Role of Tyrosine Kinase Jak2 in Prolactin-induced Differentiation and Growth of Mammary Epithelial Cells

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Genetic studies in mice have established a critical role for prolactin receptors and transcription factor Stat5 in mammary gland differentiation. However, the enzymatic coupling between prolactin receptors and Stat5 in this process has not been established. In addition to Jak2, several other tyrosine kinases reportedly also are associated with prolactin receptors and may phosphorylate Stat5. Because Jak2 null mice die in utero, we targeted Jak2 in an ex vivo model of prolactin-induced mammary epithelial cell differentiation to determine the role of Jak2 in regulation of cell differentiation and growth. Two independent targeting strategies were used to suppress Jak2 in immortalized HC11 mouse mammary epithelial cells: 1) stable expression of a specific Jak2 antisense construct and 2) adenoviral delivery of a dominant-negative Jak2 gene. We now demonstrate that Jak2 is essential for prolactin-induced differentiation and activation of Stat5 in normal mouse mammary epithelial cells. Furthermore, suppression of Jak2 in HC11 cells was associated with constitutive activation of oncoprotein Stat3 and a hyperproliferative phenotype characterized by increased mitotic rate, reduced apoptosis, and reduced contact inhibition. Collectively, our data suggest that Jak2 is differentiation-inducing and growth-inhibitory in normal mammary epithelial cells, observations that may shed new light on the role of the Jak2-Stat5 pathway in breast cancer.

Prolactin is a principal differentiation factor for human and mouse mammary epithelial cells and is required for milk production (1). However, prolactin may also stimulate mammary epithelial cell growth and act as a mammary tumor promotor (1, 2). Identifying the roles of individual signaling molecules and pathways activated by prolactin in normal mammary epithelial cells is therefore needed to better understand the role of prolactin in breast cancer.

Prolactin activates tyrosine kinase Jak21 (3) and transcription factor Stat5 (4) in target cells, including Nb2 lymphocytes, ovarian cells, and mammary cells. Specifically, the Jak2-Stat5 pathway is expected to mediate prolactin-induced mammary epithelial cell differentiation (5). Although genetic studies have established a critical role for Stat5 in mouse mammary gland differentiation (6, 7), corresponding genetic evidence is not yet available for Jak2, because Jak2 null mice die in utero (8, 9) and conditional Jak2 null mice have not been established. Although Jak2 has been regarded as the principal tyrosine kinase activated by prolactin (3), the picture has been complicated by evidence that prolactin also can activate other tyrosine kinases, including Src family kinases (10, 11), focal adhesion kinase (12), Tec kinase (13), and the ErbB-2 receptor tyrosine kinase (14). Experimental testing of the importance of Jak2 for prolactin-induced differentiation of mammary epithelial cells is therefore warranted. Furthermore, because Jak2 is oncogenic in hematopoietic cells (15), it is also critical to establish the role of Jak2 in regulating growth of normal mammary epithelial cells.

In this study, we targeted Jak2 in an ex vivo model of prolactin-induced mammary epithelial cell differentiation to determine the role of Jak2 in cell differentiation and growth. Two distinct targeting strategies were used to suppress Jak2 in immortalized HC11 mouse mammary epithelial cells. First, an effective Jak2 antisense construct was generated and stably introduced into HC11 cells. Second, a functional dominant-negative Jak2 mutant was generated and introduced into HC11 cells by adenoviral delivery. We now report that Jak2 is essential for prolactin-induced differentiation and activation of transcription factor Stat5 in normal HC11 mouse mammary epithelial cells. Importantly, suppression of Jak2 in HC11 cells was associated with a hyperproliferative phenotype characterized by increased mitotic rate, reduced apoptosis, and reduced contact inhibition. In addition, constitutive activation of Stat3 was associated with suppression of Jak2 in HC11 cells. Collectively, our data suggest that Jak2 mediates growth-suppressive and differentiation-inducing effects on normal mouse mammary epithelial cells. These observations may provide important new insight into the role of the prolactin-activated Jak2-Stat5 pathway in breast cancer.

MATERIALS AND METHODS

Hormones and Antibodies—Ovine prolactin (NIDDK-oPRL-19, AFP-9221A) and human prolactin (NIDDK-hPRL-SIAFP-B2, AFP-2968A) were kindly provided by Dr. A. F. Parlow under the sponsorship of the National Hormone and Pituitary Program, NIDDK (National Institutes of Health), the NICHD (NIH), and the U.S. Department of Agriculture. Human epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Placid, NY). Dexamethasone and insulin were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology. Monoclonal anti-phosphotyrosine-Stat5 antibody and polyclonal rabbit antisera to Jak1, Jak2, Jak3, Tyk2, Stat1, Stat3, and Stat5 were obtained from Advantech BioReagents (Conroe, TX).

