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# Epithelial-Specific and Stage-Specific Functions of Insulin-Like Growth Factor-I during Postnatal Mammary Development

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**Postnatal development of the mammary gland requires interactions between the epithelial and stromal compartments, which regulate actions of hormones and growth factors. IGF-I is expressed in both epithelial and stromal compartments during postnatal development of the mammary gland. However, little is known about how local expression of IGF-I in epithelium or stroma regulates mammary growth and differentiation during puberty and pregnancy-induced alveolar development. The goal of this study was to investigate the mechanisms of IGF-I actions in the postnatal mammary gland and test the hypothesis that IGF-I expressed in stromal and epithelial compartments has distinct functions. We established mouse lines with inactivation of the *igf1* gene in mammary epithelium by crossing *igf1*/loxP mice with mouse lines expressing Cre recombinase under the control of either the mouse mammary tumor virus long-terminal repeat or the whey acidic protein gene promoter. Epithelial-specific loss of IGF-I during pubertal growth resulted in deficits in ductal branching. In contrast, heterozygous reduction of IGF-I throughout the gland decreased expression of cyclins A2 and B1 during pubertal growth and resulted in alterations in proliferation of the alveolar epithelium and milk protein levels during pregnancy-induced differentiation. Reduction in epithelial IGF-I at either of these stages had no effect on these indices. Taken together, our results support distinct roles for IGF-I expressed in epithelial and stromal compartments in mediating growth of the postnatal mammary gland. (*Endocrinology* 147: 5412–5423, 2006)**

GROWTH AND DIFFERENTIATION of the mammary gland involves complex interactions between the epithelial and stromal compartments. The contributions of the epithelial and stromal compartments in mediating mammary development has been the focus of numerous studies, which have revealed differential expression and function of certain growth factors and steroid hormones or their receptors in each compartment. However, the potential for differential functions of molecules expressed in both compartments has not been clearly elucidated in most cases.

The role of epithelial and stromal-derived growth factors in mediating mammary development is particularly of interest for the IGFs because previous studies demonstrated a complex pattern of IGF-I and IGF-II expression in both epithelial and stromal compartments during postnatal development of the mammary gland (1, 2). Initial investigations of IGF-I expression in the postnatal mammary gland demonstrated that IGF-I is expressed in the nonepithelial portion of the mammary gland in early puberty during which it is regulated by GH (3, 4). Based on these studies, IGF-I was long considered a stromal growth factor in the postnatal mammary gland. However, in subsequent studies, we demonstrated that IGF-I mRNA is expressed in the epithelial cells of the terminal end buds (TEBs) as well as the stromal compartment during pubertal ductal growth (1, 2). In addition, IGF-I mRNA is expressed in alveolar and ductal epithelium during late pregnancy ages. The spatially and temporally restricted expression of IGF-I in the epithelium led us to propose the hypothesis that epithelial and stromal IGF-I has distinct functions. Consistent with distinct functions of IGF-I in the two compartments, we recently demonstrated that several members of the family of IGF binding proteins (IGFBPs), which function to regulate and localize the IGFs, are differentially expressed in the epithelial and stromal compartments (5). The pronounced expression of several IGFBPs in cells immediately adjacent to the growing ductal structures suggests that the IGFBPs may partition the IGFs for specific actions in the epithelial and stromal compartments.

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; Cre, Cre recombinase; FF, two floxed alleles; FN, floxed/null; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; HRP, horseradish peroxidase; IGFBP, IGF binding protein; IGF-IR, IGF type I receptor; L2, d 2 of lactation; MEC, mammary epithelial cell; MMTV, mouse mammary tumor virus; p, pregnancy day; RPA, ribonuclease protection assay; Stat, signal transducer and activator of transcription; TEB, terminal end bud; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; WAP, whey acidic protein; WF, one wild-type and one floxed allele.

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Functional studies support a role for IGF-I as a positive mediator of mammary epithelial cell (MEC) growth. IGF-I is a known mitogen for MECs and breast cancer cells *in vitro* (6–11). Our previous studies using a whole organ culture system further support a role for IGF-I in cell cycle regulation in mammary ductal epithelial cells (11, 12). These studies also suggested that IGF-I coordinates with the epidermal growth factor -related ligands in promoting proliferation of MECs in the intact gland. Studies on exogenous addition of IGF-I to mammary fat pads and systemic deletion of IGF-I in mice support an essential role for this growth factor in postnatal mammary development (13, 14).

Whereas previous studies support an essential role for IGF-I in the postnatal mammary gland, little is known about how local expression of IGF-I regulates mammary growth. The goal of this study was to investigate the mechanisms of IGF-I actions in mammary epithelial growth and test the hypothesis that IGF-I expressed in stroma and epithelial compartments has distinct functions during normal pubertal ductal growth and pregnancy-induced alveolar development of the mammary gland. We used an established mouse line carrying *igf1*/loxP alleles, which was used previously to create liver-specific and pancreatic-specific *igf1* gene inactivation (15–21). Here we report analyses of mammary glands from mice with inactivation of the *igf1* gene in mammary epithelium established by crossing the *igf1*/loxP mice with mouse lines expressing the bacteriophage Cre recombinase (Cre) recombinase under the control of either the mouse mammary tumor virus (MMTV) long-terminal repeat or the whey acidic protein (WAP) gene promoter (22, 23). We provide data supporting distinct roles for epithelial and stromal IGF-I during postnatal mammary development.

## Materials and Methods

### *Generation and analysis of transgenic mice*

All animal experimentation protocols were approved by Penn State Hershey Medical Center Institutional Animal Care and Use Committees and were conducted in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The *igf1*/loxP mouse line and the mammary expressing Cre transgenic mouse lines (MMTV-Cre and WAP-Cre) have been previously described (15, 22, 23). MMTV-Cre (line A) mice were bred with *igf1*/loxP mice to generate mice carrying the MMTV-Cre transgene and two *igf1* floxed alleles (FF). Due to recombination of the floxed allele in the female germline of MMTV-Cre/*igf1*-floxed mice, it was possible to generate animals containing one null allele and one floxed allele at the *igf1* gene locus. These mice were then maintained as an *igf1* floxed/null (FN) line and used to generate additional mice for analysis. To avoid further germline recombination, mice were generated for pubertal analysis by breeding female *igf1*-FN mice to male mice carrying the MMTV-Cre transgene and either one wild-type and one floxed *igf1* allele (WF) or FF. Littermates were used for all analyses. Genotypes were determined by transgene amplification using tail DNA as previously described (15, 22, 23).

To study the effects of Cre-induced recombination during puberty, MMTV-Cre/*igf1* floxed double-transgenic mice were ovariectomized at 4 wk of age. Mice were allowed to recover from surgery for 3 wk. Mice were then given daily ip injections of 17 $\beta$-estradiol benzoate (1 $\mu$g) and progesterone (1 mg) in 50 $\mu$l of sesame oil (all reagents were from Sigma, St. Louis, MO). After 14 d, mice were killed, and abdominal and thoracic mammary glands were removed. A subset of MMTV-Cre animals were injected ip with 0.01 ml/g body weight of cell proliferation labeling reagent [5-bromo-2′-deoxyuridine (BrdU); Amersham Pharmacia Biotechnology, Piscataway, NJ] 2 h before the animals were killed. For histological analysis, abdominal glands were fixed in 4% paraformaldehyde for 2 h, paraffin embedded, and sectioned at 4–6 $\mu$m. Additional glands were snap frozen in an isopentane dry ice bath for isolation of RNA and DNA.

The effects of epithelial IGF-I on pregnancy-induced development were studied using WAP-Cre /*igf1*-floxed double-transgenic mice. Mice were bred to generate FF or FN genotypes in the presence or absence of the WAP-Cre transgene. WAP-Cre double transgenic mice were analyzed at d 2 of lactation (L2). Additional FN and wild-type mice were analyzed at pregnancy d 5.5 (p5.5) and 14.5 (p14.5).

