IGF-2 Is a Mediator of Prolactin-Induced Morphogenesis in the Breast

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Summary

The mechanisms by which prolactin controls proliferation of mammary epithelial cells (MECs) and morphogenesis of the breast epithelium are poorly understood. We show that cyclin D1$^{-/-}$ MECs fail to proliferate in response to prolactin and identify IGF-2 as a downstream target of prolactin signaling that lies upstream of cyclin D1 transcription. Ectopic IGF-2 expression restores alveologenesis in prolactin receptor$^{-/-}$ epithelium. Alveologenesis is retarded in IGF-2-deficient MECs. IGF-2 and prolactin receptor mRNAs colocalize in the mammary epithelium. Prolactin induces IGF-2 mRNA and IGF-2 induces cyclin D1 protein in primary MECs. Thus, IGF-2 is a mediator of prolactin-induced alveologenesis; prolactin, IGF-2, and cyclin D1, all of which are overexpressed in breast cancers, are components of a developmental pathway in the mammary gland.

Introduction

The systemic hormones estrogen, progesterone, and prolactin (Prl) control cell proliferation and morphogenesis in the breast (Nandi, 1958). The cellular and molecular mechanisms underlying their growth-promoting functions remain poorly understood. These hormones act directly on cells in the mammary gland and have indirect effects on this organ through their impact on other endocrine tissues.

The mouse provides a suitable model system with which to study the regulation of growth and differentiation of the breast, both because this animal can be genetically manipulated and because its mammary glands are readily accessible for experimental manipulation. Moreover, tissue recombination techniques make it possible to distinguish between systemic and local effects of germline mutations. Thus, recombination between mammary epithelial cells (MECs) of one genotype and mammary stroma of a second genotype within a wild-type host makes it possible to determine whether the mammary gland phenotype of a mutant mouse is mediated by cell-autonomous processes that are intrinsic to MECs, intrinsic to the mammary stroma, or secondary to defects in other tissues that control breast development at a distance (Brisken et al., 1998).

Prl blood levels are elevated during the luteal phase of the menstrual cycle (Franchimont et al., 1976), increase during late pregnancy and lactation, and are highest following bouts of nursing (Horseman, 2001). The hormone acts pleiotropically on a variety of target tissues (Bole-Feysot et al., 1998), explaining the observation that female mice lacking both copies of the Prl receptor gene (PrlR$^{-/-}$ mice) have multiple defects including a complex set of endocrinological abnormalities and infertility (Binart et al., 2000; Ormandy et al., 1997).

Recently, we described the behavior of PrlR$^{-/-}$ MECs engrafted in the mammary fat pads of wild-type mice; these fat pads had previously been cleared of endogenous epithelium (Brisken et al., 1999). When induced to become pregnant, these chimeras completed mammary ductal elongation and side branching but failed to develop the alveoli that normally accompany late-stage pregnancy. This behavior demonstrated that the defect in alveologenesis derived from the inability of PrlR$^{-/-}$ MECs to respond to the high levels of circulating Prl that are present late in pregnancy and hence reflected cell-autonomous behavior of MECs. In addition, the engrafted PrlR$^{-/-}$ epithelium in these mice failed to differentiate properly into milk-secreting cells. Together, these observations indicated that Prl signaling directly controls both the increase in MEC number during alveologenesis and the differentiation of alveolar MECs into milk-secreting cells.

Although the anterior lobe of the pituitary gland normally produces the bulk of Prl in the female body, the hormone is also synthesized locally in the breast epithelium (Reynolds et al., 1997). Local Prl synthesis as well as expression of the PrlR is upregulated within breast carcinomas (Clevenger et al., 1995). While the physiological relevance of the local Prl secretion remains unclear, these observations suggest that localized deregulation of Prl signaling within the breast may contribute to breast carcinogenesis. Consistent with this notion are observations that blocking Prl signaling interferes with the growth of various breast cancer cell lines (Fuh and Wells, 1995; Ginsburg and Vonderhaar, 1995), and that mice lacking the Prl gene show a delay in polyoma middle-T antigen-induced breast tumorigenesis (Vomachka et al., 2000).

Prl acts as a potent mitogen and morphogen on the

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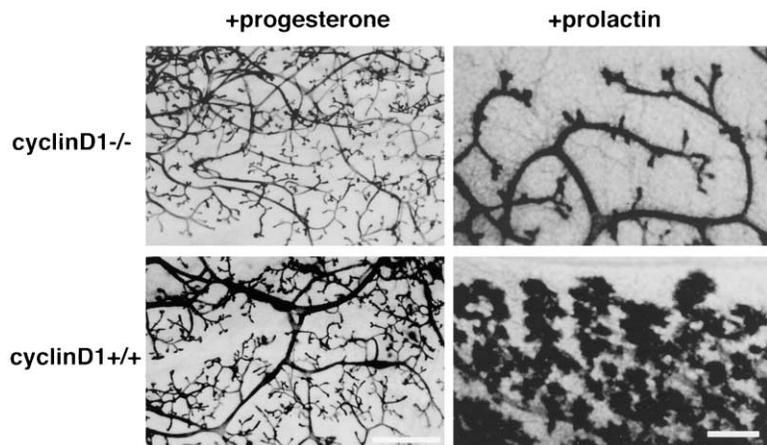
+progesterone   +prolactin

Figure 1. Response of *Cyclin D1*$^{-/-}$ Mammary Epithelium to Progesterone and Prolactin

Cyclin D1$^{-/-}$ and *Cyclin D1*$^{+/+}$ MECs were engrafted to the cleared fat pads of 3-week-old females. Eight weeks later, the recipients were stimulated for 10 days with either progesterone, left panels, or Prl, right panels. Shown are wholemount preparations of the engrafted mammary glands. Note that both grafts respond to progesterone stimulation with extensive side branching but differ in their response to Prl; the wt epithelium shows extensive alveolar development, which is absent from the *Cyclin D1*$^{-/-}$ graft. Identical results were obtained with six successfully engrafted mice in each group. Left bar, 500 μm; right bar, 400 μm.

mammary epithelium in vivo (Nandi, 1958), but the mechanisms by which it induces cell proliferation remain unclear. To gain further insight into the mechanisms underlying Prl function in vivo, we conducted a screen that selects specifically for genes that function downstream of the PrlR. Our findings indicate that an extracellular factor, IGF-2, acts as a downstream mediator of PrlR signaling and upstream regulator of cyclin D1 expression. These results provide a route by which Prl can act in a mitogenic fashion during alveologenesis and carcinogenesis and links Prl signaling, IGF-2, and cyclin D1 expression, all of which are deregulated during tumorigenesis, to a common signaling pathway.