HC11 Cell Ex Vivo Model of Mammary Epithelial Cell Differentiation

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1 The abbreviations used are: Jak, Janus kinase; Stat, signal transducer and activator of transcription; Prl, prolactin; PrlR, prolactin receptor; Adv, adenovirus; m.o.i.: multiplicity of infection; Wt, wild type; Dn, dominant-negative; EGF, epidermal growth factor; mAb, monoclonal antibody; pAb, polyclonal antibody; DAPI, 4′,6-diamidino-2-phenyl-indole; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PBS, phosphate-buffered saline.

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Cell Culture and Transient Transfections—Conduct expression and functional tests were performed by transient transfection of COS-7 cells (ATCC, Manassas, VA). COS-7 cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mm t-glutamine, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively) at 37 °C with 5% CO2. Several independent transfecions were performed using LipofectAMINE 2000 according the manufacturer’s protocol and were kept without fetal calf serum for 24–48 h following by stimulation of transfected cells with EGF.

TUNEL Assay—In situ detection of apoptotic cells was performed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Cells were air-dried on glass slides and fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 1 min on ice. The slides were rinsed with PBS several times, and the samples were then processed for TUNEL labeling using the fluorescein-based In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. Samples were rinsed three times with PBS, mounted, and analyzed under a fluorescence microscope.

Anchorage-independent Survival Analysis—Confluent HC11 cells were trypsinized into a single cell suspension. A total of 700,000 cells per well were plated in soft agarose culture dishes that had been coated with 0.8% agarose. Cells were collected at various time points and washed in PBS, and cell aggregates were dispersed by trypsinization. Parallel samples were analyzed for apoptosis by TUNEL staining and flow cytometry for hypodiploid cells.

Anti-phosphotyrosine-Stat5 Immunochemistry—HC11 cells were fixed in 4% paraformaldehyde-PBS at room temperature for 20 min.

Antisense Jak2 Construct and Generation of Stably Expressing HC11 Clones—After testing several alternatives, an effective and specific Jak2 antisense oligonucleotide was determined to be 5′-TGTCCTTCAAA-GACACCGAAAAATCTACAGGGACCTATCTCAGTTGGTGA-3′. This targeting sequence was verified to be unique to Jak2 by searching the NCBI GenBank™ data base. The sequence targets a region of Jak2 mRNA encoding the amino acid sequence PMRRGALGPSGFEDRF within the hinge region between the JH1 and JH2 domains (18). The sense control nucleotide sequence is 5′-TACCCACATGAGATGTGCT-3′.

Allele-specific amplification of the PCR products was conducted on a DNA sequence analyzer (Bresagen, Brea, CA).
The cells were gently detached by a cell scraper in PBS. The detached cells were stretched into monolayer sheets in warm PBS and adhered to poly-lysine pretreated glass slides. Before immunocytochemical staining, sample slides were pretreated with an antigen-unnmasking procedure by boiling in an antigen-retrieval solution for 10 min. The slides were incubated at 4°C overnight by using a 1:3000 dilution of the primary anti-phospho-Tyr-Stat5 monoclonal antibody AX1 (Advantex Bioreagents, Conroe, TX). For secondary detection the Histomouse kit (Zymed Laboratories Inc., South San Francisco, CA) was used, and active Stat5 was visualized with aminomethyl carbazole and counterstaining with hematoxylin. The prolactin-treated COS-7 cells cotransfected with Stat5a and prolactin receptor expression constructs were used as positive control, and subtype-specific mouse IgG and PBS were used for negative controls (not shown).

RESULTS

Prolactin-induced Differentiation of HC11 Mouse Mammary Epithelial Cells Correlates with Activation of Tyrosine Kinase Jak2—Confluent, growth-arrested HC11 mouse mammary epithelial cells can be induced to differentiate in vitro by prolactin in medium supplemented with glucocorticoids and insulin (16). This differentiation process leads to formation of mammospheres, which are acinar-like structures that have been shown to express milk proteins (19). HC11 cells have been widely used as an ex vivo model of mammary gland epithelial cell differentiation (20, 21). We took advantage of this model to determine whether Jak2 was critical for prolactin-induced differentiation.

The time-dependent differentiation of HC11 cells induced by prolactin as measured by the appearance of mammospheres is presented in Fig. 1A. Mammospheres were detectable within 1 day of prolactin treatment, and additional mammospheres continued to form over a period of 4 to 5 days of culture, reaching a plateau at a density of ~15 mammospheres per cm². Although glucocorticoids and insulin are required supplements in the differentiation medium, mammosphere formation critically requires prolactin as demonstrated by a concentration-dependent effect of prolactin ranging from 0 to 20 nM (Fig. 1B). To determine expression and activation patterns of Jak tyrosine kinases at the initiation of differentiation treatment, HC11 cells were treated with or without prolactin for 30 min and harvested. Individual Jak kinases were immunoprecipitated from cell lysates and immunoblotted for phosphotyrosine and reprobed for Jak protein levels. These analyses showed that prolactin treatment of HC11 cells correlated with selective activation of Jak2 and not of other members of the Jak tyrosine kinase family (Fig. 1C). Specifically, phosphotyrosine immunoblotting of immunoprecipitated Jak proteins established that only Jak2 became detectably tyrosine-phosphorylated in response to prolactin. Furthermore, of the four Jak kinases, Jak2 was the only Jak family member expressed at significant levels in HC11 cells. Control experiments verified that the antibodies used for immunoprecipitation and immunoblotting of the various Jak tyrosine kinases were effective against mouse isoforms (data not shown). We therefore conclude that prolactin-induced differentiation of HC11 mammary epithelial cells correlated with selective activation of Jak2 tyrosine kinase.

