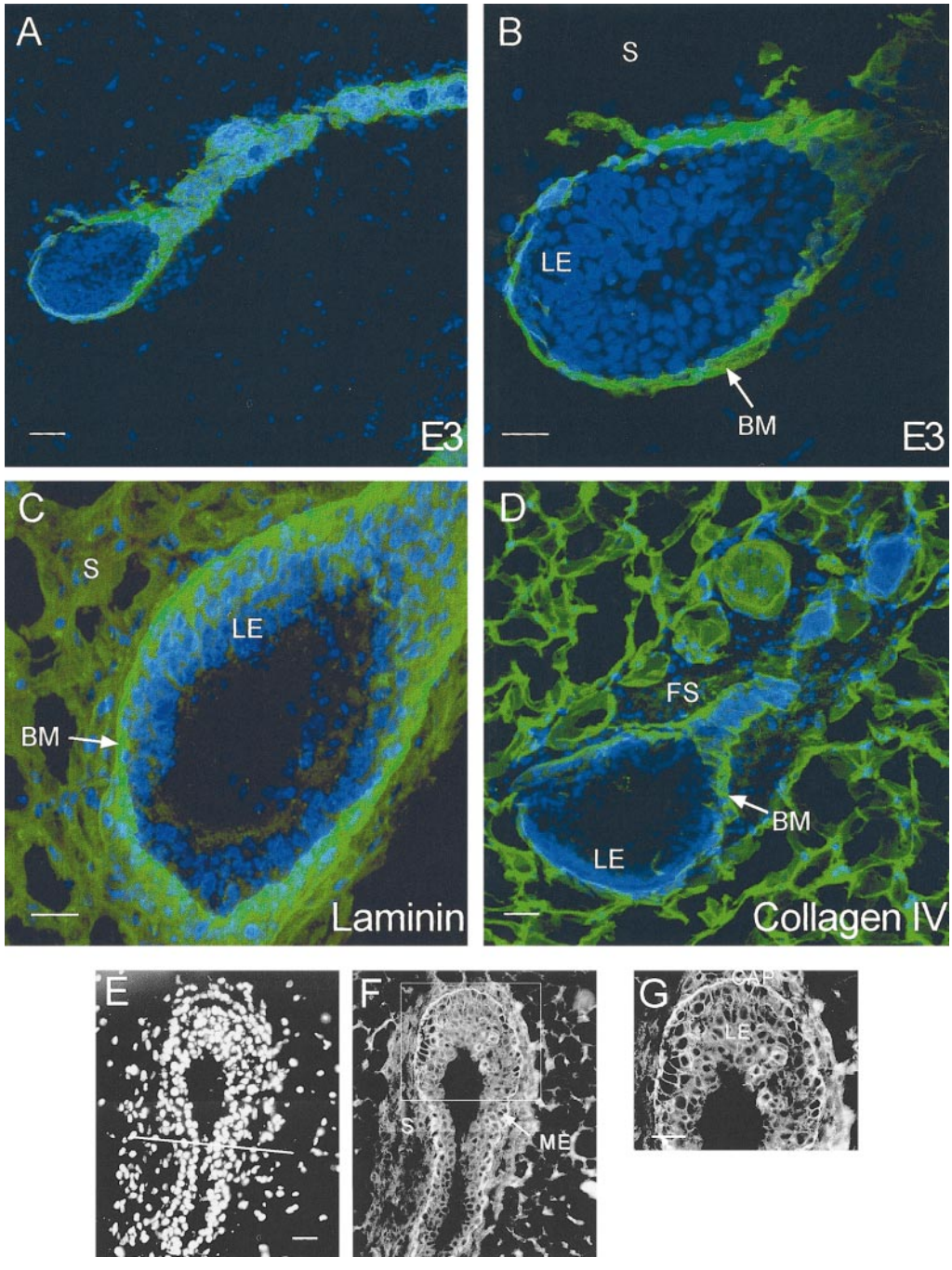

FIG. 1. Immunolocalisation of extracellular matrix molecules and β1 integrins in the virgin mammary gland. (A–D) Thick cryosections (30 μm) of virgin mammary gland were stained for (A, B) the E3 domain of the laminin α1 chain, (C) laminin-1, and (D) collagen IV. (B) is a higher power image of (A). Sections were counterstained with Hoechst 33258 (blue) to detect nuclei. Projections of 10 z-sections (3 μm steps) obtained by confocal microscopy are shown. Note staining for (A, B) E3 in a complete sheath of basement membrane surrounding the end bud, but not in the stroma compared (C) with laminin-1 showing staining in both the basement membrane and the surrounding stroma. (D) Collagen IV is present in the end bud basement membrane as well as in the fibrous stroma encasing the duct. Note the lack of fibrous stroma at the end bud tip. (E–G) Thin cryosection (7 μm) of virgin mammary gland stained (E) with Hoescht 33258 and (F) with anti-β1 integrin antibody. Line in (E) indicates approximate demarcation between end bud and subtending duct. (G) An enlargement of the boxed area in (F). Strong staining for β1 integrins is seen at the basal surface of the end bud epithelium and at sites of cell–cell interaction in the basal layer. Luminal epithelial (LE), cap (CAP) and myoepithelial (ME) cells as well as stroma (S) are positively stained. BM, basement membrane; FS, fibrous stroma. Bars, 50 μm (A and C); 25 μm (B, D, E, F); 20 μm (G).

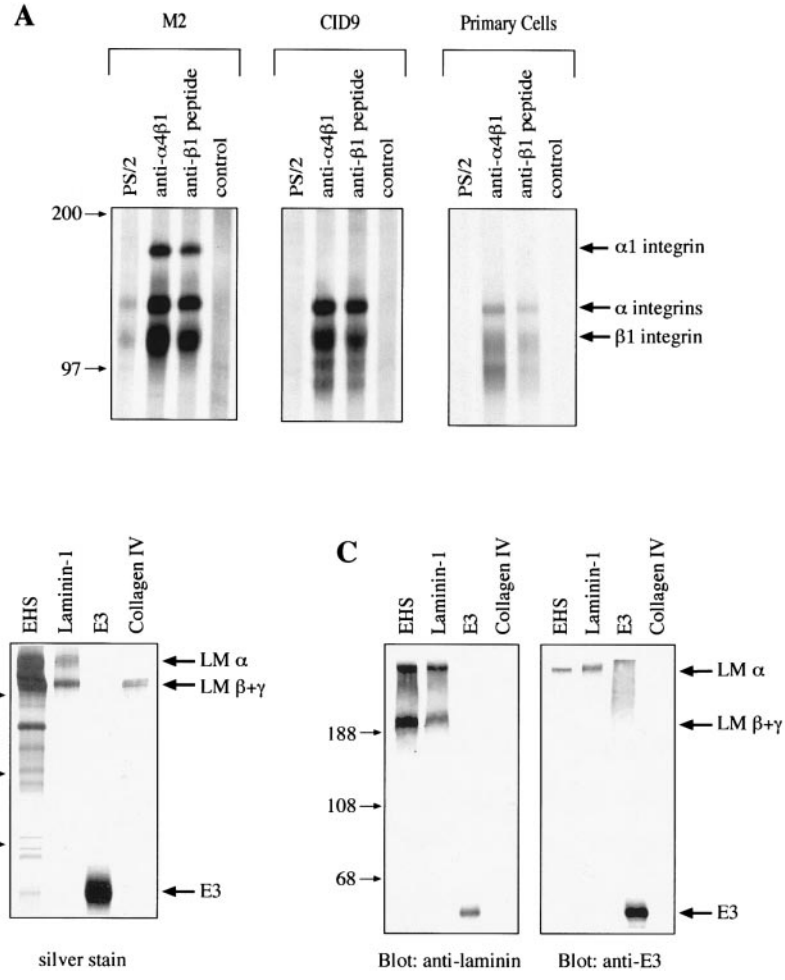

FIG. 2. Immunoreactivity of the anti-β1, anti-laminin, and anti-E3 antibodies. (A) M2 melanoma cells, CID-9 cells, and mouse mammary epithelial cells were radiolabelled overnight with [^{35}S]methionine. Equal amounts of TCA-precipitable counts (5×10^6 cpm/lysate) were immunoprecipitated with 10 μg PS/2 (anti-α4 integrin antibody), polyclonal anti-α4β1 integrin antibody, anti-β1 integrin cytoplasmic domain peptide antibody, or control IgG and analysed by gel electrophoresis under nonreducing conditions and autoradiography. The polyclonal anti-α4β1 antibody precipitated a similar profile of integrins to the anti-β1 integrin peptide antibody. In mammary cells and CID-9 cells the identity of the bands with a lower molecular weight than β1 integrin is unknown but is likely to represent precursor proteins. Neither CID-9 nor primary mammary epithelial cells expressed α1 or α4 integrin subunits, both of which were present in M2 cells. Thus in mammary tissue the anti-α4β1 antibody is specific for β1 integrins. (B, C) 0.2 μg of purified collagen IV, laminin-1 E3 fragment, or EHS matrix was separated by SDS–PAGE under reducing conditions and analysed by silver staining (7.5% SDS–PAGE; B) or immunoblotting with anti-laminin or anti-E3 fragment polyclonal antibodies (4.0–7.5% SDS–PAGE; C). Note that the anti-laminin antibody reacts with α, β, and γ laminin chains as well as the purified E3 fragment of the laminin α1 chain, whereas the anti-E3 antibody reacts only with the α1 chain or the E3 fragment. Neither antibody shows any reaction with collagen IV. Molecular weight markers are indicated by arrows.

previously reported (Sonnenberg *et al.*, 1986). The distribution pattern of both α6 and β1 suggests integrin involvement in the interaction of mammary myoepithelial cells with basement membrane and possibly in cell–cell interactions. To further investigate the role of integrins in virgin mammary development we required function-blocking integrin antibodies. A suitable reagent for α6 had already been raised (Sonnenberg *et al.*, 1986), and we developed our own antibody to mouse β1 integrin.