Results

Response of *cyclin D1*$^{-/-}$ Epithelium to Pregnancy Hormones

cyclin D1$^{-/-}$ female mice fail to lactate due to a developmental defect in the mammary gland (Fantl et al., 1995; Sicinski et al., 1995), which is intrinsic to the mammary epithelium (Fantl et al., 1999). Both Prl and progesterone are implicated in specific stages of mammary gland morphogenesis during pregnancy, a time when the *cyclin D1*$^{-/-}$ phenotype becomes apparent. To determine whether the morphogenetic and mitogenic effects of either of these hormones were dependent on cyclin D1 function, we established either *cyclin D1*$^{-/-}$ or wild-type (wt) MECs as grafts in the mammary stroma of wt hosts cleared of the endogenous epithelium. Subsequently, the recipients were treated with either Prl or progesterone for 10 days, and the responses of the transplanted cells were assessed by wholemount microscopy. Progesterone was administered to ovariectomized female mice by daily injections (Lydon et al., 1995). To achieve stably elevated blood levels of the short-lived Prl, we resorted to using transplants of pituitary glands from adult mice of the same genetic background engrafted under the kidney capsule of the host. This ectopic localization of the pituitary gland causes a selective increase in Prl levels similar to those seen during pregnancy during the first 10 days after transplant (Huseby et al., 1985).

As shown in Figure 1, left panels, the *cyclin D1*$^{-/-}$ and the wt MECs underwent extensive branching in response to progesterone, indicating that this particular morphogenetic step and associated proliferation occur normally in response to progesterone stimulation. We note here that in the course of conducting a large series of experiments, we have never observed alveologenesis when wt mice of this genetic background are treated in this way. However, when stimulated with Prl, the wt grafts (Figure 1, bottom right) developed alveolar structures, which failed to form in the *cyclin D1*$^{-/-}$ grafts (Figure 1, top right). We concluded that cyclin D1 function is required to enable MECs to respond to the morphogenetic effects of Prl. On the other hand, responsiveness to progesterone is unaffected by the absence of *cyclin D1* in MECs. With these observations in mind, we undertook to study the signaling pathway through which Prl acts to trigger alveologenesis.

Screening for Prl Target Genes

We initially sought to determine the signaling mechanism by which the ligand-activated PrlR is able to induce cyclin D1 synthesis in MECs. Control experiments (Figure 2A) showed that direct application of Prl to cultured wt MECs induced relatively little cyclin D1 synthesis within 8 hr when compared to the actions of a series of growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin. The presence of an intact Prl-PrlR signaling system in these cells was indicated by the successful induction of β-casein mRNA following exposure to Prl (data not shown). Together, these observations suggested that the mechanisms controlling *cyclin D1* transcription were intact in these cultured MECs and that it was unlikely that the PrlR signals directly to the controllers of cyclin D1 transcription via an intracellular signaling cascade.

We reasoned that an alternative signaling mechanism might involve the Prl-induced synthesis of some intermediary signaling molecule that, once produced, proceeds to induce cyclin D1 synthesis. Consistent with this model were our observations that cyclin D1 protein, while only weakly induced 8 hr after Prl exposure (Figure 2A), was significantly induced 18 hr after stimulation of growth factor-deprived primary MECs with Prl (Figure 2B). To explore the possible involvement of such an intermediary signaling molecule, we compared the transcription profiles of pairs of contralateral cleared mammary fat pads that had been reconstituted with *PrlR*$^{-/-}$
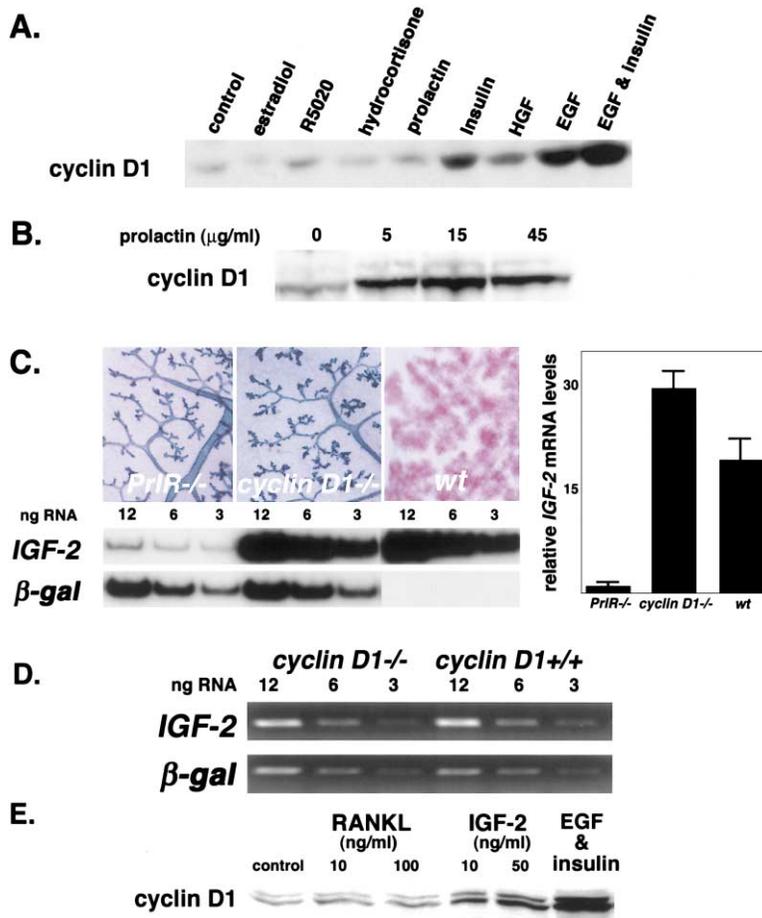
Figure 2. Establishing a Hierarchy between Prl, IGF-2, and cyclin D1

(A) Regulation of cyclin D1 protein expression in primary MECs. Primary MECs were growth factor deprived for 36 hr and subsequently stimulated with various mammotropic factors for 8 hours: estradiol (20 nmol), R5020 (20 nmol), hydrocortisone (1 μg/ml), Prl (5 μg/ml), insulin (10 μg/ml), HGF hepatocyte growth factor (10 ng/ml), and EGF epidermal growth factor (5ng/ml). Cyclin D1 protein levels assessed by Western blotting with anti-cyclin D1 antibodies.

(B) Regulation of cyclin D1 protein expression by Prl. Primary MECs were growth factor deprived for 36 hr and subsequently stimulated with different concentrations of Prl for 18 hr. Cyclin D1 protein levels assessed by Western blotting with anti-cyclin D1 antibodies.

(C) $PrlR^{-/-}$ and $Cyclin D1^{-/-}$ mammary epithelia: morphogenetic block and $IGF-2$ mRNA expression levels. Mammary epithelium from $PrlR^{-/-}ROSA26^{+/-}$ and $Cyclin D1^{-/-}ROSA26^{+/-}$ mice was engrafted to cleared inguinal fat pads of 3-week-old females. Eight weeks later, the recipients were mated, and at day 16.5 of pregnancy, the engrafted glands and an unmanipulated gland were analyzed. Top panels show glands subjected to X-gal staining and wholemounted. $PrlR^{-/-}ROSA26^{+/-}$ (left) and $Cyclin D1^{-/-}ROSA26^{+/-}$ epithelia (center) show fully branched ductal systems but lack alveoli. In contrast, the unmanipulated wt gland (right) shows extensive alveolar development. Bottom panels show $IGF-2$ mRNA expression in the mutant transplants: RNA samples in three serial dilutions were subjected to RT-PCR with IGF-2 (upper panel) and β-gal (lower panel) specific primers. Densitometry revealed that the IGF-2 signal is 30-fold increased in the $Cyclin D1^{-/-}ROSA26^{+/-}$ versus the $PrlR^{-/-}ROSA26^{+/-}$ transplant. The same results were obtained with three further pairs of engrafted mammary glands. Right panel: $IGF-2$ mRNA expression levels in engrafted and control glands were also measured by Real Time PCR and normalized to mRNA levels of the epithelial marker $Keratin 18$. Results from three experiments were averaged and plotted. Y-axis: arbitrary units.

