

Ablation of $\beta 1$ integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation

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Integrin-mediated adhesion regulates the development and function of a range of tissues; however, little is known about its role in glandular epithelium. To assess the contribution of $\beta 1$ integrin, we conditionally deleted its gene in luminal epithelia during different stages of mouse mammary gland development and in cultured primary mammary epithelia. Loss of $\beta 1$ integrin *in vivo* resulted in impaired alveologenesis and lactation. Cultured $\beta 1$ integrin-null cells displayed abnormal focal adhesion function and signal transduction and could not form or maintain polarized acini. *In vivo*, epithelial cells became detached from the extracellular matrix but remained associated with each other and did not undergo overt

apoptosis. $\beta 1$ integrin-null mammary epithelial cells did not differentiate in response to prolactin stimulation because of defective Stat5 activation. In mice where $\beta 1$ integrin was deleted after the initiation of differentiation, fewer defects in alveolar morphology occurred, yet major deficiencies were also observed in milk protein and milk fat production and Stat5 activation, indicating a permissive role for $\beta 1$ integrins in prolactin signaling. This study demonstrates that $\beta 1$ integrin is critical for the alveolar morphogenesis of a glandular epithelium and for maintenance of its differentiated function. Moreover, it provides genetic evidence for the cooperation between integrin and cytokine signaling pathways.

Introduction

The interaction between a cell and its microenvironment is critical in determining responses to both external and internal stimuli, regulating functions that include migration, proliferation, apoptosis, and differentiation. Integrins are the major class of receptors for the ECM and link cellular adhesion to the actin cytoskeleton and signal transduction pathways (Hynes, 2002). $\beta 1$ integrin heterodimers are widely expressed and control various developmental processes, including neurogenesis, chondrogenesis, myoblast fusion, and skin and hair follicle morphogenesis (Brakebusch et al., 2000; Graus-Porta et al.,

2001; Aszodi et al., 2003; Schwander et al., 2003). However, the function of $\beta 1$ integrins in the maintenance of a differentiated tissue such as glandular epithelium is not well understood.

The mammary gland consists of a network of luminal ductal and alveolar epithelium that is surrounded by and interacts with myoepithelial cells and is subtended by an ECM termed the basement membrane (BM). Development of the mammary gland occurs primarily after birth, with the ductal network extending to fill the mammary fat pad. During pregnancy, cells within alveoli proliferate and differentiate, achieving functional capacity with the synthesis of milk proteins at lactation. At involution, the majority of the epithelium undergoes apoptosis and remodeling to result in a tissue closely resembling that of the nulliparous gland (Hennighausen and Robinson, 2001).

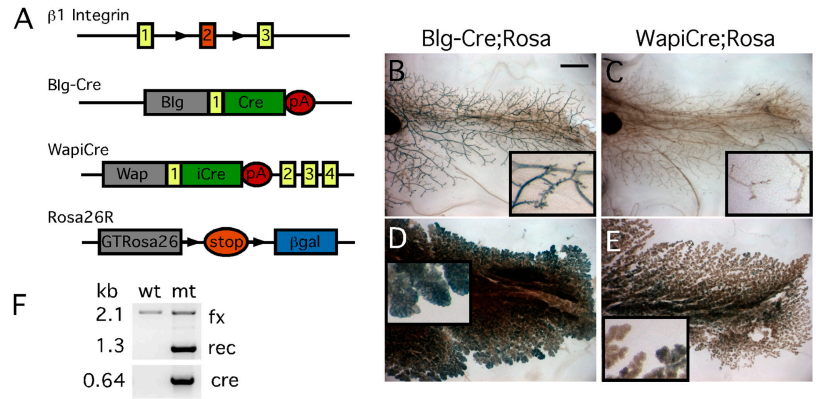
Cell-matrix adhesion regulates mammary cell structure and function in culture models. Inhibition of mammary epithelial cell contact with the BM alters morphology, survival,

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Abbreviations used in this paper: Ad β gal, β -galactosidase adenovirus; AdCre, Cre-recombinase adenovirus; Blg, β lactoglobulin; BM, basement membrane; ILK, integrin-linked kinase; Prl, prolactin; Wap, whey acidic protein.

Figure 1. Temporal deletion of $\beta 1$ integrin during mammary gland development. (A) Schematic diagram of $\beta 1$ integrin allele, Blg-Cre transgene, WapiCre transgene, and Rosa26R alleles. (B–E) X-gal staining of whole mammary glands from Blg-Cre^{Tg/+};Rosa26R^{fx/fx} (B and D) and WapiCre^{Tg/+};Rosa26R^{fx/fx} mice (C and E). (B) 12-wk nulliparous. (C) 18-wk nulliparous. (D and E) Lactation day 2. Blg-Cre directs Cre expression to mammary epithelium from at least 12 wk of age throughout pregnancy and lactation, whereas WapiCre-induced Cre expression is restricted to differentiated epithelium. (F) PCR analysis of Cre-mediated recombination in tissue from mammary glands of Itg $\beta 1$ ^{fx/fx};Blg-Cre^{+/+} (wt) and Itg $\beta 1$ ^{fx/fx};Blg-Cre^{Tg/+} (mt) mice at day 2 of lactation. The 2.1-kb product represents the floxed allele, 1.3-kb the recombined allele, and 0.64-kb the Blg-Cre transgene. Bar, 2 mm.



proliferation, and differentiation (Barcellos-Hoff et al., 1989; Streuli et al., 1991; Pullan et al., 1996). Culturing cells on a reconstituted BM matrix or on laminin restores many of these functions (Streuli et al., 1995b). Interaction of mammary cells with these ECMs is mediated by integrins, and luminal epithelium expresses the $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 1$ heterodimers (Prince et al., 2002). The importance of $\beta 1$ integrins in mammary cell function has been demonstrated by the use of function-blocking antibodies, which inhibit differentiation (Streuli et al., 1991). However, because antibody-induced integrin clustering can also result in its activation (Akiyama et al., 1994), the role of $\beta 1$ integrin in mammary cell function remains unclear.

Far less is known about the role of integrins in mammary gland development and cell function in vivo. Mammary glands formed from either $\alpha 3$ or $\alpha 6$ integrin-null epithelia are fully developed and functional (Klinowska et al., 2001). The only in vivo evidence for a role of these integrins in mammary gland development comes from studies using a dominant-negative approach, where the $\beta 1$ cytoplasmic domain was fused to the extracellular domain of the T cell differentiation antigen CD4 (Lukashev et al., 1994). In these studies, $\beta 1$ integrin was implicated in the control of mammary cell proliferation, survival, and differentiation (Faraldo et al., 1998, 2002). However, because the transgene can act as either an agonist or an antagonist depending on the level of expression (Lukashev et al., 1994), these data are difficult to interpret.

To understand the role of $\alpha\beta 1$ integrins in the regulation of mammary epithelial cell function and to determine whether this class of adhesion receptors is indeed involved in the differentiation response, we examined the consequence of conditionally deleting $\beta 1$ integrin in mammary epithelia. Two strategies were used. First, we examined the role of $\beta 1$ integrin in vivo by crossing LoxP-flanked $\beta 1$ integrin (Itg $\beta 1$ ^{fx/fx}) mice (Graus-Porta et al., 2001) with those expressing Cre under the control of either the β lactoglobulin (Blg) or whey acidic protein (Wap) promoter (Selbert et al., 1998; Wintermantel et al., 2002); here $\beta 1$ integrin was deleted from mammary epithelia before pregnancy or after the initiation of mammary differentiation. Second, we determined the effects of deleting $\beta 1$ integrin in culture by infecting primary mammary epithelial cells from Itg $\beta 1$ ^{fx/fx} mice with Cre-recombinase adenovirus (AdCre).

Several recent investigations have used a conditional-null approach to study the role of $\beta 1$ integrins in the development

and function of specific tissues; however, none has yet identified a link with epithelial cell differentiation in vertebrate systems. In the epidermis, conditional ablation of $\beta 1$ integrin leads to defects in the assembly of BM proteins, resulting in altered proliferation and the consequent defects in hair follicle morphogenesis, as well as impaired cytoskeletal dynamics, leading to reduced migration and wound reepithelialization after injury (Brakebusch et al., 2000; Grose et al., 2002; Raghavan et al., 2003). However, the spatial and temporal differentiation of keratinocytes is unaffected (Raghavan et al., 2000). In mesenchymal tissue such as bone, $\beta 1$ integrin ablation in chondrocytes causes chondrodysplasia through abnormal adhesion of cells to collagen and fibronectin and decreased proliferation via defects at the G1/S boundary of the cell cycle (Aszodi et al., 2003), whereas in muscle, $\beta 1$ integrin deletion leads to defective myoblast fusion and sarcomere assembly (Schwander et al., 2003). In neuronal tissues, integrin ablation also has a dramatic phenotype, e.g., in the neural crest-derived peripheral nervous system, where $\beta 1$ integrins are required for accurate timing in Schwann cell migration and myelination, and for maintaining processes around axons, as well as in neuromuscular junction maturation (Feltri et al., 2002; Pietri et al., 2004).

