Targeting Janus Kinase 2 in Her2/neu-Expressing Mammary Cancer: Implications for Cancer Prevention and Therapy

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Abstract

The Janus kinase 2 (Jak2) is essential for normal mammary gland development, but this tyrosine kinase and its main effector, signal transducer and activator of transcription 5, are also active in a significant subset of human breast cancers. We have recently reported that Jak2 controls the expression and nuclear accumulation of cyclin D1. Because this particular D-type cyclin has been suggested to be a key mediator for ErbB2-associated mammary tumorigenesis, we deleted *Jak2* from ErbB2-expressing mammary epithelial cells prior to tumor onset and in neoplastic cells to address whether this tyrosine kinase plays a role in the initiation as well as progression of mammary cancer. Similar to cyclin D1–deficient mice, the functional ablation of Jak2 protects against the onset of mammary tumorigenesis. In contrast, the deletion of *Jak2* from neoplastic cells or the acute, ligand-inducible down-regulation of this tyrosine kinase in an orthotopic transplant model did not affect the growth and survival of cancer cells. The constitutive activation of ErbB2 signaling, which is an initial event in the formation of mammary cancer, was able to override the functional role of Jak2 in regulating the expression of Akt1 and cyclin D1. This might be a compensatory mechanism that explains why Jak2 is a relevant target for preventing the initiation but not the progression of ErbB2-associated mammary cancer. [Cancer Res 2009;69(16):6642–50]

Introduction

In the developing mammary gland, the Janus kinase 2 (Jak2) is essential for the tyrosine phosphorylation and activation of the signal transducer and activator of transcription 5 (Stat5a and Stat5b) in response to prolactin signaling (1, 2). Developmental studies on genetically engineered mice which lack Jak2 and Stat5 conditionally at defined stages of mammogenesis showed that the Jak2/Stat5 signaling pathway plays a pivotal role in the proliferation and differentiation of alveolar cells during pregnancy (2, 3). Experimental evidence from different laboratories shows that PRL signaling through the Jak2/Stat5 pathway is important for the expression and functionality of cyclin D1 in proliferating mammary epithelial cells. It has been suggested that PRL-R signaling might *indirectly* modulate cyclin D1 expression through up-regulation of insulin-like growth factor-II (4). The expression of cyclin D1 is also *directly* regulated by PRL signaling through

the Stat5-mediated transcriptional activation of the cyclin D1 promoter (5). Our own studies using Jak2-deficient mammary epithelial cells and their isogenic wild-type controls suggest that signaling through this Janus kinase controls not only the expression of the cyclin D1 mRNA but, more importantly, Jak2 regulates the accumulation of the cyclin D1 protein in the nucleus through modification of the Akt1/GSK3β pathway, which mediates the phosphorylation and nuclear export of cyclin D1 (6). The notion that cyclin D1 is a key target of the Jak/Stat pathway is supported by the fact that females deficient in cyclin D1 exhibit impaired mammary gland development similar to Jak2 and Stat5 conditional knockout mice (7, 8).

The most pertinent functions of the PRL-R/Jak2/Stat5 signaling cascade and cyclin D1 are confined to alveolar progenitors that reside at the terminal ends of the ductal tree, a region known as lobular units in mice or terminal duct lobular units in humans (9). This specific region in the breast seems to contain hormone-responsive epithelial cells that exhibit an elevated susceptibly to neoplastic transformation depending on the reproductive status and the genetic predisposition. We previously demonstrated that in mice, alveolar progenitors facilitate mammary tumorigenesis in multiparous females overexpressing ErbB2 (Her2/neu; ref. 10). A growth inhibition of this epithelial subtype through targeted deletion of the *cyclin D1* gene has been shown to significantly reduce the onset of oncogenic Ras and ErbB2-induced mammary tumorigenesis (11, 12). Because signaling through Jak2 is important for the expression and nuclear accumulation of cyclin D1 in mammary epithelial cells, it was reasonable to propose that, like cyclin D1 deficiency, lack of Jak2 might significantly delay or abolish the occurrence of ErbB2-associated mammary cancer in mice. To address this hypothesis, we generated two mouse models that overexpress the wild-type ErbB2 receptor in mammary epithelia that are conditionally deficient in Jak2. In addition to examining whether signaling through Jak2 and Stat5 contributes to mammary cancer initiation, the targeted deletion of the *Jak2* gene in fully neoplastic cells permitted us to determine whether inhibiting the activation of this pathway is also therapeutically relevant to treat the established disease. The key findings of this study clearly show that the importance of Jak2 as a preventive or therapeutic target for breast cancer varies greatly during tumor initiation and progression.

