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PRINCIPAL INVESTIGATOR: Tamas Nagy, DVM

CONTRACTING ORGANIZATION: Cornell University
Ithaca, New York 14853

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Tamas Nagy, DVM

E-Mail: tn35@cornell.edu

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14. ABSTRACT
I was able to detect the expression of the FIP200 transgene by RT-PCR in the double-transgenic animals from one single transgenic lineage. However, I could not detect the expression of the transgenic protein in the doxycycline-treated double-transgenic animals. Faced with this insurmountable technical difficulty, I realized that I had to modify my experimental approach. Since our original plan was to inhibit the activities of FAK indirectly using its inhibitor, FIP200, I decided to directly delete FAK in the mammary epithelium of mice employing the Cre-loxP method. The necessary genetically modified mouse lines were already available in our lab. So far I analyzed virgin and lactating female mice in which FAK is specifically deleted in the mammary epithelium. No morphological abnormalities were found in the mammary gland of virgin mice however, lactating mice have severe lobulo-alveolar hypoplasia in the mammary gland.

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Introduction

Human breast cancer is a multistep neoplastic process [1], during which integrins are indispensable mediators from the initial neoplastic transformation [2], through local invasion [3] and in the metastatic spread [4]. The focal adhesion kinase (FAK), one of the major mediators of integrin signaling [5], has been implicated both *in vitro* and *in vivo* in the development of human breast cancer [2], [6], [7], [8].

Due to technical difficulties with transgene expression, I had to modify my research approach. In my new approach I decided to directly delete FAK in the mammary epithelium of mice employing the Cre-loxP method [9]. The genetically modified mouse strains were already available in our lab. So far I analyzed virgin and lactating female mice in which FAK is specifically deleted in the mammary epithelium. No morphological abnormalities were found in the mammary gland of virgin mice however, lactating mice have severe lobulo-alveolar hypoplasia in the mammary gland.

Body

Aim 1. Generation of mammary gland-specific FAK conditional knock-out mice, examination of FAK deletion and the phenotype in virgin animals, Months 13-17

a. Genotypic screening of the FAK conditional knock-out mice (PCR).

Rationale

No detailed investigation exists on FAK expression in the mammary epithelium in vivo. Since wild-type FAK increases DNA synthesis, accelerates G1/S transition, and upregulates cyclin D1 expression [10] it is expected that FAK expression is especially important in the lobulo-alveolar development of the mammary gland during pregnancy. It has been shown that cyclin D1 deletion in mice leads to dwarfism and pregnant females have severe lobulo-alveolar hypoplasia affecting the mammary gland and, as a consequence, have difficulty sustaining their litters [11].

In order to delete FAK in the mouse mammary epithelium I chose to employ the Cre-LoxP method. I chose two different promoters to specifically target the Cre-recombinase expression to the mammary epithelium. The MMTV (Mouse Mammary Tumor Virus) and the WAP (Whey Acidic Protein) promoters have been used extensively and have distinct differences, which affords one to investigate different aspects of FAK expression in the mammary gland. According to a previous comprehensive report [9], in adult MMTV-*Cre* mice the transgene expression is restricted to the epithelium of the striated ducts of the salivary gland and to both the secretory (alveolar) and non-secretory (ductal) mammary epithelial cells. In these mice Cre expression is present in virgin, pregnant, and lactating animals. In contrast, expression of the Cre-recombinase under the control of the WAP promoter is restricted to the secretory epithelial cells of the mammary gland during the last quarter of pregnancy (from day 14) and throughout lactation. The use of these different promoters enabled me to determine the physiological state (virgin, pregnancy, or lactation) in which normal expression of FAK is indispensable for undisturbed mammary gland development.

Materials and Methods

Our lab has produced a genetically modified mouse strain in which the third coding exon of both *FAK* alleles is flanked by two unidirectional *loxP* sites ($FAK^{flox/flox}$ mice, floxed mice). Cre-mediated deletion of exon 3 leads to a frame-shift mutation due to direct splicing from exon 2 (which contains the ATG initiation codon) to exon 4, and the resulting protein will be truncated (~70 aa) and nonfunctional (lacking the majority of FAK sequences) [12]. The MMTV-*Cre* mouse strain and the WAP-*Cre* mouse strain were both obtained from the National Cancer Institute (Frederick, MD).

First the $FAK^{flox/flox}$ alleles were introduced to both the MMTV-*Cre* and WAP-*Cre* mouse strains. Among the F1 offspring the MMTV-*Cre*; $FAK^{flox/wt}$ and WAP-*Cre*; $FAK^{flox/wt}$ males were chosen and each was mated to a $FAK^{flox/flox}$ female. This latter cross yielded males with the desired genotype: MMTV-*Cre*; $FAK^{flox/flox}$ (MMTV-*Cre* FAK conditional knock-out, MFCKO) and WAP-*Cre*; $FAK^{flox/flox}$ (WAP-*Cre* FAK conditional knock-out, WFCKO). Additionally mice with other genotypes were born in this step that served as controls: $FAK^{flox/flox}$ (Flox control, FCNT), MMTV-*Cre*; $FAK^{flox/wt}$ WAP-*Cre*; $FAK^{flox/wt}$ (Cre controls, CCNT) and $FAK^{flox/wt}$.

DNA isolation from the tail of the offspring have been described elsewhere [13]. FAK alleles were genotyped in a Polymerase Chain reaction (PCR) using the following primer pair: P1, 5'-GCTGATGTCCCAAGCTATTCC-3' and P3, 5'-AGGGCTGGTCTGCGCTGACAGG-3'. Using this primer combination the PCR reaction amplifies 1.6-kb, 1.5-kb, and 550-bp fragments from the $FAK^{flox/flox}$, wild-type, and FAK deleted alleles, respectively after 30 cycles of 94°C (3 min), 67°C (2 min), and 72°C (4 min). Primers CreF, 5'-CGCAGAACCTGAAGATGTTCGCGATTA-3' and CreR 5'-TCTCCCACCGTCAGTACGTGAGATATC-3' were used in another PCR reaction, which generated a 400-bp fragment after 35 cycles of 95°C (30 s), 60°C (30 s), and 72°C (30 s) in samples were the Cre transgene was present [12]. The PCR products were resolved in 1.2% agarose gels containing ethidium-bromide and the specific bands were identified by size against molecular weight markers. (Fig. 1).

Results

We have not encountered any difficulties during this phase of the investigation. The genotyping results are reproducible.

b. Morphological analysis of mammary gland-specific FAK deletion in virgin mice (physical examination necropsy and histology).

Rationale

The medically and pathologically proper evaluation of a new genetically modified mouse strain should always include physical examination, necropsy, and histology. Ancillary procedures (clinical pathology, imaging, etc.) should also be considered, especially if clinical signs exist. It is also important to examine both sexes, animals of different age, and physiological status (i.e. virgin, pregnant, and lactating). In certain circumstances, specific challenges have to be introduced (i.e. drugs or exercise) for the development of a certain phenotype.

Visual inspections and physical examinations should be performed often, starting immediately after birth, at weaning, before mating, and multiple times during pregnancy and lactation (if applicable).

Once the animal is euthanized, a complete necropsy should be performed by a veterinary pathologist. The major organs should be removed, inspected, and fixed along with the rest of the carcass. Histological samples should include the major organs (at least at the beginning) and any lesions (if present).

The histological examination should involve the major organs and lesions (if present). Once the affected organ(s) is (are) found, histology can focus only on the diseased organ(s).

Materials and Methods

All mice were housed in the Transgenic Mouse Core Facility at the College of Veterinary Medicine, Cornell University (Ithaca, NY) under specific pathogen-free conditions in accordance with institutional guidelines. All experimental procedures were approved by Cornell University's Institutional Animal Care and Use Committee. Physically normal, healthy MMTV-*Cre*; $FAK^{flox/flox}$ males were mated to $FAK^{flox/flox}$ females. In a similar fashion, WAP-*Cre*; $FAK^{flox/flox}$ males were

mated to $FAK^{flox/flox}$ females. Mating pairs were set up in the evenings and the mice were observed daily in the morning and females were checked for the presence of vaginal plugs. The day on which the vaginal plug was observed was considered 0.5 day post coitum (dpc).

Litters were inspected on the day they were born and daily thereafter. Newborn mice were first handled at 10 days of age when they were tattooed and tail samples were collected for genotyping. Mice were subjected to physical examination at weaning (21 days of age). Mice were group-housed in cages afterwards (maximum 5 animals of the same sex from the same litter in one cage).

Littermate female MFCKO/WFCKO and FCNT pairs of mice were euthanized at 4 weeks of age, 5 weeks of age, and at 8 weeks of age using CO_2. After euthanasia the left inguinal (#4) mammary gland was removed from the animal, spread on a glass slide, and fixed in Carnoy's fixative (6 parts 100% ethyl-alcohol, 3 parts chloroform, and 1 part glacial acetic acid) at room temperature for 16 hours for mammary gland whole-mount preparation. The rest of the body underwent a complete necropsy and tissues along with the rest of the carcass were fixed in 4% formaldehyde at 4 ºC for 16 hours. The following day the whole-mount preparation was washed in 70% ethyl-alcohol for 15 minutes at room temperature, after which the alcohol was gradually changed to distilled water. Afterwards the mammary tissue was stained in carmine alum stain for 16 hours at room temperature. The formalin fixed tissues were first washed in PBS (3 times, 20 minutes each), transferred to 65% ethyl-alcohol (30 minutes) and finally moved to 70% ethyl alcohol for long-term storage at room temperature.

The mammary whole-mounts were examined under a Leica S6D dissecting microscope. The formalin-fixed tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined histologically using an Olympus BX41 microscope. Photomicrographs were taken with an Olympus DP70 camera.