Our work extends these previous studies to demonstrate a key role for $\beta 1$ integrin in epithelial differentiation and expression of tissue-specific genes. Moreover, it identifies a mechanism for this role, which is through the integration of signaling networks between the integrin and cytokine pathways. Thus, we provide genetic evidence for an integrin-dependent effect on gene expression-related differentiation processes in a mammalian system in vivo. Our data support previous studies on integrin dependence of gene expression in other models (Martin-Bermudo and Brown, 1999) and strengthen the notion that in certain cell types, signaling pathways driven by soluble factors are dependent on their integrin-mediated positional identity.

Results

Conditional deletion of $\beta 1$ integrin in mammary epithelia in vivo

$\beta 1$ integrin knockout mice have an embryonic lethal phenotype, thus preventing assessment of mammary gland development (Fassler and Meyer, 1995; Stephens et al., 1995). To determine the specific role of $\beta 1$ integrin during the proliferative and dif-

differentiation stages of mammary development, we crossed $Itg\beta 1^{fx/fx}$ mice with transgenic lines expressing Cre under the control of the Blg and Wap promoters (Fig. 1 A). The specificity of Cre-mediated recombination in Blg-Cre and WapiCre mice was determined by crossing these mice with Rosa26R mice, which express LacZ after Cre recombination (Soriano, 1999). Blg-Cre-mediated recombination was detected in the ductal epithelium of nulliparous mice from the age of 12 wk (i.e., after pubertal development), with the majority of epithelial cells positive for X-gal staining (Fig. 1 B). Cre recombination had occurred by all stages of pregnancy and lactation in Blg-Cre^{Tg/+};Rosa26R^{fx/fx} mice with a mean of 80% (range 50–100%) of epithelial cells positive for X-gal staining (Fig. 1 D). In contrast, WapiCre mice demonstrated a temporally restricted Cre-activity pattern, with X-gal staining detected in ~60% of epithelia from midpregnancy and throughout lactation but not in nulliparous mice up to the age of 18 wk or until the 12th d of pregnancy, when alveoli have already begun to form (Fig. 1, C and E). Expression of both Blg-Cre and WapiCre recombination was restricted to luminal mammary epithelial cells.

These data define Blg-Cre mice as a good model for examining gene function throughout pregnancy and lactation, as the targeted gene will be deleted before the initiation of pregnancy. Because the WapiCre promoter is only expressed after differentiation has been initiated in midpregnancy, we also crossed these mice with $Itg\beta 1^{fx/fx}$ animals to examine the effects of $\beta 1$ integrin deletion in differentiated luminal epithelial cells. Using these two models, we assessed the role of $\beta 1$ integrin in mammary epithelial cell function during the proliferative and differentiation phases of mammary gland development.

Analysis of $\beta 1$ integrin deletion in $Itg\beta 1^{fx/fx}$;Blg-Cre^{Tg/+} mice (Fig. 1 and see Fig. 3) or $Itg\beta 1^{fx/fx}$;WapiCre^{Tg/+} mice (see Fig. 7) was performed at the DNA and protein levels. PCR analysis of mammary glands at day 2 of lactation identified a recombined 1.3-kB product in mutant mice along with the unrecombined 2.1-kB allele, which is present in the stromal and myoepithelial cells that do not undergo Cre-mediated recombination. Wild-type mice only contained the unrecombined 2.1-kB allele (Fig. 1 F). Examination of $\beta 1$ integrin protein by immunofluorescence demonstrated lateral and basal expression in the luminal epithelium and basal expression in myoepithelial cells, which is consistent with previous findings (see Fig. 3 A and Fig. 7, K and M; Prince et al., 2002). In mutant mice, $\beta 1$ integrin was lost from the lateral and basal surfaces of the luminal epithelium (see Fig. 3 B and Fig. 7, L and N, white arrows) but could still be detected in myoepithelial cells, which subtend the luminal epithelium. These data demonstrate temporal deletion of $\beta 1$ integrin from luminal epithelial cells during mammary gland development.

Ablation of $\beta 1$ integrin expression results in impaired lobuloalveolar development and lactogenesis

$Itg\beta 1^{fx/fx}$;Blg-Cre^{Tg/+} females displayed a lactation phenotype that varied from a total failure of lactation to being able to support litters, although pups from these dams displayed dramatically reduced growth rates and were much smaller than age-matched

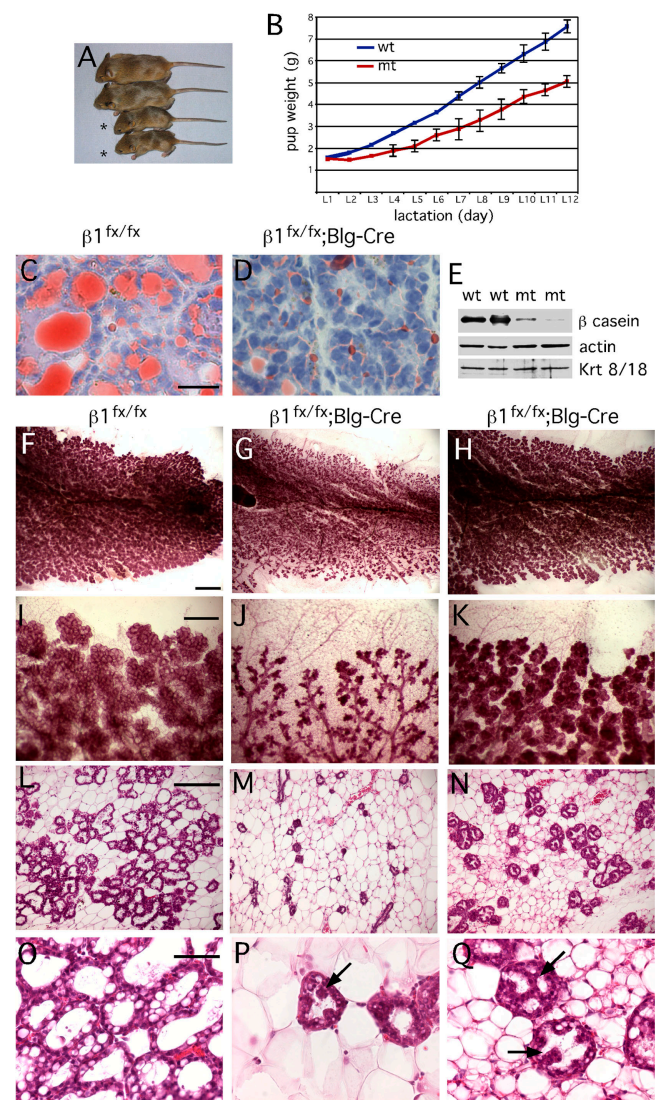


Figure 2. Defects in mammary gland development. Analysis of mammary gland development in $Itg\beta 1^{fx/fx}$;Blg-Cre^{Tg/+} mice. (A and B) Lactation defects in mutant mice. (A) Some mutant females can lactate enough to support pup survival, but pups are reduced in size (as demonstrated by asterisk; lactation day 13) compared with pups nursed by wild-type females (lactation day 12). (B) Growth-rate analysis of pups from wild-type and mutant females that were able to support some of their litter. Error bars show \pm SEM. (C–E) Oil Red O staining of milk fat (C and D) and western analysis of milk protein (E) in mammary glands from wild-type and mutant mice at day 18 of pregnancy. (F–K) Whole mount analysis of wild-type (F and I) and mutant mice with failed lactation (G and J) or reduced lactation capacity (H and K) demonstrates failed lobuloalveolar development in $\beta 1$ integrin-null mammary glands. (L–Q) Histological analysis of mammary glands from wild-type (L and O) and mutant mice with failed lactation (M and P) or reduced lactation capacity (N and Q) demonstrated reduced alveolar density. Arrows in P and Q demonstrate disruption of alveolar integrity with epithelial cells protruding into the luminal space of alveoli of mutant mice. Bars: (C and D) 25 μ m; (F–H) 2 mm; (I–K) 0.5 mm; (L–N) 200 μ m; (O–Q) 50 μ m.

controls (Fig. 2, A and B). A lack of milk was evident in the stomachs of pups from the most severely affected females, with these pups dying from dehydration within 72 h despite repeated suckling (unpublished data). Examination of two key components of milk, β casein and milk fat, confirmed that lactogenesis was compromised in the absence of $\beta 1$ integrin in vivo (Fig. 2, C–E).

Normal ductal elongation and side branching occurred in $\text{Itg}\beta 1^{\text{fx/fx}};\text{Blg-Cre}^{\text{Tg/+}}$ mice (not depicted), but there was a significant decrease in lobuloalveolar development during pregnancy (not depicted) and at the second day of lactation (Fig. 2, F–K). 5 of 10 females were unable to support pups, and these mice displayed the most severe mammary defects, with a dramatic inhibition in the formation of lobuloalveoli. For example, lactation failed in the mammary gland of the mouse shown in Fig. 2 J and was partial in the gland shown in Fig. 2 K. The defect in alveolar development was even more apparent in histological sections at the second day of lactation, when $\text{Itg}\beta 1^{\text{fx/fx}};\text{Blg-Cre}^{\text{Tg/+}}$ mice displayed decreased alveolar density (Fig. 2, L–Q).

