p130Cas as a New Regulator of Mammary Epithelial Cell Proliferation, Survival, and HER2-Neu Oncogene–Dependent Breast Tumorigenesis

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Abstract

To investigate the mechanisms through which p130Cas adaptor protein is linked to tumorigenesis, we generated mouse mammary tumor virus (MMTV)-p130Cas mice overexpressing p130Cas in the mammary gland. MMTVp130Cas transgenic mice are characterized by extensive mammary epithelial hyperplasia during development and pregnancy and by delayed involution at the end of lactation. These phenotypes are associated with activation of Src kinase, extracellular signal-regulated kinase 1/2, mitogen-activated protein kinase, and Akt pathways, leading to an increased rate of proliferation and a decreased apoptosis. A double-transgenic line derived from crossing MMTV-p130Cas with MMTV-HER2-Neu mice expressing the activated form of the HER2-Neu oncogene develops multifocal mammary tumors with a significantly shorter latency than the HER2-Neu parental strain alone. Mammary epithelial cells isolated from tumors of double-transgenic mice display increased tyrosine phosphorylation, c-Src, and Akt activation compared with cells derived from HER2-Neu tumors. In addition, p130Cas down-regulation by RNA interference increases apoptosis in HER2-Neu-expressing cells, indicating that p130Cas regulates cell survival. Consistently with the double-transgenic mice model, p130Cas is overexpressed in a significant subset of human breast cancers and high levels of p130Cas in association with HER2 expression correlate with elevated proliferation. These findings provide evidences for a role of p130Cas as a positive regulator of both proliferation and survival in normal and transformed mammary epithelial cells. Its overexpression contributes to HER2-Neu-induced breast tumorigenesis, thus identifying this protein as a putative target for clinical therapy. (Cancer Res 2006; 66(9): 4672-80)

Introduction

p130Cas, originally identified as a highly phosphorylated protein in cells transformed by v-Src and v-Crk oncogenes, is an adaptor molecule, which upon growth factor receptor and integrin activation functions as a regulator of cell motility and invasion (for reviews, see refs. 1–3). Although many data on the role of p130Cas in basic cell functions have been collected by in vitro analysis, nearly no results exist on its involvement in in vivo development and transformation. This lack of information is due to the fact that the p130Cas knockout animals die at a very early stage of embryonic development, thus preventing a detailed analysis of the role of p130Cas in the animals (4).

The relevance of p130Cas in mechanisms required for transformation was firstly suggested by the inability of activated Src to transform p130Cas-deficient mouse embryo fibroblast. Reintroduction of p130Cas in these cells was sufficient to restore Src-mediated, anchorage-independent growth (5) and the development of an invasive cell phenotype (6, 7). Moreover, the BCAR1 gene, the human hortologue of p130Cas, was isolated for its ability to induce in vitro resistance to antiestrogen drugs (8) in breast cancer cells. Patients with primary breast tumors, which express high levels of p130Cas, seem to experience more rapid disease recurrence and greater risk of resistance to the antiestrogen therapy (9).

We recently showed that in estrogen-dependent T47D cells, p130Cas associates to the estrogen receptor α to form a complex containing c-Src kinase, Crk, and p85 subunit of phosphatidylinositol 3-kinase (PI3K) (10). Association of p130Cas with the estrogen receptor is dependent on c-Src kinase activation and its overexpression in breast carcinoma T47D cells increases estrogen-dependent Src and mitogen-activated protein kinases (MAPK) kinase activities and cyclin D1 expression. Consistently, the suppression of p130Cas expression by RNA interference strongly impairs estrogen ability to activate extracellular signal-regulated kinase 1/2 (Erk1/2) MAPK and cyclin D1 expression, underlying the importance of p130Cas in the mitogenic response to estrogens (10). In this work, we evaluated the in vivo involvement of p130Cas in mammary epithelial development and remodeling. We also examined its role in tumorigenesis by crossing mouse mammary tumor virus (MMTV)-p130Cas transgenic mice with MMTV-HER2-Neu animals and evaluating p130Cas expression in a panel of human breast carcinomas.

Materials and Methods

Transgenic mice generation. See Supplementary Data.

Morphologic and immunohistochemical analysis of transgenic mice. Mice having litters of eight pups were included in the study. The fourth inguinal glands were used for morphologic analysis, cell proliferation, and apoptosis assays. For whole-mount staining, mammary glands were

flattened on microscopic slides, fixed overnight in Carnoy's solution (75% ethanol, 25% acetic acid), and stained with carmine alum. For histologic analysis, glands were fixed overnight in 4% paraformaldehyde, dehydrated in ethanol, cleared in chloroform, and embedded in paraffin. Sections of 5 μm were dewaxed and stained with H&E.

For Ki67 staining, sections were cut and dewaxed, rehydrated, and washed in TBS 0.1% Triton X-100 supplemented with NaN$_3$ to quench peroxidases at room temperature for 10 minutes. The sections were heated in 10 mmol/L Na citrate solution to unmask the antigen, saturated with 5% goat serum, and incubated with 1:50 Ki67 primary monoclonal antibodies (mAb; Novocastra, Newcastle upon Tyne, United Kingdom) overnight at 4°C. Sections were washed thrice 5 minutes each and incubated with PAP anti-mouse 1:200 (DAKO, Glostrup, Denmark) for 30 minutes at room temperature and treated with StreptAB-Complex/HRP (DakoCytomation Denmark, Glostrup, Denmark) according to the instructions of the manufacturer. The 3,3′-diaminobenzidine reaction was followed under the microscope.

Terminal deoxyribonucleotide transferase–mediated nick-end labeling assay on tissue sections and cell culture. The *In situ* Cell Death Detection kit (Roche Diagnostics, Monza, Italy) was used on formalin-fixed inguinal mammary gland 5 μm sections that are previously incubated with proteinase K (1 μg/mL) for 15 minutes at room temperature. After rinsing in PBS, 50 μL terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) reaction mixture was added to each section for 60 minutes at 37°C. In the first washing, Hoechst staining was added to label all the nuclei. Ten random fields per section were documented by photomicroscopy. The percentage of TUNEL-positive epithelial cell nuclei relative to the total number of the epithelial cell nuclei was also documented. For cell culture staining, cells were plated on glass coverslips, fixed, and processed as above.

Protein analysis and antibodies. Protein extracts were prepared from thoracic mammary glands using boiling Laemmli sample buffer 2× without dyes. For Western blot analysis, equal amounts of proteins were run on SDS-PAGE and processed for Western blotting with the following primary antibodies directed to p130Cas (1H9; ref. 10), Myc epitope mAb 9E10 (American Type Culture Collection/LGC Promochem, Milano, Italy); pErk1/2, pAkt, Akt, pGsk3-β, Gsk3-β, p-Src, pPTEN, and PTEN (Cell Signalling, Danvers, MA); Erk1/2, c-Src, Bcl-2, Bcl-xL, and pTyr-PY99 (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase-conjugated secondary antibodies were purchased from Amersham. Blots were developed with chemiluminescent system (Euroclone, Pero, Italy).