Jak2 Antisense Blocks Prolactin-induced Differentiation of Stably Transfected HC11 Clones—As one strategy to suppress Jak2 function and test the importance of Jak2 in prolactin-induced differentiation of HC11 cells, we generated an antisense construct to inhibit Jak2 protein expression. The genetic engineer of this Jak2 antisense construct, which targets a region of 51 bp unique to the Jak2 transcript, is described in detail under “Materials and Methods.” This region is located within the hinge region between the JH2 pseudokinase and JH1 kinase domains of Jak2. Functional testing of the antisense construct was first carried out using COS-7 cells and cotransfection experiments with a V5- His tagged Wt-Jak2 construct. We determined that the Jak2 antisense construct effec-
plasmid or HC11 clones. HC11 cells were transfected with vector sense control validation of efficiency of Jak2 antisense construct in stably transfected blotting for presence of increasing amounts of antisense—each case, total levels of transfected DNA were kept constant by com-
codning a 51-nucleotide antisense mRNA specific to encoding V5-epitope-tagged Jak2 and an increasing amount of plasmid COS-7 cells were cotransfected with a constant amount of plasmid Jak2 antisense construct by transient cotransfection in COS-7 cells. cotation of stably transfected HC11 clones.

Jak2

were incubated with prolactin for 4 days. Phase contrast images of Jak2

were tested for levels of tyrosine phosphorylated Jak2 and levels of Jak2

for 30 min. Jak2 was immunoprecipitated from whole cell lysates and

selected, and clones were incubated with or without prolactin (10 nM)

or sense (A Vs H11006

the cultures, and represent mean values (± S.E.) of three independent

ages of cultures on day 4 of treatment (Fig. 2D). In contrast, mammosphere formation remained intact in parental HC11 and vector-sense control cells. Mammosphere formation was quantified by counting, and the data from three independent experiments were expressed as number of mammospheres per cm² of culture surface (Fig. 2D). The inhibitory effect of Jak2 suppression on cell differentiation was equally pronounced after extended treatment with prolactin for up to 7 days (data not shown). We therefore conclude that suppression of Jak2 levels by stable expression of a Jak2 antisense construct blocked prolactin-induced differentiation of HC11 cells.

Construction and Functional Testing of a Dominant-negative Jak2 Mutant—To further test by an independent strategy whether Jak2 was essential for prolactin-induced mammary differentiation, we generated a dominant-negative Jak2 protein by deletion of the C-terminal kinase domain as described under “Materials and Methods.” As a functional test of this construct, transient transfection assays in COS-7 cells were used to examine the ability of this kinase-deleted mutant Jak2 to block prolactin-induced activation of Stat5 by Wt-Jak2. COS-7 cells were cotransfected with plasmids encoding PrlR, Stat5α, and Jak2 forms as indicated and stimulated with or without prolactin for 30 min (Fig. 3A). In whole cell lysates of mock transfected, negative control cells, immunoblotting revealed no detectable endogenous Stat5, and therefore no response to prolactin stimulation (Fig. 3A, lanes a and b). When Stat5α and prolactin receptor were cotransfected into COS-7 cells, modest but detectable prolactin-stimulated Stat5α phosphor-ylation was observed, presumably mediated by low levels of endogenous Jak2 (Fig. 3A, lanes c and d).

This inducible Stat5α activation was inhibited by cotransfection with Dn-Jak2, which migrated with the expected size of 90 kDa in SDS-PAGE (Fig. 3, lanes e and f, top and bottom panels, respectively). Cotransfection of Wt-Jak2 with Stat5α led to basal tyrosine phosphorylation of Stat5α that was markedly enhanced by prolactin treatment (lanes g and h). However, further cotransfection of Dn-Jak2 with Wt-Jak2 showed complete inhibition of both basal and prolactin-induced Stat5α activation (lanes i and j). This effect was not due to reduced levels of Stat5 or Wt-Jak2 as demonstrated by reprobing with anti-Stat5 or anti-V5 antibodies, respectively (Fig. 3A, middle and lower panels). Furthermore, these and other immunoblotting experiments showed that Dn-Jak2 effectively inhibited Wt-Jak2 function at equivalent protein levels, providing direct evidence for dominant rather than a simple competitive inhibitory effect of the kinase-deleted Jak2 mutant. Thus, we conclude that the engineered Dn-Jak2 functioned as predicted.

