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Mammary Epithelial-specific Deletion of the Focal Adhesion Kinase Gene Leads to Severe Lobulo-Alveolar Hypoplasia and Secretory Immaturity of the Murine Mammary Gland*

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Integrin-mediated cell adhesion and signaling is required for mammary gland development and functions. As a major mediator of integrin signaling, focal adhesion kinase (FAK) has been implicated to play a role in the survival, proliferation, and differentiation of mammary epithelial cells in previously studies *in vitro*. To assess the role of FAK *in vivo*, we created mice in which FAK is selectively deleted in mammary epithelial cells. The mammary gland FAK conditional knock-out (MFCKO) mice are viable, fertile, and macroscopically indistinguishable from the control littermates. In virgin MFCKO mice, mammary ductal elongation is retarded at 5 weeks of age but reaches the full extent by 8 weeks of age compared with the control mice. However, the MFCKO females are unable to nurse their pups due to severe lobulo-alveolar hypoplasia and secretory immaturity during pregnancy and lactation. Analysis of the mammary epithelial cells in MFCKO mice showed reduced Erk phosphorylation, expression of cyclin D1, and a corresponding decrease in proliferative capability compared with the littermate controls. In addition, phosphorylation of STAT5 and expression of whey acidic protein are significantly reduced in the mammary glands of MFCKO mice, suggesting defective secretory maturation in these mice. Therefore, the combination of the severe lobulo-alveolar hypoplasia and defective secretory differentiation is responsible for the inability of the MFCKO females to nurse their pups. Together, these results provide strong support for a role of FAK in the mammary gland development and function *in vivo*.

The mammary gland is composed of epithelial stroma embedded in the mammary fat pad. Most of the development of the mammary gland occurs during the postnatal life of all mammals. The rudimentary ducts are present at birth and start growing under the influence of ovarian hormones at the onset of puberty. Soon terminal end buds appear, and the process of ductal elongation commences (1). At the onset of puberty, the mammary ducts begin to branch, and alveolar buds appear at the ends of these branches. These alveolar buds are the precursors of the secretory units called alveoli that form during pregnancy and are responsible for milk production. After weaning, the mammary gland undergoes involution during which the mammary gland regresses to an almost pre-pregnant state (2). Because most of its development occurs after birth and a rapid expansion of mammary epithelial cells during pregnancy, the mammary gland is a particularly attractive model system to study cell proliferation during mammalian development. Using this model system coupled with the mouse knock-out technology, a number of signaling proteins and intracellular pathways have been shown to play important roles in the regulation of mammary epithelial cell proliferation *in vivo* (3, 4).

Like other epithelial and endothelial cells, mammary epithelial cells require integrin-mediated adhesion to the extracellular matrix for their survival and proliferation. Integrins have been shown to control mammary epithelial cell proliferation, survival, and secretory differentiation either alone or in co-operation with growth factor receptors. In the absence of continuous, integrin-induced survival signals, normal, non-transformed mammary epithelial cells undergo apoptosis (5). Furthermore, secretory differentiation of mammary epithelial cells is mediated by a unique co-operation between integrin- and prolactin signaling. In the prolactin signaling pathway prolactin binds to its receptor, which in turn becomes activated to cause Janus kinase 2 activation, which leads to signal transducer and activator of transcription 5 (STAT5)[3] phosphorylation, dimerization, and translocation into the nucleus. However, this process cannot proceed unless cells are anchored onto laminin and have normally functioning integrin signaling pathways (6). Studies using fibroblasts *in vitro* have suggested that integrin-mediated cell adhesion to extracellular matrix controls the progression of cell cycle through the G_1 phase by up-regulating various cell cycle regulators such as cyclins D1, A, E, and cyclin-dependent kinase (Cdk) 2, 4, and 6 and down-regulating Cdk inhibitors (p21 and p27) (7, 8). More recently, several laboratories have shown that conditional knock-out of β1 integrin in the

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[3] The abbreviations used are: STAT5, signal transducer and activator of transcription 5; FAK, focal adhesion kinase; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; KO, knock-out; TEB, terminal end bud; WAP, whey acidic protein; MFCKO, mammary gland FAK conditional knock-out; MMTV, murine mammary tumor virus; BrdUrd, bromodeoxyuridine.

mammary epithelium led to defective alveolar development and mammary epithelial cell differentiation, suggesting that integrin-mediated cell adhesion and signaling play an important role in mammary gland development and function *in vivo* (9–11).

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase implicated in mediating signal transduction by integrins as well as other cell surface receptors (12–16). Integrin-mediated cell adhesion to the extracellular matrix leads to FAK activation and autophosphorylation in a variety of cell types. Upon its activation and autophosphorylation at Tyr-397, FAK associates with a number of other kinases and adaptor molecules including Src family kinases, p85 subunit of phosphatidylinositol 3-kinase, phospholipase Cγ, Grb7, Shc, paxillin, p130cas, and MT1-MMP. FAK also functions as a regulated scaffolding protein to mediate Src phosphorylation of several key substrates like paxillin, p130cas, and MT1-MMP through association with both the kinase and substrate (17, 18). FAK interactions with these signaling molecules and the subsequent activation of several downstream signaling pathways have been shown to regulate cell spreading and migration, cell survival, and cell cycle progression (12–16).

Consistent with the numerous observations for an important role of FAK *in vitro*, FAK gene inactivation in mice resulted in an early embryonic lethality with major defects in the axial mesoderm and cardiovascular system (19, 20). Although these results clearly indicated a critical role of FAK in embryonic development, the embryonic lethality excluded investigation of the functions and mechanisms of FAK signaling in postnatal developmental processes *in vivo* such as mammary gland development. To overcome such problems, several groups including us have generated the floxed FAK ($FAK^{flox/flox}$) mice with the FAK gene flanked by two loxP sites (21–23). Using the floxed FAK mice, we report generation of a mammary epithelial cell-specific FAK conditional knock-out mouse by crossing them with transgenic mice expressing Cre recombinase under the control of the MMTV promoter. We found that specific inactivation of FAK in mammary epithelial cells led to severe lobulo-alveolar hypoplasia and inhibition of secretory maturation during pregnancy. As a result, these females cannot lactate, and their offspring will die unless cross-fostered to a control lactating female. These findings suggest that FAK plays an important role in the mammary gland development and function *in vivo*.

EXPERIMENTAL PROCEDURES

Chemicals, Antibodies, and Other Materials—Carmine and aluminum potassium sulfate were purchased from Sigma-Aldrich. Carmine alum staining solution was made by combining 1 g (0.2%) of carmine and 2.5 g (0.5%) of aluminum potassium sulfate in 500 ml of distilled water and boiling the solution for 20 min. The volume of the solution was adjusted to 500 ml after boiling and was filtered, and 1 crystal of thymol was added as a preservative. The solution was kept at 4 °C until use. Xylene was purchased from Sigma-Aldrich as well. Permount mounting solution was obtained from Fisher, and coverslips were procured from Corning Glass Co. (Corning, NY).

Antibodies were purchased from the following companies. Rabbit polyclonal anti-FAK antibody (C20, catalog no. sc-558), anti-estrogen receptor (catalog no. sc-542), and anti-progesterone (catalog no. sc-538) were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Rabbit polyclonal anti-phospho-STAT5 (catalog no. 71-6900) was from Zymed Laboratories Inc., South San Francisco, CA. Rabbit polyclonal anti-phospho-p44/42 mitogen-activated protein kinase (Erk1/2) antibody (catalog no. 9101) was from Cell Signaling Technology, Inc., Danvers, MA. 5-Bromo-2′-deoxyuridine (BrdUrd) staining kit (catalog no. 93-3943) was from Zymed Laboratories Inc., ApopTag® Peroxidase In Situ Apoptosis Detection kit (catalog no. S7100) was purchased from Chemicon International (Temecula, CA).

