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The Janus Kinase 2 Is Required for Expression and Nuclear Accumulation of Cyclin D1 in Proliferating Mammary Epithelial Cells

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Using a conditional knockout approach, we previously demonstrated that the Janus kinase 2 (Jak2) is crucial for prolactin (PRL) signaling and normal mammary gland development. PRL is suggested to synchronously activate multiple signaling cascades that emerge on the PRL receptor (PRLR). This study demonstrates that Jak2 is essential for the activation of the signal transducer and activator of transcription 5 (Stat5) and expression of Cish (cytokine-inducible SH2-containing protein), a Stat5-responsive negative regulator of Jak/Stat signaling. However, Jak2 is dispensable for the PRL-induced activation of c-Src, focal adhesion kinase, and the MAPK pathway. Despite activation of these kinases that are commonly associated with proliferative responses, the ablation of Jak2 reduces the multiplication of immortalized mammary epithelial cells (MECs). Our studies show that signaling through Jak2 controls not only the transcriptional activation of the *Cyclin D1* gene, but, more importantly, it regulates the accumulation of the Cyclin D1 protein in the nucleus by altering the activity of signal transducers that mediate the phosphorylation and subsequent nuclear export of Cyclin D1. In particular, the levels of activated Akt (protein kinase B) and inactive glycogen synthase kinase-3β (*i.e.* a kinase that regulates the nuclear export and degradation of Cyclin D1) are reduced in MECs lacking Jak2. The proliferation of Jak2-deficient MECs can be rescued by expressing of a mutant form of Cyclin D1 that cannot be phosphorylated by glycogen synthase kinase-3β and therefore constitutively resides in the nucleus. Besides discriminating Jak2-dependent and Jak2-independent signaling events emerging from the PRLR, our observations provide a possible mechanism for phenotypic similarities between Cyclin D1 knockouts and females lacking individual members of the PRLR signaling cascade, in particular the PRLR, Jak2, and Stat5. (*Molecular Endocrinology* 21: 1877–1892, 2007)

POSTNATAL GROWTH AND differentiation of mammary epithelial cells (MECs) are controlled by the concerted action of hormones and local growth factors as well as their downstream effectors (1–3). Although many hormone receptors are expressed and activated by their corresponding ligands in MECs during all phases of mammogenesis, biologically relevant functions of these receptors and their downstream targets seem to be limited to specified epithelial subtypes that emerge during distinct developmental stages. Specifically, the peptide hormone prolactin (PRL) induces a basal activation of downstream targets in luminal epithelial cells within the ductal compartment of the mammary gland (4). However, genetically engineered mouse models deficient in PRL or the prolactin receptor (PRLR) (5, 6) provided experimental evidence that essential functions of PRL signaling are primarily restricted to alveolar progenitors that reside at the terminal end of mature mammary ducts. The numeric expansion and differentiation of these alveolar progenitors occur predominantly during pregnancy and lactation. Females lacking two alleles of the PRLR specifically in MECs exhibited normal morphogenesis of ducts but lack milk-producing alveoli (7, 8).

In a variety of PRL-responsive cell types, PRLR signaling is suggested to synchronously activate multiple signaling cascades such as the Janus kinase 2 (Jak2) and the signal transducer and activator of transcription 5 (Stat5) (9), the Ras-MAPK pathway (10, 11), Akt (protein kinase B), and the phospholipase C-protein kinase C pathway (12, 13). PRL binding to its receptor leads to receptor dimerization and autophosphorylation of the receptor-associated Jak2 (14). In addition, Jak2 phosphorylates the receptor, thereby creating docking sites for Src homology 2 (SH2)-domain pro-

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Abbreviations: ActB, β-Actin; Akt, protein kinase B; Cdkn2a, cyclin-dependent kinase inhibitor 2a; CIS1, cytokine-inducible SH2 domain-containing protein-1; Cish, cytokine-inducible SH2-containing protein; DAPI, 4′,6-diamidino-2-phenylindole; Fak, focal adhesion kinase; GSK-3β, glycogen synthase kinase-3β; Jak2, Janus kinase 2; MEC, mammary epithelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphatidylinositol 3-kinase; PRL, prolactin; PRLR, prolactin receptor; SH2, Src homology 2; Stat, signal transducer and activator of transcription; TBS, Tris-buffered saline.

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teins such as Stat5, Src, Fyn, and Tec. Subsequently, Jak2 phosphorylates both Stat5 forms (Stat5a and Stat5b), which triggers their conformational changes and the formation of transcriptionally active dimers. Stat5a and Stat5b homodimers or heterodimers translocate into the nucleus, in which they bind to specific DNA sequence motifs and activate the transcription of target genes. Phenotypic abnormalities observed in *Stat5a*$^{-/-}$ (15), *Stat5a/b*$^{-/-}$ (8, 16, 17), *PRL*$^{-/-}$ (5), and *PRL-R*$^{-/-}$ (6) mutant mammary glands are quite similar, suggesting that Stat5 is a key player in PRL signaling *in vivo*. The identification of target genes of PRLR signaling through Jak2 and Stat5 are subject of current investigations (18, 19). The expression of milk protein genes such as *Wap* (whey acidic protein) and *β-casein* clearly depends on the PRL-induced activation of Stat5a and Stat5b in differentiated alveolar cells *in vivo* (8, 15). Conversely, Jak2/Stat5 target genes that specifically mediate the proliferation of undifferentiated alveolar precursors in response to PRL signaling during pregnancy have not been intensively studied. Among regulators of the cell cycle, Cyclin D1 might be a target of PRL signaling because females deficient in Cyclin D1 exhibit impaired mammary gland development similar to Stat5 knockout mice (20, 21). The proposed underlying mechanisms that link biologically relevant functions of Stat5 and Cyclin D1 in the developing mammary gland are quite diverse. It has been suggested previously that Cyclin D1 expression is directly regulated by PRL signaling through the Stat5-mediated transcriptional activation of the *Cyclin D1* promoter (22, 23). Conversely, Brisken *et al.* (24) proposed that signaling through the PRLR has an indirect effect on Cyclin D1 expression through up-regulation of IGF-II. Thus far, microarray expression studies, however, did not provide experimental evidence that *Cyclin D1* is a direct or indirect transcriptional target of PRL signaling and Stat5 (18, 19).

The enzymatic coupling between the PRLR and Stat5 *in vivo* and biologically relevant functions of Jak2 in mammary epithelia were difficult to elucidate because of the prenatal lethality of Jak2 conventional knockouts (25, 26). To circumvent embryonic lethality, we recently generated conditional knockout mice that lack Jak2 specifically in MECs at defined developmental stages (27, 28). We demonstrated that the excision of the *Jak2* gene uncouples signaling from the PRLR to its downstream mediator Stat5 in the presence of normal and supraphysiological levels of PRL. Mutant females that lack *Jak2* throughout the mammary ductal compartment were unable to lactate as a result of impaired alveolar proliferation and differentiation. This observation suggests that other components of the PRLR signaling cascade or other growth factors and their signal transducers, which include other Jak family members, were unable to compensate for the loss of Jak2.

Despite a wealth of knowledge about PRL signaling, there are contradictory reports about the role of Jak2 for individual, PRL-induced signaling pathways (29, 30). A direct comparison of these conflicting reports is difficult because mechanistic aspects of PRLR signaling were studied in diverse cell culture models that either do not express the full-length PRLR or do not require PRLR signaling for normal growth and differentiation. In this report, we describe the generation of mammary epithelial cell lines conditionally deficient in Jak2 and their isogenic, Jak2-expressing controls to examine essential functions of Jak2 for the activation of individual signal-transduction pathways that emerge from the PRLR during ligand stimulation. We demonstrate that Jak2 is essential for the activation of Stat5 and expression of the cytokine inducible SH2-containing protein (Cish), a Stat5-responsive negative regulator of Jak/Stat signaling. However, Jak2 is dispensable for the PRL-induced activation of c-Src, focal adhesion kinase (Fak), and the MAPK pathway. Despite activation of these kinases that are commonly associated with proliferative responses, the ablation of Jak2 reduces the multiplication of immortalized MECs. Our studies show that signaling through Jak2 controls not only the expression of the *Cyclin D1* mRNA, but, more importantly, it regulates the accumulation of Cyclin D1 protein in the nucleus by inhibiting signal transducers that mediate the phosphorylation and nuclear export of Cyclin D1. The proliferation of Jak2-deficient MECs can be rescued by expressing a mutant form of Cyclin D1 that constitutively resides in the nucleus.

RESULTS

Generation of Isogenic Normal Mammary Epithelial Cell Lines with and without Jak2

Normal MECs undergo growth senescence *ex vivo* after a few doublings. Retroviral gene transfer and expression of the simian virus 40 large T or the human papillomavirus oncoproteins E6 and E7 are frequently being used in many laboratories to inactivate p53 and pRB and to generate immortalized cell lines. This methodology, however, targets multiple pocket proteins besides p53 and pRB. To circumvent this problem, we now use a targeted null mutation of the cyclin-dependent kinase inhibitor 2a (*Cdkn2a*) locus (31) to obtain immortalized primary cells directly from animal models generated in our laboratory. Through an elaborate breeding scheme, we generated females that carry two *Jak2* floxed alleles in a homozygous *Cdkn2a* knockout background (*Jak2*$^{fl/fl}$ *Cdkn2a*$^{-/-}$). We used a method by Medina and Kittrell (32) to isolate and culture MECs from midpregnant Jak2/Cdkn2a double-mutant females. We chose midpregnancy for the derivation of MECs because 1) the well-known COMMA-1D cell line and its derivatives (*e.g.* HC11 cells) were obtained at the equivalent stage of gestation (33, 34), and 2) alveolar progenitors that amplify significantly at this developmental stage are dependent on Jak2 *in vivo* (27, 35). *Jak2*$^{fl/fl}$ *Cdkn2a*$^{-/-}$ MECs adapted quickly to the culture conditions and did not

exhibit signs of early growth senescence. Over a course of approximately 6 months, we derived pure epithelial subcultures free of contaminating fibroblasts that exhibit a typical morphology of MECs and expression of cytokeratins (Fig. 1A).