(D) $IGF-2$ expression in mammary glands engrafted with $Cyclin D1^{+/+}ROSA26^{+/-}$ and $Cyclin D1^{-/-}ROSA26^{+/-}$ mammary epithelium harvested at day 12.5 of pregnancy. Three serial dilutions of RNA from the engrafted glands were subjected to RT-PCR with IGF-2 (upper panel) and β-gal (lower panel) specific primers. The amplification products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. The same results were obtained with three further pairs of engrafted mammary glands.

(E) Cyclin D1 induction by IGF-2 and RANKL. Primary MECs were growth factor deprived for 36 hr and subsequently stimulated with RANKL, IGF-2, or EGF and insulin for eight hours. Cyclin D1 protein levels assessed by Western blotting with anti-cyclin D1 antibodies.

MECs in one fat pad versus $cyclin D1^{-/-}$ MECs in the other. The host mice were wt, and after engrafting, were allowed to become pregnant.

As described above and illustrated in Figure 2C, top panels, the morphogenetic block shown by MECs of these two genotypes was very similar. Hence, these two types of mammary glands, assayed at an identical day of pregnancy, are presumed to carry comparable numbers of cells in comparable states of proliferation. We reasoned that the only differences in gene expression pattern between the mammary glands of these two genotypes should involve those genes that lie downstream of PrlR signaling and upstream of cyclin D1 expression. More specifically, we looked for genes that were expressed at high levels in the $cyclin D1^{-/-}$ but were expressed at low or undetectable levels in the $PrlR^{-/-}$ grafts.

The donor $cyclin D1^{-/-}$ and $PrlR^{-/-}$ MECs in these grafting experiments were both derived from mice that were also heterozygous for the $ROSA26$ transgene (Friedrich and Soriano, 1991) and hence expressed β-$galactosidase$ (β-gal) constitutively. This enzyme and its mRNA served as internal controls to ensure that similar amounts of engrafted epithelia were being compared in these expression analyses. Serial dilutions of RNA derived from a group of engrafted recipient mice at day 16.5 of pregnancy were reverse-transcribed and levels of β-gal expression were measured by semiquantitative PCR. Three pairs of samples in which the contralateral transplants showed comparable amounts of β-gal expression were pooled for cRNA probe preparation and hybridization on 6.5 K Affymetrix (B-chip) mouse expression arrays. This analysis was performed in duplicate.

Out of the 6,500 transcripts surveyed, 319 were expressed at more than 3-fold higher levels in the $cyclin D1^{-/-}$ than in the $PrlR^{-/-}$ grafts; 430 transcripts were

downregulated more than 3-fold. Previous observations had indicated that, in contrast to the $PrlR^{-/-}$ epithelium, the *cyclin D1$^{-/-}$* epithelium retains its ability to undergo differentiation as manifested by its ability to synthesize milk proteins late in pregnancy (Fantl et al., 1999). Indeed, many genes encoding proteins secreted with the milk or involved in milk production such as metabolic enzymes, calcium transport, and intracellular vesicular trafficking genes were found to be differentially expressed, with their transcripts being found at far higher levels in the fat pads engrafted with *cyclin D1$^{-/-}$* MECs than in the fat pads engrafted with $PrlR^{-/-}$ MECs. In addition, genes involved in signal transduction, the construction of the cytoskeleton, and the extracellular matrix were preferentially expressed in the *cyclin D1$^{-/-}$* recombinants.

An overview of the genes whose expression levels differed by more than 10-fold is given in Table 1. Among the secreted factors that were particularly highly expressed in the *cyclin D1$^{-/-}$* grafts was insulin-like growth factor-2 (IGF-2), which was found at a level 13.2-fold higher in the glands engrafted with *cyclin D1$^{-/-}$* epithelia than in the counterparts engrafted with $PrlR^{-/-}$ epithelia. The expression levels of another growth factor, HB-EGF, differed by 4.5-fold.

To validate the initial findings, we assessed relative IGF-2 and HB-EGF expression levels in four additional matched pairs of engrafted glands by quantitative RT-PCR. After normalization for β-*gal* expression, *IGF-2* mRNA levels were consistently 12- to 30-fold higher in the cyclin D1$^{-/-}$ transplanted glands than in the $PrlR^{-/-}$ counterparts (Figure 2C, bottom panels), whereas HB-EGF levels differed by about 3-fold (data not shown). Other growth factors represented on the arrays such as FGF1, FGF15, HGF, EGF, and TGFα differed less than 3-fold between the two types of chimeric glands. We also extended our analysis to additional growth factors such as amphiregulin, IGF-1, and RANKL (receptor activator of NF-κb ligand) that are not represented on the arrays but are developmentally regulated in the mammary gland. We found no difference in their expression levels between contralateral grafts (data not shown). This indicated that the strongest upregulation of the growth factors tested was that of IGF-2. Taken together, these data provided evidence that IGF-2 synthesis in MECs is under the control of the PrlR and thus Prl.

Role of Cyclin D1 in Regulating IGF-2 Expression

Our screen was based on the assumption that those genes that were expressed at higher levels in the fat pads reconstituted with *cyclin D1$^{-/-}$* epithelium than in the fat pads reconstituted with $PrlR^{-/-}$ epithelium are transcriptionally activated by Prl signaling. It was, however, formally possible that the difference in *IGF-2* gene expression levels was independent of Prl signaling and resulted instead from a lack of repression in *cyclin D1$^{-/-}$* grafts. To address this possibility, we assessed *IGF-2* mRNA expression levels in the contralateral mammary glands of a wild-type host that had been cleared and reconstituted with either *cyclin D1$^{-/-}$ROSA26$^{+/-}$* epithelium or *cyclin D1$^{+/+}$ROSA26$^{+/-}$* epithelium. We assessed a sample at day 12.5 of pregnancy before the time when the morphological difference between the

cyclin D1$^{-/-}$ and the wt counterpart becomes apparent. As shown in Figure 2D, the expression levels of *IGF-2* mRNAs were comparable when normalized to β-*gal* expression levels in the two glands, indicating that cyclin D1 does not act to repress *IGF-2* expression. Taken together, these various observations led us to conclude that *IGF-2* expression in the MECs of pregnant mice is strongly dependent on signals emanating from the PrlR.