### *Quantitative PCR*

RNA and DNA were isolated from frozen mammary glands using the TriReagent (Molecular Research Center, Inc., Cincinnati, OH) as described by the manufacturer with the addition of the back extraction for DNA isolation. The extent of recombination for each gland was determined by performing quantitative PCR using $^{32}$P-$\alpha$-dCTP (PerkinElmer Corp., Boston, MA) and quantified using PhosphorImager (Molecular Dynamics, GE Healthcare Biosciences Corp., Piscataway, NJ) analysis. Previously designed primers were used to detect the recombined allele (15). An additional forward primer was designed to exon 4: 5′-AAT TCG CCA ATG ACA AGA CG-3′. PCR was performed to determine the linear range of amplification for each set of primers, and standard curves were determined for each primer set to identify primer pairs that amplified at equivalent rates within the same linear range. Quantitative PCR using 100 ng DNA was performed in triplicate, and PhosphorImager analysis with ImageQuant 3.3 (Molecular Dynamics, GE Healthcare Biosciences Corp.) was used to determine OD for each band. The average recombination index for each animal was calculated and expressed as the percentage recombined null allele [null band/(null band + floxed band + wild-type band) $*$ 100]. The average recombination index for each genotype was determined. The mice were grouped according to genotype as determined by PCR on tail DNA.

### *Whole-mount staining and morphometric analyses*

Abdominal mammary glands were used for whole-mount staining with iron-hematoxylin as previously described (5). The extent of ductal branching was quantified using Scion image (Scion Corp., Frederick, MD) to determine the number of intersecting branches along a line drawn midway between the leading edge of the ducts and the lymph node. Complexity was measured as the number of branches per unit length. Scion image was used to measure the percentage of the fat pad occupied by epithelium by measuring the area of the entire fat pad and the area occupied by epithelium. Similar measurements were made to determine ductal extension relative to fat pad length.

### *Ribonuclease protection assays (RPAs)*

RPAs were performed as instructed by the manufacturer using 10–20 $\mu$g RNA from each gland (PharMingen, San Diego, CA). RNA probes were prepared using linearized plasmid DNA containing exon 4 of IGF-I (18), $\beta$-casein (24), or WDNM1 (24). Templates for glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and mCyc-1 were obtained from PharMingen. PhosphorImaging analysis was used to quantify bands, and the average OD of cyclin mRNA was determined after normalization of each sample to GAPDH.

### *Western immunoblot analysis*

Frozen mammary glands were pulverized and placed immediately in protein extraction buffer (8 ml/g tissue) [40 mM Tris, 276 mM NaCl, 20% glycerol, 2% Nonidet P-40, 4 mM EDTA, 1 mM EGTA, 2 mM NaF, 2 mM Sodium orthovanadate, 40 mg/ml phenylmethylsulfonyl fluoride, protease inhibitor cocktail (1:100; Sigma)] and homogenized on ice. Homogenates were placed in chilled tubes and centrifuged at 8000 $\times$ *g* for 10 min at 4 C. The cleared tissue lysates were transferred to a chilled tube. Protein concentrations were determined by the bicinchonic acid protein assay (Pierce, Rockford, IL) and 15–50 $\mu$g of lysate used for Western blot analysis. The samples were boiled for 5 min, subjected to electrophoresis through a 4–20% sodium dodecyl sulfate polyacrylamide gel (Sigma, St. Louis, MO) or 4–12% Tris Acetate NuPage Gel (Invitrogen, Carlsbad, CA), and transferred onto nitrocellulose. Membranes with transferred

proteins were incubated for 1 h in blocking solution containing Tris-buffered saline with 0.05% Tween 20 and 5% nonfat powdered milk. Membranes were incubated overnight in blocking solution at 4 C with the following antibodies: anti-β-casein (S-15, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antiserum to mouse milk-specific proteins (1:1000, Accurate Chemical and Scientific Corp., Westbury, NY), anticyclin D1 (M-20, 1:250; Santa Cruz Biotechnology; or clone DCS6, 1:500; Cell Signaling, Danvers, MA), antiphospho-signal transducer and activator of transcription (Stat)-5a/b (Y694/Y699) clone 8–5-2 (1:500; Upstate Biotechnology, Charlottesville, VA), anti-STAT5 (1:500, BD Biosciences, San Jose, CA), anticytokeratin 18 (1:100; Research Diagnostics, Concord, MA), anticytokeratin 5 (1:500; Covance, Berkeley, CA), or anti-β-actin (1:5000; Sigma). Membranes were washed with Tris-buffered saline containing 0.05% Tween 20 and then incubated for 1 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Jackson ImmunoResearch, West Grove, PA). Bands were visualized using enhanced chemiluminescence (PerkinElmer Life Sciences) followed by exposure to film (XAR-5; Kodak, Rochester, NY) or image acquisition using the Biochemi system (UVP BioImaging Systems, UVP, Inc.) and LabWorks Software (UVP, Inc., Upland, CA). Visualized bands were quantified using Scion Image software.

### *Histology and immunohistochemistry*

For histological procedures, glands were removed, fixed in 4% paraformaldehyde for 2 h, and paraffin embedded. Five-micrometer sections were deparaffinized and stained with hematoxylin and eosin according to standard protocols. To detect IGF-I peptide expression in floxed *vs.* recombined glands, paraffin sections were deparaffinized and dehydrated through a graded series of xylenes and ethanol followed by antigen retrieval in 10 mM Na citrate buffer using a pressure cooker method. Serial sections were incubated either with a rabbit polyclonal antibody to IGF-I (prediluted, Abcam Inc., Cambridge, MA) or a rabbit polyclonal IgG isotype control (prediluted; Abcam) overnight at 4 C followed by incubation with a biotinylated goat antirabbit IgG (H+L) secondary antibody (1:250; Vector Laboratories, Burlingame, CA). Immunoreactivity was detected using streptavidin-HRP (Ready To Use, Vector Laboratories) for 30 min at room temperature followed by incubation with *NovaRED* (Vector Laboratories) substrate solution for 5 min at room temperature. Sections were counterstained with methyl green (Vector Laboratories), dehydrated, and mounted in CytosealXYL (VWR Scientific, West Chester, PA).

To detect Ki67-positive cells, antigen retrieval was performed in 10 mM Na citrate buffer as above. Sections were incubated with a rabbit antibody to Ki67 (1:50; Vector Laboratories) overnight at 4 C followed by incubation with a biotinylated goat antirabbit secondary antibody (1:1000; Jackson ImmunoResearch). Immunoreactivity was detected using streptavidin-HRP (1:500) in 3% BSA for 1 h at 37 C followed by inactive diaminobenzidine/$NiCl_2$/phosphate buffer (pH 6.2). Sections were incubated in diaminobenzidine activated by the addition of $H_2O_2$ for 10 min at room temperature in the dark. Sections were counterstained with methyl green, dehydrated, and mounted in CytosealXYL (VWR Scientific). For detection of apoptotic nuclei by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay, sections were deparaffinized as above and processed to detect DNA fragmentation according to recommended protocols (in situ cell death detection kit, Fluorescein; Roche, Indianapolis, IN).

### *Statistical analyses*

For multiple group comparisons, statistical analyses were conducted using ANOVA followed by Fisher's protected least significant difference *post hoc* test. For comparisons between IGF-I heterozygous and wild-type glands, analyses were performed using a two-sample Student's *t* test.

## Results

### *Cre expression induces lox P recombination in pubertal mammary glands*

The MMTV-Cre and WAP-Cre transgenic mouse lines were shown in previous studies to induce greater than 50% recombination in the mammary epithelium with limited mosaicism (22). Therefore, in addition to crossing the Cre-expressing lines with mice carrying two floxed *igf1* alleles, we also generated mice with one floxed *igf1* allele and one null *igf1* allele to provide the highest likelihood of complete recombination in the mammary epithelium. The ability to generate mice containing a null allele through MMTV-Cre expression is due to Cre-induced lox P recombination in the female germline of the MMTV-Cre mouse line (22). The single- and double-transgenic lines were used to analyze mice with either two or one functional *igf1* alleles in the presence or absence of mammary epithelial Cre expression driven by either the MMTV-long-terminal repeat or WAP promoters. In addition, we analyzed mammary development in the *igf1* heterozygous mice in which IGF-I expression is reduced by 50% in both stroma and epithelium.