Materials and Methods

Mouse models. Mouse mammary tumor virus (MMTV)-Cre and WAP-Cre transgenics (13) as well as Rosa-LacZ reporter mice (14) were crossed with Jak2 conditional knockout mice. The generation of genetically engineered animals with *Jak2* conditional knockout alleles (*Jak2*$^{fl/fl}$) and the PCR protocols to determine the presence of the *Jak2* floxed, *Jak2* recombined/null, and *Jak2* wild-type alleles have been described previously (2, 15). Mice overexpressing the unactivated rat ErbB2 under regulation of the MMTV long-terminal repeat (16) were obtained from The Jackson
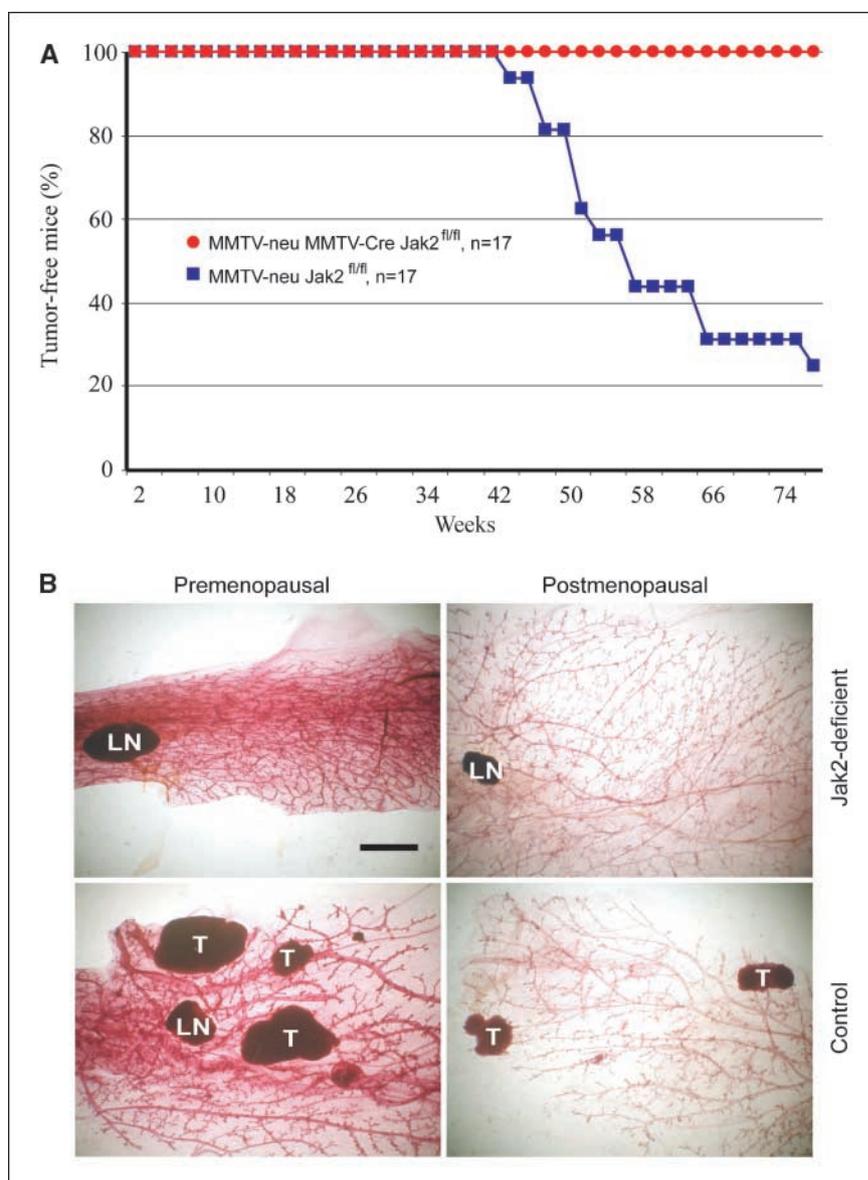
doi:10.1158/0008-5472.CAN-09-0746

Figure 1. Complete inhibition of ErbB2/Neu-induced mammary tumorigenesis through deletion of *Jak2. A,* Kaplan-Meier survival curve. *B,* mammary gland whole-mounts from premenopausal and postmenopausal Jak2-deficient females and their controls. *T,* neoplastic lesion; *LN,* lymph node; *bar,* 0.5 cm.

Laboratory. The targeted alleles and transgenes were carried in an FvB background (>50–97%), which facilitates Her2/neu-associated mammary tumorigenesis (10, 17). All animals used in this study were treated humanely and in accordance with institutional guidelines and federal regulations.

Cell cultures and expression vectors. Mammary cancer cells were derived from tumor-bearing MMTV-neu *Jak2^{fl/fl}* females and cultured in DMEM/F12 medium as described previously (6). The generation of a retroviral vector expressing Cre recombinase was published by Krempler and colleagues (18). Forty-eight hours after infection, cells were selected in complete medium containing increasing concentrations (3–7 μg/mL) of puromycin (Sigma). Mammary cancer cells were treated with 10 nmol/L of PRL for 20 min to induce the phosphorylation of Stat5. A retroviral construct expressing the tetracycline-controlled transactivator (*tTA*) was cloned by inserting the *tTA* coding sequence into the blunted *Eco*RI site of the pBabe-puro vector. A myc-tagged cDNA of human *Jak2* was cloned into the blunted *Bam*HI site of the retroviral pRevTRE vector (Clontech Laboratories, Inc.). After generating hygromycin (100 μg/mL) and puromycin double-resistant clones that express exogenous Jak2 in a doxycycline-controlled manner, we used the AdCreGFP vector (Vector BioLabs) to knock out both endogenous *Jak2* alleles. A count of vital

cells as well as a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth assay was performed according to a standard protocol (18). HC11 cells were cultured as described previously (19) and transfected with the pcDNA3 expression vector containing the activated mutant form of the ErbB2/neu oncogene (kindly provided by Dr. W.J. Muller, Royal Victoria Hospital, Montreal, Quebec, Canada). Cells were treated for 48 h with the Jak2 tyrosine kinase inhibitor tyrphostin (AG490; 50 μmol/L; Calbiochem).

Immunostaining. A basic protocol for immunohistochemistry on paraffin-embedded mammary gland specimens was described elsewhere (2). We used the following primary antibodies: α-cyclin D1 (Ab-4) from Lab Vision, α-pAkt1 (Ser[473]; 736E11) and α-ErbB2 from Cell Signaling Technology, α-phosphorylated Stat5A/B (Tyr[694/699]) from Upstate Biotechnology, and α-Ki-67 (Tec-3) from Dako. For visualization of the specific targets, we used corresponding biotinylated secondary antibodies and Vectastain Elite ABC kits (Vector Laboratories).

