Control of mammary myoepithelial cell contractile function by $\alpha 3\beta 1$ integrin signalling

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In the functionally differentiated mammary gland, basal myoepithelial cells contract to eject the milk produced by luminal epithelial cells from the body. We report that conditional deletion of a laminin receptor, $\alpha 3\beta 1$ integrin, from myoepithelial cells leads to low rates of milk ejection due to a contractility defect but does not interfere with the integrity or functional differentiation of the mammary epithelium. In lactating mammary gland, in the absence of $\alpha 3\beta 1$, focal adhesion kinase phosphorylation is impaired, the Rho/Rac balance is altered and myosin light-chain (MLC) phosphorylation is sustained. Cultured mammary myoepithelial cells depleted of $\alpha 3\beta 1$ contract in response to oxytocin, but are unable to maintain the state of post-contractile relaxation. The expression of constitutively active Rac or its effector p21-activated kinase (PAK), or treatment with MLC kinase (MLCK) inhibitor, rescues the relaxation capacity of mutant cells, strongly suggesting that $\alpha 3\beta 1$ -mediated stimulation of the Rac/PAK pathway is required for the inhibition of MLCK activity, permitting completion of the myoepithelial cell contraction/relaxation cycle and successful lactation. This is the first study highlighting the impact of $\alpha 3\beta 1$ integrin signalling on mammary gland function.

The EMBO Journal (2011) 30, 1896–1906. doi:10.1038/emboj.2011.113; Published online 12 April 2011

Subject Categories: cell & tissue architecture; development

Keywords: contraction; integrin; mammary gland; myoepithelium; Rho-GTPases

of proliferation, survival, differentiation, motility and other cellular functions (Hynes, 2002; Vincente-Manzanares *et al*, 2009). Integrin-mediated signalling has an essential role in the activation of small Rho-GTPases, such as Rho and Rac, major regulators of actin cytoskeleton and cell–cell and cell–ECM adhesion (reviewed in Huveneers and Danen (2009)).

The mammary epithelium consists of two layers of epithelial cells: the luminal and basal layers. In functionally differentiated mammary gland, i.e., during lactation, luminal cells produce and secrete milk, whereas basal cells, also referred to as myoepithelial cells, contract to force milk out of the body. Mammary myoepithelial cells are characterized by a dual differentiation program. On the one hand, these cells express numerous markers shared by basal cells from various stratified epithelia. On the other hand, they express high levels of smooth muscle (SM)-specific contractile and cytoskeletal proteins responsible for the contractile activity of the myoepithelium. Inactivation of the gene coding for myocardin-related transcription factor regulating the expression of the SM-contractile proteins perturbs differentiation of the mammary myoepithelial cells resulting in impaired lactation (Li *et al*, 2006; Sun *et al*, 2006).

The basal epithelial cell layer interacts directly with the mammary basement membrane, a specialized ECM structure underlying the mammary epithelium, and integrins of the $\beta 1$ family and $\alpha 6\beta 4$ are major ECM receptors expressed by mammary epithelial cells (Taddei *et al*, 2003). Several studies have suggested that $\beta 1$ integrins participate in the control of growth and differentiation of the mammary epithelium *in vivo* and have an essential role in the maintenance of the mammary stem cell population (Faraldo *et al*, 1998; Li *et al*, 2005; Taddei *et al*, 2008). The $\beta 1$ integrin chain binds various α subunits, and the ECM ligand specificity of the resulting dimer depends on the nature of the α subunit. Different integrin dimers may activate different intracellular signalling pathways affecting cellular functions in a context-dependent manner (Huveneers and Danen, 2009). The laminin receptor $\alpha 3\beta 1$ is one of the major integrins expressed in basal mammary epithelial cells. Germinal deletion of the $\alpha 3$ integrin chain leads to early postnatal lethality due to abnormal kidney and lung development (Kreidberg *et al*, 1996). Several recent studies using conditional $\alpha 3$ -knockout mice have addressed the functions of $\alpha 3\beta 1$ integrin in various tissues. Deletion of the $\alpha 3$ integrin chain from the epidermis indicated that $\alpha 3\beta 1$ integrin inhibited directional migration and wound healing in the skin (Margadant *et al*, 2009). $\alpha 3\beta 1$ integrin was found to control expression of the pro-angiogenic factor MRP3 in keratinocytes, promoting endothelial cell migration and wound angiogenesis (Mitchell *et al*, 2009). Conditional deletion from endothelial cells revealed a role for $\alpha 3\beta 1$ integrin in the regulation of angiogenesis (da Silva *et al*, 2010). $\alpha 3\beta 1$ integrin was found to interact with netrin1 and to modulate routes of interneuronal migration in the developing cerebral cortex (Stanco *et al*, 2009). Analysis of the role

Introduction

Integrins are $\alpha\beta$ heterodimeric adhesion receptors that connect the extracellular matrix (ECM) to the actin cytoskeleton within structures called focal adhesions. These structures stabilize the cytoskeleton and serve as a platform for the assembly of multimolecular complexes mediating activation of intracellular signalling pathways involved in the control

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Received: 12 October 2010; accepted: 15 March 2011; published online: 12 April 2011

played by $\alpha 3 \beta 1$ integrin in mammary morphogenesis, based on the transplantation of epithelium into the cleared mouse mammary fat pad, led to the conclusion that $\alpha 3 \beta 1$ integrin might be dispensable for mammary development (Klinowska *et al*, 2001). However, this study neither examined the potential function of $\alpha 3 \beta 1$ integrin during lactation, nor investigated the impact of $\alpha 3 \beta 1$ -mediated signalling events on the functional activity of the mammary epithelium.

To study the role played by $\alpha 3 \beta 1$ integrin in mammary epithelium *in vivo*, we deleted *itga3* from the mammary basal cell layer using Cre-Lox approach. We show here that ablation of *itga3* neither interfered with mammary morphogenesis, nor impeded the functional differentiation of mammary epithelial cells. However, mice harbouring $\alpha 3 \beta 1$ -deficient myoepithelium could not lactate normally due to a myoepithelial cell contractility defect associated with altered Rho/Rac balance. These results suggest that $\alpha 3 \beta 1$ integrin-mediated signalling events have an essential role in the control of the amplitude and duration of the force developed by the myoepithelial cell contractile apparatus in lactating mammary gland and provide the first evidence highlighting the impact of $\alpha 3 \beta 1$ integrin signalling on mammary gland function.