Results

The MMTV-*Cre* and WAP-*Cre* FAK conditional knock-out mice are phenotypically normal and fertile. Compared to littermate controls both the males and the virgin females exhibit normal behavior. Necropsy did not disclose any lesions. Histopathology of the examined organs (uterus, skeletal muscle, peripheral nerve, ovary/testis, kidney, adrenal gland, spleen, pancreas, liver, heart, mammary gland, salivary gland, skin, stomach, duodenum, jejunum, ileum, cecum, colon, esophagus, trachea, thyroid gland, parathyroid gland, heart, lung, peripheral lymph nodes, and brain) did not reveal any abnormalities.

c. Analysis of mammary gland development and assessment of FAK deletion in the in the mammary epithelium in virgin mice (necropsy, whole-mount, light microscopy, and immunohistochemistry).

Rationale

Since no report is available on the effects of deletion of FAK in the mammary epithelium, it is important to monitor the postnatal development of the mammary gland in its entirety. The Cre recombinase under the regulation if the MMTV promoter is expressed in the mammary epithelium early on before the unset of puberty; therefore it was important to determine if FAK deletion interferes with the development mammary gland prior to puberty (mostly ductal elongation and branching take place in this phase). With regard to the Cre recombinase expression under the WAP promoter, it is known that the Cre recombinase in this construct is only expressed in the last quarter of the pregnancy and throughout lactation. Based upon this knowledge it is reasonable to expect that FAK deletion in the WAP-*Cre*; $FAK^{flox/flox}$ animals would have no effect on the development of the virgin mammary gland.

Materials and Methods

Sample preparation (euthanasia, whole-mount preparation, and formaldehyde-fixation) has been described before. Immunohistochemistry with anti-FAK rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) was used to assess the efficiency and specificity of FAK deletion in various organs of MMTV-*Cre*; $FAK^{flox/flox}$ female and male mice. Formalin-fixed paraffin-embedded tissue sections were deparaffinized with xylene washes (3 times, 5 minutes each) and rehydrated in graded ethyl-alcohol solutions. The endogenous peroxidase activity of the tissues was quenched with incubation in 0.3% H_2O_2 in methanol for 30 minutes at room temperature. Then the tissue sections were subjected to heat-activated antigen-retrieval protocol in a 2100 Retriever antigen-retriever machine (PickCell Laboratories, Leiden, Holland) according to the manufacturer's protocol. To decrease non-specific signals, the sections were incubated first with the Avidin solution, then with the Biotin solution of the DAKO Biotin Blocking System (DAKO, Carpinteria, CA) according to the manufacturer's recommendation. The sections then were incubated with Protein Block Serum Free solution (DAKO, Carpinteria, CA) for 10 minutes at room temperature. The anti-FAK antibody was diluted at 1:200 in the above mentioned Protein Block solution and was applied to the tissue sections and the incubation was carried out in a humid chamber for 3 hours at 37 ºC. After washing with PBS (3 times, 5 minutes each) the sections were incubated for 30 minutes at 37 ºC in a humid chamber with biotinylated goat-anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA) diluted at 1:200 in PBS. After washing with PBS (3 times, 5 minutes each) the sections were incubated for 20 minutes at room temperature in a humid chamber with horseradish-peroxidase-conjugated streptavidin (Vector Laboratories, Inc., Burlingame, CA). After washing with PBS (3 times, 5 minutes each) the sections were then incubated with 3,3`-diaminobenzidine (DAB) solution prepared from 3'3`-diaminobenzidine tablet set (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's recommendation. Tissues from the MFCKO and FCNT animals were incubated with the DAB solution for exactly the same length of time. In the negative control slides the primary antibody was substituted with identically diluted normal rabbit IgG.

Results

Comparison of whole-mounts from littermate MFCKO and FCNT females did not reveal any significant and sustained differences in virgin mice at 4 weeks, 5 weeks, and at 8 weeks of age (1 complementary pair for each age group) (Figures 2-4.). Similarly, no morphological differences were seen in mammary whole-mounts from WFCKO and FCNT females at 9 weeks of age (1 complementary pair) (Figure 5).

Immunohistochemistry revealed minimal FAK expression in the mammary epithelium of the MMTV-*Cre*; $FAK^{flox/flox}$ virgin females and strong expression of FAK in the mammary epithelium of $FAK^{flox/flox}$ virgin females (Figure 6). In contrast, the WAP-Cre; $FAK^{flox/flox}$ females had similar levels of FAK expression in the mammary epithelium as did the $FAK^{flox/flox}$ females (data not shown).

FAK expression was greatly reduced in the striated ductal epithelium of the salivary gland in MMTV-*Cre*; $FAK^{flox/flox}$ mice (in both males and females) (Figure 7). Similarly FAK expression in the epidermis was moderately reduced in MMTV-*Cre*; $FAK^{flox/flox}$ mice (in both males and females, data not shown).

d. Expansion of the colony of the mammary gland specific FAK knock-out mice.

Rationale

In order to have litters consisting of only conditional knock-out (MMTV-*Cre*; $FAK^{flox/flox}$, MFCKO and WAP-*Cre*; $FAK^{flox/flox}$, WFCKO) and control ($FAK^{flox/flox}$) offspring the following mating pairs were used: MMTV-*Cre*; $FAK^{flox/flox}$ male crossed with $FAK^{flox/flox}$ female and WAP-*Cre*; $FAK^{flox/flox}$ male mated to $FAK^{flox/flox}$ female.

Materials and Methods

In the MMTV-*Cre* FAK conditional knock-out strain we now have 6 mating pairs (MMTV-*Cre*; $FAK^{flox/flox}$ male and $FAK^{flox/flox}$ female) to generate animals for experiments and for supply. In the WAP-*Cre* FAK conditional knock-out strain there are 4 mating pairs.

Results

So far 78 mice were born to several MMTV-*Cre*; $FAK^{flox/flox}$ male and $FAK^{flox/flox}$ female mating pairs. To the WAP-*Cre*; $FAK^{flox/flox}$ male $FAK^{flox/flox}$ female mating pairs, 29 mice were born. Much less MFCKO females were born (14%) in the MMTV-*Cre* strain, than expected (25%), while the frequency of MFCKO males (36%) surpasses the expected value (25%). The total number of animals in the WAP-*Cre* strain is much less, but it seems that FCNT animals (both males and females) were born with a greater frequency than expected. The details on the respective genotypes and sex are found in Table 1.

Aim 2. Effects of FAK deletion on the lobulo-alveolar development of the mammary gland during pregnancy and lactation, Months 18-24

a. Mating mammary gland specific FAK conditional knock-out and control females.

Rationale

Mating pairs were set up at 8 weeks of age. Mating strategy, daily observations, and vaginal plug detections have been described in detail before.

Materials and Methods

The details on genotyping have been described before.

Results

The length of gestation in the MFCKO, WFCKO, and FCNT females was 19 days which is within normal limits for mice. Based upon the genotypically complementary mating pairs consisting of littermate MFCKO and FCNT females I found that while litter sizes were comparable, pups born to MFCKO females did not survive, whereas the vast majority of pups born to FCNT females always survived (Table 2.). In contrast, WFCKO females can sustain normal-sized litters and the weaned pups are normal (Table 3).

b. Analyze mammary gland development during pregnancy (whole-mount, histology, immunohistochemistry, and western blotting).

Rationale

Since wild-type FAK increases DNA synthesis, accelerates G1/S transition, and upregulates cyclin D1 expression [10] it is expected that FAK expression is especially important in the lobulo-alveolar development of the mammary gland during pregnancy. It has been shown that cyclin D1 deletion in mice leads to dwarfism and pregnant females have severely inhibited lobulo-alveolar development of the mammary gland and have difficulty sustaining their litters [11].

Materials and Methods

The appropriate methods (necropsy and sample collection for whole-mounts, histology, and immunohistochemistry) have been described in detail before.

Results

Investigations are ongoing. Based upon the phenotype of the lactating MFCKO females, I expect that the lobulo-alveolar growth will be greatly inhibited in MFCKO females during pregnancy.

c. Analyze mammary gland development and assess milk production during lactation.

Rationale

It is not know if FAK expression is directly involved in the regulation of milk production. Knowing the key role FAK plays in cellular function, it is conceivable that milk production is among them.

Materials and Methods

In addition to the previously described sample collection methods (mammary whole-mounts, formalin-fixed tissues, histology, and immunohistochemistry) mammary gland, liver, salivary gland, and lung lysates were collected from lactating females. Organs were snap-frozen in liquid nitrogen and then were ground using a mortar and a pestle. Finely ground tissue was lysed at 4 ºC for 15 minutes in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) supplemented with protease inhibitors (1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF). Lysates were centrifuged to remove insoluble cellular matter and total protein concentration was measured with the Bio-Rad Protein Assay (Hercules, CA). Protein lysates were resolved on SDS-PAGE, transferred onto nitrocellulose membranes and were subjected to western blotting using antibodies against FAK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and viculin (Sigma Chemical Company, St. Louis, MO).