Mice and Genotyping by PCR—Floxed FAK mice have been described previously (21). MMTV-Cre transgenic mice (line F) (24) were obtained from the NCI, National Institutes of Health (Bethesda, MD). Mice were housed and handled according to local, state, and federal regulations, and all experimental procedures were carried out according to the guidelines of Institutional Animal Care and Use Committee at Cornell University and the University of Michigan. Mice genotyping for *FAK* and *Cre* alleles were performed essentially as described previously (21, 24).

Whole Mount Preparation—The fourth abdominal mammary gland was used for whole mount preparations. All procedures below were carried out at room temperature. The fourth abdominal mammary gland was excised during necropsy and spread between two Colorfrost® glass slides (VWR International, Inc., Rochester, NY) squeezed together by two 2-inch office binder clips. The top glass slide was removed after 10 min, and the tissue was fixed in Carnoy's fixative (6 parts 100% ethanol, 3 parts chloroform, and 1 part glacial acetic acid) for 4 h. Subsequently, the tissue was washed in 70% ethanol for 15 min, and the ethanol was changed gradually to distilled water then was finally rinsed in distilled water for 5 min. Staining was carried out overnight in carmine alum stain. The tissue was then dehydrated in graded alcohol solutions (70, 95, and 100%; 30 min each) and was cleared in two changes of xylene (30 min each), mounted, and coverslipped using Permount. Whole mounts were observed under a Leica dissecting microscope (Leica Microsystems GmbH, Wetzlar, Germany), and digital images were recorded using a SPOT FLEX® color digital camera (Diagnostic Instruments, Inc. Sterling Heights, MI) using a SPOT software package (Version 4.5, Diagnostic Instruments, Inc. Sterling Heights, MI).

Histology and Immunohistochemistry—Mice were euthanized using CO_2, and a complete tissue set was harvested during necropsy. Fixation was carried out for 16 h at 4 °C using freshly made, pre-chilled (4 °C) PBS-buffered formalin. After fixation, tissues were washed in PBS for 20 min for 3 times at room temperature, transferred into 65% ethanol (30 min), and finally transferred to 70% ethanol for storage. The tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin for routine histological examination or left unstained for later immunohistochemistry. Hematoxylin- and eosin-stained sections were examined under an Olympus BX41 light microscope (Olympus America, Inc., Center Valley,

PA), and images were captured with an Olympus digital camera (model DP70) using a DP Controller software (Version 1.2.1.108).

For immunohistochemistry, unstained tissue sections were first deparaffinized in 2 washes of xylene (5 min each) and then were rehydrated in graded ethanol solutions (100, 95, and 70%). After heat-activated antigen retrieval (model Retriever 2000, PickCell Laboratories B.V., Amsterdam, Holland) according to manufacturer's specifications, sections were treated with blocking solutions; first with Avidin-Biotin Block (Dako Corp., Carpinteria, CA) then with Protein Block Serum Free (Dako Corp.). Sections were then incubated with the primary antibody at 37 °C for 3 h in a humid chamber, washed in PBS 3 times (2 min each), then incubated with the biotinylated secondary antibody (1:200 dilution, ABC Elite Kit, Vector Laboratories, Burlingame, CA) in a humid chamber for 30 min at 37 °C and washed in PBS similarly as before. Finally, sections were incubated with horseradish peroxidase-streptavidin (ABC Elite kit, Vector Laboratories) for 15 min at room temperature in a humid chamber and washed with PBS similarly as before. As the last staining step, 3,3′-diaminobenzidine (SIGMA FAST® DAB with Metal Enhancer, Sigma-Aldrich) was added to the sections and incubated at room temperature until a macroscopically appreciable light brown color developed in the sections (generally 30 s to 5 min). Sections from littermate MFCKO and control animals were processed together and exposed to 3,3′-diaminobenzidine (DAB) for exactly the same length of time. After incubation with DAB, sections were lightly counterstained will Gill's hematoxylin. Histological examination and digital photography were carried out as described previously in this paper.

BrdUrd Incorporation Assay—BrdUrd (Sigma-Aldrich) was injected intraperitoneally at 100 mg/kg of body weight 3 h before euthanasia. Euthanasia, necropsy, and tissue processing for histology have been described in detail in previous sections of this report. Unstained tissue sections were depraffinized, rehydrated as previously described, and stained using a BrdUrd staining kit (Zymed Laboratories Inc.®). Sections were observed under a light microscope, and BrdUrd-positive mammary epithelial cells were identified by a veterinary pathologist, and their number was expressed as a percentage of all mammary epithelial cells. Statistical analysis (two-tailed *t* test) was performed using average percentages from three independent experiments, and the difference between MFCKO and control proliferation rates was interpreted as significant when the *p* value was below 0.05.

Short-term Hormonal (Estrogen and Progesterone) Treatment of Mice—All materials for this procedure (hormones and sesame oil) were purchased from Sigma-Aldrich. First, a 10-μg/ml stock solution of estrogen (β-estradiol 3-benzoate) was made using sesame oil. The estrogen stock solution was kept at −20 °C until use. For injections, 10 μl of the estrogen stock solution was added to 10 ml of sesame oil in which 100 mg of progesterone powder was dissolved previously (E_2+P working solution). Forty-eight hours before sacrifice, 100 μl of E_2+P working solution (containing 1 μg E_2 and 1 mg P) was injected subcutaneously in the interscapular area of each female mouse (littermate control and MFCKO). BrdUrd injection, euthanasia, necropsy, and tissue processing have been described previously in this report.

SDS-PAGE and Western Blotting—Tissue samples were harvested during necropsy, flash-frozen in liquid nitrogen, and ground with a mortar and pestle, and proteins were extracted using a triple detergent buffer (150 mM NaCl, 50 mM TRIS·Cl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.02% sodium azide, 1 mM sodium vanadate, pH 8.0) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Tissue homogenates were incubated on ice for 10 min, and the lysates were cleared at 4 °C by centrifugation in a bench-top microcentrifuge (Fisher) at 13,000 rpm for 10 min. Cleared supernatants were transferred into a clean microcentrifuge tube, and protein concentration was determined using Bio-Rad protein assay reagent. Protein samples were resolved with polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking in Blotto, membranes were incubated with the primary antibody (1:1000) for overnight at 4 °C, washed, and incubated with the appropriate horseradish-conjugated secondary antibody (1:5000) for 1 h at room temperature. An Enhanced Chemiluminescent (ECL) kit was used to visualize the signal (Pierce).

RESULTS

Deletion of FAK in the Mammary Epithelium of Mice—To investigate the potential role of FAK in mammary gland development and function *in vivo*, we employed the Cre/loxP conditional knock-out system to create mammary epithelial cell-specific deletion of *FAK* gene in mice. The floxed FAK ($FAK^{flox/flox}$) mice harboring two unidirectional *LoxP* sites flanking the third coding exon in both FAK alleles were prepared and characterized in our laboratory recently (21). Cre-mediated excision of exon 3 results in a frameshift mutation, which produces a small truncated and non-functional peptide (~70 amino acids) lacking the majority of FAK sequences. To obtain mammary epithelial cell-specific FAK-conditional KO mice, the floxed FAK mice were intercrossed with the MMTV-Cre mice, which express the Cre recombinase in the mammary epithelial cells in both virgin and pregnant female mice (24). The offspring were genotyped using primers specific for various FAK alleles (*i.e.* floxed, wild type, and deleted) and with primers specific for *Cre*. First-generation males from this mating harboring $FAK^{flox/+}$; *MMTV-Cre* genotype were mated to $FAK^{flox/flox}$ females, and the offspring were genotyped as before. Consistent with the expectation that mammary gland-specific deletion of FAK should not affect survival of the mice, offspring with the genotypes of $FAK^{flox/flox}$; *MMTV-Cre* (mammary gland FAK conditional knock-out, designated as MFCKO), $FAK^{flox/+}$; *MMTV-Cre,* $FAK^{flox/flox}$ (designated as control), and $FAK^{flox/+}$ were obtained at the predicted 1:1:1:1 mendelian ratio. Furthermore, both sexes of MFCKO mice were found to be grossly and behaviorally normal and indistinguishable from the control mice of similar age based on physical examination.

