The CaM Kinase, \textit{Pnck}, Is Spatially and Temporally Regulated during Murine Mammary Gland Development and May Identify an Epithelial Cell Subtype Involved in Breast Cancer\(^1\)

Heather Perry Gardner, Seung I. Ha, Carol Reynolds, and Lewis A. Chodosh\(^2\)

Department of Molecular and Cellular Engineering [H. P. G., S. I. H., L. A. C.] and Division of Endocrinology [L. A. C.], Diabetes and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6160, and Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905 [C. R.]

ABSTRACT

While screening for protein kinases expressed in the murine mammary gland, we identified previously a Ca\(^{2+}\)/calmodulin-dependent kinase, \textit{Pnck}, that is most closely related to CaMKI. In this report, we show that \textit{Pnck} is temporally regulated during murine mammary development with highest levels of expression observed late in pregnancy, concomitant with the decreased cellular proliferation and terminal differentiation of the mammary epithelium. Consistent with this finding, \textit{Pnck} is up-regulated in confluent mammary epithelial cells and is down-regulated as serum-starved cells are stimulated to reenter the cell cycle. In the mammary gland, \textit{Pnck} is expressed in an epithelial-specific and markedly heterogeneous manner, suggesting that the expression of this kinase may be restricted to a particular mammary epithelial cell type. Potentially related to its heterogeneous \textit{in vivo} expression pattern, \textit{Pnck} expression is oncogene-associated in murine epithelial cell lines derived from mammary tumors arising in different transgenic mouse models of breast cancer; cell lines derived from mammary tumors initiated by \textit{c-myc} or \textit{int-2/Fgf3} express \textit{Pnck}, whereas cell lines initiated by \textit{neu} or \textit{H-ras} do not. In an analogous manner, expression of the human homologue of \textit{Pnck} is restricted to a subset of human breast cancer cell lines. Moreover, \textit{Pnck} was found to be highly overexpressed in a subset of human primary human breast cancers compared with benign mammary tissue. Together, our data suggest that \textit{Pnck} may play a role in mammary development, and that expression of this kinase may be restricted to a mammary epithelial cell type that is transformed in a subset of human breast cancers.

INTRODUCTION

A woman’s lifetime risk of developing breast cancer is intrinsically related to reproductive events, particularly those that affect the differentiated state of the breast. Results from both human epidemiology and animal model systems indicate that an early first full-term pregnancy results in a permanent change in the breast that confers a decreased risk for the subsequent development of breast cancer (1–4). The findings that aborted pregnancies, the majority of which occur prior to the third trimester, are not protective against breast cancer and that lactation has only a minimal protective effect compared with full-term pregnancy suggest that parity-induced protection against breast cancer results from physiological changes that occur late in pregnancy (5, 6). As a consequence, the protective effect of parity has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis (2, 3). Nevertheless, the molecular and cellular basis for this phenomenon is unknown. As such, understanding the developmental changes that occur in the breast late in pregnancy is essential for understanding the protected state of the breast associated with parity.

In an attempt to better understand the relationship between development and carcinogenesis in the breast, we previously carried out a screen designed to identify protein kinases that are expressed in the murine mammary gland during development and in mammary tumor cell lines (7–10). This resulted in the identification of the novel serine/threonine kinase, \textit{Pnck}\(^3\), so named to reflect its temporally and spatially regulated pattern of expression in the mammary gland as described in this report. \textit{Pnck} is a member of the CaM-dependent family of protein kinases and is most closely related to CaMKI (9). However, no significant homology is detected between \textit{Pnck} and members of the CaM kinase family outside of the highly conserved catalytic and regulatory domains, suggesting that \textit{Pnck} may have functions unique to this family of molecules.

