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Expression of Ovine Insulin-Like Growth Factor-1 (IGF-1) Stimulates Alveolar Bud Development in Mammary Glands of Transgenic Mice

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To determine whether murine mammary growth is modulated by local insulin-like growth factor-1 (IGF-1) production, expression of recombinant IGF-1 was directed to the mammary glands of transgenic mice using an ovine prepro IGF-1 cDNA under control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter. Bioactivity of recombinant IGF-1 in transgenic mouse milk extracts was demonstrated by a concentration-dependent increase in [^{3}H]thymidine incorporation in clonal bovine mammary epithelial cells (MAC-T) compared with control mouse milk extracts; moreover, addition of excess recombinant human insulin-like growth factor binding protein-3 (rhIGFBP-3) abolished the increase in [^{3}H]thymidine incorporation attributed to recombinant IGF-1 in transgenic mouse milk. Recombinant IGF-1 was produced in mammary tissue of virgin and pregnant transgenic mice, and secreted into milk of lactating mice. However, recombinant IGF-1 was not detected in serum from transgenic mice; and ligand blot analysis of serum insulin-like growth factor binding proteins (IGFBPs) indicated no differences owing to transgene presence. In peripubertal virgin mice at 49 d of age, the frequency of appearance of mammary alveolar buds was significantly higher in MMTV-IGF-1 than in CD-1 mice, and was unaffected by ovariectomy or estradiol treatment. In conclusion, mammary synthesis of recombinant IGF-1 enhances the rate of development of alveolar buds in mammary glands of virgin transgenic mice.

Key Words: Insulin-like growth factor-1 (IGF-1); transgenic mice; mammary gland; development; alveolar buds.

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Introduction

Insulin-like growth factor-1 (IGF-1) has been implicated in many biological processes including prenatal and postnatal development of a number of tissues *(1)*. In vitro observations on the mitogenicity of IGF-1 suggest an important role in growth stimulation of mammary epithelial cells, but not necessarily a regulatory one *(2)*. IGF-1 is synthesized in mammary stroma *(3)* and is generally considered to act in a paracrine fashion via epithelial cell IGF-1 receptors. Clonal bovine mammary epithelial cells expressing recombinant IGF-1 exhibited an accelerated growth rate *(4)*.

Significant physiological effects of hIGF-1 overexpression directed by a metallothionein promoter in transgenic mice have been described, although its impact on mammary growth was not reported *(5)*. Using transgenic mouse models to examine the effects of local mammary IGF-1 synthesis, researchers showed that expression of recombinant IGF-1 under control of the whey acidic protein promoter inhibited normal involution *(6,7)*, whereas high levels of α_{s1}-casein promoter-directed IGF-1 expression in the mammary glands of transgenic rabbits was without significant effect on mammary function *(8)*. However, the effects of mammary-specific synthesis of IGF-1 in the developing mammary gland have not been described. When IGF-1-containing implants were placed into the mammary glands of hypophysectomized, gonadectomized, estradiol-treated immature rats for 5 d, Ruan et al. *(9)* showed that IGF-1 mimicked the action of growth hormone (GH) in mammary development by stimulating epithelial cell differentiation into terminal end buds and alveolar structures. Similarly, using whole mouse mammary gland organ culture, Richert and Wood *(10)* suggested that activation of the IGF receptor may mediate significant ductal and alveolar growth. Taken together with evidence that mammary IGF-1 gene expression displays developmental regulation during fetal and neonatal stages *(11,12)*, these findings indicate that IGF-1 may play an important role in early mammary development.

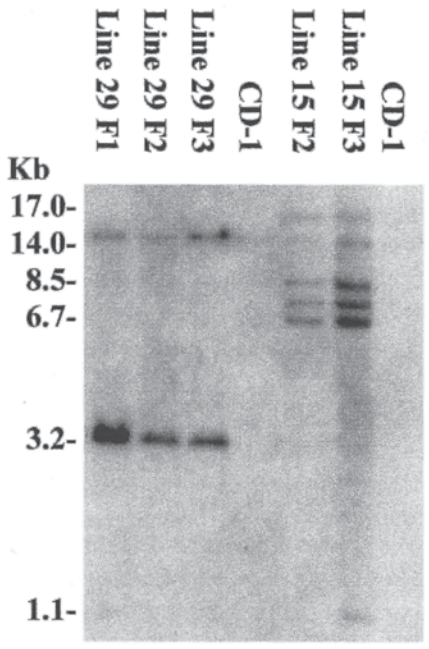

Fig. 1. Southern analysis of transgene copy number in transgenic MMTV-IGF-1 and nontransgenic CD-1 mice. Blots were hybridized with a [^{32}P]-IGF-1 cDNA as described in Materials and Methods and exposed for 10 d. Each lane contains 10 µg DNA digested with *Bam*HI. Sizes are shown in kb.

Our objective was to examine the effects of increased mammary IGF-1 synthesis on development of the peripubertal mouse mammary gland, using a transgenic mouse model expressing recombinant ovine IGF-1 under control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter. In this article, we report the transgene expression of biologically active IGF-1 in virgin, pregnant, and lactating mice and describe its effects on peripubertal mammary gland development.