Crossing of MMTV-p130Cas with MMTV-HER2-Neu mice and analysis of *in vivo* tumor formation. BALB/c mice MMTV-HER2-Neu were previously described (11). p130Cas/HER2-Neu double-transgenic mice were generated by crossing MMTV/p130Cas and MMTV-HER2-Neu. We first created an outbreed FWB/C57 strain and analyzed the first generation by PCR for the presence of the transgenes. The mice that were positive for both the transgenes were included in further analyses. The incidence of tumors was evaluated twice weekly with the investigators being blind as to mice genotype from week 1 to 19.

Isolation of primary epithelial cells from mammary gland tumors. Cells from tumors were isolated as described in ref. (12). Briefly, tumors were excised from mammary glands of MMTV/HER2-Neu and MMTV/p130Cas/HER2-Neu mice and finely chopped. Tumor cell aggregates were then incubated in trypsin (0.25% in EDTA; Life Technologies) for 2 hours at 37°C, washed in DMEM, centrifuged at low speed, and then plated in 20% fetal bovine serum/DMEM. Protein extracts were prepared in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.1% 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mmol/L EDTA, supplemented with protease inhibitors], separated, and analyzed in Western blotting as described above.

p130Cas down-regulation by RNA interference. The TUBO carcinoma cells expressing the rat p185 HER2-Neu (13) were routinely cultured in DMEM supplemented with 20% FCS. TUBO cells were transfected with small interfering RNA (siRNA) corresponding to DNA target sequence TTGACTAATAGTCTACATTTA of mouse p130Cas or nonsilencing control siRNA (Xeragon-Qiagen, Milano, Italy) by TransMessenger Transfection

Reagent (Qiagen) as described by instructions of the manufacturer. After 48 hours, cells were extracted in 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% NP40 and protease inhibitors, and analyzed by Western blotting as reported above. In parallel experiments, cells were detached by 10 mmol/L EDTA treatment, plated on glass slides for 2 hours in the presence of 10% serum, fixed with 4% paraformaldeyde for 10 minutes, and subjected to TUNEL assay as described above.

Immunohistochemistry for human breast cancer samples. Tissue arrays were prepared using the Tissue Arrayer instrument (ATA-100; Chemicon International, Temecula, CA) from 150 invasive breast carcinomas. Four cores were obtained for each tumor in the areas of higher cellularity.

Breast tumor tissues were routinely fixed in 10% formaldehyde buffer (pH 7.4) for 24 hours, paraffin-embedded, and processed for immunohistochemical analysis. p130Cas mAb 1H9 (7 μg/mL; ref. 10) was used on human sections for 2 hours at 37°C after preincubation with normal goat serum for 1 hour at room temperature (1:20). Staining was detected with EnVision+ System-HRP Labelled Polymer anti-mouse (DakoCytomation Denmark) and developed with the LiquidDAB Substrate Pack (BioGenex, San Ramon, CA). Nuclei were counterstained with Mayer hemallum.

Staining HER2-Neu in breast cancer samples was done by incubating polyclonal anti-HER2 (rabbit polyclonal DAKO; anti HER2/neu) 1:20 on human sections followed by incubation with EnVision+ System-HRP Labelled Polymer antirabbit (see above). p130Cas staining was done as described above (11).

Statistical analysis and reproducibility. Results are expressed as the means ± SE. Statistical analysis of the data was done using a Student's *t* test. For the statistical analysis of tumor occurrence in double-transgenic mice, the Mantel-Haenzel test was used as described by Boggio et al. (14). For human samples, the significance was calculated by Yates-corrected χ^2 test (×2).

Results

p130Cas overexpression leads to mammary gland hyperplasia by increased proliferation and activation of specific signaling pathways. To dissect the role of p130Cas in *in vivo* mammary gland physiology and pathology, two independent lines of p130Cas-MMTV transgenic mice were generated (see Supplementary Fig. S1). In mammary glands of virgin transgenic mice at 6 weeks of age, whole-mount analysis showed secondary ducts growth with increased branching and end-bud density (Fig. 1A, *left, top* and *bottom*), indicating that early ductal development and branching was enhanced compared with control females by high levels of p130Cas. The effect of overexpression of p130Cas on mammary gland branching was quantified by counting terminal end buds in whole-mount preparation from transgenic and wild-type (wt) mice, showing that at the virgin stage, transgenic mice displayed 50% more end buds than the wt animals (Fig. 1B). Histologic sections of mammary glands at 10 and 18 days of pregnancy cut in proximity of the central lymph node confirmed the presence of a more extensive lobuloalveolar development in the transgenic mice compared with wt littermates (Fig. 1C and D; see also Supplementary Fig. S2A), quantified as a 50% and a 30% increase, respectively (see Supplementary Fig. S2B and S2C). After parturition, at 3 days of lactation, the extent of lobuloalveolar development in the transgenic mice was strongly increased compared with wt controls (Fig. 1E). Indeed, the epithelium of transgenic glands almost completely filled the fat pad, exhibiting a totally disorganized structure with respect to the ordinate arrangement of the wt epithelium. Furthermore, the transgenic glands were characterized by areas of multilayered epithelium surrounding the secretory ducts (see *black arrow*). Therefore, MMTV-p130Cas transgenic mice showed extensive hyperplasia of epithelial structures, increasing the possibility that p130Cas
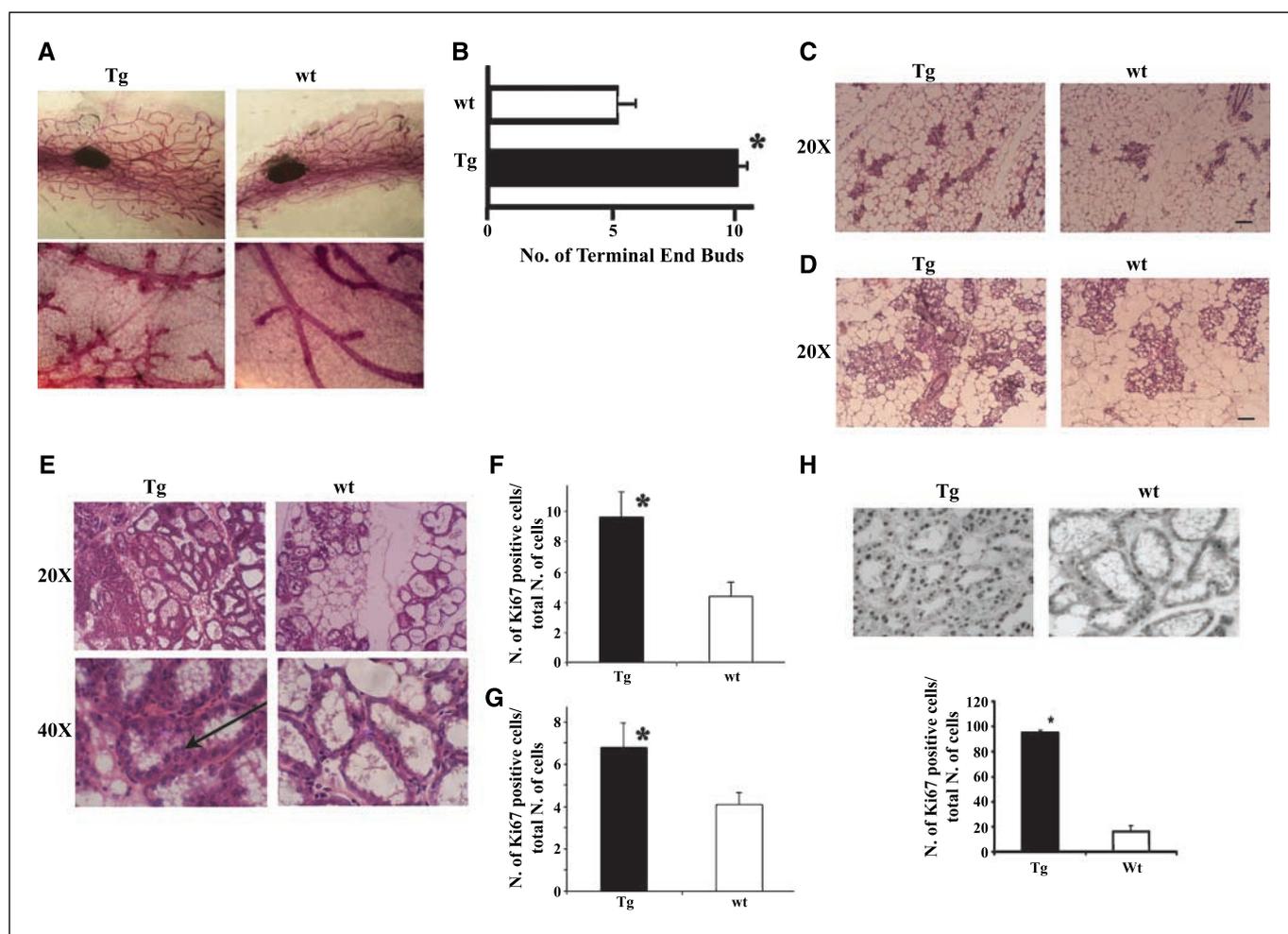
Figure 1. p130Cas overexpression leads to precocious development and mammary gland hyperplasia. *A, top,* representative whole-mount analysis of mammary glands of transgenic (*Tg*) and wt mice at 6 weeks of age. *Bottom,* higher magnification (×20) showing ductal branching in whole-mount preparation from transgenic and wt mice. *B,* number of terminal end buds counted in several fields of constant area in transgenic (*black column*) versus wt (*white column*) mammary glands. *C,* H&E-stained sections from transgenic and wt mice at 10 days of pregnancy were cut at the level of central lymph node. Bar, 40 μm. *D,* H&E-stained sections from transgenic and wt mice at 18 days of pregnancy were cut at the level of central lymph node. Bar, 40 μm. *E,* H&E-stained sections from transgenic and wt at 3 days of lactation at ×20 and ×40 magnification. *Black arrow,* multilayered and disorganized ductal epithelium in the transgenic sections. Ki67-positive cell number counted over total number of cells in transgenic and wt at 10 (*F*) and 18 (*G*) days of pregnancy. *P* < 0.01. *H, top,* Ki67 staining of sections derived from transgenic and wt mice at 3 days of lactation. *Bottom,* Ki67-positive cell number was counted over the total number of cells. *P* < 0.01. Representative of the analysis of 10 mice from each transgenic line and from 5 wt.