To evaluate the deletion of FAK in the mammary epithelial cells of MFCKO mice, protein extracts were prepared from mammary glands of virgin, pregnant, and lactating MFCKO and the control mice and used for Western blotting analysis. In

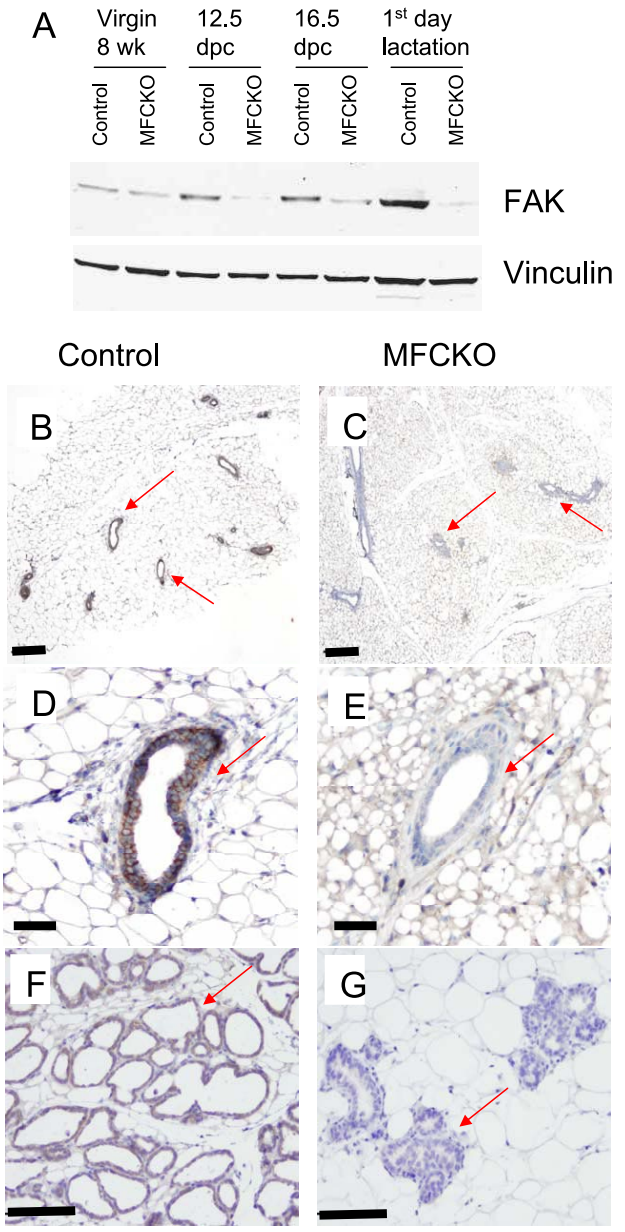

FIGURE 1. **Mammary epithelial cell-specific deletion of FAK in MFCKO mice.** *A*, protein lysates were prepared from mammary glands of virgin, pregnant, and lactating control or MFCKO mice as indicated. They were then analyzed by Western blotting using antibody again FAK (*top*) or vinculin (*bottom*). *B–G*, Mammary glands harvested from control (*B*, *D*, and *F*) or MFCKO (*C*, *E*, and *G*) mice as 8-week-old virgins (*B–E*) or at the first day of lactation (*F–G*) were sectioned and subjected to immunohistochemistry with anti-FAK. FAK is detected in the mammary epithelial cells (*arrows*) in the control but not MFCKO mice. The *scale bars* are 500, 100, and 200 μm for *panels B* and *C*, *D* and *E*, and *F* and *G*, respectively. *dpc*, days post-coitum.

virgin mice, FAK was detected in the mammary gland of the control mice at a low level, and this is reduced in the mammary gland of MFCKO mice (Fig. 1*A*). In pregnant and lactating mice, FAK expression is increased significantly in mammary gland of the control mice but remained at a very low level in the samples from MFCKO mice. Similar loading in the control and MFCKO lanes was verified by Western blotting with anti-vinculin, as shown in the bottom panel. The increase in the expression level of FAK in the mammary gland of the control mice correlated with the expansion of mammary epithelial cells during pregnancy and lactation (1). Therefore, the significantly reduced expression of FAK in pregnant and lactating MFCKO mice, in which mammary epithelial cells constitutes the major components of the mammary gland, strongly suggested an efficient deletion of FAK in the mammary epithelial cells. The residual amount of FAK detected in the MFCKO mice was not changed throughout the different stages and could be due to the low level expression of FAK in other cell types (*e.g.* adipocytes) and the presence of small amount of endothelial cells and fibroblasts, which express FAK in the mammary gland where the MMTV-Cre is not expressed (24).

In the virgin mice the number of epithelial cells within the mammary fat pad is quite small (about 5–10%) compared with stromal cells; therefore, only a relatively small reduction in FAK expression was detected using the lysates from the whole mammary glands (see the *left two lanes*, Fig. 1*A*). To further verify efficient MMTV-Cre-mediated deletion of FAK in the mammary epithelial cells, especially in virgin mice, immunohistochemical analysis of mammary glands from the MFCKO and control mice were performed using anti-FAK antibodies. As shown in Fig. 1, *B–E*, a strong staining for FAK is detected in the mammary epithelial cells of the control mice (Fig. 1, *B* and *D*) but not that of the MFCKO mice (Fig. 1, *C* and *E*) of the virgin mice. Similar results were obtained when mammary glands from lactating control and MFCKO mice were analyzed (Fig. 1, *F* and *G*). Together, these results suggest an efficient FAK deletion in the mammary epithelial cells of MFCKO mice.

FAK Deletion in the Mammary Epithelium Results in Reduced Branch Points and Mild Retardation of Mammary Ductal Elongation in Virgin Mice—To investigate the effect of FAK deletion on mammary gland development, mammary glands were harvested from virgin MFCKO and control mice at 4, 5, and 8 weeks of age and examined by both whole mount staining and histology. A 4-week-old mouse is considered sexually immature, and at this age the mammary ducts barely invade the mammary fat pad beyond the mammary lymph node. Comparison of mammary glands of MFCKO and control mice at this stage showed a reduced number of branches and slightly decreased invasion of the mammary fat pad by the mammary ducts in the cranial direction relative to the centrally located mammary lymph-node for MFCKO mice (Fig. 2*A*). Consistent with the reduced number of branches and ducts, fewer terminal end buds (TEBs) were observed in the MFCKO mammary glands. Despite the reduced numbers, however, the structure of the TEBs of MFCKO mice appears to be normal, with lumens clearly seen by whole mount staining. Histological analysis confirmed that the morphology (Fig. 2*B*) and the average size (Fig. 2*C*) of TEBs in the MFCKO and control mice are comparable.