Results

Characterization of MMTV-IGF-1 Transgenic Mice

Six transgenic founder mice were generated by pronuclear microinjection and identified by PCR analysis. Transgenic mouse lines were established from each of the six founder animals. Southern blot analysis of F_1, F_2, and F_3 offspring from two founders is shown in Fig. 1. After *Bam*HI digestion of tail DNA, all mice showed an endogenous 14-kb band. Transgenic line 15 and line 29 mice showed a faint band at 1.1 kb corresponding to the middle *Bam*HI-digestion product of the transgene (Fig. 2). Line 29 mice showed an additional band at 3.2 kb. Line 15 mice showed four additional bands at 6.7, 7.5, 8.5, and 17 kb, corresponding to the variable-length 5'-segment of the integrated transgene copies following *Bam*HI digestion. These results indicate the presence of four copies of the transgene in line 15 and one copy in line 29; further, these bands were present in F_1, F_2, and F_3 animals, indicating that the transgene copies were stably inherited.

To determine initially whether each transgenic line was expressing the transgene in mammary tissue, extramammary tissues and the fourth inguinal mammary glands from 80-d mice on d 14 of lactation were screened for the presence of recombinant IGF-1 mRNA by Northern blotting and RT-PCR. Mammary tissue from transgenic mice produced a transgene IGF-1 mRNA distinguishable from the endogenous IGF-1 mRNA found in liver tissue (Fig. 3). Mammary RNA from control mice was not loaded equally on this blot compared to transgenic mice, possibly contributing to the apparent absence of endogenous IGF-1 expression in nontransgenic mammary tissue; also, this may reflect a limitation of detection by Northern analysis. The spleen, salivary gland, kidney, and brain expressed mRNA for the IGF-1 transgene (depending on the line) at lower levels in comparison with lactating mammary tissue (Table 1). Transgene expression in brain tissue was detected in one out of two line 15 mice. Mammary expression of transgene IGF-1 ranged from 47 to 140% of endogenous liver IGF-1 mRNA for both transgenic lines. Transgene expression was not detected in liver or heart tissue in either line.

To determine whether the transgene mRNA was translated in mammary tissue, tissue homogenates were analyzed by radioimmunoassay using a mouse antihuman IGF-1 monoclonal antibody (MAb) that detected ovine and bovine, but not mouse IGF-1. Recombinant IGF-1 was detected at low levels of approx 2 ng/100 mg in the fourth inguinal mammary glands of virgin mice of transgenic lines 15 and 29. On d 12 of pregnancy, transgene expression was increased to approx 4 ng/100 mg. To induce greater activity of the glucocorticoid-responsive MMTV promoter, a test group of virgin mice were treated with dexamethasone. Dexamethasone-treated transgenic virgin mice from lines 15 and 29 responded with mammary recombinant IGF-1 production of 11.8 ± 0.6 and 21.9 ± 0.4 ng/100 mg tissue ($n = 2$/line). This acute dosage of dexamethasone was chosen based on its reported ability to induce expression of MMTV-LTR fusion genes over a short term *(13)*.

Lactating mice from transgenic lines 15 and 29 secreted 2859 ± 374 and 1008 ± 109 ng/mL recombinant IGF-1 into milk on d 16 of lactation, respectively ($n = 2$–4). Recombinant IGF-1 was not detected in plasma from transgenic virgin, pregnant, or lactating females. Plasma IGF-1 concentrations averaged 362 ± 33 and 218 ± 20 ng/mL in 60-d virgin and d 12 pregnant CD-1 mice, respectively ($n = 6$), and did not differ in transgenic mice ($p > 0.10$). Native milk IGF-1 concentrations in CD-1 mice ($n = 4$) averaged 552 ± 69 ng/mL on d 16 of lactation.

Profiles of insulin-like growth factor binding proteins (IGFBPs) in serum from control and transgenic animals were evaluated using Western ligand blotting. Four bands corresponding to:

1. 39–43 kDa IGFBP-3.
2. 32 kDa IGFBP-2.
3. 28 kDa IGFBP-1.
4. 24 kDa IGFBP-4.

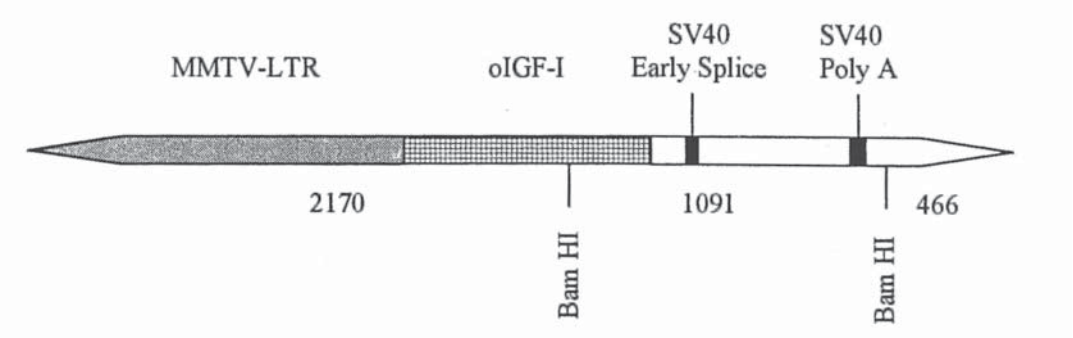

Fig. 2. Map of the MMTV-IGF-1 transgene (3.7 kb). Segments are the MMTV-LTR promoter and oIGF-1 cDNA that were assembled in the expression vector pMSG as described previously (4). SV40 early splice and poly A sites are indicated. The sizes (kb) of the fragments generated by *Bam*HI digestion of the construct are shown.