Results

$\alpha 3 \beta 1$ integrin deletion from myoepithelial cells does not interfere with functional differentiation of the mammary epithelium

To delete the gene coding for $\alpha 3$ integrin chain, mice harbouring floxed *itga3* alleles (*itga3*^{F/F}) were crossed with transgenic mice expressing Cre recombinase under control of the keratin 5-promoter (K5Cre) active in the basal epithelial cells (Ramirez *et al*, 2004). As expected, Cre-driven recombination was detected in the epidermis and mammary gland (Figure 1A). Ductal branching morphogenesis was not affected in mutant mice, although *itga3* was found to be deleted from the mammary basal epithelial layer of 5-week-old animals (Supplementary Figure S1A–C). Immunoblotting and immunofluorescence analyses confirmed efficient deletion of the $\alpha 3$ integrin chain from the mammary epithelium of 1 day-lactating mutant mouse mammary glands (Figure 1B and C).

Overall, mutant mammary tissue organization was similar to that of control animals (Figure 1C and D). The expression and distribution of various integrin subunits ($\alpha 6$, $\beta 1$ and $\beta 4$) and ECM components (processed and unprocessed $\gamma 2$ laminin chain) were similar in control and mutant glands (Supplementary Figure S2A; data not shown). Ultrastructural analysis of the mutant mammary epithelium revealed no defect in basal myoepithelial cell adhesion to the basement membrane (Supplementary Figure S2B). These data suggest that, in the absence of $\alpha 3 \beta 1$, other integrin dimers present at the surface of the basal myoepithelial cells ensure adhesion to the ECM.

The functional differentiation of the mammary epithelium appeared normal in mutant glands, as shown by evaluation of the expression levels of milk and SM-contractile protein genes characteristic of luminal and myoepithelial cells, respectively, by quantitative RT-PCR (Figure 1E). Mammary epithelial cell proliferation was not affected in K5Cre;*itga3*^{F/F} glands, and hardly any apoptotic cells were detected with anti-cleaved

caspase 3 antibody at the onset of lactation in control and mutant mammary epithelium (Supplementary Figure S2C–E; data not shown). Thus, the deletion of $\alpha 3 \beta 1$ integrin from myoepithelial cells did not affect the integrity and functional differentiation of the mammary epithelium.

K5Cre;*itga3*^{F/F} mice display lactation failure due to low rates of milk ejection

K5Cre;*itga3*^{F/F} mutant mice were viable and fertile. However, the females were unable to feed their litters. About half the pups nursed by K5Cre;*itga3*^{F/F} females died within 4 days, and the surviving pups displayed severe growth retardation (Figure 1F and G). Milk was visible in the stomachs of the pups fed by control females, whereas the pups nursed by K5Cre;*itga3*^{F/F} females appeared to have empty stomachs (Figure 1F). This defect was not intrinsic to the neonates, which developed normally after weaning or when nursed by control females. Control neonates fostered by lactating K5Cre;*itga3*^{F/F} females also failed to grow normally (data not shown). The mutant females presented no obvious abnormal maternal nurturing behaviour.

As milk was produced but not available to neonates, we hypothesized that the contractile activity of mutant myoepithelial cells was impaired, leading to a low rate of milk ejection. This hypothesis was supported by the results of morphological analyses of the lactating mouse glands. Most of the mutant glands analysed on days 7 and 18 of lactation contained large dilated ducts engorged with milk. In addition, collapsed alveoli and gland fragments displaying complete secretory tissue regression were found in mutant epithelium examined on day 18 of lactation (Figure 2A; data not shown). These morphological changes in the mutant glands were similar to those resulting from milk stasis, which is known to induce premature involution (Quarrie *et al*, 1996).

High myosin light-chain phosphorylation level is maintained in lactating K5Cre;*itga3*^{F/F} mouse mammary glands

The contraction of myoepithelial cells, like that of SM cells, is regulated by myosin light-chain (MLC) phosphorylation, which induces myosin ATPase activity, the binding of myosin to actin and contraction (Hartshorne *et al*, 1989). The subsequent dephosphorylation of the MLC by its specific phosphatase (MLCP) leads to relaxation. Immunofluorescence analysis of MLC distribution showed this protein to be present in large amounts in the myoepithelial cells of control and mutant mammary epithelium (Figure 2B, upper panels), whereas immunolabelling with antibodies against phosphorylated MLC was slightly stronger in mutant myoepithelial cells than in control cells (Figure 2B, middle and lower panels). Immunoblotting analysis performed with protein extracts prepared from control and mutant mammary epithelium on the first day of lactation clearly demonstrated that MLC phosphorylation levels were abnormally high in mutant myoepithelial cells (Figure 2C).

Consistently, on the first day of lactation, the mutant myoepithelial cells presented extensions slightly shorter than those of control cells (data not shown) and, by day 5 of lactation, in most regions of the mutant glands, myoepithelial cells displayed a characteristic hypercontractile phenotype, with a compact cell body and short thick extensions (Figure 2D and E). In control tissue, most myoepithelial

Figure 1 Conditional deletion of the *itga3* gene from mammary basal epithelial cells does not interfere with mammary development, but leads to lactation failure. (A) PCR analysis of genomic DNA isolated from back skin (BS) and mammary gland (MG) of 5-week-old *itga3^{F/F}* and K5Cre;*itga3^{F/F}* virgin females. The primers used detect Cre recombinase (Cre), the recombined exon 1 of *itga3* (Exon 1) and LoxP sequences (LoxP). Primer sequences are provided in the Supplementary data. The vertical bars in the middle panel-photograph separate parts of the gel that have been spliced together to remove intervening lines. (B) Immunoblotting analysis of α3 integrin expression levels in mammary gland from 1 day-lactating control and K5Cre;*itga3^{F/F}* females. β-Tubulin was used as the loading control. (C) Double immunofluorescence labelling of sections through inguinal 1 day-lactating mouse mammary gland with antibodies against α3 integrin and αSMA. (D) H&E staining of sections through 1 day-lactating inguinal mammary glands. (E) Relative expression levels of basal and luminal cell differentiation markers in 1 day-lactating mouse mammary glands. Quantitative RT-PCR: K14, cytokeratin 14; K18, cytokeratin 18; *csn2*, β-casein; *wap*, whey acidic protein; *acta2*, α-smooth muscle actin; *myh11*, smooth muscle-myosin heavy chain; *tpm1*, tropomyosin α; *cnn1*, smooth muscle-calponin. The levels represent mean ± s.e.m. for independent RNA preparations obtained from four control and five mutant glands (F) Photographs of pups fed by control and mutant females on postnatal days 1 (upper panels) and 15 (lower panels). Arrows indicate stomachs, full of milk in pups fed by control females and empty in pups fed by mutant females. (G) Pup survival curve in the litters fed by control (*n* = 15) and mutant (*n* = 11) females. Bars: 200 μm in (C), 75 μm in (D) and 1 cm in (F).

cells appeared more relaxed, with a significantly larger surface area and longer extensions (Figure 2D and E). Taken together, these observations suggest that the MLC kinase/phosphatase activity balance is altered in mutant glands.