Results

In contrast to the virgin mice, lactating (1st day of lactation) MMTV-*Cre* FAK conditional knock-out mice cannot sustain their litters and show distinct gross and histologic abnormalities. In Flox control mice at 1st day of lactation the mammary glands are expanded (turgid), often mottled tan-red, and the mammary ducts are frequently filled with milk appearing as arborizing white streaks at the periphery of the glands. The pectoral glands are easily distinguishable from the surrounding fascia due to their enlargement. In contrast, FAK conditional knock-out mice have less distinct, small, uniformly tan mammary glands with no gross evidence of milk production at 1st day of lactation. The pectoral glands are often indistinguishable from the surrounding fascia. The pups born to the MFCKO females are of normal size and behavior, however they have no milk in their stomach. The pups born to the FCNT females all contain adequate amount of milk in their stomach. Examination of mammary gland whole mounts revealed that at the 1st day of lactation the MFCKO mammary glands have essentially normal ductal architecture, but the lobulo-alveolar units are sparse and smaller compared to the FCNT mammary glands (Figure 8). Mammary gland histology revealed that the MFCKO mammary glands are still mostly composed of adipose stroma and the parenchyma primarily consists of dilated ductular profiles and small clusters of alveoli around some ducts. The individual alveoli in the MFCKO mammary glands are small, lined by cuboidal epithelial cells, and have very little secretum within. In contrast, in the FCNT mammary glands the individual alveoli are larger in diameter, are lined by flattened, vacuolated epithelial cells, and contain flocculent, eosinophilic material (milk). The histologic diagnosis for the MFCKO mammary gland is diffuse severe lobulo-alveolar hypoplasia (Figure 9). This morphology is compatible with minimal secretory

activity, which explains the failure of the MFCKO females to sustain their litters. So far, 3 pairs of littermate MFCKO and FCNT females were examined and the MFCKO females all showed diffuse sever lobulo-alveolar hypoplasia.

Immunohistochemistry revealed minimal FAK expression in the mammary epithelium of the MMTV-*Cre*; $FAK^{flox/flox}$ females at 1st day of lactation (Figure 10A).

Similarly to the results seen in immunohistochemitry, western blotting showed markedly reduced FAK expression in the MMTV-*Cre*; $FAK^{flox/flox}$ mammary gland lysates compared to $FAK^{flox/flox}$ control mammary gland lysates (Figure 10B).

In contrast to the lactating MMTV-*Cre* FAK conditional knock-out mice, lactating WAP-*Cre* FAK knock-out mice have no significant morphological anomalies in the mammary gland or elsewhere in the body. No differences were seen in mammary whole-mounts (Figure 11) and in histological sections of the mammary gland (Figure 12).

Western blotting revealed moderately reduced FAK expression in the WAP-*Cre*; $FAK^{flox/flox}$ mammary gland lysates compared to $FAK^{flox/flox}$ control mammary gland lysates (Figure 13). So far 1 pair of WFCKO and FCNT animals was analyzed.

d. Analyze mammary gland involution after weaning.

Rationale

A report from our lab provided evidence that FAK promotes the expression of certain matrix metalloproteases [14]. It is known that matrix metalloprotease expression is essential for normal mammary gland involution [15]; therefore we expect that FAK deletion in the mammary epithelium will have an adverse effect on mammary gland involution. However this effect might be masked by the pre-existing lobulo-alveolar hypoplasia in the pregnant mammary gland.

Materials and Methods

Various sample collection methods (mammary whole-mounts, formalin-fixed tissues, histology, and immunohistochemistry) have been described previously. I will also use gelatin zymography [16] to assess matrix metalloprotease function.

Results

Investigations are ongoing.

Bulleted List of Key Research Accomplishments

- **The Focal Adhesion Kinase (FAK) was specifically deleted in the mammary epithelium with the Cre-LoxP method using two different Cre transgenic mouse strains (MMTV-*Cre* and WAP-*Cre*).**

- **The specificity of FAK deletion in the mammary epithelium was confirmed multiple times with immunohistochemistry and western blotting. Deletion using the MMTV-*Cre* strain resulted in uniform and almost complete deletion of FAK in the mammary epithelium in all examined virgin and lactating females. Deletion using the WAP-*Cre* strain resulted in moderately diminished FAK immunoreactivity in the mammary epithelium of lactating females.**

- **Using either the MMTV-*Cre* or WAP-*Cre* strains for FAK deletion, the mammary epithelial-specific FAK conditional knock-out virgin females and males are phenotypically normal and fertile.**

- **Females, in which the MMTV-*Cre* transgene was used to specifically delete FAK, can carry their offspring to term, but they are unable to lactate due to a severe, diffuse lobulo-alveolar hypoplasia affecting their mammary gland. The pups born to these females invariably die of inanition and hypothermia within a day.**

- **Females, in which the WAP-*Cre* transgene was used to specifically delete FAK, can carry their offspring to term and they can sustain their litters until weaning without any difficulty. The size of these litters is normal and the pups in these litters are of normal size and weight.**

- **Based upon the previous observations, I concluded that FAK is indispensable in the proliferative phase (first 14 days) of the lobulo-alveolar development of the murine mammary gland.**

Reportable Outcomes

During my investigations I created two unique genetically engineered mouse strains. In the MMTV-Cre FAK conditional knock out (MFCKO) mouse strain FAK is specifically deleted in the ductal and alveolar epithelium of the mammary gland and in the epithelium of the striated ducts of the salivary gland. Virgin MFCKO females and MFCKO males have no phenotypic changes as a result of the genetic modification. Pregnant and lactating MFCKO females present with a severe lobulo-alveolar hypoplasia in the mammary gland. In the WAP-Cre FAK conditional knock out (WFCKO) mouse strain FAK is specifically deleted in the alveolar epithelium of the mammary gland. No phenotypic abnormalities were recorded in virgin and lactating females and in males so far.

Conclusions

In this annual report I gave a detailed account on the creation and phenotypic evaluation of two genetically modified mouse strains in which FAK is specifically deleted in the mammary epithelium using MMTV-*Cre* and WAP-*Cre* transgenic mouse strains.

I used immunohistochemistry and western blotting to prove that FAK was specifically deleted in the mammary epithelium. Deletion of FAK using the MMTV-*Cre* strain resulted in uniform and almost complete absence of FAK in the mammary epithelium in all examined virgin and lactating females. Deletion using the WAP-*Cre* strain resulted in moderately diminished FAK immunoreactivity in the mammary epithelium of lactating females. The virgin animals in both conditional knock-out strains are phenotypically normal and fertile.

MMTV-*Cre* directed deletion of FAK results in severe lobulo-alveolar hypoplasia of the mammary gland in lactating females rendering them unable to lactate.

WAP-*Cre* mediated FAK deletion does not cause any appreciable defect in the mammary gland during pregnancy and lactation and these females can sustain normal-sized litters.

The mammary phenotype in the lactating MMTV-*Cre* FAK conditional knock-out females is akin to that in the cyclin D1 total knock-out mice [11], pointing to an essential role for FAK during the lobulo-alveolar development of the mammary gland during pregnancy. Unlike some of the cyclin D1 total knock-out female mice however, the mammary specific FAK knock-out females cannot sustain their litters. This phenotype in the FAK knock-out females was expected, although with less severity.

Unlike the MMTV promoter, the WAP promoter has a distinct temporal expression pattern: it is not active until the last quarter of pregnancy (after the 14^{th} day) [9]. This fact, combined with the observation that the WAP-*Cre* FAK knock-out females that do not have any appreciable defect in the mammary gland during lactation, suggests that FAK activity in the mammary epithelium is not required in the last quarter of pregnancy, rather it is essential for the proliferative phase of the lobulo-alveolar development of the murine mammary gland (up to the 14^{th} day of pregnancy). The observation that FAK activity is of primary importance in rapidly dividing cells is in harmony with observations made in cell culture [10].

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Appendices

	Number (percentage of total) FCNT ♀ animals	Number (percentage of total) FCNT ♂ animals	Number (percentage of total) MFCKO/WFCKO ♀ animals	Number (percentage of total) MFCKO/WFCKO ♂ animals
MMTV-*Cre* strain	14 (18%)	25 (32%)	11 (14%)	28 (36%)
WAP-*Cre* strain	10 (34.5%)	10 (34.5%)	5 (17%)	4 (14%)

Table 1. **Genotypes of progeny according to sex from MMTV-*Cre*; $FAK^{flox/flox}$ x $FAK^{flox/flox}$ and WAP-*Cre*; $FAK^{flox/flox}$ x $FAK^{flox/flox}$ intercrosses.**

Reproductive data of littermate females			
Litters to FCNT females		Litters to MFCKO females	
MFCKO-90	9 pups (all alive)	MFCKO-93	8 pups (all dead)
MFCKO-54	5 pups (all alive)	MFCKO-59	7 pups (all dead)
MFCKO-30	7 pups (all alive)	MFCKO-32	No pups (did not get pregnant)
MFCKO-27	5 pups (all alive)		
MFCKO-16	7 pups (all alive)	MFCKO-7	Pups were born, but all died.
		MFCKO-9	4 pups were born, but all died.