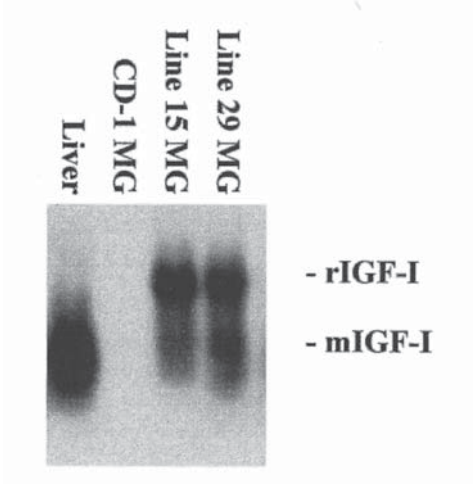

Fig. 3. Northern analysis of tissue-specific IGF-1 expression in tissues of mice at d-14 lactation. Total RNA was prepared from CD-1 liver and mammary tissue of one nontransgenic CD-1 and one transgenic mouse from lines 15 and 29 and loaded at 20 µg/lane. Blots were hybridized overnight with a [^{32}P]-IGF-1 cDNA and exposed to film for 12 d. The lower band corresponds to endogenous mouse IGF-1, and the upper band derives from transgene IGF-1 expression.

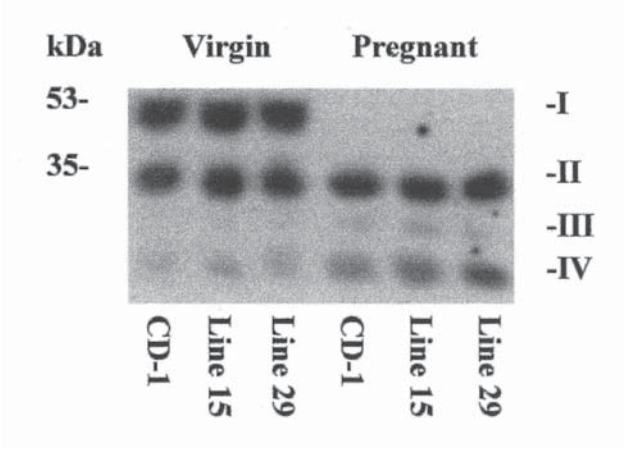

Fig. 4. Western ligand blot analysis of plasma pools (n = 6/lane) from nontransgenic or MMTV-IGF-1 mice at 60 d of age or d 12 of pregnancy. Blood samples were obtained via cardiac puncture. Plasma was collected and electrophoresed on an SDS-PAGE gel, blotted to nitrocellulose, and probed with [^{125}I]-IGF-1. Four bands corresponding to (I) 39–43 kDa IGFBP-3, (II) 32 kDa IGFBP-2, (III) 28 kDa IGFBP-1, and (IV) 24 kDa IGFBP-4 were detected in pooled plasma from CD-1 and MMTV-IGF-1 mice. Blots were exposed for 7 d.

Table 1
Tissue-Specific mRNA Expression of the MMTV-IGF-1 Construct in Tissues from Transgenic Mice at d 14 Lactation (n = 2)

	rIGF-1 Expression, % of mammary rIGF-1 expression in arbitrary densitometric units during lactation	
Tissue	Line 15	Line 29
Mammary (lactating)	100	100
Spleen	0	45
Brain	13	0
Kidney	0	10
Salivary gland	0	6
Heart	0	0
Liver	0	0

were detected in pooled plasma from CD-1 and MMTV-IGF-1 mice (Fig. 4) *(14)*. Differences in abundance of IGFBPs were not detected between control and transgenic animals in individual or pooled samples; however, pregnancy dramatically reduced serum levels of IGFBP-3 and increased IGFBP-4 levels compared with virgin mice.