To delete conditional knockout alleles from cultured cells, our laboratory constructed a retroviral vector expressing Cre (enterobacteria phage P1, cyclization recombinase) (pBabe-Cre-puro). We repeatedly demonstrated that the low-level expression of Cre from this retroviral construct is not toxic, and the coexpression of the puromycin selection marker is useful to achieve a near 100% recombination efficiency in culture (36–38). Immortal *Jak2*$^{fl/fl}$ MEC cultures were infected with pBabe-Cre-puro or the original pBabe-puro retroviral vector as a control. The selection of infected cells was performed in increasing concentrations of puromycin. Next, we determined the excision rate of *Jak2* using PCR, which is very sensitive to detect recombined but also unaltered (floxed) alleles (Fig. 1B). In addition, we performed a Jak2 immunoblot to verify the absence of the Jak2 protein in *Jak2*$^{-/-}$ MECs (Fig. 1C). The combined results of both studies confirmed that the Cre-mediated conversion of the *Jak2* floxed gene into a null allele was virtually complete in these immortalized MECs.

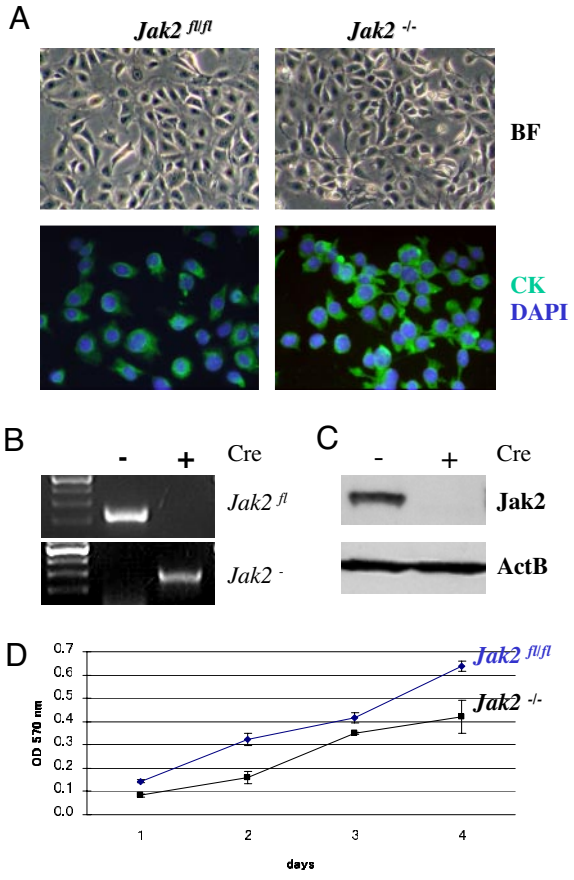

Fig. 1. Generation of Immortalized Mammary Epithelial Cell Lines Lacking Jak2

A, Phase-contrast bright-field (BF) image (*top*) and immunocytochemistry against pan-cytokeratin (CK; *bottom*) of immortalized MECs from Jak2 conditional knockout mice that were infected with a pBabe-puro retroviral control vector (*Jak2*$^{fl/fl}$) or a pBabe-Cre-puro construct to delete the *Jak2* gene (*Jak2*$^{-/-}$). Magnification, ×200. B, PCR assay to verify the loss of the *Jak2* floxed allele (*Jak2*^{fl}) and the presence of a *Jak2* recombined knockout allele (*Jak2*$^{-/-}$) in cells expressing Cre recombinase. C, Western blot analysis to confirm the ablation of the Jak2 protein in conditional knockout cells expressing Cre recombinase. ActB served as loading control. D, MTT color assay to determine growth rates of Jak2-deficient cells and their isogenic wild-type controls. The OD_{570nm} values (±SE) correspond to total number of cells within experimental groups. All time points were analyzed in triplicates.

Jak2 Deficiency Affects the Proliferative Capacity of Immortalized MECs

Immortalized *Jak2*$^{fl/fl}$ MECs and their Jak2-deficient derivatives lack p19/Arf and p16/Ink4a. These two negative growth regulators encoded by the *Cdkn2a* locus play an important role in the activity of the Cyclin D1-Cdk4/6 complex and the p53-mediated cell cycle checkpoint control (31, 39). Because entry into the S phase requires hormones and local growth factors, ablating the G_1/S checkpoint control could, at least in part, render Jak2-deficient cells less responsive to hormones and their downstream mediators. To experimentally address whether Jak2 deficiency has an effect on the proliferation of immortalized MECs, we grew Jak2-deficient cells and their controls in serum-rich media supplemented with epidermal growth factor and insulin and monitored their multiplication using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. A comparison of the growth curves illustrated in Fig. 1D demonstrated that cell proliferation was significantly reduced in Jak2-deficient cells despite the lack of p19/Arf and p16/Ink4a. The results of this study were also confirmed using live cell count over a period of 5 d (see Fig. 9D). Hence, like alveolar progenitors *in vivo* (27), immortalized MECs in culture require cytokine and hormone signaling through Jak2 for optimal numeric expansion.

Jak2 Is Dispensable for the PRL-Induced Activation of c-Src, Fak, and the MAPK Pathway

The p42/44 MAPKs are associated with cell proliferation in response to growth factor stimulation. These kinases also appear to be linked to PRL-induced proliferation of normal and neoplastic MECs [for a summary of the literature on this subject, see the recent review by Clevenger *et al.* (40)]. Because we observed that Jak2 deficiency affects the proliferative capacity of alveolar progenitor *in vivo* as well as MECs in culture, we decided to examine the activation of c-Src, Fak, and selected members of the MAPK signal transduction pathway in Jak2-deficient MECs and their isogenic Jak2 wild-type controls. As shown in Fig. 2A,

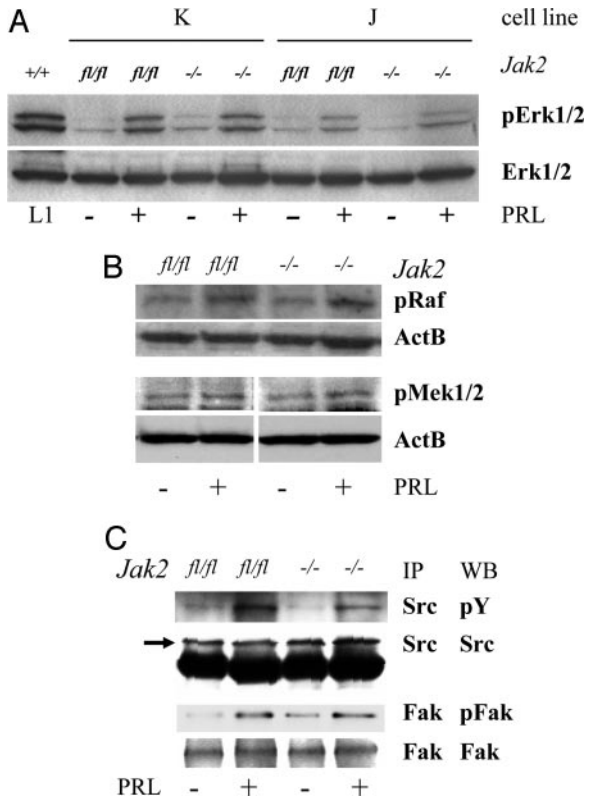

Fig. 2. Western Blot to Determine the PRL-Induced Activation of Members of the MAPK Signal Transduction Pathway (A and B) as well as c-Src and Fak (C) in Jak2-Deficient Cells and Their Isogenic Wild-Type Controls

Cells were treated for 20 min with 10 nM recombinant mouse PRL before analysis of the various signal transducers. IP, Immunoprecipitation; WB, Western blot; L1, first day of lactation.

PRL was clearly able to activate the MAPKs (*i.e.* phosphorylated ERK1/2) in two independent primary mammary epithelial cell lines. More importantly, Jak2 deficiency had no effect on the PRL-induced phosphorylation of the MAPKs. This important finding was subsequently verified *in vivo*. Like their wild-type controls, mammary gland-specific Jak2 conditional knockout mice (*MMTV-Cre Jak2*$^{f/lfl}$) exhibited an increase in phosphorylated ERK1/2 after administration of exogenous PRL (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). These observations suggested that PRLR-mediated signal transduction through the MAPKs occurred independently of Jak2 activation. Consistent with these results, other upstream kinases of this signaling cascade, in particular Raf (v-raf-leukemia viral oncogene 1) and MEK (MAPK kinase), exhibited a similar increase in phosphorylation after ligand stimulation in the presence or absence of Jak2 (Fig. 2B).

The c-Src tyrosine kinase plays an integral role in modulating growth factor receptor signaling and integrin function. The latter is regulated, in part, through alteration of the activity of Fak. It has been reported previously that c-Src can be phosphorylated by the PRLR in hepatocytes, transfected COS cells, and breast cancer cell lines (41–43). We can demonstrate that this mechanism of Src activation also occurs in untransformed, primary MECs treated with PRL (Fig. 2C, lanes 1 and 2). Like the MAPKs, the activation of c-Src remains inducible by PRL in the absence of Jak2 (Fig. 2C, lanes 3 and 4). This observation is consistent with findings of previous reports that express dominant-negative Jak2 or a mutant PRLR lacking the Jak2 binding site in COS cells or chicken fibroblasts (29, 42). Equivalent to c-Src, Fak could also be activated by PRLR signaling in a Jak2-independent manner in our isogenic mammary epithelial cell lines (Fig. 2C).