At 5 weeks of age the mammary gland starts ductal elongation (1), which is completed as the animal reaches full sexual maturity at 8 weeks of age (1). Whole mount analysis of 5-week-old females revealed significant retardation in the invasion of the mammary fat pad by mammary ducts in the cranial direction in MFCKO mice (Fig. 3, *A* and *C*). Similar to the mammary glands at 4 weeks of age, less branching points were observed in the MFCKO mice. At 8 weeks of age, however, a similar extent of ductal invasion was observed for the MFCKO and control

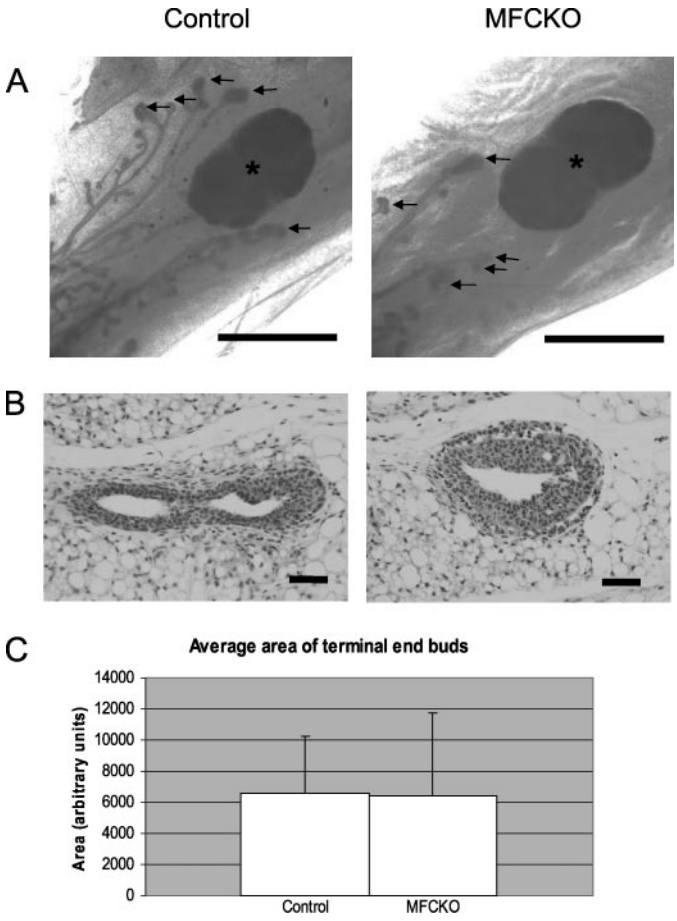

FIGURE 2. **Analysis of mammary glands from control and MFCKO mice at 4 weeks of age.** *A*, carmine alum-stained mammary whole mounts from littermate control (*left panels*) and MFCKO (*right panels*) females at 4 weeks of age. Note the reduced number of the ducts and decreased invasion of mammary ducts (ends marked by *arrows*) in the MFCKO glands (relative to the position of lymph nodes, as marked by *asterisks*) compared with control glands. However, lumens are visible in the TEBs of both control and MFCKO glands. The *scale bars* are 1 mm. *B* and *C*, mammary gland sections from control (*left*) and MFCKO (*right*) female mice at 4 weeks of age were stained with hematoxylin and eosin and viewed under microscope (*bars*, 100 μm). Note that the morphology of TEBs is comparable; the apparent size difference between control and MFCKO TEBs is due to different plane of section (*B*). The areas of the lumens of TEBs of control and MFCKO mammary glands are quantified and shown in *panel C*.

mice (Fig. 3, *B* and *C*). Nevertheless, the pattern of reduced branching persisted in the MFCKO mammary glands at 8 weeks (Fig. 3*D*). Taken together, these data suggest that whereas the overall organization of the mammary tree and the structure of TEBs are unaffected, the rate of invasion of the mammary fat pad and the total number of branch points in the mammary tree are reduced in MFCKO mammary glands.

FAK Deletion in the Mammary Epithelium Causes Severe Lobulo-Alveolar Hypoplasia during Pregnancy and Lactation—Mammary gland epithelium undergoes rapid morphogenesis during pregnancy and lactation, during which the terminal end buds fully differentiate to form lobulo-alveolar structures where milk is synthesized and secreted (3). To investigate the potential role of FAK in mammary gland morphogenesis and functions during pregnancy and lactation, female MFCKO and control mice were mated and observed during pregnancy and after delivery. The length of pregnancy was 19 days in both MFCKO and control mice, and the average litter size was also comparable. However, although pups born to a control female had stomachs filled with milk, little or no milk was found in the stomach of pups born to MFCKO females (Fig. 4*A*), and these pups would die shortly after birth unless cross-fostered to a lactating control female. These results suggested potential defects in the mammary gland morphogenesis and function of the MFCKO mice during pregnancy and lactation.

To examine the underlying causes of the inability of the MFCKO mice to lactate, mammary glands at the first day of lactation were harvested from MFCKO and control female mice and analyzed morphologically both by carmine-alum staining of the whole mount and by histology. In control mice the mammary glands were expanded (turgid), often mottled tan-red, and the mammary ducts appeared as arborizing white streaks at the periphery of the glands (larger mammary ducts engorged with milk; data not shown). The thoracic glands, which are normally almost indistinguishable from the surrounding fascia, were readily appreciable from the surrounding fascia due to their enlargement. In contrast, MFCKO mice had less distinct, small, uniformly tan mammary glands with no gross evidence of milk production. The thoracic glands were often indistinguishable from the surrounding fascia. Whole mount staining with carmine-alum showed that MFCKO mammary glands had normal ductal architecture, but the lobulo-alveolar units were sparse and smaller compared with the control mammary glands (Fig. 4, *B* and *C*). Light microscopic examination of 5-μm sections stained with hematoxylin and eosin from control mammary glands showed large lobulo-alveolar units (Fig. 5*A*) composed of many (3–8) individual alveoli that were lined by flattened, vacuolated epithelial cells and contained flocculent, eosinophilic material (milk), as expected (Fig. 5*C*). In contrast, the MFCKO mammary glands were still mostly composed of adipose stroma, and the parenchyma primarily consisted of dilated ductular profiles and small clusters of alveoli around some ducts (Fig. 5*B*). The individual alveoli in the MFCKO mammary glands were smaller, lined by cuboidal epithelial cells, and had very little secretum within (Fig. 5*D*). Together these results suggested that deletion of FAK in the mammary epithelial cells significantly impaired mammary gland morphogenesis and function as a result of severe lobulo-alveolar hypoplasia during pregnancy and lactation.

Proliferative Capability Is Markedly Reduced in MFCKO Mammary Epithelium—To explore the mechanisms of mammary epithelial hypoplasia in the MFCKO mice, we first measured the rate of apoptosis in virgin, pregnant, and lactating mice to determine whether increased apoptosis might have contributed to the defects in these mice. As expected, few apoptotic cells (<1%) were found in virgin, pregnant, or lactating mice. Furthermore, there were no significant differences in the fraction of apoptotic cells in mammary glands from the MFCKO and control mice (data not shown), suggesting that mammary epithelial hypoplasia in the MFCKO mice was not due to differential apoptosis in the mutant and control mice.