Adenoviral Delivery of Dn-Jak2 into HC11 Cells Blocks Prolactin-induced Differentiation—As the second approach to effectively inhibit the Jak2 tyrosine kinase in HC11 cells, we then generated a replication-defective adenovirus for high efficiency gene delivery of Dn-Jak2 into HC11 cells. Detailed description of this construct is presented under “Materials and Methods.” Functional testing of Adv-Dn-Jak2 was first carried out in HC11 cells using prolactin-stimulated Jak2 tyrosine

struct. A vector sense-control clone (HC11-Vs) was also selected for use as a second control cell line in addition to the parental line. Stable clones A and B were selected for further study from several positive clones, and showed markedly reduced Jak2 protein levels by immunoprecipitation and Western blot analysis (Fig. 2B, lower panel). Correspondingly, basal and prolactin-induced Jak2 tyrosine phosphorylation was lost in clones A and B (Fig. 2B, upper panel). When examined in differentiation assays, suppression of Jak2 levels in clones A and B was associated with complete disruption of prolactin-induced mammosphere differentiation, as illustrated by representative images of cultures on day 4 of treatment (Fig. 2C). In contrast, mammosphere formation remained intact in parental HC11 and vector-sense control cells. Mammosphere formation was quantified by counting, and the data from three independent experiments were expressed as number of mammospheres per cm² of culture surface (Fig. 2D). The inhibitory effect of Jak2 suppression on cell differentiation was equally pronounced after extended treatment with prolactin for up to 7 days (data not shown). We therefore conclude that suppression of Jak2 levels by stable expression of a Jak2 antisense construct blocked prolactin-induced differentiation of HC11 cells.

FIG. 2. Antisense Jak2 blocks prolactin-induced differentia-
tion of stably transfected HC11 clones. A, validation of efficiency of Jak2 antisense construct by transient cotransfection in COS-7 cells. COS-7 cells were cotransfected with a constant amount of plasmid encoding V5-epitope-tagged Jak2 and an increasing amount of plasmid encoding a 51-nucleotide antisense mRNA specific to Jak2 mRNA. In each case, total levels of transfected DNA were kept constant by compensating with empty pcDNA3 vector as indicated. Levels of Jak2 protein were monitored in cell lysates by anti-V5 immunoblotting in the presence of increasing amounts of antisense-Jak2. Parallel immunoblotting for β-actin was used to verify equal loading of cell lysates. B, validation of efficiency of Jak2 antisense construct in stably transfected HC11 clones. HC11 cells were transfected with vector sense control plasmid or Jak2 antisense plasmid using LipofectAMINE 2000. A vector sense (Vs) control clone and two antisense clones (A and B) were selected, and clones were incubated with or without prolactin (10 nM) for 30 min. Jak2 was immunoprecipitated from whole cell lysates and tested for levels of tyrosine phosphorylated Jak2 and levels of Jak2 protein by immunoblotting. C, prolactin-induced HC11 cell differentiation is disrupted in Jak2 antisense expressing clones. Parental HC11 cells, Vs-control clone, and Jak2 antisense-expressing clones A and B were incubated with prolactin for 4 days. Phase contrast images of representative fields show failure of mammosphere formation in Jak2 antisense-expressing clones. D, quantification of differentiation-suppressive effect of Jak2 antisense in stable HC11 clones. The effect of stable expression of Jak2 antisense on prolactin-induced HC11 cell differentiation was documented by counting of mammospheres after 4 days of treatment. Data are expressed as density of mammospheres in the cultures, and represent mean values (± S.E.) of three independent experiments. ND, not detected.
Dn-Jak2 is a dominant-negative mutant of Jak2 that inhibits prolactin-induced HC11 cell differentiation. A, validation of efficiency of Dn-Jak2 construct by transient cotransfection in COS-7 cells. COS-7 cells were cotransfected with p38 antisense to inactivate Stat5, Staj5a, Wt-Jak2, and/or Dn-Jak2 as indicated. Total amounts of DNA transfected were maintained constant by compensating with empty pcDNA3 vector. Parallel cultures were incubated in the presence (+) or absence (−) of prolactin (10 nM) for 30 min, and whole cell lysates were examined for tyrosine phosphorylated Stat5 (upper panel), Stat5a protein levels (middle panel), or V5-epitope tagged Wt-Jak2 and Dn-Jak2 (lower panel). B, validation of efficiency of adenoviral delivery of Dn-Jak2 to inhibit Jak2 activation in HC11 cells. HC11 cells were either mock infected (no adenovirus), infected with either Adv-Control (no insert; m.o.i. 25), or with two increasing doses of Adv-Dn-Jak2 (m.o.i. 5 and 25). Twenty-four h after infection, cells were incubated with (+) or without prolactin (10 nM) for 30 min. Jak2 was immunoprecipitated from whole cell lysates and basal and prolactin-activated Jak2 tyrosine phosphorylation was examined. In HC11 cells, both basal and prolactin-activated Jak2 tyrosine phosphorylation was inhibited by Dn-Jak2 at m.o.i. values of 5 and 25, whereas infection with control virus had no effect. Reblotting of samples for V5-tagged Dn-Jak2 protein verified specific and dose-dependent expression of Dn-Jak2 in HC11 cells (Fig. 3B) and that Adv-Dn-Jak2 was functional.