In summary, our studies on Jak2-deficient MECs show that the ligand-induced dimerization of the PRLR is able to activate c-Src, Fak, and members of the MAPK pathway in the absence of functional Jak2. Because the activation of these particular signal transducers is suggested to stimulate cell growth, we conclude that they do not play the predominant role in the reduction of the proliferation rate in response to Jak2 deficiency.

Jak2 Deficiency and Activation of Stat Proteins

The expression and activation of Stat proteins vary significantly during particular stages of mammary gland development (44). Gene targeting studies revealed that, among the seven Stat family members, only Stat5a and Stat3 are essential for mammary gland development (15, 45). As described previously, Stat5a is an important mediator of PRL signaling in MECs. PRL is also able to induce the activation of Stat3 in MCF7 and T47-D breast cancer cells (46). In contrast, normal PRL-responsive MECs exhibit little or no expression of activated Stat3 *in vivo* during pregnancy and lactation. Stat3 is significantly activated in secretory epithelial cells by proapoptotic signaling events during postlactational involution and remodeling (44, 45). Conversely, Xie *et al.* (47) recently suggested that Jak2 has an antiproliferative role in untransformed HC11 cells. The authors described that the suppression of Jak2 using dominant-negative or antisense constructs was associated with a constitutive activation of Stat3 and a hyperproliferative phenotype. In this report, we show that immortalized MECs lacking Jak2 exhibited a reduced proliferation rate compared with their Jak2-expressing isogenic controls (Fig. 1C) (see Fig. 9D). We also determined that Jak2 deficiency does not cause a constitutive activation of Stat3 as reported by Xie *et al.* (supplemental Fig. S2). Moreover, Stat3 is also not consistently activated by PRL in two different pairs of Jak2-deficient MECs and their isogenic wild-type controls. The marginally induced phosphorylation of Stat3 by PRL in one cell line was also independent of Jak2. In conclusion, differences in the activation of Stat3 are not responsible for the

reduced proliferation rate of Jak2-deficient MECs compared with their isogenic wild-type controls.

To address whether immortalized, Jak2-deficient MECs lose the PRL-inducible activation of Stat5, we treated serum-starved *Jak2*$^{-/-}$ MECs and their *Jak2*$^{fl/fl}$ isogenic controls for 20 min with mouse PRL and monitored the level of Stat5a tyrosine phosphorylation (Fig. 3A), the nuclear accumulation of Stat5 (Fig. 3B), and the binding of transcriptionally active Stat5 to DNA recognition sites (data not shown). The results of these experiments along with analyses of Stat5-responsive downstream genes (Fig. 4) consistently demonstrated that the presence of Jak2 is mandatory for the PRL-mediated phosphorylation of Stat5 and the activation of Stat5 downstream targets. In brief, Stat5 does not exhibit an increase in tyrosine phosphorylation in response to PRL stimulation in Jak2-deficient MECs (Fig. 3A, lane 7), and active Stat5 was not observed in the nuclei of serum-starved MECs or in PRL-induced Jak2-deficient cells using immunofluorescence staining (Fig. 3B). Tyrosine-phosphorylated Stat5 was detected only in control MECs expressing Jak2 when treated with PRL (Fig. 3A, lane 5), and phospho-Stat5 was predominantly nuclear in these cells (Fig. 3B). The lack of PRL-induced Stat5 activation in the absence of functional Jak2 was confirmed in HC11 cells treated with the Jak2 tyrosine kinase inhibitor AG490 (supplemental Fig. S4A). In summary, MECs that lack Jak2 lose their ability to activate Stat5 in response to PRL stimulation. The necessity of Jak2 for the activation of Stat5 is not altered by mutations associated with immortalization (*i.e.* loss of Cdkn2a) or *ex vivo* growth conditions (*i.e.* disruption of the three-dimensional epithelial architecture and lack of the stromal environment).

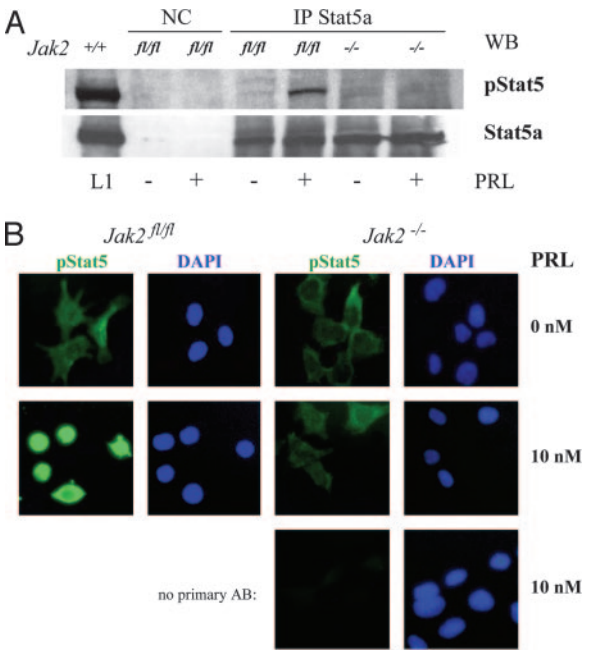

Fig. 3. PRL-Induced Activation of Stat5 in MECs Lacking Jak2 (*Jak2*$^{-/-}$) and Their Isogenic Wild-Type (*Jak2*$^{fl/fl}$) Controls

A, Immunoprecipitation (IP) and Western blot (WB) analysis of Stat5a, which is the predominant and physiologically relevant Stat5 isoform in MECs. Mammary gland tissue from the first day of lactation (L1) served as a positive control to assess the phosphorylation status of Stat5a. NC, Negative controls of the IP without primary Stat5a antibody. B, Immunofluorescence against the phosphorylated form of Stat5 to verify the correct nuclear localization of the activated form. For immunoprecipitation/Western blot and immunofluorescence, cells were treated for 20 min with 10 nM recombinant mouse PRL before analysis of the Stat5 activation. Note that Jak2 deficiency abolishes the PRL-induced phosphorylation of Stat5a (A, lane 7) and nuclear accumulation of active Stat5 (B, *bottom right*; magnification, ×400). AB, Antibody.

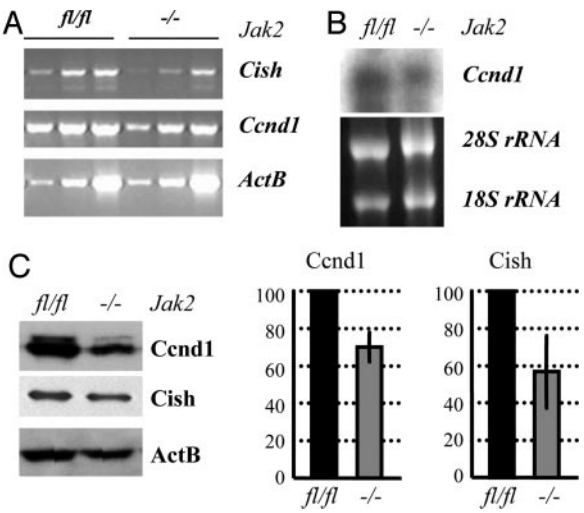

Fig. 4. Expression Analysis of Cish and Cyclin D1 (Ccnd1) in Proliferating MECs Lacking Jak2 (*Jak2*$^{-/-}$) and Their Isogenic Wild-Type Controls (*Jak2*$^{fl/fl}$)

ActB mRNA and protein serve as loading control in the PT-PCR and Western blot analyses. A, Semiquantitative RT-PCR analysis was performed using the same input of reverse-transcribed cDNAs but variable cycle numbers of PCR amplification. B, Northern blot analysis of *Cyclin D1* (*Ccnd1*) mRNA expression. The 18S and 28S ribosomal RNA serves as a control for equal loading and integrity of the RNA. C, Western blot analysis to verify the reduced expression Cish and Cyclin D1 proteins in Jak2-deficient cells compared with their isogenic wild-type controls. *Bar graphs* illustrate the relative expression levels of Cish and Cyclin D1 in three different cultures (*Jak2*$^{fl/fl}$ controls set as 100%; *error bars* represent the SE). The expression levels of both proteins were normalized against ActB. The analysis was performed on cells that were maintained in a growth factor-rich medium under subconfluent (growing) conditions.