We next assessed the proliferative rate in mammary glands of virgin MFCKO and control mice. To stimulate mammary epithelial cell proliferation that mimic pregnancy but in a more controlled manner, 8-week-old (sexually mature) virgin mice

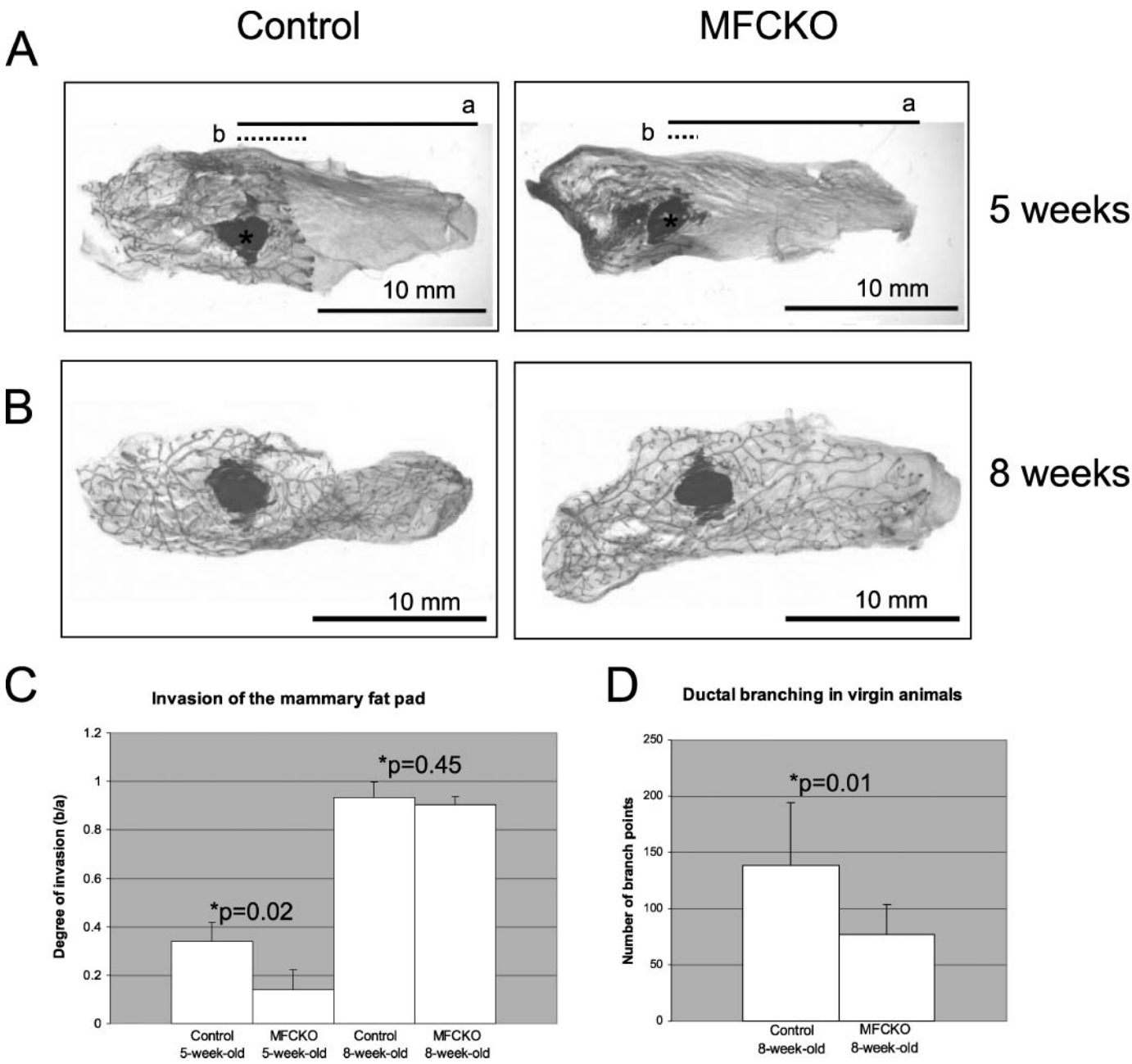

FIGURE 3. **Reduced branch points and invasion of the mammary fat pad by mammary ducts in MFCKO mammary glands.** *A* and *C*, using the mid-point of the mammary lymph-node as an anatomical landmark (highlighted with an *asterisk* in each whole mount), invasion of the mammary fat pad by mammary ducts cranially (*b*, *dotted line*) and the length of the cranial half of the fourth abdominal mammary fat pad (*a*, *solid line*) were determined under the microscope (*A*). The *scale bars* are 10 mm. The b/a ratio was used as measurements of ductal invasion and is shown here for the whole amounts from virgin control and MFCKO females at 5 and 8 weeks of age as indicated ($n = 3$ littermate pairs for each) (*C*). *B* and *D*, mammary glands from 8-week old control and MFCKO females were analyzed as in *A* (*B*), and the branch points are also determined under a microscope and shown in *panel D*. *, *p* values in *C* and *D* ($n = 3$) represent a comparison between control and MFCKO whole mounts at the same age.

were treated for 48 h with 17β-estradiol benzoate and progesterone or with vehicle (25) before injection with BrdUrd followed by euthanasia, as described under "Experimental Procedures." Proliferation of mammary epithelial cells was then examined by determination of BrdUrd incorporation using immunohistochemical staining with anti-BrdUrd antibody. In vehicle alone-treated mice, little BrdUrd incorporation was found, and there was no appreciable difference between MFCKO and control mice (data not shown). This stimulation with 17β-estradiol benzoate and progesterone was very short; therefore, it did not lead to significant expansion of the mammary tree (data not shown), and the treated glands were morphologically (whole mount and histology) indistinguishable from the non-treated ones (data not shown). Nevertheless, the acute estrogen-progesterone treatment led to a significant increase in the rate of BrdUrd incorporation in mammary epithelial cells, and this increase was greatly reduced in the MFCKO mice compared with control mice (Fig. 6, *A*–*C*). Immunohistochemical analysis of these samples showed that the levels of both estrogen (Fig. 6, *D* and *E*) and progesterone

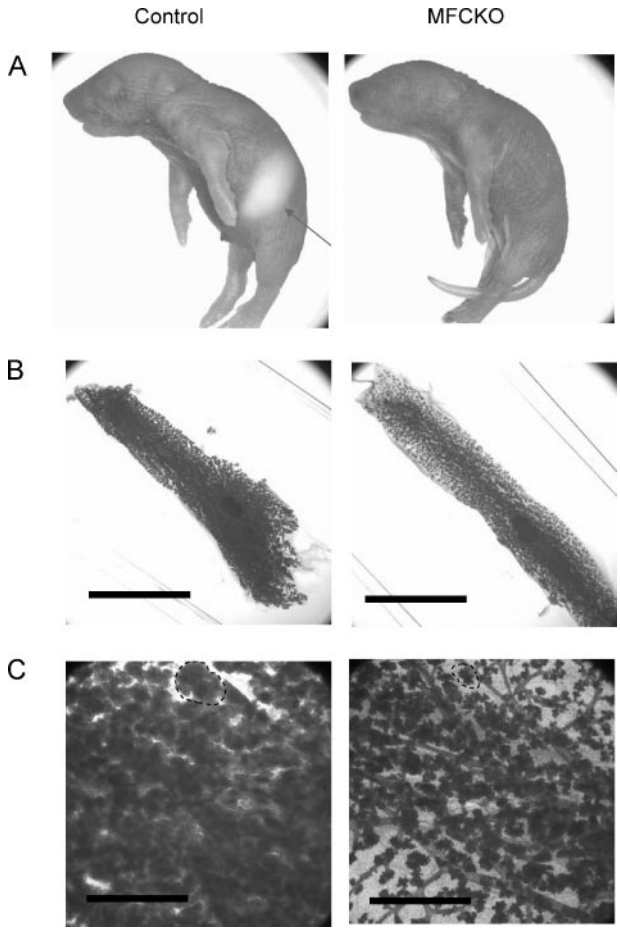

FIGURE 4. **Inability of MFCKO female to nurse the pups due to lobulo-alveolar hypoplasia.** *A*, subgross images of 1-day-old neonatal mice born to either control (*left*) or MFCKO (*right*) female. The *arrow* highlights a milk-filled stomach within the extended abdomen of the pup in *A*. Note that there is no milk in the stomach, and the abdominal wall caudal to the ribcage is sunken of the pup in *B*. *B* and *C*, low (*B*) and high magnification (*C*) carmine alum-stained mammary whole mounts from littermate control (*left panels*) and MFCKO (*right panels*) females at the first day of lactation. Note that the control mammary glands have abundantly developed lobulo-alveolar units (one such unit is *outlined* with *dots* in *C*, *left panel*), whereas the MFCKO mammary glands have sparse and smaller lobulo-alveolar units (one typical unit is *outlined* in *C*, *right panel*). The *scale bars* are 10 and 1 mm in *B* and *C*, respectively.