To determine whether Dn-Jak2 would block prolactin-induced differentiation of HC11 cells, cells were infected with or without Adv-DN-Jak2 as described and mammosphere formation in response to prolactin treatment was monitored. While prolactin-induced differentiation remained intact in mock and Adv-control infected cells, Dn-Jak2 effectively disrupted prolactin-induced mammosphere formation in HC11 mammary cells (Fig. 3C). The effect of Dn-Jak2 was dose-dependent and could not be attributed to general protein overexpression, because infection with Adv-LacZ did not disrupt mammosphere formation (Fig. 3D). Therefore, based on two independent approaches that involved either Dn-Jak2 or antisense to inactivate Jak2, we conclude that Jak2 is required for terminal differentiation of mammary epithelial cells. To our knowledge, these data provide the first direct evidence that Jak2 tyrosine kinase activity is critical for prolactin-induced differentiation of mammary epithelial cells.

Disruption of Jak2 Activity Is Associated with Inhibition of Stat5a Tyrosine Phosphorylation in HC11 Cells—Transcription factor Stat5 is critical for terminal differentiation of mammary cells and for lactogenesis as determined from genetic studies in mice (6, 7). Furthermore, Stat5 is recognized to be a substrate of Jak2 in the context of the prolactin receptor complex (22). To experimentally determine whether inhibition of Jak2 would block prolactin-induced Stat5 activation in HC11 cells, we tested the effect of Adv-Dn-Jak2 on prolactin-induced Stat5 tyrosine phosphorylation. Stat5 activation was measured by protein immunoblotting of samples from whole cell lysates using a monoclonal anti-phosphotyrosine-Stat5 antibody (Fig. 4A). Whereas prolactin-induced Stat5 activation was readily detectable in mock-infected cells or cells infected with Adv-control (lanes a–d), prolactin-induced Stat5 activation was inhibited in a dose-dependent manner in cells infected with Adv-Dn-Jak2 (Fig. 4A, lanes e–h, upper panel). Furthermore, inhibition of Stat5 activation correlated with Dn-Jak2 levels as detected by anti-V5 immunoblotting and was not due to reduction in Stat5 protein levels (Fig. 4A, middle and bottom panels, respectively).

Inhibition of prolactin-induced Stat5 tyrosine phosphorylation by Jak2 suppression was verified at the subcellular level by anti-phosphoTyr-Stat5 immunocytochemistry. HC11 cells infected with Adv-control and treated with prolactin showed marked nuclear tyrosine phosphorylation of Stat5, whereas infection of cells with Adv-Dn-Jak2 markedly inhibited Stat5 phosphorylation (Fig. 4B, panels 1 and 2). Likewise, HC11 cell clones stably expressing antisense-Jak2 displayed only minor levels of prolactin-stimulated Stat5 phosphorylation as detected by anti-phosphoTyr-Stat5 immunocytochemistry (Fig. 4B, panel 3; only clone A shown). Finally, infection of clone A with Adv-Dn-Jak2 led to an even more pronounced inhibition of prolactin-induced Stat5 phosphorylation as a readout. Cells were mock infected or infected with virus carrying no insert (Adv-control), or Adv-Dn-Jak2 at increasing multiplicity of infection (m.o.i.). Twenty-four hours later, cells were incubated with or without prolactin for 30 min, and Jak2 phosphotyrosine levels were examined. In HC11 cells, both basal and prolactin-activated Jak2 tyrosine phosphorylation was inhibited by Dn-Jak2 at m.o.i. values of 5 and 25, whereas infection with control virus had no effect.
Twenty-four hours later all cells were exposed to prolactin for 30 min, delivery of Dn-Jak2 inhibited prolactin-induced Stat5 tyrosine phosphorylation in HC11 cells by anti-phosphotyrosine-Stat5 immunoblotting. HC11 cells were either mock infected or infected with either Adv-Control (no insert; m.o.i. 25), or with two increasing doses of Adv-Dn-Jak2 (m.o.i. 5 and 25). Twenty-four h after infection, cells were incubated with (+) or without (−) prolactin (10 nM) for 30 min. Whole cell lysates were examined for tyrosine phosphorylated Stat5 (upper panel), V5-epitope tagged Dn-Jak2 (middle panel), or Stat5 protein levels (lower panel). A, adenoviral delivery of Dn-Jak2 inhibits prolactin-induced Stat5 tyrosine phosphorylation in HC11 cells by immunocytochemistry. Parental HC11 cells or HC11 clone A, which stably expresses antisense Jak2, were infected with either Adv-Control (m.o.i. 25; first and third panels, respectively) or Adv-Dn-Jak2 (m.o.i. 25; second and fourth panels, respectively). Twenty-four hours later all cells were exposed to prolactin for 30 min, followed by fixation and immunocytochemistry for activated Stat5 using anti-Stat5 pTyr antibodies.

prolactin-induced Stat5 tyrosine phosphorylation (Fig. 4, panel 4). Therefore, both molecular approaches to inhibit Jak2 activity also inhibited prolactin-induced Stat5 activation. Collectively, these observations support the concept that the Jak2-Stat5 pathway is a differentiation-inducing axis in mammary epithelial cells.