Ca\(^{2+}\) is a key intracellular signaling molecule that exerts some of its effects by binding to calmodulin and activating CaM kinases. Calmodulin, in turn, has been implicated in development. For example, point mutations in the \textit{Drosophila} calmodulin gene result in defects in development including pupal lethality and ectopic wing vein formation (11). Furthermore, calmodulin expression is regulated during cardiac development, and overexpression of calmodulin in murine cardiomyocytes results in cardiomyocyte hypertrophy (12). Like calmodulin, CaM kinases have been proposed to play diverse roles in development including CaMKIV in T-cell maturation and CaMKII in cell cycle regulation (13–16). However, developmental roles for multifunctional CaM kinases, including CaMKI, have not been defined.

We previously characterized the temporal and spatial patterns of \textit{Pnck} expression during murine development (9). In murine embryos, \textit{Pnck} expression is highest in developing brain, bone, and gastrointestinal tract. In adult mice, high levels of \textit{Pnck} expression are found in the brain, uterus, ovary, and testis. Interestingly, within several tissues \textit{Pnck} expression is limited to particular epithelial or stromal compartments, and within these compartments, \textit{Pnck} expression is further restricted to a subset of cells (9). As such, the tissue-specific and spatially restricted patterns of \textit{Pnck} expression suggest that this kinase may be involved in a variety of developmental processes.

In this report, we demonstrate that the CaM kinase, \textit{Pnck}, is spatially and temporally regulated during murine mammary development with highest levels of expression observed late in pregnancy as alveolar epithelial cells exit the cell cycle and undergo terminal differentiation. Potentially related to this temporal pattern of expression, \textit{Pnck} is up-regulated in confluent mammary epithelial cells and down-regulated as serum-starved cells are stimulated to reenter the cell cycle. We further show that \textit{Pnck} expression in the mammary gland is restricted to a subset of epithelial cells during development and that \textit{Pnck} is expressed in an oncogene-associated manner in cell

\(^1\) The abbreviations used are: \textit{Pnck}, pregnancy up-regulated nonubiquitous CaM kinase; CaM, Ca\(^{2+}\)/calmodulin; MMTV, mouse mammary tumor virus.

\(^2\) To whom requests for reprints should be addressed, at 612 Biomedical Research Building III, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104-6160. Phone: (215) 898-1321; Fax: (215) 573-6725; E-mail: chodosh@mail.med.upenn.edu.

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\(^*\) To whom requests for reprints should be addressed, at 612 Biomedical Research Building III, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104-6160. Phone: (215) 898-1321; Fax: (215) 573-6725. E-mail: chodosh@mail.med.upenn.edu.

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lines derived from murine mammary tumors with defined initiating events. Similarly, expression of the human homologue of Pnck is restricted to a subset of human breast tumor cell lines and is highly overexpressed in a subset of primary human breast cancers. Taken together, our data suggest that Pnck may be expressed within a mammary epithelial cell type that is involved in differentiation as well as transformation.

MATERIALS AND METHODS

Animal and Tissue Preparation. FVB mice were housed under barrier conditions with a 12-h light/dark cycle. After sacrifice at the indicated developmental time points, the #3, #4, and 5 mammary glands were harvested. For RNA analysis, the lymph node embedded in mammary gland #4 was removed prior to harvest. Time-matings were set up such that all mice were sacrificed at ~16 weeks of age for comparison to adult nulliparous females. Day 0.5 postcoitus was defined as noon of the day on which a vaginal plug was observed. Time points at day 2 and day 7 of regression were obtained after removing pups at day 9 of lactation. Time points at day 28 of regression were obtained after 21 days of lactation. Tissues from 10 to 20 mice were pooled for each developmental time point. Tissues used for RNA analysis were snap frozen on dry ice. Tissues used for in situ hybridization analysis were embedded in OCT compound.