overexpression can positively control the extent of proliferation. To test whether the intensive hyperplasia observed might be due to p130Cas-dependent control of proliferation, sections of mammary glands at different stages were stained with antibodies to Ki67 proliferation marker. Transgenic mice glands at 10 and 18 days of pregnancy displayed a significant increase in the percentage of Ki67-positive cells compared with the wt littermates (Fig. 1*F* and *G*). Interestingly, at 3 days of lactation, when differentiation displaces proliferation, 95% of cells from transgenic animals were still positive for Ki67 staining compared with the 20% of the cells of wt glands (Fig. 1*H, top* and *bottom*), indicating that the lobulo-alveolar hyperplasia of transgenic mice is due to a neat increase in proliferation.

To investigate whether specific signaling pathways were involved in the increased epithelial density, protein extracts were prepared from the fourth inguinal glands of both wt and transgenic mice. Western blot analysis with antibodies to p130Cas showed that in wt mice the levels of the endogenous protein peaked at the beginning of pregnancy, whereas in the transgenic animals the levels were elevated in all stages (Fig. 2*A*). Interestingly, the extent of activation of c-Src kinase, evaluated by using antibodies to its active form (p-Tyr[416]), was strongly increased in transgenic females at virgin and pregnancy stages compared with the wt (Fig. 2*A*), demonstrating for the first time a role for p130Cas as an *in vivo* regulator of Src kinase activity. In addition, transgenic mice showed an increased level of phosphorylation of Erk1/2 MAPK and of expression of cyclin D1. Finally, the levels of activated Akt that is known to promote cell survival (15–17) were higher in transgenic than in wt samples throughout all the developmental stages.

At 3 days of lactation in the wt animals, p130Cas was barely detectable (Fig. 2*B*), whereas it was highly expressed in transgenic littermates. The levels of active c-Src were hardly visible in the transgenic females, indicating that c-Src was activated mainly during the early phases of pregnancy (see Fig. 2*A*). The levels of active phosphorylated Akt and of focal adhesion kinase (not shown) were comparable between transgenic and wt animals,
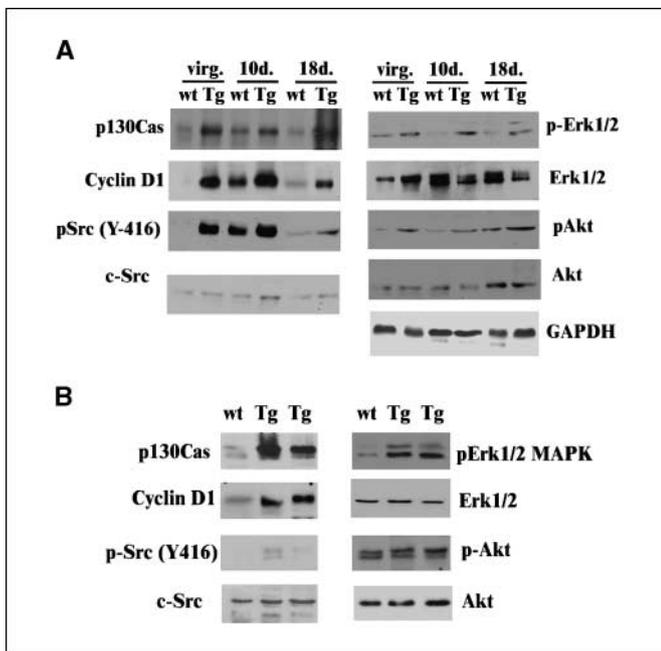
Figure 2. Signaling pathways activated by p130Cas overexpression. *A,* protein extracts from transgenic mammary glands and wt at virgin stage (*virg.*), 10 days (*10d.*), and 18 days (*18d.*) of pregnancy were analyzed with the indicated antibodies. *B,* protein extracts from transgenic and wt mice at 3 days of lactation were processed as above.

whereas phosphorylated Erk1/2 MAPK and cyclin D1 expression were lifted up in the transgenic glands. All these results indicate that p130Cas transgenic glands display a hyperplastic phenotype that mainly correlates with an early activation of c-Src kinase and sustained levels of Erk1/2 MAPK.