Characterisation of the Anti-β1 Integrin and Anti-laminin Antibodies

Immunoprecipitation. Our polyclonal anti-β1 integrin antibody was raised against purified mouse α4β1 integrin (Edwards and Streuli, 1999). In immunoprecipitation studies using [^{35}S]methionine-labelled K-1735 M2 melanoma cells, the CID-9 mammary epithelial cell line, and primary mouse mammary epithelial cells, this antibody precipitated

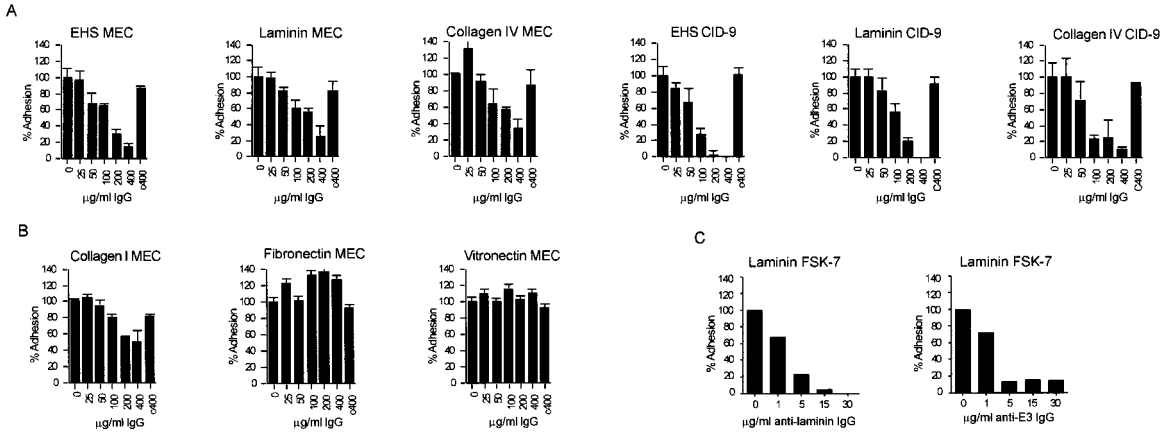

FIG. 3. Anti-β1, anti-laminin, and anti-E3 antibodies block mammary cell adhesion to extracellular matrix. (A, B) Anti-β1 integrin antibody blocks cell adhesion to basement membrane but not stromal matrices. (C) Anti-laminin and anti-E3 antibodies block adhesion to laminin. Secondary mouse mammary epithelial cells (MEC), CID-9, or FSK-7 cells were incubated for 60 min at 37°C in the presence of the indicated concentrations of (A, B) anti-β1 integrin or control IgG (indicated by c400) or (C) anti-laminin or anti-E3 antibodies in 96-well dishes coated with (A) basement membrane components, i.e., EHS matrix, purified laminin-1, or collagen IV; (B) stromal components, i.e., collagen I, fibronectin, vitronectin; or (C) laminin. After the dishes were washed, adherent cells were stained with crystal violet and then solubilised and the absorbance was read at 595 nm. Adhesion is plotted as a percentage of maximum in the absence of inhibiting antibody. Bars in (A, B) indicate SEM.

a similar profile of integrins to an antibody raised against the COOH domain of the β1 integrin subunit and did not detect any other proteins (Fig. 2A). Both antibodies precipitated β1-associated α integrins that migrate at ~150 kDa and, in M2 cells, α1 integrin (Delcommenne and Streuli, 1995). α4 integrin has previously not been detected in mammary epithelial cells (D'Ardenne *et al.*, 1991; Delcommenne and Streuli, 1995) and is normally associated with cells of haematopoietic origin (Hemler *et al.*, 1987). We confirmed this using PS/2, a monoclonal antibody specific for the mouse α4 integrin subunit, which precipitated α4β1 integrin from M2 cells but not from CID-9 or primary mouse mammary epithelial cells (Fig. 2A). In addition, PS/2 did not immunostain epithelial cells in sections of mammary gland tissue but did stain cells within lymph nodes (data not shown). Thus, as far as we can determine, α4 integrin is not expressed in normal mammary gland. This indicates that in this tissue, the anti-β1 integrin antibody is specific only for β1 integrin subunits.

Immunoblotting studies showed that the affinity-purified anti-laminin-1 antibody recognised only laminin α, β, and γ chains in purified laminin-1 and in EHS matrix, not collagen IV (Figs. 2B and 2C). Similarly, affinity-purified anti-E3 antibody detected only laminin α1 chain and purified E3 fragment, not collagen IV or other laminin chains (Figs. 2B and 2C).

Effects on cell adhesion in vitro. The anti-β1 integrin antibody blocked adhesion of both first-passage mouse mammary epithelial cells and a mammary epithelial cell line to a reconstituted basement membrane matrix (EHS matrix) and to its purified components, laminin-1 and collagen IV (Fig. 3A). The antibody also partially blocked adhesion to the stromal ECM protein, collagen I, but had no effect on the adhesion of mammary cells to fibronectin or vitronectin, possibly because the cells on these substrata adhered via β3 integrins (Fig. 3B). Addition of anti-β1 integrin antibody, with or without lyophilisation and resuspension, to subconfluent monolayers of CID-9 cells caused cells to detach from basement membrane, fibronectin, collagen I, or bovine serum albumin coated coverslips (data not shown). Cells did not detach in the presence of control IgG or with no antibody added. Thus, this reagent is a novel function-blocking antibody specific for mouse β1 integrins, and it both interferes with attachment of mammary cells to ECM proteins and disrupts established adhesion of cells in culture.

The anti-laminin-1 and anti-E3 antibodies blocked adhesion of mammary epithelial cells to purified laminin (Fig. 3C) but not to collagen IV (data not shown). GoH3 anti-α6 integrin antibody has previously been demonstrated to have function-blocking activity (Sonnenberg *et al.*, 1988), which we have confirmed in studies with T47D mammary epithelial cells (data not shown).

Antibody release from Elvax pellets. To study the effects of the anti-integrin and anti-laminin antibodies on development *in vivo*, each was separately incorporated into slow-release plastic (Elvax) pellets and implanted into the developing mouse mammary gland (Silberstein and Daniel, 1982). To determine antibody release kinetics, and to test activity on release, a pellet containing approximately 300 μg anti-β1 integrin IgG was placed in 100 μl PBS and incubated at 37°C. The PBS was replaced every day. These

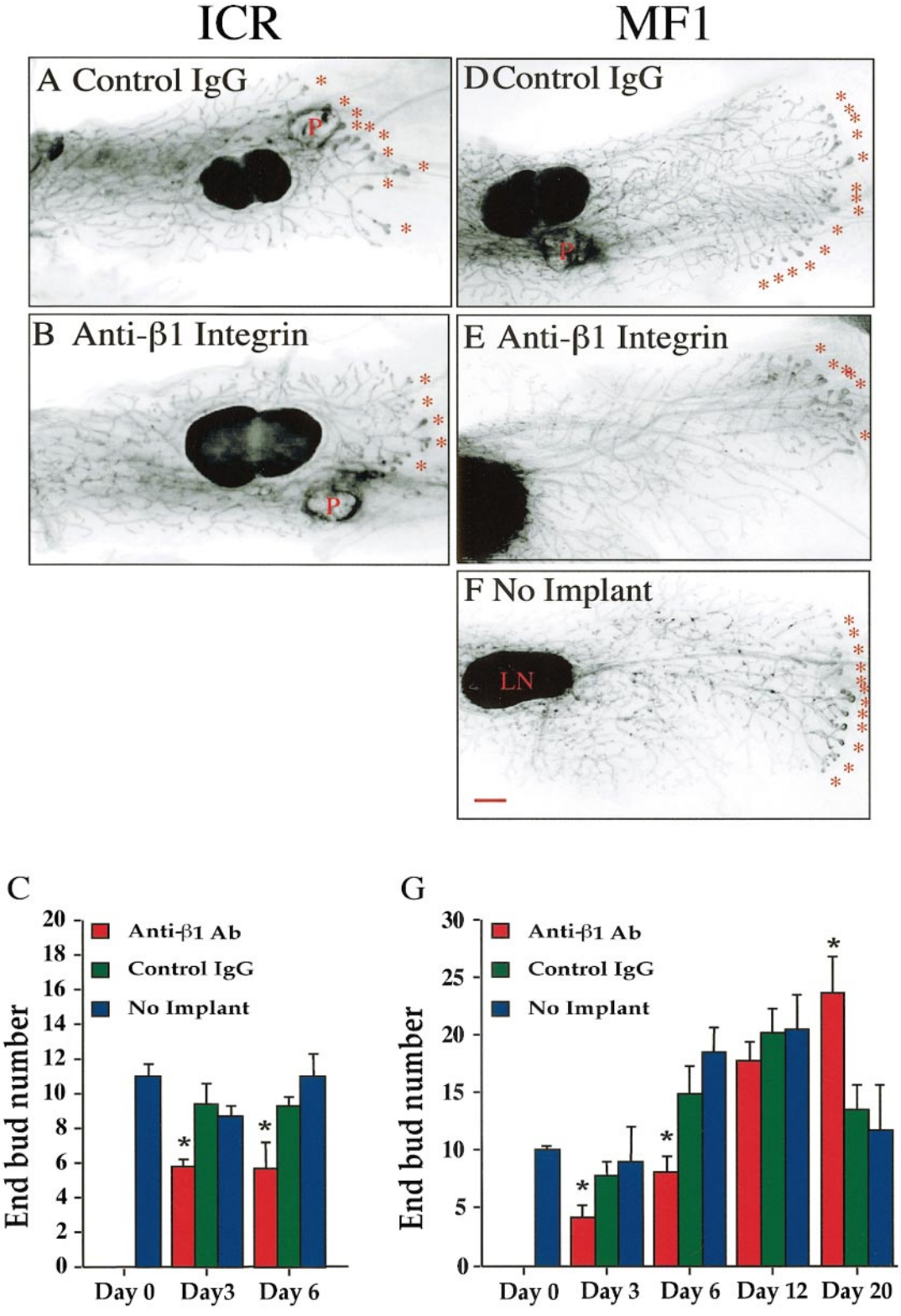

FIG. 4. The presence of anti-β1 integrin antibody reduces the number of end buds in the virgin mammary gland of (A–C) ICR or (D–G) MF1 mice. (A, B, D, E, F) Photomicrographs of whole-mounted glands after 6 days in the presence of (A, D) control IgG or (B, E) anti-β1 antibody or (F) with no implant, stained with haematoxylin. Asterisks indicate end buds, P indicates pellet, LN indicates lymph node. In

extracts were then used to immunoprecipitate integrins from [^{35}S]methionine-labelled CID-9 cell extracts. In this solution assay, the majority of integrin antibody was released within 1 day and continued to be released for at least a further day (data not shown).