Table 2. **Litter size and viability of pups born to littermate MFCKO and FCNT females.**

Reproductive data of littermate females			
Litters to FCNT females		Litters to WFCKO females	
WFCKO-50	6 pups, all died (inattentive mother)	WFCKO-53	8 pups, all alive
WFCKO-17	9 pups weaned 6 pups weaned 8 pups weaned	WFCKO-19	7 pups weaned 8 pups weaned 8 pups weaned

Table 3. **Litter size and viability of pups born to littermate WFCKO and FCNT females.**

A

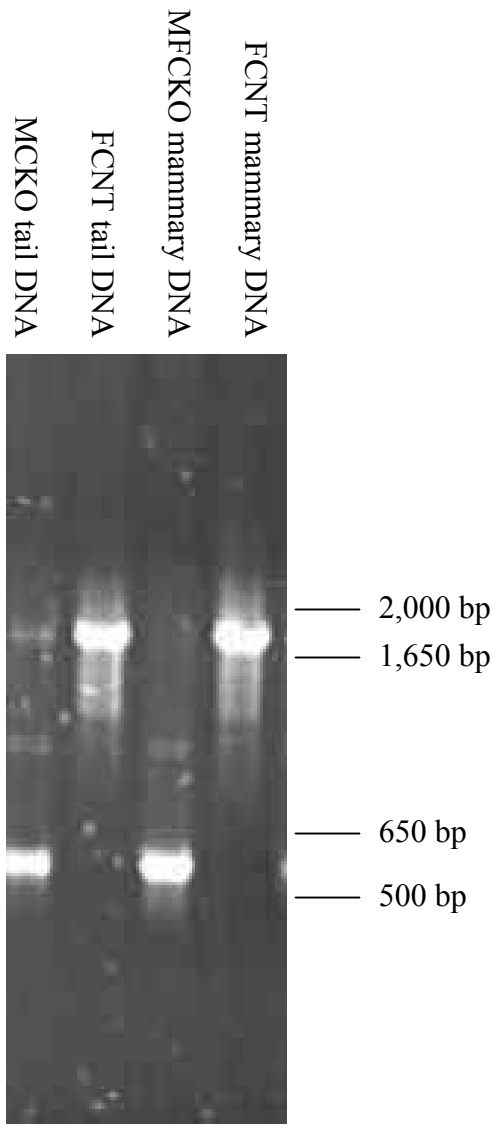
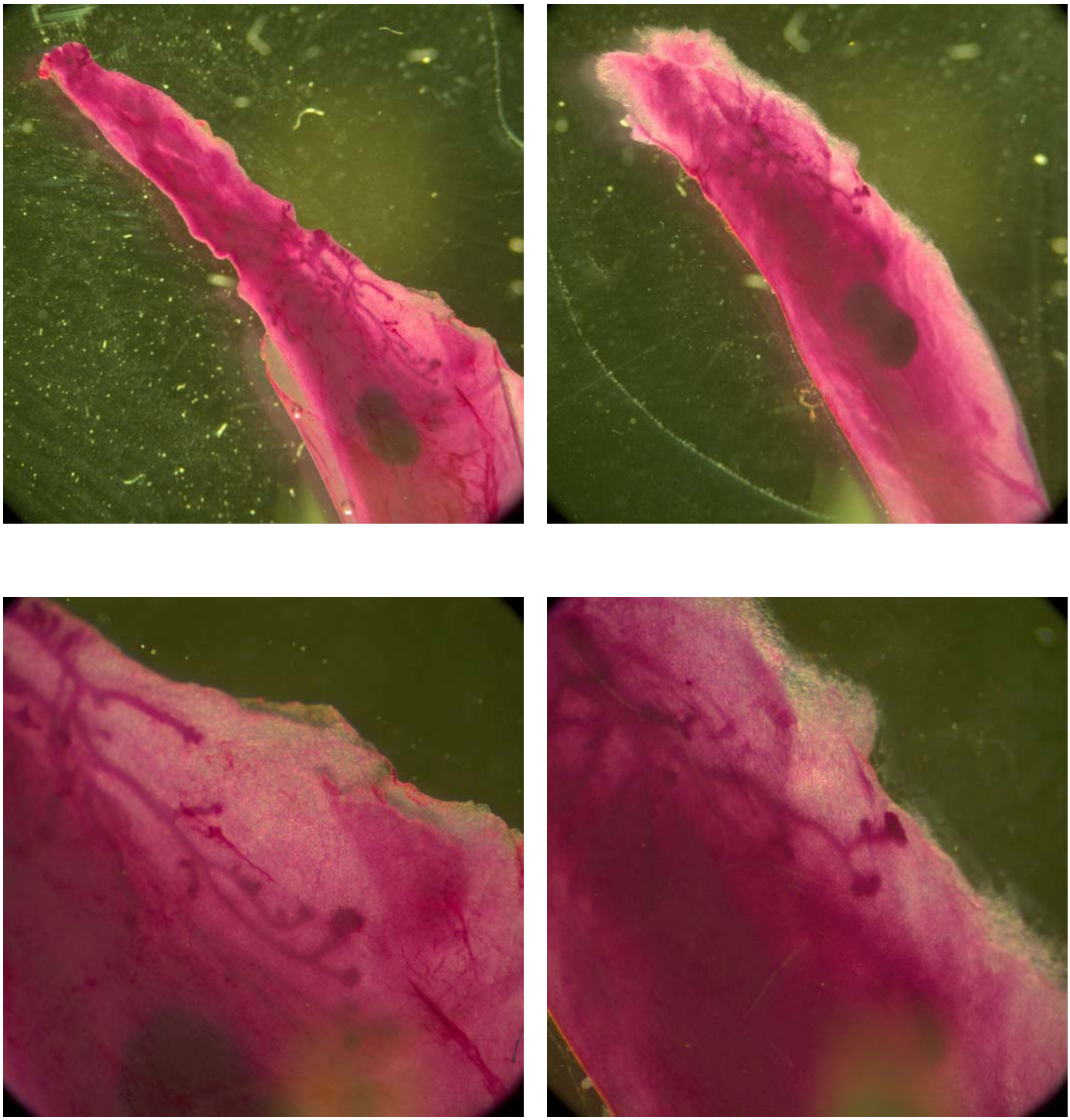
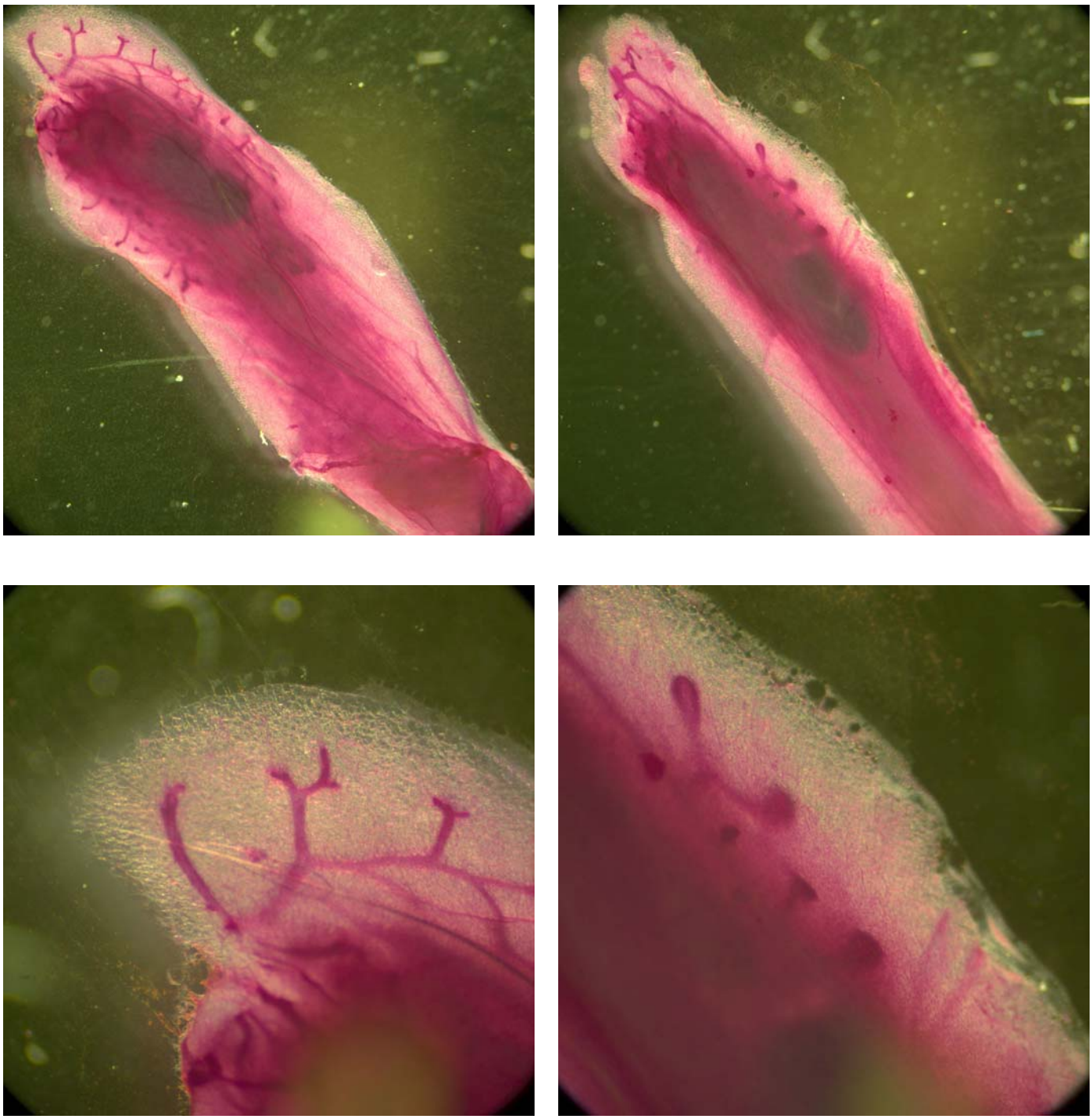


Figure 1. Detection of the FAK floxed and deleted alleles in the genomic DNA from FCNT and MFCKO mice, respectively.