Targeted Inactivation of Jak2 in HC11 Cells Resulted in a Hyperproliferative Phenotype—Terminal differentiation of cells is associated with exit from the cell cycle and inhibition of cell proliferation. To determine the effect of Jak2 on growth characteristics of HC11 cells, we compared the growth rates of HC11 clones A and B to those of parental HC11 cells and the sense-control clone. As shown in Fig. 5A, HC11 clones A and B exhibited higher growth rates than parental HC11 or the sense-control clone. In fact, the growth rates of clones A and B stably expressing Jak2 antisense were approximately double that of parental or sense-control HC11 cells. The increased growth rates of Jak2-suppressed clones A and B were also correlated with increased rates of mitosis during exponential growth, as visualized by propidium iodide staining of cells (Fig. 5B). Flow cytometry of cells during exponential growth verified a markedly increased proportion of cycling cells with a corresponding reduction in cells in the G0/G1 phase in clones A and B when compared with control cells (Fig. 5C). Specifically, whereas almost 50% of parental or vector-sense control cells were in G0/G1, less than 25% of clones A and B were in G0/G1. For these cell cycle experiments, cells were harvested and measured at ~50% confluency. The data suggest that suppression of Jak2 is associated with increased cycling of HC11 cells and higher growth rate during exponential growth.

We then examined the cell cycle characteristics of supern confluent cultures. Intriguingly, Jak2-deficient clones A and B consistently grew to a density nearly 3-fold greater than that of parental or vector sense-control HC11 cells (Fig. 6A). Furthermore, during supernconfluence, Jak2-deficient clones displayed reduced growth suppression and retained a markedly elevated S phase population compared with parental HC11 cells and vector sense-control cells (Fig. 6B). Specifically, 15–21% of parental or vector sense-control cells were in S-phase during supern confluent conditions, whereas ~30% of antisense Jak2 clones A and B remained in S-phase.

We also introduced Dn-Jak2 into HC11 cells by adenoviral gene transfer and assessed its effect on cell cycling. Supern confluent cells that had been serum-deprived for 48 h were cycling only to a very low extent (<4% in S or G2/M) in mock infected or control virus-infected cells (Fig. 6C, left two panels). However, a dose-dependent increase in the fraction of cycling cells was observed in supern confluent, serum-deprived cells overexpressing Dn-Jak2 (Fig. 6B, right two panels). These observations are consistent with a general growth-suppressive effect of the Jak2-Stat5 pathway in HC11 cells. The data are also consistent with reduced contact inhibition following suppression of Jak2.

Inhibition of Jak2 Suppresses Apoptosis of HC11 Cells Induced by Anchorage-independent Culture Conditions—The HC11 cell is a nontransformed mammary epithelial cell line that does not survive under anchorage-independent conditions. To investigate whether inhibition of Jak2 would affect the rate of apoptosis induced by culture under anchorage-independent culture conditions, we first examined apoptosis rates in HC11 cells stably expressing antisense Jak2. Parental HC11 cells, Vs-control cells, and clones A and B were cultured on 0.8% agar in normal growth medium, collected after 12 and 36 h, and assayed for apoptosis by flow cytometry. The hypodiploid fraction of HC11 cells at 12 h was markedly lower in Jak2-suppressed clones A and B than in parental or vector control cells (Fig. 7A), and although the number of apoptotic cells increased in both control cells and Jak2-suppressed cells over the next 24 h, Jak2-suppressed cells showed consistently reduced rates of apoptosis. Examination of apoptosis in parallel samples using TUNEL staining of fragmented DNA at 12 h verified reduced number of apoptotic cells in Jak2-suppressed clones, whereas phase-contrast and DAPI staining verified equal cell numbers in the selected fields (Fig. 7B). However, extended cultures revealed that suppression of Jak2 levels in HC11 cells did not confer long term survival under anchorage-independent culture conditions (data not shown).