To examine antibody release and distribution *in vivo,* Elvax pellets containing the different antibodies or their controls were implanted into the No. 4 mammary glands of 5-week-old mice, close to or just in front of the advancing end buds. Cryosections of glands harvested at various times after implantation were stained by immunofluorescence using an FITC–conjugated secondary antibody (data not shown). All the antibodies tested were still present in the implanted glands after 6 days and showed a uniform distribution throughout the gland. Control IgG was present throughout the mammary gland, but was diffuse and did not stain any particular area of the tissue. In contrast, anti-β1 integrin IgG was strongly localised to the basal surfaces of ductal epithelium, indicating that the anti-β1 integrin antibody crossed the basement membrane and contacted the epithelial cells. Antibody was not seen in the contralateral unimplanted gland and therefore did not appear to enter the bloodstream. The antibody was eventually cleared from the mammary gland, since no IgG was detected using this method 10 days after implantation. In similar experiments performed with pellets containing anti-α6 integrin or anti-laminin antibodies, staining was localised to the surface of epithelial cells and to the basement membrane, respectively.

Together our data indicate that all the antibodies used in this study are function-blocking since they block mammary cell adhesion *in vitro,* and their adhesion-perturbing effects are substrate specific. Antibody is released from Elvax pellets in an active state *in vitro* and is able to penetrate the mammary gland *in vivo* and localise to epithelial cells in regions where integrins are associated with cell–ECM interactions.

Anti-β1 Integrin Antibody Dramatically Alters Mammary Gland Morphogenesis in vivo

End bud number. To determine the effect of function-blocking anti-β1 integrin antibody on mammary gland morphogenesis *in vivo,* one of the fourth (abdominal) pair of mammary glands of 5-week-old mice was implanted with an Elvax pellet containing approximately 300 μg anti-β1 integrin antibody. Contralateral No. four glands were implanted with control rabbit IgG or were left unimplanted. In the first set of experiments the ICR mouse strain was used. Pellets were left in place for up to 6 days before the glands were removed for whole-mounting and haematoxylin staining (Figs. 4A and 4B). The number of end buds in control and anti-β1 integrin implanted mammary glands was quantitated. Compared to the number at day 0, the start of the experiment, a dramatic (48%) reduction in the number of end buds was seen after 3 days with anti-β1 integrin antibody ($P < 0.001$; Fig. 4C). In addition, compared with IgG, a 39% reduction in the number of end buds at day 3 and day 6 of anti-β1 integrin implantation was observed ($0.05 > P > 0.01$).

The mammary gland ductal network expands over this 6-day period (from postnatal day 35 to 41) to fill the fat pad more completely, but in ICR mice the number of end buds remained fairly constant during this time and was not significantly affected by control IgG (Fig. 4C, compare end bud number with no implant on day 0 with that on day 6). However, throughout this time there were fewer end buds in the presence of antibody, indicating that inhibiting integrin function caused a decrease in end bud number. This reduction in end bud number did not appear to be localised to the immediate vicinity of the pellet. End buds that remained were seen close to the pellet (Fig. 4B) and further away from it (Fig. 4E).

To examine whether the results were strain dependent and to extend our data, we repeated these experiments using MF1 mice. A similar β1-integrin-dependent inhibition of end buds was observed. Results similar to those using the ICR strain were obtained (Figs. 4D–4G). In contrast to ICR, end bud number in MF1 mice doubled between postnatal days 35 and 47 before declining and was not statistically affected by the presence of normal IgG (see Fig. 4G; no implant and IgG bars). Again a reduction in the number of end buds after 3 days treatment with anti-β1 integrin IgG was observed (41% of those at day 0, $P < 0.001$). The number of end buds was reduced in comparison to the normal IgG controls by 47% on day 3 and 46% on day 6 post-pellet implantation ($0.05 > P > 0.01$). So in MF1 mice as well as in ICRs, we have demonstrated that blocking β1 integrin function results in the regression of existing end buds as well as the inhibition of new end bud formation.

It is important to note that anti-β1 integrin antibody did not affect the overall integrity of the ductal network within the mammary gland; its action appeared specific and affected only end buds. We also discovered that the inhibition was transient since 12 days after pellet implantation the gland began to recover. We had already shown that at 12

(E), the implanted pellet was located to the bottom left of the lymph node and was just out of frame of the photograph. Scale bar, 900 μm. (C, G) Graphs showing the effect of Elvax pellets containing anti-β1 integrin antibody or preimmune IgG on the average number of end buds per mammary gland in virgin (C) ICR or (G) MF1 mice compared with unimplanted glands over up to 20 days *in vivo.* Asterisks indicate statistically significant differences by Student's *T* test. ICR and MF1 day 3 with β1 pellet vs day 0 ($P < 0.001$) and day 3 IgG ($0.05 > P > 0.01$), and day 6 β1 vs day 6 IgG ($0.05 > P > 0.01$). MF1 day 20 β1 vs day 20 IgG ($0.05 > P > 0.01$). Numbers of glands in each group are 29, 8, 8, 6, 6, 6, and 5, respectively, for ICR and 49, 11, 10, 9, 10, 10, 7, 4, 4, 4, 4, 4, and 4 for MF1. Bars indicate SEM.

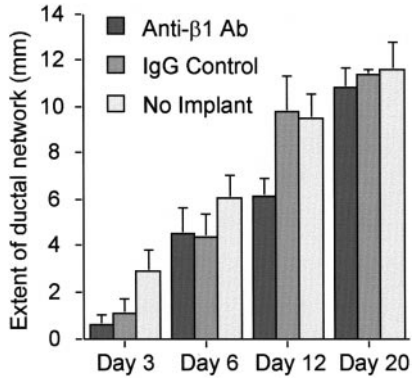

FIG. 5. The presence of anti-β1 integrin antibody reduces the extent of the mammary ductal network. Graph showing the effect of Elvax pellets containing anti-β1 integrin antibody or control IgG on the extent of the ductal network in virgin MF1 mice compared with unimplanted glands over 20 days *in vivo*. Bars indicate SEM. Numbers of glands in each group are 11, 10, 3, 11, 8, 7, 4, 4, 3, 4, 2, and 2, respectively.

days, the antibody was depleted from the gland, as it was not detected by immunofluorescence. In MF1 mice, the end bud number with anti-β1 integrin implanted for 12 days was only fractionally lower than that of IgG controls (Fig. 4G). By 20 days postimplantation, glands with anti-β1 integrin antibody still had a large number of end buds, indicating their continuing recovery from growth inhibition in the presence of antibody. By contrast, end buds in the IgG implanted controls had regressed, leaving only terminal ductal structures, since insufficient gland-free stroma remained for continued ductal elongation. In further experiments to examine the effect of using higher doses of antibody (600 μg per implant) for up to 3 days, we found that a similar (approximately 50%) amount of inhibition of growth was seen (data not shown). Since our antibody is polyclonal, future experiments to test whether monoclonal anti-mouse integrin antibodies inhibited end bud structures completely would be beneficial. No significant effect earlier than 3 days post-pellet implantation was observed by whole-mount analysis or in H&E-stained sections.

Extent of mammary ductal network. To assess the extent of the ductal network, the distance from the middle of the central lymph node of the gland to the tip of the furthest migrated duct was measured on whole-mounted mammary glands. We found that the decrease in the number of end buds seen at 6 days post-pellet implantation (Fig. 4) translated into a decrease in the extent of the ductal network several days after this (Fig. 5, day 12; compare β1 integrin with IgG and no implant). The recovery in the number of end buds seen at day 12 in the anti-β1 integrin-treated glands (Fig. 4G) was reflected by a subsequent recovery of the extent of the ductal network to control levels by day 20 postimplantation (Fig. 5). This supports the current view that ductal elongation occurs through proliferation of cells in the end bud and extension at this region. Since end bud number recovers at around this time (Fig. 4G), we would expect this to be reflected in an increased ductal network several days later, and indeed this is what was observed (Fig. 5; compare β1 integrin with IgG or control on day 20).

Together, the *in vivo* morphological studies indicated that the anti-β1 integrin antibody caused end buds to regress significantly up to 6 days postimplantation, leading to a measurable difference in the elongation of the ductal network by day 12. As the antibody in glands implanted with Elvax pellets became depleted, the glands recovered and the final extent of growth appeared normal.

Although the anti-α4 integrin component of the polyclonal anti-α4β1 serum should not affect mammary cells as they do not express α4, it may have affected other structures within the gland since, for example, α4 integrin is expressed on the surface of circulating lymphocytes. To control for this possibility, pellets containing 300 μg PS/2 (a function-blocking monoclonal anti-mouse α4 integrin antibody) or control rat IgG2b were implanted into the mammary glands of 5-week-old mice, exactly as before, and left for 6 days. No effect on the number of end buds or on the distance of ductal migration was observed (data not shown).

Taking these results together we conclude that functional β1 integrins are required for maintenance of end bud structure and morphological development of the mammary gland.

Implanted Glands Display No Obvious Differences in Cellular Organisation, Proliferation, or Apoptosis

Our data show that implanting anti-β1 integrin antibody into developing mammary gland produced obvious macroscopic effects and we therefore attempted to determine the mechanism underlying these changes using several approaches. It was possible that the antibody caused an inflammatory reaction in the gland or alternatively it might have increased apoptosis or decreased cell proliferation in developing end buds, thereby causing them to regress. H&E-stained paraffin sections of glands implanted with anti-β1 integrin antibody showed no obvious infiltration of inflammatory cells, indicating that the effects on ductal morphology were unlikely to be due to an immune response. The lack of PS/2 staining in treated glands also confirmed the absence of lymphocytes (data not shown).