FCNT MFCKO

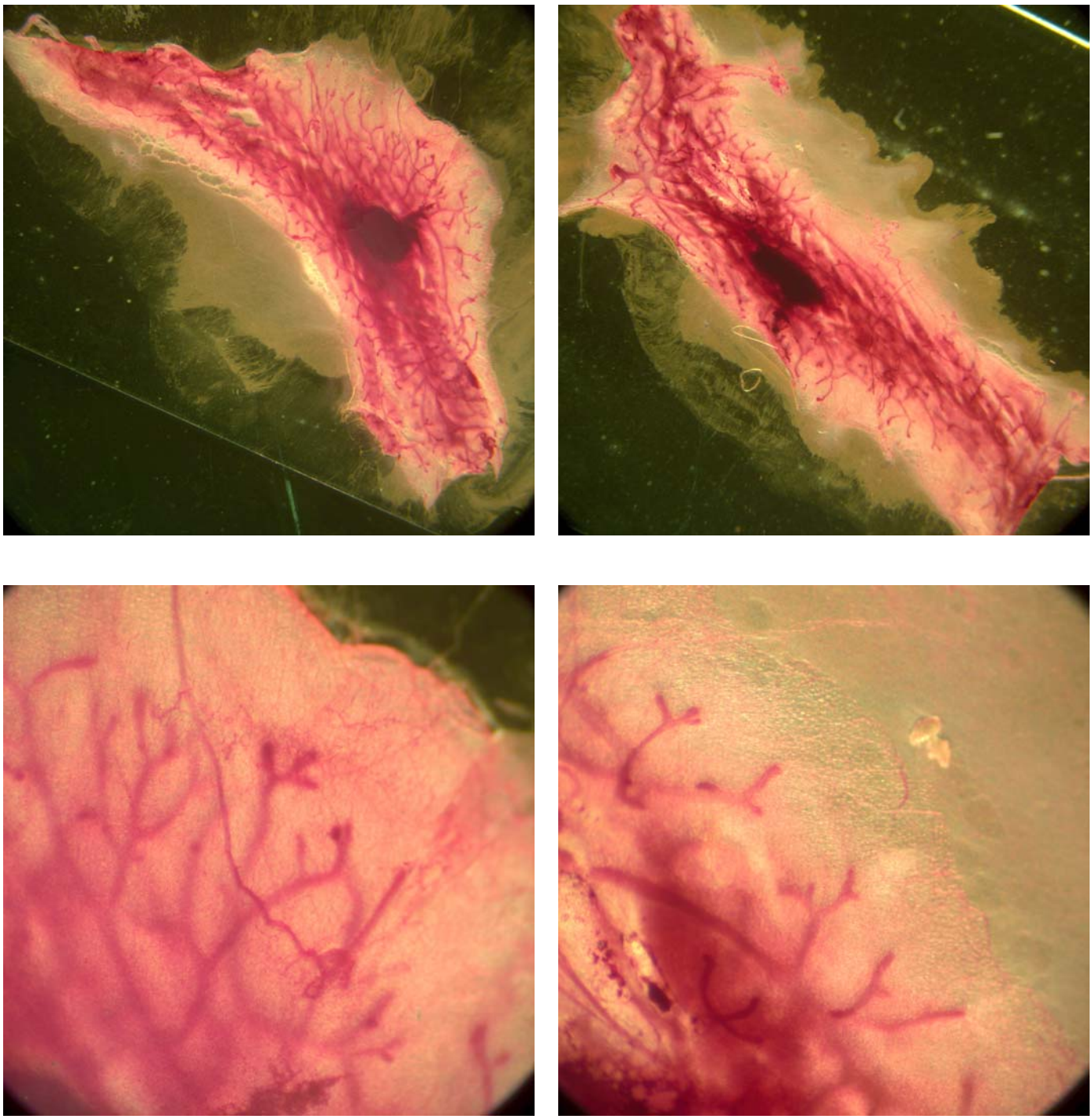
Figure 2. Mammary wholemounts of littermate 4-week-old MFCKO and FCNT female mice.
Note that there is virtually no difference in the ductal architecture. (Magnification, upper panels 6.3x, lower panels 40x.)



FCNT MFCKO

Figure 3. Mammary whole-mounts of littermate 5-week-old MFCKO and FCNT female mice.

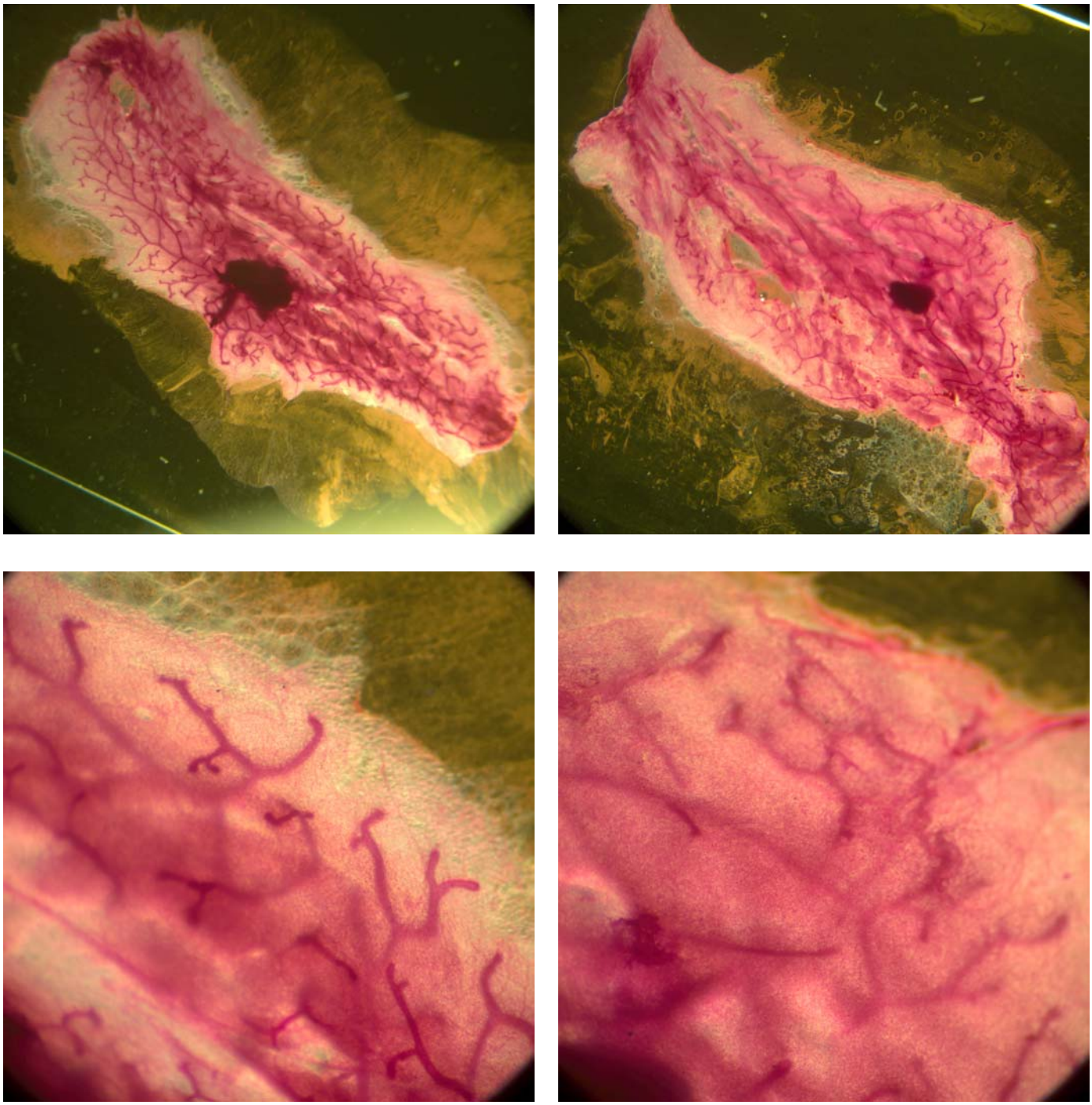
There is a mild retardation of ductal outgrowth in the MFCKO mammary gland. Additionally the terminal end buds are moderately enlarged in the MFCKO gland. (Magnification, upper panels 6.3x, lower panels 40x)



FCNT MFCKO

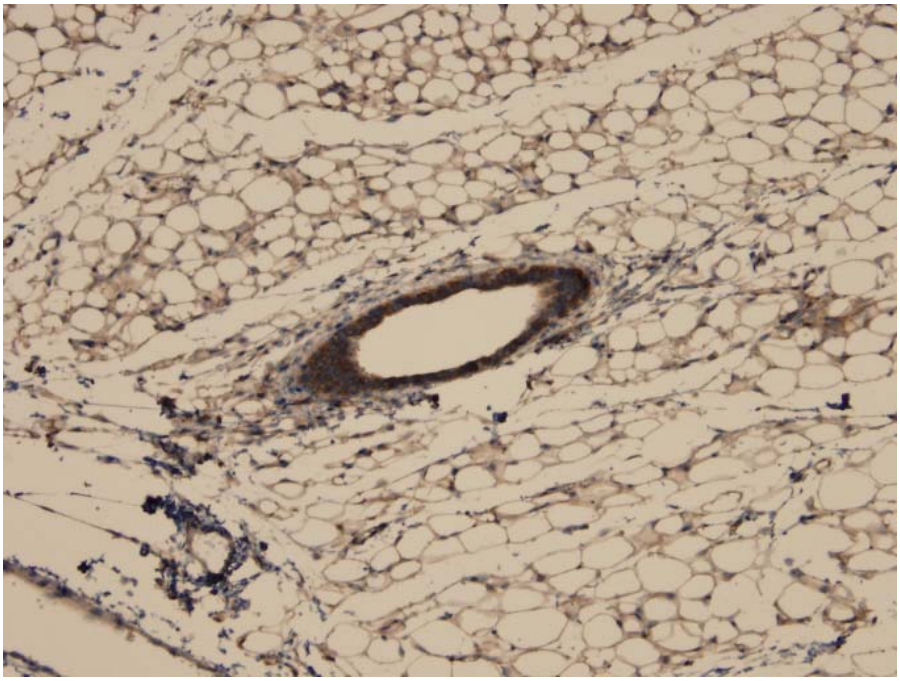
Figure 4. Mammary whole-mounts of littermate 8-week-old MFCKO and FCNT female mice.