Immunoprecipitation and Western blot analysis. The preparation of whole-cell extracts of clarified cell lysates and tissue homogenates as well as the experimental procedures for immunoprecipitation and Western blot analysis were described previously (6).
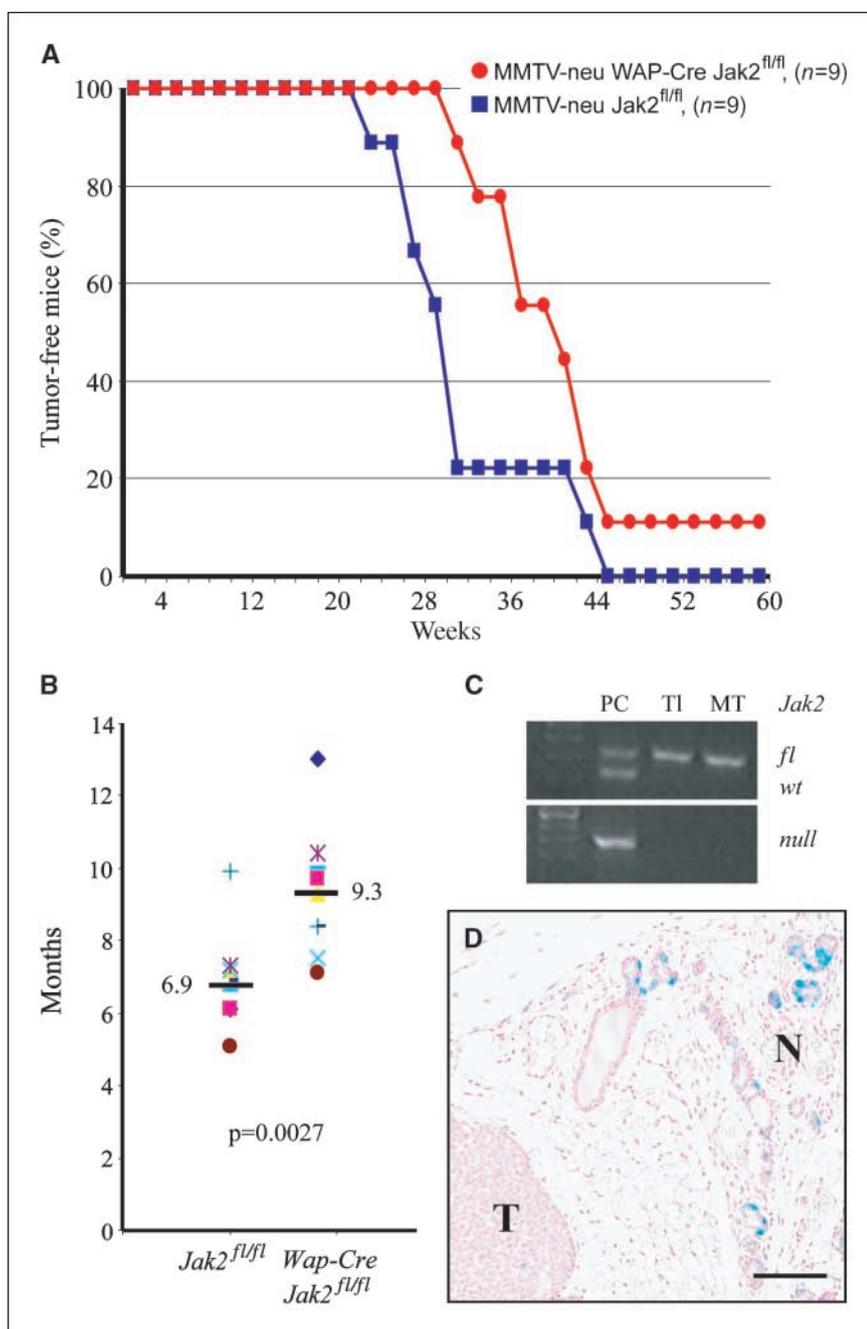
Figure 2. The onset of MMTV-neu–induced mammary cancer requires Jak2. *A,* Kaplan-Meier survival curve. *B,* mean age of tumor onset (*horizontal bars*). Each marker represents the age of onset of the first palpable tumor per mouse (*P* value, *t* test). *C,* PCR assay to detect the *Jak2* floxed allele and the recombined *Jak2*-null allele in genomic DNA of a mammary tumor (*MT*) from a MMTV-neu WAP-Cre *Jak2$^{fl/fl}$* female; *Tl,* tail DNA of tumor-bearing animal; *PC,* positive control (mammary tissue of WAP-Cre *Jak2$^{fl/wt}$* female). *D,* histologic section of a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–stained mammary cancer (*T*) with adjacent normal tissue (*N*) from a multiparous MMTV-neu WAP-Cre *Jak2$^{fl/fl}$* female (*bar,* 100 μm).

Results

Expression of ErbB2, cyclin D1, and pAkt1 in MMTV-neu transgenic mammary epithelia that lack Jak2. We generated a mouse model that overexpresses the wild-type ErbB2 receptor in mammary epithelia that are conditionally deficient in Jak2 to address whether this member of the Janus kinase family is critical for mammary cancer initiation and progression. In the initial phase of this study, we examined possible effects of Jak2 deficiency on the expression of the transgenic ErbB2 (Her2/neu) receptor, which is driven by the MMTV long-terminal repeat in this animal model (16). The expression of MMTV-neu, which is confined to the mammary epithelium, was examined by immunohistochemistry in mammary glands of Jak2-deficient females (MMTV-neu MMTV-Cre *Jak2$^{fl/fl}$*) and their littermate

controls expressing Jak2 (MMTV-neu *Jak2$^{fl/fl}$*) on day 11.5 of pregnancy (Supplementary Fig. S1). We preferred this experimental approach over Western blot analysis because the ratio of stromal cells and epithelial cells are not identical in Jak2 conditional knockout mice and their controls. In addition, the expression of the MMTV-neu transgene, which is elevated by pregnancy, may vary between epithelial subtypes. As shown in Supplementary Fig. S1*A*, ErbB2 was expressed highly in epithelial cells of ducts and alveoli in mammary glands of Jak2 wild-type females. In particular, the developing alveoli exhibited a slightly stronger immunoreactivity compared with ducts. The transcriptional activation of the MMTV long-terminal repeat *in vivo* does not require active Stat5a, the predominant Stat protein in the mammary gland (20). This might explain why Jak2 deficiency did

not abolish the MMTV-driven expression of ErbB2 (Supplementary Fig. S1*C*). However, Jak2 conditional knockout mice lack developing alveoli during pregnancy, and consequently, they did not exhibit an elevated expression of transgenic ErbB2 in this epithelial subtype during pregnancy. It is evident from the histologic analysis that the overexpression of the wild-type ErbB2 receptor did not restore the expression and nuclear localization of cyclin D1 or the activation of Akt1 (Supplementary Fig. S1*E–L*) and it did not rescue the developmental defect caused by Jak2 deficiency.

Jak2 deficiency in the epithelial compartment prior to tumor onset protects against ErbB2/neu-associated mammary cancer. After confirming that Jak2 deficiency does not abolish the expression of the MMTV-neu transgene in the mammary epithelium, we monitored a cohort of 17 Jak2 knockout females expressing MMTV-neu and 17 Jak2-expressing littermate controls over a period of 18 months to determine whether lack of Jak2 prevents Her2/neu-associated mammary tumorigenesis. As illustrated in the Kaplan-Meier survival blot (Fig. 1*A*), not a single Jak2 knockout female developed a palpable mammary lesion within the experimental timeline. In contrast, the vast majority of control mice exhibited at least one solid tumor. In addition to these primary cancers, we collected unaffected mammary glands from both groups at the experimental end point (i.e., the day of tumor resection or after 18 months in tumor-free animals). The whole-mount analysis of these glands revealed that premenopausal or postmenopausal Jak2 conditional knockout mice did not develop any microscopic lesions (Fig. 1*B*), suggesting that Jak2 deficiency prevents very early events that occur during neoplastic progression. In contrast, the vast majority of glands from premenopausal Jak2-expressing controls contained at least one, frequently two or more,

cancerous lesions. Multiple microscopic tumors were also observed in the few postmenopausal control females that did not exhibit any palpable lesions by 18 months of age.