At the cellular level, no gross differences in morphology were observed by H&E staining in the end buds of control animals or in the remaining end buds of anti-integrin-treated glands (Figs. 6A and 6B). End buds did not show a disrupted organisation of cells as they do in the presence of anti-cadherin antibody (Daniel *et al.*, 1995). The tube within a tube structure of myoepithelial cells surrounding the epithelial core was retained in treated and control tissue (Figs. 6C and 6D). In fact, in the end buds that remained there was no obvious difference in morphology and no increase in the thickness or distribution of the surrounding matrix. Immunofluorescent staining of cryosections of

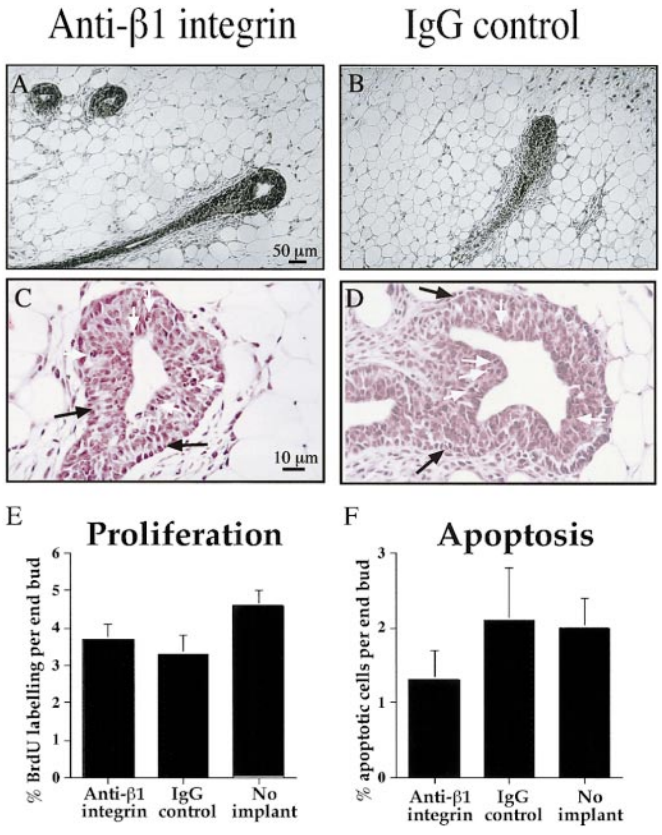

FIG. 6. Treated glands have no obvious inflammatory infiltrate, do not exhibit any aberrant organisation of cells, and do not show altered levels of proliferation or apoptosis in end buds. H&E-stained paraffin sections of (A, C) anti-β1 antibody or (B, D) control IgG-treated glands. Black arrows indicate myoepithelial cells. White arrows indicate apoptotic cells. Graphs showing the effect of anti-β1 antibody or control IgG on (E) the proliferation and (F) apoptotic indices in end buds of virgin MF1 mice compared with unimplanted glands over 6 days *in vivo*. Proliferation and apoptotic indices were calculated as the percentage of BrdU labelled cells or apoptotic figures to the total number of nuclei within the end bud sections. Bars indicate SEM. Numbers of end buds counted for each group were (E) 36, 29, and 32 and (F) 18, 13, and 18, respectively.

glands with antibodies against E-cadherin (ECCD-1) and α6 integrin (GoH3) showed no difference in the distribution of these markers in tissue implanted with anti-β1 antibody compared with controls (data not shown). Glands implanted with anti-β1 integrin antibody also showed similar cell proliferation (as shown by BrdU incorporation; Fig. 6E) and apoptotic indices (as shown by apoptotic figures seen in H&E-stained sections; Fig. 6F) in end buds compared with unimplanted or IgG implanted glands. These results suggest that in the structures we have been able to analyse, the anti-β1 integrin antibody did not affect proliferation or apoptosis. Therefore, to examine the effect of anti-β1 integrin antibody in more detail, we switched to an *in vitro* model of mammary ductal development.

Anti-β1 Integrin Antibody Perturbs Tubulogenesis of Mammary Cells in Culture

Extensive branching morphogenesis of TAC-2 mouse mammary epithelial cells can be induced in three-dimensional collagen gel culture where the cells deposit their own laminin-containing basement membrane in response to HGF and form elaborately arborised tubules (Soriano *et al.*, 1995). We therefore assessed whether anti-β1 integrin antibody could affect this tubulogenic process. Addition of increasing concentrations of anti-β1 integrin antibodies to TAC-2.1 cells grown in the presence of HGF resulted in a progressive inhibition of tube formation (Fig. 7). At 50 μg/ml the anti-β1 integrin antibody totally suppressed morphogenesis, resulting in the formation of spherical cell colonies (Fig. 7F). Quantitative analysis demonstrated that the anti-β1 integrin antibody induced a significant ($P < 0.001$) dose-dependent decrease in total cord length (Fig. 7G).

We also examined whether the anti-β1 integrin antibody could inhibit spontaneous cord formation. When suspended in collagen gels in the absence of HGF, TAC-2.1 cells formed small colonies with morphologies ranging from spherical aggregates to short, poorly branched cords (Soriano *et al.*, 1996). Incubation with increasing concentrations of anti-β1 integrin antibody resulted in a dose-dependent inhibition of spontaneous cord elongation (Fig. 7G). In addition, the anti-tubulogenic activity of the anti-β1 integrin antibody was not restricted to TAC-2 cells, since it inhibited spontaneous cord formation by two distinct subclones (Montesano *et al.*, 1998) of the EpH4 mammary epithelial cell line (Fialka *et al.*, 1996; data not shown).

Our results *in vivo* suggested that as antibody became depleted from the gland, the number of end buds and the extent of the ductal network recovered. We therefore assessed whether the inhibitory effect of the anti-β1 integrin antibody was also reversible in the culture model. TAC-2 cells were grown for 6 days in the presence of HGF to allow the formation of branched cords (Figs. 8A and 8B), at which time anti-β1 integrin antibody was added to the cultures. This induced the retraction of cell cords towards the centre of the colony (Figs. 8C and 8D), resulting in the formation of stubby cords (Fig. 8E) or rounded cell aggregates (Fig. 8F). Cord formation resumed promptly after antibody washout (Figs. 8G and 8H), giving rise to thick, branched cords with a three-dimensional morphology similar to that of the original colonies, thus demonstrating the reversibility of the antibody effect.

Together these data show that in a culture model of mammary tubulogenesis that closely mimics the formation of ducts *in vivo*, functional β1 integrins are required for duct formation. This effect was reversible both *in vivo* and in the culture model.

Anti-β1 Integrin Antibody Does Not Affect Tyrosine Phosphorylation of the c-Met Receptor

Mammary morphogenesis is dependent not only on β1 integrins but also on HGF, both *in vivo* (Yang *et al.*, 1995)

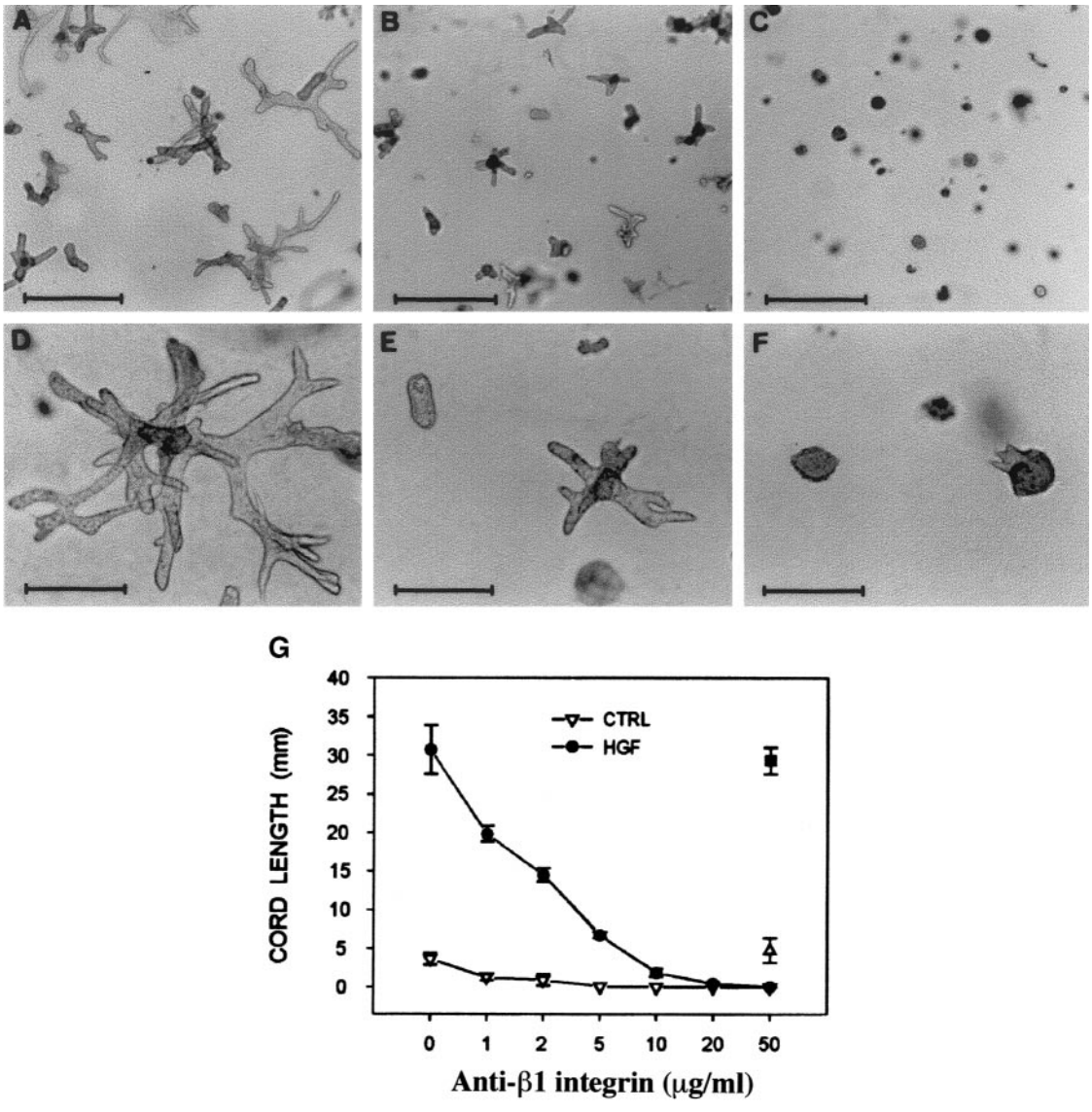

FIG. 7. Antibody against β1 integrins inhibits the tubulogenic activity of HGF. TAC-2.1 cells suspended in collagen gels were grown for 9 days in the presence of (A, D) 10 ng/ml HGF alone, (B, E) HGF and 2 μg/ml anti-β1 integrin antibody, or (C, F) HGF and 10 μg/ml anti-β1 integrin antibody. Note that TAC-2.1 cells incubated with HGF alone form branching cords, but that addition of 2 μg/ml anti-β1 integrin antibody results in a marked reduction in cord length. Addition of 10 μg/ml anti-β1 integrin antibody totally suppresses branching morphogenesis and results in the formation of spherical cell colonies. Bars, 500 μm (A–C); 200 μm (D–F). (G) Quantitative analysis of the effect of anti-β1 antibody. TAC-2.1 cells in collagen gels incubated for 9 days with 10 ng/ml HGF and the indicated concentrations of anti-β1 integrin antibody (filled circles) or control IgG (filled square), anti-β1 integrin antibody alone (inverted open triangle), or control IgG alone (open triangle). In each of at least three separate experiments, three randomly selected fields were photographed, and the total additive length of all cords in each field was determined as described under Materials and Methods.