Bioactivity of Recombinant IGF-1

To compare the mitogenic activity in milk from transgenic mice and control mice, milk extracts from a mouse of line 29 and from nontransgenic mice were cultured with cells of the MAC-T bovine mammary epithelial cell line. The enhanced mitogenic activity of transgenic mouse milk relative to control mouse milk was demonstrated by a greater stimulation of [^{3}H]thymidine incorporation in MAC-T cells ($p < 0.05$) (Fig. 5). Using a pool of milk extracts obtained from different mice, a subsequent study

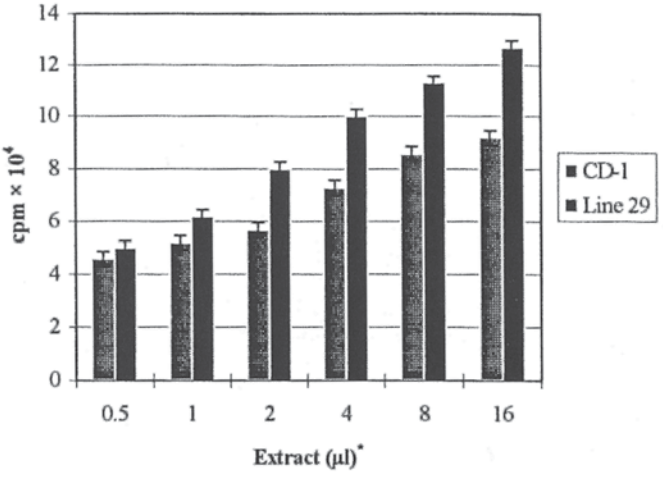

Fig. 5. Mean [^{3}H]thymidine incorporation into MAC-T cells in response to pooled milk extracts from CD-1 and transgenic MMTV-IGF-1 mice (n = 2/line). MAC-T cells were cultured in 24-well plates for 16 h followed by a pulse with 1 µCi [^{3}H]thymidine/well for 2 h. Bars represent means ± SEM from 4 wells at each concentration. *MMTV-IGF-1 differs from CD-1 controls at 2 µL extract and greater ($p < 0.05$).

Table 2
Effect of rhIGFBP-3 (320 ng/mL) on Mean [^{3}H]Thymidine Incorporation into MAC-T Cells in Response to Basal Medium (Dulbecco's Modified Eagle Medium, DMEM), rhIGF-1, or Milk Extracts (20 µL/well) (means ± SEM, n = 2/pool)

	[^{3}H]thymidine incorporation, cpm × 10^4	
Treatment	–BP3	+BP3
DMEM	1.6 ± .09	1.5 ± .07
rhIGF-1 (1 ng/well)	5.3 ± .46	1.6 ± .04[a]
rhIGF-1 (20 ng/well)	14.3 ± .70	2.2 ± .12[a]
Line 29 pool	11.1 ± .39	1.7 ± .23[a]
Line 15 pool	10.0 ± .41	1.7 ± .13[a]

[a]Different from treatments without added IGFBP-3 ($p < 0.001$).

confirmed the ability of extracts to stimulate cell proliferation as well as the ability of excess recombinant human insulin-like growth factor binding protein-3 (rhIGFBP3) to inhibit the mitogenic activity in milk extracts (Table 2).

[^{3}H]Thymidine Incorporation

The relative number of cells actively synthesizing DNA in explants of mammary tissue from nontransgenic and transgenic pregnant mice was compared by measurement of [^{3}H]thymidine incorporation over a 1-h period. Virgin mice were injected with [^{3}H]thymidine, and mammary tissue was recovered 1 h later. Subsequent analysis indicated no differences in [^{3}H]thymidine incorporation among CD-1 and MMTV-IGF-1 virgin mice or pregnant mice (Table 3).

Table 3
Mean [^{3}H]Thymidine Incorporation (cpm × 10^2/µg DNA) into Mammary Explants from 60- or 90-d Virgin or d 12 Pregnant CD-1 and Transgenic MMTV-IGF-1 Lines 15 and 29 Mice (Means ± SEM, n = 6/line)

	[^{3}H]thymidine incorporation cpm × 10^2/µg DNA		
Mouse line	60 d	90 d	Pregnant
CD-1	50 ± 3.5	46 ± 11.2	296 ± 36.0
MMTV-IGF-1			
Line 15	34 ± 5.4	31 ± 5.4	215 ± 28.9
Line 29	45 ± 8.9	48 ± 8.8	279 ± 24.6

Table 4
Effect of Ovariectomy and Hormone Treatment (NH = Placebo, E2 = Estrogen, Dex = Dexamethasone) on Presence of Alveolar Buds in Mammary Glands of CD-1 and MMTV-IGF-1 Mice

		Treatment[a]		
Mouse line	Total[a]	NH	E2	Dex[b]
CD-1	7/34	4/12	3/10	0/12
MMTV-IGF-1	24/34[c]	9/12	9/11	6/11

[a]Results are expressed as the number of animals exhibiting alveolar structures/total number of animals.

[b]Reduced overall number of mice exhibiting alveolar buds compared with NH ($p = 0.05$).

[c]Different from CD-1 ($p < 0.01$).

Not surprisingly, [^{3}H]thymidine incorporation was markedly increased in tissue from pregnant mice ($p < 0.001$).