Closer examination also revealed defects in the integrity of epithelial structures. Whereas the alveoli of wild-type females contain a simple layer of luminal epithelia surrounding a hollow lumen (Fig. 2 O), the alveoli of $\text{Itg}\beta 1^{\text{fx/fx}};\text{Blg-Cre}^{\text{Tg/+}}$ mice were disorganized and contained clumps of epithelial cells bulging into what would normally be luminal space (Fig. 2, P and Q). These protrusions often spanned the width of the alveoli and consisted of multiple layers of cells (Fig. 2 Q). Interestingly, the protuberant cells remained attached to other cells and had not completely “fallen” into the lumens.

Immunofluorescent staining of sections from glands of $\text{Itg}\beta 1^{\text{fx/fx}};\text{Blg-Cre}^{\text{Tg/+}}$ mice at day 2 of lactation demonstrated that, despite deletion of $\beta 1$ integrin (Fig. 3, A and B), epithelial polarity of mammary alveoli was essentially normal. The tight junction marker ZO-1 was located on the apical surface of luminal epithelia (Fig. 3, A and B), whereas laminin was present at the basal surface (Fig. 3, C and D). β catenin was predominately on the lateral surface at the site of cell–cell contacts, although some basal and some apical staining was also evident (Fig. 3, E–G). As indicated by the histological sections (Fig. 2, P and Q), the majority of alveoli had epithelial cells protruding into the lumen and retained cell–cell contacts as determined by β catenin staining (Fig. 3, D and F, arrows); even in these morphologically disrupted alveoli, the basal markers laminin and $\beta 4$ integrin were located normally (Fig. 3 D and not depicted). Interestingly, these protruding cells were not apoptotic as demonstrated by normal nuclear appearance (Fig. 3 F) and caspase 3 staining (not depicted), retained cell–cell adhesions as demonstrated by β catenin, and located ZO-1 to the apical surface adjacent to the lumens (Fig. 3 B and not depicted). However, BM cell adhesion had clearly been disrupted in the cells given their failure of attachment to the laminin substratum; this was more severe in some cases (Fig. 3, D and F) than in others (Fig. 3, B and G), although in each case some of the most apically located luminal cells were displaced from the BM. These data demonstrate that $\beta 1$ integrin is essential for normal lobuloalveolar development and lactation. It is also required for proper alveolar epithelial morphogenesis.

Deletion of $\beta 1$ integrin results in disruption of focal adhesions and multicellular morphogenesis

To confirm that $\beta 1$ integrin has a key role in alveolar morphogenesis, we assessed focal adhesion function and multicellular morphogenesis of mammary epithelial cells in the absence of

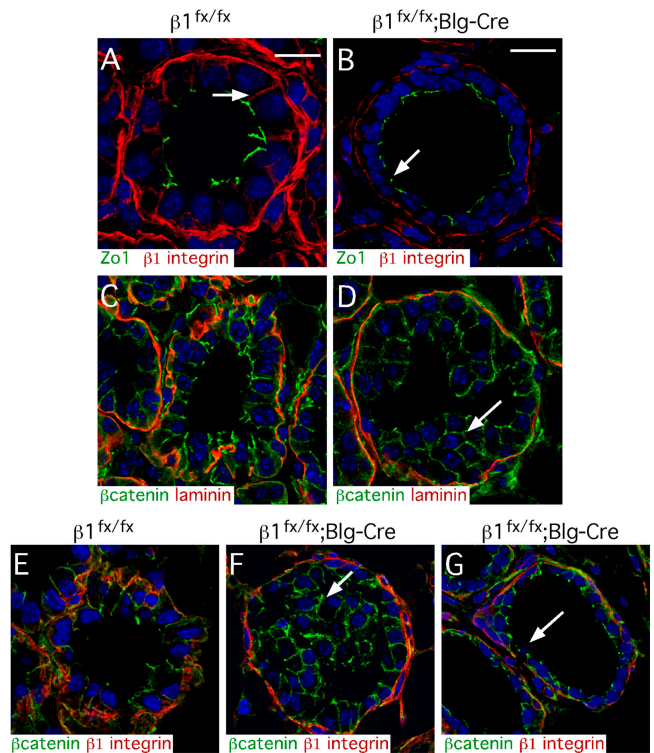


Figure 3. Defects in alveolar structure. Immunofluorescent analysis of alveolar structure from $\text{Itg}\beta 1^{\text{fx/fx}};\text{Blg-Cre}^{\text{Tg/+}}$ (A, C, and E; wild type) and $\text{Itg}\beta 1^{\text{fx/fx}};\text{Blg-Cre}^{\text{Tg/+}}$ (B, D, F, and G; mutant) mice at day 2 of lactation. B and G are from the mouse shown in Fig. 2 P, whereas D and F are from the mouse shown in Fig. 2 Q. (A and B) $\beta 1$ integrin (red) is lost from luminal cells of mutant mice. Arrows indicate lateral staining. Apical localization of ZO-1 (green) in wild-type and mutant alveoli. (C and D) Laminin (red) is correctly deposited in mutant alveoli. Arrow indicates cells protruding into lumen of mutant alveoli with cell–cell staining of β catenin (green). (E–G) Cell–cell and lateral localization of β catenin (green) in wild-type (E) and mutant alveoli, where cells have detached from the BM and protrude in the lumen (F) or are attached to BM (G). $\beta 1$ integrin (red) is basal and lateral in alveoli of wild type and basal only in mutant mice. Bars: (A) 13 μm ; (B–G) 19 μm .

$\beta 1$ integrin in culture using our established culture model. Cells were harvested from $\text{Itg}\beta 1^{\text{fx/fx}}$ mice and infected with either AdCre or β -galactosidase adenovirus (Ad β gal) before being cultured on a stromal ECM (type I collagen) or a BM matrix. 24 h after infection with either AdCre or Ad β gal, no differences in morphology were observed between cells plated on collagen, which is consistent with the time required for adenoviral-expressed proteins to be translated (Fig. 4, A and B): at this time, normal levels of $\beta 1$ integrin were present (not depicted). Between 36 and 48 h after infection, defects in cell morphology became apparent, with AdCre-infected cells displaying a rounded phenotype compared with Ad β gal-infected controls (Fig. 4, C and D).

$\beta 1$ integrin is normally localized to focal adhesions, which are protein complexes that connect the ECM with the actin cytoskeleton (Fig. 4 M). However, in AdCre-infected cells, these focal adhesions largely disappeared, corresponding to the loss of $\beta 1$ integrin, although some small adhesion complexes containing integrin-linked kinase (ILK) and talin remained (Fig. 4 N). Despite the considerable reduction of focal adhesions, AdCre-infected cells continued to express markers for other forms of adherent junctions, such as tight junctions (e.g., ZO-1; Fig. 4, O