Using a genetic labeling approach, we previously showed that MMTV-neu–induced mammary tumors arise predominantly from hormone-responsive alveolar progenitors (10). Because alveolar bud formation in nulliparous Jak2-deficient females is impaired (2), it was therefore possible that Jak2 deficiency simply eliminates the epithelial subtype that is required for tumorigenesis in the MMTV-neu model. To address whether Jak2 deficiency or simply the absence of alveolar progenitors is responsible for the lack of mammary tumors in this model, we assessed the onset of tumorigenesis in females that specifically lack Jak2 in alveolar progenitors that undergo a pregnancy-mediated differentiation program (Fig. 2). For this experimental approach, we used the WAP-Cre transgene, which is expressed in alveolar cells during later stages of pregnancy. During pregnancy, this transgene also targets alveolar cells, which remain in the mammary gland after completion of the first gestation cycle (21). In contrast to the MMTV-Cre–based model, a WAP-Cre–mediated deletion of *Jak2* in females carrying the MMTV-neu oncogene occurs *after* the formation and numerical expansion of alveolar cells. A subset of nine multiparous MMTV-neu WAP-Cre *Jak2^{fl/fl}* females and nine multiparous Jak2-expressing littermate controls were monitored for the occurrence of mammary tumors over a period of 18 months. It is evident from the Kaplan-Meier survival blot (Fig. 2*A*) and the average age when tumors became palpable (Fig. 2*B*), that the deletion of *Jak2* in a subset of alveolar cells was sufficient to delay mammary carcinogenesis. It is important to note that MMTV-neu–induced mammary carcinogenesis requires an FvB genetic background (17), and the expression of the WAP-Cre transgene

Figure 3. Jak2 deficiency does not inhibit the growth of neoplastic Her2/neu-expressing mammary cells. *A,* PCR assay and Stat5 immunoprecipitation/Western blot analysis to verify the complete ablation of *Jak2* from Her2/neu-expressing cancer cells. *B,* expression analysis of D-type cyclins (*Ccnd*) and cyclin E (*Ccne*) in Jak2-deficient cancer cells and their controls. β-Actin (*ActB*) served as a loading control. *C,* growth of Her2/neu-expressing mammary cancer cells lacking Jak2 and their isogenic controls in an orthotopic transplant of athymic nude mice. *D,* PCR assays to verify the presence of *Jak2* floxed alleles or *Jak2* null alleles in individual mammary tumors after orthotopic transplantation.
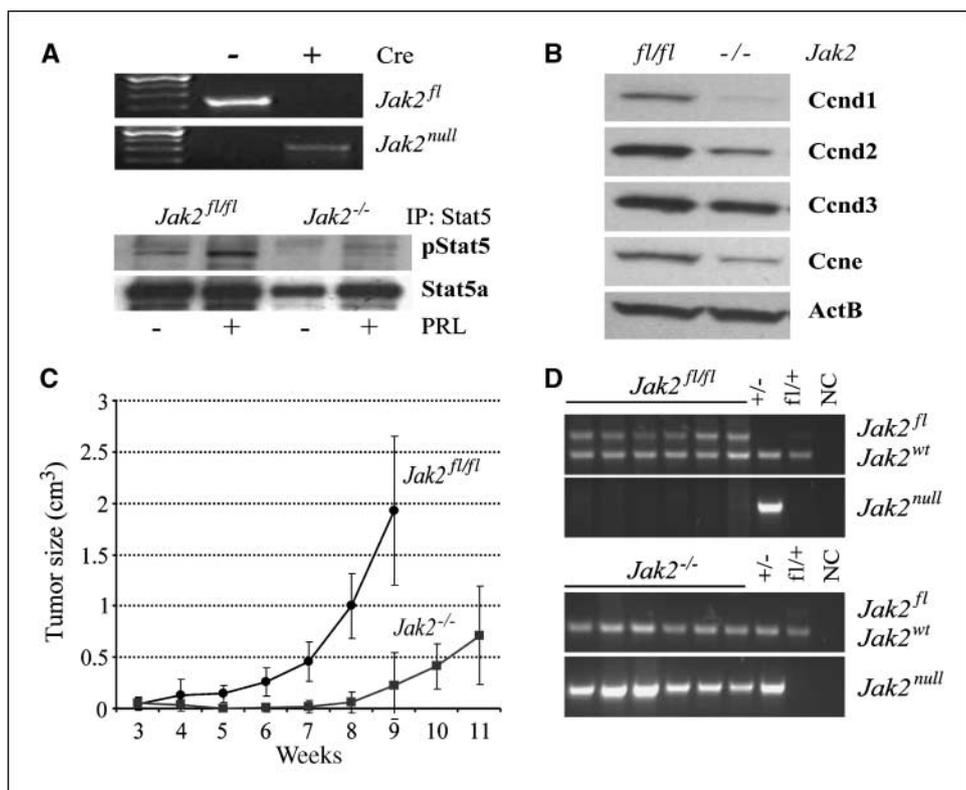
is very mosaic in this particular strain compared with other genetic backgrounds.[3] Using PCR analyses on tumors derived from MMTV-neu WAP-Cre Jak2^{fl/fl} females, we observed that mammary cancers arose solely from epithelial cells that exhibited an unrecombined Jak2 floxed allele (Fig. 2C). The assumption that tumors originated from Jak2-expressing cells was confirmed in experimental animals that also carried the Rosa-LacZ Cre/lox reporter allele in addition to the MMTV-neu transgene in the Jak2 conditional knockout. As shown in Fig. 2D, LacZ-positive alveolar progenitors were present in normal epithelial cells (N) adjacent to the tumor (T). The primary lesion itself did not contain any β-galactosidase–expressing tumor cells, suggesting that these cells were neoplastic descendents from normal cells that did not express Cre recombinase. The inclusion of a larger cohort of 18 primiparous females into this study revealed that there was no statistically significant difference in tumor latency between primiparous and multiparous Jak2-deficient mice (Supplementary Fig. S2A). This suggests that, unlike in the Jak2 wild-type controls (Supplementary Fig. S2B), additional gestation cycles did not accelerate tumorigenesis in the conditional knockouts. This interesting observation supports the notion that alveolar progenitors, which multiply during subsequent gestation cycles in a Jak2/Stat5-dependant manner, are the primary targets for neoplastic transformation in this breast cancer model.