p130Cas-overexpressing glands show delayed involution due to impaired apoptosis. We examined next the effect of the overexpression of p130Cas in mammary epithelium during involution. Transgenic and wt female mice were allowed to lactate for 7 days, the pups were removed, and the mothers were sacrificed after 2 days. At this time of involution, in wt animals, the single layer of epithelial cells of the alveolar structures has started to collapse and numerous apoptotic bodies have become evident in the ductal lumens (Fig. 3A, *red arrowhead*). In contrast, in transgenic mice, the alveoli were preserved and appeared distended (Fig. 3A, *left, bottom*). Interestingly, the tight clusters of multilayered epithelium seen during lactation (see Fig. 1E) were still present in the involuting glands of transgenic mice (Fig. 3A, *black arrow*). As involution proceeds, the alveoli in wt mammary glands collapsed (Fig. 3A, *black arrowheads*), whereas they were still visible in transgenic mice, although apoptotic bodies as well as adipocytes were present in the luminal spaces (data not shown), suggesting that involution has also started in the transgenic animals. However, at 21 days after weaning in p130Cas transgenic mice, the involution process did not result in a full loss of alveolar structures as observed in the wt animals. Indeed, transgenic mice phenotype reminds a midpregnancy stage (data not shown).

As shown by Ki67 staining at 2 days of involution, transgenic glands still retained 45% of Ki67-positive cells (Fig. 3B), indicating that a consistent amount of proliferation is preserved. In parallel, in p130Cas transgenic mice, only 5% of cells were TUNEL positive, compared with the 10% to 15% displayed by the wt, indicating that

apoptosis is severely impaired by p130Cas overexpression (Fig. 3C). Consistently, biochemical analysis of protein extracts showed that in p130Cas transgenic involuting glands Erk1/2 MAPK as well as Akt and Gsk-3β were still phosphorylated (Fig. 3D), indicating that both the MAPK and the AKT pathways were active. To investigate whether the delay of the initial phases of involution correlated with expression of antiapoptotic proteins, such as Bcl-xL and Bcl-2 (18–20), Western blot analysis done on protein extracts after 2 days of involution showed that Bcl-xL and Bcl-2 were highly increased in transgenic compared with wt mice (Fig. 3D), indicating that the antiapoptotic proteins are up-regulated by p130Cas overexpression. Therefore, these data show that p130Cas-overexpressing glands display a delayed involution due to both preserved proliferation and decreased apoptosis.

p130Cas and HER2-Neu coexpression in mammary epithelium accelerates the onset of tumor formation. p130Cas transgenic mice did not develop tumors in the first 12 months of life, suggesting that the overexpression of the protein is not sufficient to trigger cancer. To analyze p130Cas involvement in breast cancer, MMTV-p130Cas animals were crossed with MMTV-HER2-Neu transgenic mice, which express the HER2-Neu
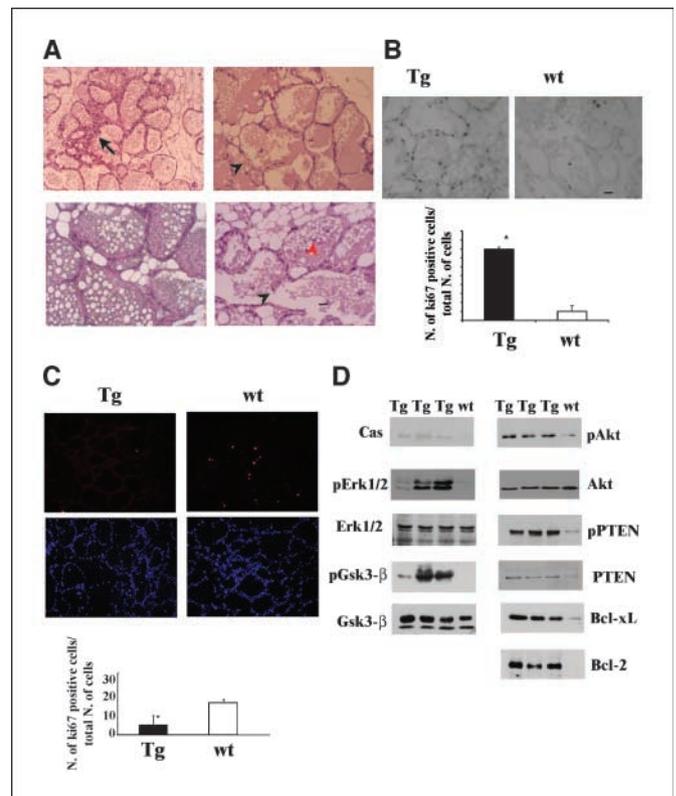

Figure 3. p130Cas overexpression leads to delayed involution and impaired apoptosis. *A,* H&E-stained sections from 10 transgenic (*left*) and 10 wt (*right*) mice at 2 days of involution at ×20 (*top*) and ×40 (*bottom*) magnifications. *Arrow,* multilayered structure in transgenic glands. *Black arrowheads,* loss of epithelial integrity in wt glands; *red arrowhead,* presence of apoptotic nuclei in the wt sections. *B,* Ki67 detection on section four transgenic and five wt mice at 2 days of involution (*top*). Ki67-positive cell number in transgenic (*black column*) and wt (*white column*) were counted over the total number of cells (*bottom*). $P < 001$. *C,* TUNEL analysis on sections of involuting glands of four transgenic (*left*) and 6 wt (*right*) mice. TUNEL-positive cells in transgenic (*black column*) and wt (*white column*) were counted over the total number of cells. $P < 0.05$. For each animal, 10 sections were analyzed. *D,* protein extracts from transgenic mammary glands and wt at 2 days of involution were analyzed with the indicated antibodies.

oncogene mutated at position 664 in the transmembrane domain (22) and provide one of the most aggressive model of mammary carcinogenesis (11). HER2-Neu and p130Cas/HER2-Neu mice were palpated weekly to detect mammary tumors. As shown in the graph of Fig. 4*A*, 10% of the double-transgenic p130Cas/HER2-Neu mice developed palpable tumors as early as 12 weeks of age. At 15 weeks, 80% of the double-transgenic mice presented tumors. In contrast, HER2-Neu mice at 15 weeks of age were still tumor-free and 35% of them started to develop tumors at 16 weeks. In 80% of mice, tumors presented only at 18 weeks. Thus, p130Cas overexpression expedites tumor latency, likely by accelerating the onset of tumor formation in the double-transgenic mice, providing evidences that p130Cas and HER2-Neu oncogene synergize in *in vivo* transformation of mammary epithelium. To investigate which signaling pathways are involved in the accelerated tumor formation in double-transgenic animals, we generated primary cell cultures from p130Cas/HER2-Neu and HER2-Neu tumors. Western blot analysis of cell extracts in Fig. 4*B* confirmed that p130Cas was highly overexpressed in the p130Cas/HER2-Neu–derived cells. Moreover, in the same cells, levels of tyrosine-phosphorylated proteins as well as of active Src were increased, whereas Erk1/2 MAPK activities were unaffected. Phosphorylation of PTEN, Akt, and Gsk3-β, which indicates activation of the PI3K/Akt pathway, was also much more induced in p130Cas/HER2-Neu–derived cells compared with HER2-Neu cells. Taken together, these data indicate that p130Cas contributes to the accelerated HER2-Neu induced transformation mainly by activating c-Src, Akt, and tyrosine kinases.