A quantitative PCR assay was used to determine the recombination index for mice of select genotypes expressing Cre-recombinase in the presence of a floxed *igf1* allele (Fig. 1). The recombination index in the FN glands was 47.9%, with minimal variation within the genotype (expected recombination is 50%), whereas WF mice had no detectable recombination within the mammary gland (Fig. 1A). The addition of the MMTV-Cre transgene to WF animals resulted in mice with a significant increase in the percentage of the null allele 43.4% ($P < 0.0001$; Fig. 1A). Similarly, the addition of the MMTV-Cre transgene to FN mice increased the recombination index from 47.9% in FN glands to 70.8% in FN/Cre glands ($P < 0.001$; Fig. 1A). Stromal cells in the FN or WF glands do not express Cre; these cells remain 50% (FN) or 0% (WF) recombined in the presence of epithelial Cre expression. Thus, the percentage of recombination shown in the presence of MMTV-Cre underrepresents the actual percentage of recombination in the epithelial cells. These data indicate that expression of the MMTV-Cre transgene induced highly efficient lox P recombination in the mammary epithelium resulting in *igf1* alleles lacking exon 4.

DNA isolated from L2 mammary glands was similarly used to determine the extent of Cre-induced recombination in glands from floxed *igf-1* mice carrying the WAP-Cre transgene. The expression of the WAP-Cre transgene induced significant recombination by L2 (Fig. 1B). Mammary glands from mice carrying two *igf1* floxed alleles (FF) showed negligible recombination, whereas the addition of the Cre transgene (FF/Cre) induced recombination resulting in 34.3% null *igf1* alleles ($P < 0.0001$; Fig. 1B). The percentage of the null allele in FN animals was 50.7%, as expected in the absence of Cre expression, which increased to 68.7% in the presence of the WAP-Cre transgene (FN/Cre) ($P < 0.001$; Fig. 1B). In both cases, the coexpression of the Cre transgene resulted in a significant increase in the percentage of null *igf1* alleles in the lactating mammary gland.

To determine alterations in IGF-I mRNA expression in the genetically altered mammary glands, we performed a ribonuclease protection assay specific for exon 4 of the *igf1* allele, which is deleted in the presence of Cre recombinase. Glands from mice heterozygous for the recombined allele (FN) had a significant decrease in IGF-I mRNA (40% of WF IGF-I mRNA levels; $P = 0.003$ *vs.* WF; Fig. 1C). However, the addition of the MMTV-Cre transgene did not further de-

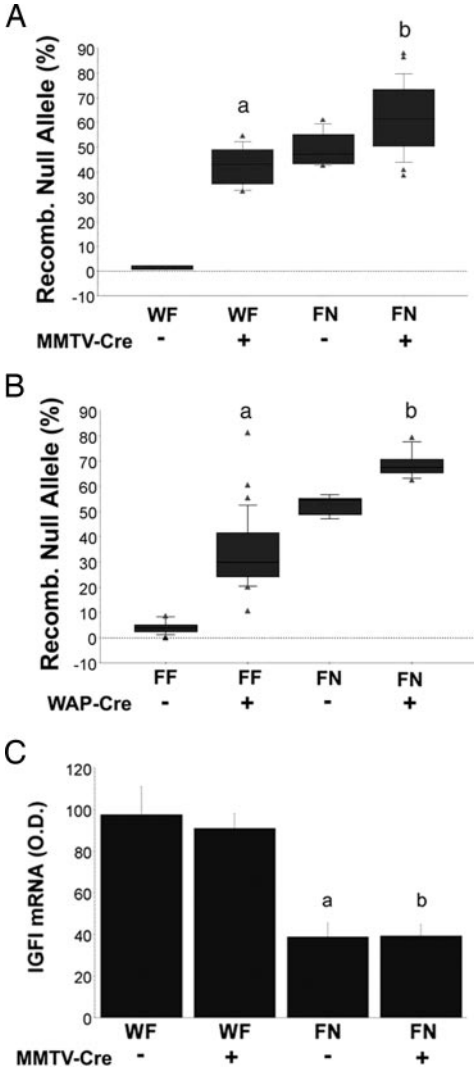


FIG. 1. Percentage of Cre-induced recombination and expression of IGF-I mRNA in transgenic mammary glands. Cre expression from the MMTV promoter or the WAP promoter induced lox P recombination in mammary glands carrying floxed-*igf1* alleles during puberty (A) and at L2 (B). An equivalent amount of DNA isolated from mammary glands of transgenic mice was used for quantitative PCR to determine the extent of Cre-induced recombination in select genotypes. Values are expressed as the percentage of the null allele. C, Expression of IGF-I mRNA levels determined by an exon-4-specific ribonuclease protection assay using RNA isolated from whole gland. Values are represented as arbitrary OD units (mean ± SE) after adjustment to values obtained from hybridization of GAPDH mRNA within each sample. The presence of MMTV-Cre or WAP-Cre is indicated below the graphs. a, $P < 0.0001$ *vs.* WF; b, $P < 0.001$ *vs.* FN.

crease IGF-I expression likely due to the high level of IGF-I expression in the stromal compartment and relatively restricted expression of IGF-I in the epithelial compartment. Similar results were obtained for pregnant glands with or without expression of the WAP-Cre transgene (data not shown).

### *MMTV-Cre-induced recombination reduces immunoreactive IGF-I in epithelial cells*

To confirm loss of epithelial IGF-I in the Cre-expressing glands, we used an antibody to IGF-I to determine IGF-I protein expression in epithelial and stromal compartments. IGF-I protein is detected throughout the stromal compartment of pubertal stage glands from all genotypes (Fig. 2, A–C). Staining specificity was determined using an IgG isotype control (Fig. 2D). IGF-I immunoreactivity was also detected in epithelial cells in TEBs and terminal ductal regions of the growing ductal structures in wild-type glands or glands containing two floxed *igf1* alleles (Fig. 2, A and B). The addition of the MMTV-Cre transgene to the FF genotype caused a significant reduction in epithelial IGF-I immunoreactivity (Fig. 2C). IGF-I protein was still strongly expressed in stromal cells surrounding the epithelial structures in the FF/Cre glands (Fig. 2C).

### *Epithelial IGF-I is necessary for normal branching during ductal growth*

Morphological alterations due to loss of IGF-I during puberty were analyzed using whole-mount preparations of the no. 4 inguinal gland from transgenic mice treated for 14 d with estrogen and progesterone. The IGF-I heterozygous glands, which had 40% of normal IGF-I mRNA throughout the gland, showed no alterations in the total fat pad area or in ductal elongation (data not shown). Similarly, reduction of epithelial IGF-I had no effect on these parameters. The percentage of fat pad occupied by epithelium was reduced by approximately 20–35% in the genotypes containing epithelial expression of Cre; however, this was statistically significant only in the FF glands containing Cre ($P < 0.05$; Fig. 3A). Neither 60% reduction of IGF-I throughout the gland (WN) nor 40–50% loss of epithelial IGF-I (WF/Cre) resulted in alterations in branching complexity (Fig. 3B). In contrast, FF/Cre and FN/Cre glands, both genotypes with greater

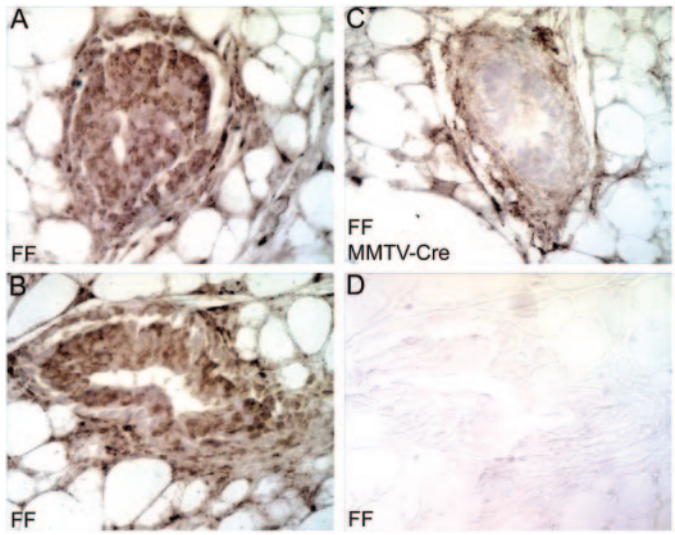


FIG. 2. Expression of IGF-I protein in transgenic mammary glands with epithelial deletion of IGF-I. Immunohistochemical analyses of IGF-I protein was performed on sections from pubertal glands of virgin estrogen-progesterone-treated mice, which carried two floxed *igf1* alleles (FF) either without (A, B, and D) or with (C) MMTV-Cre expression. A and B, FF gland immunostained for IGF-I showing staining in stroma as well as epithelium. C, FF/Cre gland immunostained for IGF-I showing stromal staining but lacking significant epithelial staining. D, Adjacent section to B showing IgG isotype antibody staining in place of the IGF-I antibody as a control.

