

Local Insulin-Like Growth Factor-II Mediates Prolactin-Induced Mammary Gland Development

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Prolactin (PRL) is a major determinant of mammary epithelial cell proliferation during alveolar development in sexually mature and pregnant mice. To date, it has not been clear whether PRL effects these responses alone or by also invoking the action of autocrine/paracrine growth factors. In this study, we provide evidence that part of the effect of PRL on mammary gland growth is mediated by IGF-II. During sexual maturity and in early pregnancy, the level of IGF-II mRNA in the mammary gland was increased concurrent with increased PRL receptor expression. The level of IGF-II mRNA was reduced in mammary tissue from PRL receptor^{-/-} mice during early pregnancy, and explants of mouse mammary gland and HC11 mammary epithelial cells both increased their expression of IGF-II after exposure to PRL *in vitro*. These findings coincided with the demonstration that IGF-II stim-

ulated alveolar development in mammary glands in whole organ culture. PRL was most efficacious in stimulating IGF-II gene transcription from promoter 3 of the mouse IGF-II gene *in vitro*. Insight into the mechanism by which PRL induced IGF-II expression was provided by the fact that it was blocked by the Jak2 inhibitor AG490 and the MAPK inhibitor PD98059. Finally, induction of insulin receptor substrate (IRS)-1 in the mammary glands of PRL-treated mice and induction of IRS-1 and IRS-2 after treatment with PRL plus progesterone indicates that these molecules are induced by PRL as potential signaling intermediates downstream from IGF-I/insulin receptors. Together, these data demonstrate a role for IGF-II as a mediator of PRL action in the mouse mammary gland during ductal branching and alveolar development. (*Molecular Endocrinology* 17: 460-471, 2003)

MAMMARY GLAND GROWTH is a dynamic process that occurs at specific stages during postnatal development. With the onset of puberty, mammary ducts ramify into the mammary fat pad to establish a ductal tree that subsequently gives rise to alveolar structures during pregnancy (1).

It is well established that this course of development is coordinately regulated by key endocrine hormones and locally derived growth factors. Of the endocrine hormones, the pituitary-derived hormone prolactin (PRL) is a primary regulator of alveolar development (2-5) through its stimulation of mammary epithelial cell (MEC) proliferation, as demonstrated *in vivo* (6) and *in vitro* (7). In parallel, PRL may have antiapoptotic effects that lead to increased MEC survival (8). These

functions of PRL are in addition to its well recognized effects on milk protein synthesis (9). Recently, we demonstrated that the expression of PRL receptor (PRLR) mRNA is up-regulated and heterogeneously distributed in the mammary ducts of mature, nulliparous female mice (6). Furthermore, PRL-stimulated proliferation of MEC is accompanied by proliferation of adjacent stromal cells that also express various isoforms of PRLR mRNA in a development-specific fashion (6). Combined, these data suggest that PRL acts upon a subpopulation of MEC and stromal cells to promote alveolar development through an as yet undefined autocrine/paracrine mechanism(s).

IGF-II is a member of the insulin/IGF family that is mitogenic for normal (10-12) and neoplastic (13) MEC. IGF-II is generally implicated as a locally derived growth factor that functions in embryonic tissues (14). However, several lines of evidence support a role for IGF-II during normal postnatal mammary gland development. IGF-II mRNA is heterogeneously expressed by MEC in the ductal epithelium of nulliparous mice (15). Similarly, IGF-II mRNA is expressed within the parenchyma and stroma of the ovine mammary gland during allometric growth and is regulated by ovarian

Abbreviations: AD, Alveolar development; CFP, cleared fat pad(s); CHO, Chinese hamster ovary; Dex, dexamethasone; E, estrogen; EGF, epidermal growth factor; ER, E receptor; FBS, fetal bovine serum; h, human; GAPDH, glyceraldehyde-3-phosphate; IGF-BP, IGF binding protein; IGF-IR, IGF-I receptor; IR, insulin receptor; IRS, IR substrate; MEC, mammary epithelial cell; o, ovine; P, progesterone; PR, P receptor; PRL, prolactin; PRLR, PRL receptor; r, recombinant; RT, reverse transcription.

function (16). Furthermore, IGF-II is expressed by both breast cancer cells (17) and stromal cells adjacent to breast tumors (18, 19). Transgenic overexpression of IGF-II in the mammary glands of mice from the ovine β -lactoglobulin promoter results in epithelial hyperplasia (20), whereas overexpression of IGF-II from the Moloney murine leukemia virus promoter results in delayed involution and apoptosis of MEC and the sustained expression of Akt (21).

Considerable debate has surrounded the question of how IGF-II acts on target cells. Although the mannose-6-phosphate receptor represents an exclusive receptor for IGF-II, it targets IGF-II for lysosomal degradation without evoking a cellular response (22). In addition, the IGF-I receptor (IGF-IR) has generally been considered the main pathway for IGF-II action because IGF-II displays a low affinity for the insulin receptor (IR; Ref. 14). Indeed, IGF-IR is expressed in the normal mouse mammary gland during development (15). However, several disparate results arising from mouse genetic models for IR, IGF-IR, and IGF-II have continued to imply a role for the IR in IGF-II action. This paradox can now be explained by the demonstration of two IR isoforms, IR-A and IR-B (23). IR-A, but not IR-B, is a high-affinity receptor for IGF-II that is expressed in several tissues including breast tumors (24). Binding of IGF-II to IR-A subsequently activates signaling pathways downstream of IR-A that include IR autophosphorylation, tyrosine phosphorylation of IR substrates (IRS) and Shc, and activation of Akt, leading to the specific induction of mitosis in target cells (23, 25).

On the basis of the recognized actions of PRL and the known functions of IGF-II, we hypothesized that IGF-II functions as an autocrine/paracrine effector of PRL action in the normal mouse mammary gland. Here, we demonstrate that PRL induces transcription of IGF-II in MEC via a PRLR-dependent pathway that leads to the stimulation of alveolar development. We also find that PRL induces IRS-1 and IRS-2 in the mouse mammary gland, indicating a role for IGF-II as a local effector of PRL action during mammary gland development.