p130Cas suppression by RNA interference restores apoptosis in HER2-Neu-expressing cells. To further assess the relevance of p130Cas in HER2-Neu-induced tumorigenesis, p130Cas expression was down-regulated by RNA interference in mouse TUBO cells, a carcinoma stable cell line derived from MMTV-HER2-Neu mice, expressing high levels of the HER2-Neu oncogene (13). Upon transient transfection with siRNA oligonucleotides, the levels of p130Cas were decreased of 70% by specific siRNAs compared with the noninterfering ones (Fig. 5*A*). To evaluate the effect of p130Cas down-regulation on cell survival, cells were subjected to TUNEL analysis. Interestingly, in cells treated with p130Cas-specific siRNA, the number of TUNEL-positive nuclei was increased 3-fold compared with TUBO cells transfected with noninterfering oligonucleotides (Fig. 5*B* and *C*), indicating that partial ablation of p130Cas enhances apoptosis. Moreover, Western blot analysis of cell extracts shown in Fig. 5*D* shows that in cells treated with p130Cas-specific siRNA, the levels of phosphorylated Src, Akt, and Gsk3-β were reduced compared with the control. In addition, in the same cell extracts, the amount of tyrosine-phosphorylated proteins was reduced, whereas Erk1/2 MAPK phosphorylation was unaffected, thus indicating that p130Cas silencing affects cell signaling pathways, mainly controlling cell survival.

p130Cas/Bcar1 is up-regulated in human breast carcinomas. The murine model presented above strongly suggests that p130Cas is involved in the progression of cancer. Immunohistochemical analysis was thus done on human breast tissues. p130Cas staining was detected in atypical ductal hyperplasia specimens but not in normal tissue (Fig. 6*A, left*). The hyperplastic duct, strongly stained with p130Cas antibodies, presented regions of multilayered epithelial cells that closely resembles the structures described in p130Cas transgenic mice at 3 days of lactation (see above, Fig. 1*E*). In addition, 117 of 150 malignant tumors examined (77%) expressed p130Cas with a homogeneous and diffuse pattern
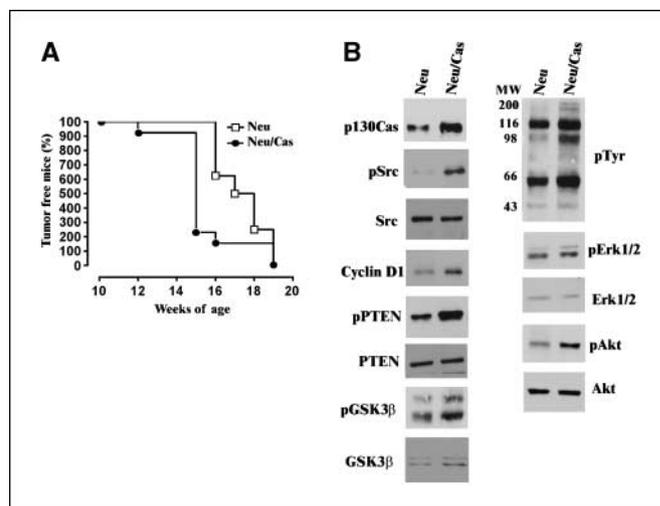

Figure 4. Kinetics of tumor occurrence in p130Cas/HER2-Neu and HER2-Neu mice. *A,* tumor formation in p130Cas/HER2-Neu (*gray line* and *black circles*) and HER2-Neu (*black line* and *empty squares*) mice. Twenty mice were analyzed for each group. The difference of occurrence between the two groups is statistically significant, $P < 0.001$. *B,* independent epithelial cell culture were derived from four distinct tumors excised from p130Cas/HER2-Neu and HER2-Neu mice. Western blot analysis of protein extracts was done with the indicated antibodies and representative results are shown. *MW,* molecular weight markers.

(Fig. 6*A, right*) and the expression was also confirmed at the level of mRNA by *in situ* hybridization (Supplementary Fig. S3). In carcinomas not expressing p130Cas, staining of the protein was confined to small vessels inside the tumor (Supplementary Fig. S3). Interestingly, by staining on consecutive sections of HER2-Neu-positive tumors, p130Cas was found to codistribute with HER2-Neu membrane protein (Fig. 6*B* and *C*), showing that the same cell can overexpress the two proteins. Moreover, statistical analysis showed that in the presence of p130Cas, HER2-Neu overexpression correlates with high Ki67 staining (>24%; see Table 1), indicating that the concomitant presence of p130Cas and HER2-Neu is significantly related to an elevated index of proliferation. Therefore, these data show that p130Cas expression is elevated in a large subset of human breast cancers and, in association with high levels of HER2-Neu, is linked to variables relating to enhanced tumor biological aggressiveness and proliferation.

Discussion

Our present study states that overexpression of p130Cas protein affects the development of the mammary gland by inducing epithelial cell hyperplasia and delayed involution. In addition, p130Cas overexpression in a large subset of human breast cancers and its synergism with the HER2-Neu oncogene in pathologic transformation suggest that p130Cas might be a crucial player in the onset of tumorigenesis. These functional data are supported by p130Cas-mediated activation of signaling pathways, which results in sustained proliferation and decreased apoptosis. Conversely, p130Cas depletion by siRNA leads to increased apoptosis by down-regulation of survival signaling pathways of transformed epithelial cells.

In *in vitro* cell systems, p130Cas has been shown to control the ability of cells to migrate and invade (1, 2), although its *in vivo* mechanisms have been thus far poorly understood. To investigate the molecular mechanisms activated by p130Cas overexpression in mammary gland development and tumorigenesis, we generated

MMTV-p130Cas transgenic mice. Expression of the transgene, already detectable in virgin animals, was scaling up during pregnancy and reached its maximal level during lactation and a certain amount of p130Cas protein was still present during the early phases of involution. Morphologically, epithelial hyperplasias were found in nearly 100% of the lactating animals examined but was evident also in virgin mice and during pregnancy. The hyperplastic phenotype correlated with a substantial increase in the amount of cell proliferation as shown by the Ki67 staining, suggesting that the overexpression of p130Cas might profoundly affect signaling pathways leading to cell cycle progression. Indeed, our data show that p130Cas expression directly influences *in vivo* the extent of activation of c-Src, Erk1/2 MAPK, and expression of cyclin D1. Interestingly, regulation of c-Src activity is strongly affected by p130Cas overexpression. As shown in the results, whereas in wt animals c-Src kinase activity peaked at 10 days of pregnancy, p130Cas overexpression results in an earlier and higher activation of endogenous c-Src starting from the virgin stage until the late phases of pregnancy. This kinetics of activation suggests that Src pathway can be a major player in the setting of hyperplasia by p130Cas. Consistently, transgenic models of activated Src in the mammary gland show extensive hyperplasia (23), similar to p130Cas transgenic mice. On the basis of *in vitro* data, we can speculate on the mechanisms through which p130Cas acts as a positive *in vivo* regulator of c-Src kinase activity. In epithelial cells, an enhancement of Src kinase activity might be achieved through the formation of a molecular complex between c-Src and p130Cas, which contributes to stabilize c-Src in its active conformation (10). Alternatively, p130Cas might expose potential binding sites for effector proteins, such as focal adhesion kinase, Crk, and tyrosine

phosphatases (2). Although in our transgenic mice, the levels of phosphorylated focal adhesion kinase are not modulated (not shown), the role of other proteins relevant for the activation of c-Src kinase cannot be excluded.