Jak2 Deficiency Causes a Reduction in Cyclin D1 Protein Expression *in Vitro* and *in Vivo*

Initially, we determined the expression profile of more than 39,000 transcripts using Affymetrix (Santa Clara, CA) Mouse Genome 430 2.0 microarrays to identify Jak2/Stat5 target genes (Creamer B. A., K. Sakamoto, and K.-U. Wagner, unpublished data). In addition to

genes that are involved in cell growth and proliferation, our initial study focused on the expression levels of negative regulators of Jak/Stat signaling to demonstrate their dependence on PRL signaling. These target genes serve as internal controls to monitor the loss of Jak2 and Stat5 activation. The microarray study revealed that the mRNA messages for *Cish* [also known as CIS1 (cytokine-inducible SH2 domain-containing protein-1)] and *Cish2* were reduced approximately 50%. The down-regulated expression of *Cish* was verified by semiquantitative RT-PCR and Western blot analysis (Fig. 4, A and C) in proliferating MECs in culture and by immunohistochemistry *in vivo* (Fig. 5). Although the reduction in the Cish protein level was moderate in proliferating Jak2-deficient MECs compared with their controls (15–20%) (Fig. 4C), the differences in the Cish expression levels appeared to be much more substantial *in vivo* (Fig. 5) and in MECs cultured at confluency (reduction by 80–90%) (supplemental Fig. S3A). This suggests that the expression of Cish is controlled by other pathways in addition to Jak2/Stat5 signaling. In contrast to negative regulators of Jak/Stat signaling, the expression of genes involved in the control of the cell cycle varied considerably on the microarrays. The expression of *Cyclin D1*, for example, was consistently lower in the Jak2-deficient MECs, but the differences were statistically insignificant. Because microarray-based expression studies often underestimate the level of fold change, we reexamined the expression of *Cyclin D1* (*Ccnd1*) mRNA using semiquantitative RT-PCR (Fig. 4A) and Northern blot analysis (Fig. 4B). The results of both assays demonstrated that signaling through Jak2 is required for maximal transcriptional activation of the *Cyclin D1* gene in proliferating MECs. Our results are consistent with recent reports that demonstrated that Stat5 is able to recognize γ-interferon activation sites within *Cyclin D1* regulatory elements and that PRL stimulation leads to an increase in the expression of a reporter gene under regulation of the *Cyclin D1* promoter (22, 23). However, the difference in the expression of Cyclin D1 was more pronounced on the level of the protein (Fig. 4C), suggesting that signaling through Jak2 controls not only the expression of the *Cyclin D1*

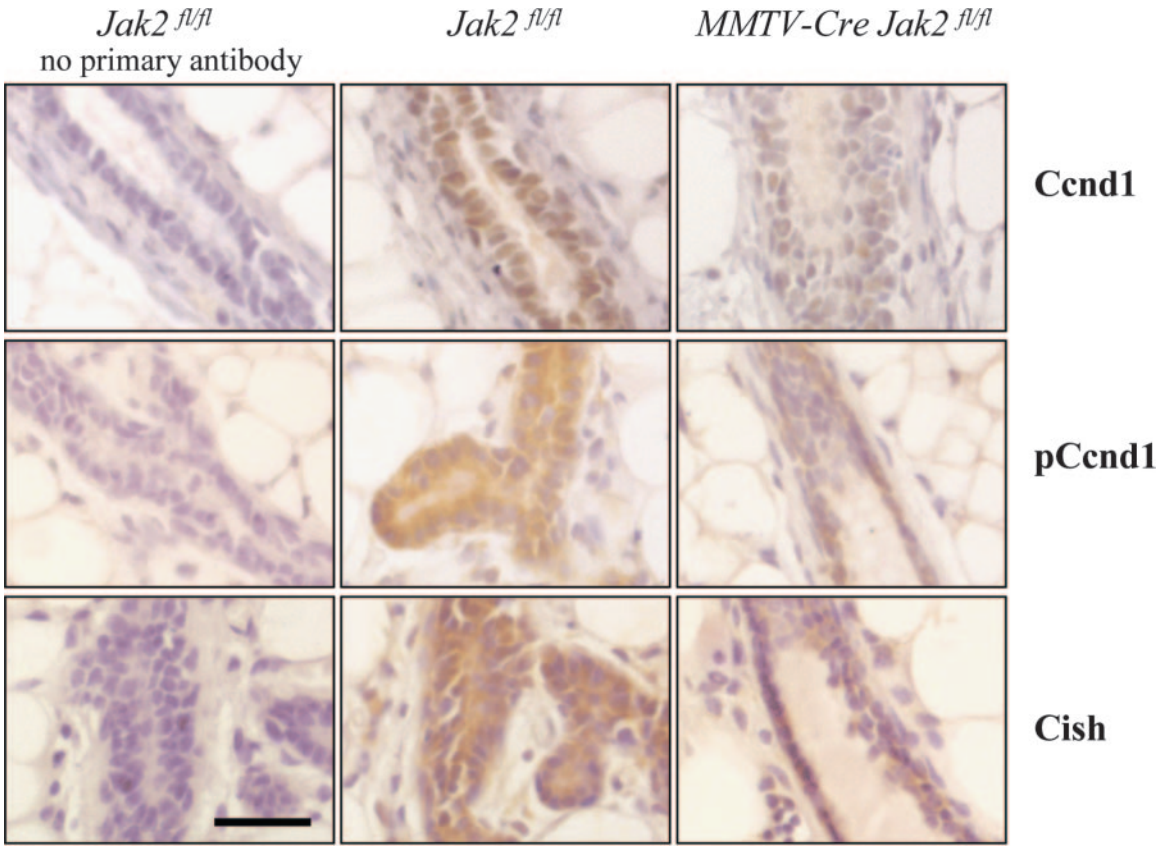

Fig. 5. Immunohistochemistry to Monitor the Expression of Cish as well as Cyclin D1 and Its Phosphorylated Form in Mammary Epithelia of Jak2 Conditional Knockout (*MMTV-Cre Jak2*$^{fl/fl}$) Females and Wild-Type (*Jak2*$^{fl/fl}$) Littermate Controls

Mammary gland specimens were retrieved from females at d 11.5 of gestation (Cish) or nulliparous females that were treated with estrogen and progesterone for 48 h to synchronize the stage of estrus cycle and stimulate the proliferation of undifferentiated alveolar precursors (Cyclin D1). Note that high levels of Cyclin D1 expression are confined to the nucleus of wild-type MECs and that the phosphorylated form is almost exclusively present in the cytoplasm of epithelial cells. Like Cyclin D1, the expression of Cish is confined to the mammary epithelial compartment of wild-type mice and is significantly reduced in the mammary gland of Jak2-deficient females. *Scale bar*, 30 μm.

gene but also the translation of the message and post-translational modification (see next paragraph). This assumption is supported by the fact that the difference in Cyclin D1 protein expression becomes even more evident in cells that reach confluency (supplemental Fig. S3A). In the confluent state, the mRNA message of the *Cyclin D1* gene is equal but barely detectable by Northern blot analysis in both Jak2-deficient cells and their controls (data not shown). Next, we analyzed the expression of Cyclin D1 in the mammary gland of tissue-specific knockout mice (Fig. 5) to verify that the down-regulated expression of this pivotal cell cycle regulator is not a cell culture phenomenon. Unlike in *Jak2*$^{fl/fl}$ littermate controls, the expression of Cyclin D1 protein in mammary ducts of tissue-specific knockout females (*MMTV-Cre Jak2*$^{fl/fl}$) was markedly reduced. This was the case for active Cyclin D1 (Fig. 5, *top*), which accumulates in the nucleus, as well as its phosphorylated form, which mainly resides in the cytoplasm (Fig. 5, *middle*). Finally, the reduction of Cyclin D1 protein as a consequence of impaired Jak2 function was confirmed in HC11 cells treated with the Jak2 tyrosine kinase inhibitor (supplemental Fig. S4B). In conclusion, the pharmacological inhibition of the Jak2 as well as the conditional knockout of the *Jak2* gene in cultured MECs and in the mammary glands of adult females resulted in a significant reduction in Cyclin D1 protein levels as well as Cyclin D1 targets (supplemental Fig. S5), suggesting that the expression of Cyclin D1 is controlled by Jak2-dependent, cell-autonomous mechanisms.

Expression of Activated Akt and Akt-Mediated Inhibition of Glycogen Synthase Kinase-3β (GSK-3β) Are Decreased in Jak2-Deficient MECs

To investigate the expression of Cyclin D1 protein in response to PRLR signaling, we starved Jak2-deficient cells and their wild-type controls overnight and treated them with PRL for 0–24 h. We observed a dramatic difference in Cyclin D1 protein expression between both experimental groups (Fig. 6A). The Cyclin D1 protein appeared to be up-regulated in the control cells after 8 h of stimulation with PRL, and Cyclin D1 was significantly reduced in the Jak2-deficient cells regardless of PRL administration. This observation suggests that Jak2 is essential to maintain a high steady-state level of Cyclin D1. In addition, we examined the levels of the Thr-286 phosphorylated form of Cyclin D1, which has been shown to be targeted for nuclear export and proteosomal degradation (48, 49). Similar to total Cyclin D1, the level of the phosphorylated form is significantly lower in the Jak2 knockout cells compared with the wild-type controls (Fig. 6A). This observation is in line with the immunohistochemical analysis of phosphorylated Cyclin D1 in the mammary gland of Jak2 conditional knockout mice (Fig. 5). We also noticed that, after 4 h of treatment, PRL causes temporarily a modest reduction in the amount of Cyclin D1 protein and a slightly larger

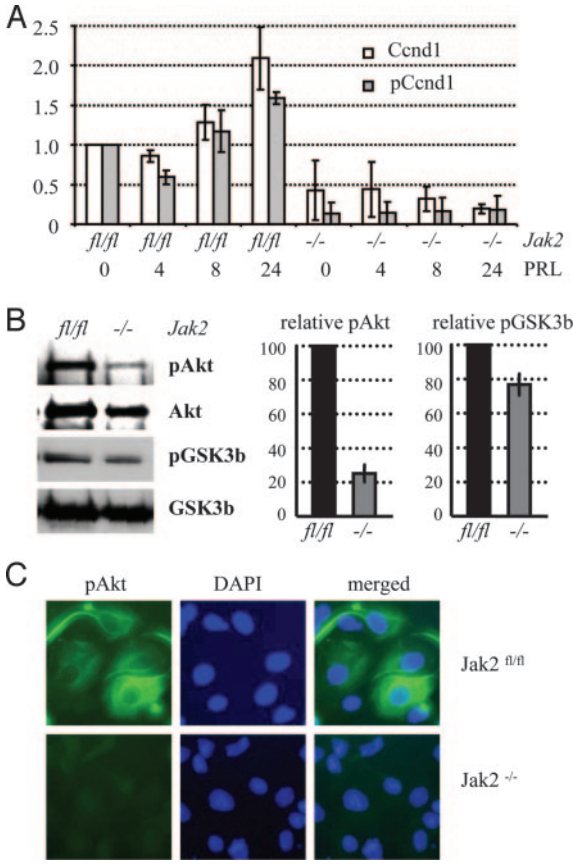

Fig. 6. Expression of Cyclin D1, Akt, and GSK-3β in Proliferating MECs Lacking Jak2 (*Jak2*$^{-/-}$) and Their Isogenic Wild-Type Controls (*Jak2*$^{fl/fl}$)