RESULTS

As a first step toward determining the role and function of IGF-II in the mouse mammary gland, we determined the developmental profile of IGF-II mRNA expression during postnatal development in nulliparous and pregnant BALB/c females. These changes were quantified by competitive RT-PCR using the PCR mimic kit from CLONTECH Laboratories, Inc. (Palo Alto, CA) that used various amounts of mimic DNA to titrate IGF-II and corresponding glyceraldehyde-3-phosphate (GAPDH) cDNA levels. Typical data obtained using this method are shown in Fig. 1; the concentration of mimic at the intercept point indicated the amount of cDNA (in attomoles) in

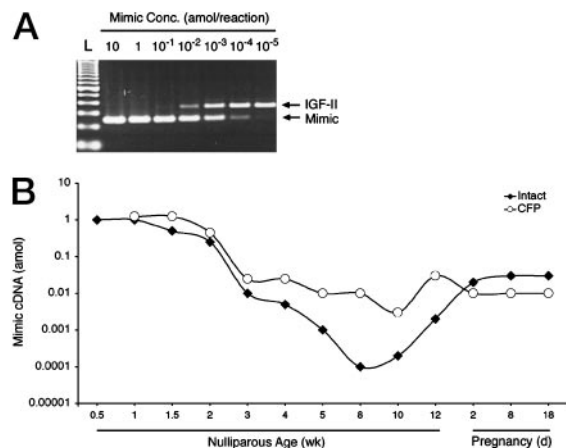


Fig. 1. Quantification of IGF-II mRNA Levels in the Mouse Mammary Gland during Development

A, Typical result of competitive RT-PCR with a cDNA mimic for determination of IGF-II mRNA levels. Different amounts of mimic cDNA bearing IGF-II PCR primer sequences were added to PCRs with a consistent amount of RT product. Quantification and plotting of band intensity enabled the amount of IGF-II PCR product to be determined from the intercept point. B, Profile of IGF-II mRNA in the mouse mammary gland and CFP during development. Total RNA from pooled tissue samples (at least $n = 6$ /point) was collected at the indicated stages of nulliparous development and pregnancy, then analyzed for IGF-II mRNA expression by competitive RT-PCR, and expressed relative to the corresponding GAPDH mRNA level.

the reverse-transcribed sample. The level of IGF-II mRNA in the intact mammary gland at different stages of nulliparous and gestational development is shown in Fig. 1B. Expression of IGF-II mRNA was particularly high in the mammary glands of neonatal females and declined dramatically during prepuberty. Thereafter, IGF-II mRNA levels increased after puberty and demonstrated a sustained increase after the onset of pregnancy. We also examined IGF-II expression in cleared mammary fat pads (Fig. 1B) because the mammary glands in prepubertal and early pregnant female mice are also composed of adipose and connective stromal tissue. As anticipated, expression of IGF-II within the mammary glands of neonates was primarily within the mammary fat pad and showed the same precipitous decline with age. This finding agreed with *in situ* hybridization studies that showed abundant IGF-II expression within the adipose and connective tissues of the mammary fat pad in neonates (data not shown). Interestingly, the decline in IGF-II mRNA levels was less pronounced in the mammary fat pad relative to the intact glands and demonstrated a smaller increase in expression beyond 9 wk of age. Higher expression of IGF-II in the intact mammary gland compared with the mammary fat pad during pregnancy concurs with the finding that IGF-II is heterogeneously expressed in the mammary epithelium of sexually mature and pregnant female mice as revealed by *in situ* hybridization (our unpublished data and Ref. 15).

These results prompted us to examine which factors regulate the induction of IGF-II during branching morphogenesis and alveologenesis in the intact mammary gland of sexually mature and pregnant female mice. Given our recent finding that PRLR expression is increased and heterogeneously distributed in the mouse mammary gland at these stages, we hypothesized that IGF-II expression within the mammary gland is PRL-regulated. Data from transcript profiling of PRLR^{-/-} mice (Affymetrix, Santa Clara, CA) indicated that IGF-II expression was reduced in the mammary glands of PRLR^{-/-} mice (data not shown). To confirm this finding we compared IGF-II expression in mammary tissues from wild-type (WT) and PRLR^{-/-} mice during early pregnancy by quantitative RT-PCR (LightCycler, Roche Diagnostics Corp., Indianapolis, IN). Because PRLR^{-/-} females have impaired fertility, we addressed this issue by transplanting both WT and PRLR^{-/-} MEC into the contralateral fat pads of Rag1^{-/-} mice that were killed at different stages of early pregnancy before any detectable genotypic differences in alveolar development.

On d 2 of pregnancy, the expression of IGF-II mRNA in PRLR^{-/-} transplants was 75% of that in WT tissue (Fig. 2). On d 4, the level of IGF-II mRNA in PRLR^{-/-} transplants was 7-fold higher than in transplants bearing WT MEC. This difference reflected increased expression of IGF-II in the Rag1^{-/-} cleared fat pad (CFP) relative to both the intact Rag1^{-/-} mammary glands and the Rag1^{-/-} CFP bearing WT epithelial trans-

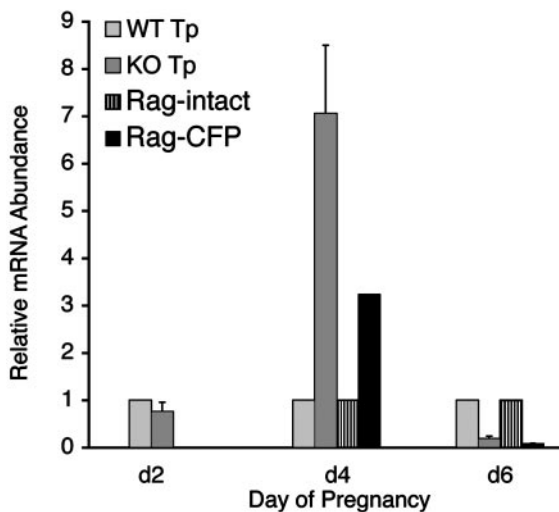


Fig. 2. Expression of IGF-II mRNA in Mammary Tissues from WT and PRLR-Deficient Female Mice

WT or PRLR^{-/-} (KO) epithelium was transplanted into Rag1^{-/-} cleared mammary fat pads and then collected as outgrown transplants (Tp) on d 2, 4, or 6 of pregnancy. Intact mammary glands or CFP from Rag1^{-/-} host females were also analyzed. Data are expressed as relative mRNA abundance determined by quantitative real-time RT-PCR and the respective fold-increase/decrease in PRLR-deficient tissues relative to WT transplants. Each mean (\pm SEM) is for five to six transplants.

plants. In keeping with observations for CFP vs. intact tissues during puberty (Fig. 1), these data indicate a mechanism for epithelial suppression of stromal IGF-II expression that is mediated by epithelial PRLR. By d 6 of pregnancy, transplants bearing WT MEC had 5-fold higher expression of IGF-II mRNA, compared with transplants of PRLR^{-/-} MEC into Rag1^{-/-} CFP, and a level of expression comparable to that in the intact Rag1^{-/-} mammary gland. Comparable levels of gene expression in Rag1^{-/-} CFP and PRLR^{-/-} transplants indicates that epithelial expression of IGF-II expression could not be activated in the absence of epithelial PRLR.

Taken together, these data support a role for epithelial PRLR in modulating IGF-II expression within both the mammary stroma and epithelium during the course of ductal branching and alveolar development.