In addition to c-Src activation, p130Cas-overexpressing glands show increased levels of phosphorylated Erk1/2 MAPK, which persist till the parturition and are maintained during lactation, likely contributing to the increased proliferation and the hyperplastic phenotype. Interestingly, perturbation of integrin β1 function in mammary gland results in reduced epithelial cell proliferation during pregnancy and lactation (24), which has been ascribed to a lack of MAPK activation (25). Furthermore, signals originated from integrin-dependent adhesion control Erk1/2 MAPK activation (26) and expression of the integrin linked kinase induces mammary gland hyperplasia accompanied by constitutive activation of Erk1/2 MAPK (27), further demonstrating the strict correlation between MAPKs and hyperplasia.

p130Cas transgenic glands also display impaired involution as shown by a slower lobuloalveolar loss of epithelial mammary tissue and by a reduced number of apoptotic cells in the transgenic glands compared with the wt likely due to a prosurvival effect of the p130Cas protein. Several mechanisms, such as activation of the PI3K/Akt pathway and expression of antiapoptotic proteins, might contribute to inhibit mammary gland involution (20). Transgenic mice expressing a normal or an activated form of Akt showed that Akt is a major player in maintaining survival stimuli (15–17, 28). In p130Cas transgenic mice, the persistent activation of Akt, Gsk3-β, and Erk1/2 MAPK along with the inactivation of PTEN and the raise of antiapoptotic proteins, such as Bcl-2 and Bcl-xL, biochemically supports the delay of involution due to a
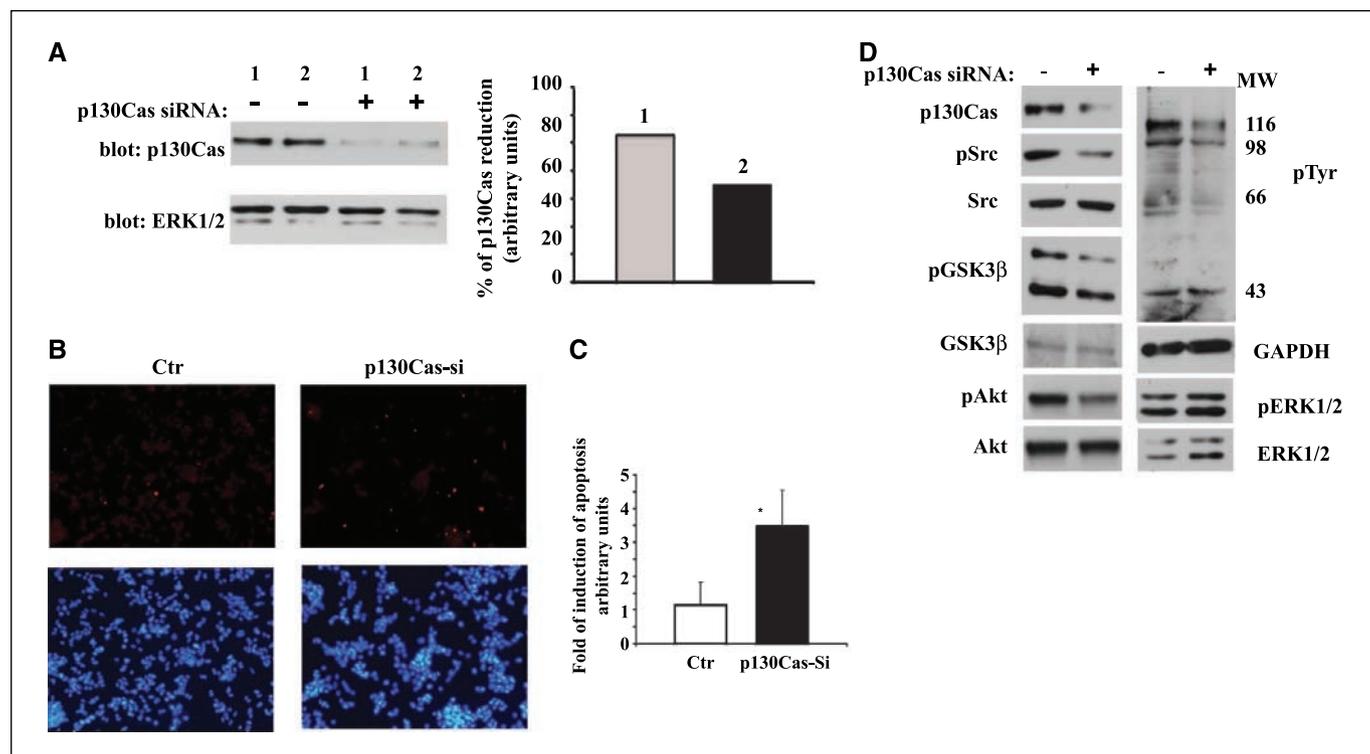