Effect of IGF-2 on Cyclin D1 Expression

While the above data indicated that the PrlR is required for the induction of IGF-2 by MECs, it did not reveal whether IGF-2, once synthesized, could proceed to induce cyclin D1 synthesis. To address whether cyclin D1 expression is under the direct control of IGF-2, we stimulated primary MECs that had been deprived of growth factors with IGF-2 and assessed cyclin D1 protein levels 8 hr later. We also stimulated the cells with RANKL, a factor recently implied to be an important mediator of cyclin D1 expression in the mammary gland (Cao et al., 2001). We observed that the extent of cyclin D1 induction is dependent on the concentration of IGF-2, amounting to a 6-fold increase at an IGF-2 concentration of 50 ng/ml. However, RANKL failed to induce cyclin D1 protein synthesis in these cells (Figure 2E) when applied at the concentration that has been shown to activate downstream signaling in these cells (Cao et al., 2001). This indicated that IGF-2 can directly upregulate cyclin D1 protein in MECs. Taken together, these observations indicated that the PrlR is required for the synthesis of IGF-2 by MECs and that this growth factor, once synthesized and released from MECs, can act in an autocrine or paracrine fashion to elicit cyclin D1 synthesis.

Role of IGF-2 in Mediating Prl Function

The scheme proposed above implies that IGF-2 acts as an important mediator of PrlR signaling during alveologenesis. In particular, these observations suggested that Prl induces IGF-2 and that the latter induces cyclin D1 synthesis, enabling alveologenesis to proceed. This thinking prompted us to determine the role of IGF-2 as a possible mediator of Prl-induced alveologenesis. More specifically, we asked whether ectopic expression of IGF-2 could rescue the defect in alveologenesis observed in MECs lacking the PrlR and thus lacking Prl signaling.

To test this notion, we infected MECs derived from 10-week-old $PrlR^{-/-}$ female mice of 129SV/C57Bl6 mixed genetic background with a retrovirus expressing IGF-2. The resulting cultures, a mixture of infected and noninfected MECs, were used to reconstitute cleared mammary fat pads of 3-week-old wt female mice. Previous work had shown that infection of MECs with a retrovirus expressing β-gal does not affect in vivo morphogenesis (Edwards et al., 1996). This indicated that use of a retroviral vector per se would have no effect on alveologenesis. As a positive control, we determined whether the alveologenesis phenotype of the $PrlR^{-/-}$ MECs could be rescued by infection with a retrovirus vector expressing the PrlR. Eight weeks after injecting these MECs into cleared fat pads, host mice were mated, and the mammary glands of the resulting pregnant mice were examined.

Table 1. Putative Prolactin Target Genes

Genes Related to Milk Secretion

X93037	*M. musculus* mRNA for WDNM1 protein
W18308	Mouse ferritin heavy chain gene
x04673	Adipsin
X61431	ACYL-COA-BINDING PROTEIN
W44201	Sim. to PROTEIN TRANSPORT PROTEIN SEC23
Y00516	Mouse mRNA for aldolase A:FRUCTOSE-BISPHOSPHATE ALDOLASE A
M32599	Mouse glyceraldehyde-3-phosphate dehydrogenase mRNA
AA071776	Sim. to GLUCOSE-6-PHOSPHATE ISOMERASE (PHOSPHOGLUCOSE ISOMERASE)
J05277	Mouse hexokinase mRNA
x04490	Casein beta
L09104	*M. musculus* glucose phosphate isomerase mRNA, 3′ end
M21285	Mouse stearoyl-CoA desaturase gene
AA117004	Sim. to ER LUMEN PROTEIN RETAINING RECEPTOR (KDEL RECEPTOR) (P23)
x02520	Lactate dehydrogenase 1, A chain
W09506	Sim. to FATTY ACID-BINDING PROTEIN

Calcium Metabolism

X97991	Calcitonin
M27844	Parvalbumin
W20937	Sim. to CALCIUM-TRANSPORTING ATPASE SARCOPLASMIC RETICULUM TYPE

Cytoskeleton

x51438	Vimentin
AA168865	Sim. to ACTIN 1 (FRAGMENT)
X13297	ACTIN, AORTIC SMOOTH MUSCLE
J04953	Mouse gelsolin gene
X14425	Mouse mRNA for profilin:PROFILIN I
U20365	*M. musculus* smooth muscle gamma actin mRNA

Growth Factors

AA002605	Mouse insulin-like growth factor II (IGF-II) gene, 5′ flank

Extracellular Matrix

X04017	Mouse mRNA for cysteine-rich glycoprotein SPARC
x14194	Nidogen
x17069	Mouse COL1A2 mRNA for pro-alpha-2(I) collagen
x65582	Procollagen, type VI, alpha 2
U08020	*M. musculus* alpha 1 type I collagen gene, partial cds and 3′ flanking region
ET61037	Lectin, galactose binding, soluble 1
x72862	*M. musculus* gene for beta-3-adrenergic receptor::Adrenergic receptor, beta 3
X73523	Mouse mRNA for matrix Gla protein (MGP)
W75072	Procollagen, type IX, alpha 2

Signal Transduction

x15358	Sim. to INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 4 PRECURSOR (IGFBP-4)
W65899	Sim. to GUANINE NUCLEOTIDE-BINDING PROTEIN G(I)/G(S)/G(T) BETA SUBUNIT 2
D10024	Mouse mRNA for protein-tyrosine kinase substrate p36 (calpactin I heavy chain)
X58251	*M. musculus* mRNA for E-selectin ligand-1
L23108	*M. musculus* mRNA for GTP-binding protein
X85788	*M. musculus* mRNA for DCC tumour suppressor
L09192	Cathepsin D
M16358	RAB1, member RAS oncogene family

Heat Shock

AA163643	Sim. to HEAT SHOCK COGNATE 71 KD PROTEIN
U73744	*M. musculus* heat shock 70 protein (Hsc70) gene
AA105022	Sim. to HEAT SHOCK PROTEIN HSP 90-BETA (HSP 84) (HSP 90)

Miscellaneous

W41817	*M. musculus* cytochrome c oxidase subunit VIII precursor (Cox8l) mRNA
Z83368	*M. musculus* RPS3a gene
M76131	Mouse elongation factor 2 (ef-2) mRNA, 3′ end
AA154007	Sim. to POL POLYPROTEIN; REVERSE TRANSCRIPTASE
X05021	Murine mRNA with homology to yeast L29 ribosomal protein gene
x54691	Cytochrome C oxidase, subunit IV
W88176	Sim. to THIOL-SPECIFIC ANTIOXIDANT PROTEIN (PRP)
Z50159	*M. musculus* mRNA for Sui1
M24263	Mouse testosterone 16-alpha-hydroxylase (CB) gene
AA138107	Mouse COX7c1 mRNA for cytochrome c oxidase VIIc (EC 1.9.3.1)
D00466	Mouse apolipoprotein E mRNA
x82067	*M. musculus* thioredoxin-dependent peroxide reductase (tpx) mRNA