These data suggestive of PRL-regulated IGF-II expression during pregnancy were confirmed using defined conditions *in vitro*. Explants of mammary glands from 18-d pregnant mice were cultured in hormone-free medium in the presence of various concentrations of ovine (o)PRL, then harvested 3 d later to determine IGF-II mRNA levels by semiquantitative RT-PCR. Although a low concentration of 50 ng/ml oPRL was without effect, potentially due to refractoriness to elevated serum PRL at this stage of pregnancy, 100–1000 ng/ml stimulated increased IGF-II mRNA expression compared with unsupplemented cultures (Fig. 3).

Given that PRLR are primarily expressed by MEC, in addition to low levels by stromal cells (6), we tested whether PRL stimulates epithelial cells to express IGF-II in an autocrine/paracrine manner. When HC11

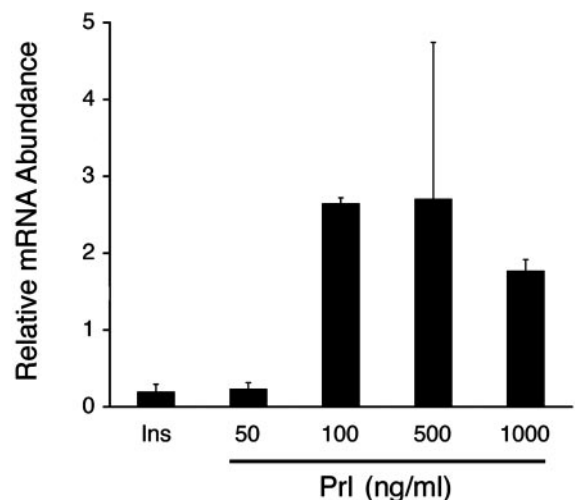


Fig. 3. PRL Induces IGF-II mRNA in Mammary Tissue from Pregnant Mice *in Vitro*

Explants of mammary tissue from mice at d 18 of pregnancy were cultured for 3 d in the presence of the indicated concentrations of oPRL. Total RNA was analyzed for IGF-II expression by semiquantitative RT-PCR and expressed relative to corresponding GAPDH mRNA levels. Data are means \pm SEM (n = 3). Ins, Insulin.

MECs were treated for 3 d with various doses of oPRL, there was considerable accumulation of IGF-II mRNA in a dose-dependent manner as detected by both semiquantitative and real-time RT-PCR (Fig. 4, A and B). This coincided with a marked induction of β -casein mRNA (data not shown). Similar results were obtained using recombinant human (rh)PRL (Fig. 4A). We also tested whether GH could induce IGF-II expression by HC11 cells. Treatment of HC11 cells with oGH stimulated IGF-II expression even more effectively than oPRL and at lower doses (Fig. 4B), whereas hGH was similarly more effective than hPRL or oPRL (Fig. 4A). The ability of PRL to induce IGF-II (Fig. 4C) and β -casein (data not shown) gene expression was independent of whether fetal bovine serum (FBS) was used to culture these cells.

Given that PRL regulates alveolar development *in vivo* and IGF-II may act downstream of PRL action, we tested whether IGF-II could participate in the stimulation of alveolar development *in vitro*. We used a defined, whole organ culture system that has previously been used to demonstrate effects of PRL and IGF-I (26) on alveolar development. In addition, we tested the effects of both rhIGF-II and rh Δ (1–6)IGF-II that is not sequestered by IGF binding proteins (IGFBPs). Under these conditions, alveolar development was significantly stimulated after culture with both forms of IGF-II at 200 ng/ml, but not at 50 ng/ml (Fig. 5).

The IGF-II gene is frequently transcribed as several splice variants that arise from differential usage of three alternative promoters positioned between exons 1 and 3 (14). We therefore tested the ability of PRL to activate each of these promoters in Chinese hamster ovary (CHO) cells cotransfected with a hPRLR long-form construct (Fig. 6). As previously identified, the relative transcriptional activity from the three IGF-II gene promoters was P3>P2>P1. The ability of PRL to activate IGF-II transcription was tested in both the presence and absence of dexamethasone (Dex), given that glucocorticoids have been shown to facilitate transcriptional activation of these promoters (27). Relative to untreated cells, PRL had no effect on P1 but stimulated 25% and 65% increases in transcription from P2 and P3, respectively. Although Dex had no appreciable effect on IGF-II transcription, PRL in the presence of Dex further stimulated luciferase activity to levels that were 15, 50, and 100% higher, respectively, than that in control cells. Therefore, although PRL alone, or in combination with Dex, was able to activate all IGF-II promoters via a presently unknown pathway(s), it was most efficacious on the P3 promoter. The specificity of PRL-induced IGF-II expression was confirmed by the demonstration that PRL had no effect on luciferase activity (in the absence or presence of Dex) when the hPRLR construct was omitted from the transfections (data not shown).

Specific pharmacological inhibitors of PRLR signaling were used to gain insight into the mechanism by which PRL induces IGF-II gene expression. Use of