Jak2 is not required for ErbB2/neu-driven mammary cancer progression. Because Jak2 plays an important role in mammary cancer prevention, we subsequently needed to address whether Jak2 is also required for the growth and proliferation of ErbB2-overexpressing cancer cells in order to make a prediction about the applicability of targeting Jak2 in a therapeutic setting. For this purpose, we cultured mammary cancer cells derived from Her2/neu-expressing control females (MMTV-neu Jak2^{fl/fl}) and introduced the Cre recombinase through retroviral gene transfer. This approach resulted in the generation of isogenic cancer cell lines with or without Jak2. After the initial selection of these cells and their wild-type controls carrying a pBabe control vector with puromycin, we noticed that Jak2-deficient cells did not undergo growth arrest and resumed normal proliferation. A PCR assay and immunoprecipitation/Western blot analysis confirmed that Jak2 was absent and lacked a PRL-induced phosphorylation of Stat5a in Cre-expressing cells (Fig. 3A). The data from this immunoblot analysis does not only verify that these cells lack functional Jak2, it also suggests that an elevated level of oncogenic ErbB2 signaling does not lead to an activation of Stat5 in the absence of Jak2. Because the continuous expression of the oncogene is required for the survival of ErbB2-associated cancer cells (22), it is evident that lack of Jak2 had no effect on the expression of the MMTV-neu transgene. The analysis of Jak2-deficient tumor cells and their controls revealed that the ablation of Jak2 resulted in a significant decrease in expression of cyclin D1 and cyclin D2, and to a lesser degree, cyclin D3 (Fig. 3B). Despite the consequential reduction in the level of cyclin E, these cells exhibited a normal numerical expansion in culture. To assess whether Jak2 is required for tumor growth in vivo, we transplanted 5×10^5 tumor cells with and without Jak2 into the collateral no. 4 fat pads of athymic nude mice. The Jak2-expressing controls exhibited a slightly better engraftment, which resulted in an accelerated expansion of the resulting tumors (Fig. 3C). Despite a delay in engraftment,

Jak2-deficient cells were able to form palpable lesions, and the subsequent analysis of genomic DNA from these tumors revealed that they were all comprised of cancer cells that carried Jak2-null alleles (Fig. 3D). The Jak2 wild-type allele, which was present in the PCR assay in both experimental groups, originated from stromal cells and blood vessels of the wild-type host. This allele was not present in the engrafted Jak2^{fl/fl} or Jak2^{−/−} cancer cells.

The delayed engraftment of Jak2-deficient cells and the smaller size of the resulting mammary tumors at similar time points might imply that the ablation of this kinase reduced the growth of tumor cells in vivo. Surprisingly, the histopathologic examination of both types of cancers showed that Jak2-deficient cells had many mitotic figures and even a significantly higher average number of Ki67-positive cells compared with their wild-type controls (Supplementary Fig. S3). In addition, cancer cells lacking Jak2 exhibited an elongated shape that resulted in a higher density of cells within a given tumor volume. These observations clearly suggest that Jak2 deficiency does not reduce the proliferative capacity and growth of Her2/neu-expressing cancer cells in vivo.

The acute, ligand-mediated down-regulation of Jak2 does not affect the growth and survival of mammary cancer cells in vivo. Like their parental cell lines in culture, transplanted Jak2-deficient tumor cells exhibited a markedly reduced expression and nuclear localization of activated Stat5 in response to prolactin stimulation in vivo (Supplementary Fig. S4). The expression of cyclin D1, however, varied greatly between tumors within the same experimental group, and consequently, there was no consistent difference in the expression of this cell cycle regulator between Jak2-deficient tumors and their Jak2-expressing controls. The infiltration of stromal cells and blood vessels into the tumors might cause some variation in the expression of cyclin D1. While performing a clonogenic assay, we also found that individual cancer cells from a primary tumor express different levels of cyclin D1 prior to and after the sustained ablation of Jak2 (Supplementary Fig. S5). Hence, clonal amplifications after cancer cell engraftment might also contribute to variations in the expression of cyclin D1 in the resulting tumor.

As shown in Fig. 3C, the sustained ablation of Jak2 prior to transplantation caused a slight delay in the engraftment of tumor cells. Despite using multiclonal primary cultures in this in vivo assay, it is still possible that the deletion of Jak2 in culture had an effect on the clonality and the outcome of this study. More importantly, we asked whether an acute down-regulation of Jak2 in vivo could cause a different biological response compared with the sustained ablation of Jak2 in culture prior to transplantation. To address these issues, we designed an experimental approach that allowed us to down-regulate the expression of Jak2 in a ligand-controlled manner after cancer cell engraftment in established tumors (Fig. 4A). For this purpose, we generated a mammary cancer cell line from a primary mammary tumor that expresses exogenous Jak2 in a doxycycline-controlled fashion in the absence of both endogenous Jak2 alleles (Fig. 4B). The ligand-induced ablation of Jak2 resulted in reduced expression and activation of Stat5, but the cyclin D1 level did not decrease in these tumor cells after acute down-regulation of Jak2 (Fig. 4C), and Jak2-deficient cells and their Jak2-expressing controls have virtually the same proliferative capacity in culture (Fig. 4D). After transplanting these cells into recipient mice, we administered doxycycline to a subset of animals when mammary tumors became palpable (<0.3 cm in diameter). This approach eliminated variations in tumor cell engraftment between experimental groups. The results of this study show that the acute down-regulation of Jak2 had no significant

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[3] Matulka-Brandl and Wagner, unpublished.

