β1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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Basel, 2005

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

Auf Antrag von

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Basel, den 05.April, 2005

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Summary

Integrins are cell adhesion receptors which mediate interactions between the extracellular matrix and the actin cytoskeleton. They are heterodimers composed of α and β subunits. As adhesion receptors, integrins are important for cell-cell and cell-matrix interactions and therefore are essential for the structural integrity of an organ. Moreover, integrin-extracellular matrix interactions play important roles in the coordinated integration of external and internal cues that are essential for proper development. β1 integrin is the most widely expressed integrin and controls various developmental processes, including neurogenesis, chondrogenesis, skin and hair follicle morphogenesis, and myoblast fusion.

To determine the role of β1 integrin in normal development of the mouse mammary gland, with a particular emphasis on how β1 integrins influcence proliferation, differentiation and apoptosis; we examined the consequence of conditional deletion of β1 integrin in mammary epithelia. *Itgβ1*$^{flox/flox}$ mice were crossed with WAPiCre transgenic mice, which led to specific ablation of β1 integrin in luminal alveolar epithelial cells. In the β1 integrin mutant mammary gland, individual alveoli were disorganized resulting from alterations in cell-basement membrane associations. Activity of focal adhesion kinase was also decreased in mutant mammary glands. Luminal cell proliferation was strongly inhibited in β1 integrin mutant glands, which correlated with a specific increase of p21^{Cip1} expression. In a p21^{Cip1} null background, there was a partial rescue of the proliferation defect, as measured by incorporation of Bromodeoxyuridene into S-phase cells. These data provide *in vivo* evidence linking p21^{Cip1} to the proliferative defect observed in β1 integrin mutant glands. A connection between p21^{Cip1} and β1 integrin as well as focal adhesion kinase was also established in primary mammary cells and an established cell line. Finally, transplanted mammary tissue from β1 integrin mutant females failed to repopulate recipient mammary glands, suggesting for the first time that β1 integrin may be required for the maintenance of mammary progenitor cells.

Overall, we found β1 integrin has multiple roles in mouse mammary gland development. Ablation of β1 integrin in luminal alveolar cells affects proliferation at early lactation, and the integrity of alveolar lumen structures during lactation. The results also suggest that β1 integrins are necessary for mammary progenitor cell proliferation and/or survival during mammary gland remodeling.

β1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli.

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Bibliography

- **Aims of the Ph.D. Project**

- To determine the role of β1 integrin in the normal development of mouse mammary gland.
- To investigate the molecular mechanisms for the phenotypes we observed in vivo.
- Establish an in vitro system that mimics the effects of deletion of the integrin β1 in the mouse.

Results

- Conditional deletion of β1 integrin in the mammary gland.
- Function of mammary gland was impaired in β1 integrin mutant mice.
- β1 integrin-mutant mammary glands have defects in alveolar integrity.
- β1 integrin mutant mammary glands show an increase in apoptosis at involution, but not during lactation.
- Loss of β1 integrin correlates with reduced mammary cell proliferation and upregulation of $p21^{cip1}$
- Ablation of β1 integrin impairs focal adhesion signalling.
- Loss of either β1 integrin or FAK induces $p21^{Cip1}$ upregulation in primary mammary cells.
- Absence of p21cip1 rescues impaired BrdU incorporation in mammary cells of β1 integrin mutant mice.
- Using an in vitro model to further mimic the proliferation defect after loss of β1 integrin.
- Loss of β1 integrin leads to a defect in alveolar cell proliferation during a second pregnancy.

Conclusion

Discussion

- Functional differentiation was normal in integrin β1 mutant glands
- β1 integrin deletion results in loss of epithelial integrity
- Loss of β1 integrin is not sufficient to trigger apoptosis in alveolar cells.
- β1 integrins have a key role in the proliferation of mammary epithelial cells in vivo and in culture
- A possible role for β1 integrins in the mammary stem cells
- β1 integrins in breast cancer

Materials and Methods

- Antibodies
- Mouse strains and generation of mammary specific β1 mutant mice.
- Pup Weight Analysis
- Milk Annalysis
- Mammary gland whole mounts
- Histology and immunofluorescence
- Electron microscopy
- Isolation and assays with primary mammary cells
- Transplantation of mammary epithelium into cleared fat pads of recipient mice
- Lysate preparation, immunoprecipitation and western blot analysis.
- MDA-MB-231 cell culture, siRNA transfections and luciferase assays
- Flow cytometric analysis
- Extraction of RNA and RT-PCR analysis of $p21^{Cip1}$

Reference

Introduction

Part I Integrins: The basic machinery for cell adhesion

In order to function as a tissue, epithelial cells must have the right shape and structure to pack together with their neighbors. Therefore, tissue formation in normal physiology requires cell adhesion. Cell adhesion governs, e.g., embryonic morphogenesis, angiogenesis, organogenesis, inflammation and tissue repair. Two types of adhesion, cell-cell adhesion and cell-matrix adhesion, are relevant for these processes. In this chapter, I will introduce the various cell adhesion junctions and adhesion receptors with an emphasis on integrins, the major extracellular matrix receptors.

1.1 Cell adhesion receptors

Cell-cell interactions, as well as cell-ECM interactions, are indispensable for normal tissue architecture. In mammals, adhesion between epithelial cells is generally mediated by three types of junctions: tight junctions (TJs), adherens junctions (AJs), and desmosomes, which together constitute the intercellular junctional complex (Perez-moreno et al. 2003). The complexes contain transmembrane receptors, usually glycoproteins that mediate binding at the extracellular surface and determine the specificity of the intracellular response.

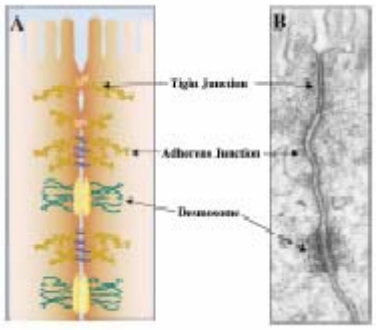


Fig.1. Composition of Three Types of Intercellular Junctions (A). Diagram of the three major types of intercellular junctions in epithelial cells. (B) Electron micrograph depicting the ultrastructure of adherens junctions, desmosomes, and tight junctions between two murine intestinal epithelial cells. (Perez-Moreno M. et al. 2003)

Each of the junctions possesses unique morphological characteristics, composition, and functions. Tight junctions are one mode of cell-cell adhesion in epithelial and endothelial cellular sheets. They act as a primary barrier to the diffusion of solutes through the intercellular space, create a boundary between the

apical and the basolateral plasma membrane domains, and recruit various cytoskeletal as well as signalling molecules at their cytoplasmic surface. The adherens junction is a cellular structure found near the apical surface of polarized epithelial cells. Recent evidence has uncovered a key role for AJs not only in directing coordinated cellular organization and movements within epithelia, but also in transmitting information from the environment to the interior of cells (Perez-Moreno M. et al**,** 2003). Desmosomes are prominent cellular structures especially abundant in tissues that experience mechanical stress, e.g. in skin.
Cell adhesion involves specific biomolecules such as memebrane anchors and receptors, extracellular ligands and cytoskeketal components. An important step in the formation of cell adhesion complexes is the clusting of the adhesion receptors involved, such as integrins or cadherins. Cadherins constitute a large family of glycoproteins comprised of an extracellular domain responsible for cell-cell interactions, a transmembrane domain, and a cytoplasmic domain that frequently is linked to the cytoskeleton. Cadherins are found in both adherens junction and demosomes and play a key role in calcium-dependent cell-cell interactions. AJs are cadherin-dependent adhesive structures that are intricately linked to the actin microfilament network. E-cadherin is typically the cadherin found in the adherens junction. While desmosomes are formed by interactions between desmosomal cadherins linked to intermediate filaments. At the adherens junctions the intracellular domain of cadherins specifically interacts with catenins; catenins serve to link the cadherin to the actin cytoskeleton and also function in cellular signaling (Wheelock and Johnson, 2003). Catenin is present in two compartments: a membrane-associated form couples E-cadherin to the cytoskeleton, and a cytoplasmic form is associated with a Wnt signaling complex that includes the serine/threonine kinase GSK3β, axin, APC, and β-catenin.

Cadherins have been implicated in a number of signaling pathways that regulate cellular behavior. It was found that cadherin function is critical in normal development, and alterations in its function have been implicated in tumorigenesis (Hajra & Fearon 2002). Changes in the normal expression pattern of the E-cadherin/catenin complex have been found in various human cancers. In breast cancer, generally speaking, partial or total loss of E-cadherin expression correlates with loss of differentiation characteristics, acquistion of invasiveness, increased tumor grade, metastatic behavior and poor prognoses. While forced expression of E-cadherin decreased proliferation of different mammary carcinoma cell lines, suggesting that E-cadherin is a potent tumor suppressor of breast cancer (Berx &Van Roy, 2001).

Both in cell-cell adhesions and cell-ECM adhesions, the associated cytoplasmic proteins of the adhesion receptors structurally link them to the cytoskeleton, thereby establishing molecular lines of communication to other cell-cell junctions and to cell-substratum junctions. The linkage of cell-cell junctions to the cytoskeleton allows single cells of an epithelial sheet to function as a coordinated tissue. Additional companion proteins connect structural and signaling elements, and thus intercellular junctions function to integrate a number of cellular processes ranging from cytoskeletal dynamics to proliferation, transcription, and differentiation.

1.2 Integrins and cell adhesion

As we already mentioned, tissue formation in animals requires both cell-cell contacts and cell-matrix contacts. At the cytoplasmic face, both of the contacts are anchored by direct contact between the transmembrane proteins and microfilaments. Cell-cell contacts are specified by transmembrane proteins of the cadherin family, while cell-matrix contacts contain proteins of the integrin family. It is becoming increasingly clear that integration of information received from cell-cell signaling, cell-matrix signaling, and growth factor signaling determines ultimate cellular phenotype and behavior.

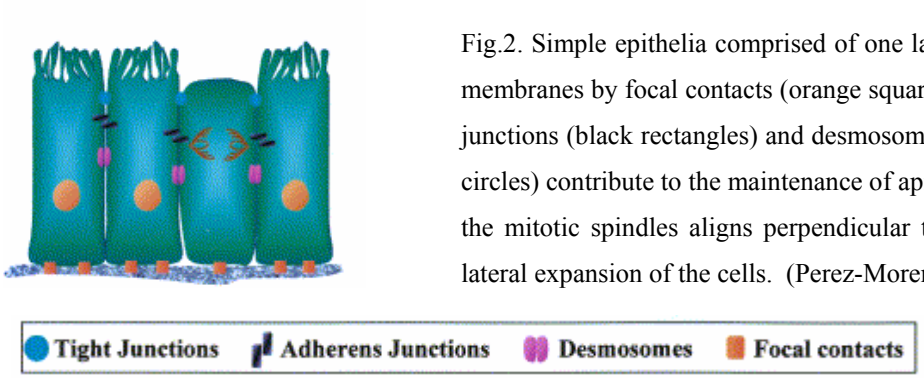


Fig.2. Simple epithelia comprised of one layer of cells attaches to the basement membranes by focal contacts (orange squares) and to adjacent cells via adherens junctions (black rectangles) and desmosomes (pink ovals). Tight junctions (blue circles) contribute to the maintenance of apical-basolateral polarity. The plane of the mitotic spindles aligns perpendicular to the basement membrane allowing lateral expansion of the cells. (Perez-Moreno.M.et al., 2003)

1.2.1. Cell-matrix contacts

Cell–matrix contacts are specialized zones at the cell surface, where activated or clustered adhesion receptors bind to their extracellular matrix (ECM) ligands and link intracellularly to components of the cytoskeleton. Cell–matrix contacts thus bridge the extracellular and intracellular milieux and are fundamental features of the cells and tissues of multicellular organisms. Different cell–matrix contacts have been characterised by their morphology or by biochemical composition, or a combination of both criteria. Cell-matrix contacts can exist as focal complexes, focal adhesions, fibrillar adhesions, three

dimensional matrix adhesions and hemidesmosomes. Some matrix contacts, such as hemidesmosomes, are specific to particular differentiated cell types, while others occur in many cell types.

The best characterized and largest of these structures is the focal adhesion (FA; also known as a focal contact). These adhesions are transient in nature, and form in many types of cells that are cultured on a substrate coated with ECM. All adherent cells bind to the ECM through integrins—transmembrane receptors that bind to specific motifs on numerous ECM proteins (Ruoslahti E. 1996). The binding of integrins to the ECM causes them to cluster and leads to the recruitment of a battery of cytoplasmic signaling and structural proteins to form FAs at the site of integrin clustering. Numerous structural proteins (e.g., vinculin, talin, α-actinin, and paxillin) act as scaffolding proteins that strengthen cell adhesion by anchoring FAs to the actin cytoskeleton (Miyamoto et al. 1995). Other types of cell-matrix adhesions that have been further identified—focal complexes, fibrillar adhesions and three dimensional matrix adhesions—are structurally similar to FAs but differ subtly in composition and morphology (Cukierman et al. 2001; Zamir E et al. 2000). Hemidesmosome are extremely large structures that form strong bonds between epitheial cells and the underlying interstitial ECM through a chain of molecular interactions. Instead of linking to the actin-based cytoskeleton, hemidesmosomes contain adaptor proteins which bind to intermediate filaments. The cell-matrix contacts are actually dynamic assemblies with above twenty proteins in a complex and all these contacts have essential roles in normal physiology and there are many contexts in which abnormalities of cell–matrix contacts lead to chronic and life-threatening diseases.

1.2.2.Integrins are the major ECM receptors

As we previously discussed, cells adhere to the ECM and to each other through specific classes of transmembrane adhesion receptors. These receptors bind to ligand extracellularly and provide an anchor to the intracellular cytoskeleton via cytoplasmic scaffolding proteins. Linkages between external cellular contacts, adhesion receptors, and cytoskeleton provide a means for bidirectional communication between the inside and outside of a cell. The major transmembrane ECM receptors in these cell-ECM adhesion sites belong to the integrin family. In addition to integrins, several other membrane molecules were recently reported to localize to focal contacts, including proteoglycans (Zimmermann and David, 1999), glycosaminoglycan receptors (Borowsky and Hynes, 1998), as well as signaling molecules (Yebra M et al. 1999), however, the role of these components in mediating or regulating adhesion is unclear.

Integrins comprise a large family of cell surface receptors that are found in many species, ranging from sponges to mammals. They are composed of two subunits, α and β. There are several α- and β- subunit isoforms; to date 8 α and 8 β subunits have been identified. The receptors always contain one α chain and one β chain and each α β combination has its own binding specificity and signaling properties. A specific ECM molecule can nevertheless be bound by different types of integrins, and specific integrins can bind to different types of ECM molecules. For example, functionally, β1 integrins are a set of cellular receptors for extracellular matrix proteins that include fibronectin, collagen, and laminin.

Integrin α and β subunits contain a large extracellular domain responsible for ligand binding, a single transmembrane domain and a cytoplasmic domain. The short cytoplasmic domain of integrins binds a variety of intracellular proteins including actin binding proteins like vinculin or talin, and also some signaling kinases like focal adhesion kinase (FAK). The extracellular domains of integrins also contain multiple binding sites such as the RGD binding site (about 50% of the integrins). As integrins bind to their ligand, they change from an inactive to an active configuration and become clustered in the plane of the cell membrane and associate with a cytoskeleton and signaling complex that promotes the assembly of actin filaments. The reorganization of actin filaments into large stress fibers, in turn, causes more integrin clustering, thus enhancing the matrix binding and organization by integrins in a positive feedback system. As a result, ECM proteins, integrins, and cytoskeletal proteins assemble into aggregates on each side of the membrane. Well-developed aggregates can be detected by immunofluorescence microscopy and are known as focal adhesions and ECM contacts (Burridge & Chrzanowska-Wodnicka, 1996). In this manner, integrins serve as integrators of the ECM and cytoskeleton, the property for which integrins are named.

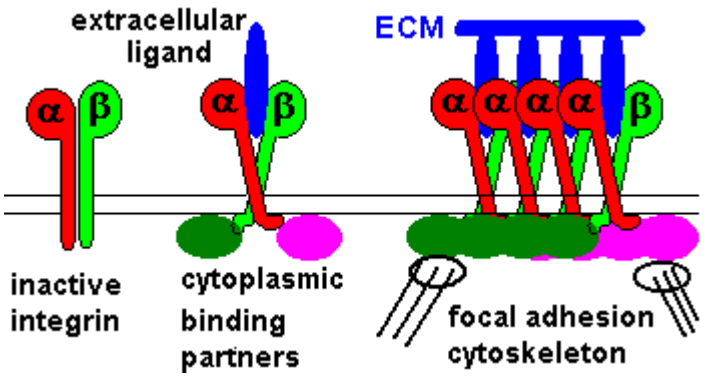


Fig.3. Integrins can adopt inactive and active configurations, which differ by change in relative orientation of the α– and β– subunits. The active orientation has enhanced affinity for both external and cytoplasmic ligands. Binding of ligand on either side promotes the change to active form, so cytoplasmic ligands can promote binding to ECM, and ECM binding can enhance interaction with cytoplasmic ligands or binding partners. (Schwartz, M.A. 2001).

1.3 Downstream pathways of integrins

Integrin-ligand interactions are accompanied by clustering and activation of the integrins on the cell surface, which is also accompanied by the transduction of signals into intracellular signal transduction pathways that mediate a number of intracellular events. Integrins transduce a great many signals. The majority of signalling molecules implicated in ECM–integrin interactions appear to be rather ubiquitous mediators of signal transduction. For example, Miyamoto et al. (Miyamoto er al. 1998) showed that at least 20 different proteins, including Rho GTPases, Raf, Ras, FAK, and MAPKs such as extracellular-signal-regulated kinases (ERKs), can be recruited to the ECM ligand/integrin-binding site. In an attempt to understand how these intracellular mediators may contribute to specialized patterns of gene expression and cell behaviour, we will focus primarily on FAK, shc and ERK/MAPK pathways.

1.3.1 The FAK/Src Pathway

Integrins activate various protein tyrosine kinases, including focal adhesion kinase (FAK), Src family kinases, and Abl, and a serine-threonine kinase, integrin-linked kinase (ILK) (Giancotti & Ruoslahti, 1999). The integrin-dependent pathways involving FAK and Src-family kinases have been studied in some detail.

Focal adhesion kinase (FAK) is a tyrosine kinase which is commonly found in integrin mediated focal adhesions. It is a critical component of the focal adhesion and provides both structural and kinase activity to the focal contact. The FAK pathway is activated by most integrins. The activation of FAK is not well understood, but it is coupled to the assembly of focal adhesions. FAK may be recruited to nascent focal adhesions because it interacts, either directly or through the cytoskeletal proteins talin and paxillin, with the cytoplasmic tail of integrin β subunits. Upon activation, FAK autophosphorylates Tyr397, creating a binding site for the Src homology 2 (SH2) domain of Src or Fyn. The Src kinase then phosphorylates a number of focal adhesion components. The major targets include paxillin and tensin, two cytoskeletal proteins that may also have signaling functions, and p130CAS, a docking protein that recruits the adapter proteins Crk and Nck. FAK also combines with, and may activate, phosphoinositide 3-OH kinase (PI 3-kinase), either directly or through the Src kinase. Finally, there is evidence that Src phosphorylates FAK at Tyr925, creating a binding site for the complex of the adapter Grb2 and the Ras GAP exchange factor mSOS. These interactions link FAK to signaling pathways that modify the cytoskeleton and activate mitogen-activated protein kinase (MAPK) cascades (Giancotti & Ruoslahti, 1999).

A number of observations strongly suggest that activation of FAK by integrins plays a central role in initiating many of the signals that regulate growth. For example, mutation of tyrosine residues critical for FAK autophosphorylation prevents integrin-mediated proliferation. Also, oncogenic transformation of cells, which abolishes the requirement for anchorage-dependent growth, activates FAK. Consistent with this, introduction of constitutively active FAK leads to cell transformation, anchorage-independent growth and the suppression of apoptosis (Boudreau NJ & Jones PL, 1999).

1.3.2 The Fyn/Shc Pathway

It is becoming clear that, like binding to the ECM, integrin signalling is determined by both α and β subunits. Several integrins interact, through the extracellular or transmembrane domain of their α–subunit, with other membrane proteins. For example, in addition to activating FAK, some β1 and αv integrins also activate the tyrosine kinase Fyn and, through it, the adapter protein Shc. In this pathway, caveolin-1 appears to function as a membrane adapter, which couples the integrin α subunit to Fyn. Upon integrin binding to ECM, Fyn becomes activated, and its SH3 domain interacts with a proline-rich site in Shc. Shc is then phosphorylated by Fyn at Tyr317 and combines with the Grb2-mSOS complex. Although most integrins interact with caveolin-1 and Fyn, only a subset of integrins can activate Fyn and thereby recruit Shc: Perhaps these integrins are associated with an activator of Fyn, such as a phosphatase that removes the phosphate group from the autoinhibitory tyrosine residue in Fyn. Yes and Lck are known to be enriched in rafts and may mediate the activation of Shc when Fyn is not expressed.

The cytoplasmic domain of the β-subunit also contributes to the specificity of integrin signalling. For example, the unique, long cytoplasmic tail of integrin β4 allows α6β4 to recruit Shc by a mechanism distinct from that used by β1 and αv integrins: upon α6β4 binding to the ECM, the β4 tail is phosphorylated on tyrosine residues by an integrin-associated kinase and binds to Shc directly (Giancotti & Ruoslahti, 1999).

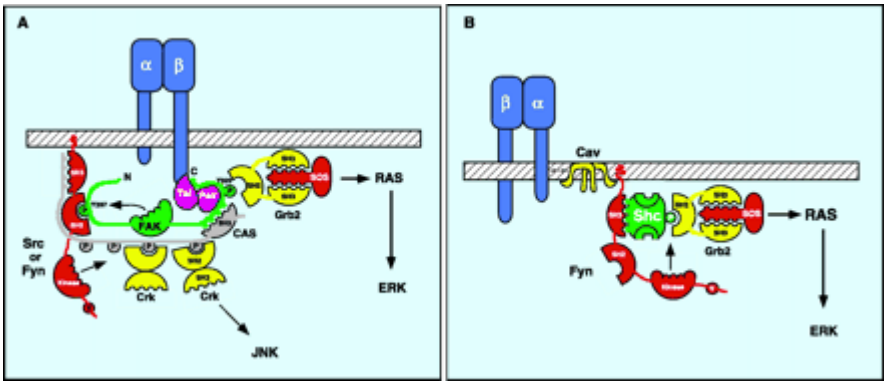


Fig.4.Model of the (A) FAK and (B) Shc pathways. (Giancotti & Ruoslahti, 1999)

1.3.3 The Erk-MAPK Pathway

The Activation of mitogen activated protein kinase (MAPK) occurs after integrin-ligand binding (RGD peptides, fibronectin, laminin), resulting in the translocation of Erk from the cytoplasm to the nucleus. We already mentioned that both FAK and Shc can contribute to the activation of the Ras–extracellular signal-regulated kinase (ERK) MAPK cascade. MAPK can also be activated by integrin linked kinase (ILK) in a FAK independent pathway. The relative contribution of each pathway may depend on the cell type and perhaps also on how far the adhesion process has progressed. In many cell types, Shc appears to be responsible for the initial high-level activation of ERK upon cell adhesion. FAK, which is activated more slowly, may sustain the ERK activation. The integrins that do not activate Shc are weak activators of ERK and cell proliferation. The ability of integrins to activate ERK may be especially important when the concentration of growth factors available to the cell is limited. In this setting, proliferation is likely to require costimulation of ERK through integrins and growth factor receptors (Giancotti & Ruoslahti, 1999).

1.3.4 Partnership with growth factors

Integrins are not only signal on their own but are also necessary for optimal activation of growth factor receptors. The receptors for insulin, platelet derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) are optimally activated by their ligands only under appropriate cell attachment conditions (Giancotti & Ruoslahti, 1999). Treatment of endothelial cells with beads coated with an Arg-Gly-Asp (RGD) tripeptide or with fibronectin leads to coaggregation not only of β1 integrins and FAK, but also of high affinity receptors for basic fibroblast growth factor (bFGF) in the newly assembled focal adhesions (Miyamoto S et al.1996). The aggregation of the growth factor receptors results in their partial activation, possibly bringing growth factor signaling closer to a threshold of manifest activity and enabling cross talk between integrins and growth factor receptors. Although a systematic analysis has not been conducted, certain integrins appear to be preferentially associated with specific growth factor receptors. Thus, the αvβ3 integrin can be immunoprecipitated in complexes with the insulin, PDGF, and VEGF receptors (Soldi R., et al 1999), whereas α5β1 and perhaps other β1 integrins associate with the EGF receptor (Miyamoto S et al.1996)

1.4 Control of cell shape, growth and survival by integrins

The formation of cell adhesion complexes by integrins assures substrate adhesion as well as targeted location of actin filaments and signalling components, and hence is essential for establishing cell polarity, directed cell migration, and maintaining cell growth and survival.

1.4.1. Integrins and cell shape: the association of actin and integrins.

Ligand binding to integrins leads to integrin clustering and recruitment of actin filaments and signalling proteins to the cytoplasmic domain of integrin. Actin cytoskeletal structures include cortical actin, stress fibers, lamellipodia, and microspikes. Actin stress fibers are linked to integrins at the inner surface of the plasma membrane involving a focal adhesion complex including α-actinin, Focal adhesion kinase, talin, vinculin, and zyxin. Signaling through integrins depends on the formation of these focal adhesions, dynamic sites in which cytoskeletal and other proteins are concentrated and which regulate migration and the shape of a cell.

Remodelling of preexisting actin filaments into the different actin filaments structures is mainly controlled by members of the Rho family of GTPase. Among them, CDC42 induces filopodia, Rac induces lamellipodia, and Rho induces focal adhesions and associated stress fibers. It is known that integrins can activate the Rho-family of small guanine nucleotide –binding proteins and then regulate cell speading and migration (Ren X.D et al. 1999).

It is well appreciated that alterations in ECM–integrin interactions cause changes in cell shape and behaviour. For example, TENASCIN-C, an extracellular matrix (ECM) glycoprotein, interacts with αvβ3 integrins to modify smooth muscle cell shape (Jones PL et al. 1997). However, recent studies have demonstrated that ECM-dependent changes in cell shape and three-dimensional tissue architecture determine cell function by modulating integrin signalling pathways. For example, when cultured on an exogenous basement membrane, normal mammary epithelial cells adopt a polarized cuboidal morphology, become quiescent and express high levels of β-casein. Although expression of β-casein depends upon basement-membrane (BM) laminin interacting with β1 integrins and activation of a tyrosine phosphorylation signalling cascade, the rounding and clustering of the cell is also a necessary condition for the milk production; if mammary epithelial cells are forced to spread on laminin, while maintaining their interaction with β1 integrins, expression of β-casein is suppressed (Roskelley, C. 1994). Thus cell

shape could impact upon integrin-dependent signalling pathways and appears to profoundly modulate the processing of signals generated by identical ECM–integrin interactions.

Recent work has revealed that the integrin–actin cytoskeleton connection is highly dynamic and subject to many regulatory processes. In healing skin wounds for example, integrin-mediated cues promote the reorganization of the cytoskeleton of keratinocytes at the wound edge resulting in directed migration and wound closure. Loss of ß1-integrins on keratinocytes leads to impaired as well as non-directed migration resulting in severely delayed re-epithelialization (Grose *et al*., 2002). Furthermore, it has become clear that the interaction between integrins and the actin cytoskeleton is differentially regulated in different locations of the cell. At the leading edge of migrating cells, integrins bind the ECM, recruit the actin cytoskeleton and initiate local reorganization of the actin network, promoting different types of membrane protrusion. At the rear of the cell, integrins detach from the ECM, dissolve the link to the cytoskeleton and are, at least partially, recycled to the front of the cell (Ballestrem *et al*., 2001). Taken together, these data indicate that the interaction of the cytoskeleton with the adhesion receptors plays an important role in cell polarity, spreading and motility.

1.4.2. Integrins and cell cycle control

1.4.2.1. Overview of cell cycle control mechanisms

In the past decades extensive studies using cultured cells and genetic model organisms have strongly contributed to the elucidation of the mechanisms that regulate cell proliferation and the the cell cycle. A synopsis is given below. Basically, the cell cycle is divided into four phases: G1 (gap 1), S (DNA synthesis), G2 (gap2) and M (mitosis). The individual phases of the cell cycle allow for a controlled replication of the genome, organelles and other cellular components.

Here we would like to discuss mainly the mechanisms regulating G1 to S phase progression on which my work has focused. Cyclins and cyclin dependent kinase (CDKs) are known to be major players in mediating the progression and passage through these two phases of the cycle. CDKs are inactive in the absence of their cyclin partners, and they are activated by the binding of their partner cyclins. In mammalian cells, the major complex present in G1 is Cyclin D/CDK (4 or 6). During the late G1 phase, Cyclin E is actively expressed and binds CDK2 forming the Cyclin E/CDK2 complex. Cyclin A is induced at or near the G1/S boundary; it binds to CDK2 in S phase and involved in S phase progression. In addition to their binding to Cyclins, the activity of CDKs is also regulated by the presence of Cyclin dependent kinase inhibitors (CKI).

Mitogenic growth factors promote G1 phase cell cycle progression by stimulating the formation or activation of Cyclin D-cdk4/6 and Cyclin E-cdk2. These mitogenic effects typically involve increases in Cyclin D expression and decreases in CKI expression. The active enzymes then phosphorylate the retinoblastoma protein (pRb) and its family member, p107. Hypophophorylated pRb and p107 form complexes with members of the E2F family, which act as transcriptional repressors. PRb and p107 phosphorylation results in disruption of pRb/E2F and p107/E2F complexes, allowing for the induction of E2F dependent genes such as Cyclin A (DeGregori J.T; 1995).

As we discussed above, CKIs are involved in the modulation of the CDK activity during the cell cycle. This family of proteins can be divided in two broad categories: cip/kip family (p21cip1, p27kip1, and p57kip2) which bind to cyclin E-cdk2, cyclin A –cdk2, and cyclin D-cdk4/6, and the INK4 family (p15, p16, p18, and p19) which bind only to cyclin D-cdk4/6. The members of the Ink4 group inhibit cdk4 and cdk6 by promoting their dissociation from Cyclin D, while the Cip/Kip members inhibit all CDKs in a concentration dependent manner (sherr, C.J, 1996). Overexpression of any member of the CIP/Kip family causes a G1 block/arrest in transfected cells; while overexpression of Ink4 members such as p16 causes a reduction of the Cyclin D/cdk4 complex levels (Quelle D.E.et al. 1995)

The p21 and p27 proteins are necessary for the formation and stabilization of CyclinD/CDK4 complexes (Cheng, M., et al.,1999). The titration of these two CKI relieves the inhibition of Cyclin E/CDK2 complexes. This, in turn, grants the progressive accumulation of Cyclin E/CDK2 activity and the further hyperphosphorylation of Rb proteins. The system generates a hierarchical program of CDK activation since the increase of CDK2 activity during G1 requires inactivation of both the cip/kip proteins and is therefore dependent on the prior activation of the Cyclin D pathway. Once CDK becomes active it triggers the degradation of p27 by targeting it for phosphorylation and subsequent ubiquitination (Vlach, JS Hennecke & B.Amati, 1997). This event has two major effects: The destabilization of Cyclin D/CDK(4,6) complexes and the resulting release of p21 which is able to inhibit Cyclin E/cdk2. The temporary titration of p21 by Cycline E/CDK2 allows the formation of CyclinA/CDK2 complexes, which will then mediate S phase progression. P21 and p27 differ mostly in their expression kinetics. Generally p27 levels are high in quiescent cells and decrease in late G1, the protein levels are thought to be regulated by proteasome mediated degradation (Vlach, JS Hennecke & B.Amati, 1997). P21 levels instead, are low in quiescent cells and increase during the late G1 phase (Macleod, KF et al. 1995). In addition to its normal regulatory mechanisms, p21 levels can be induced by the p53 pathway. The INK4 proteins are thought to mediate CDK activity modulation through the same pathway. Upon an anti-mitogenic stimulus, such as TGF-β,

INK4s are expressed and promot the dissociation of CyclinD/CDK4, which causes the releases of p21 and the inhibition of cdk2 activity (Reynisdottir,I. et al. 1995).