Mammary myoepithelial cell contraction requires activation of RhoA/ROCK pathway

In non-muscle and SM cells, MLC phosphorylation is controlled essentially by MLCK (Ikebe and Hartshorne, 1985;

Somlyo and Somlyo, 2003; Webb, 2003). The RhoA effector Rho kinase (ROCK) has also been reported to phosphorylate MLC *in vitro*, but the main effect of Rho/ROCK activation is the inhibitory phosphorylation of MLCP (Somlyo and Somlyo, 2003).

During lactation, mammary myoepithelial cell contraction is induced by pulsatile release of the cyclic peptide oxytocin (OT), which binds to a promiscuous G protein-coupled receptor (OTR). The downstream signalling pathways leading

Figure 2 Deletion of $\alpha 3 \beta 1$ integrin from the basal epithelial cell layer leads to milk stasis, sustained MLC phosphorylation. (A) Photograph of thoracic mammary glands from 18 day-lactating control and K5Cre;*itga3*^{F/F} females (upper panels) and H&E staining through inguinal mammary glands from 7 day-lactating females (lower panels). Arrows and asterisks indicate engorged dilated ducts and collapsed alveoli, respectively. (B) Double immunofluorescence labelling of sections through 1 day-lactating mouse inguinal mammary glands with antibodies against MLC or P-MLC and α SMA. (C) Immunoblotting analysis of P-MLC levels in mammary gland extracts from 1 day-lactating control and mutant females. (D) Immunofluorescence labelling of sections through 5 day-lactating mouse inguinal mammary glands with antibody against α SMA. (E) Diagram showing the surface area of myoepithelial cells at day 5 of lactation. The levels shown represent mean $\pm$ s.e.m. of surface area of 12 and 16 control and mutant myoepithelial cells, respectively, from 10 independent randomly chosen fields in each case. ** $P < 0.01$. Bars: 2.5 mm and 100 μ m in (A), upper and lower panels, respectively, 200 μ m in (B), and 10 μ m in (D).

to myoepithelial cell contraction remain largely unclear (reviewed in Reversi *et al* (2006)).

To define whether ROCK has a role in myoepithelial cell contraction, fragments of mammary tissue dissected from 1 day-lactating mammary glands were treated with ROCK inhibitor and then incubated with OT. The changes in myoepithelial cell shape following OT treatment were analysed after labelling with anti-K5 antibody and phalloidin, by immunofluorescence microscopy. As previously reported, OT treatment induced major cell shape changes (Moore *et al*, 1987). Relaxed myoepithelial cells had thin elongated processes, whereas OT-treated contracted cells had a compact spoke-shaped body with short, thick extensions (Figure 3A and B). Pre-treatment with ROCK inhibitor (Y27632) abolished the myoepithelial cell response to OT (Figure 3C). Treatment with the inhibitor only did not lead to any visible cell shape changes (Figure 3D). Similar results were obtained with fragments of mammary tissue dissected from mutant glands (data not shown). These data suggest that OT-stimulated mammary myoepithelial cell contraction requires RhoA pathway activation.

Mammary myoepithelial cells depleted of $\alpha 3 \beta 1$ integrin are unable to complete the contraction/relaxation cycle

To define the molecular mechanisms involved in the regulation of mammary myoepithelial cell contraction by $\alpha 3 \beta 1$ integrin, we used a mouse myoepithelial cell line carrying conditional alleles of *itga3* (MEC- $\alpha 3$ +) established in our

laboratory. These cells express myoepithelial markers, including K5, α -SMA and P-cadherin, and are negative for luminal markers, such as K8 (Supplementary Figure S3A–C). The adenoviral delivery of Cre recombinase, followed by cell sorting with anti- $\alpha 3$ antibody, yielded myoepithelial cells lacking $\alpha 3 \beta 1$ integrin (MEC- $\alpha 3$ –) (Figure 4A and B; Supplementary Figure S3D). Flow cytometry analysis and adhesion assays revealed no major change in the surface expression of other integrin subunits or in adhesion properties of $\alpha 3 \beta 1$ -deficient cells (Supplementary Figure S3D and E).

In agreement with the hypercontractile phenotype of mutant mammary myoepithelial cells observed *in vivo*, MEC- $\alpha 3$ – showed an increased capacity to contract 3D collagen gels as compared with MEC- $\alpha 3$ – cells (Supplementary Figure S4A). However, both, MEC- $\alpha 3$ – and MEC- $\alpha 3$ – contracted collagen gels regardless of the presence of OT (not shown) precluding us from further analysis of the molecular mechanisms underlying the abnormal contractile phenotype of $\alpha 3 \beta 1$ -deficient cells, in this model.

The contractile response of MEC- $\alpha 3$ – and MEC- $\alpha 3$ – to OT was analysed using time-lapse video microscopy. We have found that both, MEC- $\alpha 3$ – and MEC- $\alpha 3$ – cells responded to OT by a decrease in surface area in a dose-dependent manner (Figure 4C and E, upper panels), and that most MEC- $\alpha 3$ –, but not MEC- $\alpha 3$ – cells responded to lower doses of OT (Figure 4C). Analysis of OT receptor transcript levels by quantitative RT-PCR revealed no statistically significant difference between MEC- $\alpha 3$ – and MEC- $\alpha 3$ – (Figure 4D),

Figure 3 OT-induced contraction of mammary myoepithelial cells requires activation Rho/ROCK signalling pathway. Double immunofluorescence labelling of 1 day-lactating mouse mammary gland fragments with phalloidin (Phal) and anti-K5 antibody. (A) Control, untreated tissue. (B) OT-treated tissue. (C) Before addition of OT, mammary tissue was treated with ROCK inhibitor (Y27632). (D) ROCK inhibitor-treated tissue. Arrows point to the thin elongated extensions displayed by relaxed myoepithelial cells, and arrowheads indicate the short and thick extensions of contracted cells. Bar: 15 μ m.

suggesting that the capacity of $\alpha 3$ – cells to respond to lower doses of OT was not due to increased expression of OT receptor.