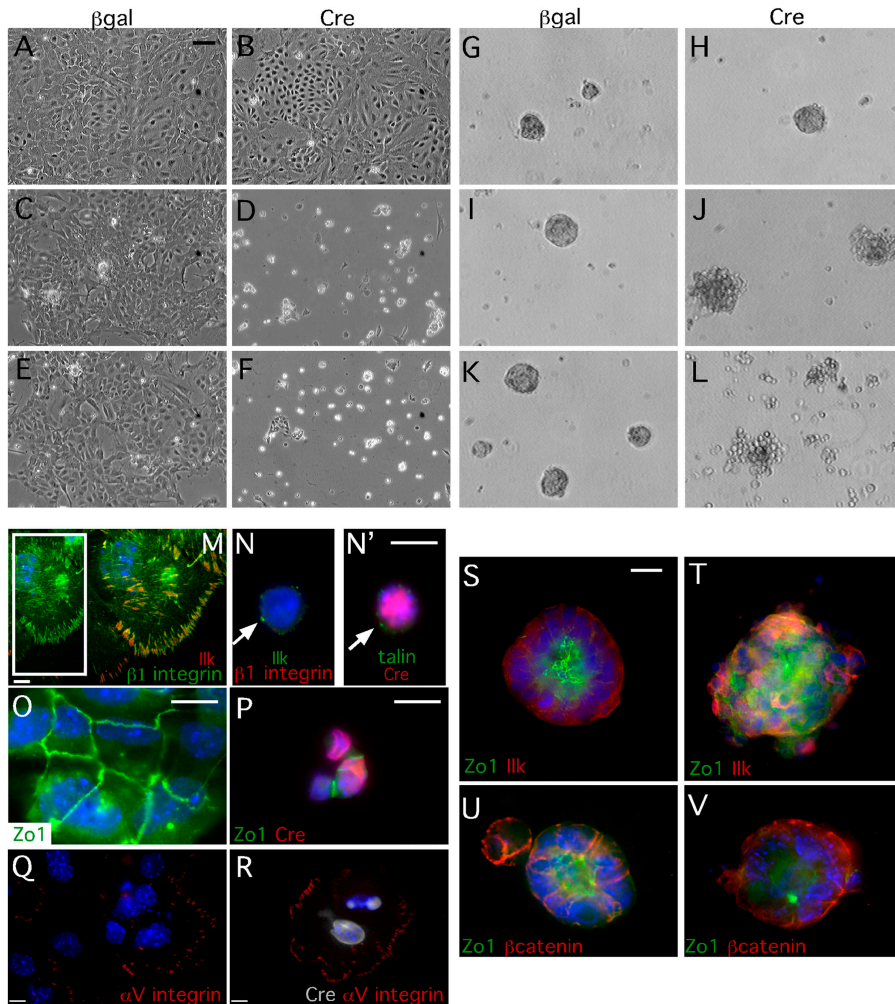


Figure 4. $\beta 1$ integrin is required for epithelial morphogenesis. Primary mammary epithelial cells from $Itg\beta 1^{flx/flx}$ mice were infected with either βgal - or Cre-expressing adenovirus; plated on collagen (A–F and M–P), vitronectin (Q–R), or BM matrix (G–L and S–V); and examined by phase-contrast microscopy (A–L) and immunofluorescent staining as shown in M–V. Time after infection was 24 h (A, B, G, and H), 24 h plus trypsinized and replated for a further 24 h (E, F, K, and L), or 48 h (all other panels). (A–F) Cells plated on collagen form a monolayer (A, C, and E), but the cells round up 48 h after integrin deletion (D) or do not spread when replated (F). Cultures were well washed before phase-contrast microscopy. (G–L) Cells plated on the BM matrix form three-dimensional polarized epithelial structures termed acini (G, I, and K), which are not maintained 48 h after integrin deletion (J). When cells already lacking integrin are plated on the BM matrix, they are unable to initiate acinar formation within the first 48 h of culture (L). (M–P) Deletion of $\beta 1$ integrin results in impaired focal adhesion contacts and a cell-rounding phenotype on collagen. In βgal -infected cells, $\beta 1$ integrin (inset, green) in focal adhesions colocalizes with ILK (M, red). In Cre-infected cells, smaller focal contacts are present (arrows) as demonstrated by ILK and talin staining (green), but the larger focal adhesions are lost (N). ZO-1 (green) is expressed in mutant cells (P). (Q–R) Focal adhesion complexes are maintained after deletion of $\beta 1$ integrin when plated on vitronectin. Similar to βgal -infected cells (Q), Cre-infected cells (gray) are well spread and display αv integrin-containing (red) adhesions (R). (S–V) Deletion of $\beta 1$ integrin results in disruption of epithelial polarity with a failure of acini formation and structure on the BM matrix (lateral cross sections through individual acini). In control acini, ILK (red) is basal (S), β catenin (red) is lateral (U), and ZO-1 (green) localizes to the apical cell surface, where hollow lumens form (S and U). This distribution is disrupted after integrin deletion (T and V). Bars: (A–L) 13 μm ; (M and Q–R) 10 μm ; (N–P) 6.5 μm ; (S–V) 13 μm .

and P), indicating that deletion of $\beta 1$ integrin does not simply result in a global disruption of all cellular adhesions.

When AdCre-infected cells that showed no apparent phenotype 24 h after infection were treated with trypsin and replated back onto collagen (Fig. 4 F), they displayed focal adhesion defects similar to infected cells that were maintained continuously on collagen for 48 h; although these cells adhered to the collagen substratum, they were unable to spread on it. The infected cells were also unable to spread on collagen IV (Fig. 5 C). Together, these data demonstrate that without $\beta 1$ integrin, primary mammary epithelial cells cultured in two-dimensions on collagen fail to either initiate or maintain focal adhesion complexes, resulting in defective form and structure of the cell. $\beta 1$ integrin-null cells did not lose their intrinsic capacity to spread on ECM proteins because when they were plated onto vitronectin, they spread well and assembled peripheral adhesion complexes containing αv (Fig. 4, Q and R) and $\beta 3$ integrin subunits (not depicted).

Mammary epithelial cells cultured on a BM matrix form three-dimensional acini, which are polarized spheres with hollow lumina that resemble alveoli formed during the later stages

of pregnancy (Aggeler et al., 1991). The requirement of $\beta 1$ integrin for the maintenance and initiation of this process was tested by plating cells either directly onto the BM matrix after virus infection or onto collagen for 24 h and then replating them onto the BM matrix. AdCre- and Ad βgal -infected cells began to form acini within 18–24 h after infection, and no differences were observed between the two populations at this time (Fig. 4, G and H). From 24 to 36 h after infection, the acini formed from $\beta 1$ integrin-null cells began to disrupt and individual cells could be seen dissociating from the polarized structure (Fig. 4 J). Cells lacking $\beta 1$ integrin before culture on BM displayed a more severe phenotype; instead these cells remained as either individuals or groups of loosely clustered cells for 48 h (Fig. 4 L).

Acini formed from Ad βgal -infected cells localized the tight junction protein ZO-1 to the apical surface, whereas focal adhesion proteins such as $\beta 1$ integrin or ILK were predominately on the basal and, to a lesser extent, lateral surfaces (Fig. 4 S and see Fig. 8 B). Ad βgal and AdCre infection was confirmed by antibody staining (Fig. 4, N and P) and by Western blot (Fig. 5). In experiments where 100% of cells were infected with AdCre, $\beta 1$ integrin-null cells failed to polarize properly: ZO-1 and ILK

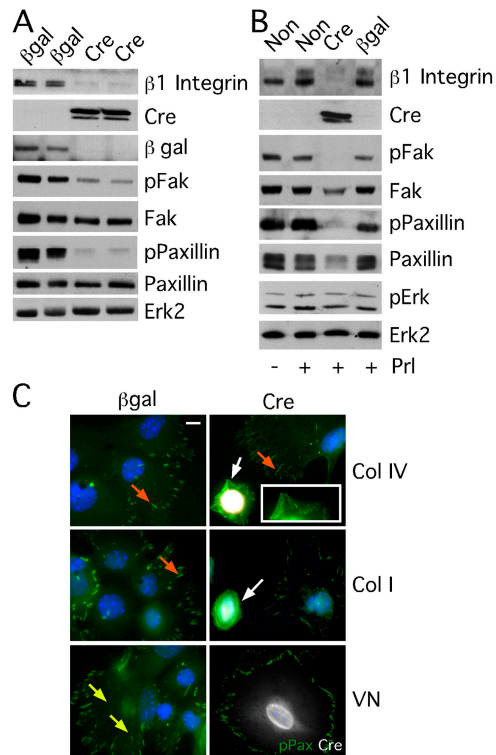


Figure 5. Disruption of focal adhesion protein signaling. Western analysis of protein lysates from $\text{Itg}\beta 1^{\text{lox/lox}}$ mammary epithelial cells infected with either βgal - or Cre-expressing adenovirus 48 h after infection. Cells cultured on either collagen in growth medium (A) or on BM matrix in differentiation medium with (+) or without (-) Prl (B). Expression of Cre resulted in reduction of $\beta 1$ integrin levels and decreased amounts of phosphorylated FAK and paxillin regardless of substratum. (C) Small focal regions containing phosphopaxillin are detected in Cre-expressing $\text{Itg}\beta 1^{\text{lox/lox}}$ cells (white arrows and inset, showing only phosphopaxillin) cultured on either collagen type IV (Col IV) or I (Col I). Neighboring uninfected cells (suboptimal doses of AdCre virus were used for this immunofluorescence experiment) or control-infected cells are spread and contain substantial phosphopaxillin-containing adhesion complexes (orange arrows). When plated on vitronectin (VN), both Cre- and βgal -infected cells spread and formed focal adhesions containing phosphopaxillin, although the smaller, more centrally located signaling complexes (yellow arrows) are lost from Cre-infected cells. Bar, 10 μm .

were mislocalized from the apical and basal surfaces and were instead present on all cell surfaces (Fig. 4 T). The total disruption of acini formation was reduced by decreasing the percentage of AdCre-infected cells to 50–75% (Fig. 4 V). In these cultures, acini were formed, and although ZO-1 appeared to be slightly reduced in intensity, it was correctly located on the apical surface (Fig. 4 V). Basal markers of polarity such as ILK were also correctly located (unpublished data).

Thus, integrin deletion both in vivo and in the culture model results in acini containing incorrectly localized cells and disrupted morphogenesis. $\beta 1$ integrins are therefore required for the multicellular glandular morphogenesis that normally occurs in association with BM.