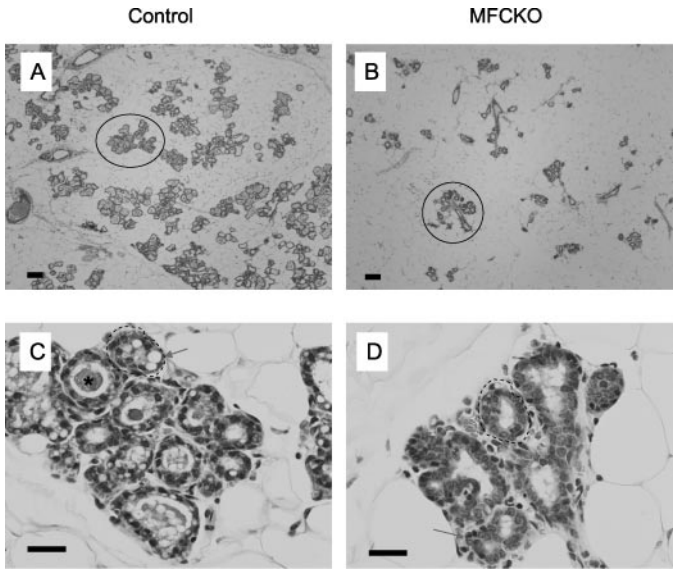

FIGURE 5. **Histological analyses of the mammary epithelium in MFCKO females.** Mammary gland sections from control (*A* and *C*) and MFCKO (*B* and *D*) female mice on the first day of lactation were stained with hematoxylin and eosin and viewed at low (*A* and *B*; *bar* = 200 μm) or high (*C* and *D*; *bar* = 50 μm) magnifications. Note that the lobulo-alveolar units are larger (*e.g. circled*) and more numerous in the control gland (*A*), whereas they are smaller (*e.g. circled*), and there is a severe paucity of them in the MFCKO gland (*B*). At high magnification, the alveoli of the control gland (*outlined* with a *dotted line*; *C*) are slightly larger, have abundant flocculent, amphophilic material (*asterisk*), and are lined by vacuolated (*arrow*) epithelial cells (evidence of producing fatty secretory material; that is, milk). In contrast, the individual alveoli of the MFCKO gland (*outlined* with a *dotted line*; *D*) are small, devoid of secretory material, and are lined by low cuboidal (*arrow*) epithelial cells (evidence of secretory inactivity).

receptors (Fig. 6, *F* and *G*) are not reduced in the MFCKO mice compared with the controls, suggesting that the reduced proliferative responses of the mammary epithelial cells in the MFCKO mice is not due to possibly decreased expression of estrogen or progesterone receptors in these mice.

We also examined mammary glands from pregnant MFCKO and control mice by histology. Consistent with a reduction in proliferation, fewer mitotic figures were found in the mammary epithelial cells of MFCKO mice compared with that of control mice (Fig. 7, *A* and *B*). In addition, well developed lumen in mammary acini was observed in the control mammary glands as expected, but these were not evident MFCKO at the similar stage of pregnancy, suggesting a delay in the maturation of mammary gland in the mutant mice (Fig. 7, *A* and *B*). Together, these results suggested that deletion of FAK in mammary epithelial cells impaired their proliferation, which is the most likely cause of lobulo-alveolar hypoplasia in the MFCKO mice during pregnancy.

Several FAK targets and downstream pathways have been implicated in mediating regulation of cell cycle progression by FAK. In particular, Erk activation and cyclin D1 expression have been well established to be key components of the FAK downstream targets in the regulation of cell proliferation in a number of previous studies (26). To evaluate the role of Erk and cyclin D1 for the deficient proliferation of mammary epithelial cells in the MFCKO mice, mammary glands from littermate female MFCKO and control mice at 12.5 days post-coitum were harvested, and protein extracts were analyzed by Western blotting. As shown in Fig. 7*C*, phosphorylation of Erk was significantly decreased, and the expression level of cyclin D1 was moderately reduced in the mammary glands of MFCKO mice compared with that in the control mice. These results suggested that Erk activation and subsequent expression of cyclin D1 also play a key role in FAK-regulated cell proliferation of mammary epithelial cells during pregnancy *in vivo*.

FAK Deletion Prevents Pregnancy-induced Secretory Differentiation of the Mammary Epithelium—In addition to the reduced proliferation, histological analysis suggested potential defects in secretory maturation of the MFCKO mammary glands (see Fig. 5*D*). To further investigate the effects of mammary epithelial-specific FAK deletion on pregnancy-induced secretory differentiation of the mammary gland, we examined phosphorylation status along with its subcellular localization of STAT5 and the expression level of the whey acidic protein (WAP) during pregnancy and lactation. STAT5, a member of the prolactin signaling pathway, is activated by Janus kinase 2