Figure 4. Acute, ligand-mediated down-regulation of Jak2 in Her2/neu-expressing mammary cancer cells that lack both endogenous *Jak2* alleles. *A,* experimental design for the generation of Jak2 conditional knockout cells that express exogenous Jak2 in a doxycycline (*Dox*)-controlled manner. *B* and *C,* Western blot analyses to monitor the doxycycline-regulated repression (*tet-off*) of myc-tagged Jak2 as well as the expression of cyclin D1 and phosphorylation of Stat5a. Immunoprecipitation (*IP*) was carried out with a Stat5a-specific antibody. β-Actin (*ActB*) served as loading control; *wd,* doxycycline withdrawal. *D,* viable cell count over a 5-d period (illustrated in a logarithmic scale) to determine growth rates of the same Her2/neu-expressing cancer cells with and without Jak2 in a doxycycline-dependent manner.
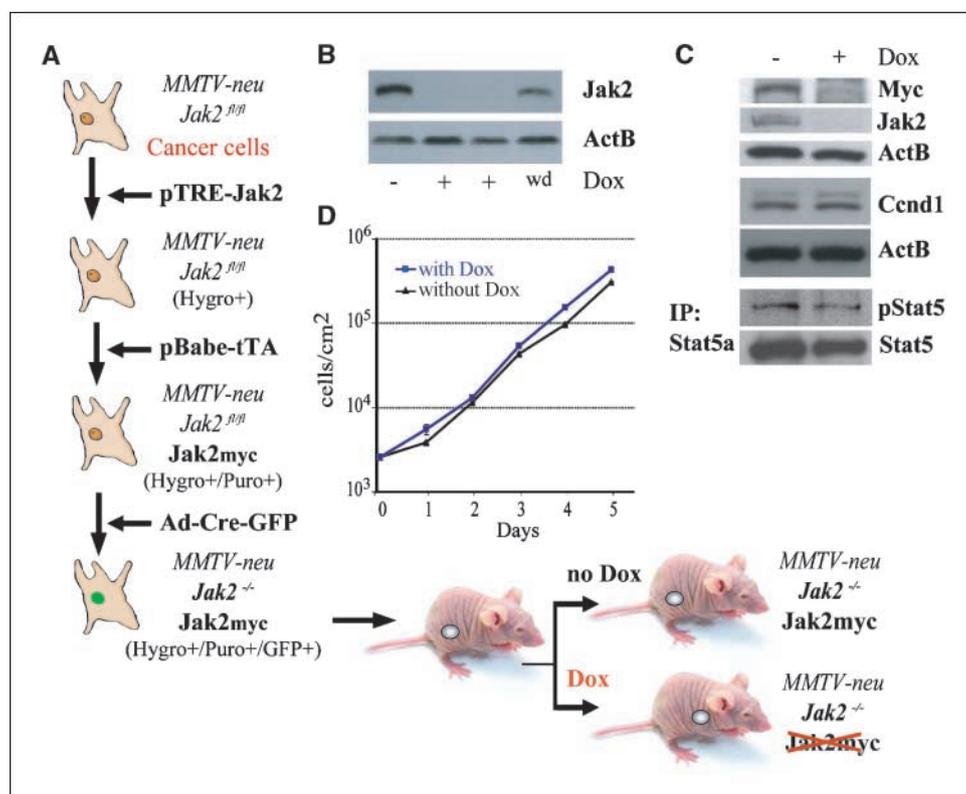
effect on the growth of these cancer cells *in vivo* (Fig. 5*A*). The correct genotype of the resulting tumors was confirmed by PCR (Fig. 5*B*). In addition, we injected PRL intraperitoneally into the mice (5 μg/g of body weight) shortly before tumor resection to verify that cancer cells exhibited reduced levels of active Stat5 as a functional readout for the ligand-inducible down-regulation of Jak2 (Fig. 5*C*). Although myc-tagged exogenous Jak2 was effectively suppressed by the ligand, there was some Jak2 protein still present, albeit at much lower levels, in doxycycline-treated animals (Supplementary Fig. S6*A*). This was expected because this kinase is highly expressed in stromal cells and blood vessels of the host. Similar to the data presented in Supplementary Fig. S3, the histologic examination of the resected tumors showed that Jak2-deficient lesions had a higher density of rapidly dividing cancer cells (Supplementary Fig. S6*B*). Despite eliminating variations in the engraftment of cancer cells, individual mammary tumors still exhibited a variation in cyclin D1 levels in both experimental groups (Supplementary Fig. S6*C*). Although it can be assumed that stromal cells that are present in the tumor express cyclin D1, it is also feasible to propose that the stromal environment and systemic factors in recipient animals are able to modify the expression of this cell cycle regulator within individual cancers. None of the animals from either experimental group exhibited metastases to the lung, which was reported to be the primary organ for the formation of secondary lesions in this animal model (16).

The constitutive activation of ErbB2 signaling supersedes the importance of Jak2 in regulating the expression and activation of Akt1. Based on the findings of our study, we asked two questions. First, why does the functional ablation of Jak2 protect against the onset of ErbB2-induced mammary tumors but not the growth of the progressing neoplastic lesion; and second, what are the compensatory mechanisms that regulate the growth

of mammary cancer cells in the absence of Jak2? Because our data showed that Jak2 was still essential for regulating the activity of Stat5 in ErbB2-expressing mammary cancer cells, other receptor-bound or oncogenic kinases which are suggested to phosphorylate Stat5 in other cell types (e.g., BcrAbl) can be excluded as possible compensatory mechanisms. In addition, we did not see an up-regulation or hyperactivation of Jak1 in response to Jak2 deficiency (data not shown). This suggests that this related Janus kinase, which is aberrantly expressed in a series of human breast cancer cell lines (23), did not substitute for the loss of Jak2.