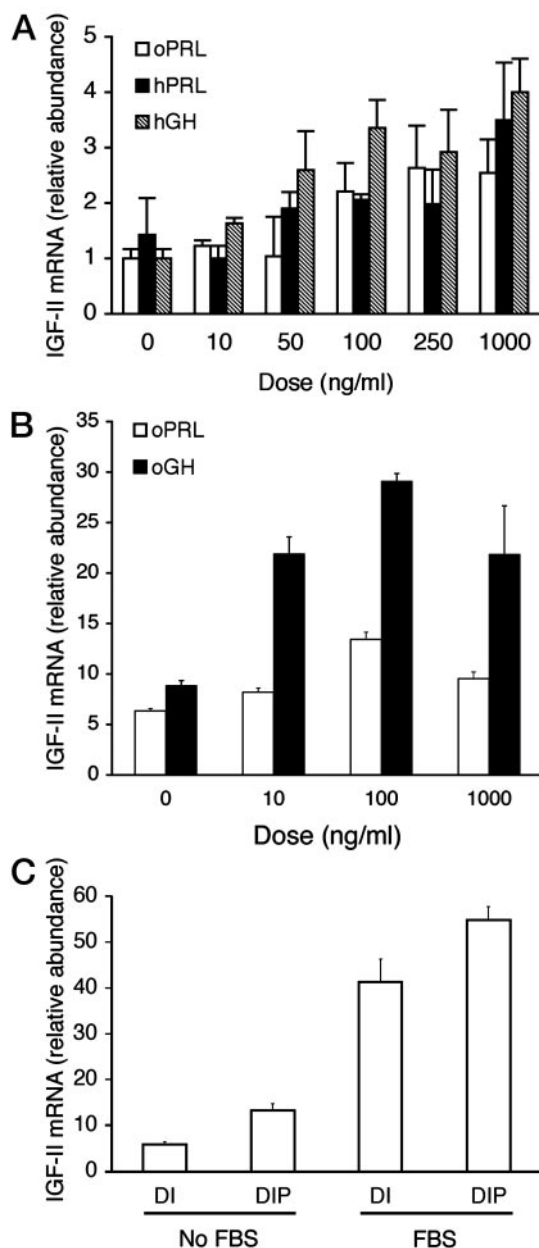


Fig. 4. PRL and GH Induce IGF-II mRNA in HC11 Mouse MECs

Cells were grown to confluence in growth medium, then cultured for 3 d in medium supplemented with insulin, Dex, and the indicated concentration of oPRL, hPRL, or hGH (A) or oPRL or oGH (B). Total RNA was analyzed for IGF-II mRNA by either SybrGreen real-time (A) or semiquantitative (B) RT-PCR and expressed relative to the corresponding level of GAPDH mRNA. Data are means \pm SEM ($n = 2$ or 3). C, PRL induction of IGF-II mRNA in cells cultured in the presence or absence of FBS. HC11 cells were treated with oPRL for 3 d in medium supplemented with or without 8% FBS and analyzed by semiquantitative RT-PCR. DI, Dex + insulin; DIP, Dex + insulin + oPRL.

AG490 to inhibit Jak2, an early intermediate signaling molecule activated by PRLR dimerization, resulted in the suppression of PRL-induced IGF-II expression

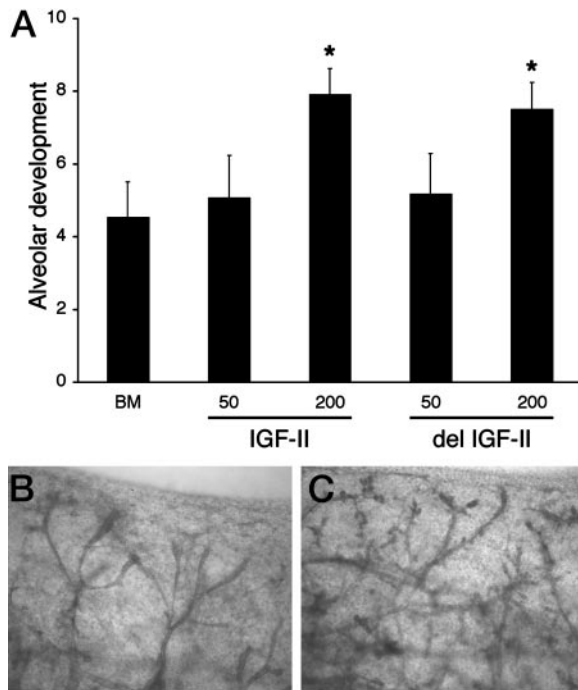


Fig. 5. Exogenous IGF-II Induces Alveolar Development in Whole Organ Culture

Mammary glands were cultured in basal medium (BM) alone or supplemented with 10 or 200 ng/ml rhIGF-II or rhdel(1–6)IGF-II. A, After 6-d culture, mammary glands were prepared as whole mounts and scored for alveolar development. Data are means \pm SEM ($n = 5$ –6 glands per treatment). *, $P < 0.05$ compared with BM mean. B and C, Photomicrographs depict morphology of mammary glands cultured in either BM (B) or 200 ng/ml rhdel(1–6)IGF-II (C).

(Fig. 7A) that coincided with its ability to block PRL-induced β -casein expression (Fig. 7B). In addition, the MAPK inhibitor PD98059 at 100 μ M was able to block PRL-induced IGF-II expression, but not β -casein expression as expected, suggesting that PRL may induce IGF-II via a Jak2-MAPK-associated pathway. Interestingly, a lower concentration of this inhibitor (5 μ M) led to increased IGF-II expression, similar to a phenomenon that has been observed previously for this inhibitor in HC11 cells (28). An additional inhibitor of PRL-induced action in MEC is epidermal growth factor (EGF; Ref. 29). In this context, when HC11 cells were treated with EGF, PRL-induced increases in both β -casein and IGF-II mRNA were abrogated (data not shown), indicating that EGF negatively regulates PRL-induced transcription of these genes by a common Jak2-associated mechanism.

Several downstream effectors of IGF action in target cells are known, including the IR substrates, IRS-1 and IRS-2. Given our finding that PRL induces the local expression of IGF-II within the mammary gland, we examined whether PRL induced IRS expression in the mammary glands of ovariectomized virgin female mice (Fig. 8). Immunoreactivity for both IRS-1 and IRS-2 was relatively low in saline-treated controls; although

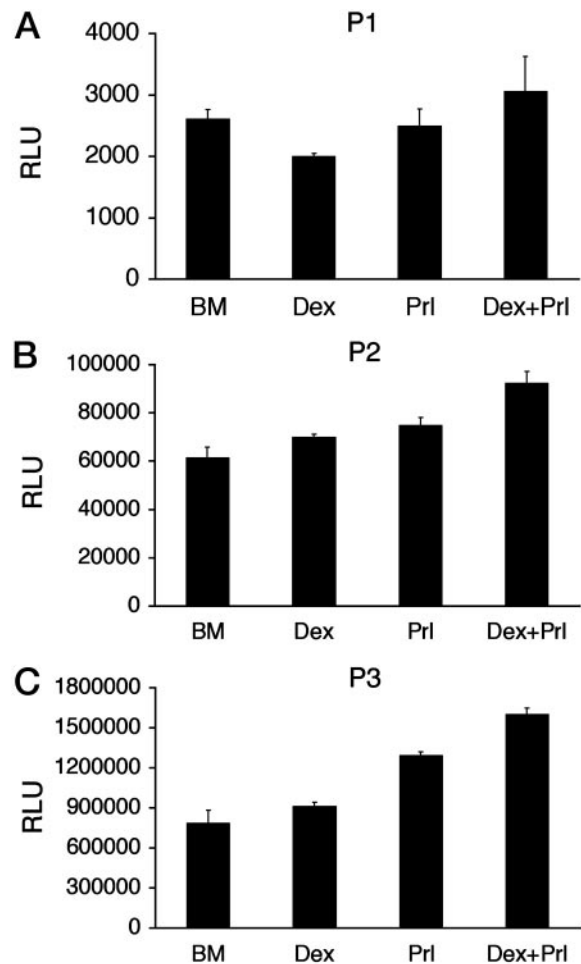


Fig. 6. Effect of PRL on Transcription from IGF-II Promoters in Transfected CHO Cells

Cells were transiently transfected with constructs carrying the P1, P2, or P3 murine IGF-II promoter upstream of a luciferase gene, along with pSV40 β -galactosidase and a human PRLR long form cDNA downstream of the elongation factor-1 α promoter. Transfected cells were treated with combinations of oPRL (1 μ g/ml) and Dex (1 μ M) for 24 h before assay of luciferase activity and correction for β -galactosidase activity. Data are means \pm SEM ($n = 3$). RLU, Relative light unit.