and in the culture model (Soriano *et al.*, 1995; Fig. 8). Since mammary cell interactions with the ECM affect the ability of soluble ligands such as prolactin and insulin to trigger their downstream intracellular phosphorylation cascades (Edwards *et al.*, 1998; Farrelly *et al.*, 1999), we asked whether integrin function was required for HGF to trigger tyrosine phosphorylation of its cell surface receptor, c-Met. TAC-2.1 cells grown in monolayer culture on collagen were stimulated for 15 min with HGF after incubation for up to 24 h with anti-β1 integrin antibody. Phosphorylation of the c-Met receptor was rapid and sustained, occurring within 6 min of HGF treatment (Fig. 9A). TAC-2 cells cultured in monolayer with 50 μg/ml anti-β1 integrin antibody became rounded, but at this concentration of antibody they did not

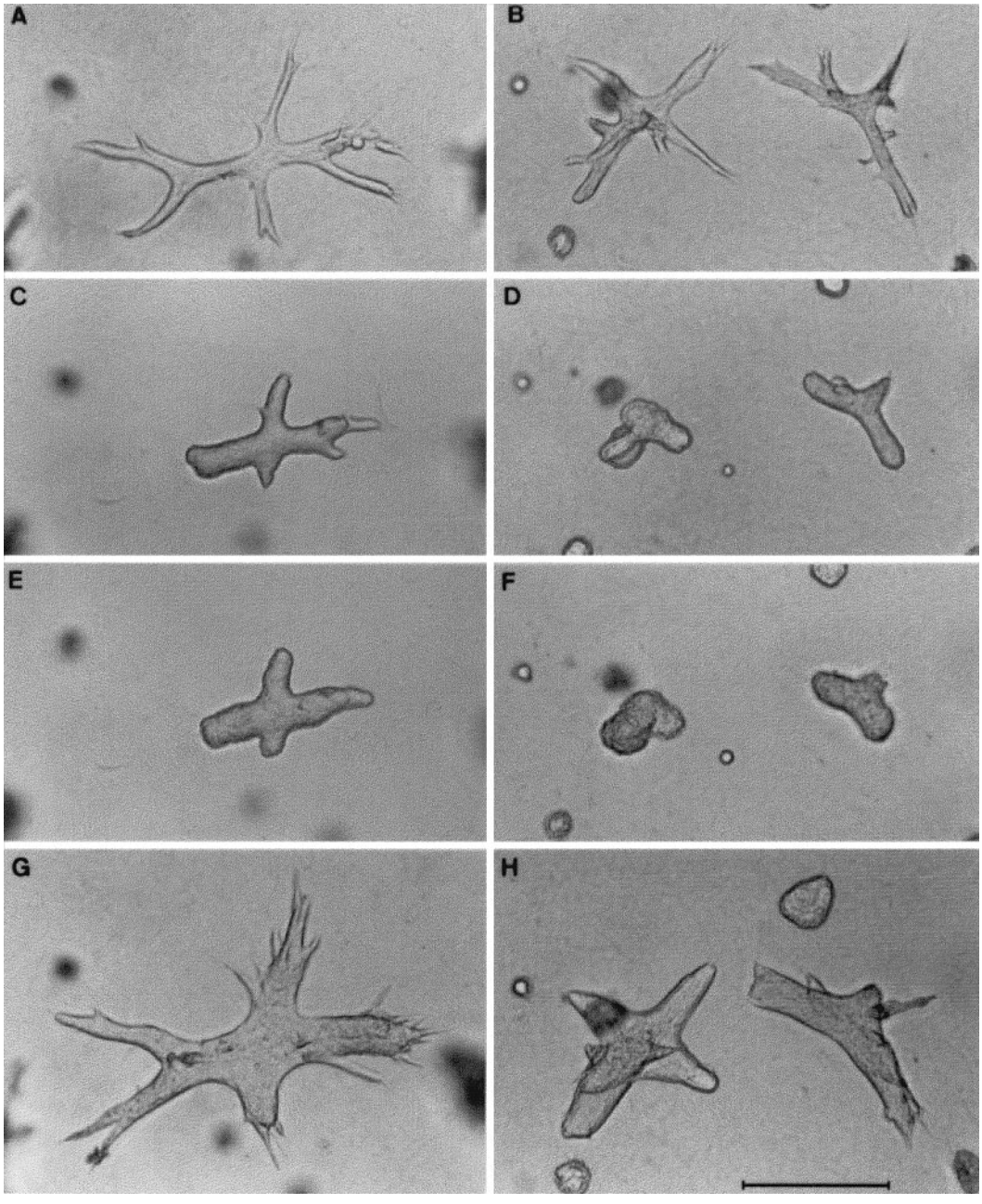

FIG. 8. The effect of anti-β1 integrin antibody is reversible. (A, B) TAC-2.1 cells suspended in collagen gels were grown in the presence of 10 ng/ml HGF alone for 6 days. At this time 50 μg/ml anti-β1 integrin antibody, together with HGF, was added to the cultures, and the same multicellular structures as those shown in (A, B) were photographed after (C, D) 12 h or (E, F) 24 h. (G, H) After 24 h the antibody was extensively washed out, and cultures were further incubated with HGF alone for 24 h. (A, C, E, G) and (B, D, F, H) show the progress of three different colonies during the experiment. Note that TAC-2.1 cells incubated with HGF alone formed branching cords, whereas addition of anti-β1 integrin antibody resulted in a marked reduction in cord length and branching after 12 h. 24 h after antibody withdrawal, elongation and branching resumed. Bar, 200 μm.

detach from the substratum. Under these conditions, tyrosine phosphorylation of c-Met was still evident after HGF treatment (Fig. 9B), and similar results were also seen when cells were grown in collagen gels in the presence of anti-β1 integrin antibody (data not shown).

These findings indicate that at least the proximal signal-

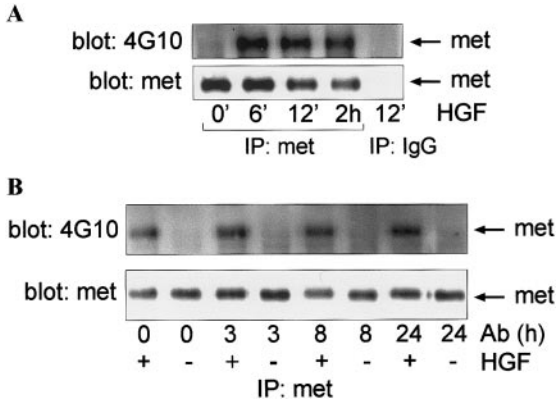

FIG. 9. Anti-β1 integrin antibody does not affect tyrosine phosphorylation of the c-Met receptor. TAC-2.1 cells were grown on collagen to subconfluency, serum-starved overnight, and treated (A) with 20 ng/ml HGF for increasing lengths of time or (B) with 50 μg/ml anti-β1 integrin or control IgG for 0–24 h and HGF for 20 min. Cell lysates were immunoprecipitated with anti-c-Met antibody, separated by 7.5% SDS–PAGE and analysed by immunoblotting with antibodies for phosphotyrosine (4G10) or the appropriate precipitating antibody. The c-Met receptor was phosphorylated 6 min after HGF stimulation and remained phosphorylated after 2 h. 50 μg/ml anti-β1 integrin antibody did not affect c-Met phosphorylation in response to HGF.

ling event in this pathway was integrin-independent. An alternate possibility is that rather than being required for signal transduction pathways involved in morphogenesis, β1 integrins may be necessary to promote physical interactions between mammary cells and their ECM. Indeed, closer inspection of Fig. 8 indicates that following integrin antibody washout, the cells formed cords along preformed pathways within the collagen gel (compare Figs. 8A and 8B with 8G and 8H). In addition, antibody treatment caused retraction of the cords into spherical aggregates, rather than just stasis of morphogenesis, suggesting a complete failure of adhesion.

Anti-α6 Integin Antibody Has No Effect on End Bud Number

To examine the role of potential α subunit partners for β1 integrin, pellets containing anti-α6 integrin monoclonal antibody were implanted into developing mammary glands as before. Blocking the function of α6 integrin had no significant effect on the number of end buds 24 h, 3 days, or 6 days after pellet implantation (Fig. 10A), nor was the morphology of the gland affected as determined by whole-mount analysis (Figs. 10B and 10C) or in H&E-stained sections (data not shown). We therefore conclude that either α6 is not the crucial partner for β1 integrin in this system or alternatively that several α-β1 heterodimers are involved and blocking the function of α6 alone is not sufficient to affect mammary development.

Anti-laminin Antibody Reduces End Bud Number while Anti-E3 Antibody Has No Effect on Mammary Morphogenesis

Since β1 integrin was required for normal mammary gland morphology, we wanted to examine whether a potential ligand was also required. Thus, pellets containing 300 μg affinity-purified polyclonal antibody raised to laminin-1 were implanted into developing mammary glands as before. A significant reduction in the number of end buds was seen after 24 h and 3 days (45 and 71%, respectively; $P < 0.01$). However, 6 days after pellet implantation development of the gland had returned to normal (Fig. 10D). No disruption in the cellular organisation of the mammary gland was seen in H&E-stained paraffin sections of treated glands (data not shown). In contrast to the results obtained with the anti-laminin antibody, pellets containing 300 μg affinity-purified anti-E3 antibody had no effect on mammary development (Fig. 10E). These results suggest that laminin is an important extracellular matrix ligand during ductal development. However, although it is possible that the anti-E3 antibody was unable to fully access the necessary epitopes within the mammary tissue, our data suggest that it is not the E3 component of the α1 chain that is the crucial domain of the laminin trimer in this system. The similarity in phenotype observed between glands treated with anti-β1 integrin and anti-laminin antibodies indicates that both laminin and β1 integrin are crucial to end bud persistence and ductal elongation in the developing mammary gland.

DISCUSSION

This is the first demonstration that both β1 integrin and laminin are required for the normal morphogenesis of virgin mammary ductal epithelium during pubertal development *in vivo*. Such experiments have not previously been possible due to the lack of function-blocking anti-mouse integrin reagents. The anti-β1 integrin antibody blocked adhesion of primary mammary cells to basement membrane proteins in culture and disrupted preestablished adhesion of primary cells cultured on ECM proteins. However, it did not affect proliferation or apoptosis of mammary cells *in vivo* or alter the proximal tyrosine phosphorylation events induced by the mammary morphogenetic growth factor HGF. Since the anti-β1 integrin antibody became localised to sites of cell–basement membrane interaction *in vivo*, we suggest that it may perturb morphogenesis via an effect on cellular traction through the ECM. The observation that mammary cells cultured within collagen gels undergo morphogenesis along preformed pathways following removal of the β1 integrin antibody supports this hypothesis. However, the inhibitory effect on development in mammary tissue was specific to end buds since the rest of the gland architecture remained intact, indicating that the antibody did not have a general adhesion-blocking phenotype *in vivo*.