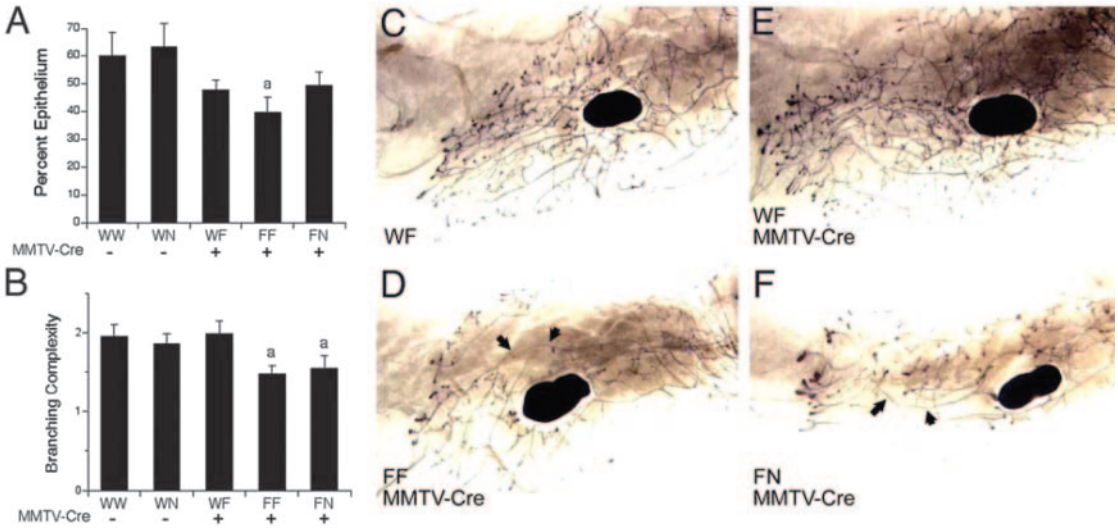


FIG. 3. Morphometric analysis of transgenic mammary glands with epithelial deletion of IGF-I. Virgin mice were ovariectomized at 4 wk of age and given daily injections of estrogen/progesterone for 14 d. A, Quantitation of the percentage of total fat pad area occupied by epithelium. B, Analysis of branching complexity in glands with greater than (D and F) or less than (C and E) 50% recombination of the *igf1* allele in mammary epithelium. WW, Wild-type/wild-type; WN, wild-type/null. The presence of MMTV-Cre is indicated. C–F, Whole-mount analysis of no. 4 inguinal glands stained with iron-hematoxylin from transgenic mice. a, $P < 0.05$.

than 40–50% reduction in epithelial IGF-I, showed decreased branching complexity ($P < 0.05$; Fig. 3B). Decreased branching complexity was apparent by the presence of long sections of ducts devoid of side branches in the FF/Cre and FN/Cre glands (Fig. 3, D and F, *arrows*), compared with WF or WF/Cre glands (Fig. 3, C and E).

### *Heterozygous reduction of IGF-I in pubertal mammary glands results in decreased levels of cyclin gene expression*

In our previous studies, we demonstrated that exogenous IGF-I induces cyclin expression and cell cycle progression of mammary epithelial cells in the intact gland in whole-organ culture (11). To determine whether reduction of IGF-I *in vivo* alters cyclin expression, we used a cyclin-specific RPA to measure changes in mRNA expression. Cyclin D1 levels were moderately reduced in the FN glands, but the reduction was not statistically significant, compared with cyclin D1 levels in the WF glands (Fig. 4A). Cyclins D2 and D3 mRNA levels were unaltered in any of the genotypes examined (Fig. 4, B and C). In contrast, cyclin A2 and cyclin B1 mRNA levels were decreased in mice heterozygous for the *igf1* null allele (FN; $P \leq 0.05$; Fig. 4, D and E). The presence of the Cre transgene in the WF, FF, or FN genotypes had no effect on cyclin expression in these analyses (Fig. 4 and data not shown). These results are consistent with our previous observations in whole-organ culture experiments on pubertal stage glands showing that exogenous IGF-I significantly induced expression of cyclins A2 and B1, important for S and $G_2$ progression, respectively (11).

### *Heterozygous reduction of IGF-I results in reduced alveolar budding and subsequent hyperplasia during alveolar development*

Our previous studies demonstrated IGF-I expression predominantly in the stromal compartment in postpubertal through midpregnant glands with little detectable IGF-I mRNA in epithelium at these times (1, 2). Based on the previous data showing a decrease in expression of $S/G_2$ cyclin expression in heterozygous glands, we continued analysis of glands from this genotype through early and midpregnant ages. Whole-mount staining of no. 4 inguinal mammary glands at p5.5 revealed reduced alveolar budding in the *igf1* (+/−) glands (Fig. 5, C and D), compared with wild-type glands (Fig. 5, A and B). Analysis of BrdU incorporation or TUNEL staining for apoptotic nuclei showed no significant differences between the two genotypes (data not shown).

At p14.5 the *igf1* (+/−) glands showed reduced alveolar density (Fig. 6, C and D), compared with wild-type glands (Fig. 6, A and B). Histological analysis of sections from *igf1* (+/−) glands confirmed the reduction in epithelial structures at midpregnancy (Fig. 6, E–H). However, a closer inspection of the alveoli of the *igf1* (+/−) glands revealed that the alveoli lacked a defined lumen and appeared more densely packed (Fig. 6, G and H) than the alveoli from wild-type glands (Fig. 6, E and F). Quantification of the number of nuclei revealed more nuclei per alveolus in the IGF-I heterozygotes (Fig. 6I). Moreover, the number of Ki67-positive cells was increased in the *igf1* (+/−) glands, compared with wild-type glands (Fig. 6J). TUNEL analysis for cells containing fragmented DNA revealed similar numbers of apoptotic cells in wild-type and *igf1* (+/−) glands (Fig. 6K).

To determine whether the increase in cell number and Ki67-positive cells was reflected in expression of cyclin genes, we used a cyclin-specific RPA to measure changes in cyclin mRNAs in the two genotypes. Expression of D-type cyclins and cyclin A2 but not cyclin B1 were significantly higher in glands from the *igf1* (+/−) mice than from wild-type mice at midpregnancy (Fig. 7, A–E). Consistent with the mRNA expression studies, cyclin D1 protein was increased in the *igf1* (+/−) glands (Fig. 7F).