IRS-1 was distributed heterogeneously at low levels, IRS-2 was barely detectable. Treatment of ovariectomized mice with oPRL for 5 d increased the expression of IRS-1, but not IRS-2, in the ductal epithelium in a heterogeneous pattern. On the basis of our recent demonstration that PRL interacts with progesterone (P) to stimulate epithelial proliferation (6), we also examined IRS levels after treatment with P plus PRL. Consistent with this finding, the most marked induction of both IRS-1 and IRS-2 followed 5-d treatment with P plus PRL. These responses were also quantified as an index of cells stained positive for IRS-1 or IRS-2 in the various treatment groups (Table 1). As indicated above, PRL induced an increase in the number of IRS-1-positive cells, and this response was increased

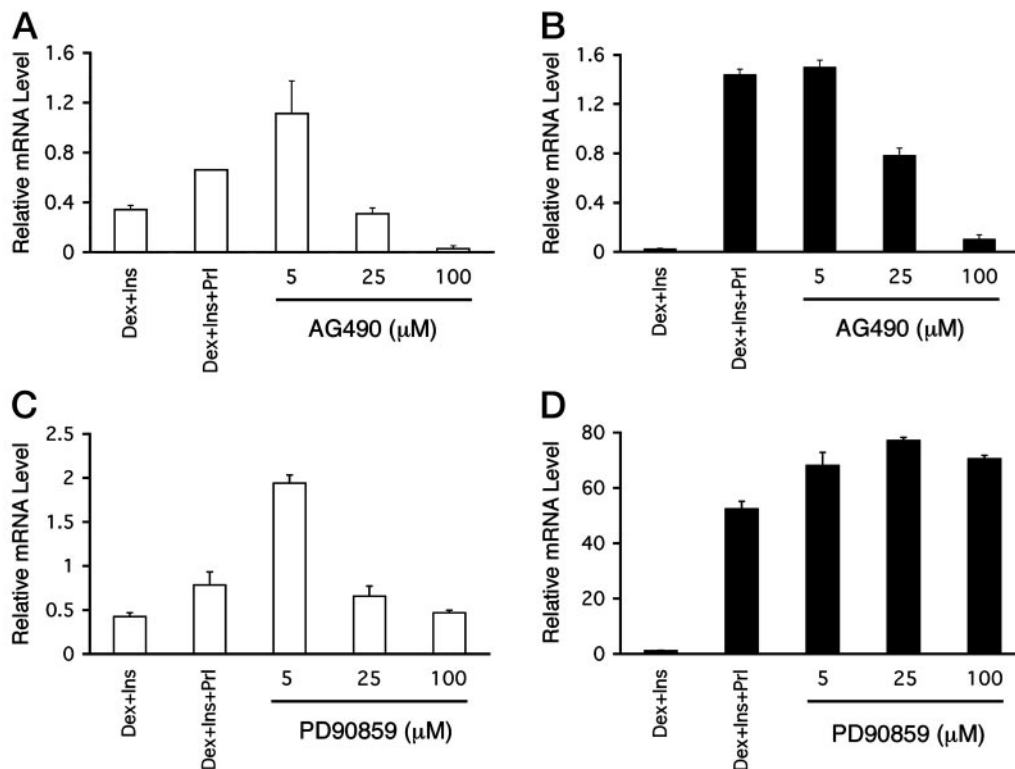


Fig. 7. Effect of Pharmacological Inhibitors on PRL-Induced IGF-II Gene Expression by HC11 Mammary Cells

Cells were grown to confluence, then cultured for 3 d in medium supplemented with insulin, Dex, and 1 μ g/ml oPRL in the presence of the indicated concentrations of AG490 (A and B) and PD90859 (C and D). Control cultures were supplemented with dimethyl sulfoxide only. Total RNA was analyzed for IGF-II (A and C, open bars) or β -casein (B and D, filled bars) mRNA by semiquantitative RT-PCR and expressed relative to the corresponding level of GAPDH mRNA. Data are means \pm SEM ($n = 3$).

in the combined presence of P plus PRL. By contrast, induction of IRS-2-positive cells was marked in the presence of P plus PRL, but not PRL alone. For both IRS-1 and IRS-2, these responses were associated with increased immunoreactivity. These findings indicate that PRL, either alone or in combination with P, induces IRS expression that potentially enhances the sensitivity of MEC to IGFs including IGF-II.

DISCUSSION

It is well documented that circulating endocrine hormones [estrogen (E), P, PRL, and GH] are the major factors that direct processes such as ductal growth, branching, and alveologenesis during the course of mammary development (1). In more recent times, it has become evident that these hormones do not necessarily deliver terminal signals but often elicit the local synthesis of additional autocrine/paracrine factors that confer differential specificity to endocrine hormone action. Such intermediates have been identified previously for E (EGF family members, Ref. 30; IGF-I, Ref. 31), GH (IGF-I, Ref. 31); and P (Wnt-4, Ref. 32). However, information has been lacking for whether local effectors of PRL action also exist. This is surprising,

given the dramatic effects of PRL on alveolar development and milk synthesis in the mammary gland (2–5).

The present data indicate that IGF-II is an autocrine effector of PRL action and induces IRS-1 and IRS-2 concurrent with ductal branching and alveolar development in the mouse mammary gland. These findings concur with several parallel functions for PRL and IGF-II in normal and neoplastic MEC. During development of the mammary glands in nulliparous and pregnant mice, both PRLR (6) and IGF-II (15) are heterogeneously distributed in MEC of the ducts. Overexpression of IGF-II in the mammary glands results in alveolar hyperplasia (20), and IGF-II is overexpressed in pregnancy-associated mammary tumors (13). Similarly, PRL is also known to be a major stimulant of alveolar development *in vivo* during pregnancy (5) and *in vitro* (4). In addition, both PRL (8) and IGF-II (21) suppress apoptosis in MEC during involution, and both PRL and IGF-II activate signaling intermediates such as Akt (21, 25, 33). In human breast cancer, the autocrine/paracrine synthesis of both PRL (34) and IGF-II (19) is increased, and both are autocrine/paracrine mitogens for breast cancer cells *in vitro* (24, 35). Furthermore, both PRL and IGF-II are recognized mitogens for normal mouse MEC *in vitro*. Taken together, these similarities between PRL and