Effects on Mammary Development

Comparison of mammary whole mounts from MMTV-IGF-1 mice with those from CD-1 mice was used to evaluate the effects of IGF-1 expression on peripubertal gland development. The single-copy line 29 was selected for this study, because transgene recombination events influencing expression were detected by Southern analysis in several line 15 individuals at the time of the experiment. Mammary whole mounts were prepared from nontransgenic and transgenic mice that had been ovariectomized, or sham-operated and subsequently treated with placebo, estradiol, or dexamethasone. Because mammary DNA synthesis at 60 d of age did not differ between control and transgenic mice, mammary development was analyzed in younger, 50-d mice following ovariectomy and hormone treatment. Histological observation of the methylene blue-stained glands showed that a greater percentage of transgenic mice (24/34, 71%) displayed alveolar buds in mammary parenchyma compared to CD-1 mice (7/34, 21%) ($p < 0.01$) (Table 4). In those mice showing alveolar buds, the buds were distinguishable throughout the ductular network

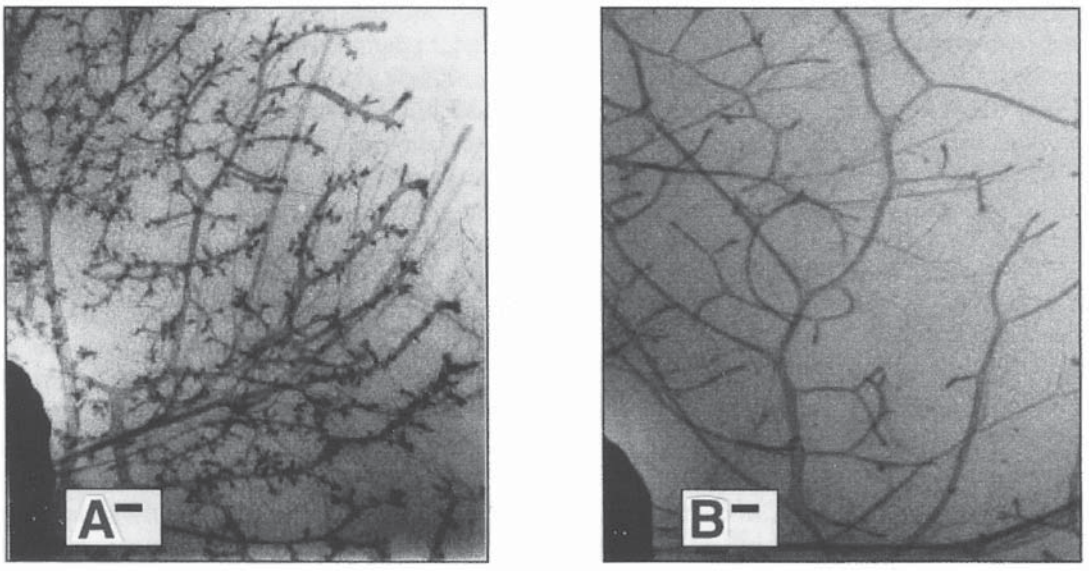

Fig. 6. Histological comparison of methylene blue-stained mammary gland whole mounts in the peripubertal period from **(A)** MMTV-IGF-1 transgenic and **(B)** CD-1 mice. Tissue was taken from 50 d virgin mice. Whole mounts were processed as described in Materials and Methods. Each bar equals 500 μm. Photos represent 24/34 transgenic line 29 and 7/34 nontransgenic CD-1 mice from a study investigating the effects of ovariectomy and hormone treatments (estradiol, dexamethasone, or placebo). Histological appearance of alveolar buds was not affected by ovariectomy or hormone treatment.

(Fig. 6). The proportion of mice showing alveolar buds was not different ($p > 0.10$) between intact and ovariectomized CD-1 (3/17 vs 4/17) or MMTV-IGF-1 mice (14/18 vs 10/16), respectively. Similarly, there was no difference between placebo- and estrogen-treated CD-1 (4/12 vs 3/10) or MMTV-IGF-1 mice (9/12 vs 9/11), respectively. However, dexamethasone reduced the number of animals exhibiting alveolar buds regardless of transgene presence ($p = 0.05$). Body weight of treatment groups was not different ($p > 0.10$).

Discussion

Production of IGF-1 within the mammary gland was achieved in this study using the model system of transgenic mice producing recombinant ovine IGF-1 under control of the MMTV-LTR promoter. Our study investigated the influence of locally produced IGF-1 on growth and development of the virgin mouse mammary gland. Ovine IGF-1, which differs from murine IGF-1 by five amino acids *(15)*, was used to evaluate the effects of IGF-1 synthesis in vivo based on our earlier studies of the role of recombinant IGF-1 produced by clonal mammary epithelial cells in vitro *(4)*. We report the characterization of transgene expression in virgin and pregnant animals, evaluation of biological activity of the recombinant IGF-1, and analysis of its effects on mammary development.