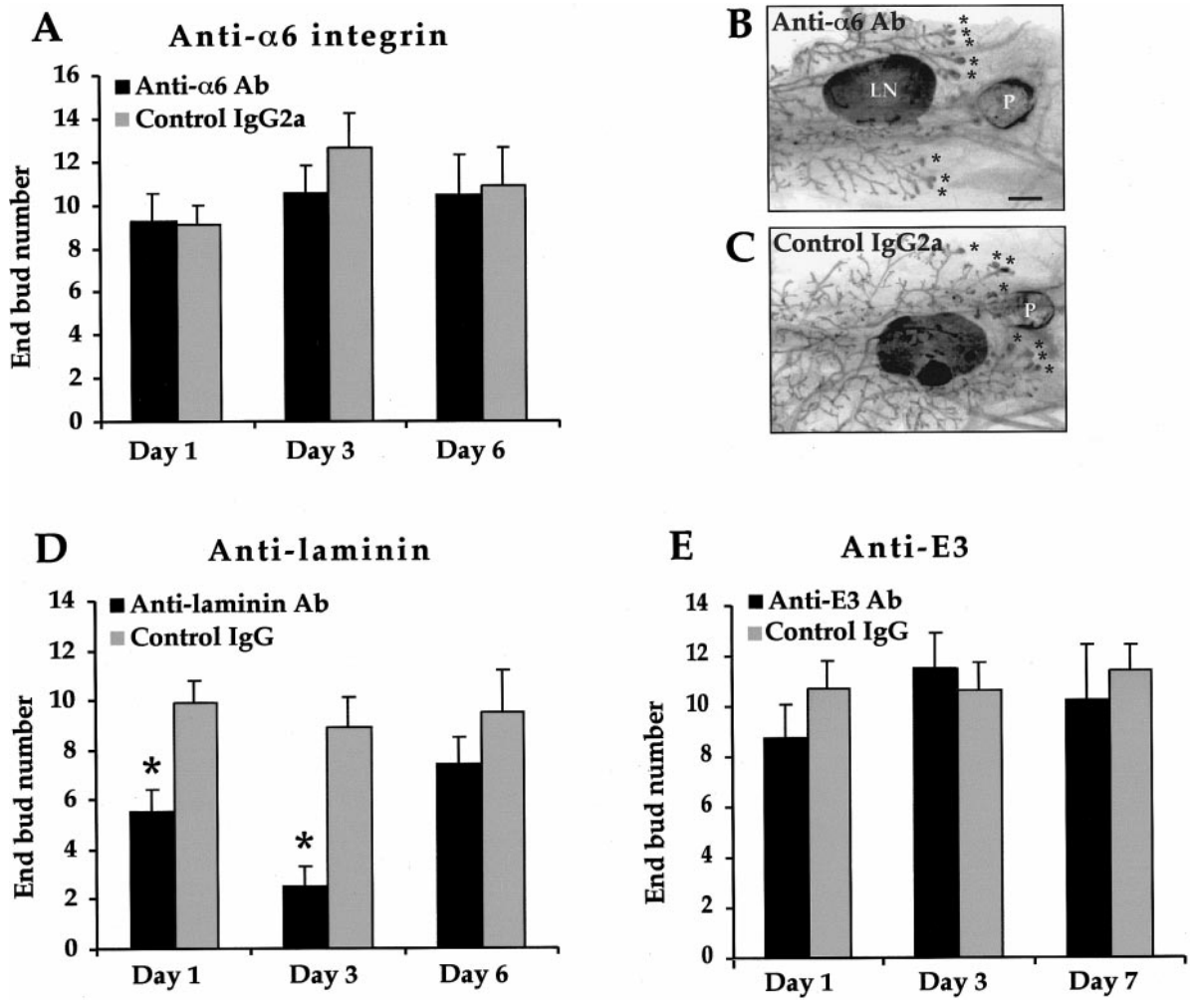

FIG. 10. The presence of anti-laminin antibody reduces the number of end buds in the virgin mammary gland of mice *in vivo,* whereas antibodies to α6 integrin or the E3 domain of laminin-1 have no effect. Graphs showing the effect of Elvax pellets containing (A) anti-α6 integrin, (D) anti-laminin, or (E) anti-E3 domain antibodies on the average number of end buds per mammary gland in virgin MF1 mice compared with pellets containing control IgG over 6 days *in vivo* (7 days for E3). Asterisks indicate statistically significantly differences by Student's *T* test ($P < 0.01$). Numbers of animals in each group are (A) 11, 11, and 7; (D) 7, 8, and 7; (E) 9, 17, and 7. Bars indicate SEM. (B, C) Photomicrographs of whole-mounted glands after 3 days in the presence of (B) anti-α6 antibody or (C) control IgG2a, stained with haematoxylin. Asterisks indicate end buds, P indicates pellet, LN indicates lymph node. Scale bar, 900 μm.

The finding that blocking laminin function *in vivo* produced a similar phenotype suggests that both laminin and β1 integrin are crucial to ductal morphogenesis.

β1 Integrins and Laminin Are Required for Mammary Gland Development in Vivo

The observation that β1 integrin null mice die in early embryogenesis underscores the crucial role of the β1 integrin family in morphogenesis (Fassler and Meyer, 1995; Stephens *et al.,* 1995). However, a study of β1 integrin function in the mammary gland using this technique has not yet been possible since the tissue largely develops postnatally. Our rationale of using antibodies has allowed us to overcome this problem and offers a complementary approach to gene knockout in which compensatory up-regulation of related genes can mask phenotypic changes.

One previous study has examined the role of β1 integrins in mammary gland function *in vivo* (Faraldo *et al.,* 1998). In that case, the development of mature mammary gland, particularly during pregnancy, was perturbed following expression of a dominant negative β1 integrin transgene expressed under the control of the MMTV promoter. In the pregnant gland, decreased cell proliferation, increased apoptosis in alveoli, and reduced milk protein expression were observed. However, development of the ductal net-

work in pubertal mammary gland was unaffected in these mice, probably due to low MMTV promoter activity at this time. One key difference in the cellular architecture of the mammary alveolus during pregnancy compared with that of the end bud or the ductal epithelium is that during pregnancy the secretory epithelial cells are in direct contact with the basement membrane rather than being separated from it by a continuous layer of myoepithelial cells. We have previously identified roles for β1 integrin in the function of mammary cells from pregnant mice using culture models (Streuli *et al.,* 1991; Farrelly *et al.,* 1999), and this has been backed up by the *in vivo* studies in pregnancy (Faraldo *et al.,* 1998). Our new data support their conclusion that β1 integrins are required for mammary gland function *in vivo.* However, they extend it by demonstrating that integrins have roles during additional stages of development, in particular for the maintenance of end bud structure in puberty. Moreover our data suggest that a β1 integrin ligand, laminin-1, is also required for maintenance of mammary end buds.

The identity of the important α integrin subunit in mammary ductal development *in vivo* has not yet been established, but our data suggest that it is unlikely to be α6 integrin. A number of other α integrin subunits, including α2 and α3 but not α1 or α7, are expressed in the mammary gland during development (Delcommenne and Streuli, 1995; Oliver *et al.,* manuscript in preparation). Therefore one possibility is that several α subunits may be involved in mammary morphogenesis, but inhibiting the function of a single α subunit may allow others to substitute. An alternate approach to identifying which α subunit(s) is involved in mammary morphogenesis comes from transplanting integrin subunit-null tissue into the mammary fat pads of wild-type syngeneic hosts, and such investigations are currently being performed in this laboratory (Klinowska *et al.,* manuscript in preparation).

Complementary to the role for integrin in the maintenance of end buds is our observation that the anti-laminin-1 antibody blocks mammary morphogenesis. Interestingly, the anti-E3 antibody does not have this effect even though it is effective in blocking adhesion of mammary cells to laminin in culture. The anti-laminin-1 antibody contains reactivity towards the γ1 chain that is present in many laminin species, while the anti-E3 antibody is specific for the α1 chain. We have preliminary data that laminin-1, laminin-3, laminin-5, and laminin-10 are expressed in the basement membrane of mammary epithelium (Oliver *et al.,* manuscript in preparation), and of these only laminin-1 and laminin-3 contain the α1 chain. It is therefore possible that laminins other than laminin-1 are important for mammary morphogenesis, but confirmation of this must await development of appropriate function-blocking antibodies or conditional knockout mice.

Important differences are observed in the integrin–ECM interactions in different developing systems. For example, antibodies to α6 integrin and the E3 fragment of laminin-1 block development of the embryonic kidney (Sorokin *et al.,* 1990, 1992) and of salivary glands (Kadoya *et al.,* 1995) in culture. However, the developing mammary gland *in vivo* does not appear to require functional E3 or α6 integrin alone for ductal morphogenesis (Figs. 10A and 10E). These data underscore the importance of studying different developmental systems to achieve a synthesis of morphogenetic mechanisms.

Problems Associated with Identifying a Morphogenetic Mechanism for β1 Integrin Requirement Using the in Vivo System

Elucidating a mechanism for the requirement of β1 integrins in ductal growth remains an important goal, although this is particularly difficult *in vivo.* In the virgin end bud, only cap cells and myoepithelial cells are in direct contact with the basement membrane, here demonstrated to contain both laminin-1 and collagen IV (Fig. 1). Thus the function-blocking anti-β1 integrin antibody may act directly on cell–basement membrane interactions.

Integrins are required for the survival and proliferation of adherent cells. In cultured mammary epithelial cells, α6 and β1 integrin antibodies induce apoptosis of isolated luminal cells derived from pregnant mice (Boudreau *et al.,* 1995; Pullan *et al.,* 1996; Farrelly *et al.,* 1999). In the virgin gland, specific cells within the end buds undergo apoptosis during normal mammary development (Humphreys *et al.,* 1996). The apoptotic cells were several cell diameters from the underlying ECM, suggesting that they may have been deleted due to lack of integrin-mediated survival signals as in the case of embryonic cavitation (Coucouvanis and Martin, 1995). Furthermore, a twofold increase in apoptosis of mammary cells in pregnancy was observed when integrin function was perturbed by a dominant-negative transgene (Faraldo *et al.,* 1998). We therefore reasoned that the anti-integrin antibody might increase apoptosis in the remaining end buds *in vivo* after antibody treatment, but found no effect of the presence of function-blocking β1 integrin antibody. In addition, the cultured TAC-2.1 cell clusters remaining within collagen gels after antibody treatment underwent morphogenesis after antibody withdrawal, indicating that if any apoptosis had occurred it was to a limited degree. Thus, the β1 integrin antibody does not appear to block morphogenesis via induction of apoptosis, which is consistent with our observation that its inhibition is reversible. One possibility is that differentiated mammary cells from pregnant mammary gland (Faraldo *et al.,* 1998; Pullan *et al.,* 1996) may be more dependent on ECM survival cues than cells from the less differentiated virgin mammary tissue, as has been shown for other cell types (Globus *et al.,* 1998).