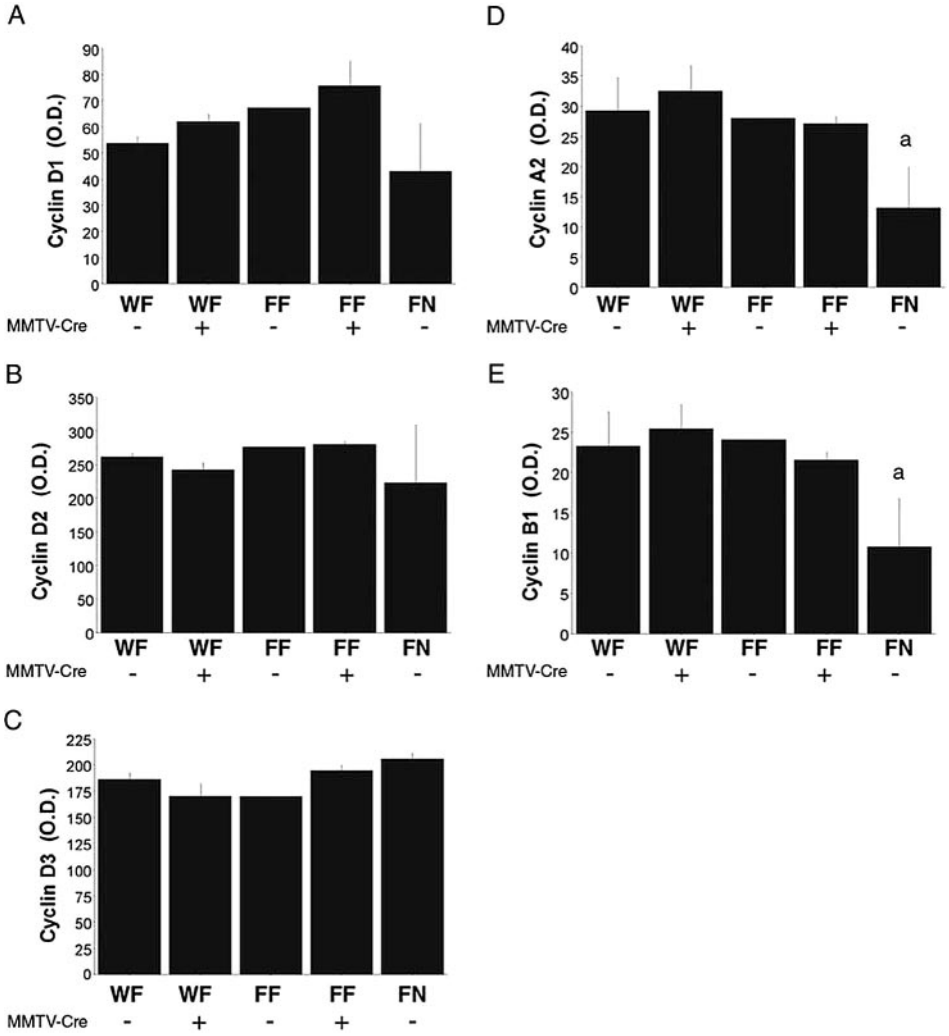


FIG. 4. Expression of cyclin mRNA in virgin E-P treated transgenic mammary glands. Virgin mice were ovariectomized at 4 wk of age and given daily injections of E/P for 14 d. Ribonuclease protection assays were performed on RNA isolated from whole gland to determine levels of cyclin mRNA for the $G_1$ cyclins, cyclin D1 (A), cyclin D2 (B), and cyclin D3 (C) and $S/G_2$ cyclins, cyclin A2 (D) and cyclin B1 (E). Values are represented as arbitrary OD units (mean ± SE) after adjustment to values obtained from hybridization of GAPDH mRNA within each sample. Genotypes are represented as for previous figures. a, $P = 0.05$ *vs.* WF.

*Milk protein expression is reduced in the absence of changes in gene expression in IGF-I heterozygous glands at midpregnancy*

To determine whether the increases in cell number and proliferation markers were accompanied by changes in the differentiation state of the cells, levels of β-casein and other milk proteins were analyzed in the p14.5 heterozygous and wild-type glands. Levels of β-casein protein were reduced by more than 50% in the *igf1* (+/−) glands ($P < 0.02$; Fig. 8, A and B). In addition to β-casein, levels of γ-casein and δ-casein were reduced in the *igf1* (+/−) glands ($P < 0.02$; Fig. 8, C and D) as well as levels of whey acidic protein ($P < 0.02$; data not shown). Levels of α-casein were unaltered in the *igf1* (+/−) glands (Fig. 8D) as were protein levels of cytokeratins 18 and 5 (Fig. 8, E–H).

To determine whether the decreases in milk protein levels were due to decreases in gene expression, glands at p14.5 were analyzed for changes in mRNA expression of two milk proteins, β-casein and WDNM-1, and changes in activation of the stat5 transcription factor. Levels of β-casein mRNA were unchanged (Fig. 9A), whereas levels of WDNM1 mRNA were moderately elevated in the *igf1* (+/−) glands at p14.5 ($P < 0.05$; Fig. 9B). Levels of phosphorylated stat5 protein were equivalent between the two genotypes at p14.5 (Fig. 9C).

Because the IGF-I heterozygous animals have systemic loss of one IGF-I allele, we determined the reduction in circulating IGF-I levels in 8-wk virgin and p14.5 pregnant *igf1* (+/−) and wild-type mice. IGF-I serum levels in the heterozygous mice were 70% of wild-type levels in 8-wk female mice (246 ± 42.2 ng/ml in *igf1* hets *vs.* 346 ± 54.4 in wt; $P < 0.05$) and were 65% of wild-type levels in the p14.5 female mice (146.5 ± 22.6 in *igf1* hets *vs.* 224.3 ± 14 in wt; $P < 0.0005$). Additionally, protein levels of IGFBP-2 and IGFBP-5, two of the most highly expressed IGFBPs in the postnatal mammary gland (5), were unaltered in the *igf1* (+/−) glands (data not shown). Similarly, analysis of IGF-II mRNA levels in mammary tissue also revealed no compensatory changes in IGF-II expression with reduction of IGF-I.

*Epithelial IGF-I in alveologenesis*

Our previous analysis indicated the reexpression of IGF-I in MECs by the later stages of pregnancy (1, 2). To determine the role of epithelial IGF-I during alveolar development, we analyzed *igf1*/Wap-Cre double-transgenic mammary glands at L2. Histological analyses of L2 glands showed no consis-

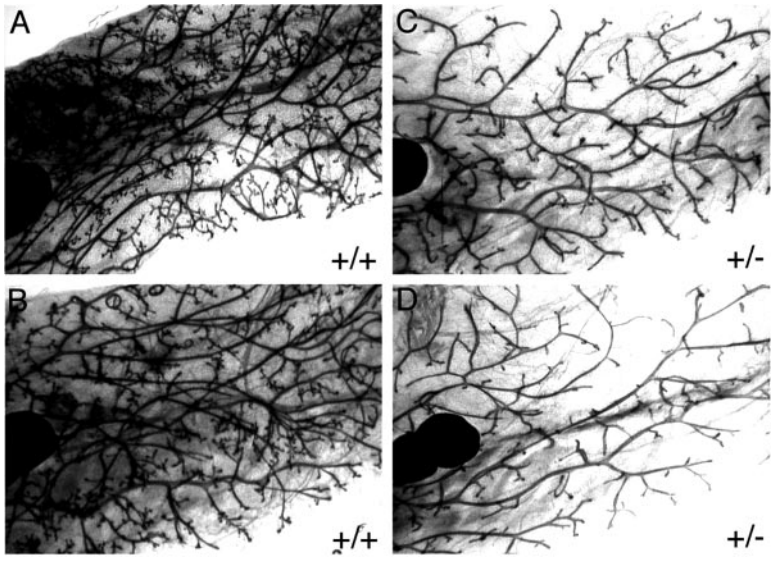


FIG. 5. Analysis of *igf1* heterozygous and wild-type glands at p5.5. Whole mounts of no. 4 inguinal mammary glands stained with iron-hematoxylin from early pregnant (p5.5) wild-type mice (A and B) and *igf1* heterozygous mice (C and D).

tent alteration in histology across the genotypes (data not shown). However, protein levels of cyclin D1 were induced at L2 in glands heterozygous for IGF-I expression (FN; $P < 0.03$; Fig. 10, A and B), similar to what we observed at p14.5. Loss of epithelial IGF-I by Wap-Cre-induced recombination had no effect on cyclin D1 levels in glands from either the FF or FN genotypes. Levels of β-casein were moderately elevated in the FN glands at L2, compared with FF glands ($P < 0.03$; Fig. 10, A and C). Loss of epithelial IGF-I in the Wap-Cre FN glands caused a further increase in β-casein protein, compared with the FN glands ($P < 0.003$; Fig. 10C).