Tissue Culture. Murine cells were cultured in DMEM medium supplemented with 10% bovine calf serum, 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human cell line nuclei were cultured in the same medium with the addition of 5 μg/ml insulin. Transformed murine mammary epithelial cell lines were derived from tumors or hyperplastic lesions that arose in transgenic mice engineered to express different oncogenes under the control of the MMTV long terminal repeat. Cell lines from MMTV-c-myc, MMTV- int-2/ Ifg3, MMTV-neuNT, or MMTV-H-ras transgenic mice have been described previously (17). NIH 3T3, NMuMG, and CL-S1 murine cells, as well as human breast tumor cell lines, were obtained from American Type Culture Cells. HC11 cells were the kind gift of Jeff Rosen (Baylor College of Medicine, Houston, TX).

Actively growing cells were harvested at ~70% confluence. Confluent cells were refed daily and harvested 3 days after confluence. For serum starvation experiments, subconfluent cells were maintained in 0.1% serum for 2 days prior to refeeding in 10% bovine calf serum and harvested at the indicated time points.

RNA Analysis. RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 μl/ml 2-mercaptoethanol, followed by ultracentrifugation through cesium chloride as described previously (18, 19) Poly(A) + RNA was selected using oligo(dT) cellulose (Pharmacia). For Northern hybridization analysis, RNA was separated on a 1% LE agarose gel and passively transferred to a Gene Screen membrane (DuPont NEN). Hybridization was performed as described using a random primed, 32P-labeled cDNA probe encompassing the untranslated region of c-myc (GenBank accession no. X01023), nucleotides 589–1287 of cytokertatin 18 (GenBank accession no. M11686), or a 1.2-kb fragment containing the entire open reading frame of cyclin D3 (19). RNase protection analysis was performed as described (19). Body-labeled antisense riboprobes were generated using [α-32P]UTP and the Promega in vitro transcription system with T7 polymerase in combination with linearized plasmids containing nucleotides 1142–1241 of β-actin (GenBank accession no. X03672), nucleotides 911–1056 of Gapdh (GenBank accession no. M32599), nucleotides 1321–1509 of murine Pnck (GenBank accession no. AF181984), or a region of human Pnck corresponding to nucleotides 538–842 of murine Pnck. Riboprobes were hybridized with RNA samples overnight at 58°C in 50% formamide/100 m doi PIPES (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subjected to autoradiography (XAR-5). β-actin or Gapdh antisense riboprobes were added to each reaction as an internal control. As a negative control, riboprobes were hybridized with tRNA and processed in parallel.

In Situ Hybridization. In situ hybridization was performed as described (19). Antisense and sense riboprobes were synthesized with the Promega in vitro transcription system using [35S]UTP and [35S]CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 1135–1509 of Pnck. Exposure times were 7 weeks in all cases.

RESULTS

Temporal Pattern of Pnck Expression during Mammary Development. In the course of screening for protein kinases with a potential role in mammary development and carcinogenesis, we isolated a CaM kinase family member, Pnck, from the mammary glands of mice undergoing early postlactational involution (7, 9). To begin to investigate the potential role of Pnck in mammary development, we examined the temporal profile of Pnck expression during the postnatal development of the murine mammary gland (Fig. 1). Pnck expression was normalized to β-actin expression to correct for dilutional effects of large scale increases in milk protein gene expression during late pregnancy and lactation. Expression levels are shown relative to 16-week-old adult virgin animals.

Fig. 1. Temporal regulation of Pnck expression during murine mammary gland development. A, RNase protection analysis of Pnck mRNA expression during postnatal murine mammary gland development. Forty μg of total RNA isolated from mammary glands at the indicated developmental time points were hybridized to 32P-labeled antisense riboprobes specific for the 3’ untranslated region of Pnck or for β-actin. B, phosphorimager quantitation of RNase protection analysis in A. Pnck expression was quantitated and normalized to β-actin expression to correct for dilutional effects of large scale increases in milk protein gene expression during late pregnancy and lactation. Expression levels are shown relative to 16-week-old adult virgin animals.