1.4.2.2. Integrins and cell cycle control

Integrins are required for growth factor signalling

Cells require anchorage to the ECM to proliferate. Integrins activate growth-promoting signaling pathways that are responsible for the anchorage requirement. Two such pathways appear to be activated by most integrins. In one of them, integrins facilitate growth factor–mediated activation of ERK. In some cells, signaling along the Ras-ERK cascade is blocked at the level of the activation of either Raf or MEK in the absence of attachment. Integrins remove this block, perhaps by activating Rac or PI 3-kinase (Frost J.A., *et al.*1997). ERK may be required for cell growth because it phosphorylates the ternary complex factor (TCF), which promotes transcription of the immediate-early gene c-Fos (Treisman R., 1996). In another pathway, integrins activate the MAPK c-Jun NH2-terminal kinase (JNK), which regulates progression through the G1 phase of the cell cycle. The activation of JNK requires the association of FAK with Src and p130CAS and the recruitment of Crk (Miyamoto S, et al. 1995). Activated JNK enters the nucleus and phosphorylates the transcription factor c-Jun, which combines with c-Fos to form the AP-1 transcription factor complex. AP-1 then regulates genes that are important for cell proliferation (Treisman R., 1996). Because most growth factors are poor activators of JNK, the ability of integrins to activate this kinase may explain why cell proliferation requires integrin-mediated adhesion.

Integrins regulate cyclin-Cdk activity

As we mentioned, progression through the G1 phase of the cell cycle requires the sequential activation of CDK4/6 and CDK2. The activities of these kinases are regulated by integrins. Thus, integrin signals are necessary for cells to traverse the cell division cycle. For example, the activation of CDK4/6 are suppressed in cells that are not anchored to ECM (Zhu X, et al., 1996). Moreover, anchorage to the ECM is necessary for the down-regulation of the CDK2 inhibitors p21 and p27 and, thus, activation of cyclin E–CDK 2 (Zhu X, et al., 1996). The reason for the accumulation of p21 and p27 in suspended cells is not known, but its effect is compounded by the decrease in Cyclin D–CDK 4/6, a complex that sequesters p21 and p27 and prevents their action on CDK2 (Giancotti FG & Ruoslahti E. 1999).

A few studies have tried to link specific integrins with specific events in anchorage-dependent cell cycle progression. Symington (Symington BE**.** 1995) showed that CDK activity and pRb phosphorylation were

stimulated when a α5β1 integrin overexpressing K562-subclone was treated with the peptide GRGDS (a ligand for α5β1 integrin). Klekotka et al. showed that the α2β1 integrin supports cell-cycle progression of mammary epithelial cells adherent to type I collagen matrices. Integrin collagen receptors containing the α2 cytoplasmic domain stimulated expression of Cyclin E and CDK2 resulting in Cyclin E/CDK2 activation in the absence of growth factors other than insulin (Klekotka PA et al. 2001). Meredith et al. (Meredith et al. 1999) microinjected a growth inhibitory form of the β1-integrin subunit into 10T1/2 fibroblasts and found that cell cycle progression was blocked in late G1, near or after the induction of cyclin E-CDK2 activity. Nevertheless, detailed studies of specific integrin on distinct Cyclin-CDK events have yet to be reported.

1.4.3. Integrins and cell survive

Apoptosis is an efficient way to physiologically eliminiate excess or damaged cells in a controlled manner that precludes an inflammatory response. Integrin-mediated cell attachment is one of the main regulators of apoptosis. Loss of attachment to the matrix causes apoptosis in many cell types. This phenomenon, referres to as "anoikis", may help maintain the integrity of tissues. (Frisch and Screaton, 2001). Specific integrin mediated attachment plays an important role in suppressing anoikis. For example, upon detachment, HUVECs rapidly die by apoptosis. However, adhesion on fibronectin protect HUVECs from TNF induced cell death (Fornaro M et al. 2003). Also, a laminin-rich basement membrane is required for long-term cultures and survive of primary mammary epithelial cells (Farrelly N et al. 1999).

Integrins suppress anoikis in attached cells by activating signaling pathways that promote survival and inactivating the ones that promote apoptosis. A number of these pathways have been partially characterized and seem to be of varying importance in different types of cells. A pathway that begins with the activation of focal adhesion kinase (FAK) by ECM bound integrins and results in the activation of phosphatidylinositol 3-kinase (PI3-K) and Akt/protein kinase B seems to be a major source of survival signals in most cells (Frisch et al. 1996b; Khwaja et al. 1997 and Matter and Ruoslahti, 2001). Akt promotes survival, at least in part, by phosphorylating and thereby inactivating two proapoptotic proteins, Bad and caspase-9 (Cardone et al.,1998 and del Peso et al. 1997). Inhibition of p53 prevents FAK deficient cells from undergoing anoikis when deprived of growth factors, suggesting that p53 mediates the death signal under FAK deficiency (Ilic D, et al. 1998**)**.

Like cell growth, anoikis can be controlled by the ECM in an integrin-specific manner. The α5β1 and αvβ3 integrins induce expression of the anti-apoptotic protein Bcl-2 in CHO cells, protecting cells from

apoptosis from stresses such as the lack of growth factors. Other integrins, including another fibronectin receptor, αvβ1, do not provide this survival effect. (Zhang et al. 1995; and Matter and Ruoslahti, 2001). Using truncated Bcl-2-regulating integrin (α5β1) screening (the cytoplasmic domain of the Bcl-2-regulating integrin was truncated to screen for cDNAs capable of enhancing expression of the Bcl-2 gene despite the disabled integrin), a mitochondrial protein, Bit1, was identified that is released into the cytoplasm during anoikis and to mediates apoptosis by regulating the functions of two Groucho family transcriptional regulators : AES and TEL. Importantly, the apoptosis induced by Bit1/AES is atypical in that caspase activation is not involved.(Jan,YW, et al. 2004). In addition, the αvβ3 integrin promotes endothelial and melanoma cell survival; this effect correlates with suppression of the p53 pathway and activation of the nuclear factor kappa B transcription factor (Stromblad S et al. 1996 & Scatena, M et al. 1998). The activation of Shc by α1β1, α5β1, and αvβ3 may also contribute to protection from apoptosis (Wary KK, et al. 1996). Furthermore, integrins and the EGF receptor coordinately prevent anoikis in epithelial cells by downregulating the BH3 domain-only proapoptotic protein Bim (Reginato et al., 2003). Thus, integrin mediated attachment to ECM is a general requirement for cell survival, but survival under special circumstances may require a particular integrin.

Anoikis is likely to be important in the maintenance of tissue architecture, as it would ensure the demise of cells that detach from their original site in a tissue. The requirement for a specific integrin as the mediator of the attachment may provide an additional safety factor, because it would facilitate the destruction of cells that have attached at an inappropriate tissue location. Tumor cells are generally resistant to anoikis and can proliferate in the absence of anchorage to ECM (Schlaepfer & Hunter, 1998). This may help to explain their propensity to leave their original site and metastasize.

1.5. Functional consequence of integrin gene mutations in mice

During the last two decades, most of the information about integrin function has been derived from in vitro cell culture systems. Gene targeting technology recently made it possible to generate mice that lack specific integrins in a constitutive or cell type-specific manner. Analyses of these mice demonstrate how integrin-mediated adhesion and signal transduction affect development and maintenance of tissues and provide additional insight into integrin functions in various diseases.

To date, 18 α and 8 β integrin subunits have been deleted in the mouse. Ablation of integrin genes leads to various phenotypes during mouse development, ranging from apparently normal mice to early lethality (Bouvard D, et al. 2001). β1 integrin is the most widely expressed integrin and controls various developmental processes. In this chapter, we will mainly focus on integrin β1 and discuss its role in early mouse development and in the different development contexts.

1.5.1 Early mouse development

Disruption of the ubiquitously expressed integrin β1 gene leads to the loss of at least 12 different integrin receptors and results in peri-implantation lethality characterized by an inner cell mass (ICM) failure. Fertilization of β1-null oocytes and the entire pre-implantation development is normal (Fässler & Meyer, 1995; Stephens LE et al. 1995). A possible explanation of the ICM failure could be the loss of β1-mediated survival signals since lack of interaction of the ectodermal cells with the BM could lead to their loss by apoptosis and arrest of additional development (Coucouvanis & Martin, 1995). Another possibility could be that the lack of β1 integrins leads to abnormal BM assembly since β1 integrin is crucial for normal expression and correct assembly of BM components into a supramolecular structure (Aumailley M et al. 2000).

1.5.2. Neurogenesis

Evidence coming from fly and worm suggest a critical role for the integrin family during brain development and for maintaining brain functions (Anton ES et al. 1999). This was also confirmed in mice. α3-null mice display a defect in neuron migration and a disorganized layering of the cerebral cortex, suggesting that this integrin is involved in radial neuronal cell migration. A very similar phenotype is observed in reeler mice, which lack the extracellular matrix protein reelin. In these mutant mice, migration of Cajal-Retzius cells is impaired, leading to an abnormal lamination of the cerebral cortex. A recent study showed that α3β1 integrin can bind reelin (Dulabon L et al. 2000). This interaction may provide a stop signal and arrest neuronal migration. Ablation of the β1 integrin gene in all cells of the developing cortex causes cortical neurons to become severely disorganized and Cajal-Retzius cells misplaced, but does not prevent the migration of neuronal precursors, however, it does cause layering defects close to the marginal zone (Graus-Porta D, 2001). The ability of neuronal precursors to migrate in the absence of β1 integrin is not consistent with the migration phenotype seen in the absence of the α3 integrin, since β1 is the only

subunit that is known to form heterodimers with α3. Perhaps an altered balance between different β1 integrin heterodimers is more detrimental than their complete absence.

Myelination in the peripheral nervous system is accomplished by Schwann cells. Myelin-forming Schwann cells synthesize abundantly the laminin receptors α6β4, α6β1 integrins and dystroglycan, and minor amounts of α2β1 integrin. Inactivation of β1 integrin specifically in Schwann cells using the Cre-loxP system has shown that β1 integrins are crucially important for Schwann cell–axon interactions. β1-null Schwann cells populates nerves, proliferate, and survive normally. However, loss of integrin β1 causes failure of ensheathing and segregating axons in development nerves and delayed myelination. (Feltri ML et al. 2002).

1.5.3. Chondrogenesis

β1 integrin is a dominant integrin β subunit expressed in the heart. Two of the four splice variants of β1 integrin; β1A and β1D are expressed on cardiac myocytes. They are identical with the exception of the last 24 amino acid residues of their respective cytoplasmic domains. The expression of β1A and β1D isoforms is developmentally regulated in cardiac cells. β1A is expressed during embryogenesis while β1D expression begins late in development and eventually becomes the dominant β1 integrin isoform expressed on adult cardiac myocytes. On the basis of in vitro analysis, integrin-mediated attachment to the ECM has been suggested to be important for controlling growth and differentiation of cardiomyocyte (Borg TK et al. 2000). Furthermore, integrins were proposed to function as mechanoreceptors that transform mechanical stimuli into biochemical signals that affect cellular function (Borg TK et al 2000). Several genetic mouse models demonstrate an important function of β1 integrin in cardiac muscle in vivo. Mice expressing β1A instead of β1D in heart show a mildly disturbed heart phenotype, whereas replacement of β1A by β1D results in embryonic lethality with a plethora of developmental defects, in part caused by the abnormal migration of neuroepithelial cells. (Baudoin C et al. 1998). Even more severe defects occur in the heart when both β1A and β1D are absent or functionally inactivated. The areas with β1-null cardiac muscle cells in the heart of β1-null chimeras become smaller with time and show signs of degeneration. Ultra-structural analysis revealed alterations in the sarcomeric architecture. In addition, transgenic mice expressing a dominant-negative form of β1 integrin, in which the extracellular and transmembrane domain of CD4 is fused to the cytoplasmic domain of β1 integrin, show hypertrophic

changes in the heart. Mice that express high levels of the transgene die around birth and display a replacement fibrosis (Keller RS et al. 2001).

1.5.4. Skin and hair follicle morphogenesis

The skin is composed of an epidermal and a dermal layer, which are separated by a basement membrane. The epidermis is made primarily of keratinocytes, while the dermis contains different cell types including fibroblasts, endothelial cells and macrophages as well as large amounts of extracellular matrix. Adhesion of the keratinocytes to the basement membrane and to each other is important for the development of skin and for the maintenance of skin integrity. During differentiation, basal keratinocytes detach from the basement membrane and migrate to suprabasal layers. This movement is accompanied by a complex change of cell–cell and cell–matrix interactions involving adhesion molecules such as cadherins and integrins (Hotchin et al., 1995; Zhu and Watt, 1996). Basal keratinocytes attach to the underlying basement membrane via integrins. In skin, several integrin receptors are expressed by the epidermal keratinocytes, including $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$. Genetic ablation of the $\alpha 6$ or $\beta 4$ integrin gene in mice resulted in a complete absence of hemidesmosomes, creating large blisters between the dermis and the epidermis (Georges-Labouesse et al., 1996; van der Neut et al., 1996). Skin-specific ablation of the $\beta 1$ integrin gene at around birth demonstrated a role of $\beta 1$ integrin in the processing of the BM components and in the growth and maintenance of hair follicles. $\beta 1$-deficient basal keratinocytes have an aberrant morphology and a reduced proliferation rate, but are still able to terminally differentiate. While ectopic expression of $\alpha 2$, $\alpha 5$ and $\beta 1$ integrin in the suprabasal layers of transgenic mice resulted in hyperproliferation, perturbed keratinocyte differentiation and a psoriasis-like phenotype (Carroll et al., 1995).

1.5.5. Myoblast fusion

Vertebrate skeletal muscle fibers express many integrin subunits in developmentally regulated patterns, including the integrin $\beta 1$ subunit and its partners α 1, 3, 4, 5, 6, 7, and (Gullberg et al., 1998). The most abundant integrin in skeletal muscle is $\alpha 7\beta 1$, which is expressed during all stages of muscle development (Bao ZZ et al. 1993). Mice with a targeted deletion of the $\alpha 7$ integrin develop a progressive muscular dystrophy after birth. The major defect is severe disruption of the myotendinous junctions (Mayer et al., 1997). Mutations that inactivated the mouse integrin $\beta 1$ subunit gene in developing myoblasts show that $\beta 1$ integrins regulate myoblast fusion and sarcomere assembly. $\beta 1$-deficient myoblasts adhere to each

other, but plasma membrane breakdown is defective. The integrin-associated tetraspanin CD9 that regulates cell fusion is no longer expressed at the cell surface of β1-deficient myoblasts, suggesting that β1 integrins regulate the formation of a protein complex important for fusion. Subsequent to fusion, β1 integrins are required for the assembly of sarcomeres. Other ECM receptors such as the dystrophin glycoprotein complex are still expressed but cannot compensate for the loss of β1 integrins, providing evidence that different ECM receptors have non-redundant functions in skeletal muscle fibers (Schwander.M et al 2003).

Part II Mammary gland development

2.1 Mammary gland is an attractive organ for development studies

Mammary gland is a specific organ for female mammals. This developed organ could deliver essential nutrients to the newborn offspring. These nutrients are assembled in a rich proteinaceous and lipid fluid termed milk. Milk is sufficiently complex that it alone is sufficient to support the development of the newborn through the critical initial stages of postnatal development and growth. Functional differentiation of the mammary gland is a crucial step in the reproductive cycle of mammals. The anatomical and morphological design of the gland as well as the regulation of its development and function is subservient to this main function.

The mammary gland consists of two primary components: the parenchyma, which forms a system of branching ducts from which secretory acini develop, and the adipose stroma, which provides a substrate within which the parenchyma develops and function. The parenchyma consists of two major epithelial structures: collecting ducts that form during puberty and are maintained throughout adulthood, and alveoli, containing the luminal, milk-secreting cells that appear during pregnancy and lactation and are lost during remodeling at involution. Alveolar growth and proliferation occurs predominantly during pregnancy, followed by functional differentiation of alveolar epithelial cells at parturition. Some additional alveolar proliferation occurs during the first few days of lactation. During lactation, the secretory cells produce large amounts of milk.

Human breasts begin developing in the embryo. In both female and male newborns, milk ducts and lobules (milk producing glands) are well formed and organized. With the beginning of female puberty, the release of estrogen in combination with progesterone causes the female breasts to undergo dramatic changes, which culminate in the fully mature form. Further maturation of the breast tissues occurs with lactation.

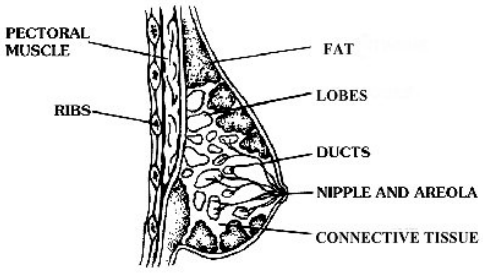


Diagram: Breast Anatomy

Fig.5.The diagram of breast anatomy. The mature female breast is composed of essentially four structures: lobules; milk ducts; fat and connective tissue (see diagram). The lobules group together into larger units called lobes. The lobes empty into the milk ducts which course through the breast towards the nipple/areolar area. There, they converge into 6-10 larger ducts called collecting ducts which enter the

Compared with human breast, a major portion of the mouse mammary gland growth occurs after birth; furthermore, the development of the gland in mice as a model system proceeds in distinct phases. In newborn mice a rudimentary system of small ducts is present which grows slowly until the onset of puberty when pronounced ductal growth occurs. Elongation of the mammary ducts mainly occurs by mitotic activity in the terminal end buds (TEBs). Development of the ducts continues in cycling virgins leading to the formation of a ductal tree which fills the entire mammary fat pad. The TEB regress to blunt ended structures, containing a single layer of luminal epithelial cells with low mitotic activity, when the ducts have reached the periphery of the mammary fat pads. Extensive ductal branching and alveolar growth occurs during pregnancy and is largely completed at parturition. Terminal differentiation of the alveolar epithelium is completed at the end of gestation with the onset of milk secretion at parturition. After weaning the entire alveolar epithelium apoptosis and the gland is being remodeled. Within a few weeks the gland has the appearance of that of a mature virgin.

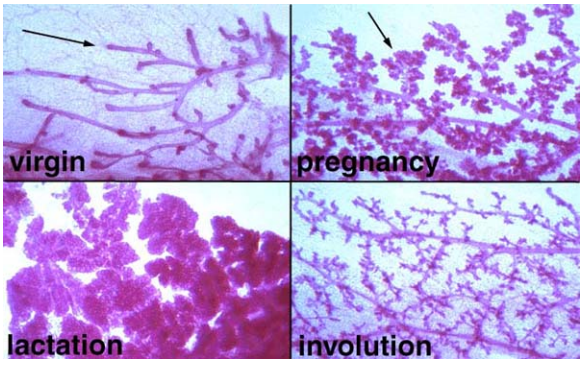


Fig.6. Whole mounts of mammary tissue from different stages of development (Lothar Hennighausen) whole mount from an imature virgin(Upper left); a 16 day pregnant mouse (Upper right); day 1 of lactation(Lower left); and 4 weeks after weaning (Lower right).

The problem of mammary gland development and function has attracted the attention of scientists for over a century. Firstly, the sole physiologic function of the mammary gland is to synthesize, assemble, and deliver copious quantities of milk to the infant upon demand. Thus, to understand the development

and function of mammary gland is an important event for the reproduction of the mammals. Furthermore, the understanding of normal gland function, development, structure and regulation can assist the investigation of the breast cancer; a disease threatens the females, for example, 180,000 woman annually in the United States. Finally, the mammary gland is a good model for the development studies when the repeated phases of growth and differentiation can be examined, since at involution the organ goes through complete remodeling. Thus the mammary gland provides a unique opportunity to evaluate the role of specific proteins in the formation and function of a transient, but highly specialized, organ. Also, in mammary gland, the complex biological and cellular interactions make the possibility to study the regulation of the same gene expression in diverse cell types.

2.2 The development of mammary epithelium

The mammary gland consists of two major epithelial structures: collecting ducts that form during puberty and are maintained throughout adulthood, and alveoli, containing the luminal, milk-secreting cells that appear during pregnancy and lactation and are lost during remodeling at involution.

2.2.1.Terminal end bud formation and ductal morphogenesis.

A branched epithelial structure includes a network of tubes that are integral to the function of a number of glandular organs (Gumbiner BM, 1992; Affolter M, et al 2003). Lung, kidney, salivary gland , and mammary gland are examples of organs that develop through branching morphogenesis. The latter is unique among these organs in that the majority of its branching is post-embryonic. Extensive branching begins in puberty in the female and ceases after expanding to the outer limits of the mesenchymal fat pad.

Branched structures are first seen in the mouse mammary gland in late embryonic development (Robinson GW et al , 1999). Expansion of the ductal tree within the fat pad occurs rapidly after 3 to 4 weeks of age, when ovarian hormones begin systemic circulation, and ceases around 10 weeks of age. During branching morphogenesis, primary ducts elongate, driven by terminal end bud proliferation, and the tree 'fans out' within the fat pad through a process of terminal end bud bifurcation and lateral side branching.

Mammary ducts are composed of a mixture of epithelial cells with distinct morphological and function lineages: the luminal cells, which form the tubular duct; and the second lineage, which give rise to myoepithelial cells to aid in the expulsion of milk from the alveoli during lactation. Both of these lineages are established simultaneously and arise from the end buds during puberty (Williams and Daniel, 1983).

The teminal end buds are bulbous structures found in pubertal animals. Elongation of mammary ducts in the immature mouse takes place as a result of rapid growth in end buds. These structures proliferate at the apex of elongating ducts and are responsible for penetration of the surrounding adipose stroma; by turning and branching, end buds give rise to the characteristic open pattern of the mammary ductal tree.

These bulb-shaped structures consist predominantly of an outer layer of epithelial cells, termed cap cells, and an inner layer of epithelial cells, termed body cells. The extent of proliferation in the TEB has been estimated to be as much as 5-fold more than that observed in mature ducts. Within the TEB, the cap cells often display the highest proliferative activity (Dulbecco R,1982, Humphreys RC 1999). The TEB is also a site of significant apoptosis in the developing gland, and it is this apoptosis that is believed to cause canalization of the developing ducts (Humphreys RC,1996). Regulation of TEB development occurs at several levels involving steroid and peptide hormones as well as local production of growth factors (Humphreys RC 1999, Kleinberg DL 1997)

Numerous studies suggest that IGF-I plays an important role in mammary gland development. Firstly, IGF-I is a potent mitogen for normal mammary epithelial cells in culture, and ductal growth can be induced in mammary gland explant cultures by IGF-I in combination with mammogenic hormones (Richert M, Wood TL 1999). Secondly, *in vivo* local administration of IGF-I induces mammary TEB development (Kleinberg DL 1998) and transgenic mice that overexpress IGF-I specifically in the mammary gland during pregnancy and lactation exhibit an increased incidence of mammary hyperplasia and tumorigenesis (Hadsell DL, 1996 & 2000). Lastly, the mRNAs for both IGF-I and the IGF-I receptor (IGF-IR) are expressed in both the mammary stroma (Walden PD, 1998) and the developing TEB (Richert M, Wood TL 1999), and studies by Ruan and co-workers (Ruan W & Kleinberg DL 2000) demonstrated that targeted deletion of IGF-I inhibits normal TEB development.

Besides hormone and growth factors, it was found that the Matrix metalloproteinases (MMPs), the stromal factors that are ideally positioned to regulate stromal–epithelial cross talk (Sternlicht & Werb, 2001), could regulate mammary gland branching morphogenesis by clearing a path for invading ducts by degrading ECM barriers and permitting ductal penetration into the mammary fat pad. During early puberty, MMP-2 supports the invasion of TEBs into the stromal fat pad, by protecting against excessive apoptosis within TEBs. Later in puberty, MMP-2 acts on the mature primary duct to repress excessive secondary lateral budding and branching. While MMP-3 acts on both primary and secondary ducts to induce secondary and tertiary branch formation (Bryony S, et al, 2003). In contrast, introduction of

exogenous tissue inhibitor of metalloproteinases-1 (TIMP-1) into pubertal mammary gland, via a pellet, retards ductal invasion (Fata et al., 1999).

2.2.2 Alveolar development and milk secretion

With pregnancy occurs, the gland begins forming side buds. Buds repeatedly form and elongate perpendicular to existing ducts to form small terminal ducts. When the mammary fat pad is filled so that there is little space between nascent buds, sac-like alveoli are formed at the end of the terminal ducts. Alveolar growth and proliferation occurs predominantly during pregnancy, followed by functional differentiation of alveolar epithelial cells at parturition. Some additional alveolar proliferation occurs during the first few days of lactation. It's was demonstrated that prolactin induced phosphorylation of Stat5 is a key event in functional mammary development and differentiation. (Details will be discussed in 2.2.5.2)

The alveoli composed of a single layer of polarized luminal, milk-secreting cells that surround an enclosed space, or lumen. The earliest signs of lumen formation are many small cavities and crevices lined with microvilli which appear at scattered sites throughout the branching cords and neck of the gland. Differentiated luminal secretory cells are well polarized and organized. The myoepithelial, another cell lineage to basket the secretory cells in the alveolus, play central role in milk ejection. Differentiated myoepithelial cells are highly contractile and their ultrastructure is reminiscent of that of smooth muscle cells.

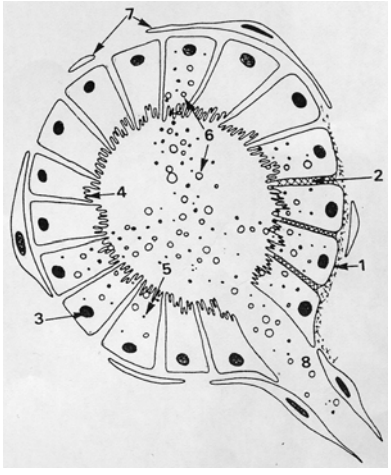


Fig.7.The phenotype of mammary alveolar epithelium in mouse. The spherical mammary alveolus, consisting of a single layer of secretory cells, is shown in schematic cross-section. Some important architectural and functional features of this structure are: (1,2) extensive interactions between cells and (1) ECM, as well as (2) other cells; (3) basal nuclei and (4) apical microvilliindicating morphological polarity; (5)casein microvilli and (6) fat droplets formed intracellularly and secreted apically into the lumen; (7) myoepithelial cells and their processes; (8) secreted milk is collected in ducts.

At the end of pregnancy, the mammary gland starts milk synthesis. Milk is an externally secreted fluid designed specifically to nourish the young. It contains vital nutrients such as proteins, carbohydrates, lipids, minerals, and vitamins together with bioactive substances. Milk is secreted more or less continuously into the alveolar lumens and stored there. Removal of milk from the alveoli is accomplished by contraction of the myoepithelial cells surrounding the alveoli and ducts. This process is called milk ejection.

Most protein components are secreted from the mammary epithelium by exocytosis. In contrast, the lipid droplets bulge against and gradually become enveloped in apical plasma membrane, finally separating from the cell as the milk fat globule. By specific gene ablation, it was found that Xanthine oxidoreductase (XOR, the rate-limiting enzyme in purine catabolism is specifically required for enveloping milk fat droplets with the apical plasma membrane prior to secretion from the lactating mammary gland. The *XOR*+/- females are unable to maintain lactation and their pups die of starvation 2 wk postpartum (Claudia Vorbach, 2002).

2.2.3 Involution

Lactogenesis represents a profound and rapid series of changes in the activity of differentiated mammary epithelial cells from a quiescent state to a fully active secretory state. In the absence of suckling or at cessation of nursing, these differentiated mammary epithelial cells are removed and the gland is remodeled to a duct system similar to that in the mature virgin. This process, named as involution, is the last stage of the mammary life cycle. The destroyed milk-producing machinery can be recapitulated in a subsequent pregnancy in preparation for another round of lactation.

Mammary gland involution goes through two distinct stages. In the first stage, lack of suckling and milk stasis results in a rapid, but reversible induction of apoptosis within the differentiated population of mammary epithelial cells, but there is no remodeling of the lobular-alveolar structure. When the lack of suckling is prolonged, the involution goes through into the second stage. The apoptosis is accompanied by a tissue-remodeling phase involving the induction of matrix-degrading enzymes and inflammatory cell infiltration. The lobular-alveolar structure of the gland is obliterated as proteinases degrade basement membrane and extracellular matrix (ECM). This mammary alveoli regress process cannot be reversed. The end result of this process is the elimination of all lobuloalveolar structures leaving behind a simple ductal tree.

In mice, apoptosis of mammary alveolar cells commences within hours of the end of sucking and peeks at day 3 and decreases thereafter, while the alveolar structure has completely degenerated after a dying period of 4 days. The apoptosis of individual alveolar cells is correlated with increased expression levels of many genes. It was found stat3, another member of the STAT family of proteins, was induced in the first phase of involution. And the mice of conditional knock out of stat3 in mouse mammary gland showed a decrease in epithelial apoptosis and a dramatic delay of the involution process upon forced weaning (Chapman RS, 2000).

2.2.4 Hormonal and growth factor regulation

Development of the mammary gland is controlled by systemic steroid and peptide hormones and local growth modulators (Topper and Freeman, 1980). Steroid hormones of the ovary and placenta were implicated very early as important stimulators of mammary gland development (Anderson R, 1974). The ductal outgrowth and, in part, alveolar proliferation is controlled by ovarian steroid hormones (Daniel and silberstein 1987). It was already determined that estrogen stimulates ductal elongation (Korach et al, 1996), while progesterone is necessary for alveolar development (Humphreys et al. 1997). Furthermore, It was established that prolactin (PRL), a 23-kDa peptide, which is mainly synthesized in lactotrophic cells of the anterior pituitary of vertebrates, are involved in lobuloalveolar differentiation, milk synthesis, and lactation (Topper and Freeman, 1980). Prolactin is essential for the transition from a proliferative to a lactating mammary gland and for the maintenance of milk secretion. The withdrawal of prolactin and oxytocin, another peptide hormone inducing the contraction of myoepithelial cells and thereby for milk ejection, causes involution of the mammary gland to a mature virgin-like state (Wagner, 1997).

Additionally, A stimulatory role in the proliferation and/or differentiation of mammary epithelial cells is suggested for most growth factors including epidermal growth factor, amphiregulin, transforming growth factor, and insulin like growth factor (Lamote.I ,2004).