The ductal architecture is indistinguishable between the MFCKO and FCNT mammary glands. The terminal end buds are have similar morphology as well. (Magnification, upper panels 6.3x, lower panels 40x)

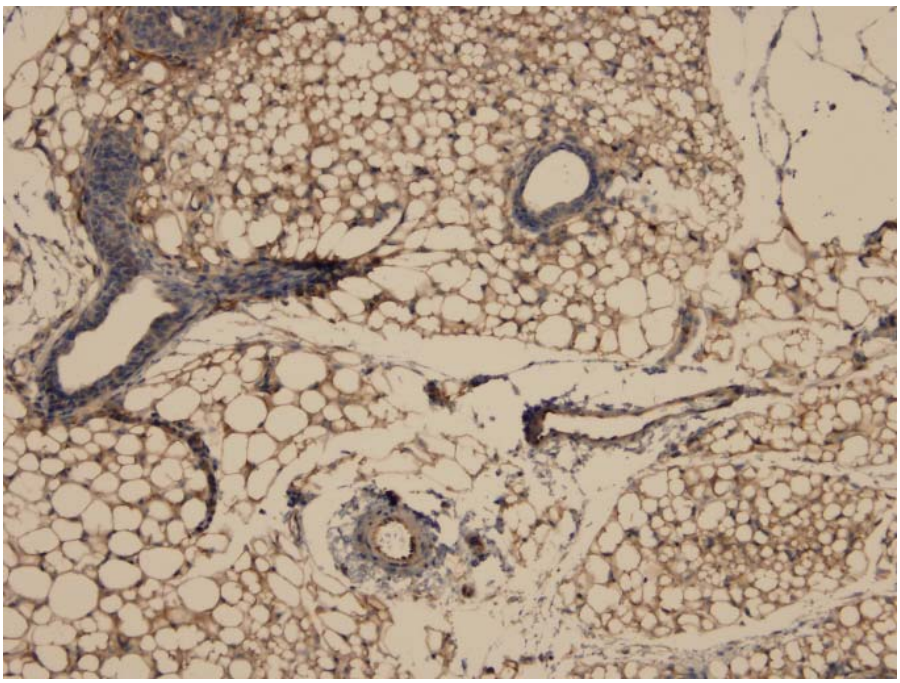


FCNT WFCKO

Figure 5. Mammary whole-mounts of littermate 9-week-old WFCKO and FCNT female mice.
The ductal architecture is indistinguishable between the WFCKO and FCNT mammary glands. (Magnification, upper panels 6.3x, lower panels 40x)

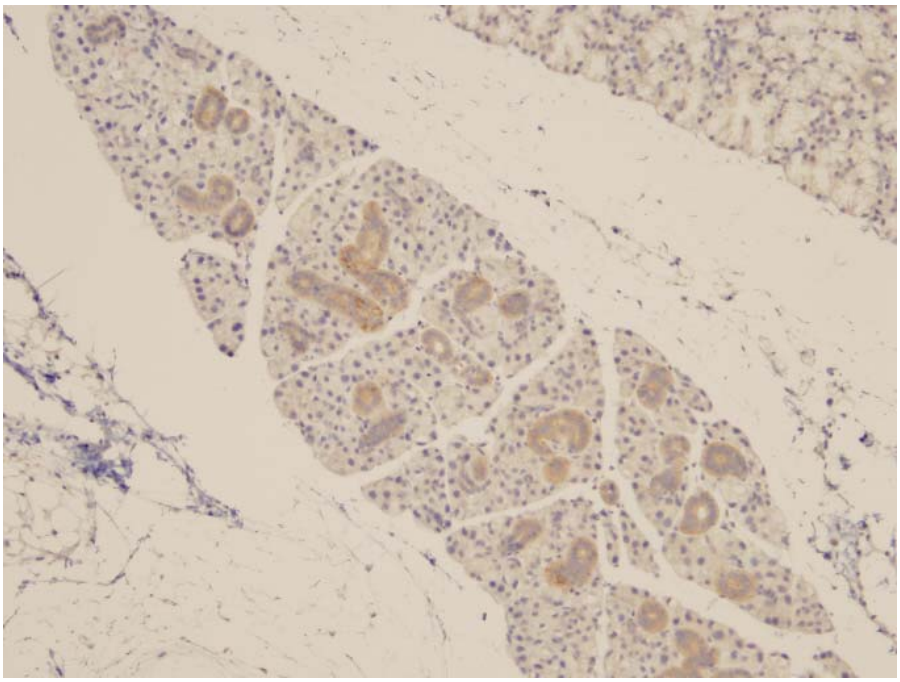


FCNT

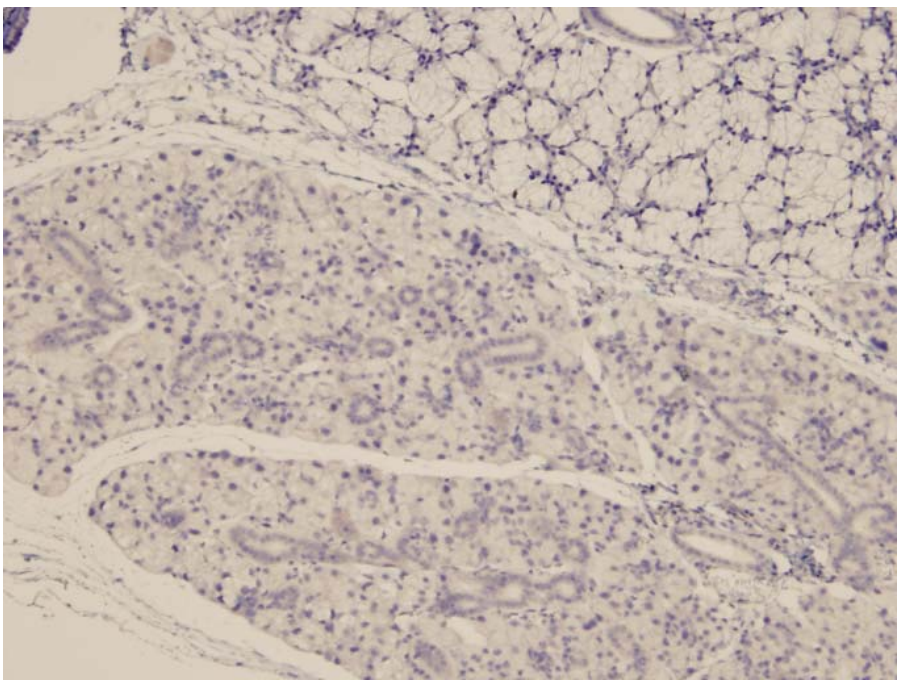


MFCKO

Figure 6. Immunohistochemistry for FAK expression in the mammary gland of littermate 8-week-old virgin MFCKO and FCNT female mice. Note the strong signal in the FCNT mammary ductal epithelium and the absence of specific immunostaining in the MFCKO epithelium. Also note the strong specific signal in arteriolar and venular endothelium in the MFCKO mammary fat pad (internal controls). Magnification: 220x

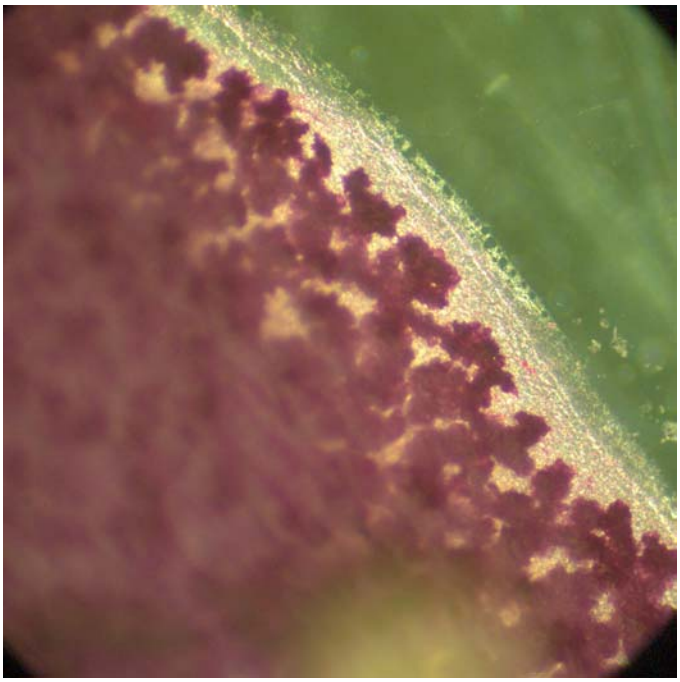


FCNT

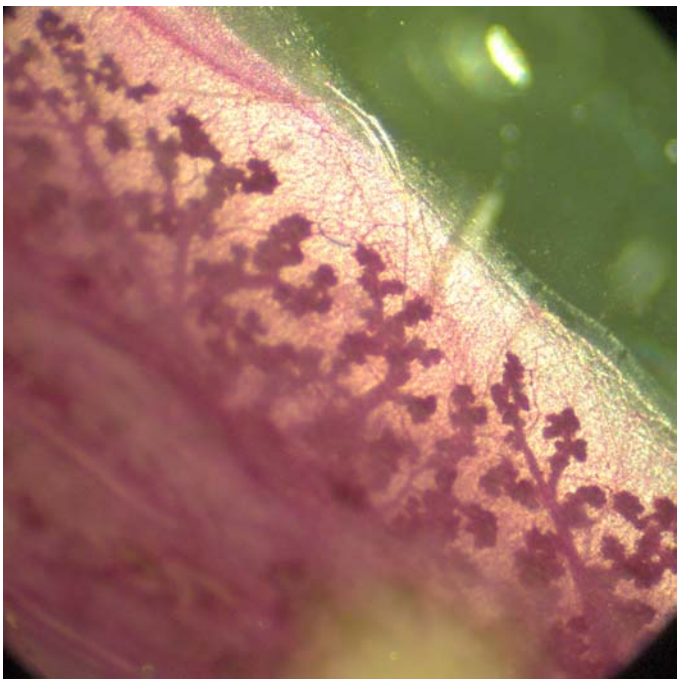


MFCKO

Figure 7. Immunohistochemistry for FAK expression in the salivary gland of littermate virgin MFCKO and FCNT female mice.
Note the strong signal in the striated ductal epithelium of the salivary gland of the FCNT animal and the absence of specific immunostaining at the same location in the MFCKO epithelium. Magnification: 220x