Tissue-specific expression of biologically active recombinant IGF-1 protein was observed in two lines of MMTV-IGF-1 mice at virgin, pregnant, and lactating stages. Previous work using the WAP-IGF-1 transgenic mouse *(6,7)* or transgenic rabbits carrying the bovine α_{s1}-casein promoter to direct IGF-1 expression *(8)* focused on transgene effects during the stages of pregnancy and lactation, when promoter activity is maximal. In contrast, the MMTV promoter utilized in our model becomes active in the virgin animal and is more strongly induced during pregnancy and lactation. Northern analysis of the tissue specificity of the transgene revealed low-level expression in salivary gland, kidney, spleen, and brain, similar to the pattern of MMTV expression noted in other studies *(16)*. However, recombinant protein was not detected in serum of transgenic mice of any stage, using a monoclonal IGF-1 antibody that does not recognize mouse IGF-1. Further, Western blot analysis of serum IGFBPs showed no differences owing to transgene expression. Absence of the 43-kDa IGFBP-3 in serum is characteristic of pregnant mice regardless of transgenesis; this degradation of IGFBP-3 was originally observed during human and rat pregnancy, and is attributed to proteolysis by a specific serum protease *(17)*. Thus, the MMTV-IGF-1 mice exhibited tissue-specific transgene expression, which allowed us to evaluate the effects of locally produced IGF-1 on the virgin mammary gland. Bioactivity of recombinant IGF-1 was demonstrated by stimulation of DNA synthesis in MAC-T cells above that derived from mitogenic activity inherent in mouse milk. Proliferative effects of recombinant IGF-1 were blocked in vitro by addition of IGFBP-3, one of the IGF-1 binding proteins secreted by mouse mammary epithelial cells, which may serve to regulate IGF-1 actions *(18)*. It follows that mammary synthesis of IGFBPs may modulate recombinant IGF-1 activity in vivo; analysis of mammary production of IGFBPs in nontransgenic and transgenic mice at different physiological stages is currently in progress.

The role of mammary-synthesized IGF-1 remains undetermined. Much evidence exists, however, to suggest that IGF-1 may be important in early mammary development *(19)*. Clearly IGF-1 is a mitogen for mammary tissue in vitro, as demonstrated using collagen gel culture of normal undifferentiated mammary epithelial cells of the mouse *(20)*, rat *(21)*, sheep *(22)*, and the cow *(23)*. The effects of IGF-1 in the immature mammary gland have been reviewed by Kleinberg *(24)*. Ruan et al. *(9)* demonstrated that implants of both intact and des(1-3)IGF-1 induced development of alveolar structures and terminal end buds in the mammary glands of hypophysectomized, castrated, and estradiol-treated sexually immature male rats. Likewise, mammary IGF-1 expression in our transgenic virgin mice induced formation of alveolar buds similar in appearance to these alveolar structures. In contrast, IGF-1 did not affect lobulo-alveolar development of mouse mammary glands in whole-organ culture *(25)*. This discrepancy may possibly be related to the different methods of in vivo transgenesis and in vitro organ culture used in the two studies.

The increased incidence of alveolar budding in nontransgenic mice in our study compared with that of Ruan et al. *(26)* may be related to the later age at which ovariectomy was performed in our animals, the shorter time period of recovery before treatments were applied, or species differences. Such alveolar buds develop around the time of puberty in response to ovarian secretions, and mature into alveolar structures during pregnancy *(27)*. Treatment with dexamethasone depressed alveolar bud development, despite its ability to induce IGF-1 transgene expression in virgin mice. Such a result may be attributed to a general catabolic effect of acute dexamethasone treatment on body tissues. Alternatively, dexamethasone has been shown to inhibit growth hormone-induced IGF-1 mRNA synthesis in the liver *(28)* and may have exerted its effect on alveolar bud development by reducing endogenous liver IGF-1 synthesis.

Evaluation of the rate of mammary growth may be an important indicator of the effects of IGF-1 on an absolute level. DNA synthesis, as measured by [^{3}H]thymidine incorporation into mammary explants, was not different between MMTV-IGF-1 and CD-1 virgin mice. The lack of difference in mammary DNA synthesis, despite the observed difference in presence of alveolar budding, suggests that IGF-1 may enhance the rate of early virgin mammary development occurring prior to 60 d of age.

During pregnancy, IGF-1 stimulated DNA synthesis or cell proliferation in mammary tissue slices from pregnant cows *(29)*, collagen gel culture of cells from pregnant heifers *(30)*, and in primary cells as well as in response to in vivo infusion of IGF-1 *(31)*. Our results did not demonstrate an effect of local IGF-1 synthesis on whole-mount appearance of transgenic mouse mammary glands during pregnancy, although additional evaluations are justified since the density of ductular and alveolar structures may render potential differences visually undetectable.

Similar to more defined mechanisms of action in other tissues, mammary production of IGF-1 under control of circulating GH may contribute to the growth and differentiation of mammary epithelium *(32)*. The relative importance of systemic vs local production of IGF-1 for the development of the mammary gland remains controversial, since mammary IGF-1 expression is considerably lower than that in liver and other reproductive tissues *(33)*. However, mammary IGF-1 expression can be influenced by exogenous GH *(34)* as well as plane of nutrition *(35)* and displays developmental regulation *(36)*, suggesting a potential role in mammary growth. In our study, epithelial cell synthesis of IGF-1 induced alveolar bud development in peripubertal mice, potentially at a faster rate or at an earlier age than in nontransgenic mice. Additional studies are in progress to evaluate the hormonal factors contributing to this effect as well as the consequences of mammary IGF-1 expression in lactating MMTV-IGF-1 mice.