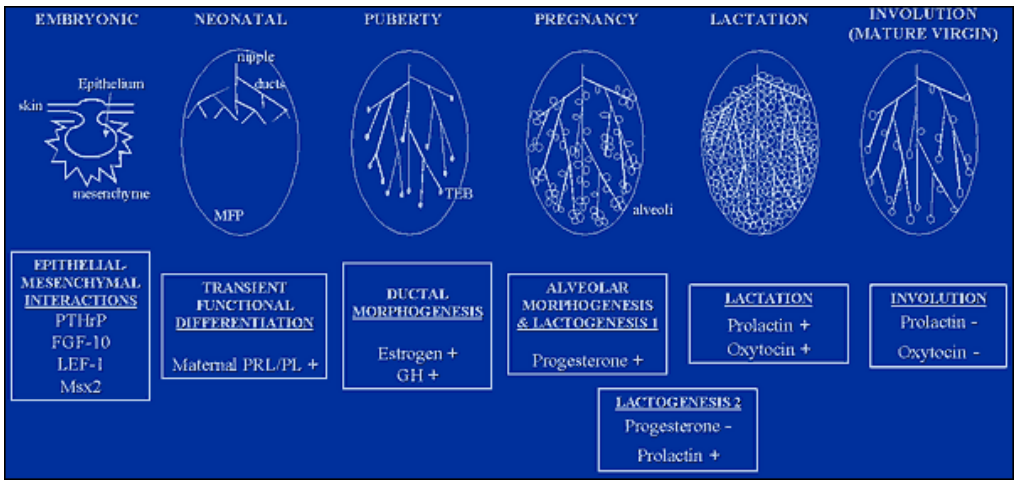


Fig.8. Overview of the regulation of mammary gland development. During embryonic development, signaling molecules important in epithelial-mesenchymal interactions include PTHrP, FGF-10, LEF-1, and Msx2. Under the influence of maternal PRL and PL, the neonatal mammary gland undergoes transient functional differentiation and produces witch's milk. Mammary gland development proceeds slowly after birth until puberty, when E and GH stimulate rapid ductal elongation. During pregnancy, progesterone stimulates alveologenesis and lactogenesis 1. At parturition, the withdrawal of progesterone is required for initiation of lactogenesis 2. Prolactin promotes lactogenesis 2 and, along with oxytocin, maintains lactation. The withdrawal of prolactin and oxytocin causes involution of the mammary gland to a mature virgin-like state. (MFP, mammar.y fat pad; TEB, terminal end bud.) *Wysolmerski; endotext.com*

2.2.5. Signaling within the cell

2.2.5.1. Proliferation and Cell cycle regulators

In the first chapter (1.4.2), we already discussed the cell growth and cell cycle control. It's known the cyclins and cyclin dependent kinase (cdks) are the major players in mediating the progression and passage through the cycle.

Four mammalian G1 cyclins have been described to date: cyclins D1, D2, D3 and cyclin E. The expression of cyclin D1 is rapidly induced following the exposure of cells to mitogens; it's levels rapidly decline after the mitogens have been removed. This, together with a very short half-life of this protein permits rapid modulation of cyclin D1 levels in response to changes in extracellular environment (Sherr, C.J. 1994). Cyclin D1 was originally cloned as an oncogene responsible for parathyroid adenomas (Motokura et al. 1991). Subsequently, the aberrant expression of cyclin D1 was documented in several human malignancies. Most striking is the frequent involvement of cyclin D1 in human breast cancers (Schmidt, E. 1996). Consistent with the oncogenic role of cyclin D1 are the observation that transgenic mice engineered to overexpress this cyclin in their breast tissue are prone to mammary adenocarcinomas (Wang, T.C. et al. 1994).

Besides basic cell cycle machinery modulators, there is an increasing list of local growth factors, such as epidermal growth factor, amphiregulin, and insulin like growth factor, has been shown to have a stimulatory role in the proliferation and/or differentiation of mammary epithelial cells. Mammary glands from adolescent AR null mice displayed striking defects in ductal outgrowth. Additional loss of EGF or TGF exacerbated the defect whereas mice lacking only EGF and TGF had normal glandular arborization, underscoring the fundamental role of AR in ductal elongation (N. Luetteke et al, 1999). Different from EGF ligand, studies in mice on ERBB expression and activating profiles revealed that signalling by EGFR is critical for ductal outgrowth(L. Troyer and D. Lee, 2001). The insulin-like growth factor (IGF) family of ligands (IGF-I and IGF-II), binding proteins (IGFBP 1–6), and receptors (IGF-IR and IGF-IIR) also play pivotal roles in growth and development of the mammary gland. And the downstream signalling pathways of IGF receptors have been elucidated. In general, IGF-IR acts through two primary cascades, the mitogen activated protein (MAP) kinase and phosphatidyl-3-kinase (PI3-K) kinase pathways. The ultimate targets of the MAP kinase and PI3-K kinase cascades include members of the Ets and forkhead transcription factor families. Regulation of transcription factors provides a mechanism by which IGF mediates a proliferative and differentiative effect (LeRoith and Roberts, 2003).

2.2.5.2 cell differentiation and Stat5a regulation.

During lactation, the secretory cells produce large amounts of milk. Inside the various milk proteins, WAP and β-casein are as known to be transcriptional regulated by prolactin-JAK-STAT signalling pathway.

Prolactin and many cytokins use STAT proteins to regulate the transcriptions of specific genes through the JAK-STAT pathway. Ligand binding triggers dimerization or oliogmerization of receptors. Receptor-associated tyrosine kinase (JAKs) cross-phosphorylate each other as well as the tyrosine residues on the receptors. Subsequently, SH2-containing latent cytoplasmic proteins from the STAT family are recruited to the receptor complex and phosphorylated by the JAKs. Two STAT proteins dimerize, translocate into the nucleus, and activate gene transcription by binding to GAS in gene promoters.

In the functional postpartum gland, high levels of activated Stat5 can be found while only small amounts of phosphorylated Stat1 and Stat3 have been detected (Liu, X., 1996). And phosphorylation of Stat5a and -5b is very low in mammary tissue of virgins and during early pregnancy but rises sharply after day 14 of pregnancy. This led to the hypothesis that the activation of Stat5 is a critical step in the terminal differentiation of mammary secretory epithelium (Liu, X., 1996).

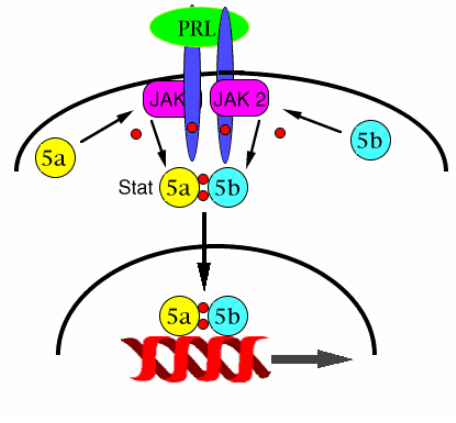


Fig.9. Prolactin signalling in the mammary gland. PRL binds to its receptor and causes the PRLR to dimerize. Receptor-associated tyrosine kinase JAK2 phophorylates the prolactin receptor and the signal transducers and activators of transcription Stat5a and Stat5b. Activated Stat5a and –5b are transported into the nucleus, bind to GAS sequences and induce transcription of target genes.

Indeed, the essential role of prolactin or STAT5 activity in mammary development and lactogenesis was confirmed in mice by genetic disruptions in mice. The inactivation of either the prolactin receptor gene (Ormandy et al., 1997) or the Stat5a gene (Liu et al., 1997, Miyoshi et al., 2001; Teglund et al., 1998) results in impaired alveolar proliferation and functional differentiation.

Similar to the prolactin receptor, it was found that members of the ERBB family have also been shown to activate STAT5 (Jones et al., 1999; Kloth et al., 2002; Olayioye et al., 2001). ERBB4 phosphorylates STAT5A at the regulatory amino acid Y694 in a STAT5A SRC-homology 2 (SH2) domain-dependent manner (Jones et al., 1999). Furthermore, ERBB4 phosphorylates STAT5A at a tyrosine(s) in addition to at Y694 (Jones et al., 1999) raising the intriguing possibility that ERBB4 regulates novel STAT5 activities through multiple phosphorylation events. Recently, genetic ablation of Erbb4 alleles within the developing mammary gland revealed a complete ablation of STAT5 activation in Erbb4$^{Flox/Flox}$Wap-Cre mammary epithelium at parturition. Consistent with disrupted STAT5 function, Erbb4$^{Flox/Flox}$Wap-Cre mammary glands at parturition failed to express the mammary epithelial differentiation marker Npt2B. Defects in epithelial functional differentiation at parturition were accompanied by a profound reduction in expression of the STAT5-regulated milk genes β casein and whey acidic protein. That suggests ERBB4 functions as an essential mediator of STAT5 signaling, and that loss of STAT5 activity contributes to the impaired functional differentiation of mammary glands observed in mice containing conditional Erbb4 deletions (Weiwen Long, 2003).

2.2.5.3 Involution and apoptosis of mammary epithelia cells

The two stages of mammary gland involution are controlled by progressive gain of death signals and loss of survival factors. The first stage of involution is controlled by local mammary-derived signals. Milk accumulation triggers increased expression of the death-inducing *bax* gene through a yet-undefined

mechanism. Importantly, this process is p53-independent (Li, M, 1996). The same stimuli mediate loss of Stat5a and 5b phosphorylation disrupting the principal pathway for prolactin signaling (Schmitt-Ney, M, 1992; Liu X, 1996). Prolactin is a lactogenic hormone that can promote cell survival. Phosphorylation of Stat3 is low during lactation and increases sharply during the first stage of involution at a time when Stat5 phosphorylation is lost (Liu.X, 1996).

The second stage of involution is ushered in by the complete loss of survival factors due to decreased levels of systemic lactogenic hormones and activation of proteinase-dependent pathways (Lund, L. R, 1996). Ensuing disruption of basement membrane and extracellular matrix (ECM) results in remodeling of the gland to a state resembling the mature virgin. Systemic lactogenic hormones are survival factors that persist through the first stage of mammary gland involution. Although these survival factors cannot overcome the dominant local apoptosis signals unleashed after removal of the suckling stimulus, they presumably prevent the gland from being remodeled (Lund, L. R, 1996; Boudreau, N, 1995). For example, glucocorticoid stimulation prevented progression of the gland into the second irreversible stage of involution, but did not block apoptosis (Li M.L 1997). Importantly, involution can be readily reversed during the first 48 h of involution, but not later.

2.3 The function of mammary stromal.

The essential functional component of mammary gland is the mammary epithelial cells. This cell is the one that synthesizes milk proteins and can occastionally become neoplastic. However, it relies on other cell types for its development and function. The mammary gland exsits as a community of epithelial cells that form adhesive interactions with extracellular matrix and the stromal cells. Such associations are essential for maintaining normal tissue homeostasis and function, and, when they break down; cells are often deleted by apoptosis.

2.3.1. Role of the stroma cells in the mammary development

The stroma is an important primary component of the mammary gland. Breast stroma accounts for more than 80% of the resting breast volume (Drife JO, 1986). The stromal provides a substrate within which the parenchyma develops and function. And it can further support subsequent mammary parenchyma development. The stroma or the supportive platform for the epithelial layer is composed of fibroblasts,

endothelial cells, smooth muscle cells, adipocytes, inflammatory cells, nerve cells and a macromolecular network of proteoglycans and glycoproteins collectively termed the extracellular matrix (ECM).

In seminal experiments by Kratochwil and Sakakura, it was suggested that the stroma play an active role in mammary gland development and function. It could direct and constrain the developmental plasticity of mammary parenchyma (Sakakura T, 1991). When Kratochwil cultured a composite of embryonic mammary epithelium and embryonic sub-mandibular (salivary) mesenchyme, the mammary tissue developed salivary gland-like lobules. Extending these experiments *in vivo*, Sakakura demonstrated that not only embryonic but also adult mammary tissue could respond in this way to salivary mesenchymal signals. Importantly, the instructive properties of the stroma did not extend to cytodifferentiation: in a pregnant host animal, salivary-like mammary transplants synthesized the milk protein α-lactal-bumin. (Sakakura T, 1976)

The inductive role of stroma is evidence by the identification and elucidation of paracrine factors, which regulate the mammary parenchyma morphogenesis, e.g., Wnt proteins, transforming growth factor β (TGFβ), and hepatocyte growth factor. For example, TGF-β1 and TGF-β3 have roles in both promotion and inhibition of branching morphogenesis that are dependent on concentration and context. HGF/SF promotes ductal outgrowth and tubule formation in the mammary gland (Pollard JW. 2001). And Wnt-2, Wnt5a, and Wnt-6 are detected in stroma at a stage preceding ductal outgrowth (Webber-Hall et al. 1994) rasing the possibility that one or more Wnt family are candidates for mediating epithelial induction by the stroma. It's likely that additional paracreine factors will be identified as more attention is focused on role of the stroma. It is likely that further knowledge of stromal-epithelial interactions would greatly enhance the understanding of mammary gland function.

2.3.2 cell–matrix interactions with the host stroma

At the mammary epithelial and stromal cells boundary, there is a separating barrier of special ECM called basement membrane (BM). It contains various cellular elements such as laminin, fibrous and non-fibrous collagens, proteoglycans, and glycoproteins. The components of basement membrane, including LM-1, nidogen, heparan sulphate proteoglycan, and collagen IV, have been detected by immunohistochemistry around the ducts and alveoli of the virgin, pregnant, and lactating mouse mammary gland (Warburton et al., 1982, 1984; Sonnenberg et al., 1986; Beck et al., 1993; Keely et al., 1995). All of these components provide mechanical support to the tissue as well as forming a dynamic, developmentally active extracellular matrix/basal lamina complex.

An intact basement membrane is essential for the proper function, differentiation and survive of the mammary epithelial cells. For example, in the adult, evidence from culture studies show that the signals required for the induction of tissue-specific differentiation during pregnancy and maintenance of function during lactation arise primarily from basement membrane. Further support for a critical role for basement membrane in the functional differentiation of the gland comes from studies in involution where degradative loss of basement membranes correlates with loss of functional activity in the epithelium. Thus the extracellular matrix in conjunction with certain cytokines plays a central role in coordinating mammary epithelial development (Bissell MJ, 1993). Furthermore, it's already established from cell culture model that ECM has an essential function in the control of mammary epithelial cell differentiation and survive. Primary mammary epithelial cells that fail to contact the BM show various alterations, including changes in survival and functional differentiation, as measured by their inability to respond to lactogenic hormones (Barcellos-Hoff et al., 1989) During involution, besides of apoptosis of epithelial cells, an important additional feature is the proteolytical degradation of the basement membrane between stroma and epithelium. Ultrastructural studies have demonstrated BM (basement membrane) disorganization 3 days after weaning (Strange et al., 1992). In addition, the activity of BM degrading matrix metalloproteinase (MMP) enzymes, which are important for gland remodelling at later stages of involution, is up-regulated 3–4 days after weaning. (Lund et al., 1996).

There are two main ways in which the ECM can affect cell behaviour. One of these is through harbouring growth factors or growth factor-binding proteins. The other is that cell-ECM interactions can directly regulate cell behavior, either through receptor-mediated signalling or by modulating the cellular response to growth factors. For example ligation of integrins, the major class of ECM receptors, profoundly affects the cellular response to mitogenic signals, indicating that cell–matrix interactions are an important determinant in the regulation of cell cycle entry (MA Schwartz, 1999; A Howe, 1998). Furthermore, the signaling pathways specific to ECM integrin can alter interactions between steroid hormones and growth factors. For example, *In vitro* studies of the effects of ECM proteins in mammary epithelial monolayer cultures derived from adult mammary gland have been studied (Haslam SZ, 2001). Interactions between steroid hormones and growth factor and between two growth factors are influenced by ECM composition. Collagen type I (Col I) and fibronectin (FN) and, to a lesser extent, laminin (LM) promote an EGF+IGF-1 synergistic effect on proliferation. No synergistic, additive or inhibitory effects of progestin or estrogen with growth factors are observed on Col I or FN. However, on LM, progestin reduces the proliferative response to growth factors (Woodward TL, 2000).

2.3.3 Stromal-epithelial interactions during tumor formation

As we all known, the essential functional component of the mammary gland is the mammary epithelial cell. This cell is the one that synthesizes milk proteins and can occasionally become neoplastic. Since we already talked about the profound roles of both stromal and ECM in regulating the normal behavior of mammary epithelium, it would not be unreasonable to propose that a breakdown in stromal structure and/or cell interactions with extracellular matrix contributes to neoplasia.

It has been shown that certain physiological changes that take place in the mammary gland stroma during involution are similar to those that occur during malignancy. These include enzyme degradation of the ECM, loss of cell adhesion, and breakdown of the basement membrane and release of growth factors. Unlike normal cells, malignant cells appear to be resistant to the apoptosis which occurs during tissue remodeling and thus these conditions may facilitate tumour cell dissemination and invasion. And the ability of tumor cells to cross tissue boundaries may be a result of misregulation of the proteinases relative to their inhibitors. Proteinases that degrade the extracellular matrix (ECM), including the serine proteinases and matrix metalloproteinases (MMPs) have been implicated in various pathological states such as inflammation, rheumatoid arthritis, and all stages of tumor progression including growth, invasion, metastasis, and angiogenesis. Werb and colleagues engineered the luminal mammary epithelium of mice to overexpress stromelysin-1 (One of the metalloproteinase), which predictably disrupted normal stromal-epithelial interactions and perturbed tissue organization and differentiation. The experiments clearly showed that a desmoplastic stroma can drive malignant transformation of an epithelium (Werb.Z, 1996). Interestingly, in many carcinoma systems, most matrix metalloproteinases (MMPs) are largely expressed by the stromal cells, whereas the tumour cells are relatively silent in MMP expression. For example, to determine the tissue source of the most relevant MMPs, HBC cell lines and HBC tissues were xenografted into the mammary fat pad (MFP) or bone of immunocompromised mice and the expression of different MMPs were measured. The data directly demonstrate tumour induction of MMP production by stromal cells in both the MFP and bone environments and suggest that MMP-13 and MT1-MMP will be relevant targets for inhibiting breast cancer progression (Lafleur MA, 2004). To test the tissue organization field theory directly, recently, Maffini and co-workers used an acute chemical carcinogen (NMU) treatment to rapidly induce tumor formation, and a mammary gland epithelial reconstitution approach to distinguish between the contribution of stromal-epithelial interactions and genetic mutations to malignancy (Maffini et al., 2004). Interestingly, none of the animals that received MECs treated with

NMU in culture developed neoplastic lesions, unless their stromal fat pad had also received a prior bolus of NMU. It's a further data to suggest that the stroma might itself constitute an important mutagenic target. The paracrine factors have been widely implicated not only in the normal mammary parenchyma morphogenesis but also in the mammary carcinogenesis. Recently, mammary fibroblasts engineered to ectopically express HGF or TGFβ1 alone or together induced mammary epithelial to develop ductal carcinoma in situ, adenocacinoma, and poorly differentiated cancer, whereas transplantation of the same epitheial cell population with wild-type fibroblasts did not (Kuperwasser, C. et al , 2004). Using the conditional inactivation of the TGFβ type II receptor gene in fibroblast, Moses and colleagues have shown that TGF-β signaling in fibroblasts modulates the growth and oncogenesis of adjacent epithelia (Bhowmick NA).

2.4 Mammary stem cells

It is evident that the mammary gland possesses a strong regeneration capacity bypass the subsequent reproductive and lactation cycles. A logical question is then which cells offer this regeneration capacity and which cells will survive and which cells will be replaced during the dry period?

The first question may be explained by the presence of a long-lived population of stem cells in mammary gland that have a near-infinite propensity to produce functional cells. Stem cells are undifferentiated, division-competent cells that reside in a given tissue, and that function to regenerate and/or replace all of the cell types that compose that tissue. The presence of pluripotent stem cells and cell-line committed progenitors in the normal mammary gland has been described by several authors in different species such as the mouse, rat, human and cow.

2.4.1.One cell, one mammary gland

Kordon and Smith suggested the existence of a population of self-renewing and pluripotent stem cells in the mammary gland of mice. Using viral integration to mark and follow individual clones, they reported that a fully differentiated mammary gland could be derived from a single cell clone. They took small fragments of mammary epithelium from CzechII MMTV-infected mice and transplanted them into cleared mammary fat pads of syngeneic hosts. These hosts were mated and 1 day after parturition about 80% of each of the reconstituted glands was removed for analysis, leaving the remainder intact for subsequent serial transplantation. As expected, most of the transplanted epithelial fragments expanded

during pregnancy to become complete and functional mammary glands. If these glands had been derived from several different progenitors, no clear pattern of MMTV-insertional events would have been detected. If, on the other hand, the outgrowths were clonal a distinct and easily detected pattern of MMTV insertion sites would have been seen, as was the case in 20 of the 30 different outgrowths examined by Southern analysis. As a control, the intact contralateral glands of the host mice were analyzed in the same way and, as would be expected for a polyclonal tissue derived from several progenitors, no clear pattern of insertional events could be seen. The inescapable conclusion of this first part of the work of Kordon and Smith was that most of the reconstituted mammary glands were clonal. Fragments of these clonal glands were then transplanted into new hosts where they were shown to retain the same pattern of MMTV insertion sites, providing evidence of self-renewal of the original stem cell. Additional MMTV insertion sites were often detected, however, suggesting the acquisition of more mutations during clonal expansion. (Kordon & Smith 1998).

2.4.2 Two lineage-specific progenitors come from one stem cell

By transgenic mouse models, Kordon and Smith also demonstrated that regeneration of the mammary secretory lobules can be affected separately from the growth and development of the mammary epithelial ducts (Kordon & Smith 1995). This suggests that mammary epithelial stem cells capable of producing two functionally distinct progenitor cells in the mammary gland epithelium: one capable of producing daughters committed to ductal formation, the other capable only of producing daughters committed to lobular function.

Two distinct epithelial cell progenitors have been identified by experiments designed to determine whether basal lobular and ductal phenotypes could develop independently under conditions imposed by a limiting dilution (Kordon & Smith, 1998). As these precursor cells are limited in their proliferation capacity, they need themselves to be renewed by cells originating from the pluripotent stem cell population. It can therefore be postulated that candidate cells for renewal are the precursor cells that are responsible for expanding and maintaining the number of mammary epithelial cells in subsequent lactation.

It was found that lobular i.e. secretory progenitor cells are present as distinct entities among the mammary epithelial cells of immature virgin female mice. Similarly, ductal epithelial progenitors are present within the same population, and both cell populations are extremely small (Kordon and Smith, 1995).

Additionally, using cre-loxp system, Wagner et al. (2002) demonstrated that a newly matured epithelial cell population which does not undergo cell death during involution or remodeling after lactation and function as alveolar progenitors. In this study, WAP-Cre transgene mice were crossed with reporter (Rosa-LacZ) mice to monitor the differentiation process of alveolar precursor cells in response to lactogenic hormones. It was also presented the genetic evidence that despite the close morphological resemblance, an involuted mammary gland is fundamentally different from a virgin gland, due to the lack of the parity-induced population in the latter.

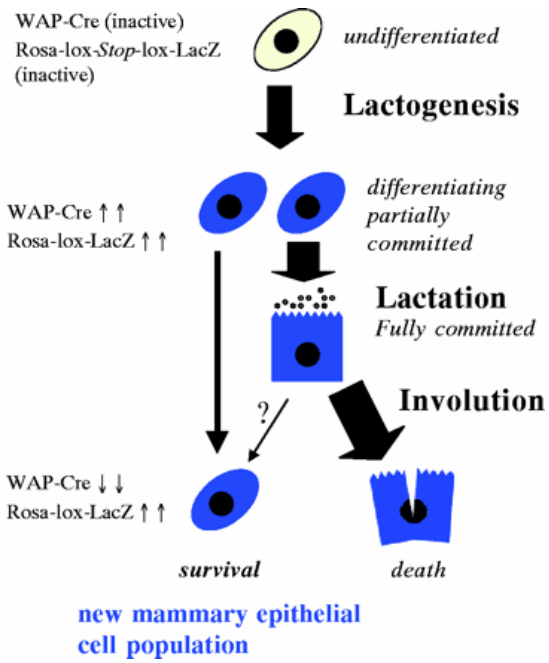


Fig.10. The basic principle of the genetic labeling of differentiating cells in the developing mammary gland using the Cre-lox technique. In this experimental setting, the transient upregulation of Cre recombinase in differentiating epithelial cells during pregnancy permanently activates a ubiquitously expressed reporter transgene (Rosa-*lacZ*), whose expression is not dependent on the differentiation status of a given cell. The reporter gene remains active in cells that no longer require high systemic hormone levels to maintain a functionally differentiated state (i.e. WAP gene expression). Hence, the permanent activation of the reporter gene (blue X-Gal staining) genetically labels differentiating cells that bypass apoptosis and remodeling at the conclusion of the reproductive cycle. The labeled cells in the remodeled (involuted) gland represent a new epithelial subtype, which is not present in nulliparous animals (Wagner et al. 2002).

2.4.3. Markers and morphology

Mammary epithelial cells of a distinctive morphology can be found in a position intermediate between the basal and luminal cells. These intermediate cells are distinguished by their pale-staining cytoplasm under both light and electron microscopy (Chepko & Smith 1997). They have few cellular organelles, pale nuclei and are found as a small light cells (SLC) and as an undifferentiated large light cells (ULLC). In rodents the results of *in vitro* and *in vivo* experiments suggest that the pale-staining or light cells situated

between the luminal and the myo-epithelial cell layers are the most likely candidates for a stem-cell population (Chepko & Smith 1997).

The isolation of stem cells from the mammary gland in human and rodents has been hindered by the lack of identified specific cell surface markers. In order to characterize putative epithelial stem cells further, a method successfully used for stem cell isolation in other tissues were used to isolate a side population (SP) from mouse mammary gland. The technique consists of staining isolated cells with Hoechst 33342 followed by flow cytometric analysis to sort a 'side population' (SP) of cells that efflux the fluorescent dye (Goodell et al. 1997). This shows that mouse mammary SP cells are enriched for expression of three putative stem-cell markers; Sca-1, α6-integrin and telomerase (Alvi et al. 2003; Welm et al. 2002). The percentage of mouse mammary SP cells was estimated to be 2–3% of all epithelial cells in one study (Welm et al. 2002) and 0.5% of total cells in the other (Alvi et al. 2003). Alvi et al. have further reported that nearly half of the SP cells were steroid receptor-positive and have produced preliminary data suggesting the presence of a similar SP population in human breast epithelium (Alvi et al. 2003).

2.4.4. Experimental approach for mammary stem cell studies

An in vivo transplantation system was used to evaluate the developmental capacities of specific mouse mammary epithelial cell populations. Mammary cleared fat pad and transplantation technique is to place a small piece of mammary tissue or mammary epithelial cells back into a fat pad that is devoid of endogenous epithelium, the injected material is capable of producing an entire new mammary epithelial tree. This approach relies on the remarkable organizational ability of both the mammary epithelium and stroma. It is an important method not only on normal mammary gland development, but also on the mammary stem cell exploration.

One example of the power of the transplantation method using transgenic animals is the experiment of Smith who investigated the developmental potentials of mouse mammary epithelial cells in WAP-LacZ mice and TGFB1 mice (Kordon EC, et al 1995). By tranplantation, the results demonstrate the phenotype is a property of the transgenic epithelium and is not due to extracellular effects mediated within the transgenic environment and suggest that distinct progenitor populations exist for alveolar cells and for ductal cells as well as for multipotent cells. Additionally, increased expression of TGFB1 preferentially affected alveolar-progenitor stem cells. Furthermore, The use of quantitative transplantation analysis

along with specific markers for stem cells would provide a more comprehensive understanding of the developmental capabilities of the mammary parenchyma at different stages as well as the effects of specific agents (i.e. TGFβ1, oncogenes) in these processes. The genetically engineered mice coupled with fluoresence-activated cell sorting analysis and transplantation into the cleared mammary fat pad is set as a model system to isolate and characterize functional mammary progenitors and stem cells

In addition, the cleared fat pad allows serial transplantation of cell populations. Although established cell lines maintained in cell culture by serial passage have provided a useful means to study the effects of drugs and biological molecules on cell function and growth, this approach is often limited by the absence of tissue interactions. In contrast, the ability to serially transplant mammary cells into the mammary fat pad allows the establishment of stable and immortalized lines of non-neoplastic mammary cells analogous to the established cell lines in vitro.

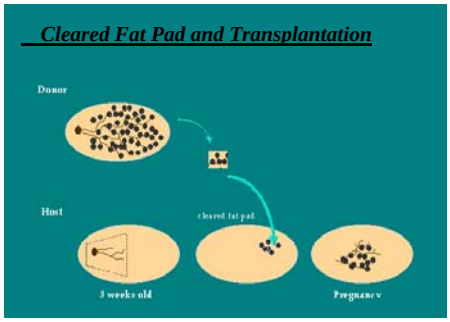


Fig.11. Cleared Fat Pad technique

2.4.5. Cancer prevention and treatment

Stem cells are important not only because of the potential that they offer for organ regeneration or replacment, but also because of the risk they pose in carcinogenesis. As far as cancer is concerned, there is indirect evidence that stem cells persist in the mammary gland throughout life, this longevity makes them susceptible to accumulating genetic damage during self-renewal, where they must be regarded as prime targets for oncogenic transformation. Thus, One implication of the 'multihit theory' of carcinogenesis is that cancer is a stem-cell disease. If this is the case, then breast-cancer prevention strategies must be targeted to mammary epithelial stem cells. Indeed, the presence of stem cells in the

mammary gland and susceptibility to carcinogens appear to be related. The greatest concentration of stem cells is in terminal end bud and alveolar bud structures during pubertal development in rodents, and it is during this period that the gland is most sensitive to carcinogens (Russo & Russo 1978a; Russo & Russo 1978b). Similar structures exist in the breasts of prepubertal and adolescent women, and it is this age group that suffered the highest rates of breast cancer after irradiation due to the atomic detonations in Japan in 1945 (McGregor et al. 1977).

In addition, little is known about whether tumours contain stem cells equivalent to normal tissues. Most current chemotherapeutic and endocrine agents induce apoptosis, but whether mammary stem cells are susceptible to programmed cell death is not known. Studies on the mouse small intestine suggest that there may be two populations of stem cells. One of these undergoes spontaneous apoptosis as part of the homeostatic mechanism restricting the number of stem cells present at any one time (Potten CS 1997). The other, smaller, population is resistant to radiation-induced apoptosis, undergoes DNA repair and, presumably, is responsible for repopulation of the damaged intestinal epithelium. If this is the case, then therapies aimed at killing proliferating cells may have little impact on this small number of tumour stem cells that remain quiescent, but which may cause tumour repopulation following treatment. Stem cells may also be responsible for local or distant recurrence that may occur several years after initial treatment of the primary tumour. It is therefore imperative that we identify tissue-specific stem cells and study their regulation in order to generate new targets for therapy that can prevent tumour stem cells from seeding tumour regrowth. In mammary gland field, the model of transplanted mouse mammary gland refined by Kordon and Smith should allow us to determine whether mammary stem cells exhibit similar properties and to develop the means to overcome resistance to apoptosis.