Importantly, the planar cell surface area did not change in response to OT, if MECs were pre-treated with an inhibitor of myosin ATPase, blebbistatin (Supplementary Figure S4B), strongly indicating that the decrease of cell surface area can serve as the readout of cell contraction.

Consistent with the results obtained with lactating mouse mammary tissue fragments, the OT-induced contraction of MEC- $\alpha 3$ + and MEC- $\alpha 3$ – depended largely on ROCK. Indeed, pre-treatment with the ROCK inhibitor abolished OT-induced contraction (Figure 4E and F; Supplementary

Movie 3). Thus, regardless of the presence of $\alpha 3\beta 1$ integrin, the myoepithelial cell contractile response to OT involved activation of the RhoA pathway.

Treatment with 10^{-9} M OT induced a contractile response in most MEC- $\alpha 3$ + and MEC- $\alpha 3$ –. This concentration was therefore chosen for further experiments. No significant difference was observed between MEC- $\alpha 3$ + and MEC- $\alpha 3$ – in terms of the time course and intensity of the primary contractile response, and a minimal surface area of 54.8 ± 1.6 and $51.9 \pm 1.4\%$ of the initial area, for MEC- $\alpha 3$ + and MEC- $\alpha 3$ –, respectively, was reached about 6 min after stimulation (Figure 4E and F; Supplementary Table I). Following contraction, MEC- $\alpha 3$ + and MEC- $\alpha 3$ – gradually recovered their initial surface area, with mean relaxation times of 20.2 ± 1.1 and 20.4 ± 0.9 min, respectively. After relaxation, the surface area of most MEC- $\alpha 3$ + remained constant, whereas about half the MEC- $\alpha 3$ – failed to maintain the state of post-contraction relaxation and underwent additional contractions (Figure 4E and F; Supplementary Table I; Supplementary Movies 1 and 2). These data are in keeping with the observation that high levels of MLC phosphorylation were maintained in lactating K5Cre;*itga3*^{F/F} mouse mammary gland. Thus, $\alpha 3\beta 1$ integrin-mediated signalling is required for the accomplishment of the contraction cycle, i.e., MLC dephosphorylation and relaxation.

To study whether the regulation of the cell contractile function by $\alpha 3\beta 1$ integrin involves its engagement at the cell surface, we pre-incubated MEC- $\alpha 3$ + cells with a function-blocking anti- $\beta 1$ integrin antibody before OT treatment. The anti- $\beta 1$ integrin antibody induced additional contractions in MEC- $\alpha 3$ + cells, phenocopying $\alpha 3$ -deficient cells, and confirming that integrin interaction with its ECM ligands is essential for the control of the contraction/relaxation cycle in the myoepithelial cells (Supplementary Figure S4C; Supplementary Table 1).

$\alpha 3\beta 1$ -mediated stimulation of the FAK/Rac/PAK pathway is required for the inhibition of MLCK and the accomplishment of the myoepithelial cell contraction/relaxation cycle

Focal adhesion kinase (FAK) controls Rho-family GTPase activation following integrin engagement (reviewed in Vicente-Manzanares *et al* (2009) and Huvneers and Danen (2009)). We therefore analysed FAK phosphorylation levels in the immunoprecipitates obtained from 1 day-lactating mouse mammary gland protein extracts, with anti-FAK antibodies. The levels of total FAK phosphorylation (as estimated with an anti-PY antibody) and FAK autophosphorylation at tyrosine 397 (Y397) were considerably lower in mutant glands than in control tissue (Figure 5A), suggesting an impaired Rho-family GTPase activation.

Previous studies in keratinocytes have shown that $\alpha 3\beta 1$ -mediated signalling may lead to Rac activation (Choma *et al*, 2004, 2007; Hamelers *et al*, 2005). In the extracts obtained from 1 day-lactating K5Cre;*itga3*^{F/F} mouse mammary glands, the level of active Rac was found to be significantly lower than in control tissue, whereas the level of Rho activation was higher in mutant gland extracts than in control (Figure 5B). In untreated and OT-treated MEC- $\alpha 3$ –, Rac activation levels were found to be significantly decreased as compared with MEC- $\alpha 3$ + (Figure 5C). As expected, OT stimulation did not affect Rac activation but resulted in significant increase of

Figure 4 Mammary myoepithelial cells depleted of $\alpha 3\beta 1$ integrin are unable to complete contraction/relaxation cycle. (A) Immunoblotting analysis of protein extracts from MEC- $\alpha 3+$, MEC- $\alpha 3-$ and from a cell line with luminal characteristics (LEC) with anti- $\alpha 3$ integrin ($\alpha 3$), anti-K5, anti-K18, anti-E-cadherin (E-cad) antibodies. β -Tubulin (β -tub) was used as a loading control. (B) Double immunofluorescence labelling of MEC- $\alpha 3+$ and MEC- $\alpha 3-$ with anti- $\alpha 3$ and anti-vinculin antibodies. (C) Diagram showing the percentage of MEC- $\alpha 3+$ or MEC- $\alpha 3-$ undergoing contraction in response to decreasing doses of OT. The levels shown represent mean $\pm$ s.e.m. obtained in four independent experiments. * $P < 0.05$; ** $P < 0.01$; NS, not statistically significant. (D) Relative expression levels of OT receptor (OTR) in MEC- $\alpha 3+$ and MEC- $\alpha 3-$. Quantitative RT-PCR analysis. The levels represent mean $\pm$ s.e.m. for three independent samples of MEC- $\alpha 3+$ and MEC- $\alpha 3-$. NS, not statistically significant. (E) Microphotographs of MEC treated with OT taken at different time points after treatment, as indicated in the top right corner. $\geq 40'$ marks micrographs taken between 40 and 45 min after OT addition. When indicated, cells were treated with Y27632, before OT. (F) Cell surface area changes following OT treatment as calculated from the experiments illustrated in (E). The levels shown represent mean $\pm$ s.e.m. obtained in four independent experiments. Bars in (B) and (E), 10 μ m.

active Rho levels in control MEC- $\alpha 3+$ and MEC- $\alpha 3-$. However, prior, as well as after OT treatment, active Rho levels in MEC- $\alpha 3-$ were similar to those of MEC- $\alpha 3+$ (Figure 5C). Western blot analyses of extracts prepared from 1 day-lactating mouse mammary glands and cultured MECs did not reveal any $\alpha 3\beta 1$ -dependent significant differences in Rac and Rho protein levels (Supplementary Figure S5A and B; data not shown). Altogether, these data indicate that the Rho/Rac activity balance is altered in $\alpha 3\beta 1$ integrin-deficient mammary myoepithelial cells.