Figure 5. p130Cas silencing by siRNA increases cell apoptosis. *A*, cell extracts from TUBO cells transiently transfected with control noninterfering siRNAs (−) or with two p130Cas siRNAs (+1, +2) were blotted with the indicated antibodies. *Right*, densitometric analysis. *B*, TUNEL analysis of TUBO cells transfected with siRNA oligo 1 or with control siRNA oligo. *C*, TUNEL-positive cells counted over the total number of cells and expressed as fold of induction. *P* < 0.01. Representative of three independent experiments. *D*, protein extracts from cells transfected with p130Cas siRNA (+) or control siRNA (−) were analyzed with the indicated tantibodies.
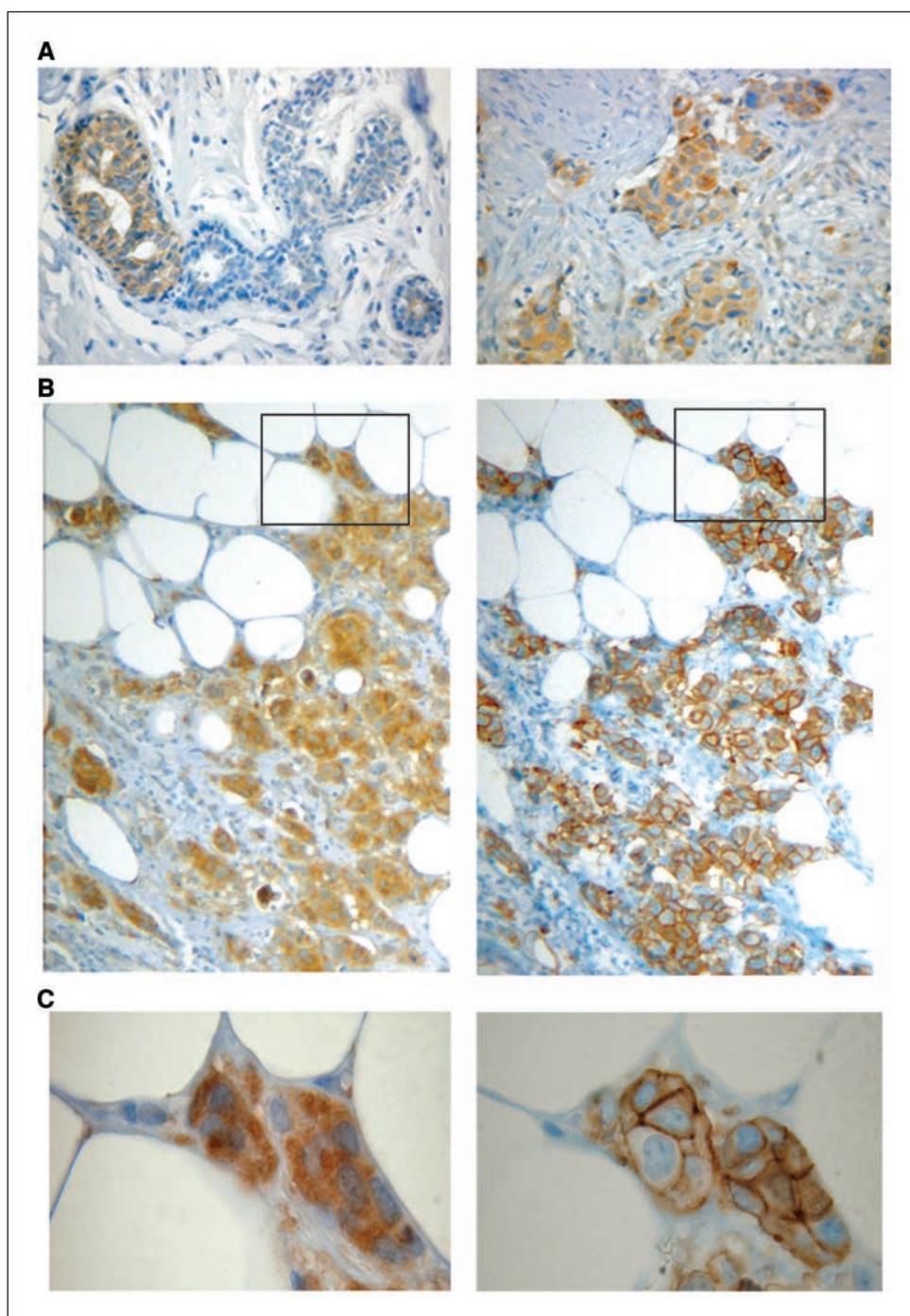
Figure 6. p130Cas/BCAR1 is up-regulated in human breast carcinomas. *A, left,* immunostaining by p130Cas mAb 1H9 of an atypical ductal hyperplasia section showing low p130Cas expression in a normal terminal ductal lobular unit (*right*) and in two small ducts (*central part*) and high p130Cas expression on a terminal ductal hyperplastic lobular unit forming arcades and rigid bridges. *Right,* homogeneous and diffuse pattern of staining of p130Cas in an invasive ductal carcinoma. *B* and *C,* immunostaining of breast cancer tissues of two consecutive sections with p130Cas mAb 1H9 antibody (*left*), or with anti-HER2/neu (rabbit polyclonal antibody (*right*). *C,* enlargements of the squared sections present in (*B*).

p130Cas-dependent antiapoptotic program (20). Moreover, our data for the first time correlate the expression of p130Cas with the Akt/PI3K pathway *in vivo*. Predicted mechanisms through which p130Cas can activate PI3K rely on the ability of p130Cas to associate either with the p85 subunit of PI3K (29) or with AND34, a known activator of the PI3K pathway (30).

Although p130Cas transgenic mice at 12 months of age show hyperplasia and delayed involution, they do not develop tumors yet. Absence of tumors in the presence of hyperplasia and delayed involution was also observed in transgenic mice expressing Akt under the MMTV promoter (17), indicating that neither p130Cas nor Akt are *per se* sufficient to trigger tumorigenesis. On the contrary, by crossing p130Cas-MMTV and MMTV-HER2-Neu mice, known to develop stochastic multifocal mammary cancers with a median latency of 17 weeks, we generated double-transgenic mice with accelerated tumor formation. In double-transgenic mice, tumors appeared already at 12 weeks of age and 80% of these mice presented tumors at 15 weeks, whereas the HER2-Neu animals were still tumor-free. Tumor cells explanted from the double-transgenic mice exhibited higher levels of tyrosine phosphorylation, as well as increased activation of Src and Akt pathways compared with HER2-Neu–derived cells. These data indicate that high levels of p130Cas synergize with HER2-Neu activity by up-regulating cellular proliferation and maintaining survival likely

Table 1. Correlation between HER2-Neu expression and proliferative index in p130Cas-positive human breast samples

	P130Cas positive	
	HER2-Neu positive (%)	HER2-Neu negative (%)
Ki67 high (>24%)	59	35
Ki67 low (<24%)	41	65

NOTE: One hundred fifty cases of human breast cancers were subjected to immunostaining to detect p130Cas and HER2-Neu and evaluate Ki67 proliferative marker. The percentages of tumors showing high and low levels of Ki67 were calculated in function of the presence of HER2-Neu and p130Cas in positive samples. Pearson $\chi^2 = 5.653$; Fisher's exact test, two-sided = 0.023.

by Src and Akt. No changes of Erk1/2 MAPK activities are observed in cells explanted from double-transgenic mice. This might be because Erk1/2 MAPKs, a known primary target of activated HER2 (31), are already strongly phosphorylated in HER2-Neu–derived cells. In this context, a further modulation on Erk1/2 MAPK activities by p130Cas overexpression might not be detectable. In addition, specific silencing of p130Cas expression by siRNA in TUBO cells, which are stable epithelial carcinoma cells derived from the HER2-Neu mice (13), leads to an increase in the amount of apoptotic cells. Indeed, the down-regulation of p130Cas caused a marked decrease in activation of Src kinase as well as of Akt and Gsk3-β, demonstrating that in cells expressing high levels of the HER2-Neu oncogene, p130Cas is required for the survival program.

Our present data underline a role for p130Cas in growth, survival, and cell transformation, but its involvement in human breast cancer is still to be determined. Breast cancer growth is regulated by coordinated actions of hormonal, growth factors, and extracellular matrix signaling pathways in which p130Cas has been shown to play a relevant role (32–38).

Our analysis of 150 tumor cases show that p130Cas is overexpressed in a high percentage of human breast cancers independently of tumor histologic type and grade. However, the statistical

analysis indicates that concomitant overexpression of p130Cas and HER2 significantly correlates with proliferation. These data show that *in vivo* p130Cas increased expression associated with the HER2-Neu pathway might enhance tumor biological aggressiveness. Indeed, the p130Cas/HER2-Neu double-transgenic model perfectly matches with this hypothesis, showing that overexpression of p130Cas and HER2-Neu accelerates the onset of tumor formation. The correlation in human breast cancer between p130Cas and HER2-Neu expression might also affect the reduced response to antiestrogens therapy in breast cancer overexpressing p130Cas (9). In fact, taking into account that in tumors overexpressing HER2-Neu, tamoxifen may lose its estrogen antagonist activity and may acquire more agonist-like activity resulting in tumor growth stimulation (39), our present evidences suggest that p130Cas might be an additional factor regulating both the response to antiestrogen and oncogenic transformation mediated by tyrosine kinase receptors. How p130Cas protein levels can be up-regulated in human breast cancer is not known; Increased expression of p130Cas mRNA shown by *in situ* hybridization suggest that the mechanism acts either at transcriptional or mRNA stabilization levels. However, there are no data available on transcriptional regulation of p130Cas gene and its protein turnover.