Part III Integrins in the normal development and carcinogenesis of mouse mammary gland

In part II, we mentioned that the mammary epithelium consists of two layers: a luminal layer of secretory cells and a basal layer of myoepithelial cells. Integrins exist in both of the layers. Immunohistochemical studies with human and mouse mammary gland tissue revealed the presence of α2, α3, α6, β1, and β4 subunits in the luminal and myoepithelial cells and of α1, α5, and αv chains exclusively in the myoepithelial cells. The α5 and αv subunits seem to be expressed at lower levels than other integrin chains, being restricted to certain developmental stages and regions of the mammary tree. As expected, most integrins are present at sites of cell-ECM interaction, on the myoepithelial cells and on the basal surface of the alveolar luminal cells. The α2, α3, α6, and β1 integrin chains have also been found on the lateral surfaces of the luminal cells at sites of cell–cell interaction where ECM proteins are not detected in normal mammary epithelium. The activation status, potential ligands (if any), and functions of the integrins present on lateral cell surfaces are not known. These integrins may remain inactive or serve for homo- or heterotypic cell–cell interactions as it has been suggested to do in other cell types (Symington BE et al. 1993).

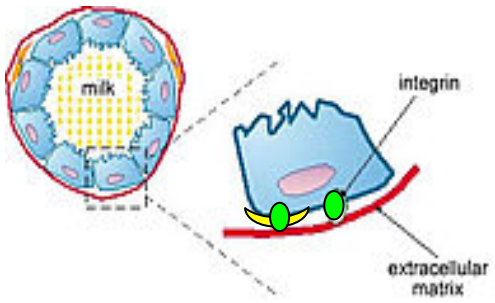


Fig.12. A schematic section through part of the mammary gland: the lactating alveolus) epithelial cells (blue) attach to the extracellular protein matrix (red) through a series of integrin proteins (green).

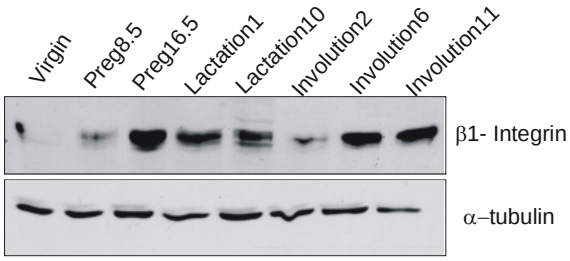
Integrins have been identified as important regulators of mammary epithelial cell growth and differentiation. Their ability to promote cell anchorage, proliferation, survival, migration, and the induction of active ECM-degrading enzymes suggests that they play an essential role in normal mammary morphogenesis, but, on the other hand, reveals their potential to promote tumor progression.

3.1 Integrins play important roles in mammary gland development

Although data concerning the regulation of integrin expression during mammary gland development are rather fragmentary, there is enough experimental evidence to conclude that they are controlled transcriptionally and post-transcriptionally. Northern and western blotting performed with isolated rat mammary epithelial cells has shown that expression of the β1 and β4 chains increases during pregnancy. During lactation, β1 is further upregulated, and its expression level reaches a peak, whereas β4 level drops. (Huang R.Y. & Ip M.M, 2001).

We observed similar changes of β1 integrin expression in the mouse stain C57Bl/6 (Fig.13). The upregulation of integrin β1 during late pregnancy and lactation suggests that integrins have important roles during these stages.

Fig.13. Expression pattern of β1-Integrin during murine mammary gland development (BL/6)



3.1.1. Control of mammary epithelial cell proliferation and survival

In normal adherent cells, activation of MAPKs and induction of proliferation in response to growth factor stimulation occurs only if cells interact with ECM. Moreover, integrin-mediated adhesion to ECM substrates is essential for cell survival (described more in Part I). Using a human breast cancer model, Weaver et al. could show that treatment of tumor cells in a 3-dimensional culture with inhibitory β1-integrin antibody or its Fab fragments led to a striking morphological and functional reversion to a normal phenotype. The newly-formed reverted acini re-assembled a basement membrane and re-established E-cadherin-catenin complexes, and re-organized their cytoskeleton. At the same time they downregulated cyclin D1, upregulated p21cip1 and stopped growing (Weaver T.M. et al. 1997). Moreover, reciprocal interactions between EGF receptor and integrin signaling pathways in the control of proliferation have been demonstrated by Wang et al. (Wang. F et al. 1998;Wang. F, et al. 2002). Inhibiting either EGF receptor or β1 integrin, in tumor cells growing in three-dimensional cultures in Matrigel, results in growth

arrest and restoration of normal morphogenesis. Similar results were obtained by inhibiting MAPKs or PI3K, the activation of which is triggered by integrins and growth factor receptors. Importantly, cell–ECM interactions can regulate expression of the EGF receptor, as cells treated with function-blocking anti-β1 integrin antibody have decreased EGF receptor levels (Reginato M.j. et al. 2003).

In the absence of a survival signal that is induced and maintained by integrins, normal, non-transformed mammary epithelial cells undergo apoptosis, termed in this case anoikis. Gilmore et al. showed that this process depends on the translocation of Bax, a proapoptotic protein of the Bcl2 family, from the cytosol to the mitochondria (Gilmore A.P et al. 2000). Adhesion signals mediated by focal adhesion kinase (FAK) prevent the activation of Bax and its translocation to the mitochondria. Bax is activated extremely rapidly after cell detachment (Wang.P et al. 2003), whereas subsequent events in the apoptosis program, such as cytochrome c release and caspase activation, occur after a considerable delay. Bim is another pro-apoptotic protein induced in mammary epithelial cells following the loss of integrin-mediated adhesion to ECM (Reginato M.j. et al. 2003). Bim appears to be a powerful cell death inducer in mammary epithelial cells, as the downregulation of its expression by the RNAi technique inhibits anoikis. The engagement of β1 integrin, activation of the EGF receptor, and activation of the ERK MAPKs are required to block Bim expression in epithelial cells, clearly demonstrating that integrins trigger survival signals in concert with growth factor receptors. Stable overexpression of the EGF-receptor blocks cell detachment-induced Bim expression, leads to the activation of ERK in detached cells, and prevents apoptosis. Thus, overexpression of the EGF-receptor may enable cells to escape anoikis; lead to a loss of anchorage dependence (Reginato M.j. et al. 2003) and this could be involved in the cancer development.

Phosphatidyl-inositol-3-kinase (PI3K) and the serine/threonine kinase Akt are required for transmission of the cell survival signal triggered by integrin mediated adhesion. PI3K is a downstream effector of insulin receptor signaling and is activated upon treatment of cells with insulin or insulin-like growth factors. After several days of primary culture, mammary epithelial cells grown on collagen I undergo apoptosis, whereas those cultured on Matrigel in the presence of insulin or insulin-like growth factors (but not prolactin or hydrocortisone) do not (Farrelly.N et.al. 1999). The α6 and β1 integrin subunits have been found to be required for mammary epithelial cell survival under these conditions. The phosphorylation of insulin receptor substrate-1, its binding to PI3K, and the phosphorylation of Akt are all enhanced in cultures on Matrigel, suggesting that cell–ECM interactions and PI3K pathway signaling via the insulin receptor control survival/apoptosis in mammary epithelial cells (Farrelly.N et.al. 1999; Lee Y.J and C. H. Streuli, 1999).

The first evidence that β1 integrins are involved in the control of cell survival and proliferation during mammary gland development in vivo came from transgenic mice expressing a chimeric molecule consisting of the cytoplasmic and transmembrane domains of β1 integrin fused to the extracellular domain of CD4 (MMTV-β1-cyto) in mammary epithelial cells. This chimeric molecule does not bind ECM ligands and in cultured cells, acts as a dominant negative mutant of β1 integrin. The mammary glands of MMTV-β1-cyto females displayed low epithelial cell proliferation and high apoptosis rates during the intensive growth phase of mid-pregnancy and early lactation, accompanied by a lack of activation of the MAPKs, ERK, and JNK (Faraldo MM et al. 1998&2001). PI3K activation upon adhesion leads to the phosphorylation of Akt, which in turn phosphorylates the pro-apoptotic protein Bad. These phosphorylation events are essential for cell survival. In the mammary glands of MMTV-β1-cyto mice, Akt and Bad were all found to be less phosphorylated than in wild-type animals, revealing a deficiency in activation of the PI3K pathway accompanying the perturbation of β1 integrin function (Faraldo MM et al. 2001). Important evidence supporting the involvement of β1 integrins in the control of mammary epithelial cell survival in vivo was obtained by Prince et al. (Prince et al. 2002). This study demonstrated that although β1 integrin was present in mammary epithelial cells at all developmental stages, the amount of ligand-associated β1 integrin detected with a conformation-sensitive antibody decreased markedly at the onset of involution prior to the peak of apoptosis. Finally, an interesting finding suggesting that integrins may transmit pro-apoptotic signals was reported by Seewaldt et al. (Seewaldt VL et al. 2001). Human mammary epithelial cells expressing p53 undergo growth arrest and form acinus-like structures after several days of culture in Matrigel, whereas cells with suppressed p53 function, under the same experimental conditions, undergo apoptosis. Cell death can be prevented by incubation with anti-β1 or anti-β3 function blocking antibodies, suggesting that α3β1 integrin may be involved in the induction of apoptosis in the cells lacking active p53 (Bachelder RE et al. 1999).

3.1.2.Control of mammary epithelial cell differentiation

Pioneering studies indicated that the cellular microenvironment is critical for mammary epithelial differentiation. For example, primary mammary epithelial cells can maintain the glucose metabolic patterns of lactating gland and secret milk proteins in response to the lactogenic hormones when the cells are cultured on a collagen I gel which is floated into the medium. However, these cells lose their differentiation when they were cultured in conventional tissue culture conditions, even in the presence of

the lactogenic hormones (Lee, E.Y.H et al. 1984). These experiments provided some of the first evidence that tissue microenvironment and cell shape are prerequisites for tissue specific gene expression. Interestingly, the induction of differentiation in cells cultured on floating collagen gels correlated with deposition of an endogenously synthesized basement membrane. Direct evidence of a role for basement membrane in differentiation came from the observation that single, isolated mammary cells could be induced to express β-casein in the presence of basement membrane proteins but not on the collagen I (streuli C.H et al. 1991).

Integrins are major receptors for extracellular matrix proteins, including basement membrane components. Studies with functional blocking integrin antibodies have been used to demonstrate their requirement for the mammary epithelial differentiation. Anti-β1 integrin antibody inhibited the ability of mammary cells embedded within basement membrane to express the milk protein β-casein, wherera other blocking antibodies such as one for E-cadherin did not block the differentiation (Streuli C.H et al. 1991). Thus, integrins act as basement membrane receptors to trigger a signalling pathway leading to milk protein expression.

Basement membrane signals could regulate mammary differentiation at the transcription level. A transcription factor, STAT5 (signal transducer and activator of transcription 5), has been shown to play a critical role in mammary gland development and in the control of milk gene expression (More details in Part II). Experiments carried out with mammary epithelial cells in culture have shown that the activation of STAT5 via the prolactin receptor pathway requires β1 integrin-mediated interaction with laminin, the major component of Matrigel. Prolactin does not induce phosphorylation of its receptor, JAK2 or STAT5, if mammary epithelial cells are cultured on interstitial collagen I (Streuli C.H et al. 1995). Moreover, both β1 and β4 integrin signals are required for β-casein gene expression by mammary cells cultured on Matrigel, suggesting that these integrins act in concert in the control of mammary epithelial cell differentiation (Muschler J, et al. 1999).

The perturbation of β1 integrin function in vivo (MMTV-β1-cyto mouse model) results in the impaired differentiation of secretory epithelial cells at the beginning of lactation and precocious dedifferentiation during involution, as estimated by levels of β-casein and WAP gene transcripts (Faraldo MM et al 1998). The precocious dedifferentiation of the secretory epithelial cells in these transgenic mice is caused by a lack of activated STAT5 in the nuclei. However, STAT5 and JAK2 phosphorylation levels are unaffected

in the mammary glands of these transgenic mice. These findings suggest that β1-integrin signaling may have an impact on STAT5 activity, not only by controlling STAT5 phosphorylation, but also by affecting the translocation of STAT5 into the nucleus. The perturbation of β1 integrin function during involution also leads to the precocious activation of NF-KB, which can compete with STAT5 for transcriptional coactivators and may therefore inhibit the STAT5-dependent transcription of milk protein genes (Faraldo MM et al 2002).

Overall, these studies convincingly demonstrate that basement membrane is essential for the development and differentiation of mammary epithelium. The integrin receptors, including β1 integrins and α6β4, are involved in the control of mammary epithelial cell terminal differentiation.

3.2 Integrins and breast carcinogenesis

3.2.1. Integrin expression and function in breast carcinogenesis

In normal breast, intense staining of many integrin subunits is seen concentrated at the basement membrane in the myoepithelial layer. In invasive carcinomas, this cell layer is most often absent and the expression of integrin subunits on the surface of carcinoma cells is diffuse. This led to the erroneous assumption that there is an overall decrease, or absence, of integrin expression in breast carcinoma. Although the expression of some integrin subunits is decreased, it is clear from many in vitro and in vivo studies that integrin receptors are expressed in breast adenocarcinomas and that they contribute significantly to the pathobiology of breast cancer.

Integrins have been implicated in several aspects of tumor progression, including tumor cell survival, induction and activation of matrix-degrading enzymes, migration, and anchorage at the sites of metastasis. In this respect, α6β1 and α6 β4, which act as laminin receptors, are the best-studied integrins. They promote the survival and migration of breast carcinoma cells. α6 expression is strongest in the myoepithelial cells and the staining is predominant at the basal surface in contact with the basement membrane. High levels of α6 integrin chains in human mammary carcinoma are clearly correlated with low patient survival rates (Friedrichs K et al. 1995). In vitro, in highly metastatic human mammary carcinoma cell lines, the inhibition of α6β1 integrin dimer formation abolishes tumor growth and metastasis. High levels of apoptosis are observed in the tumors formed by cells lacking the α6 β1 dimer. These observations provide evidence that the α6 β1 integrin promotes cell survival in mammary tumors (Wewer UM et al. 1997). The α6 β4 integrin, in addition to promoting tumor cell survival, also increases

the motility of tumor cells. Carcinoma cell lines often express high levels of α6 β4 integrin but do not form hemidesmosomes, which anchor the cells and hinder migration. In this case, α6 β4 is associated with actin microfilaments in the invasive membrane protrusions and serves to enhance motility. The α6β4 integrin is also involved in the activation of PI3K, the small GTP-ases Rho and Rac, and protein kinase A, which are all essential for carcinoma migration and invasion (Mercurio AM et al. 2001; O'Connor & Mercurio, 2001). α6β4 mediated compression enables carcinoma cells to remodel the basement membrane by a process involving the packing of basement membrane material under the cells and the mechanical removal of this material from adjacent areas (Rabinovitz I et al. 2001).

In the mammary tumorigenesis studies, α2β1 is a special in comparison with the other integrins we discussed above. The α2β1 integrin is a dual-specificity receptor that recognizes collagens I and IV and members of the laminin family. The α2 subunit is expressed basally as well as laterally in luminal epithelial cells. The expression of α2β1 is maintained in benign breast lesions such as fibrocystic disease or fibroadenomas (Zutter MM et al. 1990). However, the expression level of α2β1 decreases with the differentiation status of breast adenocarcinomas. Specifically, poorly differentiated adenocarcinomas express very low, or undetectable levels of α2β1 while moderately differentiated adenocarcinomas express intermediate levels (Zutter MM et al. 1990&1993). These correlative studies suggested that the α2β1 integrin is important for maintaining the differentiation and controlling proliferation of the breast epithelium and that its loss is essential for the progression to invasive carcinoma.

3.2.2 Integrin linked kinases

Two important enzymes that associate with focal adhesions are focal adhesion kinase (FAK) and integrin-linked kinase (ILK). Because of their ability to bind many adaptor proteins and other signaling enzymes, FAK and ILK are viewed as critical components that integrate cell–ECM interactions with cell phenotype. Besides these, they were found to be involved in the progression of breast tumor.

FAK is over-expressed in a number of human tumors, such as colon and breast carcinomas, sarcomas, ovarian carcinomas etc. A correlation between the expression level of FAK and the invasive potential of the cancer has been described in most of these studies. Possible mechanisms that lead to elevated FAK expression might involve increased dosage and amplification of the FAK gene, as observed in cell lines derived from human tumors of lung, breast and colon, or its constitutive activation, as described in malignant melanoma cells (Kahana O et al. 2002). Cancer studies show that FAK might play a role in

suppressing apoptosis. For example, down regulation of FAK with antisense oligonucleotides results in apoptosis of tumor cells in culture, as does the inhibition of its function by expression of either FRNK or the N-terminal domain (Beviglia L et al. 2003). Furthermore, breast tumor cell lines that are viable without ECM attachment also undergo apoptosis upon interruption of FAK function, demonstrating that FAK is a survival signal even in the absence of matrix signaling (Xu LH et al. 2000).
An important function of ILK is structural and, together with its associated proteins, this kinase serves to provide an essential component linking the cytoskeleton with integrin attachment sites. At the time of its original identification, ILK was proposed to be involved with neoplastic progression because ectopic expression of human ILK disrupted cell adhesion to ECM and induced transformation of intestinal epithelial cells, as judged by anchorage independent growth (Hannigan GE et al. 1996).

ILK may also be involved with neoplastic progression in the mammary gland, although no studies have yet been carried out to examine whether its expression or activity is altered in human breast cancer. In a transgenic mouse model, human ILK expressed under the control of the MMTV promoter induced epithelial hyperplasia within 6 months, and focal mammary tumors (with a range of different morphological phenotypes) appeared in 34% of animals at an average age of 18 months (White DE et al. 2001). The proliferative response in the epithelium of transgenic glands was accompanied by constitutive phosphorylation of PKB, GSK3β, and MAPK. Although it is not clear whether this phosphorylation is directly responsible for increased proliferation, other studies in MCF7 cells have indicated that ILK can increase the activity of the cyclin D1 promoter (D'Amico M et al. 2000).

3.2.3. Integrin cooperativity with growth factors

An emerging area in the study of integrin contributions to cancer is the cross-talk between these adhesion receptors and soluble growth factor and cytokine receptors. As mentioned in part II, in the normal breast integrin receptors cooperate with hormones and growth factors to promote mammary epithelial differentiation and function. An increasing number of studies indicate that integrins also cooperate with soluble factors to promote carcinoma progression.
Integrins can cooperate with growth factor receptors to enhance their signaling capabilities. For example, α6β1 and α6β4 integrins can associate with the ErbB2 protein and increase its signaling functions. The NIH3T3 cells, which have over expressed both α6β4 integrin and erbB2 receptors, but not those overexpressing a crippled ErbB-2, showed enhanced proliferation rates and invasiveness (Falcioni R. et

al. 1997). Another mechanism of integrin-growth factor receptor cooperation involves integrin activation by growth factors and cytokines. A well-studied example is the activation of the αvβ5 integrin by the insulin-like growth factor receptor (Brooks PC et al, 1997). Breast carcinoma cells that express αvβ5 adhere to but do not migrate on vitronectin *in vitro* unless they are stimulated with IGF-1 or insulin. In vivo, these cells form tumors in the absence of IGF-1, however, they only metastasize when stimulated with IGF-1 or insulin (Brooks PC et al, 1997). These data suggest that the cooperation between αvβ5 and the IGF-1 receptor may regulate migration and invasion to promote the metastatic spread of tumor cells.

3.2.4. In vitro and in vivo models

Since many integrins have the potential to promote tumor progression, antagonists of integrins, such as blocking antibodies or peptides, have been used to inhibitor the function of integrins in tumor cells. For example, vascular integrins, in particular αvβ3, are important regulators of angiogenesis, including tumor angiogenesis. It has been shown that integrin αvβ3 antagonists could suppress tumor angiogenesis and tumor progression in many preclinical tumor models. In some breast cancer cell lines, functional integrin β1 specific antibodies with the capacity to override the oncogenic events and yield tumor revertant cells have been found. For example, in a developed human breast cancer model (HMT-3522 human breast cancer cell line), treatment of tumor cells in a 3-dimensional culture with inhibitory β1-integrin antibody or its Fab fragments led to a striking morphological and functional reversion to a normal phenotype (Weaver V.M. et al. 1997). The newly formed reverted acini re-assembled a basement membrane and re-established E-cadherin-catenin complexes, and re-organized their cytoskeletons. At the same time they downregulated Cyclin D1, upregulated p21cip1, and stopped growing. Tumor cells treated with the same antibody and injected into nude mice had significantly reduced number and size of tumors in nude mice. The tissue distribution of other integrins was also normalized, suggesting the existence of intimate interactions between the different integrin pathways as well as adherens junctions. On the other hand, nonmalignant cells when treated with either α6 or β4 function altering antibodies continued to grow, and had disorganized colony morphologies resembling the untreated tumor colonies. This shows a significant role of the α6/β4 heterodimer in directing polarity and tissue structure. The observed phenotypes were reversible when the cells were disassociated and the antibodies removed (Weaver V.M. et al. 1997).

A recent paper by White et al. shows that β1 integrin is required for mammary tumor growth in vivo. Using cre-loxp recombination system to disrupt β1-integrin function in a transgenic mouse model of

breast cancer, they could show that β1-integrin expression is critical for the initiation of mammary tumorigenesis (White, DE et al, 2004). Although ablation of β1 integrin expression from cells of the mouse mammary epithelium did not impair mammary gland development during puberty, deletion of the conditional β1-integrin allele dramatically impaired mammary tumorigenesis in the MMTV/PyV MT (polyomavirus middle T oncogene) mice. In addition, the deletion of β1 integrin in cultured mammary tumor cells was found to be associated with a decrease in the phosphorylation of FAK tyrosine residues, including the c-Src binding site. The deletion of β1 integrin from these PyV MT-induced tumor cells also inhibited the capacity of these cells to proliferate and form tumors in vivo. Taken together, all these observations suggest that β1 integrin plays a critical role in both the initiation and maintenance of mammary tumor growth in vivo (White, DE et al, 2004).

Part IV: Summary and open questions

To summarize, integrins play essential roles in the development of the mammary gland. The integrin β1, one of the main integrin subunit in the mammary gland, plays important roles in mammary epithelium proliferation, differentiation, and apoptosis. Besides their functions in the normal mammary gland development, they also have potentials to promote mammary tumor progression.

Although different approaches to interfering β1 integrin expression have been applied to investigate the role of integrin β1 in the mammary gland development and mammary tumorigenesis, some limitations of these approaches have left many questions open for clarification. For example, what is the role of integrin β1 in the specific mammary cell type, or the specific time and window in the mammary gland development? What is the molecular mechanism which blocks tumor cell proliferation after β1 integrin deletion?

As mentioned, Faraldo used a dominant negative transgene (CD4-β1 integrin) to interfere with β1 integrin function in the gland (Faraldo MM et al. 1998). However, dominant negative approaches have some limitations. Furthermore, integrins are unique molecules that transduce signals in two directions. Thus, the portion of β1 integrin remaining in the dominant negative transgene namely the transmembrane and intracellular domain still has the potential to transmit signals. In addition, it is known that depending on expression level, CD4-β1 integrin can act either in a dominant negative or a dominant active manner. Thus, to understand the role of integrin β1 more precisely in the mammary gland development and clarify the molecular mechanism, a genetic approach is very appropriate.

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Aims of the Ph.D. Project

(1) **To determine the role of β1 integrin in normal development of the mouse mammary gland, with a particular emphasis on how β1 integrins influcence proliferation, differentiation and apoptosis.**

By using conditional deletion strategy, this was explored in a specific time and window *in vivo*.

(2) To investigate the molecular mechanisms for the phenotypes we observed *in vivo*.

The uncovering of such mechanisms will explain how β1 integrin affect normal development; this could provide clues for the breast cancer investigation.

(3) Based upon results obtained in vivo, to establish an *in vitro* system that mimics the effects of deletion of the integrin β1 in the mouse.

The aim is to gain experimental flexibility by using an *in vitro* model that can be more easily manipulated than the current mouse model and allow us to further investigate the molecular mechanisms underlying the *in vivo* phenotypes.

Results:

Conditional deletion of β1 integrin in the mammary gland

To inactivate the *Itgβ1* gene in the mammary gland, we crossed $Itg\beta1^{flox/flox}$ mice (Graus-Porta et al., 2001) with WAPiCre transgenic mice (Wintermantel et al., 2002). WAPiCre is specifically expressed in the luminal epithelial cells of the gland starting at midpregnancy and reaches a maximum at day 3 of lactation (Wintermantel et al., 2002)(Fig 2A panel d). WAPiCre is not expressed in the ductal tree of the virgin gland, nor is it expressed in the basal myoepithelial cells of the mammary gland (Wintermantel et al., 2002)(N. Li, unpublished observations and Fig 2A panels b & d). Littermates with the genotype $Itg\beta1^{flox/flox}$; $WAPiCre^{-/-}$ (referred to as control mice) or $Itg\beta1^{flox/flox}$; $WAPiCre^{+/-}$ (referred to as β1-integrin mutant mice) were used for all analyses.

Mammary glands from 3-day lactating females were used to examine Cre-mediated recombination. A PCR analysis carried out on DNA from mammary tissue, revealed the expected 1.3 kB product resulting from the recombined *Itgβ1* allele in mutant mice (Fig 1B). The unrecombined 2.1 kB floxed allele was detected in control mice and in mutant mice due to the presence of myoepithelial and stroma-derived cells (Fig 1B). Western analyses for β1 integrin protein revealed a strong decrease in expression in the recombined mutant glands, compared to control glands (Fig 1C), corroborating the PCR results. β1 integrin is not totally absent since expression remains in the myoepithelial cell layer.

Immunohistochemical analyses carried out on glands from control 3-day lactating females revealed the expected localization of β1 integrin to the basal layer of the myoepithelial cells, and to the basal-lateral layer of the luminal cells (Fig.2B, panels e & g; white arrow indicates lateral staining) (Prince et al., 2002; Taddei et al., 2003). In lactating glands from β1 integrin mutant females, the majority of luminal cells were negative for β1 integrin immunostaining (Fig. 2B, panels f & h), which was most obvious on the lateral surface of mutant cells (white arrow, panel g vs. yellow arrow, panel h). The remaining β1 integrin immunostaining is from the myoepithelial cells. These results demonstrate the development-specific ablation of β1 integrin in the luminal cells of the mammary alveoli.

To examine directly the functional consequences of β1 integrin ablation on cell adhesion, we performed adhesion assays using primary mammary epithelial cells prepared from 5-day lactating glands. There were no striking differences in the adhesion of control and β1 integrin mutant cells plated on poly-D-

lysine (PDL), collagen I (Cl), fibronectin (FN), and gelatin (Fig 1F). However, primary cells from β1 integrin-mutant glands displayed a severely reduced adhesion on laminin, the major β1 integrin ligand in the mammary gland (Prince et al., 2002) (Fig 3, LN).

Function of mammary gland was impaired in β1 integrin mutant mice

The body weight of litters nursed by either control or integrin β1 mutant mothers were monitored from new birth until lactation day 20 (Fig. 4A). The pup weight of pups nursed by mutant mothers began to decrease after first round lactation day 5. These pups appear visibly malnourished after first round lactation day 14. (Fig. 4A)

Interestingly, at the secondary lactation stage, the pups nursed by mutant mothers appear visible malnourished after lactation day 1, and pup weight of pups nursed by mutant dams reduced dramatically in comparison with first round lactation stage (Fig.4B).

To check the secretion and the quantity of the milk, at first round lactation-day-5 and lactation-day-14, control and mutant mice were injected with oxytoxin and milk were collected from #4 mammary glands of each mouse. Preliminary data showed that consistent with the functional pup weight analysis, control or mutant dams secret similarly volume of milk at lactation-day-5, but there is only little milk secretion by mutant mothers at lactation-day-14 (Fig.5C)

Electrophoretic separation of control or mutant milk samples showed similar milk expression patterns at lactation day 5 or lactation day 14, including milk proteins WAP and β-casein (Fig 5A). Western blot analysis also showed similar β-casein expression pattern at different lactation stages (Data not shown).

The prolactin receptor and its effectors, janus kinase 2 (Jak2) and signal transducer and activator of transcription 5 (Stat5) have essential roles in mammary gland development and functional differentiation (Liu, 1997; Brisken, 1999; Shillingford, 2002). The activity of Stat5 and Jak2 in control and β1 integrin mutant mammary glands was examined using phospho-Tyr specific antiserum. P-Stat5 levels were strongly- and P-Jak levels slightly-decreased in 3-day lactating glands of mutant females. By 14 days the activity of both proteins was near normal (Fig.5B).

ERBB families of type 1-receptor tyrosine kinases and their ligands have crucial functions during mammopoiesis. Recently, ErbB4 was found to be an essential mediator of STAT5 signaling (Long et al.2003). In wild type or mutant mouse mammary glands, erbB4 protein was localized within luminal epithelial nuclears at early lactation (fig 5D, upper panel). It was trans-localized to membrane at late lactation both in wild type or mutant glands (Fig 5D, lower panel). But the mutant gland sections had

more diffused staining. The integrity of erbB4 staining was not as good as the staining in wild type sections (Fig 5D, low panel, Right).

We already showed that expression of the milk proteins β-casein and WAP was normal in mutant mice, suggesting that the decrease in Jak2/Stat5 activity early in lactation did not have an overall effect on milk protein production. As discussed above, the phosphate cotransporter isoform Npt2b staining (Fig8, panel c&d), a marker of functional alveolar differentiation (Shillingford, 2003), which is expressed on the apical surface of secretory cells (Miyoshi et al., 2001), was normal in β1 integrin null cells. (Fig 8, panel d). Taken together, these results suggest that functional differentiation of mammary glands lacking β1 integrin is normal, and the decreased pup weight nursed by mutant mice might be due to the impaired milk secretion of the mutant mammary glands.

β1 integrin-mutant mammary glands have defects in alveolar integrity due to detachment of luminal epithelial cells

The morphology of control and β1-integrin mutant mammary glands was assessed by whole-mount analysis and hematoxylin and eosin (H & E) staining of paraffin sections. Whole-mounts showed that during pregnancy and lactation the overall organization of the ductal trees and alveoli were normal in the absence of β1 integrin (Fig.6, panel a-d). H & E stained paraffin sections also showed no significant differences in either the development of the lobular-alveolar structures or their density in control compared to β1-integrin mutant glands taken from 16.5- day pregnant or 1-day lactating mice (Fig 7A, panels a & c vs. b & d, respectively and at higher magnification panels g & i vs. h & j, respectively). However, at lactation day 5 there was a noticeable change in the alveolar integrity of the glands from β1 integrin mutant females. Luminally shed cells or bulging cells in the process of shedding were detected (Fig 7A, panel f, yellow & black arrows, respectively). In control females, there are very few detached cells and quantitation revealed an 11-fold increase in shed and bulging cells after loss of β1 integrin. (Fig 7A, panel e & f, white box). This phenotype was maintained at later stages of lactation (Data not shown). Interestingly, at secondary late-pregnancy and early lactation, the alveolar density severely reduced in comparison with control mammary glands either by whole mount analysis (Fig.6 e-h) or by H&E staining (Fig.7B:a-d). An examination of lactating mammary glands from females after a second lactation revealed the same phenotype, i.e., the integrity of the gland was disturbed and many cells were shed into the lumen of β1 integrin mutant females (Fig 7B, panels b & d, higher magnification). The density of the

lobular-alveolar structures was also decreased in the β1 integrin mutant females compared to controls at day 5 of the first lactation (Fig. 7B, panel d vs. c). These results suggest that loss of β1 integrin decreases luminal cell-matrix adhesion, resulting in loss of alveolar integrity. Moreover, the decreased alveolar density suggests that proliferation of mammary cells in the absence of β1 integrin might be affected.