It is known that mammary tumors that arise in mice expressing the wild-type ErbB2 occur through somatic activating mutations within the ErbB2 transgene itself (24). This initial event in tumorigenesis seemed to be affected in the Jak2 conditional knockout because we did not observe preneoplastic lesions (Fig. 1). We therefore reasoned that the constitutive activation of the ErbB2 receptor itself might override the functional role of Jak2 in regulating the expression and activation of Akt1 as well as the expression of cyclin D1. To experimentally address this hypothesis, we generated immortalized, nontumorigenic mammary epithelial cells (HC11) expressing the activated form of the rat Neu gene (Fig. 6*A*). The constitutive activation of ErbB2 signaling led to a significant up-regulation of Akt1 and cyclin D1 expression. The pharmacologic inhibition of Jak2 using AG490 resulted in an expected decrease in the expression and activation of Akt1 as well as a noticeable down-regulation of cyclin D1 in the control cells. In contrast, the functional ablation of Jak2 signaling had no effect on the expression and activation of Akt1 in HC11 cells expressing the activated Neu receptor tyrosine kinase. Despite a reduction in cyclin D1 upon treatment with AG490, these cells still exhibited a much higher level of this cell cycle regulator compared with uninfected control cells. This observation suggests that constitutive
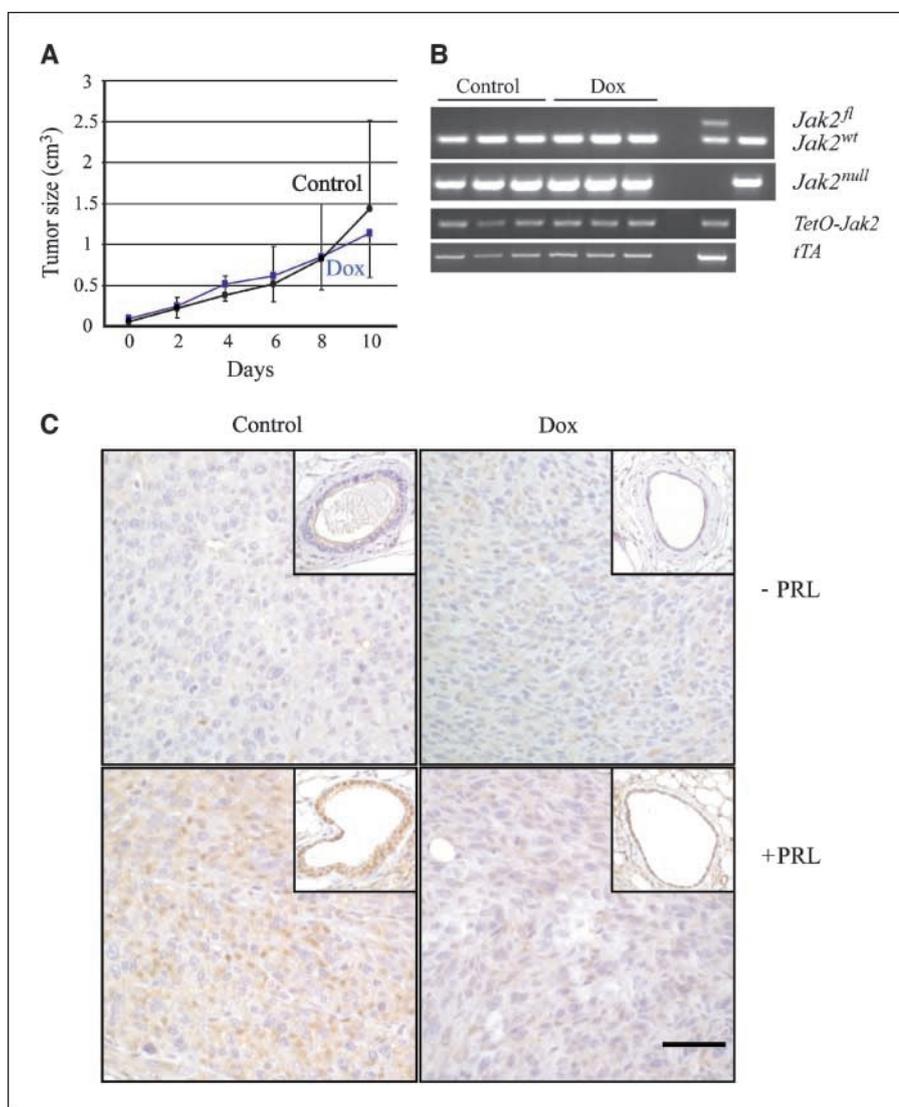
Figure 5. Orthotopic transplantation of mammary cancer cells that express Jak2 in a doxycycline (*Dox*)-regulated manner. *A*, growth of mammary cancer cells that conditionally express Jak2 in athymic nude mice that were fed doxycycline after tumor engraftment and their untreated controls. *B*, PCR assays to verify the correct genotype of cancer cells within individual mammary tumors after orthotopic transplantation and doxycycline treatment. *C*, immunohistochemistry to assess the PRL-mediated activation of Stat5 in control tumors and cancers lacking Jak2. *Insets*, the PRL-induced activation of Stat5 in normal mammary glands derived from tumor-bearing, recipient females. Slides were counterstained with hematoxylin (*bar*, 50 μm).

signaling of ErbB2 is sufficient to supersede the importance of Jak2 in regulating the expression and activation of Akt1. This observation might explain why targeting Jak2 alone is not sufficient to halt the numerical expansion of cancer cells that express the activated ErbB2 receptor.

Discussion

The conditional deletion of *Jak2* prior to neoplastic transformation shows that targeting this tyrosine kinase might be an effective strategy for preventing the onset of Her2/neu-associated breast cancer. We never observed the occurrence of Jak2-deficient mammary tumors in MMTV-Cre and WAP-Cre–based conditional knockout mice. The protective effect of Jak2 deficiency on Her2/neu-induced mammary carcinogenesis does not seem to be simply the result of an ablation of alveolar progenitors which are targets of neoplastic transformation in this breast cancer model. Our observations in multiparous females suggest that Jak2 deficiency might prevent tumor initiation by restricting the repeated numerical expansion of alveolar progenitors in response to the hormonal milieu of pregnancy.

When overexpressed or aberrantly activated, Jak2 and Stat5 may facilitate two important hallmarks of cancer, i.e., evasion from apoptosis and self-sufficiency in growth signals (25). In two recent studies, Iavnilovitch and colleagues provided experimental evidence that the overexpression of wild-type and constitutively active Stat5 can promote the occurrence of sporadic mammary cancers in mice (26, 27). The long latency and the fact that only a small subset of Stat5-overexpressing females developed mammary cancer suggest that Stat5 is a facilitator for other, more potent neoplastic changes that subsequently drive tumor progression. The notion that Stat5 is involved in cancer initiation is also supported by the fact that the ablation of Stat5a delays the incidence of mammary cancer formation in mice that overexpress transforming growth factor-α or the SV40 large T-antigen (28, 29).

Although genomic alterations in *Jak2* and *Stat5* have not been reported in neoplastic lesions of the human breast, active Stat5 is present in a significant subset of cases of breast cancer (30). In a larger clinical study, Nevalainen and colleagues (31) reported that the tyrosine phosphorylation of Stat5 in primary neoplasia was associated with a favorable prognosis and that the activation of Stat5 was frequently lost during metastatic progression. Based

on these and other reports, it was reasonable to propose that the activation of Jak2/Stat5 signaling might play a differential role during tumor initiation and progression (32). Initially, a hyperactive Jak2/Stat5 pathway might facilitate very early stages of neoplastic transformation. Because Stat5 is also known to promote epithelial cell differentiation, its sustained activation, on the other hand, might slow the progression of benign lesions to invasive breast cancer. The availability of the Jak2 conditional knockout model gave us the unique opportunity to experimentally discriminate the roles of Jak2/Stat5 signaling during tumor initiation versus cancer progression. Collectively, our study shows that Jak2 deficiency and the consequential inactivation of its main target, Stat5, do not reduce the proliferative capacity and growth of Her2/neu-expressing cancer cells *in vitro* and *in vivo*. Although it is unlikely that Jak2 could serve as the sole target for the chemotherapy of Her2/neu-positive breast cancers, it is worth investigating in future studies whether inhibiting the functionality of Jak2 can augment the efficacy of trastuzumab or pan-ErbB

tyrosine kinase inhibitors. This current study, however, did not provide clear evidence of whether inhibiting PRL-R signaling promotes EMT or cancer progression as suggested previously (33, 34). In our experimental setting, we did not observe an increase in lung metastasis in recipient females that carried Jak2-deficient tumors.