In conclusion, p130Cas is implicated in mammary gland development and tumorigenesis: Its increased expression leads to hyperplasia, delayed involution, and accelerates tumor formation in the presence of other oncogenic stimuli; whereas its down-regulation decreases cell tumor survival. Thus, our data suggest that p130Cas can be considered not only as a novel diagnostic tool to assess the morbidity of cancers in which is expressed at high levels, but also can be a new therapeutic target for treating very aggressive cancers such as those overexpressing HER2-Neu.

References

1. Bouton AH, Riggins RB, Bruce-Staskal PJ. Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. Oncogene 2001;20:6448–58.
2. Cabodi S, Defilippi P. The essence of integrin signal transduction. In: Danen E, ed. Georgetown, TX: Landes/Eurekah, PubMed. 2005.
3. Miranti CK, Brugge JS. Sensing the environment: a historical perspective on integrin signal transduction. Nat Cell Biol 2002;4:E83–90.
4. Honda H, Oda H, Nakamoto T et al. Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. Nat Genet 1998;19:361–5.
5. Huang J, Hamasaki H, Nakamoto T et al. Differential regulation of cell migration, actin stress fiber organization, and cell transformation by functional domains of Crk-associated substrate. J Biol Chem 2002;277:27265–72.
6. Hsia DA, Mitra SK, Hauck CR et al. Differential

regulation of cell motility and invasion by FAK. J Cell Biol 2003;160:753–67.
7. Brabek J, Constancio SS, Shin NY, Pozzi A, Weaver AM, Hanks SK. CAS promotes invasiveness of Src-transformed cells. Oncogene 2004;23:7406–15.
8. Brinkman A, van der Flier S, Kok EM, Dorssers LC. BCAR1, a human homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. J Natl Cancer Inst 2000;92:112–20.
9. van der Flier S, Brinkman A, Look MP et al. Bcar1/p130Cas protein and primary breast cancer: prognosis and response to tamoxifen treatment. J Natl Cancer Inst 2000;92:120–7.
10. Cabodi S, Moro L, Baj G, et al. p130Cas interacts with estrogen receptor α and modulates non-genomic estrogen signaling in breast cancer cells. J Cell Sci 2004;117:1603–11.
11. Lucchini F, Sacco MG, Hu N, et al. Early and multifocal tumors in breast, salivary, harderian and epididymal tissues developed in MMTY-Neu transgenic mice. Cancer Lett 1992;64:203–9.

12. White DE, Kurpios NA, Zuo D, et al. Targeted disruption of β1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell 2004;6:159–70.
13. Rovero S, Amici A, Carlo ED, et al. DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. J Immunol 2000;165:5133–42.
14. Boggio K, Nicoletti G, Di Carlo E, et al. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. J Exp Med 1998;188:589–96.
15. Hutchinson J, Jin J, Cardiff RD, Woodgett JR, Muller WJ. Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression. Mol Cell Biol 2001;21:2203–12.
16. Schwertfeger KL, Richert MM, Anderson SM. Mammary gland involution is delayed by activated Akt in transgenic mice. Mol Endocrinol 2001;15:867–81.

17. Ackler S, Ahmad S, Tobias C, Johnson MD, Glazer RI. Delayed mammary gland involution in MMTV-AKT1 transgenic mice. Oncogene 2002;21:198–206.

18. Walton KD, Wagner KU, Rucker EB III, Shillingford JM, Miyoshi K, Hennighausen L. Conditional deletion of the bcl-x gene from mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise cell function during lactation. Mech Dev 2001;109:281–93.

19. Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R. Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. Development 2004; 131:3469–79.

20. Green KA, Streuli CH. Apoptosis regulation in the mammary gland. Cell Mol Life Sci 2004;61:1867–83.

21. Spencer KS, Graus-Porta D, Leng J, Hynes NE, Klemke RL. ErbB2 is necessary for induction of carcinoma cell invasion by ErbB family receptor tyrosine kinases. J Cell Biol 2000;148:385–97.

22. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 1988;54:105–15.

23. Webster MA, Cardiff RD, Muller WJ. Induction of mammary epithelial hyperplasias and mammary tumors in transgenic mice expressing a murine mammary tumor virus/activated c-src fusion gene. Proc Natl Acad Sci U S A 1995;92:7849–53.

24. Faraldo MM, Deugnier MA, Lukashev M, Thiery JP, Glukhova MA. Perturbation of β1-integrin function alters the development of murine mammary gland. EMBO J 1998;17:2139–47.

25. Faraldo MM, Deugnier MA, Thiery JP, Glukhova MA. Growth defects induced by perturbation of β1-integrin function in the mammary gland epithelium result from a lack of MAPK activation via the Shc and Akt pathways. EMBO Rep 2001;2:431–7.

26. Giancotti FG, Tarone G. Positional control of cell fate through joint integrin/receptor protein kinase signaling. Annu Rev Cell Dev Biol 2003;19:173–206.

27. White DE, Cardiff RD, Dedhar S, Muller WJ. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene 2001;20:7064–72.

28. Debnath J, Walker SJ, Brugge JS. Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Biol 2003;163:315–26.

29. Li E, Stupack DG, Brown SL, Klemke R, Schlaepfer DD, Nemerow GR. Association of p130CAS with phosphatidylinositol-3-OH kinase mediates adenovirus cell entry. J Biol Chem 2000;275:14729–35.

30. Felekkis KN, Narsimhan RP, Near R, et al. AND-34 activates phosphatidylinositol 3-kinase and induces anti-estrogen resistance in a SH2 and GDP exchange factor-like domain-dependent manner. Mol Cancer Res 2005;3:32–41.

31. Dankort DL, Wang Z, Blackmore V, Moran MF, Muller WJ. Distinct tyrosine autophosphorylation sites negatively and positively modulate neu-mediated transformation. Mol Cell Biol 1997;17:5410–25.

32. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005; 5:341–54.

33. Fata JE, Werb Z, Bissell MJ. Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. Breast Cancer Res 2004;6:1–11.

34. Lamote I, Meyer E, Massart- Leen AM, Burvenich C. Sex steroids and growth factors in the regulation of mammary gland proliferation, differentiation, and involution. Steroids 2004;69:145–59.

35. Slamon DJ. Proto-oncogenes and human cancers. N Engl J Med 1987;317:955–7.

36. Clarke RB. Steroid receptors and proliferation in the human breast. Steroids 2003;68:789–94.

37. Andrechek ER, White D, Muller WJ. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. Oncogene 2005;24: 932–7.

38. Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW. The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation 2002;70:537–46.

39. Osborne CK, Shou J, Massarweh S, Schiff R. Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. Clin Cancer Res 2005;11:865–70s.

p130Cas as a New Regulator of Mammary Epithelial Cell Proliferation, Survival, and HER2-Neu Oncogene−Dependent Breast Tumorigenesis

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