Next, we examined mammary glands from 5-day lactating females with various markers to probe the integrity of the alveolar structures. Staining for the myoepithelial marker smooth muscle actin did not reveal any differences between β1 integrin mutant and control glands (Fig.8, panels a & b). Staining with a laminin-1 antiserum showed that BM localization was normal in the control and mutant glands (Fig.8, panel e & f), however, it is evident that in these glands many luminal cells have lost their ability to adhere to the BM and have been shed, or are in the process thereof (Fig 8, panel f, white arrows). E-cadherin showed a basal-lateral localization in control luminal cells. This pattern was altered in some luminal cells of β1-integrin mutant glands (Fig 8, panel g & h), which correlates with decreased E-cadherin levels in the mutant glands (Fig. 11D). The expression of β-catenin was normal in mutant glands at early latation while it was decreased at lactation day 14(Fig. 11D). We also observed a decrease in the tight junction protein ZO-1 late in lactation, which might result from decreased E-cadherin levels since assembly of tight junctions depends upon E-cadherin recruitment to adherens junctions (Data not shown). Thus, with respect to a myoepithelial cell marker, BM deposition and an apical epithelial marker, mammary glands from mice lacking luminal β1 integrin appeared normal. The major phenotype of β1 integrin mutant glands was a decreased association of the luminal cells with the laminin-1 rich BM, leading to loss of epithelial integrity and increased shedding of cells into the lumen of the gland.

It is noteworthy, however, that β4 integrin, which is localized to the basal surface of the luminal and myoepithelial cells in control glands (Fig.8, panel i), was relocalized in β1 integrin mutant glands; in additional to basal staining, β4 integrin was also detected on the apical surface of some luminal cells (Fig.8, panel j). Phalloidin was used to visualize actin, since β1 integrins connect the ECM to the actin cytoskeleton. Actin stress fibers running along the basal surface of the cells were evident in sections from control glands (Fig.8, panel k). In contrast, there were no stress fibers evident in β1-integrin mutant glands. Furthermore, strong actin staining was also evident in some lateral and apical surfaces (Fig.8, panel l), suggesting that in the absence of luminal β1 integrins, the actin cytoskeleton has become disorganized.

β1 integrin mutant mammary glands show a slight increase in apoptosis at involution, but not during lactation

Various lines of evidence point to the important role of integrin-mediated adhesion in mammary cell survival. Primary mammary cells undergo apoptosis unless cultured on laminin, or when β1 integrin function is perturbed (Pullan, 1996b; Gilmore, 2000). Accordingly, we examined mammary glands from β1 integrin mutant mice for apoptosis using the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay. Apoptotic cells were not detected in lactating glands from β1 integrin mutant mice and luminally shed cells were not TUNEL positive (data not shown). Electron microscopy (EM) was also used to investigate cells at a higher resolution. Neither cells that lost contact with their neighbors (Fig 9A, N), nor cells that had detached and were in the lumen (Fig 9B, white circled cell), showed evidence of apoptosis. In particular, the nuclei of these cells appeared normal and did not show apoptotic characteristics, such as condensed nuclear fragments (the casein micelles and milk-fat droplets are evident in the milk of β1 integrin mutant females).

Considering that apoptosis is generally low during lactation, we also examined involution, a developmental stage with high levels of cell death (Strange et al., 1992; Green et al., 2004). Litters were removed from 9-day lactating control and β1 integrin mutant females to induce involution; 24 hrs later mammary glands were isolated and examined (Prince et al., 2002). H & E stained paraffin sections revealed that involuting glands from β1-integrin mutant mice had 4-5 fold more shed cells in the lumen in comparison to controls (Fig. 10, top panels and quantitation on the right). TUNEL staining was also performed on the sections. Quantitation of positive cells, both alveolar and shed, revealed a 1.5-fold increase in apoptotic cells in β1-integrin mutant mammary glands, compared with control glands (Fig. 10, lower panels and quantitation on the right), however, this increase is not significant. It is noteworthy that not all shed cells were TUNEL positive (black vs. green arrows in panel d).

STAT3 is pro-apoptotic and is a crucial mediator of post-lactational regression (Chapman et al., 1999). Using conditional gene targeting, it has been shown that in the absence of STAT3, involution is delayed for several days, owing to a reduction in apoptosis, and this is associated with elevated levels of p53 (TRP53 -; Mouse Genome Informatics) and p21, precocious activation of STAT1, and failure to induce IGFBP5. After loss of integrin β1, at lactation day 3 and day 14, the phosphorylation of stat3 was decreased (Fig.11, panel A). We also checked the expression of p53 and another pro-apoptotic marker

Bax. In the absence of integrin β1, the expression of Bax was increased throughout lactation, while the express of p53 was only upregulated during late lactation (Fig.11, panel B).

The serine/threonine kinase PKB transmits survival signals triggered by integrin-mediated adhesion (Datta et al., 1999). The activity of this kinase was assessed using an antiserum specific for the active, phosphorylated form. There was no difference in the level of Ser 473 phosphorylated PKB in extracts from lactating glands of β1-integrin mutant mice compared to control mice. Furthermore, phosphorylation of Ser 9 of glycogen synthase kinase 3-β (GSK3β), a downstream target of PKB, was not altered in β1-integrin mutant mammary glands (Fig.11, panel B).

Taken together, the results suggest that in the absence of β1-integrin, the epithelial cells are shed more easily into the lumen of the gland. This phenomenon is apparently not due to increased apoptosis during lactation, but very likely due to decreased adherence of the luminal cells to the BM in the absence of β1 integrins, suggesting that loss of integrin β1 couldn't trigger the apoptosis in mammary luminal epithelial cells during lactation. At involution, there is a slight increase in the number of apoptotic cells, but this increase is not significant.

Loss of β1 integrin correlates with reduced mammary cell proliferation and upregulation of $p21^{Cip1}$

At the start of lactation the mammary gland undergoes a proliferative burst. To examine whether β1 integrin is involved in this process, 2-day lactating females were injected with bromodeoxyuridine (BrdU) 2 hrs before sacrifice and paraffin sections from the isolated mammary glands were examined with anti-BrdU monocloncal antibody. In control mice approximately 8% of the luminal nuclei stained positively, while only 2% of the luminal cells in mammary glands from β1 integrin mutant mice showed BrdU incorporation (Fig. 12A, panels a & b; quantitation in c). Thus, mutant glands show a strong reduction in proliferation at the start of lactation.

Progression through the cell cycle is regulated by periodic activation and inactivation of cyclin dependent kinases (CDK). To provide a mechanistic understanding of the decreased proliferation rate in the β1 integrin mutant glands, we examined the level of positive and negative CDK regulators, the cyclins and the CDK inhibitors (CKI), respectively. Cyclin D1, an important G1 regulator in the mammary gland (Sicinski et al., 1995) was slightly increased in lactating glands from mutant mice; while neither cyclin E, cyclin B1 nor CDK2 levels differed between control and β1 integrin mutant glands (Fig 12B). CKIs of the CIP/KIP family ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) were also analyzed. The levels of $p57^{Kip2}$ were similar

in control and mutant glands; $p27^{Kip1}$ levels were slightly elevated in β1 integrin mutant mammary glands taken from 14-day, but not 3-day lactating mice; $p21^{Cip1}$ was dramatically increased in β1-integrin mutant glands throughout lactation including lactation day 2 (Fig 12C & data not shown). At least at early times the increase in $p21^{Cip1}$ might be independent of p53, which was decreased in β1 integrin mutant in comparison to control mammary glands (Fig 11A). There was also a significant increase in $p21^{Cip1}$ specific mRNA in glands lacking β1 integrin (data not shown). Thus, it is possible that the proliferation defect might be due, at least partially, to the specific increase in $p21^{Cip1}$ expression.

Ablation of β1 integrin impairs focal adhesion signaling

In addition to providing adhesion, engagement of β1 integrins promotes the formation of signaling complexes that regulate F-actin accumulation; the best characterized being the focal adhesions. We examined focal adhesion kinase (FAK) whose recruitment to these structures induces downstream signaling (Schlaepfer, 1998) and paxillin a focal adhesion associated protein that is a target for active FAK and Src kinase. FAK activity, as measured by phosphorylation on Tyr397, an autophosphorylation site, was decreased in β1 mutant glands (PY397 FAK, Fig.11C). Phosphorylation of paxillin on Tyr118 was also decreased in β1 mutant glands (Fig11C). FAK expression level was slightly decreased in mutant glands taken at day 14 of lactation (Fig.11C).

Loss of either β1 integrin or FAK induces $p21^{Cip1}$ upregulation in primary mammary cells

To investigate further the link between β1 integrin, FAK and $p21^{Cip1}$, we used primary mammary cells from $Itg\beta 1^{flox/flox}$ and from $FAK^{flox/flox}$ mice. Primary cells were prepared from pregnant females and cultures were infected with an Adeno-Cre virus or a control Adeno-βgal control virus. Cell lysates from 2-day infected cultures were examined by western analyses for β1 integrin, FAK and $p21^{Cip1}$ levels. The nearly 100% infection efficiency was attested to by the low levels of both β1 integrin and FAK in Adeno-Cre infected cells (Fig 13 panels a, b & c). Importantly, each of these cultures showed a strong induction in $p21^{Cip1}$ levels (Fig 13 panels a, b& c). As we observed *in vivo*, $p27^{Kip1}$ levels were unaffected by loss of β1 integrin in primary cultures. Furthermore, similar to in the in vivo observation, we found PY-397 FAK was decreased in the primary cells after loss of integrin β1 (Fig13 panels b). Thus, intact mammary glands as well as isolated primary cells display elevated $p21^{Cip1}$ expression and impaired focal adhesion

signalling in the absence of β1 integrin. Moreover, loss of FAK function also leads to upregulation of $p21^{Cip1}$ expression.

Absence of $p21^{Cip1}$ rescues impaired BrdU incorporation in mammary cells of β1 integrin mutant mice.

To examine if $p21^{Cip1}$ up-regulation was responsible for the proliferation defect observed in β1 integrin mutant mammary glands, $p21^{Cip1}$ -/- mice were crossed with β1 integrin mutant mice to generate double mutant mice. Western Blot analysis was used to examine the expression of β1 integrin and p21Cip1 in each mice (Fig.14A). BrdU was injected into lactating females and paraffin sections from isolated mammary glands were stained with an anti-BrdU specific antibody (Fig.14B). There was a statistically significant increase in DNA synthesis in double mutant glands in comparison with the integrin β1 mutants (Fig.14B).

Using in vitro model to further investigate the proliferation mechanisms: the anti-proliferative effect of β1 integrin loss in MDA-MB-231 cells is dependent upon $p21^{Cip1}$ up-regulation

To investigate directly if loss of β1 integrin blocks proliferation via $p21^{Cip1}$ induction, we used as a model MDA-MB-231 breast cancer cells, in which β1 integrin is known to play an important role in the tumor cell phenotype (Trusolino et al., 2000; Wang et al., 2002). Transfection of β1 integrin specific siRNA strongly reduced expression of the integrin, relative to the control LacZ siRNA-transfected cells (Fig 15A). β1 integrin knockdown cells had higher levels of $p21^{Cip1}$ protein (Fig 15A) and mRNA (Fig 15B), relative to controls. Immunofluorescence revealed that PY-397-FAK was present on the membrane focal adhesions in control cells (Fig 15A, upper panel), and in β1 integrin knockdown cells PY-397-FAK was relocalized to the cytoplasm (Fig 16A, lower panel). To link FAK to $p21^{Cip1}$ induction, we used the FAK-related non-kinase (FRNK), composed of the noncatalytic carboxyl-terminal protein-binding domain of FAK, which acts in a dominant negative manner to attenuate FAK activity (Richardson et al., 1997). MDA-MB-231 cultures were transfected with a $p21^{Cip1}$ promoter luciferase reporter plasmid (el-Deiry, 1993), together with the FRNK-encoding plasmid and luciferase activity was measured; β1 integrin knockdown cells were also examined in a parallel experiment. The results showed that down-regulation of β1 integrin expression or interfering with FAK signaling led to a 2-fold and 5-fold increase in $p21^{Cip1}$ promoter activity, respectively (Fig. 16B, upper & lower panels).

Importantly, loss of β1 integrin had a strong anti-proliferative effect on MDA-MB-231 cells. In comparison to control cultures, a FACS analysis revealed that β1 integrin knockdown cells had a 41% increase in G1-DNA content and a 90% decrease in BrdU incorporation (Fig 17C, β1); a cell count showed that there was a > 50% decrease in cell number (Fig 17B, β1). In the next experiment, specific siRNA was used to probe for the role of $p21^{Cip1}$ in the anti-proliferative response observed in β1 integrin knockdown cells. To accomplish this, cells were transfected with siRNAs for β1 integrin and for $p21^{Cip1}$, either alone or in combination. Western analyses were used to assess transfection efficiencies (Fig 17A), and DNA content and cell number were monitored (Fig 17 B& C). Knockdown of $p21^{Cip1}$ had essentially no effect on G1-DNA content, BrdU incorporation (Fig 17C p21 vs. control) or cell number (Fig 17B, p21 vs. LacZ). Cells with β1 integrin and $p21^{Cip1}$ knockdown were rescued from the effect of β1 integrin loss. More specifically, G1 DNA content decreased from 88% in β1 integrin knock-down cells to 70% in double knock-down cells (56% rescue, based on 47% G1 DNA in control cells); BrdU incorporation increased from 4% in β1 integrin knock-down cells to 19% in double knock-down cells (54% rescue, based on 39% in control cells) and cell number was rescued by approximately 50%. These results suggest that β1 integrin controls proliferation by maintaining low expression of $p21^{Cip1}$; in the absence of β1 integrin signaling, $p21^{Cip1}$ expression is induced transcriptionally and cells accumulate in the G1 phase of the cell cycle.

Loss of β1 integrin leads to a defect in alveolar cell proliferation during a second pregnancy

The phenotype resulting from β1 integrin loss was more pronounced in glands from mice undergoing a second pregnancy. Wholemounts and H & E stained sections both showed that the lobulolalveolar units were sparser in the β1 integrin mutant glands compared to control glands (Fig 18A, panels a vs. b and c vs. d). Following involution, it has been shown that a portion of WAPCre expressing cells bypass apoptosis and remain in the remodeled mammary gland, where they give rise to clonal populations of alveolar cells during subsequent pregnancies (Wagner et al., 2002; Ludwig et al., 2001). Considering this, we hypothesized that the sparse phenotype was due to the presence of progenitor cells with a recombined *Itgβ1* gene in the involuted gland, which had an impaired ability to proliferate during the second pregnancy. We examined this further using the mammary transplantation technique, a method to functionally identify mammary stem cells by measuring their in vivo outgrowth potential (Kordon and Smith, 1998; Smith, 1996). Pieces of mammary tissue from β1 integrin mutant mice or control mice were

isolated from 3-day lactating glands, a time of maximal Cre expression. These tissue pieces were transplanted into the cleared fat pads of recipient syngeneic mice (6 mice for each genotype). After 10 weeks, recipient females were mated and at pregnancy-day 16, they were sacrificed and mammary gland whole mounts were prepared. As expected, control transplants were able to grow out and form ducts and/or alveolar structures (5/6) (Fig 18B, panel a). In striking contrast, transplanted tissues from b1 integrin mutant mice were unable to grow in recipient mice (6/6). These results strongly suggest that alveolar progenitor cells have an impaired ability to proliferate in the absence of β1 integrin.

β1-integrin play multiple roles in the development of mammary gland

Overall, we found β1 integrins have key roles at several stages during the development and function of the mammary gland (Fig.19). Firstly, It could regulate the proliferation of mammary alveolar cells at early lactation. During involution and tissue remodeling, it might participate in the mammary progenitor cell proliferation and/or survive. As an important cell adhesion receptor, it could involve in the glandular morphogenesis and maintenance of mammary gland integrity throughout lactation.

Fig.1 Genetic ablation of β1 integrin in mouse mammary gland.

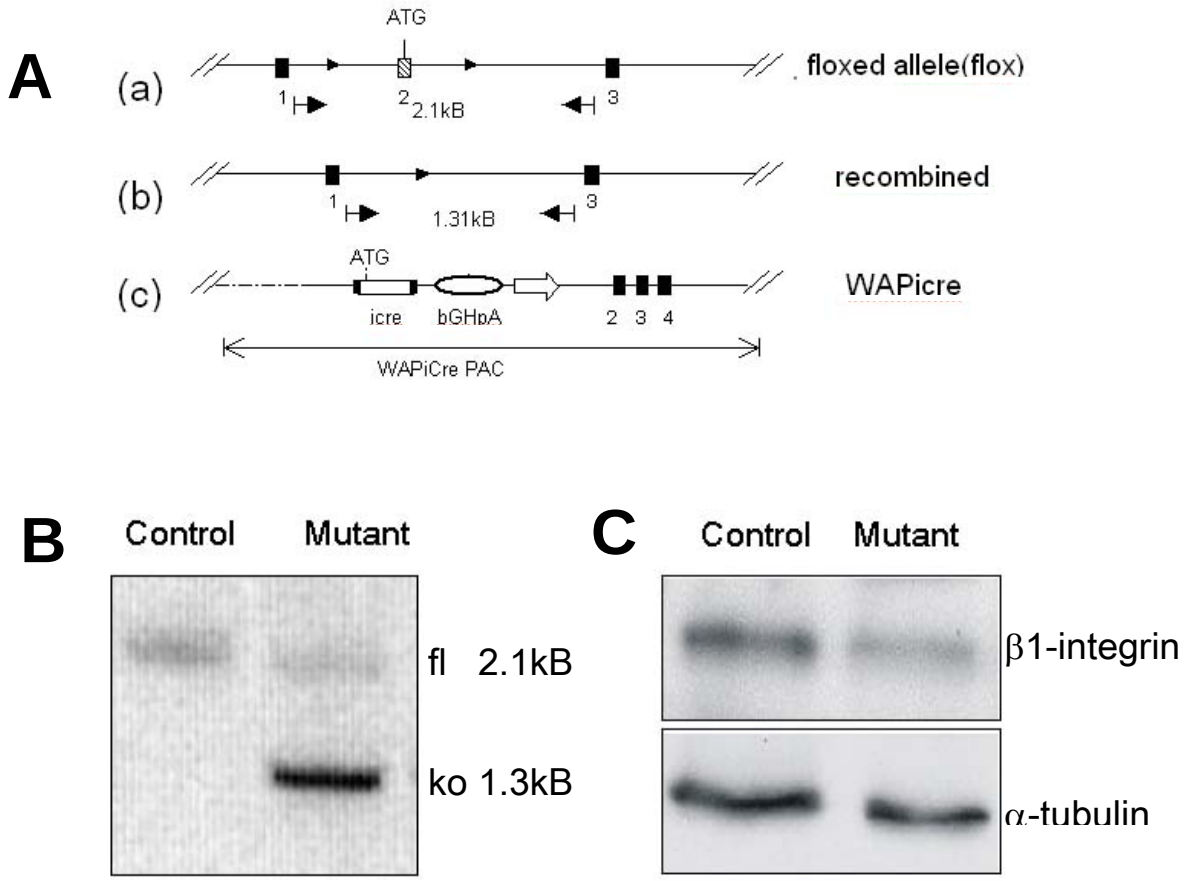

Figure 1. Genetic ablation of β1 integrin in mouse mammary gland. (A) Schematic representation of the floxed β1 integrin allele (a) and the recombined allele (b). The first coding exon of the β1 integrin allele was flanked by two loxP sites (Graus-Porta et al., 2001). The PCR primers for detection of the floxed and recombined allele and the product size are indicated. (c) The structure of the WAPiCre transgene is shown. Transgenic mice were generated using a P1-derived bacterial artificial chromosome (PAC) harboring the WAPiCre gene with at least 25 kb of 5' flanking sequences.(Wintermantel et al., 2002). (B) Cre mediated recombination was analyzed by PCR using genomic DNA from 3-day lactating mammary glands of control (*Itgβ1*$^{flox/flox}$;*WAPiCre*$^{-/-}$) or β1-integrin mutant (*Itgβ1*$^{flox/flox}$;*WAPiCre*$^{+/-}$) mice. The 2.1 and 1.3 kB bands correspond to the β1 floxed (fl) and the β1 integrin recombined (ko) alleles, respectively. (C). At lactation day 3, β1 integrin and α-tubulin expression levels were analyzed by western blotting on tisse lysates.

Fig.2 Specific ablation of β1 integrin in lactating mammary luminal epithelial cells

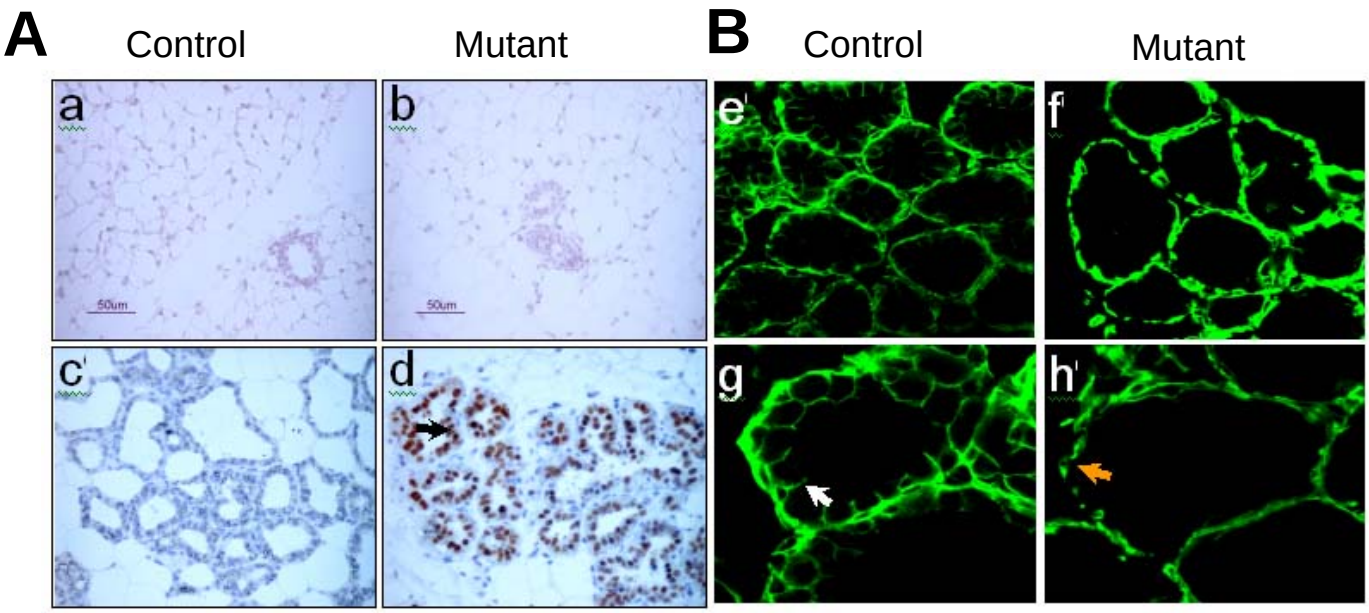

Figure 2. (A) WAPiCre displays tightly controlled expression in luminal epithelial cells. Paraffin sections from virgin (a) and 3-day lactating (c) control mice or virgin (b) and 3-day lactating (d) β1 integrin mutant mice were analyzed for Cre recombinase expression using a specific antiserum. The black arrow in (d) indicates Cre positive cells. (B) Efficient and specific ablation of β1 integrin in luminal epithelial cells. Frozen sections of mammary glands from 3-day-lactating control (e & g) and β1 integrin mutant (f & h) mice were stained for β1 integrin. The pictures in g & h were taken at a higher magnification. The white and yellow arrows indicate β1 integrin staining of luminal and myoepithelial cells, respectively.

Fig.3 Effect of β1 integrin deletion on adhesion of primary mammary epithelial cells.

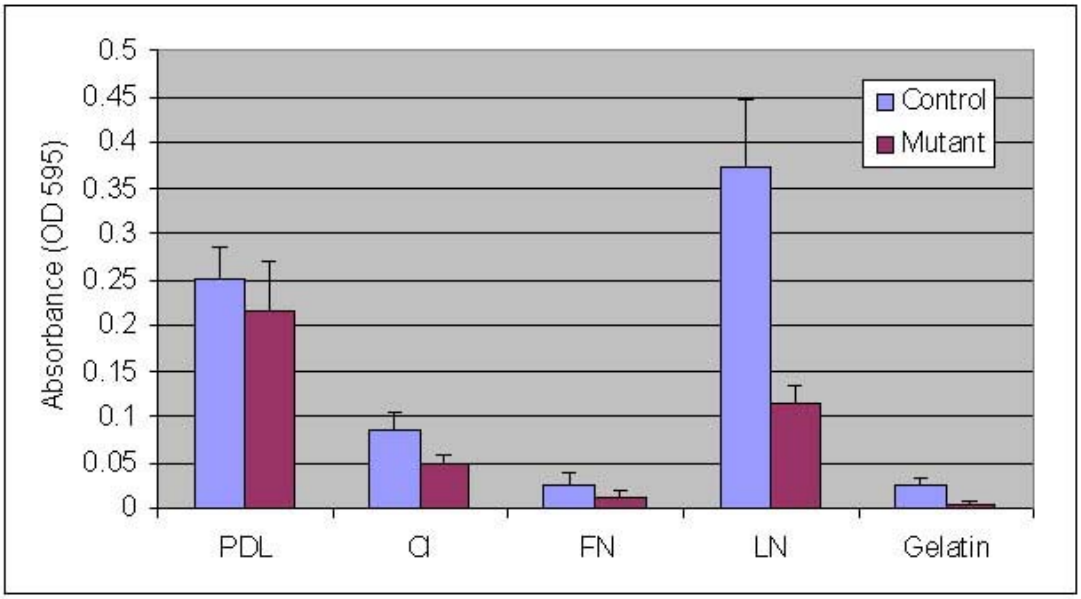

Figure 3 Loss of β1 integrin in luminal cells leads to impaired adhesion on laminin. Primary mammary cells were prepared from 5-day lactating control and β1 integrin mutant females and used immediately in adhesion assays. 7 x 10^4 primary cells were plated on 96 wells pre-coated with poly-D-Lysine (PDL), collagen type I (CI), fibronectin (FN), laminin (LN) and gelatin. After one hour, unattached cells were washed away, and adhered cells were fixed, stained with crystal violet, solubilized in 1% SDS and absorbance at 595 nm was read. The error bars represent standard deviation of the mean of triplicate samples within one experiment.

Fig.4 Pup Weight Analysis.

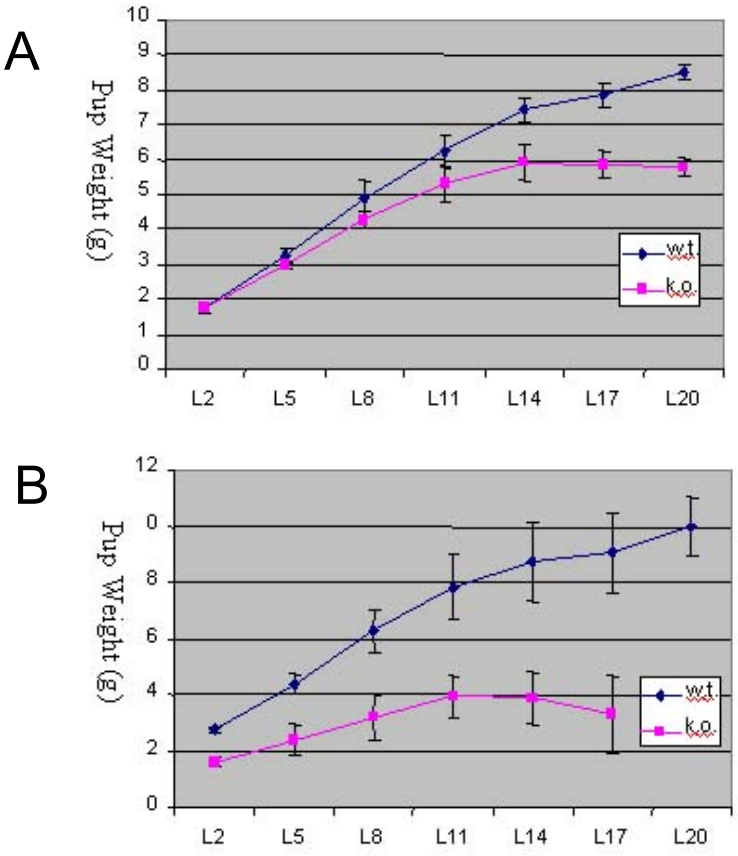

Figure 4. Average body weight increase of pups nursed by three control and three mutant mice during first lactation stage (A) and second lactation stage (B).
Each data point represents the average of 3 litters each with 8 pups (n=24,a) or 6 pups (n=18,b). Data are means±S.D. This assay is independent of the genetic background

Fig.5 Milk expression pattern.

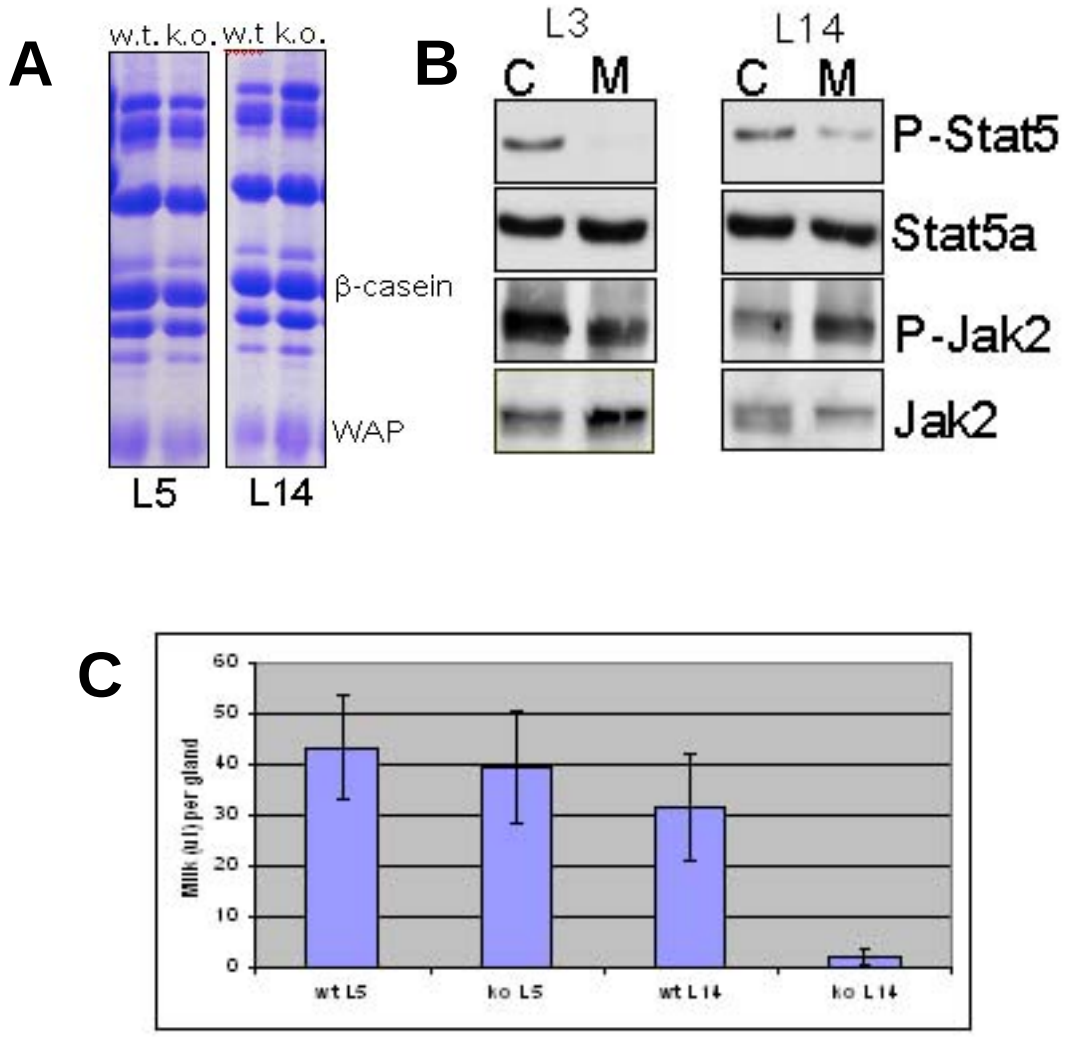

Figure 5. (A) Milk expression pattern at first lactation day 5 and day 14. The milk proteins (WAP and β-casein) are indicated by arrows. (B) Western Blot analysis of p-Stat5, stat5, p-Jak2 and Jak2 protein expression at lactation day 3 and lactation day 14. (C) Mice were milked at lactation-day-5 and lactation-day-14. And the secreted milk from the fourth mammary glands were collected and calculated at these two time points.