Genes expressed at more than 10-fold higher levels in mammary glands engrafted with cyclin DI$^{-/-}$ than with PrlR$^{-/-}$ mammary epithelium, as assessed by microarray analysis.
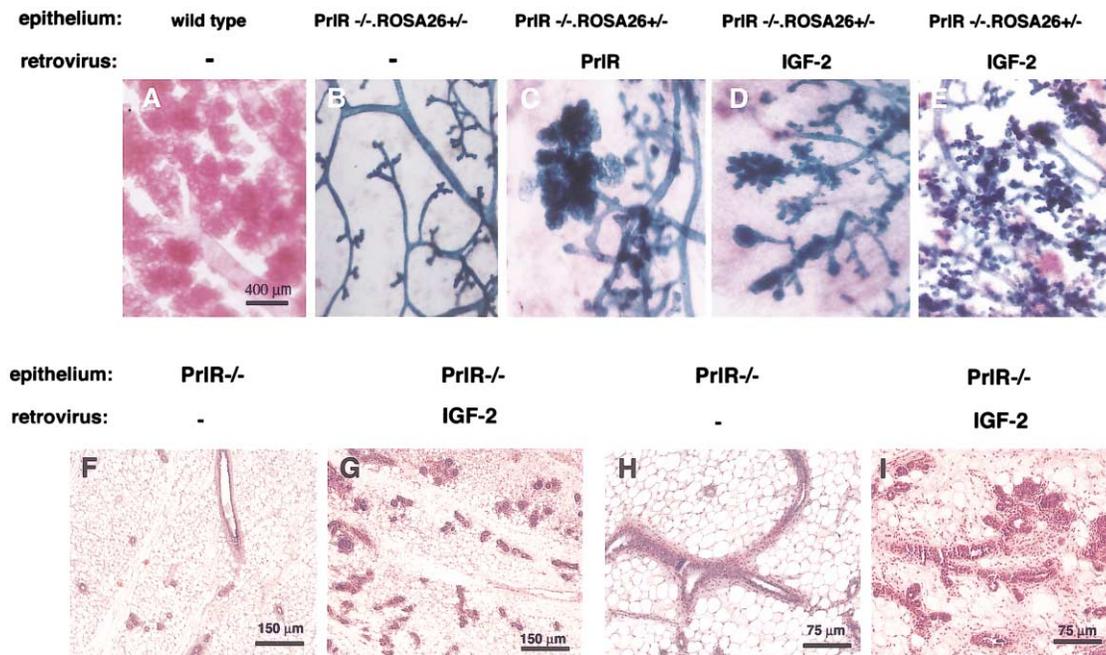
Figure 3. Rescuing the Morphogenetic Defect of *PrlR*^{−/−} Epithelium by Ectopic Expression of *IGF-2*

MECs derived from *PrlR*^{−/−}*ROSA26*^{+/−} female mice were infected with retroviruses expressing either the *PrlR* (C) or *IGF-2* (D,E,G, and I) or mock infected (B, F, and H) and subsequently engrafted into the cleared fat pads of 3-week-old females. The recipients were impregnated. At the end of pregnancy the engrafted glands and thoracic mammary gland were analyzed by X-gal staining and subsequent wholemount microscopy (top panels).

(A) Unmanipulated gland displays extensive alveolar development.

(B) Mock-infected *PrlR*^{−/−} epithelium devoid of alveolar structures.

(C) *PrlR*^{−/−} epithelium infected with *PrlR* retrovirus showing a sector with alveolar development within a ductal system devoid of alveolar structures. Note that the alveoli are as distended as in the wt control, indicating that the epithelium lining them displays secretory activity.

(D and E) *PrlR*^{−/−} epithelium infected with *IGF-2* retrovirus showing a sector containing alveolar structures within a ductal system devoid of alveoli (D), or showing more extensive alveolar sprouting (E). Note: outpouchings are not distended, suggesting that the epithelium lining them is not actively secreting.

(F–I) H&E stained histological sections of mock-infected (F and H) and *IGF-2*-infected (G and I) *PrlR*^{−/−} epithelium. Note the increase in MEC number caused by ectopic IGF-2 expression (G versus F) and the similarity of the outpouchings formed (G and I) to alveoli seen at mid pregnancy (Figure 4, bottom right panel).

Retrovirus-mediated expression of the PrlR in the *PrlR*^{−/−}*ROSA26*^{+/−} MECs enabled alveolar development to occur in discrete sectors of the reconstituted glands (Figure 3C). In contrast, engrafted *PrlR*^{−/−}*ROSA26*^{+/−} MECs that were not exposed to the PrlR retrovirus and used as a negative control in this experiment (Figure 3B) developed into a highly branched ductal tree that completely lacked alveoli, as shown previously (Brisken et al., 1999). Moreover, an unmanipulated thoracic gland of these recipient mice (Figure 3A) used as a positive control shows extensive alveolar development, typical of late-stage pregnancy. As anticipated, the alveoli in this unmanipulated gland are fully distended, indicating that the cells lining them are differentiated and actively secreting milk products. The alveolar structures whose formation has been rescued by the retroviral PrlR vector were as dilated as the alveoli formed in the thoracic gland of the host (Figure 3A), implying that these structures are secreting milk products. Thus, both the morphogenetic as well as the differentiative effects of Prl signaling could be rescued by ectopic expression of the wt PrlR in the previously *PrlR*^{−/−}MECs.

We next determined whether the defects of the *PrlR*^{−/−}MECs could be rescued with an IGF-2-expressing retrovirus vector. As shown in Figures 3D and 3E, the ectopic expression of IGF-2 in *PrlR*^{−/−} MECs led to the development of multiple out-pouchings of the mammary ductal tree. Histological analysis of *PrlR*^{−/−} grafts (Figures 3F and 3H) and *PrlR*^{−/−} epithelia ectopically expressing IGF-2 (Figures 3G and 3I) further illustrate the increase in epithelial cell number caused by IGF-2 and show that the resulting structures closely resemble normal alveoli at mid-pregnancy (see Figure 4, right bottom panel). However, in contrast to the alveolar structures seen in the mutant epithelium rescued by the PrlR, these out-pouchings of the mammary ductal tree failed to expand, indicating that the cells lining the alveoli were not actively secreting milk products and were thus not terminally differentiated. We concluded that IGF-2 can indeed function as an important downstream mediator of Prl's morphogenetic effects, whereas it does not make any discernible contribution to the differentiation of MECs leading to milk production and secretory activity.