## Discussion

The results of this study demonstrate that IGF-I has distinct functions depending on its epithelial or stromal expres-

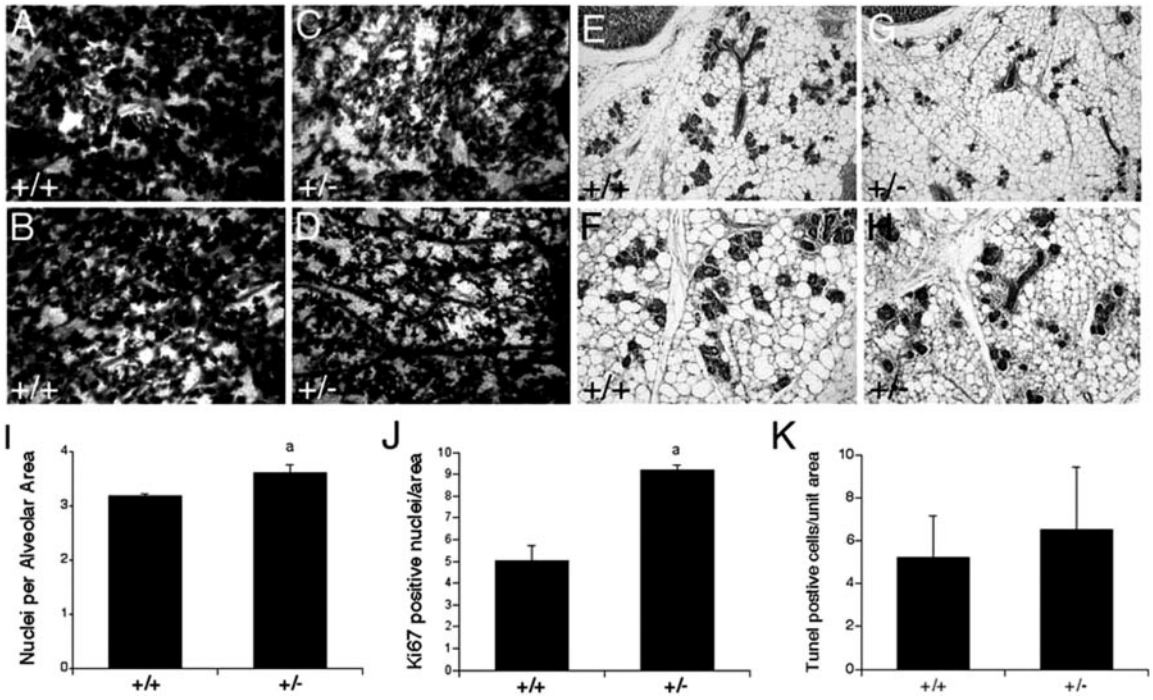


FIG. 6. Analysis of *igf1* heterozygous and wild-type mice at p14.5. Whole mounts of no. 4 inguinal mammary glands stained with iron-hematoxylin from mid-pregnant (p14.5) wild-type mice (A and B) and mice heterozygous for IGF-I (C and D). E–H, Histological analysis of no. 4 inguinal mammary glands at p14.5 from wild-type mice (E and F) or *igf1* heterozygous mice (G and H). Paraffin sections from no. 4 inguinal mammary glands were stained with hematoxylin and eosin. I, Quantitation of nuclei per alveolar area was performed on hematoxylin/eosin-stained sections from wild-type (n = 3) and *igf1* heterozygous (n = 6) glands using Scion Image analysis. J, Quantitation of Ki67-positive epithelial cells in sections of glands from the same animals analyzed in I. K, Quantitation of TUNEL-positive epithelial cells in sections of glands from the same animals analyzed in I and J. All analyses for graphs shown in I–K were performed by averaging counts from three sections per gland. +/+, wild-type (n = 3);+/−, *igf1* heterozygous (n = 6). a, $P < 0.05$ in I and $P < 0.001$ in J.

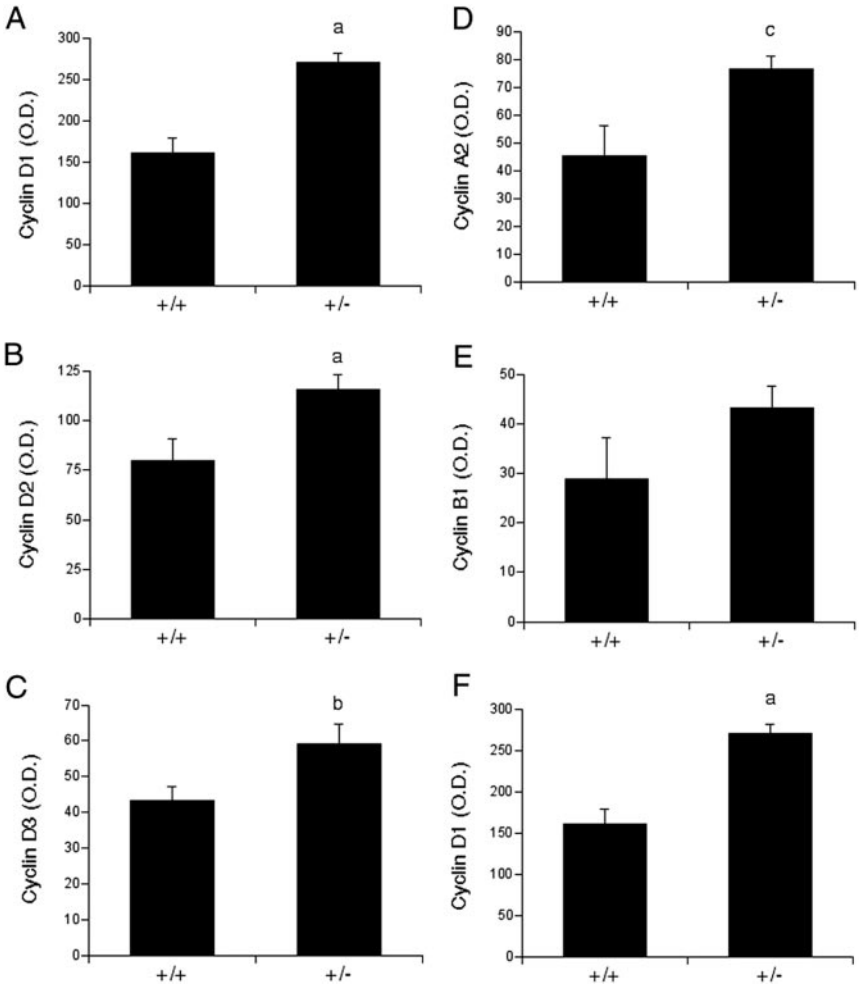


FIG. 7. Expression of cyclin mRNAs in p14.5 mammary glands from wild-type (+/+) and *igf1* heterozygous (+/−) mice. Ribonuclease protection assays were performed to determine levels of cyclin mRNAs for the $G_1$ cyclins, cyclin D1 (A), cyclin D2 (B), and cyclin D3 (C) and $S/G_2$ cyclins, cyclin A2 (D) and cyclin B1 (E). Values are represented as arbitrary OD units (mean ± SE) after adjustment to values obtained from hybridization of GAPDH mRNA within each sample. F, Analysis of cyclin D1 protein levels in wild-type and *igf1* heterozygous mammary glands from Western immunoblotting. Values are represented as arbitrary OD units (mean ± SE) after adjustment to β-actin within each sample. a, $P < 0.005$; b, $P < 0.05$; c, $P < 0.001$.

sion. These data are more remarkable in light of our previous data showing restricted sites and times of IGF-I expression in developing mammary epithelium. In these previous studies, we demonstrated that IGF-I is expressed by epithelial cells of the TEB as well as cells in the stromal compartment during pubertal ductal growth (1, 2). Here we show that IGF-I expressed in epithelial cells during puberty is essential for normal branching morphogenesis, whereas stromally produced IGF-I contributes to induction of S and $G_2$ cyclins in pubertal mammary tissue. We also show that IGF-I has a critical role in alveolar development during pregnancy, a time when IGF-I expression is predominantly in the stromal compartment (1, 2). Reduction in IGF-I levels during pregnancy results in an early delay in alveolar budding and alveolar density and a subsequent compensatory hyperplasia. In addition, IGF-I appears critical for posttranscriptional regulation of milk protein expression during alveolar development.