Next, we investigated the effect of Rac inhibition on the contractile response of MEC to OT. The treatment of control MEC- $\alpha 3+$ with Rac inhibitor NSC23766 before OT

stimulation induced additional contractions after the primary contractile response (Figure 5D and E; Supplementary Table I; Supplementary Movie 4). Similarly, the expression of dominant-negative Rac (Rac^{N17}) in MEC- $\alpha 3+$ led to additional contractions (Figure 5D and E; Supplementary Figure S5C; Supplementary Table I; Supplementary Movie 5). The p21-activated kinase (PAK) is a downstream effector of Rac signalling (Bagrodia and Cerione, 1999). Expression of dominant-negative PAK (PAK^{DN}) or its silencing with shRNA (PAK-sh) leading to approximately two-fold decrease of initial PAK protein levels, induced additional contractions in control MEC- $\alpha 3+$ cells (Figure 5D and E; Supplementary Figure S5D and E; Supplementary Table I; Supplementary

Figure 5 Analysis of the intracellular signalling pathways affected by deletion of $\alpha 3\beta 1$ integrin from mammary myoepithelial cells. (A) Immunoblotting analysis of FAK phosphorylation (PY, pan-tyrosine; Y^{397} P-FAK, tyrosine 397) levels in the immunoprecipitates obtained with anti-FAK antibodies or with non-relevant IgG using protein extracts from 1 day-lactating control and mutant mouse mammary glands. (B) Quantitative analysis of Rac and Rho activation in protein extracts from 1 day-lactating mammary glands prepared from five control and five mutant mice. The horizontal bars indicate the medians; ** $P < 0.01$. (C) Quantitative analysis of Rac and Rho activation levels in protein extracts from MEC- $\alpha 3^{+}$ and MEC- $\alpha 3^{-}$. When mentioned, cells were treated with OT. (D) Microphotographs of MEC- $\alpha 3^{+}$ treated with OT taken at different time points after treatment. NCS, MEC- $\alpha 3^{+}$ pre-treated with Rac inhibitor (NCS23766); Rac N17 , MEC- $\alpha 3^{+}$ stably transfected with a plasmid coding for a dominant-negative Rac mutant; PAK DN , MEC- $\alpha 3^{+}$ stably transfected with a plasmid coding for a dominant-negative PAK mutant; PAK-sh, MEC- $\alpha 3^{+}$ stably transfected with a plasmid coding for PAK shRNA. (E) Diagram showing the percentage of MEC- $\alpha 3^{+}$, MEC- $\alpha 3^{-}$, MEC- $\alpha 3^{+}$ treated with NCS, MEC- $\alpha 3^{+}$ expressing Rac N17 , PAK DN or PAK shRNA able to maintain their shape after a single OT-induced contraction/relaxation cycle. (F) Upper panels show MEC- $\alpha 3^{-}$ transduced with constitutively active Rac (Rac V12). Lower panels represent MEC- $\alpha 3^{-}$ stably transfected with a plasmid coding for a catalytically active PAK mutant (PAK CA). (G) Diagram showing the percentage of MEC- $\alpha 3^{+}$, MEC- $\alpha 3^{-}$ and MEC- $\alpha 3^{-}$ expressing Rac V12 or PAK CA able to maintain their shape after a single OT-induced contraction/relaxation cycle. (H) Diagram showing the percentage of MEC- $\alpha 3^{+}$, MEC- $\alpha 3^{-}$ and MEC- $\alpha 3^{+}$ and MEC- $\alpha 3^{-}$ pre-treated with MLCK inhibitor (ML7), able to maintain their shape after a single OT-induced contraction/relaxation cycle. The levels shown in (C), (E), (G) and (H) represent the mean $\pm$ s.e.m. obtained from four independent experiments. * $P < 0.05$; ** $P < 0.01$. Bars in (D) and (F), 10 μ m.

Movie 6). Thus, inhibition of the Rac/PAK pathway phenocopied the lack of $\alpha 3\beta 1$ integrin in MEC. The pre-treatment of MEC- $\alpha 3^{-}$ with Rac inhibitor had only a minor effect on the response to OT, suggesting that Rac activity might already be inhibited in the absence of $\alpha 3\beta 1$ (Supplementary Figure S5F). We then obtained MEC- $\alpha 3^{-}$ expressing a constitutively active form of Rac, Rac V12 , or a catalytically active form of PAK,

PAK CA (Supplementary Figure S5G and H). Expression of Rac V12 or PAK CA in MEC- $\alpha 3^{-}$ prevented additional contractions, with about 80% of the cells remaining stable after the primary contractile response (Figure 5F and G; Supplementary Table I; Supplementary Movies 7 and 8). Thus, activation of the Rac/PAK signalling pathway rescued the abnormal phenotype caused by a lack of $\alpha 3\beta 1$ integrin.

Several studies have demonstrated that PAK can affect myosin contractile function by phosphorylating and inhibiting MLCK (Sanders *et al*, 1999; Goeckeler *et al*, 2000; Murthy *et al*, 2003; Wirth *et al*, 2003). We, therefore, investigated whether reducing MLCK activity restored the relaxation capacity of MEC- $\alpha 3^-$ cells. As expected, pre-treatment of MECs with 10 μ M of the MLCK inhibitor ML7, prevented the OT-induced contraction (data not shown). However, treatment with a low dose of ML7 (1 μ M) did not interfere with primary contractile response of MEC- $\alpha 3^+$ and MEC- $\alpha 3^-$ and had only minor effect on MEC- $\alpha 3^+$ post-contraction relaxation (data not shown; Figure 5H; Supplementary Table I). Strikingly, treatment of MEC- $\alpha 3^-$ with 1 μ M ML7 led to the restoration of their relaxation capacity, such that $\sim 70\%$ of the cells remained stable after the primary contraction (Figure 5H; Supplementary Table I; Supplementary Movie 9). These results suggest that $\alpha 3\beta 1$ integrin-deficient cells display an increase in MLCK activity, leading to abnormally high MLC phosphorylation levels and impaired relaxation.