Disruption of signaling pathways in response to $\beta 1$ integrin deletion

To address the perturbed focal adhesion formation and epithelial morphogenesis further, we investigated the requirement for $\beta 1$ integrin in the activation of selected downstream sig-

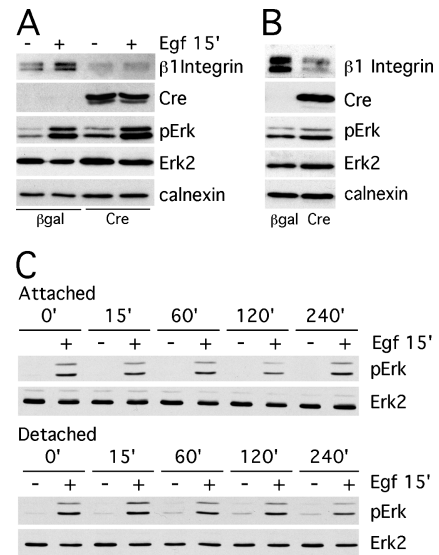


Figure 6. Erk activation in response to EGF stimulation in the absence of $\beta 1$ integrin. (A and B) $\text{Itg}\beta 1^{\text{lox/lox}}$ primary mammary epithelial cells infected with either βgal - or Cre-expressing adenovirus on collagen. (A) Cells cultured for 36 h after infection in growth medium, starved for 4 h, and stimulated with (+) or without (-) EGF 15 min before harvest. (B) Cells in growth medium for 48 h after infection. Ablation of $\beta 1$ integrin does not affect the rapid (A) or sustained (B) activation of MAPK pathway as measured by Erk phosphorylation in response to EGF. (C) Primary wild-type epithelial cells were grown on collagen for 24 h and either left on collagen (attached) or maintained in suspension (detached) for times as indicated before 15 min of EGF stimulation. Erk phosphorylation was not affected by the total loss of integrin ligation in mammary epithelial cells.

naling mediators, including the protein tyrosine kinase FAK and the adaptor protein paxillin. Infection of primary epithelial cells with AdCre virus resulted in knockdown of $\beta 1$ integrin by 48 h after infection in cells plated on collagen and the BM matrix (Fig. 5). Loss of $\beta 1$ integrin in cells cultured continuously on collagen in growth medium (similar to those cells shown in Fig. 4, C and D) resulted in decreased levels of tyrosine phosphorylation at residue 397 of FAK, which is critical for its full activation (Fig. 5 A; Schlaepfer and Hunter, 1998). Similarly, tyrosine phosphorylation of paxillin was reduced. Erk2 and calnexin were used as protein loading controls (Fig. 5, A and B; and not depicted). Thus, phosphorylation of FAK and paxillin were dramatically reduced in the absence of $\beta 1$ integrin. The primary integrin receptors for collagen are $\alpha 1\beta 1$ and $\alpha 2\beta 1$, whereas $\alpha 6\beta 1$ and $\alpha 6\beta 4$ are the main laminin receptors (Taddei et al., 2003). Although the $\beta 1$ -null cells were unable to spread well on collagen I or IV, the small number of adhesion complexes that did form retained the ability to initiate proximal signaling because they contained phosphorylated paxillin (Fig. 5 C, top). Moreover, the $\beta 1$ -null cells spread on vitronectin and had large peripheral adhesion complexes showing robust phosphopaxillin staining (Fig. 5 C, bottom), although interestingly the smaller, more centrally located phosphopaxillin adhesions are not visible in the absence of $\beta 1$ integrin (yellow arrows).

We next investigated the phosphorylation status of FAK and paxillin in cells cultured on laminin-rich BM matrix. The total levels of both these proteins were slightly lower, whereas

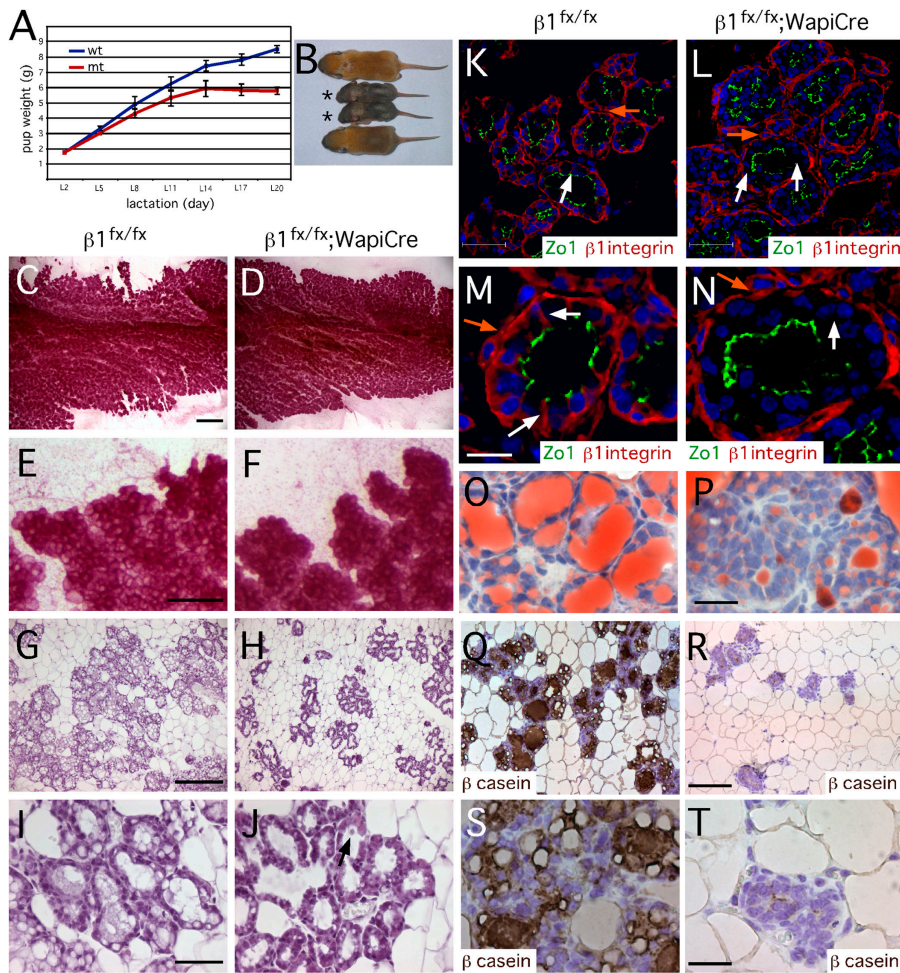


Figure 7. Impaired epithelial function in *Itgβ1^{fx/fx};WapiCre^{Tg/+}* mice. (A and B) Impaired lactogenesis was observed in *Itgβ1^{fx/fx};WapiCre^{Tg/+}* mice. (A) Pup growth-rate analysis of wild-type and mutant mice from first pregnancy. Error bars indicate \pm SEM. (B) Reduced size of pups from mutant females (asterisk) is maintained after second pregnancy. (C–F) Whole mount analysis shows no significant difference in lobuloalveolar development between wild-type and mutant mice (E and F are higher power views). (G–J) Histological analysis demonstrates a slight reduction in alveolar density from the mammary glands of mutant mice at lactation day 2. Alveolar morphology is essentially normal, although some epithelial cells detach and shed into the lumen of alveoli (J, arrow). (K–N) As with the *Blg-Cre* deletions, $\beta 1$ integrin remains in the myoepithelial population (orange arrows) but is absent in luminal cells. This is particularly noticeable on lateral cell surfaces, where the integrin normally present (K and M, white arrows) has disappeared (L and N, white arrows). ZO-1 is located on the apical surface of luminal cells in both wild-type and mutant alveoli (M and N). (O–T) Oil Red O staining of milk fat (O and P) and β casein staining (Q–T) in mammary glands from wild-type and mutant mice at day 18 of pregnancy. Milk protein (casein) and lipid (Oil Red O) is dramatically diminished in $\beta 1$ integrin-null mammary glands. Bars: (C and D) 2 mm; (E and F) 0.5 mm; (G and H) 200 μ m; (I and J) 50 μ m; (K–L) 47 μ m; (M and N) 15 μ m; (O, P, S, and T) 25 μ m; (Q and R) 100 μ m.

their tyrosine phosphorylation was reduced dramatically, even in the presence of lactogenic hormones (Fig. 5 B). Thus, deletion of $\beta 1$ integrin diminishes the activation of some of the immediate proximal downstream signaling mediators required for normal cell morphology, although the cells do retain a capacity for proximal integrin signaling when they adhere to the ECM through alternative β integrin subunits. A reduction of FAK and paxillin tyrosine phosphorylation also occurs after integrin deletion in vivo (unpublished data).

The impairment of adhesion signaling in $\beta 1$ integrin-null epithelia raised the possibility of a general inability of the cells to respond to external stimuli. To test whether MAPK signaling can be activated after reducing $\beta 1$ integrin-null levels, cells were stimulated with EGF and the phosphorylation of Erk1/2 was measured (Gilmore et al., 2002). This pathway was activated in cells plated on the BM matrix and stimulated with lactogenic hormones, regardless of the presence or absence of $\beta 1$ integrin (Fig. 5 B). However, mammary cells grown on the BM matrix do not undergo significant proliferation (unpublished data), so subsequent experiments were performed on collagen. To test rapid activation of the MAPK pathway, cells were serum starved and treated with EGF for 15 min. Although AdCre infection resulted in the knockdown of $\beta 1$ integrin (Fig. 6 A), both these and control cells responded to EGF by increasing Erk1/2 phosphorylation compared with nonstimulated cultures.

Furthermore, similar levels of pErk were observed after continuous exposure to serum and EGF (Fig. 6 B). Thus, $\beta 1$ integrin is not required for the phosphorylation of Erk in response to either pulse or steady-state exposure to EGF.