Parallel studies of HC11 cells grown under anchorage-dependent conditions also provided evidence for reduced apoptosis following delivery of Dn-Jak2 into confluent cultures, as demonstrated by TUNEL staining of cells undergoing DNA fragmentation (Fig. 7C). Representative fields from cell cultures presented by phase-contrast, TUNEL, and DAPI staining, showed that a larger number of HC11 cells underwent apoptosis when infected with Adv-control compared with Adv-Dn-Jak2 (Fig. 7C). We conclude from these experiments that suppression of Jak2 in HC11 cells inhibits cellular apoptosis under several culture conditions. Thus, the hyperproliferative phenotype of HC11 cells associated with suppression of Jak2 also involved anti-apoptotic elements.

The Hyperproliferative Phenotype Resulting from Jak2 Supp...
Fig. 5. Suppression of Jak2 tyrosine kinase in Jak2 antisense expressing HC11 cell clones is associated with a hyperproliferative phenotype. A, increased growth rate in Jak2 antisense expressing HC11 clones A and B. The growth rates of parental HC11 cells, vector-sense (Vs) control expressing control clone, and Jak2 antisense-expressing clones A and B were compared by plating cells at the same low density and following cell numbers over 72 h. Cell numbers were counted manually in a hemacytometer, and the data represent means of three independent experiments (S.E. indicated by bars). B, increased growth rate in Jak2-suppressed HC11 cells was associated with increased number of mitotic figures. Exponentially growing cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and Jak2 antisense expressing clones A and B were stained with propidium iodide to better visualize dividing cells (indicated by arrows). C, increased growth rate in Jak2-suppressed HC11 cells was associated with increased number of cycling cells by flow cytometry. Exponentially growing cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and Jak2 antisense-expressing clones A and B were stained with propidium iodide and analyzed by flow cytometry to determine fraction of cells in the various stages of cell cycle.
FIG. 6. Suppression of Jak2 in stable Jak2 antisense expressing clones of HC11 cells is associated with reduced contact inhibition. A, increased saturation density of HC11 clones A and B. Superconfluent cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and Jak2 antisense-expressing clones A and B were counted to determine cell saturation density. Cell numbers were counted in duplicate in three independent experiments. Values represent mean cell number/cm², and bars represent S.E. B, increased fraction of cycling cells in superconfluent cultures of HC11 clones A and B. Superconfluent cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and Jak2 antisense-expressing clones A and B were stained with propidium iodide and analyzed by flow cytometry. C, dose-dependent increase in cycling cells in superconfluent cultures of HC11 cells exposed to Adv-Dn-Jak2. Superconfluent cultures of parental HC11 cells were mock infected, or infected with either Adv-Control (no insert; m.o.i. 25), or with two increasing doses of Adv-Dn-Jak2 (m.o.i. 5 and 25). Forty-eight h after infection and maintenance under serum-free conditions, cells were stained with propidium iodide and analyzed by flow cytometry.
expression in HC11 Cells Is Associated with Constitutive Activation of Stat3—The observed hyperproliferative and undifferentiated phenotype of HC11 mammary epithelial cells with disrupted Jak2-Stat5 signaling is intriguing in light of the general loss of differentiation associated with progressing breast cancer cells. Because Stat3 has been shown to be an oncogene (23) and to be constitutively activated in human breast cancer (24), we examined the effect of Jak2 suppression on basal Stat3 activation in HC11 cells. Interestingly, Western blot analysis of whole cell lysates from subconfluent HC11 cells indicated that Stat3 was constitutively active in hyperproliferative clones A and B, as determined by increased Stat3 phosphotyrosine levels in the absence of increased Stat3 levels (Fig. 8A).

Similar results were obtained with introduction of Dn-Jak2 into HC11 cells by adenoviral transfer. Dn-Jak2 also led to constitutive activation of Stat3 as measured by Stat3 phosphotyrosine and Stat3 protein immunoblotting (Fig. 8B). The effect was dose-dependent and was not induced by infection with control adenovirus. Based on two independent molecular approaches, we conclude that the hyperproliferative phenotype of Jak2-suppressed HC11 cells correlated with constitutive activation of Stat3. Thus, the possibility exists that constitutive activation of Stat3 is involved in the hyperproliferative phenotype of the cells associated with Jak2 suppression. Further investigations into the role of Stat3 in proliferation of HC11 cells, and the relationship between Stat3 activation and suppression of the Jak2-Stat5 pathway, are underway.