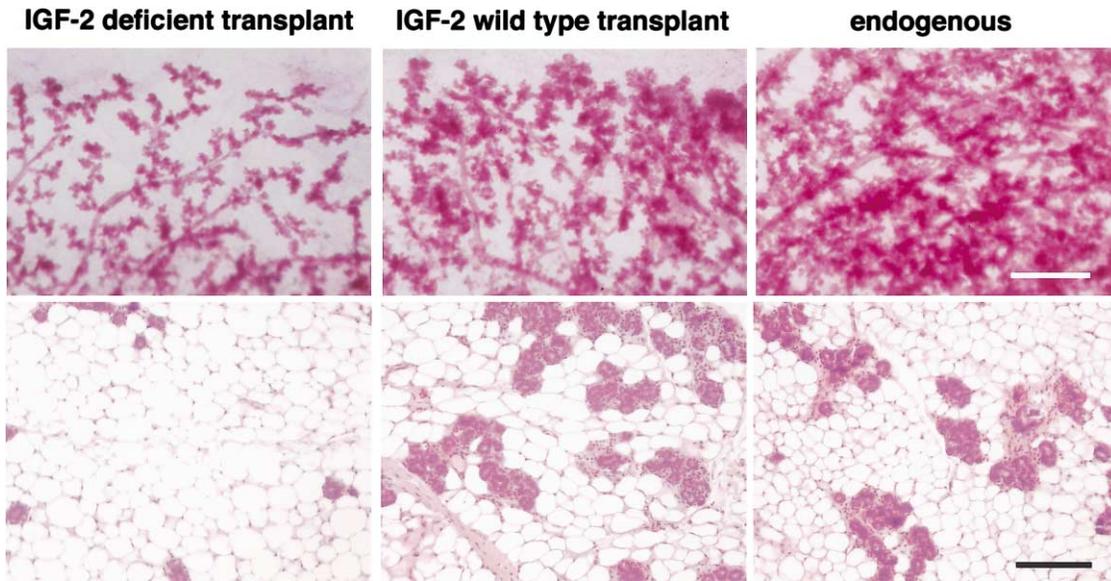When wt MECs infected with the IGF-2 retrovirus were used to reconstitute cleared fat pads of wt hosts, no

IGF-2 deficient transplant　　**IGF-2 wild type transplant**　　**endogenous**

Figure 4. Function of *IGF-2* in the Mammary Epithelium in Mid/Late Pregnancy.

Mammary epithelium from *IGF-2*$^{+/-}$ and *IGF-2*$^{+/+}$ mice was engrafted to the cleared inguinal fat pads of 3-week-old F$_1$ (129SV/C57Bl6) females. Eight weeks after surgery, the recipients were impregnated and their mammary glands were harvested at day 14.5 and analyzed by wholemount preparation (top panels) and subsequent histological sectioning and H & E staining (bottom panels). Mutant graft (left panels) shows less alveolar development than contralateral wt graft (central panels) and an unmanipulated gland of the recipient (right panels). The result is representative of ten pairs of grafts analyzed at this stage of pregnancy. Scale bar: top panels, 1 mm; bottom panels, 100 μm.

difference to the endogenous glands was noticed upon wholemount analysis (data not shown) in over 20 such grafts. This indicates that constitutive expression of IGF-2 itself is not sufficient to induce alveologenesis but does so only in the context of other changes induced by pregnancy.

Role of Endogenous IGF-2 Expression in Mammary Gland Development

To further assess the functional role of endogenous *IGF-2* expression in the mammary epithelium, we determined whether the absence of *IGF-2* expression in MECs would also impair development of the mammary epithelium. To address this question, we procured mice that carried an inactivated germline allele of *IGF-2* (DeChiara et al., 1990). The *IGF-2* gene is paternally imprinted in mammals and, as expected, females that inherit the mutant allele from their father show no expression of IGF-2 in their mammary tissue (data not shown), an absence that had previously been reported in other tissues (DeChiara et al., 1990, 1991). The IGF-2-depleted animals are initially growth retarded but breed normally, and the mutant females nurse their pups (DeChiara et al., 1990, 1991), indicating that mammary gland development is not severely impaired in the absence of IGF-2 expression. Nonetheless, it remained possible that the absence of IGF-2 significantly affected mammary epithelial morphogenesis during pregnancy.

To assess this possibility, we transplanted IGF-2-depleted and wt MECs into cleared contralateral fat pads of wt hosts and assessed alveologenesis during a subsequent pregnancy. A series of three experiments, comprising a total of ten pairs of grafts, revealed that the IGF-2 mutant epithelium showed consistently less alveolar development than the wt counterparts at day 14.5 of pregnancy as assessed by wholemount microscopy (Figure 4, top panels) and histological analysis (Figure 4, bottom panels). However, by the end of pregnancy, the contralateral transplants were indistinguishable (data not shown). Hence, the absence of IGF-2 compromises the ability of Prl to induce alveologenesis in mid-pregnancy, but it appears that other factors subsequently elicited by Prl compensate for this defect in the later stages of pregnancy.

Localization of PrlR and IGF-2 mRNA Expression in the Mammary Epithelium

To gain further insight into the connection between Prl signaling and IGF-2 synthesis, we assessed the localization of *PrlR* and *IGF-2* mRNA expression by in situ hybridization of mammary glands at different developmental stages. As shown in Figure 5, in the virgin female there was substantial expression of the *PrlR* mRNA in the luminal compartment of the mammary epithelium. Expression of *IGF-2* mRNA was barely detectable at this stage. In contrast, at day 12.5 of pregnancy, the *IGF-2* mRNA-related signal was intense and was colocalized with the *PrlR* mRNA signal in the luminal layer of MECs in the mammary epithelium. At day 16.5 of pregnancy, both mRNAs were found in the newly formed alveolar structures. We concluded that the localization of these two transcripts is consistent with a mechanism in which IGF-2 expression is induced by Prl stimulation of the same or nearby MECs.

Regulation of IGF-2 Expression by Prolactin

To test whether IGF-2 expression is indeed controlled by Prl, primary wt MECs in culture were stimulated with
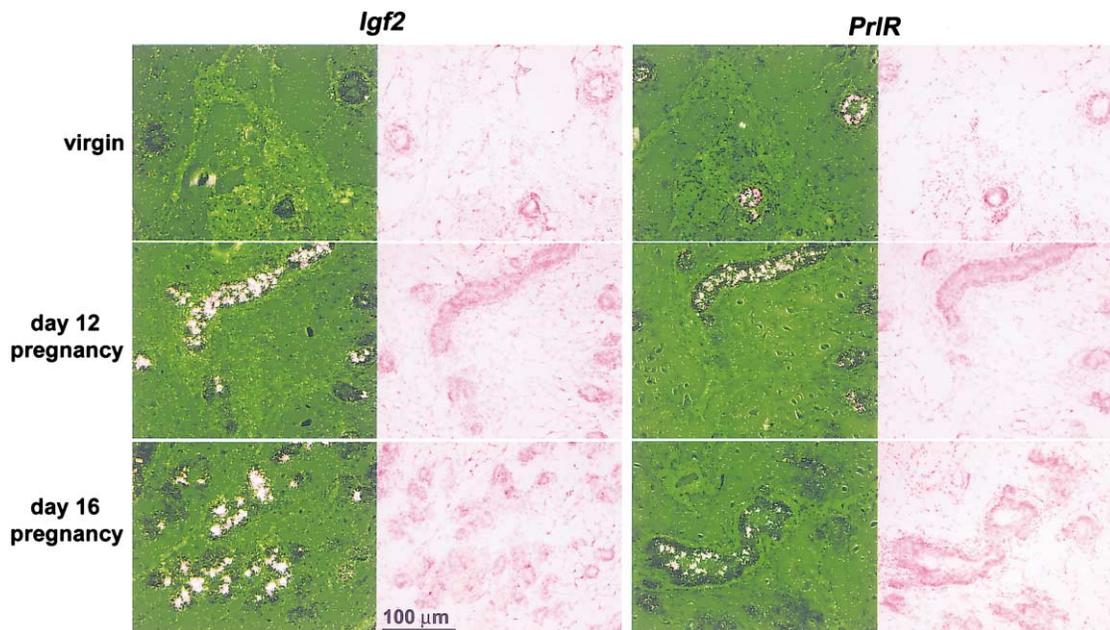
Figure 5. Coexpression of *PrlR* and *IGF-2* mRNAs in the Luminal Compartment of the Mammary Epithelium