### *IGF-I in pubertal mammary development*

Using a recombination strategy to decrease IGF-I expression specifically in mammary epithelial cells, we demonstrated that epithelial IGF-I is necessary for normal branching during pubertal mammary gland development. Branching complexity was significantly decreased in glands with greater than 50% recombination of the *igf1* allele in epithelial cells. A role for IGF-I in branching was also shown with IGF-$I^{m/m}$ mice carrying an intronic insertion at the *igf1* locus that results in a 70% reduction in tissue and circulating IGF-I (25, 26). In this study, Richards and colleagues also analyzed branching morphology in mammary glands from mice carrying a liver-specific deletion of IGF-I (LID mice), which results in a 75% reduction in circulating IGF-I but normal tissue IGF-I levels (18), including the mammary gland (26). Mammary glands from LID mice had normal branching morphology, supporting the hypothesis that the branching deficit in the IGF-$I^{m/m}$ mice was due to reduction of local IGF-I expression (26). However, in these studies, the contributions of epithelial- or stromal-derived IGF-I to ductal branching were not determined.

Our previous data demonstrated that IGF-I mRNA is expressed in the epithelial cells as well as the stroma during pubertal ages, and its expression in the epithelial compartment predominates in the TEBs (1, 2). Based on our previous studies showing IGF-I mRNA in TEBs along with the data presented here, we conclude that epithelially expressed IGF-I has a specific role in promoting branching during pubertal ductal development. Loss or reduction of IGF type I receptor (IGF-IR) in epithelium decreases ductal branching (27), suggesting epithelial IGF-I acts directly through the IGF-IR in

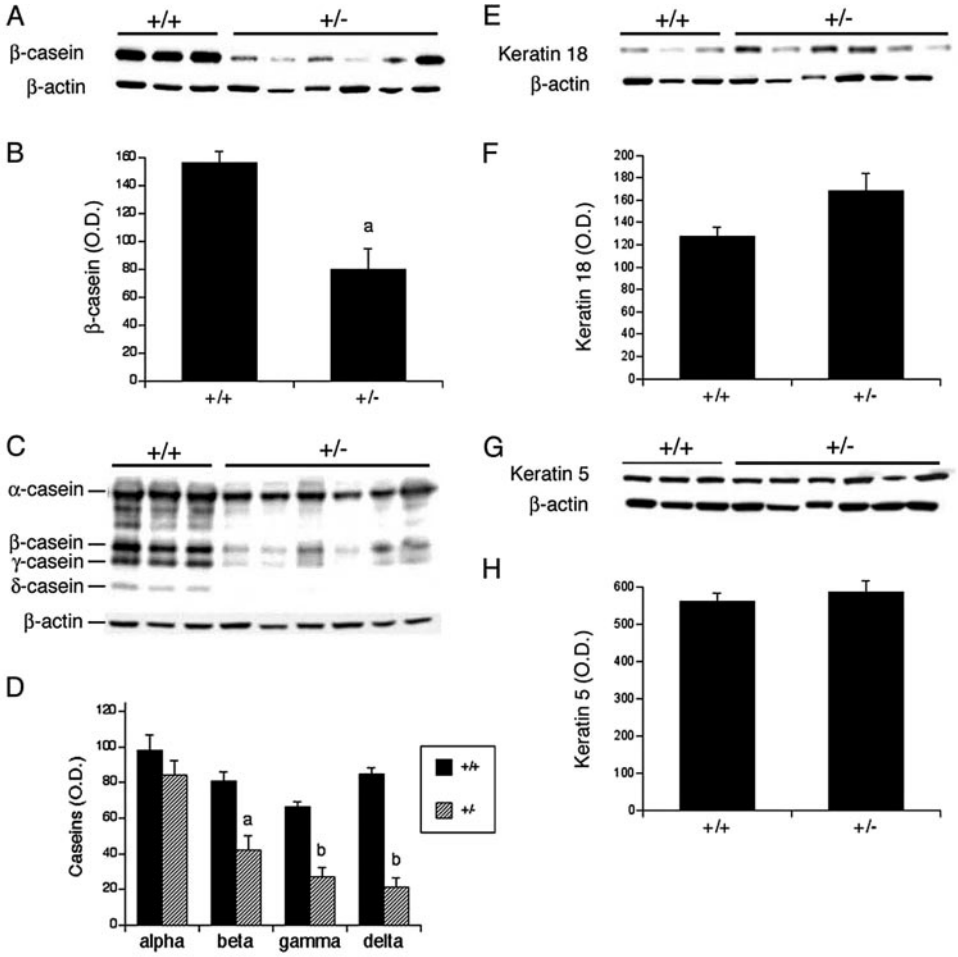


FIG. 8. Analysis of protein expression in p14.5 mammary glands from wild-type (+/+) and *igf1* heterozygous (+/−) mice. A and B, Western immunoblot analysis of β-casein protein levels. C and D, Western immunoblot analysis of milk proteins including α-casein, β-casein, γ-casein, and δ-casein. E–H, Western immunoblot analysis of cytokeratin 18 (E and F) and cytokeratin 5 (G and H). Values are represented as arbitrary OD units (mean ± SE) after adjustment to β-actin within each sample. a, $P < 0.05$; b, $P < 0.02$.

epithelial cells to promote branching. The prominent localization of IGF-I mRNA to the TEBs further suggests either that its expression in these cells is essential for branching due to autocrine/paracrine effects on the TEB itself or that it is secreted and discretely localized to act on ductal cells as the ducts penetrate the fat pad. The IGF-I immunostaining suggests that IGF-I protein is present in the TEBs as well as in the growing epithelial structures, particularly in the more terminal regions. Autocrine/paracrine actions of growth factors on ductal branching has been suggested for TGFβ, which is concentrated in the periductal extracellular matrix and locally reduced at the sites of lateral branch points (28, 29). Second, several IGFBPs, which can mediate local tissue availability of the IGFs, have discreet patterns of expression during ductal elongation in the mammary gland (2, 5, 30). Specifically, IGFBP-2 is expressed at very discreet sites along the neck of the TEB and in clusters of cells along the growing ductal structures in the pubertal gland (5). Thus, it is possible that IGFBP-2 tethers IGF-I at discreet locations along the ducts at putative branch points.

Whereas loss of epithelial IGF-I compromises ductal branching, it does not appear to affect TEB formation. Well-formed TEBs were seen at the leading edge of glands in all the genotypes analyzed. In contrast, systemic loss of IGF-I or loss of IGF-IR in the epithelium severely compromises TEB formation and ductal outgrowth (14, 27). One explanation for our results is that stromally derived IGF-I promotes TEB formation. However, our previous expression studies demonstrated high expression of both IGF-I and IGF-II mRNAs in the TEBs during ductal development. Thus, it is possible that IGF-II compensates for loss of IGF-I in TEB formation but not in ductal branching.

Whereas TEB formation was uncompromised with loss of epithelial IGF-I, at least one of the major ducts in these glands had sections completely devoid of branches, suggesting deficits in both bifurcation and side branching. The mechanisms that control ductal branching are poorly understood, although numerous factors have been reported to regulate branching morphogenesis (for reviews, see Refs. 29 and 31–33). Recently progesterone in combination with IGF-I was shown to stimulate ductal growth and branching, whereas estrogen with IGF-I promoted TEB formation, side branching, and alveolar budding (34). Combining these recent findings with our results, it is tempting to speculate that epithelial IGF-I functions in the progesterone-mediated branching pathway because we observe normal TEBs and decreased branching with loss of epithelial IGF-I. Interestingly, complete deletion of IGF-I, which results in lack of TEBs and absence of ductal growth, was rescued in part by treatment with exogenous IGF-I, suggesting that stromal IGF-I might suffice for these functions (14). However, a reduction in branching morphogenesis was evident in these glands, pos-

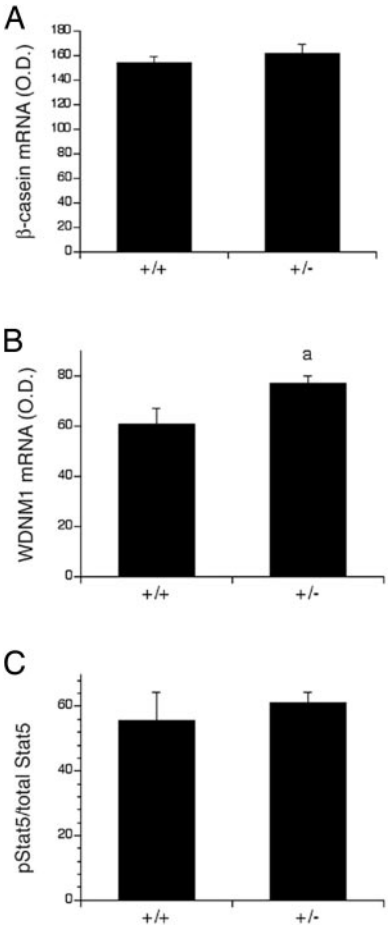


FIG. 9. Analysis of milk protein mRNAs and activation of Stat5 in p14.5 mammary glands from wild-type (+/+) and *igf1* heterozygous (+/−) mice. RPAs were performed to determine levels of β-casein mRNA (A) and WDNM1 mRNA (B). Values are represented as arbitrary OD units (mean ± SE) after adjustment to values obtained from hybridization of GAPDH mRNA within each sample. C, Western immunoblot analysis for phosphorylated stat5 and total stat5 in wild-type and *igf1* heterozygous mammary glands. Values represent arbitrary OD units (mean ± SE) after adjustment to β-actin within each sample and are represented as percentage of phosphorylated stat5 to total stat5. a, $P < 0.05$.