**DISCUSSION**

The present study used adenoviral delivery of dominant-negative Jak2 and stable expression of Jak2 antisense mRNA to identify Jak2 as a critical mediator of prolactin-induced differentiation of nontransformed HC11 mammary epithelial cells. The associated disruption of prolactin-induced Stat5 activation most likely disrupts the key molecular mechanism responsible for disrupting prolactin-induced differentiation. Furthermore, targeted suppression of Jak2 in HC11 cells led to a hyperproliferative phenotype, suggesting that Jak2 exerts a growth-inhibitory influence on normal mammary epithelial cells. Whereas suppression of Jak2 did not lead to transformation of HC11 cells, Jak2 suppression did,
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however, reduce contact inhibition and extend cell survival under anchorage-independent growth conditions. Stat3 was constitutively activated in hyperproliferative, Jak2-suppressed HC11 mammary epithelial cells, and this activation of Stat3, a known oncogene (23), may contribute to the hyperproliferative phenotype. Jak2 as a Mediator of Prolactin-induced Differentiation—We originally identified Jak2 as the prolactin-associated tyrosine kinase in Nb2 lymphoma cells (3, 25). Although other Jak tyrosine kinases reportedly are not activated by prolactin receptors, numerous studies indicate that prolactin may activate other, non-Jak tyrosine kinases. These include tyrosine kinases Fyn (10), Src (11), Tec (13), and focal adhesion kinase (12). In most cases, temporal activation profiles suggest that prolactin activates Jak2 upstream of the non-Jak tyrosine kinases, but at least one report indicated that prolactin can activate Src independent of Jak2 (26). Consequently, the notion that all prolactin-induced effects are mediated by Jak2 may not be correct. Therefore, it was of particular importance to determine whether Jak2 mediates prolactin-induced differentiation of mammary epithelial cells.

The present study now provides direct evidence that Jak2, in fact, is critical for mammary epithelial cell differentiation. After establishing that prolactin activated Jak2, and not other Jak, in HC11 cells, and that concentration-dependent induction of cell differentiation by prolactin correlated with Jak2 activation, two independent strategies were used in vitro for targeted suppression of Jak2. These included construction of two sets of novel reagents, a vector for stable or transient expression of a novel and specific Jak2 antisense-mRNA, and a replication-defective adenovirus for efficient gene delivery of Dn-Jak2. Both sets of molecular tools were validated and determined to be effective in independent experiments, and both strategies independently demonstrated that Jak2 is critical for prolactin-induced differentiation by selectively disrupting mammosphere formation. Disruption of prolactin-induced differentiation was not associated with any general cytotoxic effects, because suppression of Jak2 by either strategy led to increased proliferation rates and reduced cellular apoptosis. Furthermore, the described molecular tools may be applied to determine whether Jak2 is critical for biological effects of other cytokine receptors in a variety of cell types.

Evidence That Jak2 Phosphorylates and Activates Stat5 in HC11 Cells—Transcription factor Stat5, and especially the Stat5α isoform, is critical for mammary gland differentiation (6). Stat5 is phosphorylated on a single tyrosine residue following prolactin receptor activation, and this modification causes Stat5 to dimerize, which in turn is needed for DNA binding and transcriptional regulation (4). Jak2 is presumed to mediate prolactin-induced tyrosine phosphorylation of Stat5 (22), although Stat5 can also be phosphorylated by the Src tyrosine kinase (27). In the present study, we determined that suppression of Jak2 activity blocked prolactin-stimulated Stat5 tyrosine phosphorylation in HC11 cells, both by immunoblotting and by immunocytochemistry using an anti-phosphotyrosine-Stat5 antibody. Specifically, in Jak2-suppressed HC11 clones, Stat5 activation was significantly down-regulated. Furthermore, Stat5 activation was inhibited in a dose-dependent manner by Dn-Jak2 delivered by adenoviral infection. These findings support a view of the Jak2-Stat5 pathway as a differentiation-inducing axis in mammary epithelial cells.

Targeted Inactivation of Jak2 Resulted in Hyperproliferative Phenotype of HC11 Cells—The Jak2-deficient HC11 clones showed significantly increased growth rates, loss of contact inhibition, and prolonged survival under anchorage-independence culture conditions. Although suppression of Jak2 in HC11 cells was associated with loss of Stat5 activation, Stat3 became constitutively tyrosine-phosphorylated. Constitutive activation of Stat3 was observed both in HC11 clones stably expressing the Jak2 antisense construct and in cells overexpressing Dn-Jak2 by adenoviral delivery, raising the possibility that the Jak2-Stat5 pathway normally inhibits Stat3 activation in HC11 cells. Interestingly, a similar mutual exclusion of Stat5 activation and Stat3 activation has been observed in mammary epithelial cells within the physiological setting of mammary gland involution (28). Weaning, or artificially induced milk stasis, rapidly shuts off Stat5 activation in lactating mammary epithelial cells, and Stat3 becomes activated instead (29). Although the importance of Stat3 activation in hyperproliferative HC11 cells remains to be determined, this observation is particularly intriguing in light of the established tumor-promoting role of Stat3 (23).

In summary, the present work demonstrated that Jak2 is critical for prolactin-induced differentiation of HC11 mouse mammary epithelial cells. Equally important, the data are also consistent with an overall growth-inhibitory role of the Jak2-Stat5 pathway in mammary epithelial cells. This notion is of direct relevance to mammary tumorigenesis, because the data suggest a tumor-suppressive role of the Jak2-Stat5 pathway in the mammary gland. In general, cancer cells are characterized by enhanced growth and reduced levels of differentiation. Future work will address the possibility that progression of mammary and breast tumors involve a gradual loss of Jak2-Stat5 signaling, with a consequent increase in Stat3 activation.

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