Mammary glands were harvested from a virgin adult female mouse and from mice at days 12.5 and 16.5 of pregnancy. The glands were processed for *in situ* hybridization; adjacent sections were hybridized with ^{35}S-labeled antisense cRNA probes for *PrlR* or *IGF-2* and exposed for 6 days. H&E stained sections are shown next to the corresponding dark-field exposures. Both *PrlR* and *IGF-2* mRNA expression localizes to the luminal epithelium. Light green areas represent the dense fibrous stroma surrounding the ducts. Scale bar: 150 μm.

Prl, and IGF-2 mRNA expression levels were measured at various time points by real-time PCR. IGF-2 mRNA levels increased 2-fold within 2 hr of Prl exposure, 15-fold at 16 hr, and were 30-fold elevated by 72 hr (Figure 6, top). By contrast, expression of RANKL mRNA remained constant. To test whether the induction is specific to Prl, we analyzed IGF-2 and RANKL levels in progesterone-stimulated mammary glands. RANKL was induced 80-fold, whereas IGF-2 expression remained unchanged (Figure 6, bottom). Thus, Prl can specifically induce IGF-2 expression in MECs through a direct mechanism not involving other cell types present in the mammary gland. In the same cells, RANKL expression is positively regulated by progesterone rather than Prl signaling.

Discussion

Although the role of hormones in breast carcinogenesis is widely recognized, in vitro studies are limited to breast carcinoma cell lines (Brockman et al., 2002; Das and Vonderhaar, 1996; Schroeder et al., 2002), and the precise mechanisms by which hormones control proliferation and differentiation of MECs in vivo remain largely unknown. Here, we identify IGF-2 as a downstream mediator of PrlR morphogenetic signaling and an upstream regulator of cyclin D1 expression. This links Prl signaling, IGF-2, and cyclin D1 expression, all of which are deregulated during breast carcinogenesis in humans, to a common developmental signaling pathway, i.e., Prl → IGF-2 → cyclin D1.

Our data have revealed that, unexpectedly, Prl does not act directly on intracellular mitogenic regulatory pathways but triggers instead proliferation through a locally produced, secreted factor that appears to operate in a paracrine manner to encourage MEC proliferation. These findings suggest a model in which the Prl-mediated induction of IGF-2 in PrlR-positive MECs provides an amplification of the initial Prl signal by broadcasting it via paracrine signaling to nearby MECs. This may serve to coordinate proliferation among the MECs participating in the formation of an alveolus and also may create a means for sustaining the Prl stimulus over an extended period of time, even when Prl levels have declined, as often occurs because of pulsatile secretion of this hormone (Horseman, 2001).

The closest relative of the PrlR—the growth hormone receptor—responds to ligand by inducing IGF-1 secretion, and the latter serves to mediate some of its downstream effects (Cohen and Rosenfeld, 1996). This creates a striking parallel with the presently observed behavior of the PrlR and IGF-2. It remains to be seen whether IGF-2 mediates Prl signaling functions in various organs throughout the body or whether its role is limited to the mammary gland.

IGF-1, IGF-2, several IGF binding proteins and the IGF-1R have all been shown to be expressed in the mammary gland (Wood et al., 2000). IGF-1 has been found to mediate the actions of growth hormone and to cooperate with estrogen to promote ductal development (Kleinberg, 1998). In accord with such a role, IGF-1R$^{-/-}$ MECs transplanted to the cleared mammary fat of a wt host shows impaired ductal outgrowth but a normal response to pregnancy hormones (Bonnette and Hadsell, 2001). Hence this pathway is involved in a distinct step of mammary morphogenesis that occurs before those that are regulated by Prl and progesterone.
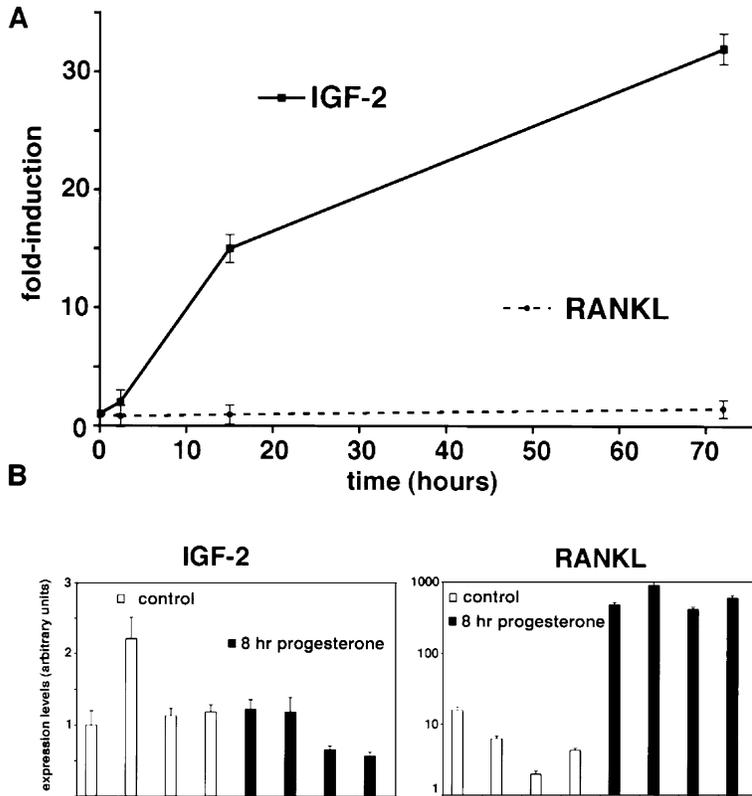
Figure 6. Induction of *IGF-2* mRNA Expression in MECs by Prolactin

MECs were stimulated with Prl (5 μg/ml) and RNA was harvested at different time points and reverse-transcribed. The cDNA was amplified with *Keratin 18*-, *IGF-2*-, and *RANKL*-specific primers and the products quantified by real time PCR. The graph shows relative *IGF-2* expression after normalization for *Keratin 18*, values are in triplicates, representative of four different experiments.

Eight 10-week old wt females were ovariectomized. After 20 days, they were injected with 200 ng estradiol. Twenty-four hours later, four of them were injected with 2 mg progesterone (P); the other four received vehicle only (control). Eight hours later, the mammary glands were harvested, and RNA was extracted and reverse-transcribed. The cDNA was amplified with *Keratin 18*-, *IGF-2*-, and RANKL-specific primers and the products quantified by real time PCR. Shown is relative *IGF-2* and *RANKL* expression after normalization for *Keratin 18*.