In concert, these data strongly indicate that, in the absence of $\alpha 3\beta 1$ integrin, Rac activation is impaired, leading to the maintenance of MLC phosphorylation and impeding myoepithelial cell relaxation. This incomplete contraction/relaxation cycle should prevent subsequent full-strength contractions, accounting for the lower milk ejection rates observed in K5Cre;*itga3*^{F/F} mice.

Discussion

In this study, to analyse the functions of $\alpha 3\beta 1$ integrin in mammary development and functional differentiation of mammary epithelial cells, we have deleted the *itga3* gene specifically from the mammary basal epithelial cell layer. In agreement with previous data, mammary ductal branching and lobulo-alveolar development were not perturbed in mutant mice (Klinowska *et al*, 2001). We also showed that, similarly to control cells, by the onset of lactation, mutant mammary myoepithelial cells attained a differentiated state and expressed SM-specific contractile proteins. As expected, the differentiation of luminal secretory cells was not affected in mutant epithelium, and expression levels of milk protein genes were similar to those of control animals. Nevertheless, the lack of $\alpha 3\beta 1$ integrin led to a perturbation of intracellular signalling events essential for myoepithelial cell contractile activity resulting in lactation failure.

In mutant mammary tissue, FAK and Rac activation were impaired, whereas MLC phosphorylation levels were significantly higher than in control tissue. Consistent with these observations, cultured mammary myoepithelial cells depleted of $\alpha 3\beta 1$ were unable to maintain the state of post-contraction relaxation. The abnormal phenotype of mutant cells was rescued by the expression of activated Rac or PAK or by inhibiting MLCK activity. These results strongly suggest that $\alpha 3\beta 1$ integrin-mediated activation of the FAK/Rac/PAK signalling pathway is required for the inhibition of MLCK activity and the relaxation of mammary myoepithelial cells.

The scheme presented in Figure 6 summarizes the intracellular signalling events essential for mammary myoepithelial cell contractile activity and illustrates the contribution of $\alpha 3\beta 1$ integrin to the control of this cellular function. Our results reveal that, in addition to the phospholipase

Figure 6 $\alpha 3\beta 1$ -mediated stimulation of the Rac/PAK pathway is required for completion of the myoepithelial cell contraction/relaxation cycle. The contraction and relaxation of myoepithelial cells require MLC phosphorylation and dephosphorylation, respectively. The binding of OT to its receptor leads to MLC phosphorylation and myoepithelial cell contraction via activation of the phospholipase C/ Ca^{2+} /calmodulin (CaM)/MLCK pathway and the RhoA/ROCK signalling pathways. MLCK directly phosphorylates MLC, whereas ROCK inhibits MLCP (Somlyo and Somlyo, 2003). The results of our study suggest that the engagement of $\alpha 3\beta 1$ integrin leads to the activation of the FAK/Rac/PAK pathway and MLCK inhibition, thereby, permitting dephosphorylation of MLC and relaxation. In $\alpha 3\beta 1$ integrin-deficient cells, Rac activation is impaired resulting in altered MLCK/MLCP activity balance and sustained MLC phosphorylation, preventing the accomplishment of the contraction/relaxation cycle.

C/ Ca^{2+} /MLCK signalling pathway (reviewed in Reversi *et al* (2006)), the RhoA/ROCK cascade has a major role in the control of OT-induced myoepithelial cell contraction. Most probably, upon OT ligation to its receptor, these two pathways operate in parallel, with MLCK directly phosphorylating MLC and ROCK inhibiting MLCP. The net outcome of MLCK and MLCP activities results in MLC phosphorylation and myoepithelial cell contraction. Further, the $\alpha 3\beta 1$ integrin-mediated cell-ECM adhesion leads to the activation of the FAK/Rac/PAK signalling pathway, and activated PAK phosphorylates and inhibits MLCK, resulting in the desensitization of myosin to Ca^{2+} and in the myoepithelial cell relaxation necessary for the completion of the contraction/relaxation cycle and further contractions in response to pulsatile OT stimulation.

Previous studies have demonstrated that $\alpha 3\beta 1$ -deficient keratinocytes fail to form stable leading lamellipodia, consistent with the impairment of Rac pathway activation (Choma *et al*, 2004). However, in this case, the abnormal phenotype is not rescued by the expression of constitutively active Rac, confirming that the physiological functions of individual integrin dimers are cell context dependent. Furthermore, in keratinocytes, the Rac guanine-exchange factor, Tiam1, has been shown to promote $\alpha 3\beta 1$ integrin-mediated cell spreading on laminin (Hamelers *et al*, 2005).

The guanine-exchange factors acting downstream from $\alpha 3\beta 1$ integrin in the control of Rac activation in myoepithelial cells remain to be determined.

Mammary myoepithelial cells express several different integrin dimers, but none seems to be able to substitute for $\alpha 3\beta 1$ in activation of the Rac/PAK pathway. Flow cytometry analysis and adhesion assays showed no major change in the surface levels of other integrins or in the adhesion properties of $\alpha 3\beta 1$ -deficient cells, strongly suggesting that the observed phenotype results from the lack of $\alpha 3\beta 1$, rather than from the upregulation of other integrin dimers.

Our findings are consistent with two recent studies reporting high rates of MLC phosphorylation and Rho activation in SM cells depleted of mediators of integrin signalling, such as α -parvin or integrin-linked kinase (ILK) (Kogata *et al*, 2009; Montanez *et al*, 2009). These studies suggested that the interaction of the ILK/ α -parvin complex with β integrin cytoplasmic tail suppresses SM cell contractility, thereby serving as an important regulator of vascular morphogenesis (Wickström *et al*, 2010). Although deregulation of the Rho/Rac balance leading to cell hypercontractility seems to be a common feature between $\alpha 3$ -deficient mammary myoepithelium and ILK or α -parvin-depleted vascular SM, $\alpha 3\beta 1$ integrin deletion from the myoepithelial cell layer did not have any visible impact on the formation of the bilayered mammary epithelium. It seems plausible that signalling pathways controlled by $\alpha 3\beta 1$ integrin and ILK/ α -parvin complex do not overlap entirely. For instance, it has been suggested that ILK/ α -parvin complex could differentially regulate signalling downstream of $\beta 3$ and $\beta 1$ integrins (Wickström *et al*, 2010).