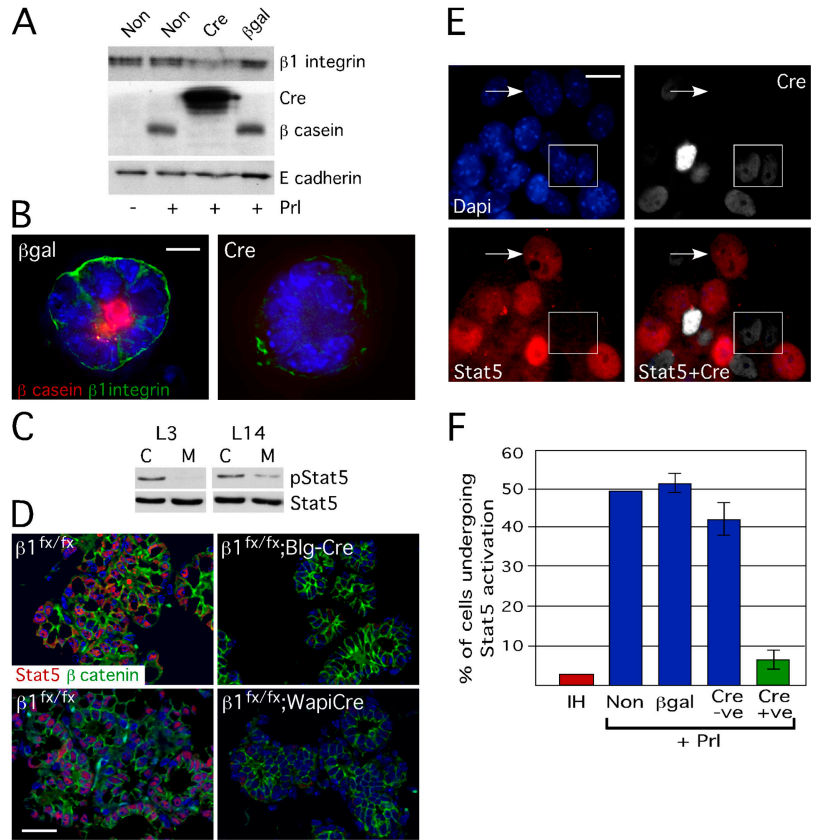
Because other β integrins may substitute for the $\beta 1$ subunit, and to rule out the possibility that the residual $\beta 1$ integrin in Fig. 6 (A and B) contributes to EGF signaling, primary wild-type cultures were trypsinized and kept in suspension (where integrins are unligated) or cultured on collagen before a 15-min stimulation with EGF (Fig. 6 C). EGF induced Erk1/2 phosphorylation in both attached and suspended cells, demonstrating activation of the MAPK-signaling cascade in the complete absence of integrin ligation. Thus, unlike some other cell systems (Bill et al., 2004), EGF-dependent activation of this pathway in primary mammary epithelial cells is not dependent on $\beta 1$ integrin or integrin ligation.

Deletion of $\beta 1$ integrin from differentiated epithelium results in impaired lactogenesis

The use of *Itgβ1^{fx/fx};Blg-Cre^{Tg/+}* mice identified a critical requirement for $\beta 1$ integrin in lactogenesis. However, because of this impaired development we could not determine if $\beta 1$ integrin is required directly for mammary differentiation or if the defective lactation occurs as a result of abnormal lobuloalveolar

Figure 8. $\beta 1$ integrin regulates Prl-mediated differentiation and Stat5 activation.

(A) Itg $\beta 1^{fx/fx}$ primary epithelial cells plated on the BM matrix and infected with AdCre resulted in the down-regulation of $\beta 1$ integrin and failed differentiation as demonstrated by impaired β casein synthesis after Prl stimulation for 24 h. Note that wild-type cells infected with AdCre virus still differentiated in response to Prl (not depicted). (B) Immunofluorescent staining of acini with antibodies to $\beta 1$ integrin (green) and β casein (red) formed from Cre- and β gal-infected Itg $\beta 1^{fx/fx}$ cells demonstrated impaired differentiation. (C) Western analysis of whole mammary gland lysates from Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ (M) mice show reduced phosphorylation levels of Stat5 compared with Itg $\beta 1^{fx/fx};WapiCre^{+/+}$ (C) controls at days 3 and 14 of lactation in their first pregnancy. (D–F) Prl-induced Stat5 nuclear translocation is disrupted after loss of $\beta 1$ integrin. (D) Stat5 (red) nuclear staining is prominent in wild type but is severely diminished in mutant alveoli from Blg-Cre and WapiCre mammary glands. β catenin (green) is localized on the lateral surfaces of luminal epithelial cells of wild-type, Blg-Cre, and WapiCre alveoli. (E) Triple immunofluorescence of Cre-infected Itg $\beta 1^{fx/fx}$ primary epithelial cells stained for Cre (gray), Stat5 (red), and Dapi (blue). The infected cells fail to translocate Stat5 to the nucleus; two examples are depicted within white box. In contrast, noninfected cells (which do not stain for Cre) do show Stat5 translocation after 15 min of Prl stimulation (arrows). (F) Quantification of the number of cells undergoing Stat5 activation after 15 min of Prl stimulation. Error bars indicate \pm SEM. Bars: (B and E) 13 μ m; (D) 40 μ m.



development. To address this, we used Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ mice to examine the role of $\beta 1$ integrin in vivo after luminal epithelial cells had become differentiated.

Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ mice were able to support their litters during lactation. However, close examination of pup growth rates revealed a significant decrease in pup weight from Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ dams (Fig. 7 A), demonstrating a defect in the functional capacity of the mammary glands. Whole mount analysis demonstrated no obvious differences in lobuloalveolar development (Fig. 7, C–F), although histological sections indicated that alveoli in Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ mice were smaller than wild-type controls (Fig. 7, G–J). Consistent with a role for $\beta 1$ integrin in the regulation of alveolar structure, increased numbers of epithelial cells were present in the lumen of alveoli from Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ mice, although this phenotype was less dramatic than that observed in Itg $\beta 1^{fx/fx};Blg-Cre^{Tg/+}$ mice. The alveoli retained a more open morphology at lactation day 2 (Fig. 7 J) than in the Itg $\beta 1^{fx/fx};Blg-Cre^{Tg/+}$ mice (Fig. 2, P and Q). The defect in the functional capacity of alveoli was not rescued by repeated pregnancies, with pups from Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ mice after the second pregnancy displaying a dramatic reduction in growth rates compared with wild-type controls (Fig. 7 B).

Immunofluorescent staining revealed that luminal cells within alveoli of the Itg $\beta 1^{fx/fx};WapiCre^{Tg/+}$ mice were correctly polarized because they positioned ZO-1 in an apical location (Fig. 7, K–N), β catenin was located at cell–cell junctions (Fig. 8 D, bottom right), and laminin was deposited at the basal cell surface (not depicted). Despite this, the cells were unable to

synthesize milk efficiently. They made low levels of milk fat, as determined by Oil Red O staining (Fig. 7, O and P), and milk proteins, as disclosed by immunostaining with an antibody to β casein (Fig. 7, Q–T). Thus, the WapiCre alveoli develop normally and the epithelial cells retain their polarity, but they fail to differentiate efficiently in vivo.

Together, these data suggest that deletion of $\beta 1$ integrin after the initiation of differentiation in vivo results in an inability to maintain a functional phenotype in late pregnancy and lactation. This indicates that $\beta 1$ integrin may be needed directly for the signaling pathways that maintain the differentiation response. We therefore tested this possibility using a culture model.

$\beta 1$ integrin is necessary for prolactin (Prl)-induced mammary epithelial cell differentiation

A direct role for $\beta 1$ integrin in Prl signaling and milk protein gene expression has not been explored using a null mutation, though it has been suggested (Streuli et al., 1995b; Edwards et al., 1998; Faraldo et al., 1998, 2002). To directly assess whether $\beta 1$ integrin is required for epithelial differentiation in response to a Prl signal, we infected primary cultures from late-pregnant Itg $\beta 1^{fx/fx}$ mice with AdCre or Ad β gal virus and plated them on the BM matrix (Fig. 8, A and B). After 24 h under growth conditions, cultures were exposed to differentiation medium with or without Prl for another 24 h, after which the production of β casein, a differentiation endpoint marker, was assessed. Because primary mammary epithelial cells undergo

little proliferation on the BM matrix in the presence of differentiation medium, this assay allowed the role of $\beta 1$ integrin in differentiation to be evaluated in the absence of proliferation (which does occur in the *in vivo* context).

β casein was not synthesized in the absence of Prl, whereas the addition of Prl resulted in the synthesis of β casein in both noninfected and Ad β gal-infected cells (Fig. 8 A). In contrast, AdCre-infected cells, in which $\beta 1$ integrin levels are significantly reduced (Fig. 8 A and Fig. 5 B), failed to undergo differentiation. The lack of β casein synthesis and secretion into the lumen of acini was confirmed by immunofluorescent staining (Fig. 8 B). Acini that maintained a polarized structure because of a heterogeneous population of AdCre-infected and noninfected cells produced β casein in $\sim 25\%$ of acini compared with 75% of β gal-infected controls. AdCre infection of 100% of cells resulted in a complete failure of differentiation. These data demonstrate that $\beta 1$ integrin is essential for Prl-mediated differentiation in mammary epithelia.

$\beta 1$ integrin-null cells fail to activate Stat5

The failure of Prl-induced differentiation in $\beta 1$ integrin-null cells suggested that this ECM receptor is required for the transduction of the Prl signaling pathway. Whole mammary gland lysates from Itg $\beta 1^{fx/fx};$ WapiCre $^{Tg/+}$ and Itg $\beta 1^{fx/fx};$ WapiCre $^{+/+}$ mice at days 3 and 14 of lactation were probed with phospho-specific antibodies to Stat5 (Fig. 8 C). A reduction in the levels of activated Stat5 was observed during both early and late lactation in mutant mice compared with wild-type controls; moreover, impaired nuclear translocation of Stat5 occurred at day 18 of pregnancy (i.e., just before lactation) in both Itg $\beta 1^{fx/fx};$ Blg-Cre $^{Tg/+}$ and Itg $\beta 1^{fx/fx};$ WapiCre $^{Tg/+}$ mice (Fig. 8 D). These results confirm that loss of $\beta 1$ integrin prevents Stat5 signaling and that a similar phenotype results in glands of mice generated with both the Blg and Wap promoters. $\beta 1$ integrin is therefore required for the activation of a transcription factor essential for the induction of milk gene synthesis *in vivo*.