The current model of growth in the virgin mammary gland has been elucidated from studies on the effect of implants containing TGFβ1, 2, or 3, where end buds shut down and assume the morphology of terminal ducts, with little cell proliferation detectable (Silberstein and Daniel, 1987; Robinson *et al.,* 1991). However, these structures

retain the capacity to become end buds and reemerge once TGFβ is removed. The morphological effect of anti-β1 integrin or anti-laminin antibodies on virgin ductal development is reminiscent of, but most likely different from, that of TGFβ. Analysis of total end bud number reveals that not only are new end buds prevented from forming but existing end buds must also regress. In contrast to the TGFβ studies, in which a thick collagenous sheath was deposited around the end bud physically inhibiting growth (Silberstein *et al.,* 1990), we did not observe massive ECM deposition by light microscopy in the presence of β1 integrin or laminin antibodies. It was therefore not possible to distinguish between end buds that had regressed through the action of anti-integrin antibody from those that had regressed naturally. A further caveat is that although we have shown that all the antibodies used are function-perturbing in culture some may be less effective *in vivo.*

Despite extensive investigation, we have been unable to pinpoint a mechanism by which blocking the function of β1 integrins affects the development of virgin mammary gland. The most likely explanation is that the inhibition of β1 integrins occurs at random within the end bud population and we are simply not observing any end buds during their regression. So although anti-β1 integrin antibody does not appear to have a significant effect on cell proliferation or survival within the end buds that remain in treated glands, it may be that proliferation and cell survival are affected in structures that would have been end buds had the function of β1 integrins not been blocked.

A Possible Role for β1 Integrin Based on Culture Studies

To simplify investigation of the mechanism by which anti-β1 integrin antibody exerts its effect on glandular development, we used a 3-D collagen gel model in which cultured cell lines undergo morphogenesis under the influence of factors such as HGF (Soriano *et al.,* 1995). The "cords" that form in the culture model differ from mammary ducts *in vivo,* notably in the absence of a complex end bud structure. It has so far not been possible to maintain end bud morphology *in vitro* (Daniel *et al.,* 1984; Richards *et al.,* 1982); however, elegant studies using end bud cells embedded in collagen and subsequently implanted *in vivo* have demonstrated that cord morphologies were seen while cells remained in contact with collagen, but that end bud structures formed as soon as cells grew into adipose tissue (Daniel *et al.,* 1984). Thus the cord morphology may be a simplified form of morphogenesis; it is not an artefact of culture, but instead may be due to direct contact with collagen I rather than the mammary stroma. In addition, cords of TAC-2.1 cells deposit basement membrane between the cells and the collagen gel (Soriano *et al.,* 1995), suggesting first that in this regard the culture model resembles the epithelial cell–basement membrane–stromal matrix arrangement seen *in vivo,* and second, that the anti-β1 integrin antibody may directly affect cell–basement membrane adhesion in culture. Studies using cultured human mammary epithelial cells either derived from carcinomas or isolated from milk have suggested a role for α2β1 integrin in the spontaneous branching phenotype seen in collagen gel cultures (Berdichevsky *et al.,* 1992; Keely *et al.,* 1995; Saelman *et al.,* 1995; Zutter *et al.,* 1995). However, in these studies it was not clear whether the cells deposited an endogenous basement membrane.

We wished to determine whether our antibody blocked HGF-induced mammary morphogenesis in culture since HGF has been shown to be important for ductal development *in vivo* (Yang *et al.,* 1995). Our findings indeed showed that the anti-β1 integrin antibody abolished HGF-stimulated morphogenesis of cells cultured in collagen gels, and these results led to two possible hypotheses for the role of β1 integrin. Initially, we postulated that integrins might be involved in crosstalk with the c-Met HGF receptor. This suggestion was built on our previous work demonstrating an involvement of integrins in the tyrosine phosphorylation events associated with the prolactin signalling pathway driving milk protein gene transcription (Edwards *et al.,* 1998; Streuli *et al.,* 1991, 1995). However, we found that although tyrosine phosphorylation of the c-Met receptor was rapidly induced after HGF stimulation, it was unaffected by incubation of cells for up to 24 h with anti-β1 integrin antibody. Thus, the effect of blocking β1 integrin on ductal morphogenesis must be either downstream of c-Met receptor phosphorylation or completely independent of it.

An alternative possibility was that a key role for integrins might reside in the physical interaction of ductal cells with the ECM. In this model, HGF supplies the stimulation for growth, while integrins provide purchase for ductal elongation. Such a hypothesis is supported by the experiments with function-blocking anti-β1 integrin antibody in the culture model. Here, the ductal cords that form under the influence of HGF retract to spherical structures in the presence of integrin antibody, but then regrow along previously formed canals within the collagen gels after antibody is removed (Fig. 8). These results are in broad agreement with recent reports that altering the strength of integrin interaction with the ECM affects HGF-induced branching of human mammary epithelial cells in culture (Alford *et al.,* 1998). That system was different from ours since the integrin-blocking antibody was added at the time of cell plating and caused cell dissociation rather than inhibition of morphogenesis. In addition, reduced HGF-induced morphogenesis in collagen gels was observed in MDCK cells expressing antisense α2 integrin, although here the high levels of apoptosis induced by expression of the antisense construct complicate interpretation of the results (Saelman *et al.,* 1995).

Thus, although there are differences between the *in vivo* and the culture models there are also important similarities. *In vivo,* end bud number is diminished by the anti-β1 integrin antibody (Fig. 4), resulting in a reduced extent of the ductal network several days later; as the antibody

becomes depleted, the glands recover (Fig. 5). In the culture model, mammary epithelial cord length is reduced by anti-β1 antibody, but cord formation resumes after antibody washout. Thus, β1 integrin is required both for duct extension *in vivo* and cord elongation in culture. Since end buds do not appear to be able to be formed within collagen gels, it remains to be determined whether the precise mechanism for integrin requirement is similar in both systems. However, given the lack of apoptosis in both systems and the lack of any obvious changes to ductal morphology *in vivo*, it is possible that integrins are required for cellular traction through the stromal matrix rather than the maintenance of normal cellular architecture.

Implications for Breast Cancer Progression

Our study has implications for deciphering breast cancer progression, since it indicates a role for integrin in mammary epithelial morphogenesis. Breast tumours have lowered levels of some integrin subunits. However, β1 subunit levels are often retained (Koukoulis *et al.*, 1991) and their ablation, for example in the aggressive MDA-MB-435 breast carcinoma line, led to fewer and smaller metastases in *nu*/*nu* mice (Wewer *et al.*, 1997). Moreover, breast cancer patients with higher levels of integrin expression in their tumours had shorter survival times (Friedrichs *et al.*, 1995). Since anti-integrin antibodies can revert tumour cells to a normal phenotype (Weaver *et al.*, 1997), and since our work shows that they block mammary morphogenesis, we suggest that therapeutic strategies to target the function of β1 integrins *in vivo* may compromise the ability of tumour cells to form metastatic outgrowths and thereby severely reduce progression to malignancy.

Summary

In summary, we have demonstrated that ductal morphogenesis in the pubertal mammary gland, both *in vivo* and in culture, depends not only on the influence of morphogenetic factors but also on cell–ECM interactions transduced by integrins. We propose that the extracellular matrix and laminin in particular together with β1 integrins are crucial to maintaining end bud structure and function and therefore that normal ductal development and mammary gland morphogenesis require functional β1 integrins.

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REFERENCES

Abraham, W. M., Sielczak, M. W., Ahmed, A., Cortes, A., Lauredo, I. T., Kim, J., Pepinsky, B., Benjamin, C. D., Leone, D. R., Lobb, R. R., *et al.* (1994). Alpha 4-integrins mediate antigen-induced late bronchial responses and prolonged airway hyperresponsiveness in sheep. *J. Clin. Invest.* **93,** 776–787.

Adler, S., and Chen, X. (1992). Anti-Fx1A antibody recognizes a beta 1-integrin on glomerular epithelial cells and inhibits adhesion and growth. *Am. J. Physiol.* **262,** F770–776.

Agbas, A., and Sarras, M. P., Jr. (1994). Evidence for cell surface extracellular matrix binding proteins in *Hydra vulgaris*. *Cell Adhes. Commun.* **2,** 59–73.

Alford, D., Baeckstrom, D., Geyp, M., Pitha, P., and Taylor Papadimitriou, J. (1998). Integrin–matrix interactions affect the form of the structures developing from human mammary epithelial cells in collagen or fibrin gels. *J. Cell Sci.* **111,** 521–532.

Ashkenas, J., Muschler, J., and Bissell, M. J. (1996). The extracellular matrix in epithelial biology: Shared molecules and common themes in distant phyla. *Dev. Biol.* **180,** 433–444.

Berdichevsky, F., Gilbert, C., Shearer, M., and Taylor Papadimitriou, J. (1992). Collagen-induced rapid morphogenesis of human mammary epithelial cells: The role of the alpha 2 beta 1 integrin. *J. Cell Sci.* **102,** 437–446.

Boucaut, J. C., Darribere, T., Boulekbache, H., and Thiery, J. P. (1984). Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. *Nature* **307,** 364–367.

Boudreau, N., Sympson, C. J., Werb, Z., and Bissell, M. J. (1995). Suppression of ice and apoptosis in mammary epithelial cells by extracellular matrix. *Science* **267,** 891–893.

Bronner Fraser, M. (1985). Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* **101,** 610–617.

Coleman, S., Silberstein, G. B., and Daniel, C. W. (1988). Ductal morphogenesis in the mouse mammary gland: Evidence supporting a role for epidermal growth factor. *Dev. Biol.* **127,** 304–315.

Coucouvanis, E., and Martin, G. R. (1995). Signals for death and survival—A 2-step mechanism for cavitation in the vertebrate embryo. *Cell* **83,** 279–287.

Daniel, C. W., Berger, J. J., Strickland, P., and Garcia, R. (1984). Similar growth pattern of mouse mammary epithelium cultivated in collagen matrix in vivo and in vitro. *Dev. Biol.* **104,** 57–64.

Daniel, C. W., Strickland, P., and Friedmann, Y. (1995). Expression and functional role of E-cadherin and P-cadherin in mouse mammary ductal morphogenesis and growth. *Dev. Biol.* **169,** 511–519.

D'Ardenne, A. J., Richman, P. I., Horton, M. A., McAulay, A. E., and Jordan, S. (1991). Co-ordinate expression of the alpha-6 integrin laminin receptor sub-unit and laminin in breast cancer. *J. Pathol.* **165,** 213–220.