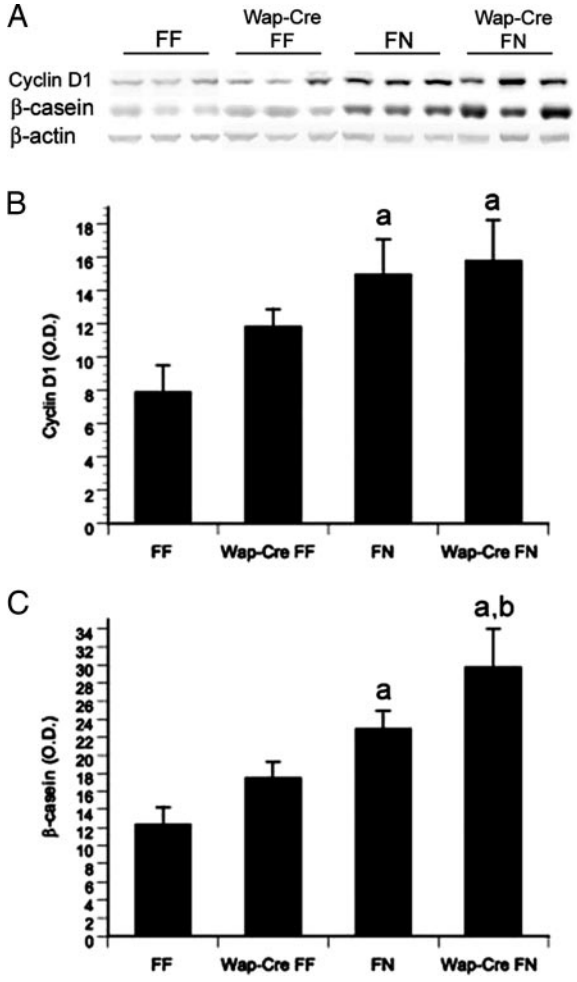


FIG. 10. Analysis of cyclin D1 and β-casein protein expression in L2 mammary glands from transgenic mice with Wap-Cre recombination of *igf1*. A, Western immunoblot analysis of cyclin D1 and β-casein protein levels in mammary glands from L2. Three representative samples are shown for each genotype. The presence of Wap-Cre is indicated. B and C, Quantitation of Western immunoblot analysis. Values are represented as arbitrary OD units (mean ± SE) after adjustment to β-actin within each sample (n = 9 for FF, n = 10 for Wap-Cre FF, n = 6 for FN, and n = 9 for Wap-Cre FN. a, $P < 0.03$ *vs.* FF; b, $P < 0.005$ *vs.* Wap-Cre FF.

sibly due to limited treatment times or the necessity for IGF-I expression in the epithelial compartment.

Whereas epithelial IGF-I regulates ductal branching morphogenesis, our data support the conclusion that stromally derived IGF-I is critical for normal expression of cell cycle regulators, particularly the S and $G_2$ cyclins. The presence of a null allele significantly decreased cyclin A2 and cyclin B1 expression levels, compared with glands with two functional *igf1* alleles. Although these analyses were conducted on mRNA from homogenized glands, sites of both BrdU incorporation and Ki67 immunostaining were observed primarily in epithelial cells. These data indicate that a 50% decrease in IGF-I throughout the mammary gland results in decreases in the expression of cell cycle regulatory components within the epithelial cells. In contrast, glands with decreased epithelial IGF-I but normal stromal IGF-I levels have wild-type levels of cyclin expression. These data complement our previous data showing that exogenous IGF-I induces cyclin expression and proliferation in MECs in intact mammary glands cultured *ex vivo* (11).

### *IGF-I in alveologenesis*

In addition to examining IGF-I during pubertal ductal growth, we examined the effects of reduced IGF-I during alveologenesis. Because expression of IGF-I in the mammary gland is predominantly stromal until late pregnancy, we focused our analysis on glands with a heterozygous reduction in IGF-I. At early stages of pregnancy, reduction of IGF-I significantly retarded the appearance of alveolar buds. Interestingly, these glands showed no significant reduction in BrdU incorporation or induction of apoptosis in the MECs. However, the reduction in IGF-I did result in alterations in proliferation by midpregnancy ages when the glands had a hyperplastic phenotype. A similar compensatory hyperpla-

sia after a time of reduced growth was recently described after inactivation of the IGF-IR in developing skeletal muscle (35). Moreover, whereas transplantation of IGF-IR null epithelium into cleared fat pads severely compromised graft efficiency, TEB formation, and ductal development, the grafts that were successful partially recovered ductal growth during pregnancy (27). Taken together, these data suggest a compensatory proliferation mechanism during pregnancy, which is independent of IGF-I and the IGF-IR.

In addition to mediating proliferation during early alveologenesis, wild-type levels of IGF-I are necessary for normal milk protein expression. However, the reduction in milk protein levels cannot be interpreted as an overall reduction in differentiation because it appears that the IGF-I effect on milk proteins occurs at the level of translation. Consistent with these results, a recent report (36) demonstrated that insulin in combination with prolactin induces β-casein protein but not mRNA expression. Because the insulin concentrations used in this study were superphysiological and at levels that stimulate the IGF-IR, these data can be reinterpreted in light of our data to support the conclusion that it is IGF-I that coordinates with prolactin to induce translation of β-casein and potentially other milk proteins.

Using Cre-induced recombination initiated during mid- to late pregnancy, we observed no measurable morphological effect from reduction of epithelial IGF-I. Lack of a significant phenotype at L2 in glands carrying the *igf1* floxed alleles and Wap-Cre transgene may be due to the low expression of IGF-I in epithelial cells until late pregnancy ages. The only significant alteration observed with the presence of the WAP-Cre transgene was a further increase in β-casein protein levels observed in the IGF-I heterozygous glands at L2. Similar to what we observed in the midpregnant glands, the alteration in β-casein at L2 was not associated with significant changes in phosphorylation or total levels of stat5 (data not shown). However, the alteration in β-casein at this stage was the reverse of what we observed at midpregnancy, in which reduction of IGF-I resulted in lower levels of milk proteins. It is possible that the elevation of β-casein protein at L2 in the IGF-I heterozygous glands and further induction with the Wap-Cre recombination in the MECs is a compensatory response in the pathways important for milk protein translation.

We have determined that IGF-I actions in postnatal mammary development are dependent on both the site of expression (epithelial *vs.* stromal) and the developmental stage. Specifically, epithelial IGF-I is essential for normal ductal branching during puberty. In contrast, stromally produced IGF-I mediates parameters of proliferation in the epithelial cells during both pubertal growth and alveologenesis. In addition, IGF-I also has a role in milk protein translation during alveologenesis. It is also interesting that whereas IGF-II is expressed in both the stromal and epithelial compartments during these stages, it cannot entirely compensate for loss or reduction of IGF-I in these compartments. Thus, it is likely that these two ligands have distinct roles in postnatal mammary development, perhaps in part through specific localization and compartmentalization by members of the IGFBPs.

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