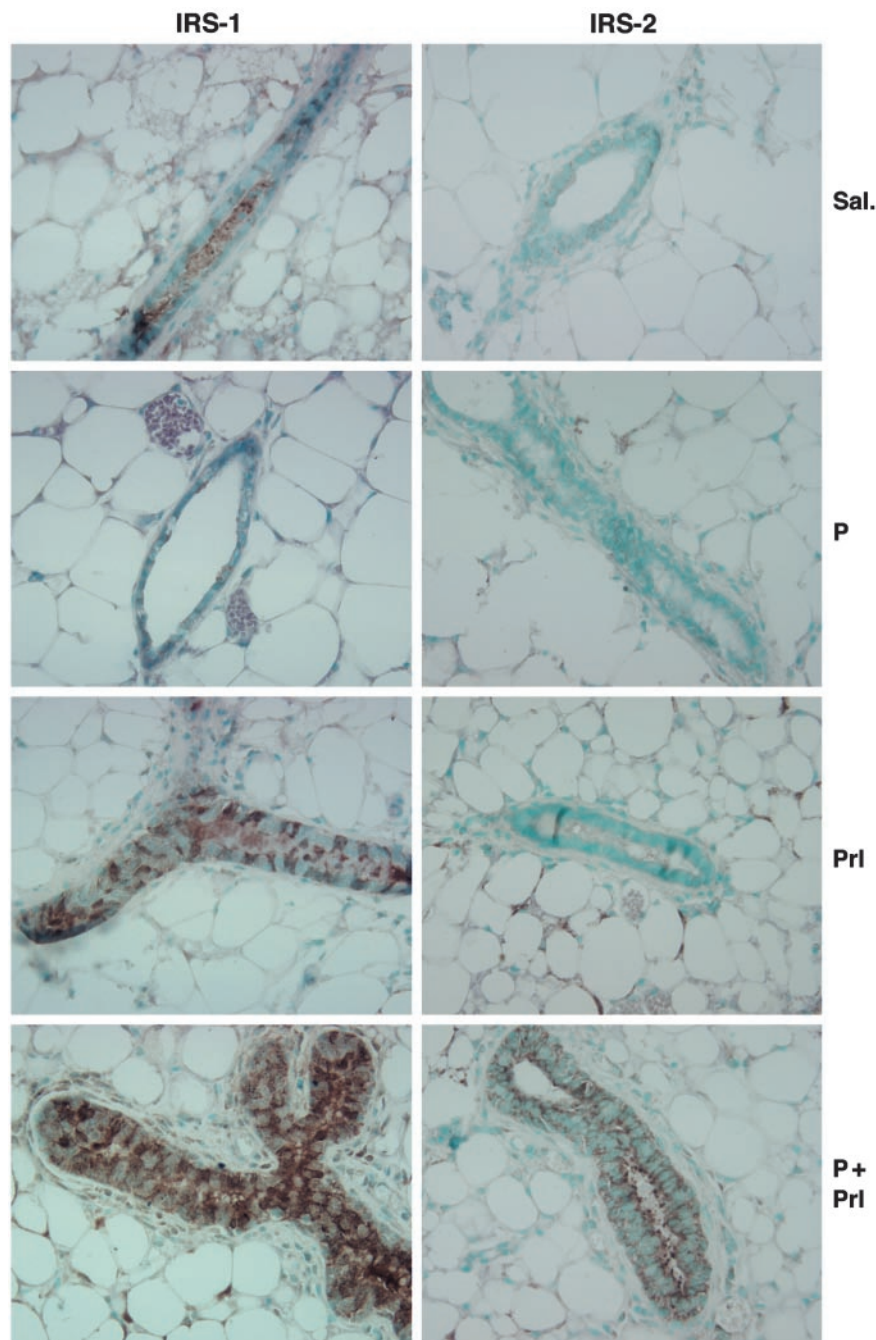


Fig. 8. Immunohistochemical Analysis of IRS-1 (*left*) and IRS-2 (*right*) in Mammary Glands from Hormone-Treated BALB/c Mice. Female mice ovariectomized at 9 wk of age were treated 1 wk later with saline (Sal), P, PRL, or P plus PRL for 5 d. Immunoreactive products for IRS-1 and IRS-2 were detected as a *brown* diaminobenzidine precipitate. Methyl green counterstain. Magnification, $\times 400$.

IGF-II implicate them in a combined role toward MEC proliferation during normal mammary development and in breast cancer.

The present data also indicate that the effects of GH and PRL on the developing mammary gland may be distinguished by their ability to differentially induce the expression of IGF-I and IGF-II, respectively. Several reports have shown that GH, in concert with E, in-

duces ductal elongation through the local stromal synthesis of IGF-I (31). By contrast, PRL does not induce the local synthesis of IGF-I (31) or ductal elongation, but instead can induce IGF-II so as to promote alveolar development (present study). This outcome is most clearly indicated in $PRLR^{-/-}$ mice that fail to undergo alveolar development (2, 3) in association with reduced expression of IGF-II.

Table 1. Results of Immunohistochemical Staining for IRS-1 and IRS-2 in the Mammary Glands of Hormone-Treated Mice

Treatment	IRS-1		IRS-2	
	% Cells positive	Staining intensity ^a	% Cells positive	Staining intensity
Saline	16 ± 2	+, +	8.4 ± 0.14	+/-, +/-
P	11.1 ± 3.9	+, +	8.42 ± 5.3	+, +/-
PRL	38.5 ± 6.5	++, ++	9.75 ± 0.35	+, +
P + PRL	59.5 ± 0.5	++++, +++++	81.9 ± 2.3	++++, +++++

Data are means ± SEM (n = 2).

^a Subjectively scored staining intensities are listed for each animal.

Divergence between the effects of GH and PRL on local IGF expression may be critical in demarcating the roles of these hormones during the course of normal mammary gland development. Our current data also suggest that GH can induce the expression of IGF-II in MEC, consistent with the knowledge that GH and PRL cooperate during lobuloalveolar development, potentially through the combined induction of IGF-I and IGF-II. Although it is possible that PRL may exert its observed effects through the GH receptor, our results for promoter transfection studies and analyses using PRLR^{-/-} mice would suggest that this is not the case. Furthermore, it is likely that PRL and GH induce IGF-II through common shared and recognized signaling pathways (36–38). In the ovine mammary gland, IGF-II mRNA was most abundant in the mammary parenchyma during a period of allometric growth (16). This abundant local synthesis of IGF-II may account for the extensive tertiary branching of mammary ducts in ruminants during this period compared with those in the mouse (39). Although IGF-II mRNA was also abundant in the mouse mammary fat pad of neonates, this expression declined precipitously in the weaning/prepubertal period. Such marked down-regulation of IGF-II gene expression during this period has also been reported in other tissues of various species (40–42). This decline probably represents either the maturation of fetal mammary mesenchyme (that expresses a high level of IGF-II) into white adipose tissue, or changing systemic control by hormones such as thyroid hormone (43). In parallel, it is tempting to speculate that high local expression of IGF-II in this period contributes to branching of the mammary ductal rudiment in neonates. It was also intriguing that proliferative epithelium within the intact mammary gland suppressed IGF-II mRNA levels relative to those in the CFP during prepuberty and puberty, and also in the presence of WT but not PRL receptor knockout epithelium during early pregnancy. This may represent a local means to down-regulate stromal IGF-II expression and associated alveolar development during a period of primarily ductal elongation. Similar evidence for the regulation of IGF expression by epithelial-stromal interactions has been identified in the normal mammary gland (16) and in breast tumors (18), although it is not clear what mechanism underlies this phenomenon. On the basis of this evidence, however, the increased expression of stromal IGF-II in the vicinity of breast tumors may

reflect a loss of this suppressive ability in tumors rather than the alternate acquisition of the ability to induce stromal IGF-II.