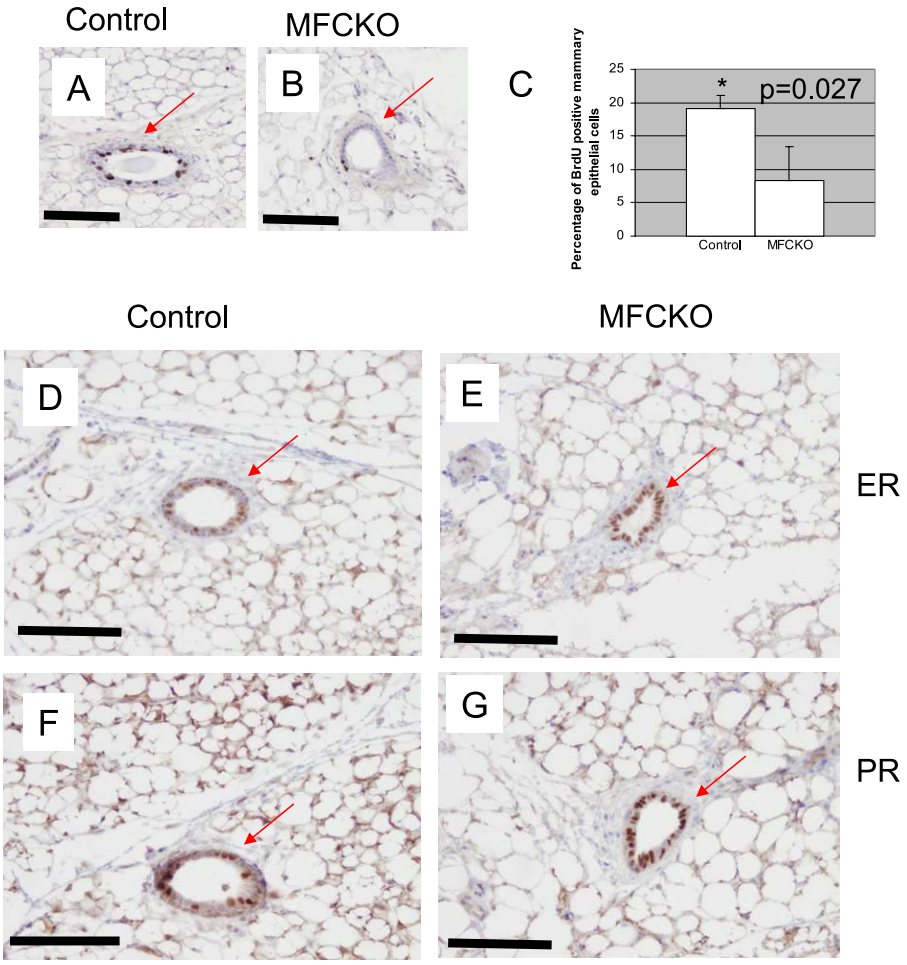

FIGURE 6. **Defective proliferative response of the mammary epithelial cells in MFCKO mice.** Control and MFCKO littermate female mice were treated with estrogen and progesterone (E2+P) as described under "Experimental Procedures." *A* and *B*, proliferation of mammary epithelial cells in the treated mice was determined by BrdUrd incorporation assays (*A* and *B*) as described under "Experimental Procedures." *C*, *bar graph* representation of percentage of BrdUrd-positive mammary epithelial cells in control and MFCKO littermate females. *, *p* value represents comparison between control and MFCKO mice. *D–G*, the sections from the control (*D* and *F*) and MFCKO (*E* and *G*) mice were also subjected to immunohistochemistry with anti-estrogen receptor (*ER*; *D* and *E*) or anti-progesterone receptor (*PR*; *F* and *G*). The *scale bars* are 200 μm, and *arrows* mark mammary epithelial cells.

(JAK2) and translocates to the nucleus upon activation by JAK2 and induces milk protein gene expression (6). WAP is a small (12 kDa) protein produced by the secretory mammary epithelial cells from the last third of the pregnancy (24). In control mice, phosphorylation of STAT5 was detected in the mammary glands during pregnancy and was increased during lactation as expected (Fig. 8*A*). In contrast, little phosphorylated STAT5 was detected in the MFCKO mammary gland either during pregnancy or lactation. Consistent with the Western blotting results, a strong signal for phosphorylated STAT5 was detected in the nuclei of the secretory epithelium of the control mammary gland, whereas there was no nuclear or cytoplasmic immunostaining in the MFCKO mammary glands (Fig. 8, *B* and *C*). Similarly, WAP was abundantly expressed in the secretory mammary epithelial cells in the control mammary gland but only showed a somewhat weaker signal in the MFCKO epithelium (Fig. 8, *D* and *E*). Together these results suggested that deletion of FAK in the mammary epithelium also reduced mammary gland differentiation, which likely also contributed to the inability of the female MFCKO mice to sustain their pups after birth.

DISCUSSION

By specifically deleting FAK in the mammary epithelial cells, this report documents for the first time an important role of FAK in the mammary gland development and functions *in vivo*. We show that deletion of FAK caused decreased mammary epithelial proliferation during pregnancy, leading to lobulo-alveolar hypoplasia. Furthermore, loss of FAK also led to an inhibition of secretory differentiation in the mammary epithelial cells. The combination of the defects resulted in an inability of the MFCKO females to nurse their pups, which die shortly after birth.

Previous studies have established FAK as a key mediator of integrin signaling in the regulation of various cellular functions *in vitro* (13–15, 16). However, it is less clear on the relative importance of FAK in integrin functions and signaling *in vivo* given the multitude of intracellular signaling pathways downstream of integrins. In particular, less is clear on the potential role of FAK in the regulation of cell proliferation and/or differentiation controlled by integrin-mediated cell adhesion *in vivo*, whereas a number of studies indicated a function of FAK in promoting cell survival and migration (21, 27). The major phenotypes of the MFCKO mice in defective mammary epithelial cell proliferation and differentiation resemble those observed in the mammary gland-specific integrin β1-conditional KO mice (9–11), which suggests that FAK plays an important role in mediating integrin regulation of mammary gland development and function *in vivo*.

It is interesting to note, however, FAK conditional KO mice appear more severe than the integrin β1 conditional KO mice, which are still able to lactate and nurse their pups at least partially (9–11). It is possible that use of different Cre transgenic mice may explain some of the differences in the severity of the phenotypes between the integrin β1 and FAK conditional KO mice. Studies by Naylor *et al.* (9) and Li *et al.* (10) used transgenic mice with Cre expression in mammary glands during pregnancy rather than before pregnancy, which may explain the less severe phenotype in these mice. Alternatively, deletion of FAK may also impact on intracellular signaling pathways besides integrins, which together with defects in integrin signaling may be responsible for the more severe phenotype in the

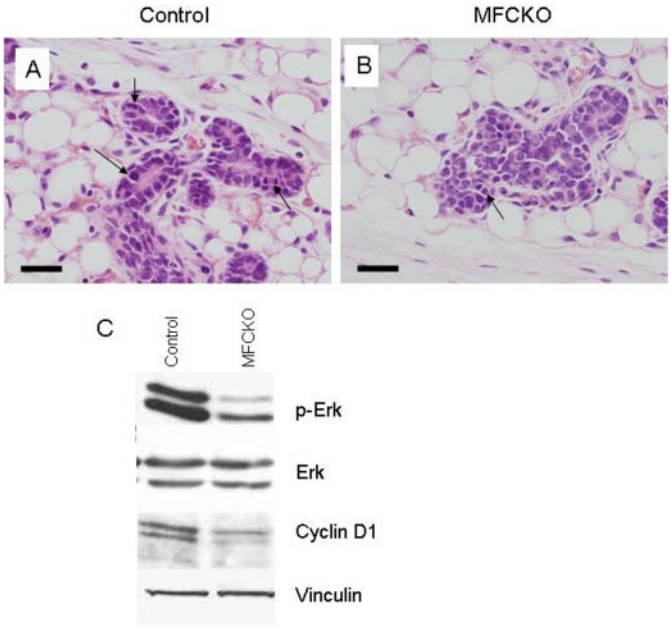

FIGURE 7. **Histological and biochemical analysis of mammary epithelial cells in pregnant MFCKO mice.** *A* and *B*, mammary gland sections from control (*A*) and MFCKO (*B*) female mice at pregnancy day 12.5 were stained with hematoxylin and eosin and viewed under a microscope (*bar* = 50 μm). Note that the lobulo-alveolar units in the control section (*A*) are larger, and the individual mammary epithelial cells are large with slightly basophilic cytoplasm containing a large nucleus with stippled chromatin. In contrast, the MFCKO gland (*B*) has small mammary lobulo-alveolar units. The individual mammary epithelial cells are small with an eosinophilic cytoplasm containing round nucleus and condensed chromatin within. Also note the number of mitotic figures (*arrows*) is more in the control section (*A*) than in the MFCKO section (*B*). *C*, protein lysates were prepared from mammary glands of control or MFCKO mice pregnancy day 12.5 and analyzed by Western blotting using various antibodies as indicated. *p-*, phosphorylated.