A, PRL-induced expression of the Cyclin D1 protein and its phosphorylated form. After withdrawal of all growth factors for 16 h, cells were treated with 10 nM mouse PRL for a period of 0–24 h. *Bar graphs* illustrate the relative expression levels of Cyclin D1 and phospho-Cyclin D1 from three Western blots. The expression of both forms was normalized to the expression of ActB in each Western blot. The relative levels of Cyclin D1 and phospho-Cyclin D1 were calculated based on the protein levels in untreated *Jak2*$^{fl/fl}$ control cells (set as 1.0). *Error bars* represent the SE. B, Expression analysis of total and phosphorylated (active) Akt as well as total and phosphorylated (inactive) GSK-3β in Jak2-deficient MECs and their wild-type controls. *Bar graphs* illustrate the relative expression levels ($\pm$ SE) of active Akt and inactive GSK-3β in three different cultures (*Jak2*$^{fl/fl}$ controls set as 100%). The expression levels of pAkt and pGSK-3β were both normalized against their corresponding unphosphorylated forms (*i.e.* total Akt and GSK-3β). C, Immunofluorescence against the phosphorylated form of Akt in MECs lacking Jak2 (*Jak2*$^{-/-}$) and their isogenic wild-type controls (*Jak2*$^{fl/fl}$). Nuclei were counterstained with DAPI (magnification, $\times$400). Cells were maintained in growth factor-rich media under subconfluent (growing) conditions.

decrease in the amount of phospho-Cyclin D1 in the wild-type cells. The latter form is known to reside in the cytoplasm. In support of this finding, we also observed that relatively more Cyclin D1 resided in the cell nu-

cleus at this time point as determined by immunofluorescence (see next section and Fig. 8).

Diehl *et al.* (50) previously suggested that the phosphorylation and proteolytic turnover of Cyclin D1 and its subcellular localization are linked through the action of the GSK-3β. GSK-3β is able to phosphorylate Cyclin D1 specifically on the Thr-286 residue, thereby triggering the nuclear export and degradation of Cyclin D1 in the proteosome. GSK-3β is a constitutively active kinase that can be phosphorylated and inhibited by cytokine stimulation through Akt. It has been demonstrated previously that PRL can activate Akt in a murine lymphoid cell line expressing the PRLR (51), and it has been suggested that PRLR signaling may activate Akt in a variety of breast cancer cell lines (52) (Monica Richert and Steven M. Anderson, personal communication). Conversely, PRL alone had only a minimal effect on the activity of Akt in HC11 cells (53). Regardless of the Jak2 expression status, PRL was unable to induce the phosphorylation of Akt in our primary MEC cultures (data not shown), suggesting that, as in HC11 cells, PRLR signaling alone is not sufficient to activate Akt and that a robust activation of Akt may require additional growth factors in untransformed MECs. Therefore, we examined the expression and phosphorylation of Akt and GSK-3β in MEC cultures that were maintained in a growth factor-rich medium (Fig. 6, B and C). The results of the Western blot analysis (Fig. 6B) and the immunofluorescence studies (Fig. 6C) showed that the protein expression of Akt, in particular its activated form, was significantly reduced in the Jak2 knockout cells compared with their wild-type controls. This finding was confirmed in HC11 cells that were treated with the small-molecule inhibitor against Jak2 (supplemental Fig. S4B). This observation suggests that the expression and activity of Akt is regulated by Jak2-dependent mechanisms in MECs. Likewise, the expression of the inactive form of GSK-3β was reduced in cells lacking Jak2. Although the relative expression level of inactive (*i.e.* phosphorylated) GSK-3β was decreased by only 20% in proliferating Jak2-deficienct cells compared with their controls, this difference in pGSK-3β expression was more pronounced (60–70% reduction) when both cell lines reached confluency (supplemental Fig. S3B). This finding implies that signaling mechanisms triggered by cell density cooperate with Jak2 and Akt in the regulation of the activity of GSK-3β. In summary, Jak2 deficiency results in a decline of active Akt and, in turn, in an increase in the relative amount of active GSK-3β. An increase in the amount of active GSK-3β, which is unmodified by Akt, is able to negatively regulate Cyclin D1 protein levels though phosphorylation, which, as Diehl *et al.* (50) have shown previously, is the signal for nuclear export and proteosomal degradation.

In MECs, the activity of Akt is regulated by the phosphatidylinositol 3-kinase (PI3K) in response to the activation of receptors that possess intrinsic or associated tyrosine kinase activity such as the PRLR (40). It has been proposed that the PI3K can be activated by PRL through multiple signal transducers, including Src family kinases and Ras (54, 55). In addition, the regulatory subunit of the PI3K [*i.e.* p85 (regulatory subunit of the PI3K)] has been shown to associate with Stats and adaptors of cytokine and growth factor receptors such as IRS1 (insulin receptor substrate 1), Gab1 and Gab2 (growth factor receptor bound protein 2-associated proteins 1 and 2), and SHP-2 (SH2-containing protein tyrosine phosphatase-2). In this report, we demonstrated that the PRLR is able to activate c-Src, Fak, and MAPKs in response to ligand stimulation in Jak2-deficient MECs. Hence, it is less likely that impaired activation of these signal transducers is the underlying cause for the significant differences in Akt activation through PI3K in the Jak2 knockout model. Although Stat3 has been shown to associate with the PI3K (56), we can also exclude this signal transducer from the study because we demonstrated that Stat3 is not activated by PRL in a Jak2-dependent manner in our MEC cultures. Recently published studies suggested that active Stat5 is able to bind to the regulatory subunit of the PI3K in hematopoietic cells (57). We therefore asked whether this interaction also occurs in MECs and in a PRL-inducible manner that requires Jak2. First, we performed reciprocal coimmunoprecipitation with antibodies against Stat5a and p85 on a mammary gland tissue lysate from a postpartum female. The results shown in Fig. 7A indicate that tyrosine-phosphorylated Stat5 was indeed able to bind to p85 *in vivo*. Conversely, the association between p85 and active Stat5a was very weak in PRL-stimulated, Jak2-expressing cultured MECs (Fig. 7B), which may be attributable to 1) relatively low levels of phospo-Stat5 in cultured cells compared with actual mammary gland tissue, or 2) the absence of an adaptor protein, which is modified by a growth factor receptor other than the PRLR that is not active in cultured cells treated only with PRL. Although administration of PRL resulted in a marginal but detectable increase in p85/Stat5a interaction in MECs expressing Jak2, there was absolutely no change in the levels of coprecipitated p85 between untreated and PRL-stimulated Jak2-deficient MECs, suggesting that Jak2 may function as a facilitator for the PI3K/Stat5 interaction, probably by activating Stat5 or by acting as a scaffold as part of a larger protein complex. The reverse coimmunoprecipitation with an antibody against p85 gave similar results (data not shown). The interaction between both signal transducers was also very weak in PRL-treated HC11 cells and undetectable in the same ligand-stimulated cells treated with the Jak2 inhibitor AG490 (data not shown). In summary, the observation that active Stat5 is able to interact with p85 in the mammary gland *in vivo* opens up the possibility that phospho-Stat5 may control cellular processes independently of its classical nuclear function(s). Because the tyrosine phosphorylation of Stat5 requires Jak2, it is likely that such a process is mediated by the PRL-induced activation of this Janus kinase. However, because the association between

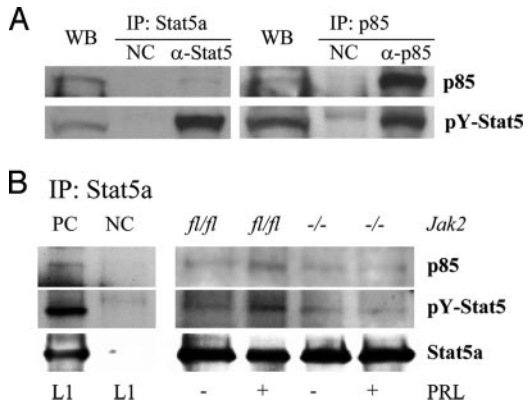

Fig. 7. Interaction of PI3K and Stat5 *in Vivo* and *in Vitro*

A, Reciprocal coimmunoprecipitation with antibodies against Stat5a and p85 on a mammary gland tissue lysate from a postpartum female. NC, Negative control for the coimmunoprecipitation without primary antibody; WB, whole-cell lysate as positive control for the immunoblot; IP, immunoprecipitation. B, Lack of PRL-inducible interaction between active Stat5 and p85 (PI3K) in Jak2-deficient MECs. Jak2-deficient MECs and their wild-type controls were treated for 20 min with 10 nM recombinant mouse PRL before being harvested. Total Stat5a was immunoprecipitated (IP) from lysates of PRL-treated cells and untreated controls. A lysate of mammary tissue from a postpartum female [lactation day 1 (L1)] was used as a positive control (PC) for the immunoblot. The identical sample without the primary Stat5a antibody was used as a negative control (NC) to assess the specificity of p85 interaction with Stat5. The immunoblot was probed with an antibody that recognizes the regulatory subunit of the PI3K (p85). Subsequently, the membrane was reprobed with antibodies against activated Stat5 (pY-Stat5) and total Stat5a (IP loading control).