Materials and Methods

Transgene

Details on the construction of the MMTV-IGF-1 transgene (Fig. 1) have been described elsewhere *(4)*. The 3.7-kb construct contains a 0.7-kb cDNA encoding an ovine exon-2 prepro IGF-1 *(37)* under control of the MMTV-LTR promoter. The construct was isolated from the expression vector pMMTV-IGF-1 *(4)* following digestion with the restriction endonucleases *Bgl*II and *Nde*I (USB, Cleveland, OH), excised from a 1% agarose gel, purified using GeneClean (BIO 101 Inc., La Jolla, CA), and filtered through a 0.45-µm spin filter.

Generation of Transgenic Mice

The linearized MMTV-IGF-1 fragments were microinjected into the pronuclei of embryos from CD-1 mice (Charles River Laboratories, Wilmington, MA) housed 4/cage in a temperature- (21 ± 1°C) and light- (12-h light/day) controlled environment. Rodent chow and tap water were available to mice ad libitum. Tail DNA was isolated from the resulting offspring and screened for presence of the transgene by PCR. Tail DNA served as the template in a 25-µL reaction including each of the following: 1X *Taq* polymerase buffer, 0.2 m*M* dNTPs, 0.4 µ*M* oligonucleotide primers, 1.5 m*M* $MgCl_2$, and 0.625 U *Taq* polymerase (Promega Corp., Madison, WI). The oligonucleotide primers 3c (5'-GATGCCAGTCACATCCTCCTCGC-3'; exon 3, nucleotides 190–212) and 4a (5'-GAGCCTTGG GCATGTCGGTG-3'; exon 4, nucleotides 383–403) amplified a 329-bp sequence in the ovine IGF-1 cDNA *(37)*. Initial denaturation of the samples was performed at 98°C for 2 min followed by 40 cycles at 56°C for 45 s, 72°C for 45 s, and 94.5°C for 45 s. Nontransgenic mouse DNA was used as a

negative control, and a dilution series of MMTV-IGF-1 plasmid standards served as a positive control.

Transgenic founders were subsequently mated with nontransgenic CD-1 mice to generate transgenic lines. All pregnant or lactating mice used in this study were mated at 6–7 wk of age and were primigravid. Transgene copy number for individuals within each line was determined by Southern blotting using 10 µg *Bam*HI-digested tail DNA. Digested samples were electrophoresed through a 0.8% agarose gel and transferred to nitrocellulose membranes (NitroPlus, MSI, Westborough, MA). Membranes were hybridized overnight at 42°C with the 0.7-kb ovine IGF-1 cDNA [^{32}P]-labeled by random priming, washed according to manufacturer's instructions, and exposed to Kodak XAR-5 film at –80°C with intensifying screens.

To evaluate the activity of the glucocorticoid-responsive promoter, a test group of virgin mice were treated with dexamethasone following steroid priming of mammary gland development. Beginning at 28–30 d of age, control and transgenic F_1 females (n = 2/line) were injected subcutaneously with estrogen (1 µg/mL) and progesterone (1 mg/mL) for 9 d *(38)*. Twelve hours after the final steroid injection, mice were injected ip with dexamethasone (Sigma, St. Louis, MO; 50 µg in saline with 1% ethanol and 1 mg/mL gum arabic) every 12 h for a total of 5 injections. Mice were sacrificed by cervical dislocation 12 h after the final injection, and tissue was removed for analysis of recombinant IGF-1 concentration.

RNA Analysis

Total RNA was extracted from tissues using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of total RNA were electrophoresed on a 1% agarose–0.66 *M* formaldehyde gel and transferred to nylon membranes (ZetaProbe, Bio-Rad, Hercules, CA) by vacuum blotting (Bio-Rad). Northern blots were processed as described above for Southern blots.

Total RNA prepared from the fourth inguinal mammary gland was used in confirming mammary transgene expression by RT-PCR. The oligonucleotide primer 6a (5'-CTGCACTCCCTCTGCTTGTG-3'; exon 6, nucleotides 426–445) was hybridized to 1 µg of total RNA in 10 µL at 95°C for 5 min followed by cooling for 15 min at 37°C *(37)*. Reverse transcription was performed in a 25-µL reaction volume of 1X first-strand buffer (50 m*M* Tris-HCl, pH 8.3; 75 m*M* KCl; 3 m*M* $MgCl_2$), 200 U of Moloney Murine Lukemia Virus (M-MLV) reverse transcriptase (Life Technologies, Gaithersburg, MD), 2.0 m*M* dNTPs, 10 m*M* dithiothreitol (DTT), and 40 U rRNasin (Promega Corp) at 37°C for 1 h. To remove RNA remaining at the end of the incubation period, samples were digested with 1 µg of RNase A for 15 min at 37°C, followed by incubation at 37°C in 0.1 *N* NaOH and 0.03 *M* EDTA. Samples were neutralized and fractionated on Sephadex G-50 spin columns before precipitation of cDNA with ethanol in the presence of ammonium acetate. The cDNA product was analyzed by PCR as described above for the detection of the MMTV-IGF-1 transgene.