Our findings combined with those from other studies collectively indicate that PRL contributes toward regulation of the mammary IGF system. For example, PRL induced the synthesis of an undefined IGFBP in explants of pregnant mammary tissue *in vitro* (44). Likewise, declining PRL during involution is implicated in the increased expression of IGFBP-5 by MEC (8). The repertoire of the actions of PRL can now be extended to it being an effector of IGF-II synthesis and MEC proliferation in sexually mature and pregnant females. Clearly, the various IGF-BPs and IGF-IR/IR subtypes, including IR-A, also possess a significant capacity to regulate these local signals. In the present study, we also identified that PRL treatment further led to the significant induction of IRS protein levels concordant with its ability to induce IGF-II expression in the mammary gland. Previously, it has been shown that both IRS-1 and IRS-2 are phosphorylated in response to IGF-II action via the IR-A (23). Hence, these data afford a mechanism for MEC proliferation and alveolar development that involves the parallel induction of IGF-II and IRS molecules by PRL, then subsequent phosphorylation of elevated IRS proteins by autocrine/paracrine IGF-II acting through IR-A. Further studies are presently underway to demonstrate this IRS phosphorylation *in situ*.

What remains to be established is whether PRL cooperates with other hormones such as P to regulate IGF-II. Recently, we showed that PRL synergized with P to induce MEC proliferation and ductal branching in sexually mature female mice that coincided with the heterogeneous colocalization of PRLR and PR in ductal MEC. Interestingly, IGF-II mRNA is also heterogeneously distributed in ductal MEC (15), as was IRS-1 and IRS-2 in the present study, where IRS-1 and IRS-2 were most markedly induced by P plus PRL. Furthermore, cells that express P receptor (PR) are frequently E receptor (ER)-positive, and these cells are frequently adjacent to ER/PR-negative cells that are division-competent. Hence, it is conceivable that, through a presently unclear association, MEC positive for ER, PR, and PRLR are activated to express IGF-II that in turn provides paracrine mitogenic stimulation for adjacent hormone receptor-negative MEC that proliferate as alveolar progenitor cells.

In conclusion, these data indicate a previously unidentified role for IGF-II as a local effector of PRL action in the mammary gland. This mode of PRL action via IGF-II likely complements other hormone-induced local growth factors to facilitate the full course of mammary development.

MATERIALS AND METHODS

Animals, Treatments, and Tissues

All procedures were conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Mice were maintained on a 12-h light, 12-h dark schedule with *ad libitum* access to laboratory chow and water.

To study developmental changes in IGF-II mRNA levels, mice were euthanized at different ages to provide mammary tissues. To address hormonal variations during the estrous cycle, pubertal and sexually mature mice were killed at diestrus as determined by vaginal appearance (45) and confirmed by vaginal lavage (46). Mice euthanized during gestation were mated at 10 wk of age, and the presence of a vaginal plug was considered d 0 of gestation.

To study changes within the mammary fat pad, endogenous mammary epithelium was surgically removed to leave a cleared mammary fat pad (47) as described previously (6). Mice were subsequently euthanized at various stages of nulliparous and gestational development. Epithelium-free mammary fat pad tissue was obtained from females at 3 wk of age and earlier by collecting the fat pad tissue dorsal to the supramammary lymph node. All tissues were snap-frozen in liquid nitrogen and stored at -80°C .

Mammary epithelium from WT and PRLR^{-/-} mice was analyzed after transplantation to Rag1^{-/-} immunocompromised hosts. After the no. 4 mammary fat pads of Rag1^{-/-} female mice were cleared of endogenous epithelium, PRLR^{-/-} and WT epithelium was transplanted into contralateral mammary fat pads. The animals were aged for 12 wk to allow the epithelium to develop within the fat pad. After this period, the animals were mated, and d 0 of pregnancy was designated after observance of a vaginal plug. Mammary tissues were collected at 2, 4, and 6 d of pregnancy when there was no detectable difference in alveolar development between genotypes ($n = 4\text{--}6$ for each time point) and were snap-frozen in liquid nitrogen before storage at -80°C .

To determine hormonal effects on IRS expression, nulliparous female BALB/c were bilaterally ovariectomized at 9 wk of age. One week later, mice were injected for 5 d with twice-daily injections (sc) of oPRL (1 $\mu\text{g}/\text{g}$ body weight-d) with or without P (1 mg/d) in 0.9% saline. Harvested mammary tissues were fixed in 4% paraformaldehyde, then embedded in paraffin for sectioning at 4 μm .

Cell Culture

HC11 normal mouse MECs were routinely maintained in growth medium comprising RPMI 1640 supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD), EGF (10 ng/ml; Collaborative Research, Waltham, MA), insulin (5 $\mu\text{g}/\text{ml}$, Life Technologies, Inc.), HEPES (4.7 g/liter), sodium bicarbonate (2.2 g/liter), glutamine, penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). For gene expression experiments, cells were plated into six-well plates at 1.5×10^5 cells per well in growth medium and were grown for 2–3 d to confluence. Cells were subsequently changed to maintenance medium [RPMI with 8% FBS, HEPES, sodium bicarbonate, penicillin/streptomycin, and insulin (5 $\mu\text{g}/\text{ml}$)] either alone or supplemented with Dex (1 μM) with or without various concentrations of oPRL, for an additional 3 d.

CHO cells were routinely grown in α -MEM supplemented with 10% FBS and penicillin/streptomycin.