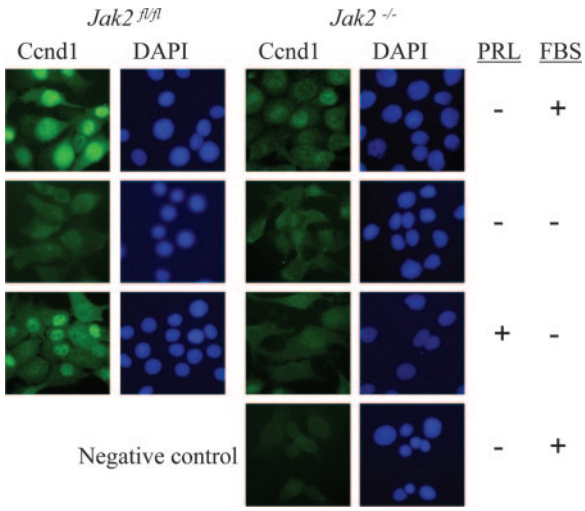

Fig. 8. Subcellular Localization of Cyclin D1 in MECs Lacking Jak2 (*Jak2*$^{-/-}$) and Their Isogenic Wild-Type Controls (*Jak2*$^{fl/fl}$)

Cells were maintained in a growth factor-rich medium [2% fetal bovine serum (FBS) supplemented with epidermal growth factor and insulin] or a growth factor-deprived medium. Additionally, MECs maintained in the absence of growth factors were stimulated for 4 h with 10 nM mouse PRL.

Stat5 and PI3K was very weak *in vitro*, it is unlikely that the lack of this weak interaction is the sole underlying mechanism for the significantly reduced activation of Akt in our Jak2-deficient culture model. It is therefore more plausible that Jak2 controls the activity of PI3K, probably through the suggested modification of its regulatory subunit p85 (58). At this point, we also cannot exclude the possibility that Jak2 could function as an essential scaffold for the suggested activation of PI3K by c-Src as mentioned above.

PRLR Signaling through Jak2 Regulates the Nuclear Localization of Cyclin D1 in Proliferating MECs

Because Jak2 is required for optimal expression of active Akt and the inhibition of GSK-3β, it was reasonable to closely investigate the direct response of PRLR signaling on the nuclear localization of Cyclin D1. Cyclin D1 was predominantly nuclear in Jak2-expressing MECs grown in a growth factor-rich medium (Fig. 8). Nuclear Cyclin D1 was also observed, albeit at much lower levels, in Jak2-deficient MECs. The withdrawal of all growth factors for 16 h resulted in mostly cytoplasmic expression of Cyclin D1 independent of the Jak2 expression status. Nuclear accumulation of Cyclin D1 was observed in wild-type cells that were treated for 4 h with PRL alone. In contrast, Cyclin D1 remained cytoplasmic and was detected only in a small subset of cells lacking Jak2, suggesting that, in proliferating MECs, PRLR signaling through the Jak/Stat pathway is important for nuclear accumulation of Cyclin D1.

Expression of a Constitutively Nuclear Form of Cyclin D1 Increases the Proliferation of Jak2-Deficient MECs

To verify whether nuclear accumulation of Cyclin D1 is able to increase the proliferation of Jak2-deficent MECs, we infected Jak2 knockout epithelial cells with a retroviral vector expressing the T286A mutant form of Cyclin D1. Because we suggested that the nuclear accumulation of Cyclin D1 is regulated by GSK-3β in a Jak2-dependent manner, we reasoned that it might be necessary to express this particular mutant form of Cyclin D1 that, according to Diehl *et al.* (48, 50), cannot be phosphorylated by GSK-3β and exported from the nucleus. After retroviral transfer and selection of cells with hygromycin, we verified by PCR that Jak2 knockout cells and their Cyclin D1 (T286A)-expressing *Jak2*$^{-/-}$ derivatives were both lacking the *Jak2* gene (Fig. 9A). Next, we determined the expression levels of Cyclin D1 and its phosphorylated form in wild-type MECs (*Jak2*$^{fl/fl}$), Jak2-deficient MECs (*Jak2*$^{-/-}$), and Jak2-deficient MECs transfected with the Cyclin D1-T286A construct (Fig. 9B). All three cell lines were maintained in a growth factor-rich medium. The West-

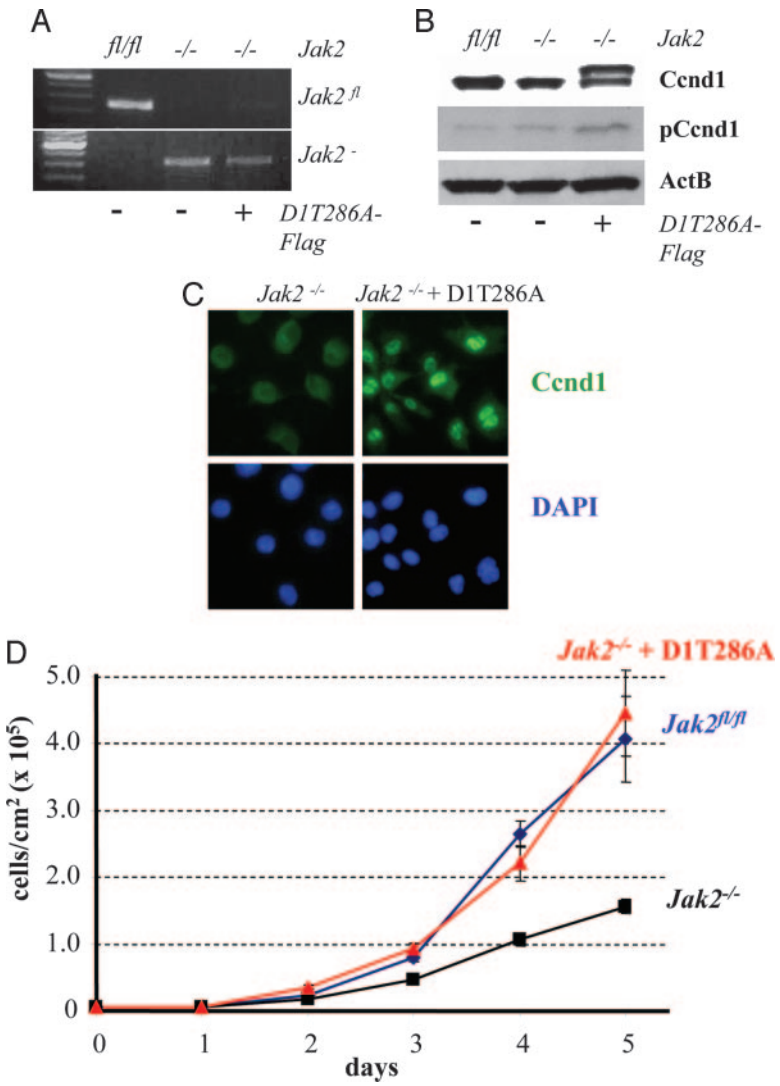

Fig. 9. Expression of a Constitutively Nuclear Cyclin D1 (Ccnd1-T286A) Increases the Proliferation of Jak2-Deficient MECs

A, PCR assay to verify the complete absence of wild-type Jak2 in MECs transfected with a Cyclin D1-T286A expression construct. B, Western blot analysis to compare the expression levels of Cyclin D1 and phosphorylated Cyclin D1 in wild-type MECs, Jak2-deficient MECs, and Jak2-deficient MECs reconstituted with Cyclin D1-T286A. Note that the mutant Cyclin D1 contains a Flag tag and, therefore, can be separated from the endogenous Cyclin D1 protein. All cell lines were maintained in a growth factor-rich medium. C, Immunostaining of Cyclin D1 in Jak2-deficient MECs with and without expression of Cyclin D1-T286A to verify the nuclear localization of the mutant Cyclin D1 protein. D, Viable cell count over a 5-d period to determine growth rates of Jak2-deficient MECs with and without expression of Cyclin D1-T286A. All time points were analyzed in triplicates; *error bars* represent the SE. Note that the expression of exogenous, mutant Cyclin D1 restores the proliferation of Jak2-deficient cells to levels found in wild-type controls.

ern blot analysis revealed that the Cyclin D1-T286A construct was highly expressed in Jak2-deficent MECs. The mutant Cyclin D1 contained an N-terminal Flag tag and, therefore, exhibited a different mobility and was easily distinguishable from the endogenous Cyclin D1 protein. As expected, the T286A mutant protein was not detected with an antibody that specifically recognizes the phosphorylated Cyclin D1 form. Jak2-deficient MECs transfected with the Cyclin D1-T286A construct exhibited bright fluorescence staining, and Cyclin D1 was predominantly nuclear (Fig. 9C). In contrast, the expression of Cyclin D1 was low in all MECs and both cytoplasmic and nuclear in MECs lacking Jak2. Next, we performed a viable cell count over a period of 5 d to determine growth rates of Jak2-deficient MECs with and without expression of Cyclin D1-T286A compared with the wild-type control (Fig. 9D). We observed an increase in the proliferation rate of Jak2 knockout MECs reconstituted with mutant Cyclin D1 that was virtually identical to the wild-type controls. We also performed an MTT assay on Jak2 knockout cells with and without mutant Cyclin D1, and the results were identical (data not shown). In summary, the expression of a constitutively nuclear Cyclin

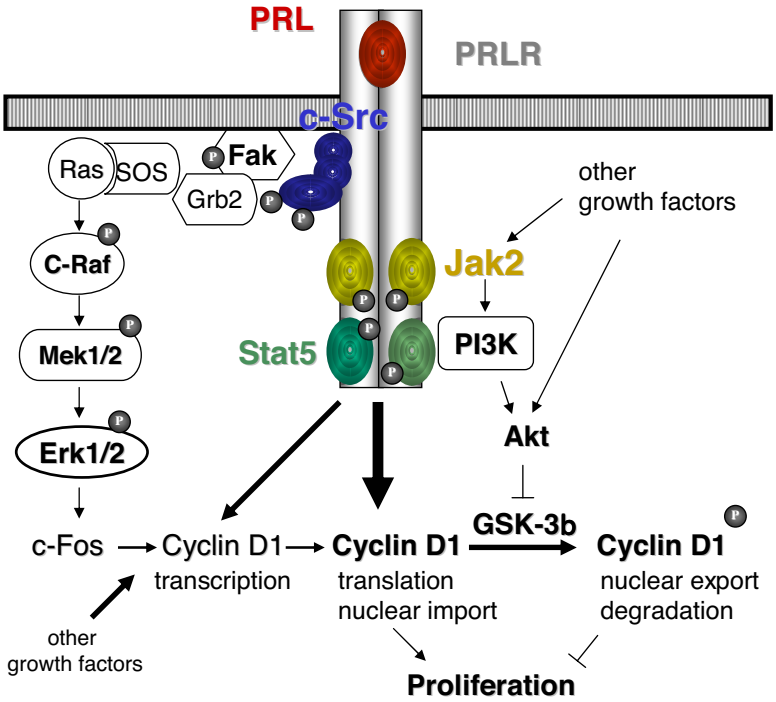

Fig. 10. Jak2-Dependent and Jak2-Independent Signaling Pathways Emerging from the PRLR and Their Suggested Impact on the Expression, Modification, and Subcellular Localization of Cyclin D1

The expression and activation status of signal transducers downstream of the PRLR displayed in bold were analyzed in this report.