To assess whether $\beta 1$ integrin acutely mediates Stat5 activation, we performed a nuclear translocation assay on primary mammary epithelial cells infected with AdCre or Ad β gal (Fig. 8, E and F). After activation, Stat5 translocates into the nucleus, where it initiates transcription of target genes such as β casein and Wap (Gouilleux et al., 1994; Liu et al., 1996). In the absence of Prl, only $2.9 \pm 0.96\%$ of the noninfected cells displayed Stat5 nuclear translocation; this increased to $49.2 \pm 0.15\%$ after 15 min of stimulation with Prl and was not affected by infection with Ad β gal virus ($51.6 \pm 2.36\%$ of cells with nuclear localization). However, the infection of cells with AdCre virus, which ablates $\beta 1$ integrin, resulted in a failure of Prl to initiate Stat5 activation, with only $6.8 \pm 2.64\%$ of cells demonstrating Stat5 nuclear localization. The use of suboptimal levels of AdCre virus during infection provided a further internal control, resulting in a proportion of the cells that were uninfected as assessed by Cre antibody staining. These Cre-negative cells behaved in a manner similar to noninfected or Ad β gal-infected controls, displaying Stat5 activation in $42.2 \pm 8.15\%$ of cells. Thus, our data show that $\beta 1$ integrin is critical for the Prl-induced activation of Stat5, a transcription factor essential for mammary

epithelial differentiation, and they provide genetic evidence for cooperation between integrin and cytokine signaling pathways.

Discussion

In this paper, we report on two phenotypes that result from ablation of the $\beta 1$ integrin gene from luminal cells of mammary epithelium. The first is that mammary gland development during pregnancy is perturbed, resulting in malformed alveoli. The second is that lactational differentiation is compromised, leading to the inability of dams to nurse their pups efficiently.

$\beta 1$ integrin is essential for alveolar development

Conditional deletion of $\beta 1$ integrin resulted in normal elongation and tertiary side branching of mammary ducts after pregnancy. This is in concordance with a recent study where normal ductal outgrowth during puberty was observed in Itg $\beta 1^{fx/fx};$ MMTV-Cre $^{Tg/+}$ mice (White et al., 2004). In our work, the lack of $\beta 1$ integrin compromised subsequent lobuloalveolar development. Sparse alveoli were observed in histological sections of mammary glands from Itg $\beta 1^{fx/fx};$ Blg-Cre $^{Tg/+}$ mice, and alveoli failed to expand into fully formed lobuloalveoli. Variation in phenotype severity (and indeed the number of epithelial cells expressing Cre, ranging from 50 to 100% in the Blg-Cre line) was observed between mice, most likely because of the mosaic expression of Cre, as has been observed in other lines (White et al., 2004). Consistent with this, both $\beta 1$ integrin-null and normal alveoli were observed in the same glands, with only those lacking $\beta 1$ integrin displaying a phenotype.

The retarded lobuloalveolar development in Itg $\beta 1^{fx/fx};$ Blg-Cre $^{Tg/+}$ mice is suggestive of a defect in epithelial proliferation. In a separate study, we found that the loss of $\beta 1$ integrin leads to decreased epithelial cell proliferation both in culture and in Itg $\beta 1^{fx/fx};$ WapiCre $^{Tg/+}$ mice (at the beginning of lactation, when a proliferative burst normally occurs) and that this is attributable to increased levels of the cell cycle inhibitor p21 and the consequent effects on cell cycle machinery (Li et al., 2005). This phenotype may reflect a general requirement for $\beta 1$ integrin *in vivo* because related proliferation defects have been noted in other $\beta 1$ integrin-null cell types such as chondrocytes (Aszodi et al., 2003). Reduced proliferation has also been noted in the mammary glands of mice expressing a dominant-negative chimeric form of $\beta 1$ integrin (Faraldo et al., 1998).

A novel phenotype that we observed *in vivo* is disrupted alveolar morphogenesis. In some cases, protuberant networks of cells formed within alveoli; these cells are wholly detached from the subtending laminin-rich BM, but retain cell-cell interactions as judged by β catenin staining, and the tight junction protein ZO-1 was localized apically, adjacent to the residual lumens. Remarkably, these cells do not undergo overt apoptosis. In contrast to ablation of $\beta 1$ integrin in skin, deletion in the mammary gland does not appear to cause defects in BM formation (Raghavan et al., 2000); this may be because $\beta 1$ integrin was targeted in luminal epithelium rather than myoepithelial cells, which are the main source of BM proteins (Gudjonsson et al., 2002).

Although we are currently trying to understand the mechanism for the protuberant phenotype, the displacement of luminal cells from the BM reflects a key role for $\beta 1$ integrin heterodimers in mediating cell–ECM adhesion in luminal mammary epithelia. This is supported by the observations that loss of $\beta 1$ integrin in the culture model resulted in defective focal adhesion function and cell rounding on two-dimensional substrata, as occurs after perturbation of $\beta 1$ integrin in other cell types (Lukashev et al., 1994), and that polarized acini could not form or be maintained on a three-dimensional laminin-rich matrix. Both $\beta 3$ and $\beta 4$ integrins are expressed in many mammary carcinoma lines and become up-regulated in primary cultures after an extended period in culture, but our data suggest that they may not substitute in vivo. $\beta 4$ integrin is normally restricted to myoepithelial (as opposed to luminal) cells in vivo (Runswick et al., 2001), and we have not been able to detect the $\beta 3$ subunit in vivo (unpublished data). Furthermore, transplanted $\alpha 6$ -null mammary epithelium lacking the $\alpha 6\beta 4$ heterodimer develops normally in vivo (Klinowska et al., 2001).

$\beta 1$ integrin controls mammary epithelial cell differentiation

Although it is possible that altered proliferation and morphogenesis of mammary alveoli lead to the failed lactogenesis of $\beta 1$ integrin–null mammary glands, two lines of evidence indicate that this effect is more direct. First, we have performed experiments similar to those with the Blg promoter, using instead the Wap promoter, which is expressed at a later time in development. Our rationale for this is that the Blg promoter is expressed in pre-pregnant ductal epithelium; thus, integrin ablation before pregnancy might inhibit the formation of proper alveoli. However, by deleting $\beta 1$ integrin after the advent of alveolar formation via the Wap promoter (which is not expressed until mid-pregnancy), we demonstrate that lactation is still disrupted in cells that are already committed to an alveolar fate and have begun to differentiate. Moreover, our data using the Itg $\beta 1^{fx/fx}$; WapCre^{Tg/+} mice indicate that milk production is compromised in alveoli where the epithelial cells retain the ability to polarize (as judged by apical distribution of ZO-1, lateral distribution of β catenin, and basal localization of laminin) and maintain a more open morphology than alveoli from the Itg $\beta 1^{fx/fx}$; Blg-Cre^{Tg/+} mice. Second, we have extracted fully differentiated cells from the mammary glands of late-pregnant Itg $\beta 1^{fx/fx}$ mice and infected them with AdCre virus. Under these conditions, and using well-established assays for mammary differentiation, the loss of $\beta 1$ integrin prevents Prl-induced milk protein expression.

Together, these experiments demonstrate a central role for $\beta 1$ integrin in the maintenance of a differentiation signal in mammary epithelium: indeed, they provide key evidence using gene deletion that integrins are required to maintain tissue-specific function in glandular epithelium. $\beta 1$ integrin has also been implicated in the control of epidermal differentiation; however, unlike the mammary gland, where $\beta 1$ integrin signaling is permissive for differentiation, in the epidermis the $\beta 1$ integrin signaling is inhibitory (Watt, 2002).

It is well known that members of the Prl signaling pathway have a fundamental role in both alveolar development and mammary differentiation (Hennighausen and Robinson, 2001). The defect in alveolar development and differentiation in $\beta 1$ integrin–null mammary glands suggests a relationship between integrin and Prl signaling. We have demonstrated that this is indeed the case because Stat5 activation is compromised in vivo during lactation and it does not translocate to the nucleus in response to Prl in the culture model. In previous work, we have argued that matrix-specific integrins are essential to control the Prl signaling cassette in mammary cells because it is not activated during culture on stromal ECM, and we have suggested that this is attributable to a dominant-negative inhibitory mechanism that prevents differentiation on inappropriate ECM (Streuli et al., 1995a; Edwards et al., 1998). Our study now provides genetic evidence that integrins have at least a permissive role in Prl receptor signaling, and we are now trying to identify a mechanism to explain the requirement of integrins. Importantly, however, the lack of integrin does not compromise all ligand-activated signaling pathways. Steady-state Erk1/2 phosphorylation in $\beta 1$ integrin–null cells cultured in growth medium is no different than that in integrin-containing cells, and acute stimulation with EGF in both integrin–null cells and wild-type cells deprived of all integrin signaling (by detachment from the ECM) leads to similar levels of Erk1/2 phosphorylation.