The reduced Rac activation in $\alpha 3\beta 1$ -deficient mammary tissue extract prepared from 1 day-lactating gland was associated with an increase in Rho activation. However, our experiments with cultured myoepithelial cells revealed a role for the Rac signalling pathway, downstream from $\alpha 3\beta 1$ integrin, in the regulation of post-contraction relaxation, and suggested that $\alpha 3\beta 1$ integrin *per se* had little or no impact on the Rho pathway activation. We found that $\alpha 3\beta 1$ -deficient cells responded to lower doses of OT than control cells. This phenomenon might result from higher levels of MLCK activity rather than increased levels of the Rho pathway activation in $\alpha 3\beta 1$ -deficient cells. The Rho and Rac pathways are known to have antagonistic roles in the regulation of various cellular functions, making important fine tuning of the Rho/Rac balance (Sander *et al*, 1999). Elevated levels of active Rho observed in mutant gland extract may result from additional *in vivo* adaptation events associated with unaccomplished contraction/relaxation cycle.

Cell contraction results from the integration of force-generating processes driven by actomyosin, actin polymerization at the cell periphery and the binding of actin filaments to the ECM via integrins at focal adhesion sites. The molecular mechanisms underlying the mechanotransduction processes remain unclear, but it has been suggested that intracellular signalling events associated with focal adhesions regulate force transmission between cells and the ECM (Gunst and Zhang, 2008; Geiger *et al*, 2009; Vincente-Manzanares *et al*, 2009). Our study provides the first *in vivo* evidence that $\alpha 3\beta 1$ integrin-mediated signalling may contribute to the control of the amplitude and duration of the force developed by the cellular contractile apparatus in response to OT. This critical role in the regulation of

OT-mediated contractility appears to be unique to $\alpha 3\beta 1$ integrin and involves Rac-mediated signalling events.

Materials and methods

Mouse strains

All animal experiments were conducted in accordance with the French veterinary guidelines and Council of Europe guidelines for experimental animal use (L358-86/609EEC). The K5Cre mice were kindly provided by Dr J Jorcano (Ramirez *et al*, 2004). The generation of mice carrying conditional $\alpha 3$ integrin alleles (*itga3^{F/F}*) has been described elsewhere (Margadat *et al*, 2009). Wild-type, *itga3^{F/+}*, *itga3^{F/F}* mice were used as control. Only females with litters of six to eight pups were selected for the analysis of mammary phenotype at lactation.

BrdU-incorporation test, tissue preparation, whole-mount staining, histology and immunostaining

Mice were injected intraperitoneally with bromodeoxyuridine (BrdU), 0.25 mg/g of body weight, 2.5 h before tissue collection. Dissected mammary glands were either embedded into cryoprotectant (Tissue-Tek[®] O.C.T) and processed for cryosections (7 μ m) and immunostained as previously described (Raymond *et al*, 2005) or spread onto a glass slide, fixed in MethaCarn and, either stained with Carmine alum in whole-mount, or embedded in paraffin. Sections (5–7 μ m thick) were used for haematoxylin/eosin or immunofluorescence staining (Teulière *et al*, 2005). Cells grown on glass coverslips were processed for immunostaining as previously described (Raymond *et al*, 2005). Cell nuclei were stained with DAPI. The list of primary antibodies used for immunostaining is provided in the Supplementary data. All secondary antibodies and phalloidin were Alexafluor conjugated (Molecular Probes). The microphotographs shown in Figure 2D were acquired with Leica SP5 confocal microscope. Leica DM6000B microscope was used for other immunofluorescence images.

Evaluation of the OT effects on alveolar myoepithelial cell shape

Mammary glands were processed as described elsewhere (Moore *et al*, 1987). Briefly, glands were chopped finely with a scalpel in a buffer containing 136 mM NaCl, 1.2 mM NaH₂PO₄, 1.2 mM MgSO₄, 5 mM KCl, 1.7 mM CaCl₂, 1.1 mM glucose, 0.03 mM Na₂ EDTA, 10 mM Hepes, pH 7.2 and, when indicated 10 μ M Y27632 (Calbiochem) for 10 min and then treated with 50 nM oxytocin (Tebu-bio) for 2 min at 37°C. Tissue fragments of ~1 mm³ in size were subsequently fixed for 30 min in 4% paraformaldehyde, then for 5 min in 100% acetone at –20°C, re-hydrated in PBS containing 1% BSA and incubated with the anti-keratin 5 antibody, phalloidin and DAPI. After incubation with the secondary antibody and washing, tissue fragments were mounted in Thermo Scientific Shandon Immu-Mount medium for observation under a Leica DM6000B microscope.

Quantitative RT-PCR

RNA was isolated from the third mammary glands of 1 day-lactating females with the RNeasy kit (Qiagen GmbH). Reverse transcription and quantitative PCR were performed as previously described (Taddei *et al*, 2008). The sequences of the primers used are provided in the Supplementary data and in Taddei *et al* (2008). The values were normalized to glyceraldehyde phosphate dehydrogenase expression levels using the comparative CT method.

Protein extracts, immunoprecipitation and immunoblotting

Proteins extracts were prepared from the fifth mammary glands of 1 day-lactating females. Tissue (125 mg/ml) was homogenized in lysis buffer (50 mM, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% SDS, 4 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate) containing a cocktail of protease inhibitors (Sigma-Aldrich). The extracts were cleared by centrifugation at 10 000 g at 4°C for 15 min. For immunoprecipitation assays, 1 mg of protein extracts was pre-absorbed with gammaBind Plus Sepharose beads (GE Healthcare) for 1 h at 4°C on a vertical rotator, incubated for 1 h with 4 μ g of primary antibody and for 2 h with gammaBind Plus Sepharose beads. The beads were then washed four times with lysis buffer, resuspended in reducing sample buffer for electrophoresis and

boiled for 5 min. The supernatants were run on a NuPAGE® Novex® 4–12% Bis Tris gel (Invitrogen). After electrophoresis, proteins were transferred onto nitrocellulose. Membranes were successively incubated with 5% BSA in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature to block non-specific binding sites and with primary antibodies in TBST, overnight at 4°C. Secondary antibodies coupled to horseradish peroxidase were purchased from Amersham. Detection was performed by chemiluminescence (Super Signal® west Pico chemiluminescent substrate, Pierce) using a Fuji Imager LAS3000. The list of the antibodies used for immunoprecipitation and immunoblotting is provided in the Supplementary data. Protein extracts from four control and mutant glands were analysed. Protein extracts from MEC were prepared as previously described (Raymond *et al*, 2005) and immunoblotting was performed as described above.