D1 was able to rescue the proliferation of Jak2-deficient MECs. This observation clearly supports the suggested role for PRL signaling and the Jak2/Stat5 pathway in the regulation of Cyclin D1 stability and nuclear retention through Akt-mediated inhibition of GSK-3β.

DISCUSSION

The peptide hormone PRL is essential for normal MEC proliferation and differentiation of alveolar precursor cells. Beside its importance for normal mammary gland development, PRL is suggested to play a role in neoplastic transformation of epithelial cells and breast cancer progression. The *PRL* gene as well as the full-length form and splice variants of the PRLR are coexpressed and up-regulated in human breast cancer cells (59, 60). This autocrine loop is suggested to modify a variety of biological responses of breast cancer cells, including cell proliferation and cell motility [for more information on this subject, refer to a comprehensive review by Clevenger *et al.* (40)]. Because of the local synthesis of PRL in breast epithelial cells, the inhibition of PRL release from the pituitary gland with drugs such as bromocryptin is not an effective strategy to prevent or treat breast cancer (60). Therefore, the inhibition of downstream targets of PRLR signaling appears to be a suitable approach for cancer prevention and therapy. To develop effective strategies, it is essential to delineate diverse signaling pathways that emerge on the PRLR and define their precise biological responses.

Jak2-Dependent and Jak2-Independent Signaling Pathways that Originate on the PRLR during Ligand Stimulation

Because the PRLR does not possess an intrinsic kinase domain, receptor-associated kinases such as Jak2 and c-Src are essential for the transmission of ligand-induced signals to other transducers that, in turn, cause a variety of biological responses. Jak2 might be crucial in this process because of its proposed role in PRLR phosphorylation, which seems to be a prerequisite for the activation of diverse signaling cascades. The central role of Jak2 for PRLR signaling is supported by genetic evidence. A knockout of the PRLR and Jak2 deficiency in MECs both result in impaired proliferation of undifferentiated alveolar precursors during pregnancy (6, 27, 35). In this report, we also demonstrate that the ablation of Jak2 reduces the proliferation of immortalized MECs in culture that lack two important regulators of the cell cycle machinery, p19/Arf and p16/Ink4a. Surprisingly, this reduced proliferative capacity of Jak2-deficient cells is not caused by impaired signaling through c-Src, Fak, and the MAPK pathway that are all commonly associated with proliferative responses after mitogen stimulation (Fig.

10). Although it has been repeatedly demonstrated that the PRLR can activate MAPKs in response to ligand binding (61–63), the involvement of Jak2 in this signaling cascade remains controversial. In MCF7 cells, for example, Jak2 was reported to associate directly with Shc (Src homology 2 domain containing protein) (63). Jak2 has also been proposed to indirectly activate MAPKs through cross talk with other receptors such as the ErbBs (64). Conversely, Fresno Vara *et al.* (29) suggested that the PRLR-mediated activation of the c-Src kinase occurs independently of Jak2 function. Conversely, the inhibition of c-Src and related family kinases appeared to have no effect on the PRL-mediated activation of Ras and MAPKs (30), and reports regarding the requirement of c-Src for the activation of both Stat5 forms are inconsistent (42, 65). A direct comparison of these conflicting reports is difficult because mechanistic aspects of PRLR signaling were studied in diverse cell culture models, including COS and pre-B-cells as well as fibroblasts that do not express the full-length PRLR or do not require PRLR signaling for normal growth and differentiation. Through targeted deletion of the *Jak2* gene in MECs, we demonstrate in this report that c-Src is indeed activated by PRLR signaling in a Jak2-independet manner. Conversely, Jak2-deficiency abolishes the PRL-induced activation and nuclear accumulation of Stat5. Therefore, the activation of c-Src kinase by PRL is not sufficient to activate Stat5. This observation is in agreement with our previous findings in mammary gland-specific Jak2 knockout mice that show that Stat5 cannot be activated by normal or extraphysiological levels of PRL. The reduced expression of the Stat5-dependent immediate early gene Cish (also known as CIS1) *in vitro* and *in vivo* provides additional evidence for the lack of Stat5 activation in response to Jak2 deficiency (Figs. 4 and 5) (supplemental Fig. S3). Cish is a member of the CIS/JAB (Jak binding protein) family of negative regulators of cytokine signaling, and its overexpression in transgenic mice results in impaired PRLR signaling and phenotypic abnormalities (*i.e.* lack of alveolar proliferation and differentiation) that are comparable with Jak2 mammary-specific knockout mice (66). Interestingly, Cish-overexpressing mice did not exhibit a deregulated expression of c-fos and c-myc in the mammary gland. This might be an indication that the activation of c-Src and MAPK are not affected in this transgenic model that specifically inhibits the Jak/Stat pathway, and, therefore, this observation corresponds to our findings described in this report that Jak2 deficiency does not abolish the PRL-inducible activation of c-Src and MAPKs.

Optimal Expression and Nuclear Localization of Cyclin D1 Require Signaling though Jak2 in Proliferating MECs

Cyclin D1 knockout mice exhibit normal ductal morphogenesis in the nulliparous mammary gland but lack pregnancy-induced multiplication of alveolar precursors (20, 21). Because of obvious phenotypic similarities between Cyclin D1 knockouts and females lacking individual members of the PRLR signaling cascade, in particular the PRLR, Jak2, and Stat5a, it is reasonable to hypothesize that Cyclin D1 might be a target of PRLR signaling through the Jak/Stat pathway. Brisken *et al.* (24) proposed that the effect of PRL on Cyclin D1 expression is indirect through up-regulation of IGF-II. However, we did not observe reduced expression of *IGF-II* mRNA in cultured MECs lacking Jak2 when grown under growth factor-rich conditions (data not shown), suggesting that IGF-II deficiency may not be the underlying mechanism for the reduced proliferative capacity of Jak2-deficient MECs. Alternatively, Brockman *et al.* (22, 23) proposed that activation and binding of Stat5 to the *Cyclin D1* promoter directly regulates the expression of this important cell cycle regulator. Indeed, we observed a reduction in *Cyclin D1* mRNA expression in MECs lacking Jak2, but the difference in the expression of Cyclin D1 was more evident on the level of the protein. We demonstrated that PRL signaling through Jak2 influences the nuclear accumulation of Cyclin D1 and reduces the relative amount of phosphorylated Cyclin D1. Consistent with this observation, the levels of activated Akt and phosphorylated (inactive) GSK-3β are reduced in Jak2-deficient MECs. It has been shown previously that GSK-3β is the central kinase responsible for nuclear export and subsequent proteosomal degradation of Cyclin D1, and its activity is regulated by Akt through mitogenic signals (50). Our findings indicate that signaling through Jak2 controls not only the expression of *Cyclin D1* mRNA but, more importantly, it controls the accumulation of the Cyclin D1 protein in the nucleus by inhibiting signal transducers that have been shown to mediate the phosphorylation and subsequently nuclear export and destruction of Cyclin D1 (Fig. 10). It has been demonstrated previously that constitutively active Akt prolonged the survival of alveolar cells in the mammary gland (67). Despite the reduction of active Akt in Jak2-deficient MECs that are grown in a growth factor-rich medium, PRL signaling alone does not significantly activate Akt in untransformed MECs. This finding suggests that signaling mediated by Jak2 does not initiate but greatly intensifies the activation of Akt through signaling cross talk with other growth factor receptors.

The Jak2-dependent regulation of Cyclin D1 expression and nuclear accumulation provides a rational explanation for why immortalizing mutations (*i.e.* the lack of the Cdkn2a locus) did not abolish the dependence of MECs from hormonal signals through Jak2 for optimal proliferation. The expression of Cyclin D1 protein, which is induced by Jak2, is required for the Cdk4/6-mediated phosphorylation of pRB regardless of whether the inhibitor of the Cyclin D1-Cdk4/6 complex (*i.e.* Ink4a encoded by the *Cdkn2a* locus) is present or not. Interestingly, reduced expression of Ink4a through DNA methylation has been reported to occur in more than one quarter of human breast can-

cers, and a similar percentile of human breast cancer cases overexpress Cyclin D1 [for more information, refer to recent reviews by Tlsty *et al.* (68) and Sutherland and Musgrove (69)]. Hence, both types of common genetic changes synergistically enhance the functionality of the Cyclin D1-Cdk4/6 complex. The targeted reduction of Cyclin D1 expression using antisense constructs seems to be sufficient to reduce the proliferation of breast cancer cells (70). In addition, genetic studies show that the deletion of the *Cyclin D1* gene abolishes the onset of Ras and Her2 (human epidermal growth factor receptor 2)/neu-induced mammary tumorigenesis (71). Because signaling through Jak2 is important for the expression and nuclear accumulation of Cyclin D1 in MECs, it is reasonable to propose that, like Cyclin D1 deficiency, lack of Jak2 might significantly delay or abolish Her2/neu-induced mammary cancer in genetically engineered models for breast cancer.