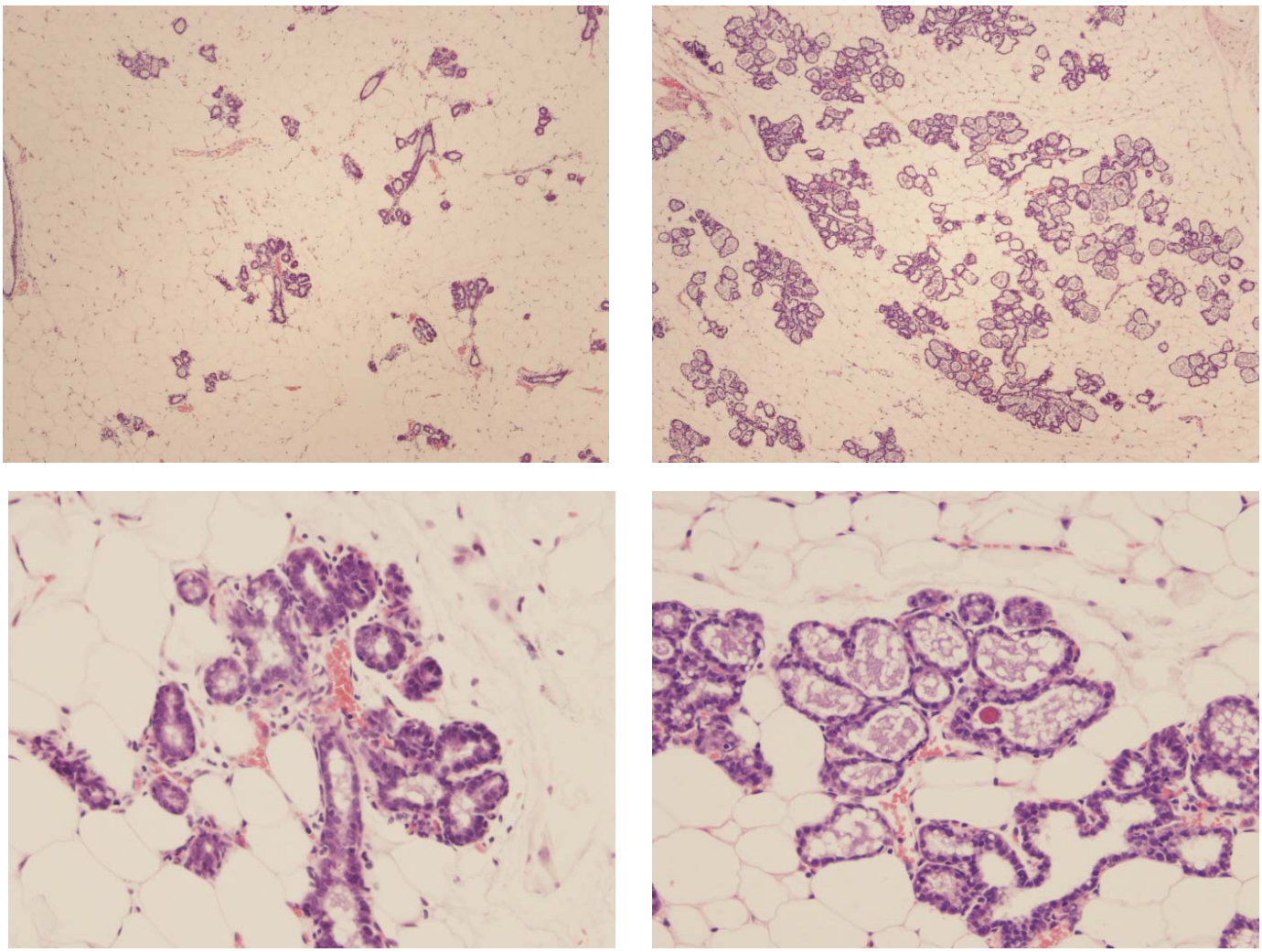


FCNT



MFCKO

Figure 8. Moderate to severe lobulo-alveolar hypoplasia in the conditional knock-out mammary glands
Note the paucity of the lobulo-alveolar units in the MFCKO mammary gland. Magnification 40x.



MFCKO FCNT

Figure 9. Moderate to severe lobulo-alveolar hypoplasia in the conditional knock-out mammary glands.
Upper panels, magnification 44x. Note that in the MFCKO gland the fat pad is sparsely populated with the lobulo-alveolar units, whereas in the FCNT gland the fat pad is mostly filled with the lobulo-alveolar units. Lover panels, magnification 220x. Individual lobulo-alveolar units are shown. Note the cuboidal epithelial lining, the absence of clear, round vacuoles in the epithelium, and the paucity of secretory material in the alveolar lumina of the MFCKO gland. The epithelium in the FCNT gland is composed of attenuated epithelial cells, which have clear, round, cytoplasmic vacuoles and the alveolar lumina are filled with flocculent, eosiniphilic material (milk).