In summary, we have shown that $\beta 1$ integrin is critical for the alveolar morphogenesis of a glandular epithelium and for the maintenance of its differentiated function. Our data highlight a central role for integrins in cytokine signaling, thus demonstrating that some signaling pathways that were once believed to result from simple ligand-dependent activation actually depend on more complex intracellular networks that take into account the integrin-mediated positional identity of cells.

Materials and methods

Mouse strains and breeding

The Itg $\beta 1^{fx/fx}$, Blg-Cre^{Tg/+}, WapCre^{Tg/+}, and Rosa26R mice have been described previously (Selbert et al., 1998; Soriano, 1999; Graus-Porta et al., 2001; Wintermantel et al., 2002). The WapCre mouse contains an improved version of the Cre recombinase under the control of the Wap promoter that minimizes gene silencing and mosaicism in cells where Wap is normally expressed. ICR mice were purchased from Harlan Olac Ltd. Cre-mediated deletion was achieved by crossing fx/fx mice with Cre Tg/+ mice to produce $fx/+$; CreTg/+ mice. $fx/+$; CreTg/+ mice were then crossed with fx/fx mice to produce fx/fx ; Cre Tg/+ (mutants) or fx/fx ; Cre +/+ (wild type) used for analysis. Genotyping was performed as previously described (Selbert et al., 1998; Soriano, 1999; Graus-Porta et al., 2001; Wintermantel et al., 2002) from DNA prepared from ear clips.

All mice were mated at ~12 wk of age. Growth-rate analysis of pups was performed by standardizing litter sizes to eight pups/litter and weighing pups at least every 2 d throughout lactation. Pups that died because of dehydration were not replaced from Itg $\beta 1^{fx/fx}$; Blg-Cre^{Tg/+} mice that displayed a lactation phenotype, and average pup weight was calculated from the maximum number of pups supported from these mice. Note that the pups nursing from mice with integrin–null mammary glands are heterozygous for the integrin flox allele, and because Cre is only expressed in mature female mammary tissue, the pups are all phenotypically normal.

Mice were housed and maintained according to the University of Manchester and UK Home Office guidelines for animal research.

Morphological and histological analysis

Whole mount analysis was performed by spreading inguinal mammary glands on polysine slides, fixing in 10% neutral buffered formalin over-

nicht, and defatting in acetone before staining with carmine alum (0.2% carmine and 0.5% aluminium sulfate) overnight. The whole mount was dehydrated using a graded ethanol series followed by immersion in Slide Bright for 1 h and stored in methyl salicylate before photography using a dissecting microscope (Leica; Naylor and Ormandy, 2002). Quantification of ductal side branching was performed as previously described (Naylor et al., 2003). Whole mounts were subsequently paraffin embedded before sectioning (5 μ m) and subjected to standard hematoxylin and eosin staining. Histology was detected with a microscope (Axioscop2; Carl Zeiss Microimaging, Inc.) using PlanNeofluar 10 \times (NA 0.3) and 40 \times (NA 0.75) lenses at RT. Images were captured with a color camera (AxioCam; Carl Zeiss Microimaging, Inc.) and analyzed with Openlab 4 software (Improvision).

X-gal staining was performed by fixing inguinal mammary glands with 4% PFA for 1–2 h at 4°C and washing three times in 100 mM sodium phosphate, pH 7.3, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP-40 for 30 min before adding 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal and incubating at RT overnight. Tissue was dehydrated with an ethanol series and cleared in Slidebright before being photographed.

Oil Red O staining was performed by staining 6- μ m mammary gland cryosections in freshly diluted Oil Red O solution (six parts 0.5% Oil Red O stock solution [Sigma-Aldrich] and four parts H₂O) for 15 min. Sections were rinsed twice with 60% isopropanol and once with water and then counterstained with hematoxylin for 1 min before being photographed.

Primary cell assays

Extraction and adenoviral infection of primary mammary epithelial cells from 15.5- to 17.5-d pregnant mice was performed as described previously (Watkin and Streuli, 2002). Cells were infected with either Cre recombinase (AdCreM1; Microbix Biosystems, Inc.) or Ad β gal (a gift from A. Ridley, University College London, London, UK) in suspension for 45 min before being plated at equal density onto either collagen I or the BM matrix (Matrigel; Becton Dickinson; Pullan et al., 1996). Cells were cultured in growth medium containing 5 μ g/ml insulin, 1 μ g/ml hydrocortisone, 3 ng/ml EGF, 10% FCS, 50 U/ml Pen/Strep, 0.25 μ g/ml fungizone, and 50 μ g/ml gentamycin in F12 medium or differentiation medium containing the same concentrations of insulin, hydrocortisone, fungizone, Pen/Strep, and gentamycin in DME/F12 medium (GIBCO BRL) with or without 3 μ g/ml Prl (ovine Prl) for the induction of differentiation as indicated (reagents were purchased from Sigma-Aldrich unless otherwise indicated). Replating experiments were performed after 24 h growth on collagen. Growth medium was removed, and cells were incubated for 10 min in HE buffer (9.5 g/L HBSS-Ca²⁺ free, 0.35 g/L NaCO₃, and 2g/L EDTA; Sigma-Aldrich) before trypsinizing (GIBCO BRL) for 2–3 min, spinning at 12,000 rpm for 4 min, and plating onto either collagen or the BM matrix as described. 24 h after infection, zVAD was added to all cultures to minimize apoptosis.

Stat5 nuclear translocation assay was performed by plating adenovirus-infected cells on collagen-coated coverslips (collagen coated for 2 h at RT, followed by three washes of PBS) in growth medium. 12 h after infection, growth medium was replaced with differentiation medium containing BM matrix (1:50) without Prl (Streuli et al., 1995b). 48 h after infection, Prl was added, and after a 15-min stimulation, cells were fixed with 4% PFA before immunostaining with Stat5a (Santa Cruz Biotechnology, Inc.) and Cre (Chemicon) antibodies.

Western analysis

Protein was extracted from cell lysates using 1 \times net buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, and 2 mM EDTA, pH 7.6) for cells on collagen or 2 \times net buffer for cells on BM matrix with 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1:100 dilution of Protease cocktail inhibitor set 1 (Calbiochem). Lysates from mammary glands were prepared by homogenizing frozen mammary tissue in 10 mM HEPES, pH 7.5, 100 mM KCl, 5% glycerol, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM Na₃VO₄, and 10 mM sodium molybdate. Lysates from mammary glands used for casein analysis were extracted in 2 \times net buffer. Immunoblotting was performed as described previously (Naylor et al., 2003). For cell cultures, equal amounts of protein were used and equivalent loading was assessed by referral to controls such as Erk2 and calnexin. For mammary gland samples, equivalent amounts of protein were determined by quantification using a protein assay (Bio-Rad Laboratories). Specific binding was detected using HRP-conjugated secondary antibodies (GE Healthcare) with Chemiluminescence reagent (PerkinElmer) and Biomax light film (Eastman Kodak Co.). Primary antibodies were as follows: β 1 Integrin, paxillin (BD Biosciences), β gal (Promega), FAK (a gift from A. Ziemiecki, University of

Bern, Bern, Switzerland), calnexin (StressGen Biotechnologies), cytokeratin 8/18 (Progen Biotechnik), β casein (Streuli et al., 1995b), phospho-FAK Y397, phospho-Jak2 Y1007/1068, phosphopaxillin Y31, Jak2 (Biosource International), E-cadherin (Qbiogene), phospho-Stat5 (Upstate Biotechnology), phospho-Erk1/2, phospho-PKB S473, PKB (Cell Signaling Technology), β actin (Sigma-Aldrich), and Erk2 (Santa Cruz Biotechnology, Inc.).

Immunostaining

Cells plated on either collagen- or BM matrix-coated coverslips were stained as described previously (Streuli et al., 1991) and photographed after mounting with Prolong anti-fade (Invitrogen). Immunostaining of mammary glands was performed on tissue that had been frozen in liquid nitrogen and stored at –80°C. 6- μ m sections were then fixed and immunostained as described previously (Klinowska et al., 2001). Immunofluorescence was detected with either a confocal microscope (for tissue sections; SP2 AOBIS; Leica) or a microscope (for cultured cells; Axioplan2; Carl Zeiss Microimaging, Inc.) using PlanApochromat 100 \times and 63 \times lenses (NA 1.40) with Immersol 518F oil at RT. 0.2- μ m z sections were captured with a camera (Orca ER; Hamamatsu) and analyzed with confocal software (Leica) or Openlab 4 software.

Antibodies used for immunostaining that were different to those used in Western analysis were as follows: α 4 β 1 integrin and laminin (Klinowska et al., 1999), GoH3 (ICN Biomedicals), ZO-1 (Chemicon), cleaved Caspase 3 (Cell Signaling Technology), ILK (Upstate Biotechnology), α -smooth-muscle actin (Sigma-Aldrich), β 4 integrin, β catenin (BD Biosciences), secondary α -mouse Cy2 or Rhx, α -rabbit Cy2 or Rhx, and α -rat Rhx (Jackson ImmunoResearch Laboratories).

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