Promoter Analysis

Luciferase reporter constructs containing promoter regions for the murine IGF-II gene (P1, P2, and P3) that have been described elsewhere (48) were kindly provided by Dr. Peter Rotwein (Oregon Health Sciences University, Portland, OR). A cDNA encoding the hPRLR long form (kindly provided by Paul A. Kelly, Institut National de la Santé et de la Recherche Médicale, Paris, France) was subcloned into the pEF6C mammalian expression vector that uses the elongation factor-1 α promoter (Invitrogen, Carlsbad, CA). CHO cells were plated into six-well plates in growth medium at 2.4×10^5 cells per well 24 h prior to transfection. Cells were then transfected for 36 h in the same medium with a total of 2 μg plasmid DNA. After transfection, cells were changed to serum-free medium (α -MEM supplemented with ITS+1; BD Biosciences, Bedford, MA) and cultured for 24 h longer in the presence or absence of oPRL (1 $\mu\text{g}/\text{ml}$) and/or Dex (1 μM). Cells were then scraped into $1\times$ cell lysis buffer and assayed for luminescence using the luciferase assay system (Promega Corp., Madison, WI) on a Lumat LB9507 luminometer. Transfection efficiency was normalized for expression from a pSV40 β -galactosidase construct (Promega Corp.) as measured using a 96-well colorimetric assay (β -Galactosidase Enzyme Assay System, Promega Corp.) on an ELx800UV microtiter plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Transfection experiments were conducted in triplicate on three occasions.

Whole Organ and Explant Culture

Whole organ culture of mouse mammary glands was conducted essentially as described (26). Briefly, BALB/c mice were primed for 9 d with E and P contained within cholesterol pellets implanted sc. Mammary glands were then floated on siliconized lens paper and cultured in Waymouth's medium (Life Technologies, Inc.) supplemented with 1% BSA, HEPES, sodium bicarbonate, penicillin/streptomycin, aldosterone, hydrocortisone and oPRL for 6 d, with medium changed on the third day. Media was further supplemented with rhIGF-II (Becton Dickinson and Co., Bedford, MA) or rhdel(1–6)IGF-II (Upstate Biotechnology, Inc., Lake Placid, NY) at either 10 or 200 ng/ml. Mammary glands were then spread on microscope slides, fixed, and prepared as whole mounts stained with alum carmine. Mammary gland alveolar development (AD) was blindly scored on the basis of a previously described approach (26) using a subjective linear scale of 1–10 (where 1 is no AD, and 10 is AD equivalent to that in mid pregnancy). For each treatment, five or six mammary glands were analyzed.

In a separate experiment, explants of mammary tissue (approximately 8 mm³) from BALB/c mice on d 18 of pregnancy were floated on siliconized lens paper and cultured in Waymouth's medium with insulin (50 ng/ml), or supplemented with various doses of oPRL for 3 d. After the culture period, explants were snap-frozen in liquid nitrogen before RNA extraction.

Quantitative and Semiquantitative RT-PCR

Total RNA was extracted from tissues or cell monolayers using Trizol before treatment with DNase I (10 U/ μg RNA; Life Technologies, Inc.). RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) primed by oligo deoxythymidine and random hexamers in a final volume of 25 μl . Negative controls for reverse transcription (RT) were generated in the absence of RT enzyme, whereas PCR-negative controls were performed in the absence of RT product.

Quantitative competitive RT-PCR using the PCR mimic construction kit (CLONTECH Laboratories, Inc.) was used to determine changes in IGF-II mRNA levels during mammary gland development in pooled tissue samples. Briefly, a competitor mimic cDNA with primer-specific 5' and 3' ends was synthesized to be of similar size to the specific IGF-II PCR product. This DNA fragment was then quantified and diluted before being added to individual PCRs at different levels to provide a competitive internal standard in the presence of 0.2 μ M each primer and PCR master mix (Roche Molecular Biochemicals, Indianapolis, IN). Reaction products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified by image analysis using NIH Image. The log ratio of IGF-II product to IGF-II mimic product was then plotted against the log number of mimic molecules added to the PCR. The amount of IGF-II cDNA was calculated by determining the X intercept where the ratio of target to mimic concentration equaled 1. Levels of IGF-II mRNA were expressed relative to the level of GAPDH mRNA that was similarly determined using a GAPDH mimic in competitive PCR.

In subsequent experiments, semiquantitative RT-PCR analysis of gene expression for IGF-II, GAPDH, and β -casein mRNA was performed using a previously validated method (6) and optimized conditions for each gene. Gene expression levels were determined from products resolved on ethidium bromide-stained agarose gels, and corrected to the corresponding level of GAPDH mRNA. Triplicate samples were analyzed in each case.

For RT-PCR analysis of IGF-II mRNA expression in PRLR^{-/-} and corresponding WT samples, real-time monitoring of PCR amplification dynamics was achieved using a LightCycler (Roche Diagnostic Systems, Inc.). Additional real-time RT-PCR analysis of HC11 mRNA levels was performed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). Reverse-transcribed RNA was amplified using the FastStart PCR kit (Roche Molecular Biochemicals) or JumpStart kit (Sigma, St. Louis, MO) in the presence of SybrGreen, and cycle amplification differences were used to determine approximate differences in mRNA copy number between samples according to the manufacturer's instructions.

The following primers were used for RT-PCR analyses of IGF-II mRNA (49) to yield a single, sequence-verified product: 5', GAGCTTGTTGACACGCTTCAGTTTGTC; and 3', ACGTTGGCCTCTCTGAACTCTTTGAG, using a 60 C annealing step. Gene expression levels were normalized to the corresponding level of GAPDH mRNA determined using primers from CLONTECH Laboratories, Inc.

Immunohistochemistry

Immunohistochemistry for IRS-1 and IRS-2 was conducted on paraffin-embedded sections of mammary tissue using rabbit antihuman IRS-1 or IRS-2 antibodies (Upstate Biotechnology, Inc.). All incubations were performed at room temperature, and all washing was performed with TBST [0.15 M NaCl, 0.01 M Tris-HCl (pH 7.4), 0.05% Tween 20] unless otherwise stated. Slides were cut at 3–4 μ m, baked overnight at 58 C, and deparaffinized using a Shandon-Lipshaw Varistain. Heat-induced antigen retrieval was performed in 0.1 M Tris-HCl (pH 9.0) for 5 min using a pressure cooker. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide solution for 5 min. Avidin/biotin blocking was performed with a blocking kit from Vector Laboratories, Inc. (Burlingame, CA). Slides were then incubated in IRS-1 antibody (1:400 dilution in Tris-buffered saline + 1% BSA) or IRS-2 antibody (1:200 dilution in TBS + 1% BSA) for 1 h, biotin-labeled secondary antibody (1:200) for 30 min, and then horseradish peroxidase-labeled avidin (1:200) for 30 min. For a negative control, slides were incubated with purified rabbit Ig (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Detection was then performed by incubation

with diaminobenzidine (DAKO Corp., Carpinteria, CA) for 15 min, followed by enhancement with 0.2% osmium tetroxide for 30 sec. Slides were counterstained with 0.05% methyl green for 30 sec, dehydrated, and mounted using cytooseal. Quantification of immunostaining results was performed as a subjective intensity score or by determining the percentage of cells that were immunostained positive in two individual mice. This immunopositivity index was determined as the number of positive cells among at least 500 epithelial cells counted over 10 or more fields.

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