Fig.5 Milk expression pattern.

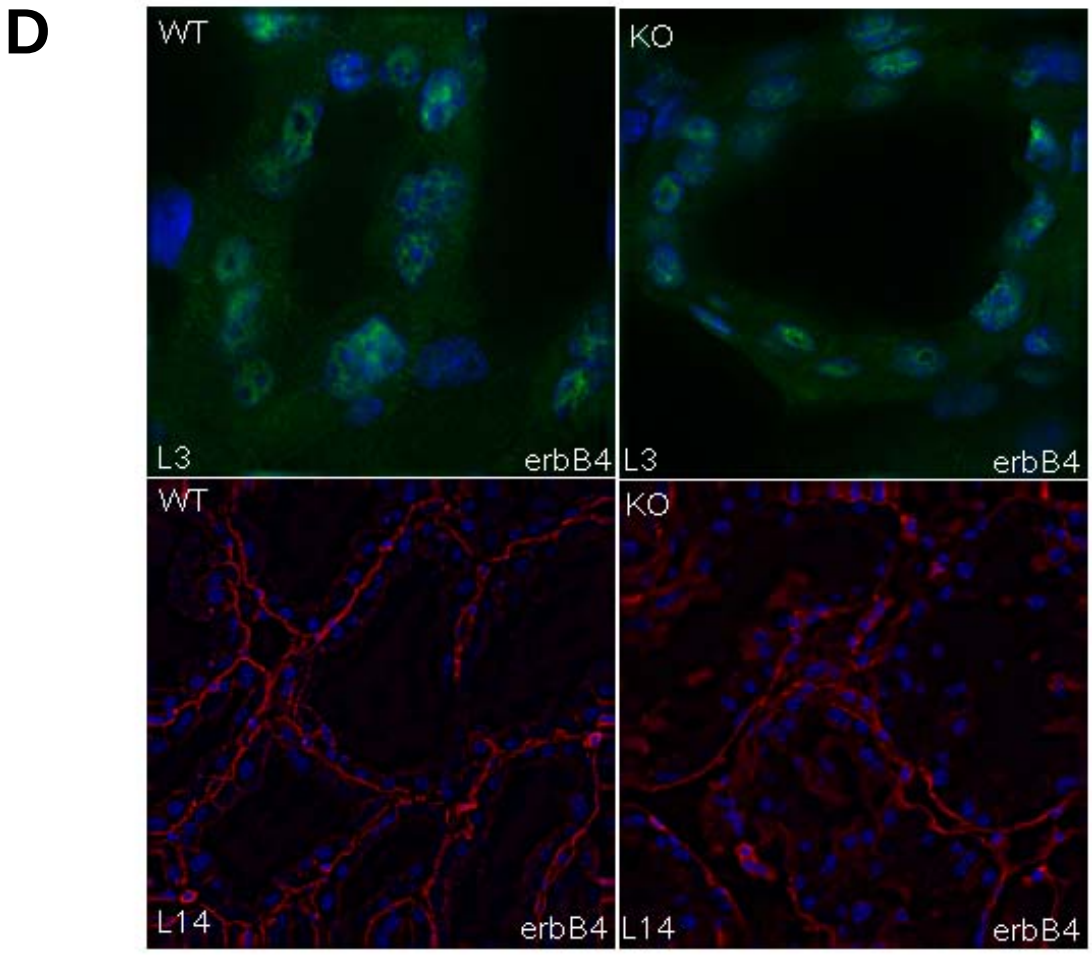

Figure 5. (D) ErbB4 staining was checked on mammary sections at first lactation day 3 and day 14. Note the nuclear localization of erbB4 at lactation day 3. At lactation day 14 it concentrated on the membrane and at this time the staining of erbB4 is more diffused in the mammary gland sections of mutant mice.

Fig.6 Whole mount analysis of control and β1 integrin mutant mammary glands.

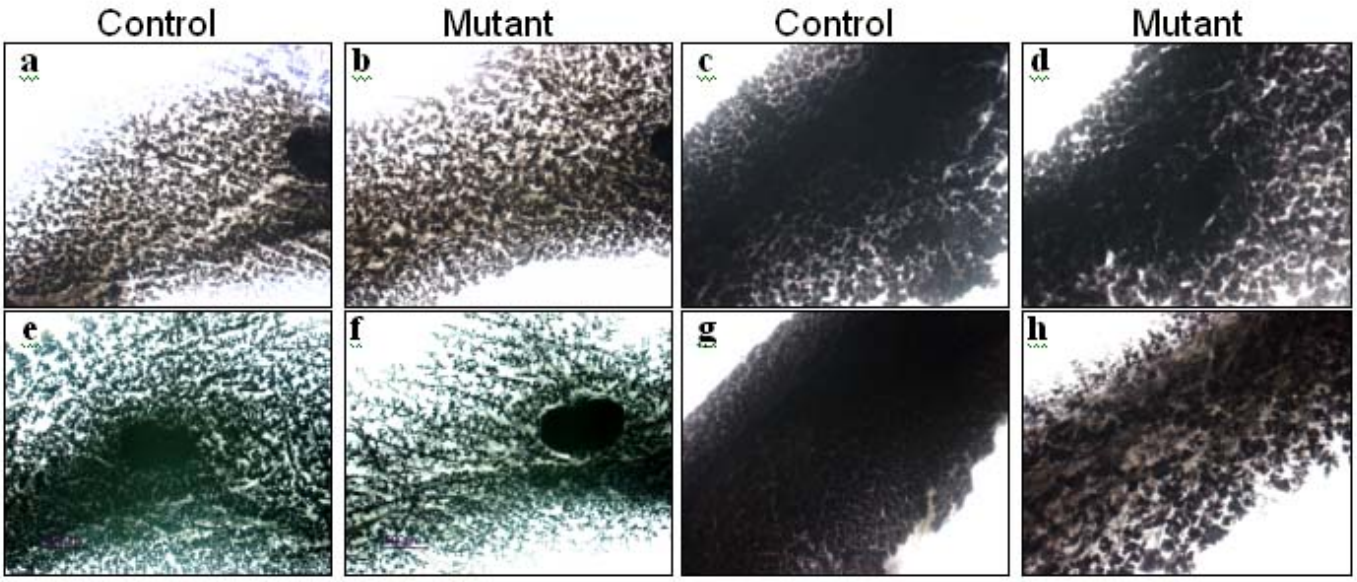

Figure 6. Whole mount analysis of mammary glands taken from control(a;e;c;g) and β1 integrin mutant(b;f;d;h) mice during the first (a-d) or second (e-h) round of pregnancy(a;b;e;f) and lactation(c;d;g;h).

Fig.7 Loss of integrin β1 leads to adhesion defects and detachment of mammary epithelial cells.

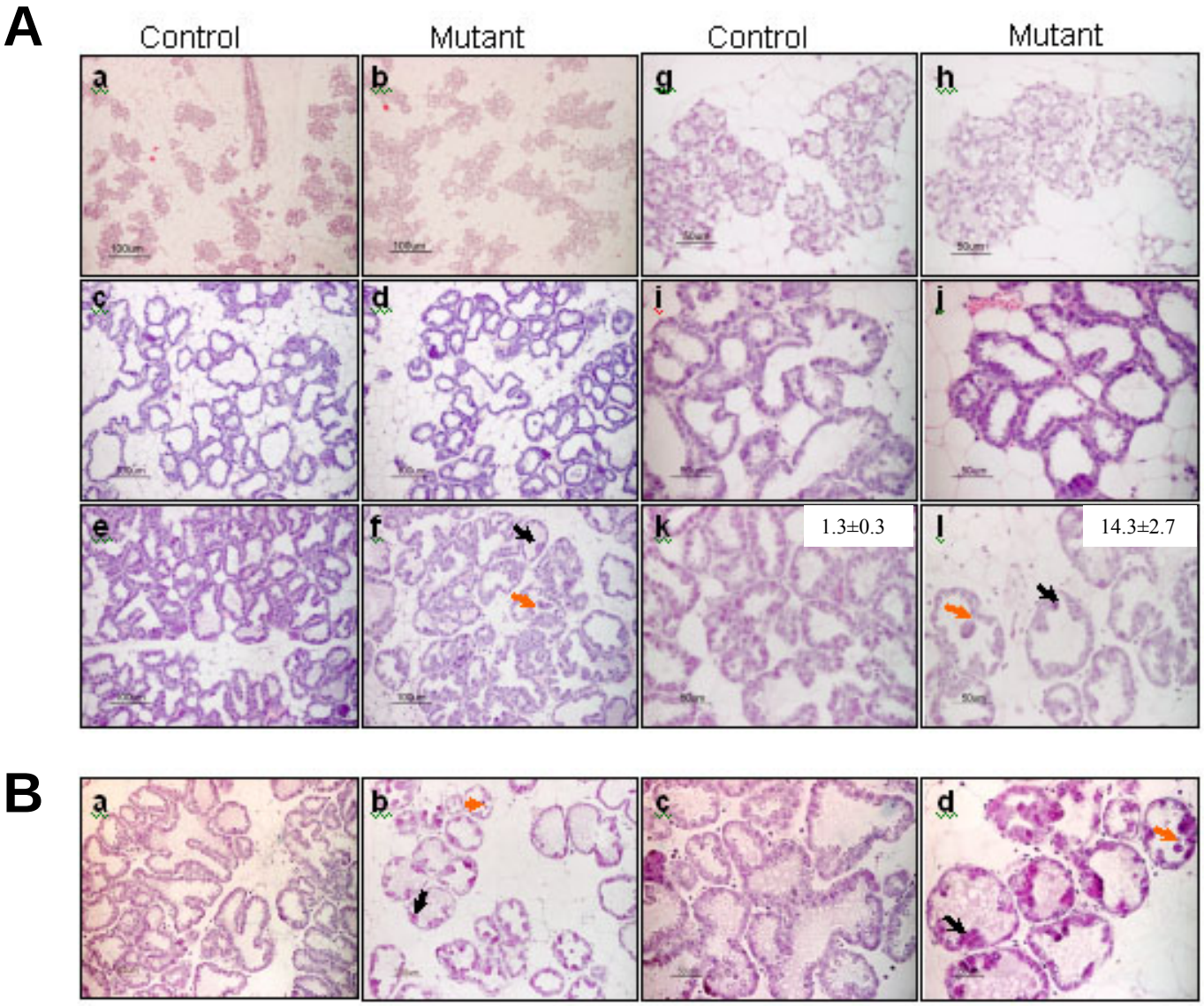

Figure 7. Mammary glands were taken from control- and β1 integrin mutant mice during the first (A) or second (B) round of pregnancy and lactation, and paraffin sections were prepared and stained with H & E. (A) The mammary glands were from mice at: day 16 of pregnancy (a, b, g & h), lactation day 1 (c, d, i & j), and day 5 (e, f, k & l). (B) The mammary glands were from mice at lactation day 5 (a-d). The black arrows in (A) panels f & l and (B) panels b and d, indicate irregularities in the alveolar architecture; the yellow arrows in (A) panels f & l, and (B) panels b & d, indicate cells shed into the lumen of β1 integrin mutant mice, a phenotype that became starting at lactation day 3 of the first pregnancy. (A) panels g-l and (B) panels c and d, were taken at 2X higher magnification than (A) panels a-f and (B) panels a and b. White boxes in k&L indicate the quantification of the displaced cells (including shed and procuding cells) from 3 pairs of control and mutant mice.

Fig.8 Immunostaining of the molecular markers on the mammary gland sections.

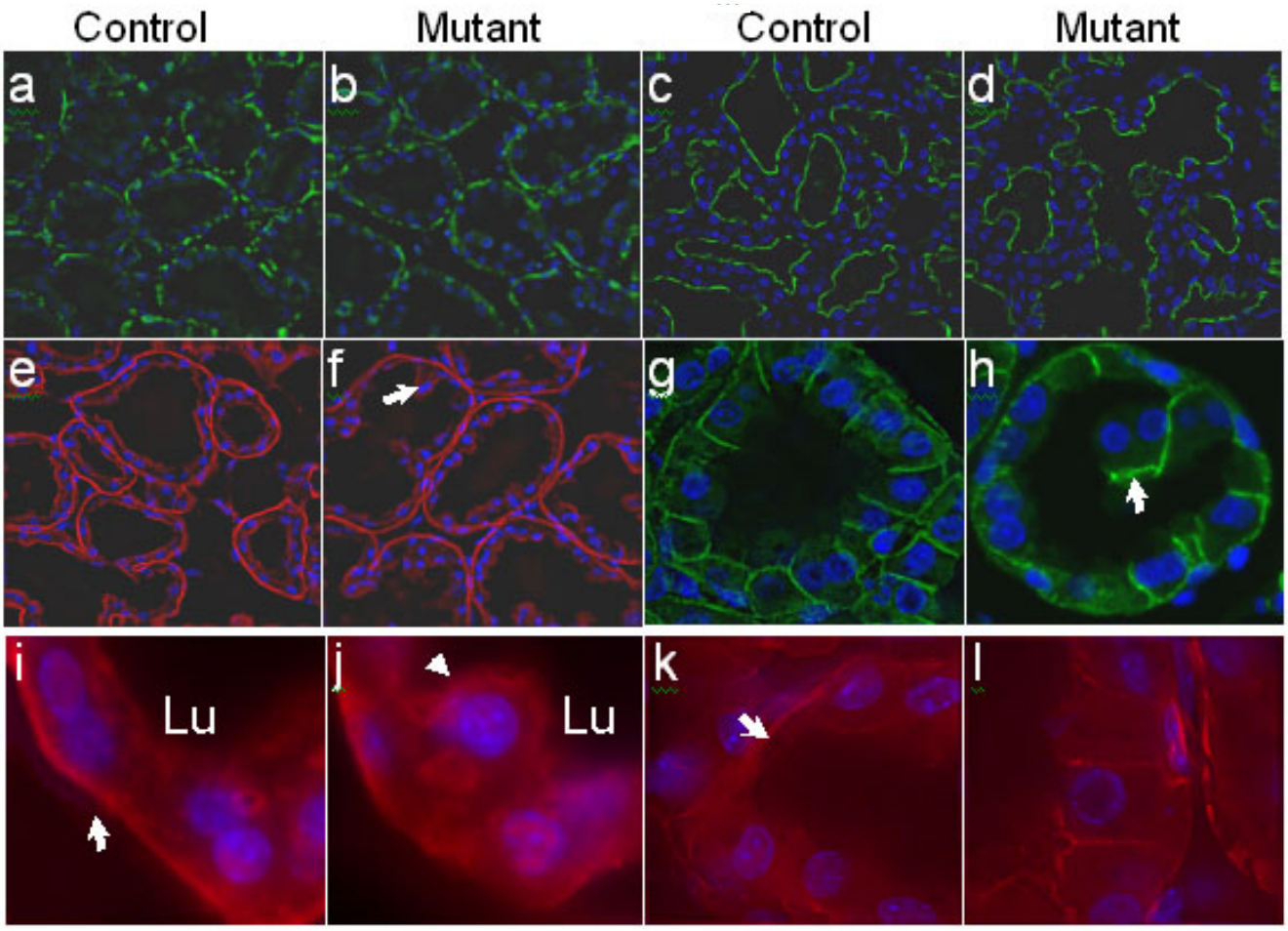

Figure 8. The Frozen sections of mammary glands from 5-day-lactating control mice (a, c, e, g, i & k) and β1 integrin mutant mice (b, d, f, h, j & l) mice were stained for: (a & b) α-smooth-muscle actin (20x); (c & d) the sodium phosphate cotransporter isoform, Npt2b (20x) (e & f) Laminin-1 (20X); (g & h) E-cadherin (60X); the arrows in panels f & h indicate cells detached from the BM; (i & j) β4 integrin (120X); the arrowhead in panel j shows relocalization of β4 integrin from the basal surface (white arrow) to the apical surface in mutant cells; (k & l) F-actin (100X); the arrow shows stress fibers in luminal cells from control mice. In panel l actin staining is localized not only on basal but also lateral surfaces of mutant cells.

Fig.9 There is no increase of apoptosis in integrin β1 mutant glands during lactation.

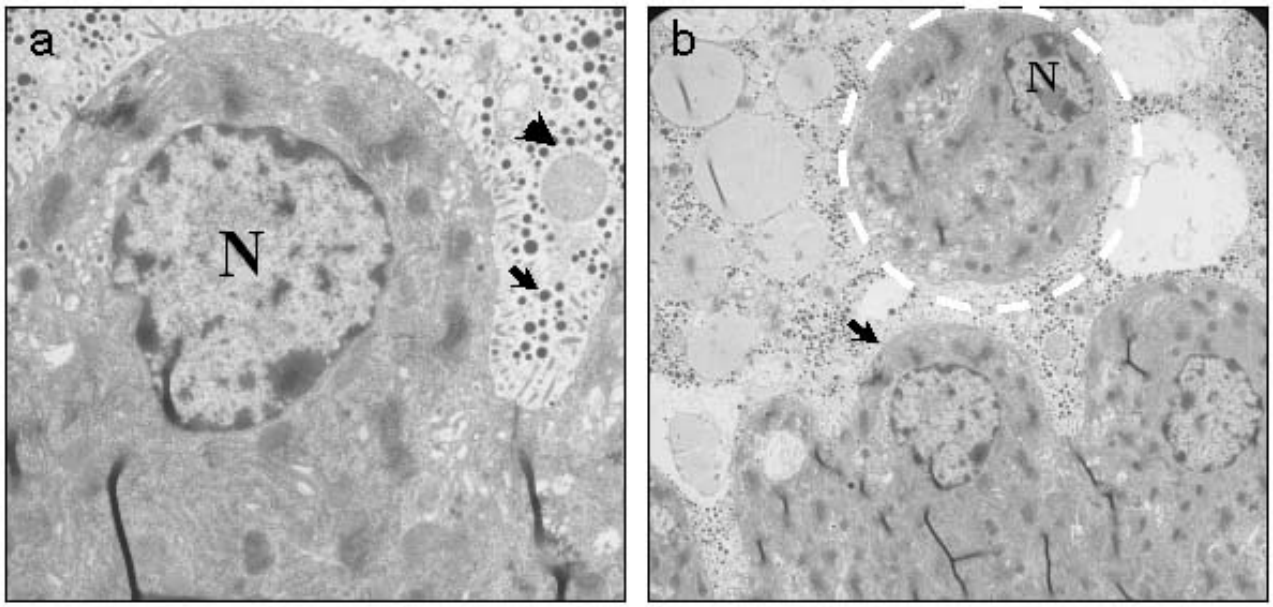

Figure 9. Sections prepared from β1 integrin mutant mammary glands of 14-day lactating females were analyzed by electric microscopy. In (a) a single luminal epithelial cell that is detaching from its neighbors is shown; the nucleus is labeled N. The black arrow and arrowhead indicate caseins micelles and a lipid droplet, respectively (X5000). In (b) a detached cell in the lumen is outlined in white. Another cell (arrow) is in the process of detaching. Neither nucleus (N) is apoptotic (X2500).

Fig.10 Loss of β1 integrin could slightly increase the apoptosis rate during involution.

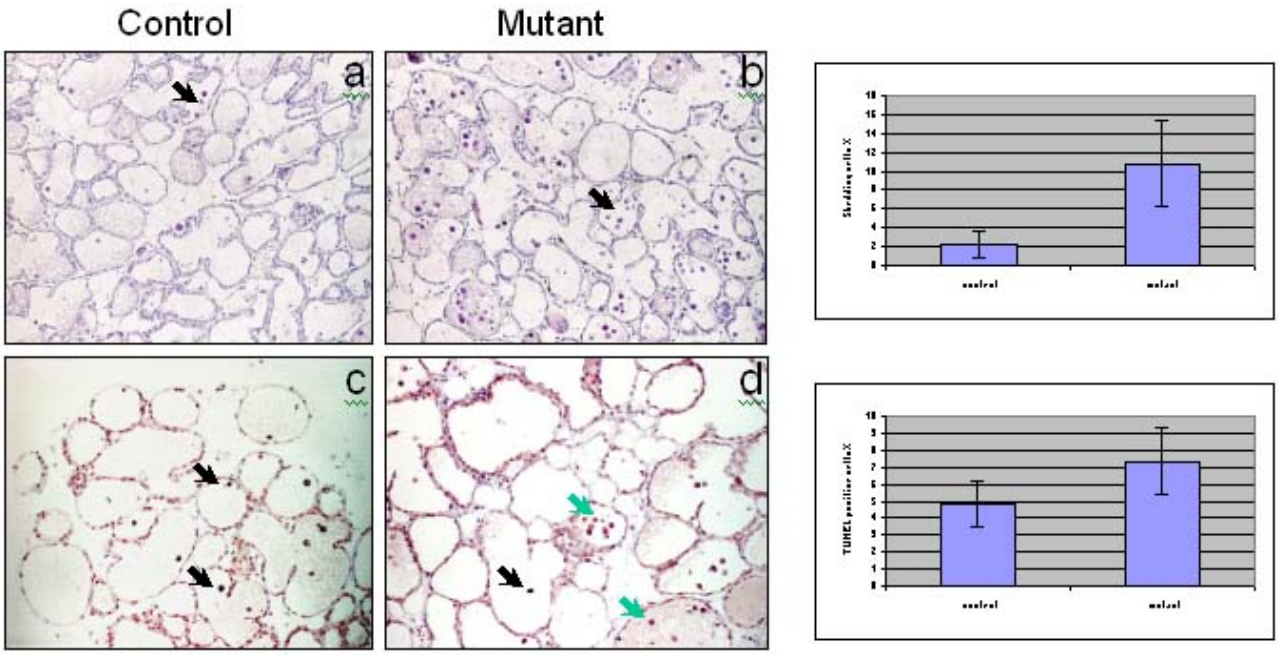

Figure 10. Paraffin sections from day 1 involuting mammary glands from control- (a & c) and β1 integrin mutant (b & d) mice were stained with hematoxylin and eosin (a & b) or were TUNEL-stained to detect apoptosis (c & d). In a & b cells shed into the lumen (black arrows) were quantified (insert on right) in sections from 3 pairs of control and mutant mice. In each case, 900-1200 nuclei per section were counted. Data are means±S.D. In c & d the black arrows show positive TUNEL staining; the green arrows show shed cell that are negative for TUNEL staining. Tunel positive cells were quantified (insert on right) in sections from 3 pairs of control and mutant mice. In each case, 900-1200 nuclei per section were counted. Data are means±S.D.

Fig.11 Changes of signalling pathways after loss of β1 integrin.

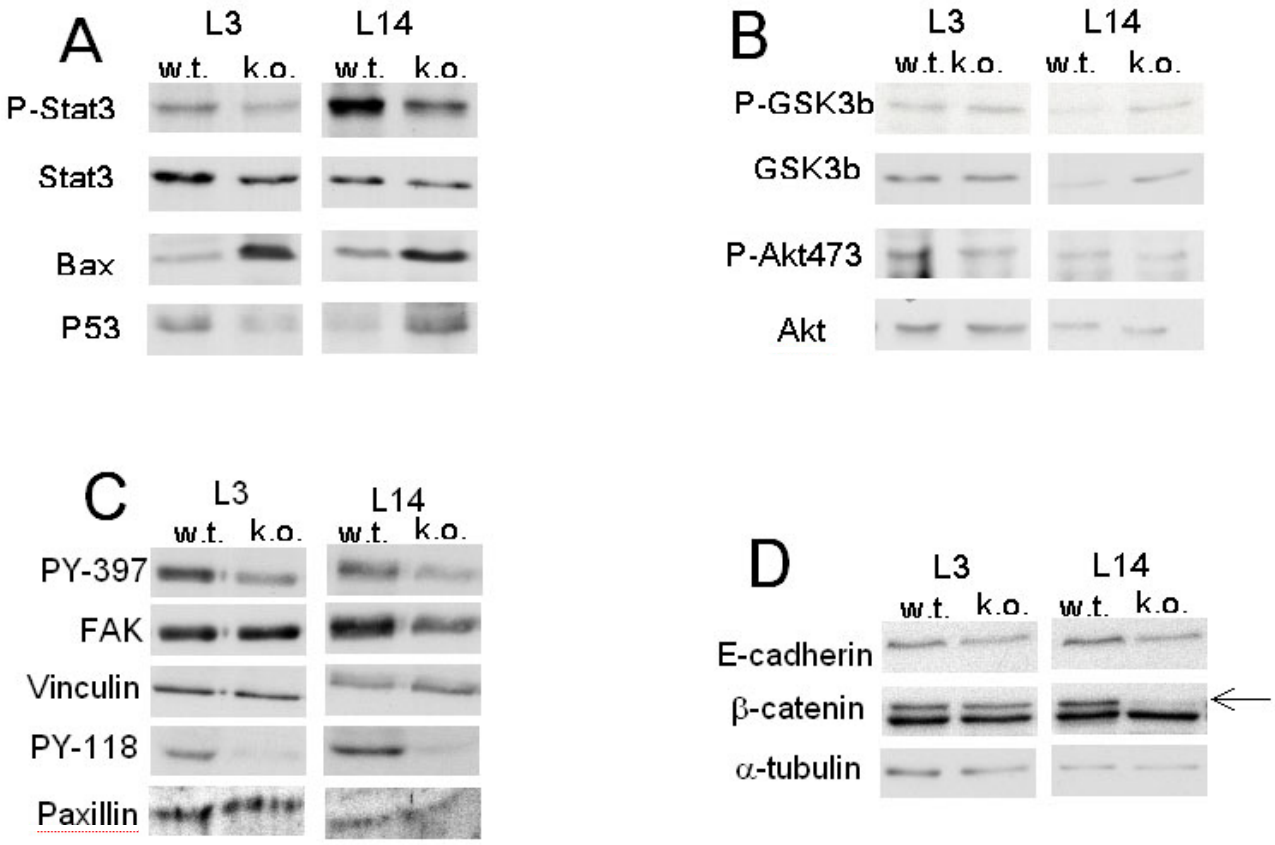

Figure 11. Analysis of proteins from 3- and 14-day lactating mammary glands. Western blotting revealed the levels of (A). p-Stat3, stat3, Bax and p53; (B).p-GSK3β, GSK3β, p-AKT47,Akt; (C). Tyr397 FAK (PY397), FAK, vinculin, Tyr118 Paxillin (PY118) and paxillin; (D). E-cadherin, β-catenin and α-tubulin protein expression.

Fig.12 Loss of β1 integrin leads to reduced mammary cell proliferation.

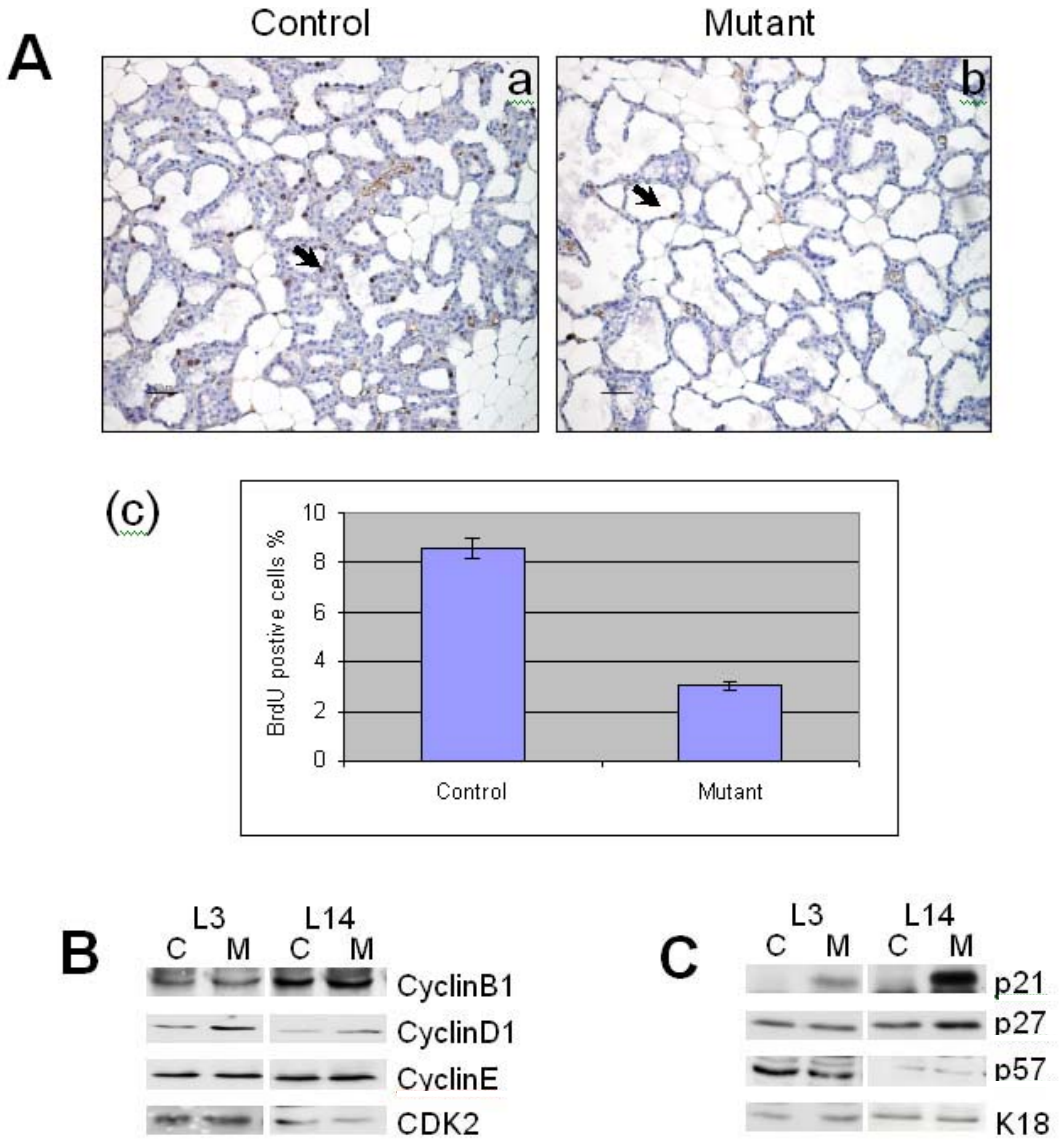

Figure 13. (A) For DNA labeling, 2-day lactating females were injected with BrdU 2 hr prior to sacrifice. Paraffin sections of mammary glands from control (a) and mutant β1 integrin mutant (b) mice were incubated with a monoclonal antibody against BrdU and stained with the ABC staining system. BrdU-positive nuclei (black arrows) were quantified (c) from 3 pairs of control and β1 integrin mutant 2-day lactating females. In each case, 900-1200 nuclei per section were counted. Data are the mean±S.D. (B & C) Analysis of cell cycle regulators from 3- and 14-day lactating mammary glands of control (C) and β1 integrin mutant (M) mice. (B) Western blotting analysis revealed that the level of Cyclins B1 and E and CDK2 were not altered in mutant mice; while Cyclin D1 expression was slightly increased. (C) Western blotting analysis revealed that the levels of $p27^{Kip1}$ and $p57^{Cip2}$ were not altered in mutant mice at lactation day 3; $p27^{Kip1}$ level was increased at lactation day 14; $p21^{Cip1}$ level was strongly increased throughout lactation; keratin 18 was used to control loading.