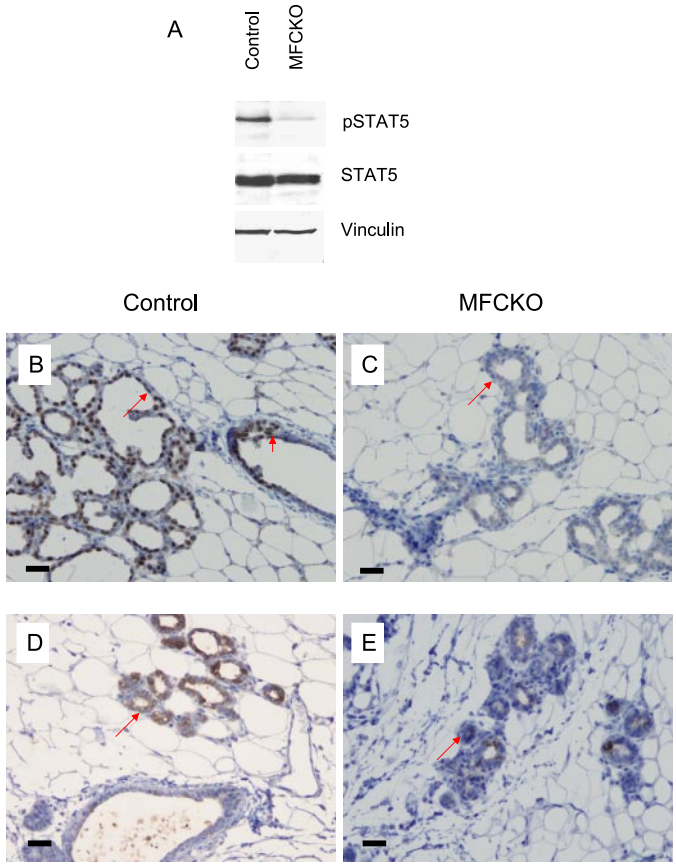

FIGURE 8. **Defective differentiation of mammary epithelium in MFCKO mice.** *A*, protein lysates were prepared from mammary glands of control or MFCKO mice on the first day of lactation and analyzed by Western blotting using various antibodies as indicated. *B–E*, mammary glands harvested from control (*B* and *D*) or MFCKO (*C* and *E*) mice at the first day of lactation were sectioned and analyzed by immunohistochemistry with anti-pSTAT5 (*B* and *C*) or anti-WAP (*D* and *E*). Although the control mammary gland (*B*) has strong nuclear immunostaining for phosphorylated STAT5 in virtually every acinar epithelial cell (*long arrow*) and to a lesser degree in the nuclei of ductal epithelial cells (*short arrow*), there is a striking absence of specific immunostaining in the mammary alveolar epithelium (*arrow*) of the MFCKO gland (*C*). Similarly, the alveolar epithelium (*arrow*) of the control gland (*D*) exhibits strong cytoplasmic staining of WAP, whereas within the alveolar epithelium (*arrow*) in the MFCKO gland (*E*) there is a paucity of WAP-specific immunostaining. Scale bars are 50 μm for *B–E*.

FAK conditional KO mice. Indeed, FAK has been shown to participate in signaling pathways initiated by growth factors and cytokines in addition to integrins (12–16).

Several previous studies *in vitro* suggested that FAK regulated cell cycle progression at least in part through activation of Erk signaling and increased expression of cyclin D1 (26, 28, 29). Consistent with these observations, we found that both Erk activation and cyclin D1 expression were reduced in the mammary glands from the MFCKO mice compared with those from control mice. Interestingly, both FAK phosphorylation and Erk activation were decreased in the mammary gland of integrin β1 conditional KO mice (9, 10), providing further support for a role of Erk signaling pathway in the regulation of mammary epithelial cell proliferation by integrin signaling through FAK *in vivo*.

Although the status of cyclin D1 expression was not examined in the integrin β1 conditional KO mice (9–11), several *in vitro* studies have established regulation of cyclin D1 expression by integrin-mediated cell adhesion (7, 8, 28). Furthermore, previous studies also suggested that FAK enhanced cyclin D1 expression levels via transcriptional activation of the cyclin D1 promoter and that cyclin D1 regulation by FAK was dependent upon integrin-mediated cell adhesion and through the FAK-mediated Erk signaling pathway (26). Therefore, it is likely that lobulo-alveolar hypoplasia observed in both MFCKO mice and integrin β1 conditional KO mice (9–11) were caused by the reduced expression of cyclin D1 in mammary epithelial cells. Consistent with such a possibility, it has been shown that normal expression of cyclin D1 is indispensable for lobulo-alveolar development of the mammary gland (30, 31). Morphologically, at least with regard to the severity of the lobulo-alveolar hypoplasia, the mammary pathology in the cyclin D1 knock-out mice closely resemble phenotypes in the MFCKO mice (30).

It should be noted, however, that the cyclin D1 knock-out females could still produce a small amount of milk to sustain fewer and markedly smaller pups compared with pups from control females. Histologically, there is severe lobulo-alveolar hypoplasia in the lactating mice, but the secretory units still show a proper degree of differentiation. The secretory maturation enables the mammary gland to produce enough milk for limited pup survival. This is likely due to the fact that although cyclin D1 is a major mediator for FAK regulation of mammary epithelial cell proliferation, other targets downstream of FAK

may be responsible for the differentiation defects observed in the MFCKO mice.

Mammary epithelium differentiation and secretory maturation are controlled by several hormones including estrogen, progesterone, and prolactin (3). In particular, prolactin signaling through its receptor and the intracellular Janus kinase 2-STAT5 pathway has been demonstrated to play a crucial role in these processes. Interestingly, integrin-mediated adhesion and signaling is required for prolactin signaling pathway in mammary epithelial differentiation as suggested by a number of recent studies. Treatment with anti-β1 integrin antibodies *in vitro* (32) or expression of a dominant negative β1 integrin transgene *in vivo* (33) has been shown to inhibit STAT5 activation and milk production. Moreover, in β1 integrin null mammary epithelial cells, prolactin cannot activate STAT5 (9). In cultured mammary epithelial cells, expression of dominant negative Rac1 also inhibited prolactin signaling. More interestingly, in β1 integrin-null mammary epithelial cells prolactin signaling can be restored upon introduction of activated Rac1. These results suggest strongly that Rac1 may play a key role in mediating integrin regulation of the prolactin signaling to STAT5 and mammary epithelial cell differentiation. However, no report is yet available on whether Rac1 deletion in the mammary epithelium will cause an inhibition of STAT5 phosphorylation and a block of secretory maturation *in vivo*.

Our data suggest that FAK may be another important mediator of integrin regulation of the prolactin signaling pathway in mammary gland differentiation and maturation. The phenotypes of pregnant MFCKO mice resembles that seen in mice with mammary-specific deletion of the prolactin receptor or STAT5 (34). Both prolactin receptor- and STAT5-null mammary gland exhibited lobulo-alveolar hypoplasia. Prolactin receptor-null epithelium had epithelial structures with a central lumen that resembled alveoli. In contrast, STAT5-null mammary epithelium had normal ductal architecture, but no alveoli were present, and no milk protein gene expression was observed. Histologically the mammary glands of MFCKO mice resembled more the STAT5-null mammary glands than the prolactin receptor-null mammary glands. Furthermore, female MFCKO mice were not able to produce milk and nurse the pups, and reduced phosphorylation of STAT5 and expression of WAP was observed in these mice. Therefore, deletion of FAK in the mammary glands may disrupt integrin regulation of prolactin signaling through STAT5, leading to the deficient mammary epithelial differentiation and secretory maturation. They also suggest that FAK likely affected the cross-talk between integrin and prolactin signaling in the intracellular signaling pathways rather than at the prolactin receptor level. It would be very interesting to determine whether FAK regulates STAT5 activity by directly phosphorylating it or through other intermediaries in mammary glands *in vivo* in future studies. It would also be interesting to investigate the relationship and potential cooperation of FAK and Rac1 in their roles of mediating integrin regulation of prolactin signaling in mammary epithelial cells given previous results suggesting cross-talk between them in the regulation of a variety of cellular functions in various cultured cells (35–37).

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