MATERIALS AND METHODS

Mouse Models

The generation of genetically engineered mice with a *Jak2* conditional knockout allele [*Jak2*$^{fl/fl}$ or *Jak2*^{tm1Kuw}] and the PCR protocols to determine the presence of the *Jak2* floxed, *Jak2* recombined/null, and *Jak2* wild-type alleles have been described previously (27, 28). *Cdkn2a* knockout mice [*Cdkn2a*^{tm1Rdp} (31)] were obtained from the National Cancer Institute Mouse Repository of the Mouse Model for Human Cancer Consortium (Frederick, MD). All animals used in the described studies were treated humanely and in accordance with institutional guidelines and federal regulations.

Primary Cell Cultures and Expression Vectors

Primary mammary epithelial cultures from midpregnant Jak2/Cdkn2a double-mutant females were prepared according to a protocol published by Medina and Kittrell (32). The generation of a retroviral vector expressing Cre recombinase was described previously (36, 37). Forty-eight hours after infection, cells were selected in complete medium containing increasing concentrations (3–7 μg/ml) of puromycin (Sigma, St. Louis, MO). Recombinant mouse PRL (AFP306C) was kindly provided by Dr. A. F. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) under the sponsorship of the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health. We used 10 nM PRL to induced PRLR signaling in Jak2-deficient MECs and their wild-type controls. The Flag-tagged, mutant Cyclin D1-T286A cDNA in a pBabe-puro retroviral vector was a kind gift from Alan Diehl (University of Pennsylvania, Philadelphia, PA). The puromycin cassette of this vector was replaced with a hygromycin resistance gene from vector pIREShyg (Clontech, Mountain View, CA). Forty-eight hours after infection with viral particles, Jak2-deficient MECs were selected in 100–200 μg/ml Hygromycin B (Invitrogen, Carlsbad, CA).

MTT Assay

To determine the growth properties of Jak2-deficient cells, we performed an MTT growth assay as described previously (36, 38, 72). The MTT was obtained from Sigma. Two times 10^4 cells of each genotype were seeded in triplicates in a 96-well microtiter plate. Absorbance was measured at 570 nm with an Elx 808 (BioTek Instruments, Winooski, VT) ELISA reader.

Immunoprecipitation and Western Blot Analysis

For the majority of immunoprecipitations and Western blots, cell pellets were lysed on ice for 30 min in 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.4 units/ml aprotinin, 1 mM NaF, and 0.1 mM sodium orthovandate. For coimmunoprecipitation of Stat5 and p85, we used the following lysis buffer: 1% Nonidet P-40, 50 mM Tris (pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.4 units/ml aprotinin, 1 mM NaF, and 0.1 mM sodium orthovandate. Protein was quantified using a Bradford assay (Pierce, Rockford, IL). Clarified tissue lysates corresponding to 2.0 mg, 500 μg, or 100 μg of total protein were immunoprecipitated with Stat5a (1:500 dilution), Src (1:50 dilution; Cell Signaling Technology, Beverly, MA), or Fak (1 μg/ml; A-17; Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Immunoprecipitates or whole-cell extracts were resolved by SDS-PAGE and blotted onto polyvinylidene fluoride membranes (Invitrogen). The membranes were blocked for 1 h in 1× Tris-buffered saline (TBS), 0.05% Tween 20, and 5% dry milk. Subsequently, membranes were incubated with primary antibodies in blocking buffer or 5% BSA in 1× TBS and 0.05% Tween 20 at 4 C overnight, washed three times for 5 min in washing buffer (1× TBS and 0.05% Tween 20), and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) in blocking buffer. Membranes were washed again three times in washing buffer and once for 5 min in 1× TBS without Tween 20. Protein bands were detected using the ECL chemiluminescence kit for Western blot analysis (GE Healthcare, Little Chalfont, UK) according to the instructions of the manufacturer. Membranes were stripped using 0.2 M NaOH for consecutive detection of various proteins. The quantification of Cyclin D1, phospho-Cyclin D1, Akt, and GSK-3β was performed by scanning blots in the linear range using the Luminescence Image Analyzer LAS-1000plus (Fujifilm, Tokyo, Japan). The following antibodies were used: α β-actin (ActB) (I-19) (1:2000 dilution), α-Cyclin D1 (72-13G) (1:1000 dilution), and α-PI3K p85α (B-9) (1:1000 dilution) from Santa Cruz Biotechnology; α-phosphotyrosine antibody (4G10; 1 μg/ml) from Upstate Biotechnology (Lake Placid, NY); phospho-ERK1/2 (1:1000 dilution), α-Stat3 (124H6; 1:1000 dilution), α-phospho-Stat3 (58E12; 1:1000 dilution), α-phospho-c-Raf (Ser338) (56A6; 1:1000 dilution), α-phospho-MEK (Ser217/221) (1:1000 dilution), α-phospho-Fak (Thr576/557) (1:1000 dilution), α-Akt, α-phospho-Akt (Ser473) (1:1000 dilution), α-GSK-3β, and α-phospho-GSK-3β (Ser9) (1:1000 dilution) antibodies from Cell Signaling Technology; and α-panERK (1:2000 dilution) antibody from BD Bioscience (San Jose, CA). The α-phospho-Cyclin D1 (Thr286) antibody was a kind gift from Dr. A. Diehl (University of Pennsylvania). The α-phospho-Stat5a/b (Y694/9) antibody (AX1; 0.5 μg/ml; Advantex Bioreagents, Conroe, TX) was kindly provided by Dr. Hallgeir Rui (Thomas Jefferson University, Philadelphia). The polyclonal α-Stat5a antiserum was a gift from Lothar Hennighausen (National Institutes of Health, Bethesda, MD).

RT-PCR Methodology and Primer Sequences

Total RNA was isolated from cell pellets using standard guanidinium thiocyanate-phenol-chloroform extraction. A SuperScript II kit from Invitrogen with oligo-dT primers was used to perform the first-strand synthesis. PCR amplification of various differentiation factors was conducted using the following

primer pairs: *Cish*, 5′-GGA CAT GGT CCT TTG CGT ACA G-3′ and 5′-TGG CTC AGT CAG AGT TGG AAG G-3′; *Ccnd1*, 5′-CAG ACG GCC GCG CCA TGG AA-3′ and 5′-AGG AAG TTG TTG GGG CTG CC-3′); *c-Fos*, 5′-CAT GAT GTT CTC GGG TTT CAA CG-3′ and 5′-CCA AGG ATG GCT TGG GCT CAG-3′; and *ActB*, 5′-TGG ATG ACG ATA TCG CTG CGC-3′ and 5′-AAG CTG TAG CCA CGC TCG GTC-3′. Two aliquots were removed during the PCR after various cycles (determined empirically for each gene) to compare amplification products in the linear amplification range.

Northern Blot Analysis

Twenty micrograms of total RNA was separated on a 1.5% formaldehyde gel and transferred to a GeneScreen Plus membrane. The Cyclin D1 transcript was detected by probing the membranes with the ^{32}P randomly labeled cDNA of Cyclin D1 (*Ccnd1*). The hybridization was performed overnight at 65 C using QuickHyb (Stratagene, La Jolla, CA). Membranes were washed in 1× SSC buffer containing 0.1% SDS and exposed for 24 h to a Kodak XOMAT-AR film (Eastman Kodak, Rochester, NY).

Immunocytochemistry and Immunohistochemistry

For immunocytochemistry, cells were fixed at −20 C in 100% methanol or 70% ethanol, washed twice in 1× PBS, and incubated for 20 min in blocking solution (1× PBS and 3% BSA). The slides were treated for several hours with a 1:100 to 1:200 dilution of the primary antibody in blocking solution. The sources for the primary antibodies (α-Cyclin D1, α-phospho-Cyclin D1, α-phospho-Stat5a/b, and α-phospho-Akt) are described above. The α-pan-cytokeratin antibody (F3418) was purchased from Sigma. The latter antibody was fluorescein isothiocyanate conjugated. After an additional washing step, the respective targets (Cyclin D1, pStat5, or pAkt) were visualized with mouse-specific or rabbit-specific Alexa Fluor 488-conjugated secondary antibodies (1:1000 dilution; Invitrogen). The slides were washed repeatedly in 1× PBS and mounted with Vectashield containing 1.5 μg 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

A basic protocol for immunohistochemistry on paraffin-embedded mammary gland specimens was described previously (27). In contrast to the staining of Cyclin D1 in cultured cells, we used a Cyclin D1 rabbit polyclonal antibody (Ab-4) from NeoMarkers (Fremont, CA), which exhibited reduced nonspecific staining. The sources for the other primary antibodies (α-phospho-Cyclin D1, α-Cish, and α-ERK1/2) are described above. For visualization of the specific targets, we used corresponding biotinylated secondary antibodies (1:200 dilution) and Vectastain Elite ABC kits (Vector Laboratories). 3,3-Diaminobenzidine was used as a chromogen, and slides were counterstained with Mayer's hematoxylin. Bright-field and fluorescence images of histological slides were taken on a Nikon (Tokyo, Japan) Labophot microscope equipped with a Nikon Coolpix 990 camera, as well as fluorescein isothiocyanate and DAPI filter sets.

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