[^{3}H]Thymidine Incorporation

Mammary tissue was obtained from female virgin mice (n = 6/stage) at 60 and 90 d and at 12 d for pregnant mice. For pregnant animals (n = 6), tissues for explants and for IGF-1 assay were obtained from the fourth inguinal glands. Approximately 150 mg of explants (1–3 mg each) were incubated in 2 mL DMEM (Life Technologies) containing 2 µCi [^{3}H]thymidine (SA 67 Ci/mol; ICN Biomedicals, Irvine, CA) in a shaking water bath at 37°C for 1 h.

Because we anticipated that preparation of homogeneous explants from virgin mouse mammary glands would be difficult, 60- and 90-d mice were injected with [^{3}H]thymidine (2 µCi/g body weight) for measurement of DNA synthesis in the mammary gland. After 1 h, the right fourth inguinal gland was removed for analysis. Incorporation of [^{3}H]thymidine into mammary DNA from both virgin and pregnant mice was used as a measure of DNA synthesis. The validity of both methods of measuring [^{3}H]thymidine incorporation described above has been previously discussed *(39)*.

Mitogenic Activity in Transgenic Mouse Milk

The mitogenic activity in transgenic and nontransgenic mouse milk from primiparous 80 d transgenic mice was compared for the ability to stimulate [^{3}H]thymidine incorporation in the MAC-T bovine mammary epithelial cell line *(40)*. Defatted milk samples from d 12 lactation were extracted to remove IGFBPs *(41)*, lyophilized, and resuspended in serum-free DMEM. The MAC-T cells were cultured in 24-well plates and serum-starved for 72 h before addition of milk extracts with or without 320 ng/mL rhIGFBP-3 (Celtrix Pharmaceuticals, Santa Clara, CA) for 16 h at 37°C. Cells were then pulsed for 2 h with 1 µCi of [^{3}H]thymidine/well. At the end of the incubation, cells were harvested for measurement of [^{3}H]thymidine incorporation *(42)*.

Radioimmunoassay for IGF-1 and Ligand Blotting

Extraction of IGF-1 from plasma, milk, and mammary tissue homogenates was performed using an acid-ethanol cryoprecipitation method according to Breier et al. *(41)*. Two hundred microliters of defatted milk or plasma were mixed with acid-ethanol extraction buffer at a ratio of 1:4. For mammary tissue, 100 mg of frozen crushed tissue were homogenized in 1 mL of extraction buffer. Following extraction, the supernatant was frozen at –20°C until radioimmunoassay for IGF-1 as previously described *(43)*. Recombinant IGF-1 and total IGF-1 in extracts were measured using a mouse antihuman IGF-1 monoclonal primary antibody *(44)* and with a rabbit antihuman IGF-1 polyclonal primary antibody (antiserum UB3-189; Hormone Distribution Program of NIDDK and the National Hormone and

Pituitary Program), respectively. The lower limit of detection for assays using the MAb was approx 10 ng/mL. Approximately 85% of IGF-1 was recovered when added to milk and plasma in this assay (data not shown).

Plasma profiles of IGFBPs were analyzed by Western ligand blotting *(45)*. Blood samples were centrifuged for 15 min at 12,000*g*, and the resulting plasma fraction was recovered. Samples (4 µL) were dissolved in reducing SDS-polyacrylamide gel sample buffer and separated overnight by 12.5% SDS-PAGE at constant current. Proteins were blotted to a nitrocellulose membrane (MSI, Westborough, MA). Blots were incubated with [^{125}I]IGF-1 and exposed to Kodak XAR-5 film at –70°C for 5–7 d with intensifying screens.

Effects of oIGF-1 Expression on Mammary Development

Effects of transgene expression on mammary development were examined using mammary gland whole mounts. Mice were randomly selected from CD-1 and transgenic mouse populations housed similarly to avoid bias owing to the stage of the estrous cycle on mammary development. Prepubertal female mice in the F_3 and F_4 generations were ovariectomized or sham-operated at 35 d of age, as early as possible following PCR analysis of transgenesis before puberty, and allowed to recover until hormone injections commenced at 42 d of age. Animals within each group (n = 6/treatment) were designated to receive one of three hormone injections im for 7 d: dexamethasone (5 µg/d), estradiol (1 µg/d), or placebo. Mice were sacrificed by cervical dislocation at 49 d of age. Fourth inguinal mammary glands were removed, spread onto microscopy slides, fixed overnight in Histochoice (Amresco, Solon, OH), and subsequently processed as described by Strum *(46)*.

Statistical Analysis

Statistical analysis was performed using the GLM procedure in SAS *(47)*. Linear contrasts were used to determine effects of the addition of milk extracts from control and transgenic mice on [^{3}H]thymidine incorporation into MAC-T cells. Chi-square analysis was used to test for transgene and treatment effects on the number of mice exhibiting alveolar buds. Contrasts were performed to test for significance in measurements of [^{3}H]thymidine incorporation. Values are presented as least-squares means ± SEM.

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