Delcommenne, M., and Streuli, C. H. (1995). Control of integrin expression by extracellular matrix. *J. Biol. Chem.* **270,** 26794–26801.

Edwards, G. M., and Streuli, C. H. (1999). Preparing a polyclonal antibody to mouse β1 integrin with function-blocking activity. *In* "Integrin Protocols" (A. Howlett, Ed.), Methods in Molecular Biology Vol. 129, pp. 135–152. Humana Press, Clifton, NJ.

Edwards, G. M., Wilford, F. H., Liu, X. W., Hennighausen, L., Djiane, J., and Streuli, C. H. (1998). Regulation of mammary

differentiation by extracellular matrix involves protein-tyrosine phosphatases. *J. Biol. Chem.* **273,** 9495–9500.

Elliott, B. E., Ekblom, P., Pross, H., Niemann, A., and Rubin, K. (1994). Anti-beta(1)-integrin IgG inhibits pulmonary macrometastasis and the size of micrometastases from a murine mammary-carcinoma. *Cell Adhes. Commun.* **1,** 319–332.

Faraldo, M. M., Deugnier, M. A., Lukashev, M., Thiery, J. P., and Glukhova, M. A. (1998). Perturbation of beta 1-integrin function alters the development of murine mammary gland. *EMBO J.* **17,** 2139–2147.

Farrelly, N., Lee, Y-J., Oliver, J., Dive, C., and Streuli, C. H. (1999). Extracellular matrix regulates apoptosis in mammary epithelium through a control on insulin signaling. *J. Cell Biol.* **144,** 1337–1348.

Fassler, R., Georges Labouesse, E., and Hirsch, E. (1996). Genetic analyses of integrin function in mice. *Curr. Opin. Cell Biol.* **8,** 641–646.

Fassler, R., and Meyer, M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev.* **9,** 1896–1908.

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

Friedrichs, K., Ruiz, P., Franke, F., Gille, I., Terpe, H. J., and Imhof, B. A. (1995). High expression level of alpha 6 integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res.* **55,** 901–906.

Globus, R. K., Doty, S. B., Lull, J. C., Holmuhamedov, E., Humphries, M. J., and Damsky, C. H. (1998). Fibronectin is a survival factor for differentiated osteoblasts. *J. Cell Sci.* **111,** 1385–1393.

Gorcyznski, R. M., Chung, S., Fu, X. M., Levy, G., Sullivan, B., and Chen, Z. (1995). Manipulation of skin graft rejection in alloimmune mice by anti-VCAM-1:VLA-4 but not anti-ICAM-1:LFA-1 monoclonal antibodies. *Transpl. Immunol.* **3,** 55–61.

Hemler, M. E., Huang, C., Takada, Y., Schwarz, L., Strominger, J. L., and Clabby, M. L. (1987). Characterization of the cell surface heterodimer VLA-4 and related peptides. *J. Biol. Chem.* **262,** 11478–11485.

Howard, J. E., Hirst, E. M., and Smith, J. C. (1992). Are beta 1 integrins involved in *Xenopus* gastrulation? *Mech. Dev.* **38,** 109–119.

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

Hynes, R. O. (1996). Targeted mutations in cell adhesion genes: What have we learned from them? *Dev. Biol.* **180,** 402–412.

Johnson, K. E., Darribere, T., and Boucaut, J. C. (1993). Mesodermal cell-adhesion to fibronectin-rich fibrillar extracellular-matrix is required for normal *Rana pipiens* gastrulation. *J. Exp. Zool.* **265,** 40–53.

Kadoya, Y., Kadoya, K., Durbeej, M., Holmvall, K., Sorokin, L., and Ekblom, P. (1995). Antibodies against domain E3 of laminin-1 and integrin alpha 6 subunit perturb branching epithelial morphogenesis of submandibular gland, but by different modes. *J. Cell Biol.* **129,** 521–534.

Keely, P. J., Fong, A. M., Zutter, M. M., and Santoro, S. A. (1995). Alteration of collagen-dependent adhesion, motility, and morphogenesis by the expression of antisense alpha(2) integrin messenger-RNA in mammary cells. *J. Cell Sci.* **108,** 595–607.

Kleinberg, D. L. (1997). Early mammary development: Growth hormone and IGF-1. *J. Mam. Gland Biol. Neoplasia* **2,** 49–57.

Koukoulis, G. K., Virtanen, I., Korhonen, M., Laitinen, L., Quaranta, V., and Gould, V. E. (1991). Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. Correlations with their functions as receptors and cell adhesion molecules. *Am. J. Pathol.* **139,** 787–799.

Lallier, T., Leblanc, G., Artinger, K. B., and Bronner Fraser, M. (1992). Cranial and trunk neural crest cells use different mechanisms for attachment to extracellular matrices. *Development* **116,** 531–541.

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

Pullan, S., and Streuli, C. H. (1996). The mammary gland epithelial cell. *In* "Epithelial Cell Culture" (A. Harris, Ed.), pp. 97–121. Cambridge Univ. Press, Cambridge, UK.

Pullan, S., Wilson, J., Metcalfe, A., Edwards, G. M., Goberdhan, N., Tilly, J., Hickman, J. A., Dive, C., and Streuli, C. H. (1996). Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. *J. Cell Sci.* **109,** 631–642.

Richards, J., Guzman, R., Konrad, M., Yang, J., and Nandi, S. (1982). Growth of mouse mammary gland end buds cultured in a collagen gel matrix. *Exp. Cell Res.* **141,** 433–443.

Robinson, S. D., Silberstein, G. B., Roberts, A. B., Flanders, K. C., and Daniel, C. W. (1991). Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. *Development* **113,** 867–878.

Saelman, E. U. M., Keely, P. J., and Santoro, S. A. (1995). Loss of MDCK cell alpha-2-beta-1 integrin expression results in reduced cyst formation, failure of hepatocyte growth-factor scatter factor-induced branching morphogenesis, and increased apoptosis. *J. Cell Sci.* **108,** 3531–3540.

Silberstein, G. B., and Daniel, C. W. (1982). Elvax 40P implants: Sustained, local release of bioactive molecules influencing mammary ductal development. *Dev. Biol.* **93,** 272–278.

Silberstein, G. B., and Daniel, C. W. (1987). Reversible inhibition of mammary gland growth by transforming growth factor-beta. *Science* **237,** 291–293.

Silberstein, G. B., Strickland, P., Coleman, S., and Daniel, C. W. (1990). Epithelium-dependent extracellular matrix synthesis in transforming growth factor-beta 1-growth-inhibited mouse mammary gland. *J. Cell Biol.* **110,** 2209–2219.

Snedeker, S. M., Brown, C. F., and Diagustine, R. P. (1991). Expression and functional properties of transforming growth factor-alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc. Natl. Acad. Sci. USA* **88,** 276–280.

Sonnenberg, A., Daams, H., Van der Valk, M. A., Hilkens, J., and Hilgers, J. (1986). Development of mouse mammary gland: Identification of stages in differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. *J. Histochem. Cytochem.* **34,** 1037–1046.

Sonnenberg, A., Modderman, P. W., and Hogervorst, F. (1988). Laminin receptor on platelets is the integrin VLA-6. *Nature* **336,** 487–489.

Soriano, J. V., Orci, L., and Montesano, R. (1996). TGF-β1 induces morphogenesis of branching cords by cloned mammary epithelial

cells at subpicomolar concentrations. *Biochem. Biophys. Res. Commun.* **220,** 879–885.

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.

Sorokin, L., Sonnenberg, A., Aumailley, M., Timpl, R., and Ekblom, P. (1990). Recognition of the laminin E8 cell-binding site by an integrin possessing the alpha 6 subunit is essential for epithelial polarization in developing kidney tubules. *J. Cell Biol.* **111,** 1265–1273.

Sorokin, L. M., Conzelmann, S., Ekblom, P., Battaglia, C., Aumailley, M., and Timpl, R. (1992). Monoclonal antibodies against laminin A chain fragment E3 and their effects on binding to cells and proteoglycan and on kidney development. *Exp. Cell Res.* **201,** 137–144.

Stephens, L. E., Sutherland, A. E., Klimanskaya, I. V., Andrieux, A., Meneses, J., Pedersen, R. A., and Damsky, C. H. (1995). Deletion of beta-1 integrins in mice results in inner cell mass failure and periimplantation lethality. *Genes Dev.* **9,** 1883–1895.

Streuli, C. H., Bailey, N., and Bissell, M. J. (1991). Control of mammary epithelial differentiation—Basement membrane induces tissue-specific gene expression in the absence of cell cell interaction and morphological polarity. *J. Cell Biol.* **115,** 1383–1395.

Streuli, C. H., and Bissell, M. J. (1990). Expression of extracellular matrix components is regulated by substratum. *J. Cell Biol.* **110,** 1405–1415.

Streuli, C. H., Edwards, G. M., Delcommenne, M., Whitelaw, C. B. A., Burdon, T. G., Schindler, C., and Watson, C. J. (1995). Stat5 as a target for regulation by extracellular matrix. *J. Biol. Chem.* **270,** 21639–21644.

Trikha, M., De Clerck, Y. A., and Markland, F. S. (1994). Contortrostatin, a snake venom disintegrin, inhibits beta 1 integrin-mediated human metastatic melanoma cell adhesion and blocks experimental metastasis. *Cancer Res.* **54,** 4993–4998.

Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., 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.

Weg, V. B., Williams, T. J., Lobb, R. R., and Nourshargh, S. (1993). A monoclonal antibody recognizing very late activation antigen-4 inhibits eosinophil accumulation in vivo. *J. Exp. Med.* **177,** 561–566.

Wewer, U. M., Shaw, L. M., Albrechtsen, R., and Mercurio, A. M. (1997). The integrin alpha 6 beta 1 promotes the survival of metastatic human breast carcinoma cells in mice. *Am. J. Pathol.* **151,** 1191–1198.

Williams, J. M., and Daniel, C. W. (1983). Mammary ductal elongation: Differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev. Biol.* **97,** 274–290.

Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K. M., Birchmeier, C., and Birchmeier, W. (1995). Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* **131,** 215–226.

Zutter, M. M., Mazoujian, G., and Santoro, S. A. (1990). Decreased expression of integrin adhesive protein receptors in adenocarcinoma of the breast. *Am. J. Pathol.* **137,** 863–870.

Zutter, M. M., Santoro, S. A., Staatz, W. D., and Tsung, Y. L. (1995). Reexpression of the alpha(2)beta(1) integrin abrogates the malignant phenotype of breast-carcinoma cells. *Proc. Natl. Acad. Sci. USA* **92,** 7411–7415.

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