Fig.13 Loss of β1 integrin or FAK leads to upregulation of p21 in primary cells.

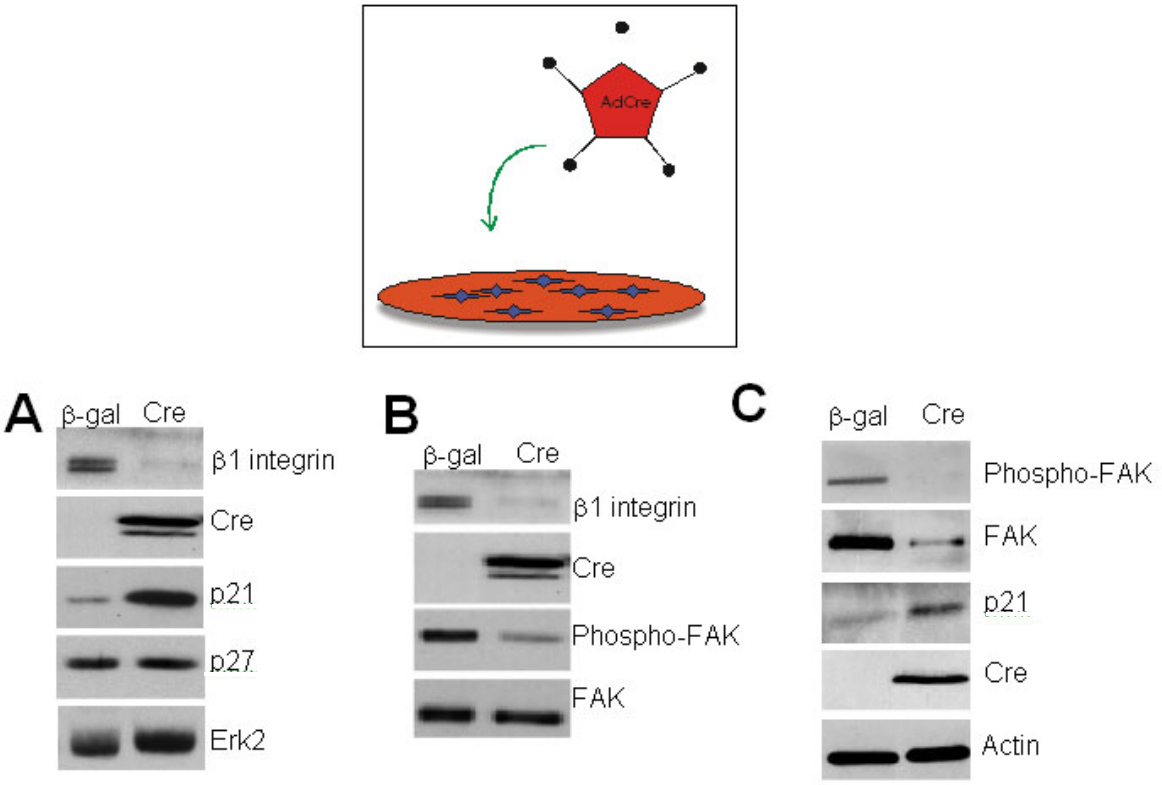

Figure.13. Loss of b1 integrin or FAK in primary mammary cells leads to upregulation of $p21^{Cip1}$. Primary mammary cells were prepared from pregnant $\beta 1^{flox/flox}$ mice (A,B) or pregnant $FAK^{flox/flox}$ mice (C) and infected with Adeno-β gal control virus or with Adeno-Cre-virus. The infection efficiency was nearly 100%, as attested to by staining parallel cultures with a Cre specific antibody. Cell lysates were prepared and analyzed by western blotting. The membranes were probed with antiserum specific for: (A) β1 integrin, Cre, $p21^{Cip1}$ and $p27^{Kip}$; Erk2 was used to control loading; (B) β1 integrin, Cre, PY-397 FAK and FAK. (C) PY-397 FAK, FAK, $p21^{Cip1}$ and Cre; actin was used to control loading.

Fig.14 Absence of p21^{Cip1} rescues the proliferation defect in β1 integrin mutant mice

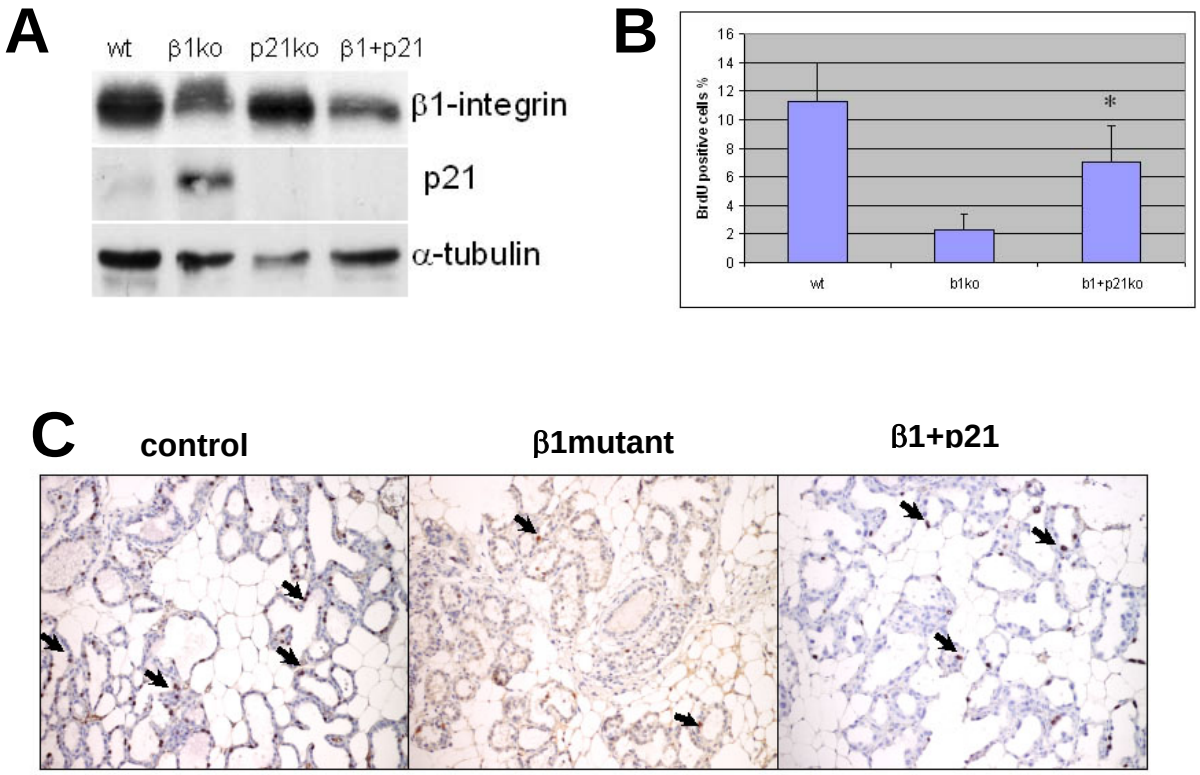

Figure 14. (A). Analysis the expression of integrin β1, p21cip1 from 2-day lactating mammary glands of control (wt), β1 integrin mutant (b1ko), p21komice and double integrin β1 mutant p21null (β1+p21) mice.

(B & C). Paraffin sections of mammary glands from 2-day lactating control, β1 integrin mutant and double p21^{Cip1}null; β1 integrin mutant mice were stained with a BrdU antibody. BrdU-positive nuclei (C, black arrows) were quantified (B). For control and β1 integrin mutant 3 of each were examined; for the double p21^{Cip1}null; β1 integrin mutants 7 mice were examined. In each case, 900-1200 nuclei per section were counted and data are the mean±S.D. The asterik denotes significant changes ($p \leq 0.05$) compared with β1 integrin mutant glands.

Fig.15 Loss of β1 integrin leads to upregulation of p21 in MDA-MB 231 cells.

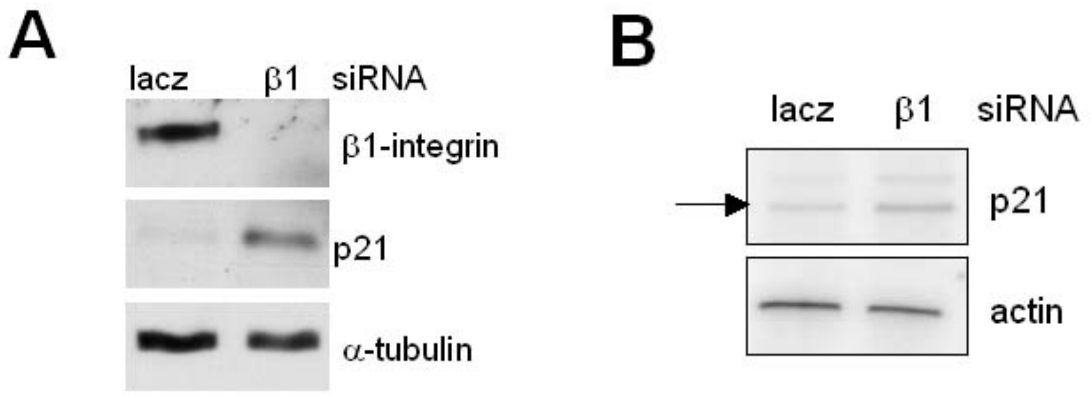

Figure 15. β1 integrin- or control lacZ- siRNA was transfected into MDA-MB-231 cells: after 4 days, (A) cell lysates were analyzed by western blotting and membranes were probed with β1 integrin, $p21^{Cip1}$ and α-tubulin specific antibodies; (B) total RNA was isolated and $p21^{Cip1}$ and actin RNA levels were quantified using specific PCR primers.

Fig.16 Loss of β1 integrin leads to mislocalization of p-FAK in MDA-MB 231 cells.

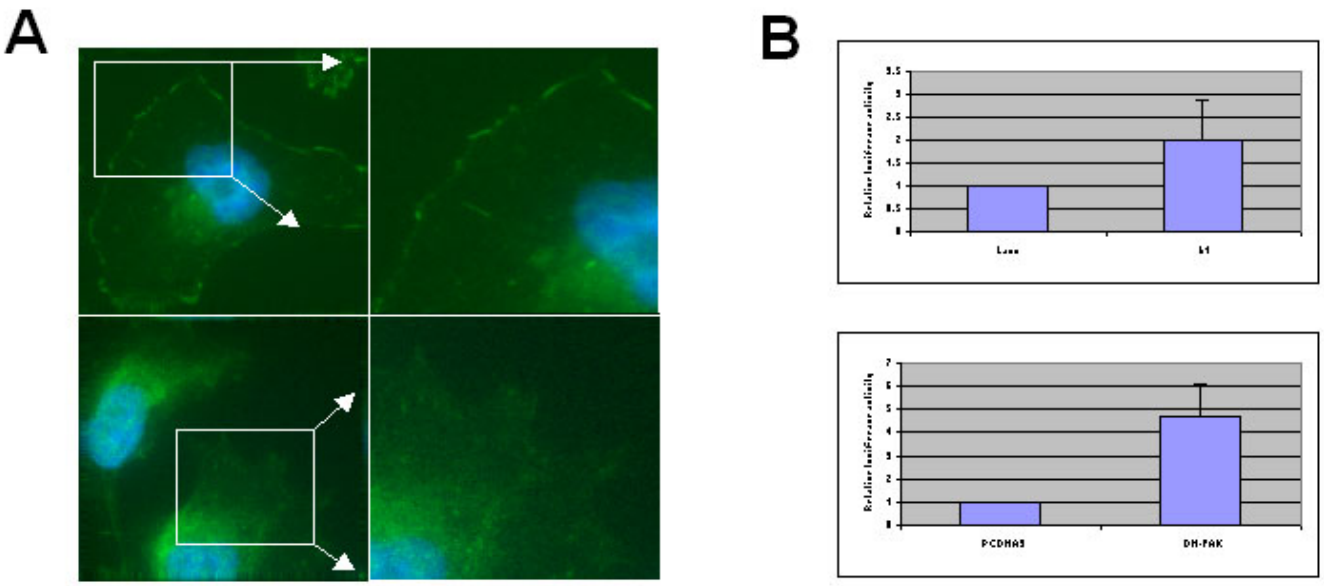

Figure 16. (A) After 3 days cells transfected with LacZ siRNA (upper panel) and β1 integrin siRNA (lower panel) were stained with PY-397-FAK antiserum. The boxed area is shown enlarged on the right. (B) (Upper panel) cultures were transfected 2 days with control LacZ siRNA or β1 integrin specific siRNA; then the WWP-Luc reporter plasmid plus control renilla plasmid were transfected and 2 days later $p21^{Cip1}$ promoter activity was measured. (Lower panel) $p21^{Cip1}$ promoter activity was measured 2 days following transient transfection of a FRNK expression plasmid (DN-FAK) or the control pCDNA3 plasmid together with the WWP-Luc reporter plasmid and the control renilla plasmid. In each experiment luciferase activity was normalized to the *Renilla* internal control.

Fig.17 $p21^{Cip1}$ was responsible for the anti-proliferative effects of β1 integrin loss.

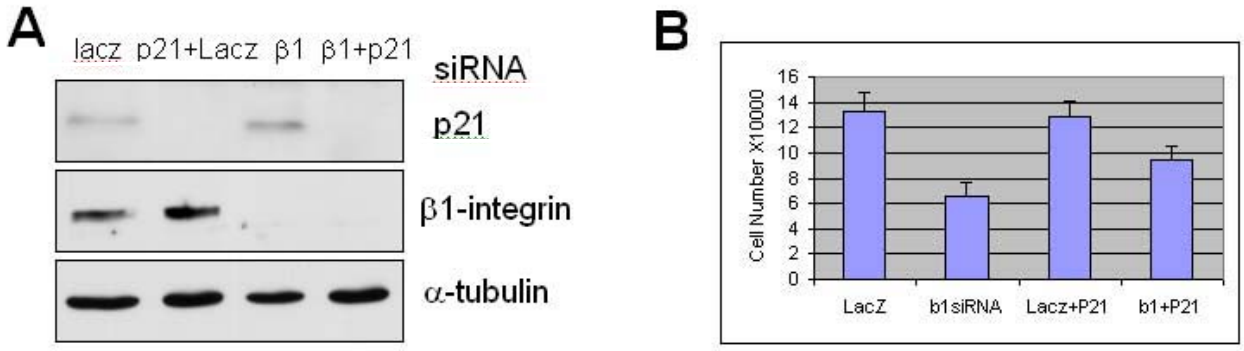

Figure 17. (A & B) Four days after transfection of siRNA for LacZ, β1 integrin, $p21^{Cip1}$ + LacZ, or β1 integrin + $p21^{Cip1}$ (A) MDA-MB231 cell lysates were analyzed by western blotting and membranes were probed with $p21^{Cip1}$, β1 integrin, and α-tubulin specific antibodies, or (B) cells were counted in triplicate. Data are presented as the mean ±S.D.

Fig.17 $p21^{Cip1}$ was responsible for the anti-proliferative effects of β1 integrin loss.

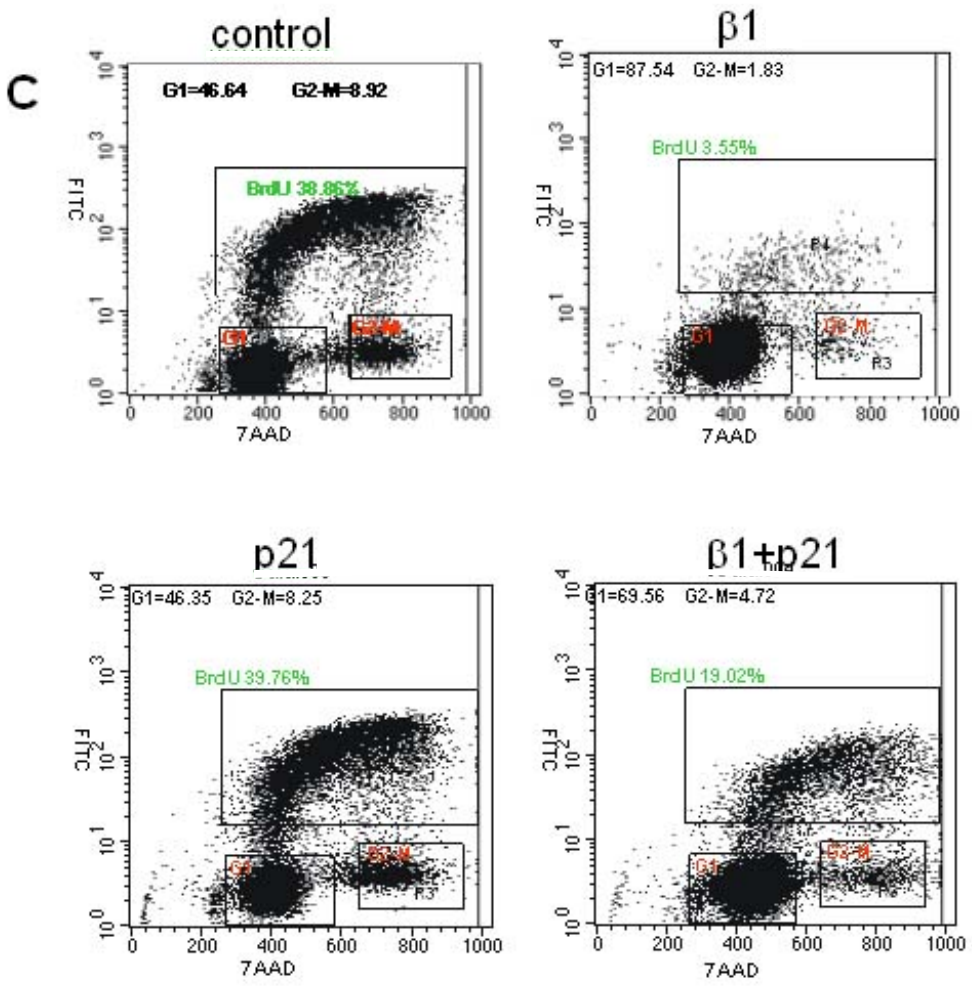

Figure 17. (C) MDA-MB231 cells were transfected with LacZ siRNA and/or β1 integrin siRNA for 3 days, then labeled with BrdU for 1hr before collecting. Harvested cells were stained with anti-BrdU-FITC to quantitate cells in S-phase and with 7AAD to stain DNA before flow cytometry was performed. The percent cells in G1 and G2-M is indicated in the top of each panel; the percentage of cells that incorporated BrdU is indicated in the box. Data are presented as the mean±S.D.

Fig.18 A possible role for β1 integrins in the maintenance of mammary stem cells.

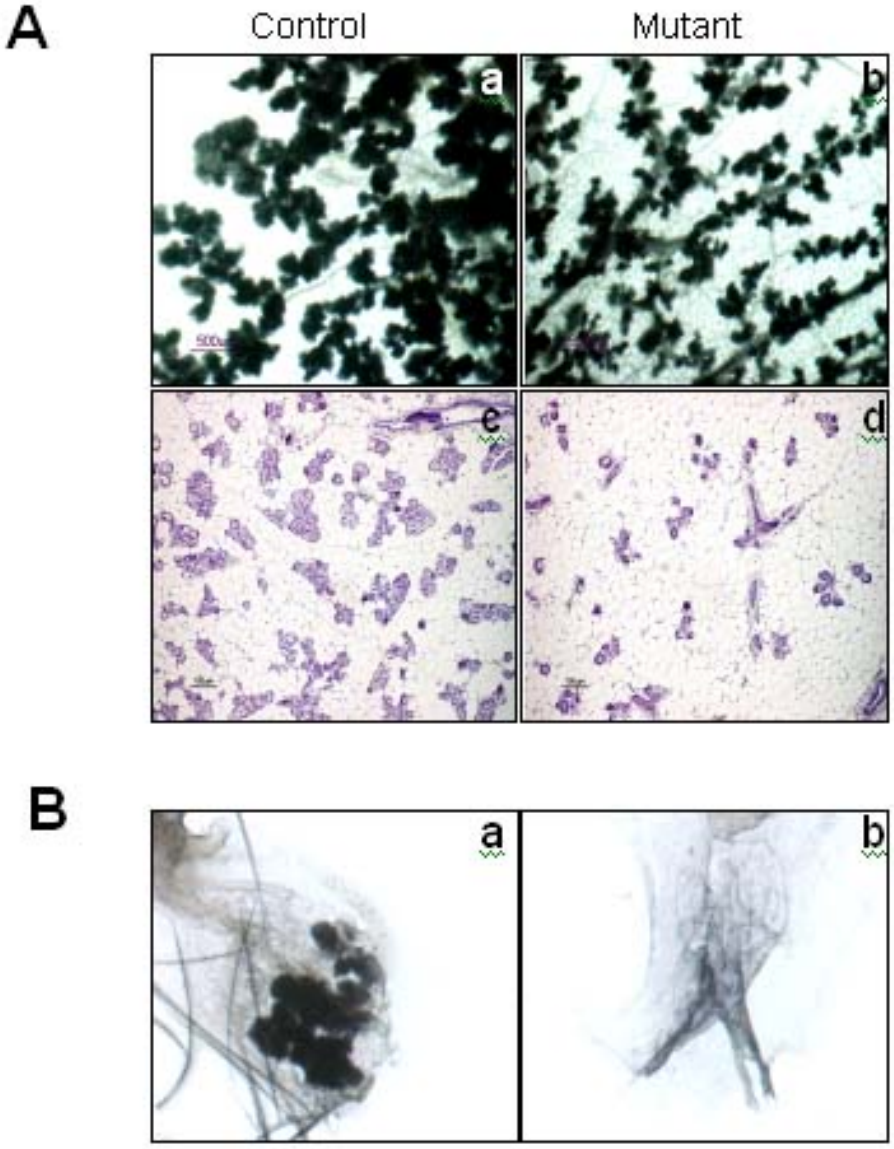

Figure 18. (A) Whole mount analysis (a-b) or histological analysis (c-d) of mammary glands taken from control and β1 integrin mutant females at day 16.5 of a second pregnancy. (Magnifications: a & b - 6X; c & d -10X). (B) Whole mount analysis of mammary transplant outgrowths. Pieces of mammary tissue taken from 3-day lactating control mice (a) or β1-integrin mutant mice (b) were transplanted into cleared fat pads of 21-day old syngeneic females. After 10 weeks the recipient female mice were mated and at pregnancy day-16 glands were removed and examined by whole-mount analysis (8X).

Fig.19 β1 integrins play multiple roles in the development of mammary gland.

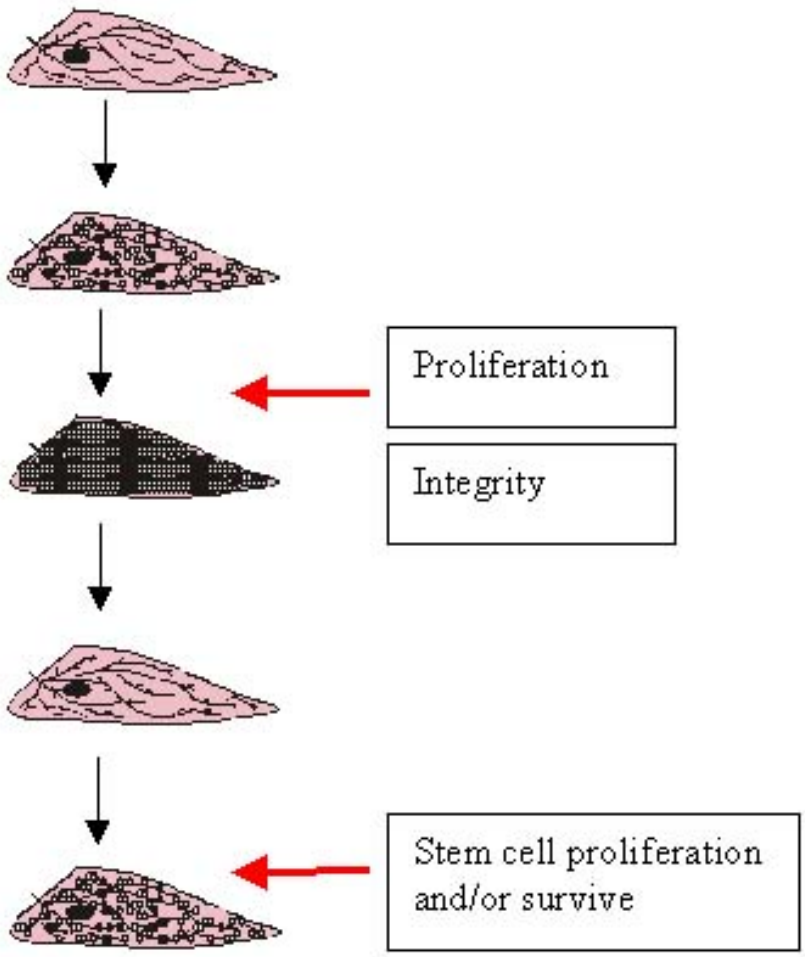

Figure 19. A model showing β1 integrins have key roles at several stages during the development and function of the mammary gland. Firstly, It could regulate the proliferation of mammary alveolar cells at early lactation. During involution and tissue remodeling, it might participate in the mammary progenitor cell proliferation and/or survive. As an important cell adhesion receptor, it could involve in the glandular morphogenesis and maintenance of mammary gland integrity throughout lactation.

Conclusion

- **Integrin β1 was specifically deleted in the mammary luminal secretory cells after cre mediated recombination.**
- **In the absence of β1 integrin, the alveolar architecture was impaired and cells were shed into the lumen.**
- **The normal proliferative burst at the start of lactation was severely reduced in the absence of β1 integrin, which correlated with specifically increased $p21^{Cip1}$ expression.**
- **Ablation of β1 integrin impairs focal adhesion signaling**
- **In mammary primary cells, ablation of β1 integrin or FAK could impair focal adhesion signalling and upregulate $p21^{cip1}$ expression.**
- **Ablation of β1 integrin in a $p21^{Cip1}$ null background partially rescued the proliferative defect in vivo.**
- **In MDA-MB-231 cells, loss of integrin β1 caused a G1 block and $p21^{cip1}$ upregulation, and knock down of $p21^{cip1}$ could partially rescue this proliferation defect.**
- **Transplanted mammary tissue from β1 integrin mutant females failed to repopulate recipient mammary glands.**

Discussion

In this report we present the first detailed molecular analysis of mice with a mammary gland-restricted disruption of the β1 integrin-encoding gene. WAPCre-mediated recombination of β1 integrin occurred specifically in luminal alveolar cells in late pregnancy and lactation, a time when several phenotypes became apparent. Firstly, luminal secretory cells of many alveolar structures became disorganized. Cells became detached from the basement membrane and were shed into the lumen of the gland; suprisingly, these cells were not apoptotic. Secondly, luminal cell proliferation was reduced strongly and $p21^{Cip1}$ levels were elevated dramatically in the β1 integrin mutant glands. A link between β1 integrin, FAK, $p21^{Cip1}$ and decreased cell proliferation was established not only *in vivo*, but also *in vitro* in both primary mammary cells and an established cell line. Finally, transplanted mammary tissue from β1 integrin mutant mice was unable to grow in recipient hosts, suggesting a possible role for β1 integrins in stem cell maintenance. Based upon these results we propose that β1 integrins have key roles at several stages during the development and function of the mammary gland (Fig.19).

The major functions of integrins are to connect the ECM to the cytoskeleton in order to propagate structural order between the two and to activate signalling cascades controlling cellular behavior (Giancotti and Tarone, 2003). The phenotypes we present here are consistent with these two main functions. We have also observed some differences to published results, e.g., with respect to apoptosis and laminin deposition following β1 integrin loss (Faraldo et al 1998). These variations are very likely due to the strategy we employed, for example genetic compared to implantation of β1 blocking antibody-containing pellets (Klinowska et al., 1999) or a DN approach (Faraldo et al 1998). Furthermore, the use of a different Cre transgene has allowed the effect of β1 integrin loss to be examined at another developmental stage. Interestingly, ablation of β1 integrin using the MMTV-Cre transgene, which is expressed in the virgin gland, did not lead to alterations in mammary gland proliferation (White et al., 2004). Similar to our results, the MMTV-dominant-negative β1 transgenic strain (Faraldo et al 1998) also displayed a proliferation defect at lactation; however, the responsible cell cycle regulator was not identified.

Functional differentiation was normal in integrin β1 mutant glands

Although we observed function impairment of mammary gland in β1 integrin mutant mice as measured by pup weight ananlysis (Fig.4), based upon molecular and morphological criteria, functional differentiation of mammary glands lacking β1 integrin was normal. Although there was a slight decrease in Jak2 and Stat5 activation (Fig 5B), this did not lead to alterations in milk protein gene expression (NL, unpublished observations), which is also consistent with the apical expression of Npt2b, a marker of functional mammary secretory cells (Fig.8, panel c&d). Furthermore, casein micelles and milk-fat droplets are evident in the milk of β1 integrin null females (Fig 9A). These results were to some extent unexpected based upon published in vitro (Barcellos-Hoff, 1989)(Streuli, 1995) and in vivo (Faraldo, 1998) results. However, it has been observed that many organs are able to differentiate in the absence of β1 integrin (Bouvard, 2001). For example, keratinocytes display altered morphology and reduced proliferation in the absence of β1 integrin, however, are still able to terminally differentiate (Brakebusch, 2000).

Moreover, WAPicre expression commences at late-pregnancy and it is possible that in most of the luminal cells, the differentiation controlling genes are already turned on before the recombination occurs. Furthermore, the integrin β1 is only absent in the luminal epithelial cells and still remained in myoepithelial cells in the mutant glands. Although the luminal epithelial cells are main functional cell type, the myoepithelial cells which help in milk injection still contain β1 integrin and its interaction with the basement membrane may play a role in mataining the differentiation of the organ.

Thus, we favor the hypothesis that the observed function impairment of the mammary gland in β1 integrin mutant mice (Fig.4) is not due to a differentiation defection, but due to the decreased mammary epithelial proliferation and impaired mammary gland integrity (disscussed below).

β1 integrin deletion results in loss of epithelial integrity

1. Detachement from the basement membrane

We show here for the first time that β1 integrin ablation triggers a loss of epithelial integrity in the alveolar structures of the lactating mammary gland. Alterations included areas of cells protruding from the luminal surface and the appearance of shed cells in the lumen, while basement membrane was normal in β1 integrin mutant glands.