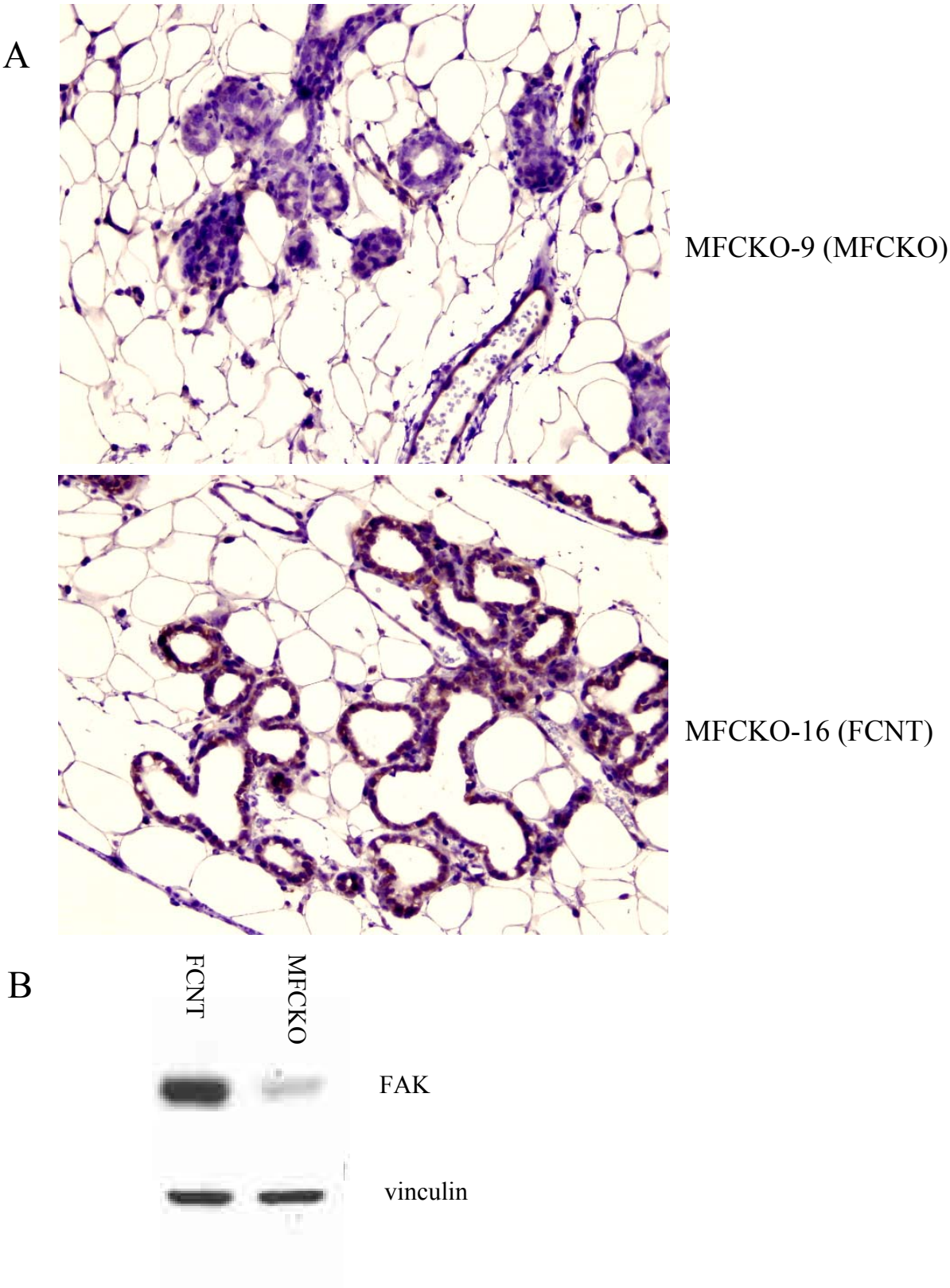
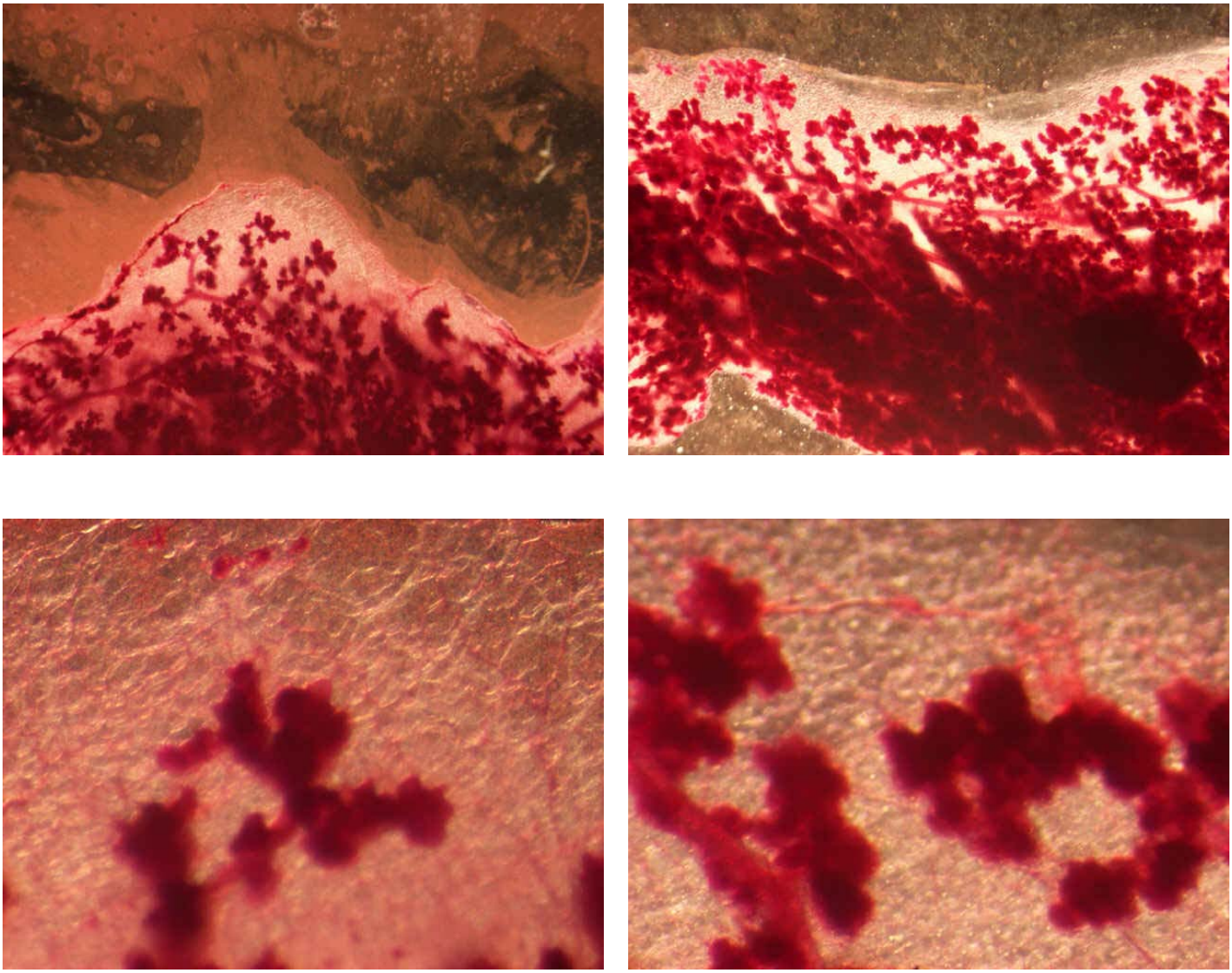
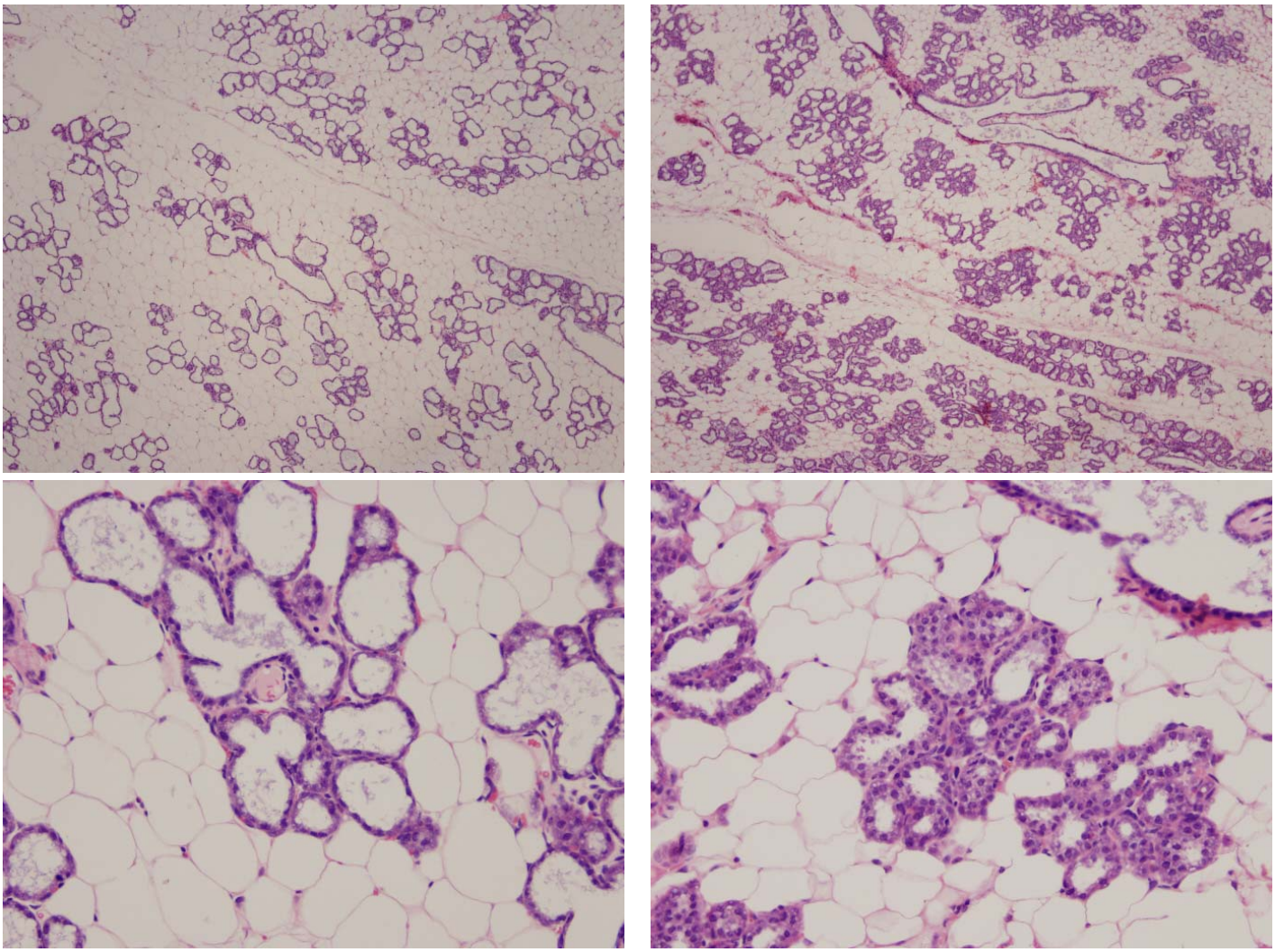


Figure 10. Immunohistochemistry and western blotting for FAK showing greatly reduced FAK expression in the MFCKO mammary gland.
(A) Immunohistochemistry, magnification 220x, Note that the vascular endothelium shows distinct staining even in the MFCKO gland (B) Western blotting of mammary gland lysates from the same animals. There is greatly reduced FAK expression in the MFCKO mammary lysate. Vinculin immunoblotting serves as loading control for SDS-PAGE



FCNT WFCKO

Figure 11. Mammary gland whole-mounts from littermate WFCKO and FCNT females at 1st day of lactation.
Note that the lobulo-alveolar units are well formed and are present in large numbers in both the WFCKO and FCNT mammary glands. (Magnification, upper panels 6.3x, lower panels 40x)



FCNT WFCKO

Figure 12. Mammary gland histology in littermate WFCKO and FCNT females at 1st day of lactation.
Note that the lobulo-alveolar units are well formed and fill most of the mammary fat pad in both the WFCKO and FCNT mammary glands. (Magnification, upper panels 44x, lower panels 220x magnification)

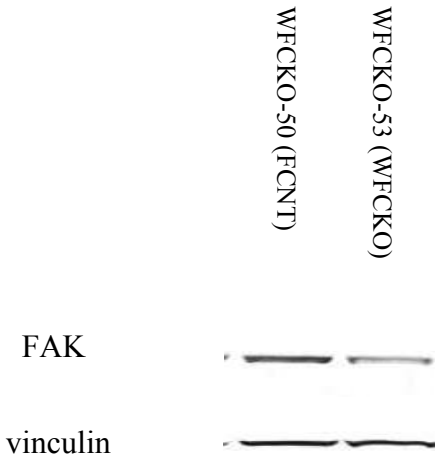


Figure 13. FAK expression levels in littermate WFCKO and FCNT females at 1st day of lactation.
There is moderately reduced FAK expression in the WFCKO mammary gland lysate. Immunoblotting for vinculin serves as loading control for SDS-PAGE.