The signaling pathway elucidated here may play an important role in the pathogenesis of many breast cancers. Thus the anti-apoptotic/mitogenic signal provided by IGF-2 expression appears to be constitutively active in tumors expressing high levels of IGF-2 (McCann et al., 1996). When viewed in terms of its effects aiding tumor progression, this IGF-2 expression may obviate the need for deregulation of other components of this pathway, notably Prl itself. Indeed, IGF-2, its cousin IGF-1 (Cullen et al., 1992), and two receptors activated by both factors, the IGF-1R (Baserga et al., 1997; Surmacz et al., 1998) and the insulinR (A form) (Morrione et al., 1997), are all found to be overexpressed in certain breast carcinomas (Pandini et al., 1999; Sciacca et al., 1999).

The IGF-2 gene has five promoters, which are used in a tissue-specific (Gray et al., 1987) and developmentally regulated fashion (Schofield and Tate, 1987). A search of the regulatory upstream region with MatInspector V2.2/Transfac 4.0 (Quandt et al., 1995) revealed 44 consensus STAT binding sites, compatible with a model in which Prl induces IGF-2 transcription through STAT5 activation. However, we find that in mammary glands engrafted with STAT5ab$^{-/-}$ epithelia, IGF-2 levels are comparable to those seen in wt mammary glands. More generally, the functional significance of these particular IGF-2 promoter sites remains unclear, and the specific identities of the promoters used during breast development and tumorigenesis remain to be established. Our finding that the induction of IGF-2 by Prl goes from 2-fold during the first 10 hr to a 60-fold increase by 72 hr of Prl stimulation is compatible with different IGF-2 transcriptional promoters being activated with distinct kinetics.

We have shown that cyclin D1 is required specifically for Prl-induced morphogenesis of the ductal epithelium and that IGF-2 functions as an intermediary between the two, being capable of inducing cyclin D1 on its own. Whether the increase in cell number seen in the course of IGF-2 induced alveologenesis is due to increased proliferation, decreased apoptosis or both remains to be assessed. Significantly, studies comparing *IGF-2* mRNA expression with the pattern of BrdU-labeled cells during mid-pregnancy (Richert and Wood, 1999) suggest that IGF-2 may function as a mitogen for MECs. However, in the context of mammary epithelial involution occurring during weaning, ectopically expressed IGF-2 has been found to exert anti-apoptotic effects (Moorehead et al., 2001). The fact that constitutive IGF-2 expression shows a striking capacity to rescue the morphogenetic defect of PrlR$^{-/-}$ MECs without rescuing the ability of the mutant epithelium to produce milk is consistent with a model whereby Prl induces morphogenesis through IGF-2 and cyclin D1 expression and controls differentiation through a second, distinct downstream signaling pathway.

While constitutive expression of IGF-2 is sufficient to rescue the morphogenetic defect of PrlR$^{-/-}$ MECs, it is clearly not the only Prl-induced growth/survival factor that can do so. The retardation in alveologenesis seen in mid-pregnancy in IGF-2-depleted epithelia is reversed later in pregnancy, ostensibly by the compensatory actions of other, still-unknown factors. An attractive candidate here is HB-EGF, whose synthesis we find also to

be dependent on PrlR action, albeit to a lesser extent. The FGF signaling pathway has also been implicated to play an important role in alveologenesis (Jackson et al., 1997), and there is evidence that the Wnt pathway acting via cyclin D1 (Hsu et al., 2001; Imbert et al., 2001) may be involved. It is conceivable that certain genes encoding molecules linked to these regulatory pathways were missed in our screen, which surveyed an arbitrary subset of 6500 mouse genes.

Recently, RANK signaling, which is important to bone remodeling and calcium homeostasis, has been linked via IKKα to cyclin D1 expression in the mammary gland (Cao et al., 2001). Previously, RANKL, the ligand of the RANK receptor, was implicated as an important stimulator of mammary gland development including alveologenesis (Fata et al., 2000). Indeed, the RANK$^{-/-}$ mammary glands show a dilated ductal system and cystic pouches (Fata et al., 2000), suggesting that the RANK/RANKL signaling pathway is important for a step or steps in mammary gland development that are distinct from those initiated by Prl signaling. This separation of function is now directly indicated by the present findings that mammary glands engrafted with PrlR$^{-/-}$ or cyclin D1$^{-/-}$ MECs show the same levels of RANKL expression and that Prl signaling specifically induces IGF-2 expression in MECs while having no effects on RANKL. RANKL expression is linked by our work directly to progesterone action. Just as importantly, while IGF-2 treatment of MECs is sufficient to directly induce cyclin D1 expression, RANKL exposure has no such effect. We therefore conclude that RANKL is an important localized mediator of progesterone action and the morphogenetic steps executed by progesterone and RANKL are essential precursors to the subsequent morphogenetic steps that are triggered by Prl.

Experimental Procedures

Mice
Mice were of C57Bl6x129SV background and genotyped as described (Brisken et al., 1998; DeChiara et al., 1990; Ormandy et al., 1997; Sicinski et al., 1995). For stimulation with progesterone, 10-week-old female mice were ovariectomized. Twenty days later, they were injected s.c. for 10 days either with 1 μg of 17-β-estradiol or 1 mg of progesterone in sesame oil per day (Lydon et al., 1995).

Mammary reconstitutions, wholemounting, X-gal-, and immunostaining were as described (Brisken et al., 1998, 2000).

Cell Culture
Primary MECs were prepared from 10-week-old virgin female mice as described (Kittrell et al., 1992). For retroviral infection, 293T cells were transiently transfected using Fugene (Roche) with plasmid DNAs. Conditioned medium was removed after 24 and 48 hr and frozen and placed on MECs at day 3 of culture (Edwards, 1996).

Retroviral Construct
IGF-2 was amplified from mammary gland polyA RNA using the following primers: F GAATTCAAGATGGGGATCCCACATC, R GAATTCCTGATGGTTGCTGGACATC. The amplified cDNA was cloned into pCR-Blunt II-TOPO (Invitrogen), sequenced and subcloned into MSCV2.2 (Hawley et al., 1994).

Quantitative Real-Time PCR (rtPCR)
Quantitative rtPCR analysis was performed using the iCycler apparatus (Bio-Rad) and SYBR Green PCR Core Reagents system (Perkin-Elmer Applied Biosystems). Results were evaluated with ICYCLER IQ REAL TIME DETECTION SYSTEM SOFTWARE (Bio-Rad).

IGF-2 F, GTGCGGAGGGGAGCTTGTTGAC; IGF-2 R, GTGGGCGTCTTTGGGTGGTA; RANKL F TGTACTTTCGAGCGCAGATG; RANKL R, CCCACAATGTGTTGCAGTTC.

In Situ Hybridization
In situ hybridization was as described (Das et al., 1994). Frozen sections were hybridized with ^{35}S-labeled antisense or sense cRNA probes for PrlR or IGF-2 for 4 hr at 45°C and exposed for 6 days.

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