Establishment of MEC line, viral transduction, stable transfection and flow cytometry

The established MEC of *itga3^{F/F}* genotype was routinely maintained in low serum medium consisting of DMEM:F12 (Gibco-BRL) buffered with 15 mM Hepes, pH 7.6 (Sigma-Aldrich) containing 10 µg/ml insulin (Sigma-Aldrich), 5 ng/ml epidermal growth factor (Sigma-Aldrich), 0.5 mg/ml bovine serum albumin (Sigma-Aldrich), 5 µg/ml linoleic acid complex (Sigma-Aldrich), 2% calf serum (HyClone), 50 µg/ml gentamycin (Sigma-Aldrich) and 20 U/ml nystatin (Sigma-Aldrich). The line was established as previously described (Medina and Kittrell, 2000). In brief, primary mammary epithelial cells obtained by collagenase/hyaluronidase digestion of inguinal glands from 16 day-pregnant *itga3^{F/F}* females were seeded into matrigel in low serum medium defined above. After 4 weeks of growth, the cells were recovered from matrigel using dispase, plated on collagen-coated plastic and maintained in culture. After ~2 months, when most cells became senescent, and only few clones were capable of expanding, the proliferating cell clones were harvested and amplified. The cell clone stably expressing myoepithelial cell markers and negative for mammary luminal markers was chosen for further studies (Supplementary Figure S3). Cre-mediated deletion of *itga3^{F/F}* alleles was performed as described previously (Raymond *et al*, 2005). Retroviral transduction was performed as described elsewhere (Sterk *et al*, 2000), and infected cells were subsequently maintained under G418 selection (500 µg/ml; #ant-gn-5, Invivogen). The retroviral construct myc-Rac^{V12} cloned in pLZRS-MS-IRES-Neo/pBR was kindly provided by Dr JG Collard. Stable transfections were performed with Lipofectamin™ 2000 (#11668, Invitrogen) according to the manufacturer's instructions. The pCAG-Myc-PAK-CAAX-IRES-GFP, pCAG-Myc-PAK-R299-CAAX-IRES-GFP and pCDNA-GFP-Rac^{N17} vectors provided by Dr M Nicolic (Jacobs *et al*, 2007) and Dr A Gautreau, respectively, were co-transfected with pGK puro plasmid. Transfected cells were selected using puromycin (10 mg/ml, #ant-pr-5, Invivogen), and the bulk population of cells was analysed in all cases. The αPAK shRNA and control shRNA plasmids were from Santa Cruz Biotechnology (0365C-270016-SH and sc-108060). Transfections were performed according to the manufacturer's instructions. Flow cytometry and cell sorting were performed, respectively, on BD FACSCalibur and FACSvantage (Becton Dickinson) flow cytometers, as previously described (Margadant *et al*, 2009).

Time-lapse video microscopy

For video microscopy, sparse cell cultures were grown on glass-bottomed microwell dishes (MatTek Corporation) for 16–20 h.

Time-lapse video microscopy (one image captured per min) was performed on Time Lapse Imaging System Biostation IM (Nikon Corporation) with an integrated incubation system (37°C, 5% CO₂). MLCK inhibitor (ML7) was used at concentrations of 1 µM and 10 µM, ROCK inhibitor (Y27632) at 10 µM; Rac1 inhibitor (NCS23766) at 100 µM; Myosin ATPase inhibitor (blebbistatin) at 25 µM (all from Calbiochem). OT (1 nM, or as indicated; Tebu-bio) was added 45–120 min after inhibitor and the response was followed during 60 min. Planar surface area was measured as readout for contractile responsiveness with Image J software. Initial surface area was defined as a mean value over 15 min. Spontaneous cell surface area fluctuation did not exceed 9.6%. A decrease in surface area was considered as contraction when the cell surface area was ≤72% of the initial area. Cells with a surface area of at least 86% of the initial surface area were considered as relaxed. Cells that were not spreading (showing refringent borders), abnormally large (usually polynucleated) or dividing, and cells that did not remain stable during the 15 min preceding the addition of OT were excluded from the analysis. The data obtained in four independent experiments are presented.

Rho-GTPases activation assay

Quantification of Rac and RhoA activation was performed using G-LISA Rac and RhoA Activation Assay Biochem Kits, following the manufacturer's instructions (Cytoskeleton, Inc.).

Statistical analysis

Statistical analysis was performed with Prism 4 software (GraphPad) using Mann–Whitney *U* test for the comparison of two groups except in Figure 2E where unpaired *t*-test was used. A value of **P* < 0.05, ***P* < 0.01 or ****P* < 0.001 denoted statistical significance.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We are particularly grateful to Dr J Jorcano for providing K5Cre transgenic mice and to Drs T Sasaki, M Nikolic, JG Collard and A Gautreau for reagents. We thank S Jannet and I Grandjean for excellent assistance with animal care, M Denoyelle and A Di Cicco for technical help. We thank the Nikon Imaging Centre at Institut Curie-CNRS for providing time-lapse video microscopy facilities. This work was supported by *La Ligue Nationale Contre le Cancer (Equipe Labelisée 2009)* and a grant from *Agence Nationale de la Recherche* ANR-08-BLAN-0078-01 to MAG. KR received funding from *La Fondation pour la Recherche Médicale* and *Institut National du Cancer*. SC was supported by a grant from *Cancéropôle Ile de France*. MAG is *Directeur de Recherche* at the *Insitut National de la Santé et de la Recherche Médicale (INSERM)*.

Author contributions: KR designed most experiments; KR and SC performed experiments and analysed results; MK and AS generated mice carrying conditional alleles for *itga3* gene; MK made first observations concerning lactation failure of mutant mice; HJ performed electron microscopy experiments; KR, SC, AS and MAG interpreted and discussed the data; KR and MAG wrote the paper; MAG designed and supervised the study.

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