The underlying mechanism(s) that substitute for the function of Jak2 in neoplastic cells might be specific to certain cancer types (e.g., activation of other Janus kinases, receptor tyrosine kinases, or aberrantly activated cytoplasmic kinases). The differential requirement of Jak2 during tumor initiation and progression in wild-type ErbB2-overexpressing mice cannot be simply explained by a compensatory mechanism that involves Jak1, a Janus kinase that has been implicated in receptor cross-talk in human breast cancer cells (23). For this cancer model, it is important to consider that activating mutations in the transgene leads to a sporadic onset of mammary cancer after a long latency (24). Although the overexpression of wild-type ErbB2 was insufficient to genetically rescue the expression of Akt1 and cyclin D1 *in vivo*, we can show that the constitutive activation of the ErbB2 receptor was able to override the functional role of Jak2 in regulating the expression and activation of Akt1 in normal mammary epithelial cells prior to neoplastic transformation. This suggested that the compensatory mechanism illustrated in Fig. 6*B* may also be crucial for the level of expression of D-type cyclins. Cyclin D1 was highly expressed in normal cells carrying mutant ErbB2/neu, and it remained up-regulated when these cells were treated with a Jak2 inhibitor.

Based on the fact that pregnancy accelerates mammary tumorigenesis in MMTV-neu females expressing Jak2, it is reasonable to suggest that Jak2 deficiency prior to neoplastic transformation restricts the repeated numerical expansion of alveolar progenitors during gestation cycles. This consequently prevents DNA replication errors and the occurrence of genetic changes, including activating mutations in ErbB2. Because signaling through Jak2 regulates the expression of cyclin D1 in untransformed mammary epithelial cells, this might explain why Jak2 and cyclin D1 knockout mice do not develop tumors in females overexpressing wild-type ErbB2 (11, 12). It is interesting to note that, similar to its ability to override Jak2 signaling, the constitutive activation of ErbB2 causes mammary cancer in a significant subset of cyclin D1–deficient females (12). These neoplastic lesions have been reported to bypass the requirement for D-type cyclins altogether through up-regulation of cyclin E. Therefore, this observation challenges the current view about the importance of cyclin D1 in the initiation of ErbB2-associated mammary cancer (11, 35, 36). It is currently impossible to weigh the significance of these individual reports because they lack a description as to whether these particular studies were performed in an FvB genetic background, which is paramount for the occurrence and latency of mammary tumorigenesis in mice overexpressing wild-type ErbB2. More importantly, these previous studies focused primarily on cancer initiation. To model cancer progression and therapy, animal models have to (*a*) develop neoplastic lesions and (*b*) express the therapeutic target (37). From this experimental standpoint, it still remains to be seen whether cyclin D1 is a genuine therapeutic target for ErbB2-expressing mammary cancers *in vivo*. The Jak2 conditional deletion model taught us that the signaling pathways which facilitate mammary tumor initiation do not necessarily possess a similar importance during tumor progression.
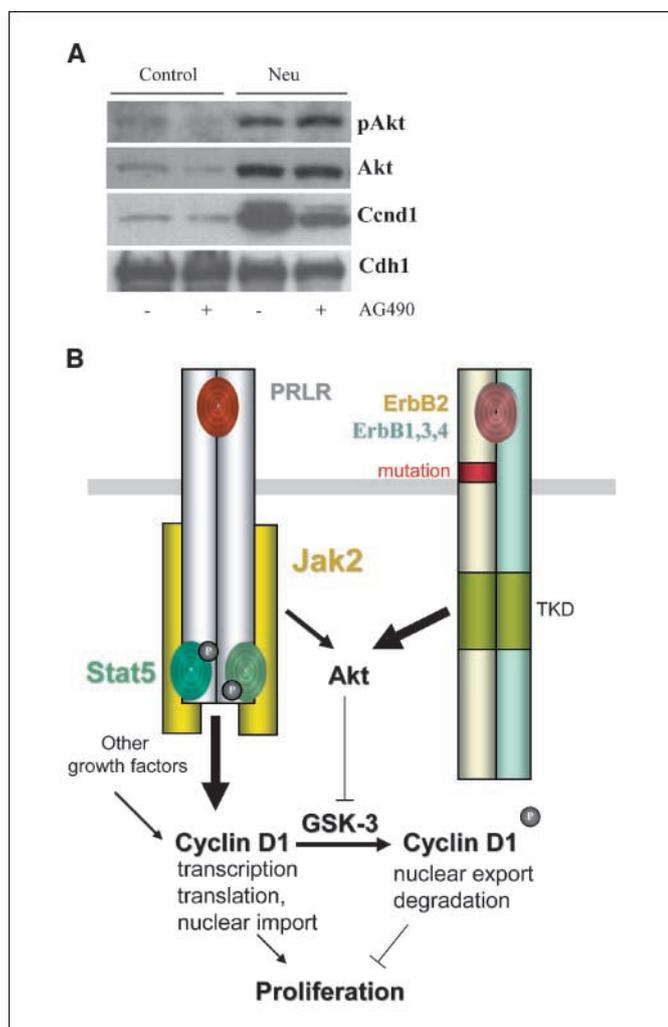

Figure 6. The constitutive activation of oncogenic Her2/neu up-regulates the expression of cyclin D1 and mediates a sustained expression and phosphorylation of Akt1 independent of Jak2 signaling in normal mammary epithelial cells. *A,* Western blot analysis to assess the expression of Akt1 and cyclin D1 in HC11 cells expressing the activated form of Her2/neu and their wild-type controls. Cells were treated with AG490 to inhibit Jak2. E-cadherin (*Cdh1*) served as loading control. *B,* schematic outline of receptor crosstalk that regulates Akt1 and cyclin D1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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