It is well known that one of the main functions of integrins is to mediate adhesion between the cells and the extracellular matrix. Indeed, in the adhesion assay, we found that primary luminal epithelial cells

prepared from β1 mutant mammary gland showed defective adhesion specifically on laminin, one of the main components of the basement membrane. Therefore, it is not surprising that *in vivo* cells start to be shed after lossing this anchor. For example, skin and hair follicle integrity is crucially dependent on β1 integrin expression on keratinocytes (Brakebusch, 2000). However, in mammary gland, this event only became visible after the lactation-day-3, at that time the WAPiCre has the peak Cre expression, and became very obvious after lactation day 5, when about 14% of the luminal cells were in the process of shedding or lost in the lumen (Fig.7h). We suggests that this phenotype is not a simple event happening only because cells have become detached, but may need other signals to occur. For example, we suspect that integrin β4 may play some roles to protect cells from being shed because more hemi- desmosomes could be formed after loss of integrin β1 because integrin β4 could compete α6 subunit (to form hemi-desmosomes) with integrin β1.

2. Relocalization of integrin β4 subunit

In the mammary epithelial cells, there are two main integrin β subunits, integrin β1 and integrin β4. Integrin β1 is present at the basolateral surfaces of both myoepithelial and luminal epithelial cells. The β4 is only present at the basal aspect of these two cell types. After ablation of integrin β1, we observed that luminal and myoepithelial cells of control and β1 integrin mutant glands display β4 integrin on their basal surfaces (Fig.8i). In addition β4 integrin was detected on the apical surface of some luminal cells in the β1 integrin mutant glands (Fig.8j). The mislocalization of integrin β4 was also observed in dominant negative integrin β1 transgenic mice where integrin β4 was revealed at the basal cell surface and, in addition, co-localized with laminin at the lateral surface of luminal epithelial cells (MA.Glukhova, 1998). We didn't observe the relocalization of laminin. The relocalization of integrin β4 revealed defects in cell polarization and may suggest the importance of integrin β1 in cell–cell junctions.

3.Decreased cell-cell association

The importance of β1 integrin in promoting both cell-extracellular matrix and cell-cell interactions is well known from, for example, the kidney (Ojakian and Schwimmer, 1994; Schoenenberger et al., 1994) and keratinocytes (Symington et al., 1993; Larjava et al., 1990). In control mammary glands, β1 integrin was present on basal and lateral surfaces of luminal cells; its absence in the mutant glands very likely contributes to weakened cell-cell interactions. E-cadherin is another protein that might be involved in

maintenance of alveolar integrity. In control glands, E-cadherin was found on lateral surfaces of most luminal cells (Fig.8g), while in mutant glands many luminal cells had little or no staining, reflecting the decrease in E-cadherin levels (Fig.11D). Conditional knock-out of E-cadherin in the mouse mammary gland revealed that E-cadherin is a survival factor since massive cell death was observed at parturition in the mutant glands (Mechanisms of Dev. 115(2002) 53-62, Rolf Kemler, 2002). Furthermore, β-catenin expression was not changed at early lactation but was decreased at lactation day 14 (Fig.11D). Integrin-dependent BM adhesion is known to reinforce E-cadherin cell-cell adhesion (Schreider et al., 2002) and catenin complex formation (Weaver et al., 1997). There was also a decrease in the tight junction protein ZO-1 late in lactation (data not shown), which might be a consequence of reduced E-cadherin levels since assembly of tight junctions depends upon E-cadherin recruitment to adherens junctions (Cereijido, 2000)
Thus, these data suggest that in the mammary gland β1 integrin function has an essential role in normal tissue architecture via its interactions with other adhesion complexes.

4.Interferance of actin cytoskeleton

Phalloidin was used to visualize actin, since β1 integrins connect the ECM to the actin cytoskeleton. Actin stress fibers running along the basal surface of the cells were evident in sections from control glands (Fig.8, panel k). In contrast, there were no stress fibers evident in β1-integrin mutant glands. Furthermore, strong actin staining was also evident in some lateral and apical surfaces (Fig.8, panel l), suggesting that in the absence of luminal β1 integrins, the actin cytoskeleton has become disorganized. Integrin-dependent BM adhesion is known to cooperate in F-actin cytoskeleton organization (Schreider, 2002) (Wang, 1999) Thus, these data suggest that in the mammary gland β1 integrin function has an essential role in normal tissue architecture via its interactions with other adhesion complexes and with the actin cytoskeleton.

5. Is the impaired milk secretion due to the change of the structural integrity?

Interestingly, functional analysis showed that the pups fed by mutant mothers had reduced pup weight in comparison to the pups fed by wild type mothers. However, functional differentiation as measured by milk protein expression was normal in integrin β1 mutant glands, while preliminary data showed that the milk secretion in mutant glands might be impaired. Based on the functional analysis and the structural integrity results, we suggest that the reduced weight observed in pups nursed by mutant mothers might be due to the impaired milk secretion. This impairment in turn might be caused by the altered structural integrity of the alveoli in mutant mammary glands.

Loss of β1 integrin is not sufficient to trigger apoptosis in alveolar cells.

Despite the evidence for weakened cell-BM interactions in the absence of β1 integrin, there was no obvious increase in apoptotic cells during lactation. Even cells shed, or in the process thereof, were alive, as attested to by their nuclear appearance and the lack of TUNEL staining (Fig 9 & data not shown). These results are in contrast to those described for primary mammary cells, which are dependent upon β1 integrin signaling for survival (Boudreau et al., 1995; Streuli et al., 1995) as well as transgenics expressing the DN β1 transgene (Faraldo et al 1998). In a similar vein, in the absence of β1 integrins basal keratinocytes underwent *in vitro*, but not *in vivo* apoptosis (Brakebusch et al., 2000). *In vivo*, there is likely to be a balance, which is well attuned to the environment, between apoptotic and anti-apoptotic factors. Primary cells could be more susceptible to apoptosis since they are removed from their normal environment. Indeed, microarray analyses carried out on mammary glands from 3-day lactating mice, a time when luminal cell-shedding began, revealed that in mutant glands anti-apoptotic genes, such as thioredoxin-dependent peroxide reductase (Ueda et al., 2002) were up-regulated, and pro-apoptotic genes including caspase-11 (Hisahara et al., 2000) were down-regulated (NL, data not shown). Western Blotting analysis also revealed the alteration in the regulations of two pro-apoptotic genes: STAT3 and Bax. STAT3 is pro-apoptotic and is a crucial mediator of post-lactational regression (Chapman et al., 1999). Using conditional gene targeting, it has been shown that in the absence of STAT3, involution is delayed for several days, owing to a reduction in apoptosis (Humphreys RC, et. al. 2002). Bax, a proapoptotic member of the Bcl-2 family of proteins, was first discovered in a screen of proteins that exhibited binding interactions with Bcl-2. Bax is likely to have pore-forming activity in the mitochondrial membranes, subject to control or prevention by association with specific antiapoptotic molecules (especially Bcl-2 and Bcl-x_L), related to its ability to bind to BH-3 domain-only containing Bcl-2 family member proteins, and induce the release of mitochondrial cytochrome *c* (Willis S. et al. 2003). In the absence of integrin β1, the expression of Bax was increased throughout lactation, while the phosphorylation of STAT3 was decreased throughout lactation (Fig.11, panel A). We also examined the serine/threonine kinase PKB, which transmits survival signals triggered by integrin-mediated adhesion (Datta et al., 1999). The activity of this kinase was assessed using an antiserum specific for the active, phosphorylated form. There was no difference in the level of Ser 473 phosphorylated PKB in extracts from lactating glands of β1-integrin mutant mice compared to control mice. Moreover, phosphorylation of Ser 9 of glycogen synthase kinase

3-β (GSK3β), a downstream target of PKB, was not altered in β1-integrin mutant mammary glands (Fig.11, panelB).

Finally, it has been documented that only a combination of unfavorable conditions induces apoptosis. For example, HC11 mammary cells when released from a laminin-rich BM (Chammas et al., 1994) become apoptotic only if exposed to serum-free medium; EGF-containing medium allowed survival of the released cells (Merto et al., 1997). *In vivo*, it is possible that other signals combined with loss of BM attachment could abolish the balance and promote apoptosis. Indeed, at the start of involution, there was a slight increase in TUNEL-positive cells in β1 integrin mutant glands, compared to control glands (Fig.10). The level of the pro-apoptotic Bax protein was also elevated in mutant glands (NL, unpublished observations). Using a conformation specific antibody, it was observed that at the start of involution there is a decrease in β1 integrin-BM interaction (Prince et al., 2002). Building upon these results and our current observations, Our results suggest that *in vivo*, cell detachment can be uncoupled from apoptosis and loss of β1 integrin in mammary luminal cells is not sufficient to trigger cell death.

β1 integrins have a key role in the proliferation of mammary epithelial cells *in vivo* and in culture

1. Integrins and proliferation control

Cells require anchorage to ECM to proliferate; β1 integrin is known to have an important role in modulating the activity of signaling pathways that regulate proliferation in many cell types, such as in basal keratinocytes (Brakebusch, 2000 & more details in introduction part I and III). In addition to the alveolar integrity impairment, the other major phenotype observed in β1 integrin mutant mice was a proliferative defect. Proliferation of secretory luminal cells occurs during pregnancy and early in lactation. We did not observe a consistent decrease in the number of BrdU incorporating cells at mid-pregnancy (NL, unpublished observations), likely due to the fact that WAPCre expression only commences at this developmental stage. However, our results clearly show that early in lactation there is a strong decrease in alveolar cell division in the absence of β1 integrin (Fig 12).

2. CDK inhibitors

Cell cycle progression is controled by cyclins and CDKs. A key event in the G1 phase of cell-cycle progression is hyperphosphorylation of the retinoblastoma (Rb) protein by active cyclin-CDK complexes, leading to release of E2F-family transcription factors from their complex with Rb. In addition to cyclin

binding, the activity of the G1 phase cyclin-CDKs is affected by the action of specific CDK-inhibitors (CKIs). It was shown that integrin signals are necessary for cells to traverse the cell division cycle, a loss of its signaling correlates well with increased levels of different CDK inhibitors. β1 deficient chondrocytes show a defect in the G1/S phase transition, which is, accompanied by upregulation of the CKIs $p16^{Ink4a}$ and $p21^{Cip1}$ (Aszodi et al., 2003). Ablation of β1 integrin in developing cerebellum revealed its role in cerebellar granule cell precursor (CGP) proliferation. In the absence of β1 integrin the CGP pool failed to expand due to $p27^{Kip1}$ upregulation (Blaess et al., 2004). Here we show that there is a specific increase in $p21^{Cip1}$ levels in lactating mammary glands from β1 integrin mutant mice. Morover, we have also provided *in vivo* evidence that $p21^{Cip1}$ is responsible for the anti-proliferative effects of β1 integrin loss. In the absence of $p21^{Cip1}$ and β1 integrin there was a partial, but statistically significant rescue in the proliferative defect (Fig 14). Using the MDA-MB-231 cells we also showed that simultaneous knockdown of $p21^{Cip1}$ in β1 integrin knockdown cells partially rescued the G1 cell cycle block induced by loss of the integrin (Fig 17). Since we observed both upregulation of Bax and $p21^{cip1}$, the downstream target gene of p53, throughout lactation, we also checked the expression of p53. After loss of integrin β1, the expression of p53 was down-regulated at early lactation and increased at late lactation. We also checked another target gene MDM2 and there are no difference of p-MDM2 or total protein expression between the control glands and the mutants. In the future it will be important to verify whether p53 play a role in the upregulation of $p21^{Cip1}$ after loss of integrin β1.

3. The role of Focal adhesion kinase (FAK) in proliferation.

FAK plays an important role in integrin-mediated signaling (Geiger et al., 2001; Parsons et al., 2000). We observed that phospho-FAK levels were decreased in β1 integrin mutant mammary glands (Fig 4E). Paxillin, a focal adhesion associated protein is a target for tyrosine kinases that are activated as a result of integrin signaling after cell adhesion. FAK in association with Src, which binds activated FAK via a SH2-Tyr397 association, phosphorylates paxillin on two major sites, one of which (Tyr118) we show here is strongly decreased in the β1 integrin mutant mammary glands (Fig 11C).

Studies carried out with the transgenics expressing dominant β1 integrin also reported that the proliferation rate was decreased at the begining of lactation. However, they showed that this growth defect induced by perturbation of β1-integrin function in the mammary gland epithelium result from a lack of MAPK activation via the Shc and Akt pathways, but the kinase activity of FAK was normal. In our studies,

we could not find consistent change in p-MAPK level (Data not shown); and the AKT pathway appears to be normal in mutant glands (Fig. 11b).

We consider it likely that FAK kinase provides the link between β1 integrin, $p21^{Cip1}$ and decreased proliferation. Firstly, using primary mammary cells from $Itg\beta 1^{flox/flox}$ and from $FAK^{flox/flox}$ mice we showed that Cre-mediated recombination of each gene led to impaired focal adhesion signalling and an increase in $p21^{Cip1}$ expression (Fig. 13). Secondly, siRNA-mediated knockdown of β1 integrin in MDA-MB-231 cells led to PY-397-FAK relocalization, increased $p21^{Cip1}$ expression and decreased proliferation. Expression of the DN FAK protein, FRNK (Richardson et al., 1997), in these cells induced the same phenotype. Finally, we determined that $p21^{Cip1}$ was responsible for the anti-proliferative effects of β1 integrin loss since simultaneous knockdown of $p21^{Cip1}$ in β1 integrin knockdown cells rescued the proliferative defect induced by loss of the integrin.

To date there is no physiological evidence linking FAK to $p21^{Cip1}$ upregulation. In fibroblast cells, FAK has been directly linked to cell division, which correlates with changes in the expression of cyclin D1 and the cdk inhibitor, $p21^{Cip1}$; while cyclin D1, but not $p21^{Cip1}$, was thought to be the primary functional target of FAK signaling pathways in cell cycle regulation (Zhao, 1998; Parsons et al., 2000). We showed here, both *in vivo* and *in vitro*, that β1 integrin has a key role in regulating cell cycle progression of luminal mammary epithelial cells, and we propose that β1 integrin signaling via FAK stimulates mammary alveolar cell proliferation, while in the absence of the integrin, $p21^{Cip1}$ transcription is stimulated leading to a proliferative block.

To date we have not found the molecular link the FAK kinase and $p21^{cip1}$. A possible candidate Hic-5 , a LIM protein with striking similarity to paxillin, has been checked in the studies, because it was reported that Hic5 could shuttle between focal adhesion sites and nuclear and it functions as a potential coactivator for Sp1 (shibanuma, 2004). Western Blotting analysis showed that the expression of Hic-5 was increased in the mutant glands during lactation. However, by immunostaining, we couldn't find obviously nuclear translocation of Hic-5 in both mammary sections and MDA-MB-231 cells (Data not shown).

A possible role for β1 integrins in the mammary stem cells

Our results also suggest that β1 integrin has an important role in proliferation and/or maintenance of mammary alveolar progenitor cells. Mammary glands taken from females undergoing a second round of

pregnancy showed a strong reduction in the density of lobulolalveolar units, suggesting that there is a reduction in the number of progenitor cells that enable rapid alveolar expansion (Fig 7A). Using the mammary transplantation technique, which allows a functional identification of mammary stem cells by measuring their *in vivo* outgrowth potential (Smith, 1996; Kordon and Smith, 1998), we could show that epithelium from β1 integrin mutant glands had a severe impairment in its ability to repopulate a mammary fat pad. These results strongly suggest that alveolar progenitors have an impaired ability to proliferate and/or suvive in the absence of β1 integrin. Considering the known role of integrins in maintenance of stem cells in, e. g, hair cell follicles (Watt, 2002), it is tempting to speculate that β1 integrins might also have a similar role in the mammary gland.

Interestingly, we observed a specific upregulation of $p21^{cip1}$, one of the CDK inhibitors, in the mutant gland. It's known that $p21^{cip1}$ is not only a protein that binds and inhibits cyclin-dependent kinases, but also recognized to play an important role in DNA repair, apoptosis, cellular senescence, terminal differentiation, and cell cycle arrest upon extracellular signalling (Poole AJ, et al. 2004). However, the molecular events orchestrating the role of $p21^{cip1}$ remain largely undefined in stem cells. For example, relative to keratinocytes from wild-type mice, keratinocytes derived from p21cip1-/-mice contain a significantly increased number of cells with clonogenic potential and high rates of attachment, two interrelated properties which have been directly connected with label-retaining stem-cell populations. These subpopulations have a lesser commitment to differentiation and generate all types of terminally differentiated keratinocytes that are present *in vivo*, not only in the interfollicular epidermis but also in the hair follicles (Topley GI, et al. 1999). On the other hand, $p21^{cip1}$ was reported as a stem cell marker in human breast epitheial cells. These cells with Hoechst dye-effluxing "side population" (SP) properties, characteristic of mammary stem cells in mice, were demonstrated to be undifferentiated "intermediate" cells by lack of expression of myoepithelial and luminal apical membrane markers. These SP cells were 6-fold enriched for ERα-positive cells and expressed several fold higher levels of the ERα, $p21^{cip1}$ and Msi1 genes than non-SP cells (Clarke RB, et al. 2005). In addition, $p21^{cip1}$ might play a role in stem cell surive. For example, $p21^{cip1}$ rescues human mesenchymal stem cells from apoptosis induced by low-density culturing (van den Bos. et al. 1998). Thus, we propose that loss of integrin β1 could affect the proliferation and/or survive of mammary stem cells, and $p21^{cip1}$ might be involved in this process. However, the functions and mechanisms underline it needs to be determined in the future.

β1 integrins in breast cancer

It is well known that integrins have important roles in cancer cell biology (Christofori, 2003). Activation and elevated expression of β1-integrin-coupled signaling effectors have been implicated in the induction of a wide variety of human cancers, including those of the breast, colon, prostate, and ovaries. In addition, the overexpression of β1-integrin-associated molecules such as ILK can result in the induction of mammary tumors in experimental mouse models (White et al. 2001). With respect to breast cancer, β1 integrin blocking antibodies induced a morphological and functional reversion of breast tumor cells growing in a 3D culture model (Weaver et al., 1997). The same antibody also blocked proliferation of MDA-MB-231 tumor cells (Wang et al., 2002). Building on these studies, we show here that in the absence of β1 integrin these cancer cells arrest in G1 due to up-regulation of $p21^{Cip1}$. Very recently the role of β1 integrin was also examined in a transgenic mammary tumor model, where it was shown that loss of this integrin interferes with the ability of polyoma middle T (MT) expressing mammary cells to proliferate (White et al., 2004). This was manifested *in vivo* by the absence of MT-induced hyperplasias/tumors in mammary glands. Based upon our results we propose that $p21^{Cip1}$ upregulation in response to β1 integrin loss might be the mechanism underlying the block in tumor cell proliferation.

Materials and Methods

Antibodies

Antibodies used for immunostaining were: α4 β1 integrin and laminin-1 (Klinowska et al., 1999), β4 integrin (Zymed), E-cadherin (BD Transduction Laboratories), PY-397-FAK (Biosource), Cre (Wintermantel et al., 2002), α−smooth-muscle actin (Sigma), BrdU (Roche), Npt2b (a gift from J.Biber, Univ Zurich)(Hilfiker et al., 1998), phalloidin-TRITC (Sigma), FITC-labeled and TRITC-labeled secondary antibodies (Molecular Probes).

Antibodies used for western analyses were: β1 integrin (Graus-Porta et al., 2001); β-catenin (BD Transduction Laboratories), FAK (Upstate Biotechnology); $p21^{Cip1}$ $p57^{Kip2}$, cyclin E, cyclin B1, Cdk2, Bax, P53 (Santa Cruz), cytokeratin 18 (PROGEN), α−tubulin (Neomarkers); PY-397-FAK (Biosource); E-cadherin, $p27^{Kip1}$ (Transduction Labs), cyclin D1 (NoVoCastro); Vinculin (Sigma). .), p-stat5(Upstate Biotechnology), stat5a polyclonal antiserum (JBC Volume 271, Number 50, Issue of December 13, 1996 pp. 31863-31868),

Mouse strains and generation of mammary specific β1 mutant mice.

The $Itg\beta1^{flox}$ mouse strain, in which the first coding exon of the integrin β1 subunit gene was flanked by two loxP sites, has been described (Graus-Porta et al., 2001) (Fig 1A panel a). Mice expressing Cre under the control of the whey acidic protein (WAP) gene promoter harbor a P1-derived bacterial artificial chromosome (PAC) carrying the improved coding sequence of Cre recombinase (WAPiCre) (Wintermantel et al., 2002) (Fig. 1A panel c). $Itg\beta1^{flox/flox}$ mice were mated with mice heterozygous for the WAPiCre transgene on a $Itg\beta1^{flox/+}$ background. Offspring were genotyped by PCR analysis using genomic DNA prepared from tail biopsies. Littermates with the genotype $Itg\beta1^{flox/flox}$; *WAPiCre-/-* (referred to as control mice) or $Itg\beta1^{flox/flox}$*; WAPiCre*+/- (referred to as β1-integrin mutant mice) were used for all analyses. β1-integrin mutant mice were generated in accord with Mendelian ratios. For the *in vivo* rescue experiment, $p21^{Cip1}$ null mice (Jackson Laboratory) were crossed with $Itg\beta1^{flox/+}$*;*$WAPiCre^{+/-}$ mice, to produce $Itg\beta1^{flox/+}$*;*$WAPiCre^{+/-}$;$p21^{+/-}$ and $Itg\beta1^{flox/+}$;$p21^{+/-}$ mice, which were further intercrossed.

In order to initiate forced involution, litters of at least 6 pups were removed from β1-integrin mutant females after 9 days of lactation (Prince et al., 2002) and the mammary glands were isolated and analyzed at the specified times. The mice were maintained and handled according to the Swiss guidelines for animal safety.

Pup Weight Analysis

We mixed newborn pups from wt or k.o. Mothers and give to eight (1st Lactation) or six (2nd lactation) litters to pseudo-mothers. The body weight increase was documented for each pup from lactation day 2 to 20. The average body weight of the litters was calculated as means±SD.

Milk Annalysis

Pups were removed for 3-4 h before milking the mothers. Mice were anesthetized with NARKETAN+Rompun (100μL/10 g body weight) and injected intraperitoneally with Oxytoxin (0.3IU; Sigma) in 200μL PBS. After a 5-10-min incubation period milk was withdrawn with a Pasteur pipette. Diluted milk was analyzed with 15% SDS-PAGE gel.

Mammary gland whole mounts

Inguinal mammary glands were dissected, spread onto a glass slide and fixed overnight in Tellyesnicky's Fixative. The slides were rinsed in water, the tissue was defatted with acetone, hydrated through graded alcohol, and stained with Iron-haematoxylin for 1.5 h, then washed in water, dehydrated, and mounted.

Histology and immunofluorescence

Inguinal mammary glands were dissected, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, then embedded in paraffin or frozen in optimal cutting temperature compound (OCT, Tissue Tek) for sectioning. Immunohistochemistry was performed on 8 μm cryosections that were fixed in 4% paraformaldehyde. The sections were stained for times ranging from 1 hr to overnight with the primary antibody that was diluted in blocking solution. H & E staining was carried out on paraffin sections of 5 μm thickness using the tissue stainer COT 20 (Medite). For BrdU labeling, 2-day lactating females were intraperitoneally injected with 100 μg BrdU (Sigma)/g body weight 2 hr prior to sacrifice. Paraffin sections of the mammary glands were incubated with a monoclonal antibody against BrdU and stained with a mouse ABC staining system (Santa Cruz). For the detection of cell apoptosis, paraffin

sections were TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) -stained using the In Situ Cell Death Detection Kit, AP (Roche).

Electron microscopy

Pieces of mammary gland from 14-day lactating females were fixed in 3% paraformaldehyde, 0.5% glutaraldehyde in 10 mM PBS (pH 7.4) for 1 hr. The tissue was rinsed in PBS then post-fixed in 1% osmium tetroxide in PBS for 1 hr and dehydrated in an ascending series of ethanol solutions followed by 15 min in acetone. Specimens were infiltrated with Epon:acetone (1:1) then Epon:acetone (2:1) for 1 hr each, followed by pure Epon for 4 hr. Polymerization was carried out at 60° C for 24-48 hr. Ultrathin sections were cut, mounted and contrasted with 6% uranyl acetate for 60 min, followed by lead acetate for 2 min. Sections were viewed under a Leo 910 transmission electron microscope.

Isolation and assays with primary mammary cells

Primary mammary cells were prepared from control and β1 integin mutant females (Pullan, 1996a). The cultures consist of > 80% epithelial cells, as attested to by pan-keratin staining. Freshly prepared cells were used to perform adhesion assays: 7 x 10^4 cells were plated in 96 well dishes pre-coated with poly-D-Lysine (500 μg/ml, Sigma), collagen type I (50 μg/ml, Roche), fibronectin (25 μg/ml, Gibco), laminin (20 μg/ml, Sigma) or gelatin (1 mg/ml, Sigma). After one hour, unattached cells were washed away and adhered cells were fixed, stained with crystal violet, solubilized in 1% SDS and the absorbance was read at 595 nm.

In order to ablate β1 integrin and FAK in vitro, primary cultures were prepared from pregnant $Itg\beta l^{flox/flox}$ and $FAK^{flox/flox}$ mice. In the $FAK^{flox/flox}$ mice, LoxP sites flank the exon that contains the ATP loop in the kinase domain and Cre-mediated excision results in a frame-shift that prevents FAK protein expression. Primary cells were infected in suspension with Adeno-Cre or Adeno-β–galactosidase virus for 45 min(Watkin et al.,2002), before plating and culturing for another two days. More than 95% infection was verified by immunostaining with antibodies to Cre or β-galactosidase. zVAD was added for the final 24 hr to protect against possible cell death.

Transplantation of mammary epithelium into cleared fat pads of recipient mice

Inguinal mammary glands of syngeneic 21-day-old females were cleared of mammary epithelium as described (Deome et al., 1959). Mammary glands from 3-day lactating control or β1 integrin mutant mice were dissected, chopped into pieces of approximately $1mm^3$ and transplanted into the cleared fat pads. Ten weeks after transplantation, recipient females were mated and at pregnancy-day 16, mice were sacrificed and mammary gland whole mounts were prepared. In this experiment, six mice were transplanted with tissue pieces from wild type and six with tissue pieces from β 1 integrin mutant mice.

Lysate preparation, immunoprecipitation and western blot analysis.

To prepare lysates from mammary glands, the frozen tissue was ground to a powder in liquid nitrogen and homogenized in lysis buffer containing 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 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM Na_3VO_4, and 10 mM sodium molybdate. Cell lysates were prepared in NP40 lysate buffer (Lane et al., 2001). Cell lysates were subjected to SDS-PAGE, then transferred to PVDF membranes, which were blocked in 10% horse serum (GIBCO) or 5% nonfat milk for 1 hr and incubated overnight at 4°C with specific antibodies. Membranes were then incubated with the secondary antibody (Amersham) and signals were detected by enhanced chemiluminescence (ECL; Amersham)

MDA-MB-231 cell culture, siRNA transfections and luciferase assays

MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco-BRL). For siRNA transfection, cells were seeded in 6-well plates and transfected 24 hrs later with siRNA duplexes (100 nM) using OligofectAMINE (Invitrogen), according to the manufacturer's protocol. The 21-mer oligoribonucleotide pairs (Qiagen) were as follows: for integrin β1 (accession number NM-033669) nucleotide 167-189, for $p21^{Cip1}$ (Accession number NM-000389) nucleotide 236-254, for control LacZ (accession number NM55068) nucleotide 4277-4297 (obtained from D. Cappellen, FMI). Two days after siRNA transfection, cells were trypsinized, counted and plated either for immunohistochemistry on collagen I coated wells or for proliferation assays. Cells were stained with a P-FAK specific antiserum 24 hr after plating; 48 hr after replating cell proliferation was measured. For transient transfections with plasmids, cells were plated at a density of 1×10^5 per well in 6-well dishes. One day later cells were transfected with a DN-FAK expression vector (FRNK, from Dr. Schlaepfer, Scripps, CA), a $p21^{Cip1}$ reporter plasmid (WWP-Luc reporter plasmid from Dr. Vogelstein) and a *Renilla*

plasmid (Promega) using the Tranfection Reagent Effecten (Qiagen). Two days later, lysates were prepared and the luciferase assay was performed with Dual-Luciferase Reporter Assay Systerm (Promega), according to standard procedures. To control for transfection efficiency, Luciferase activity was normalized to the *Renilla* control.

Flow cytometric analysis

Cells were transfected with siRNA for LacZ, β1 integrin or $p21^{Cip1}$ as described above. After 3 days cells were pulse-labeled with 10 μM BrdU for 1hr, then harvested and stained using the BrdU Flow Kit (BD pharmingen) according to the manufacturer's protocol. BrdU-positive cells were detected using a fluorescein isothiocyanate-conjugated anti-BrdU antibody and DNA was stained with 7-amino-actinomycin D. Cell cycle position of the BrdU-labeled cells was determined by two-color flow cytometric analysis.

Extraction of RNA and RT-PCR analysis of $p21^{Cip1}$

RNA was prepared by the Trizol method (GiBCO) and purified using the RNAeasy kit (Qiagen). Purified RNA was reverse transcribed and PCR amplified by standard procedures using the specific oligonucleotide primers for $p21^{Cip1}$ (F: 5'- GGACCTGTC ACTGTCTTGTA- 3'; R: 5'-CTTCCTCTTGGAGAAGATCAG -3') and actin (F:5'-CCTTCCTGGGCATGGAGTCCT-3'; R: 5'-GGAGCAATGATCTTGATCTT –3').

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Acknowledgements

First of all, I would like to thank Dr. Nancy Hynes, my supervisor. She gave me the opportunity to work in her lab and kindly support me to finish the project. Throughout my PhD studies, I really learned a lot from her.

Thanks also to Dr. Max Burger and Dr. Patrick Matthias, the other two members of my thesis committee for the interest they took in my work and for the advice they gave me during the committee meetings.

I want to thank my colleagues in the lab, including former colleagues: Andrea, Gianluca George, Kay, Magda, Nicky, Romina, Thomas; and current colleagues: Ali, Anne, David, Francisca, Ilja, Maria, Patrick, Patrizia, Regis, Susanne, Thomas, Yutaka. I have to say our lab always has stimulative discussions and nice atmosphere. I really enjoyed working with all of my colleagues. In addition, special thanks to Francisca Maurer for her kindly technical aids and many nice helps in my life.

I would like to thank Dr.Oakeley (FMI) for the microarray analyses, Ms. Sauder (Biocenter, University of Basel) for the EM work, Francisca (FMI) for helping with part of the mouse typing work. I also got technical helps from David, Melanie, Dania, Martin, Stefano (FMI) and Emma (Univ. Manchester).

I would like to thank our collaborators: Dr. Ulrich Mueller (Scripps, la Jolla) provides us the Loxp-flanked β1 integrin mice. WAPiCre mice are from Timothy Wintermantel in Dr. Gunther Schuetz lab (Heidelberg). Matthew and Franziska in Dr. Charles Streuli's lab (Univ. Manchester) performed primary cells preparation and adeno-cre infection experiments. Dr.Streuli and Dr.Mueller also give us many nice suggestions about our manuscript.

Finally, many thanks to my family: my parents, my sister and my dear husband Yu. I would not have reached this point without supports from them.