Pnck mRNA expression levels were found to be low and relatively constant in nulliparous animals between 2 and 16 weeks of age, a period that encompasses ductal morphogenesis (Fig. 1). In contrast, a 2-fold up-regulation of Pnck expression was observed early in pregnancy as compared with age-matched nulliparous animals. Pnck expression was normalized to β-actin expression to correct for dilutional effects resulting from the massive increases in milk protein gene expression that occur during late pregnancy and lactation (Refs. 7, 19, and 20; Fig. 1B). As verified by quantitative in situ hybridization analysis, normalization of gene expression to β-actin expression provides a more accurate assessment of changes in gene expression on a per cell basis than normalization solely to RNA assayed (Fig. 2).

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4 J. Hartman, unpublished results.
expression remained elevated during mid-pregnancy and attained maxi-
mal levels of expression (5-fold) late in pregnancy, concomitant with
the cessation of proliferation and terminal differentiation of the alve-
olar epithelium. Pnck expression levels returned to baseline during
lactation and early postlactational regression. Notably, steady-state
levels of Pnck mRNA were higher in the mammary glands of parous
animals after 4 weeks of postlactational involution as compared with
age-matched nulliparous animals.

**Heterogeneous Expression of Pnck in the Mammary Epithel-
lum.** To determine whether pregnancy-induced changes in Pnck
mRNA expression levels represent global changes in expression
throughout the mammary gland or changes within a subpopulation of
cells, in situ hybridization analysis was performed (Fig. 2). Consistent
with our RNase protection results, in situ hybridization confirmed that
Pnck expression peaks late in pregnancy. Furthermore, throughout
postnatal development Pnck expression was detected only in the
mammary epithelium and was strikingly heterogeneous during preg-
nancy, with highly expressing cells located adjacent to cells in which
Pnck expression was low or undetectable. The spatial heterogeneity of
Pnck expression was most marked during late pregnancy, at which
time only a small fraction of epithelial cells was observed to express
Pnck at high levels. The heterogeneous spatial pattern of Pnck ex-
pression differs from that observed for other protein kinases that we
have examined, as well as for genes such as cytokeratin 18, Gapdh,
and β-actin (7).

**Pnck Expression in Vitro.** The observation that Pnck expression
peaks late in pregnancy as alveolar epithelial cells exit the cell cycle
and undergo terminal differentiation suggested that Pnck mRNA
expression may be inversely related to mammary epithelial prolifer-
ation. To investigate this possibility, Pnck mRNA levels were ana-
lyzed in actively proliferating or confluent mammary epithelial cell
lines (Fig. 3A). This analysis revealed that steady-state levels of Pnck
mRNA were an average of 3.7-fold higher in confluent cells compared
with actively proliferating cells (Student’s t test, P < 0.01). To
distinguish whether this increase in Pnck expression was attributable
to decreased proliferation or to the establishment of cell-cell contacts
in confluent cells, Pnck expression levels were analyzed in subcon-
fluent serum-starved mammary epithelial cells as they reentered the
cell cycle after refeeding (Fig. 3B). Consistent with the up-regulation
of Pnck expression observed in confluent cells, refeeding of serum-
starved cells resulted in a rapid decrease in Pnck expression that began
within 1 h and reached a nadir at 4 h after refeeding. Identical results
were observed in a second mammary epithelial cell line (data not
shown).
PNCK Expression in Transgenic Mammary Tumor Cell Lines.

To begin to examine the potential role of PNCK in mammary tumorigenesis and to investigate the hypothesis that PNCK is expressed in an epithelial cell subtype in the mammary gland, PNCK mRNA expression was examined in a panel of mammary epithelial cell lines derived from independent adenocarcinomas arising in MMTV transgenic mice expressing either the neu/NT, c-myc, H-ras, or int-2/Fgf3 oncogenes in the mammary epithelium (Ref. 17; Fig. 4). All cell lines were proliferating at similar rates when harvested as evidenced by their similar levels of cyclin D3 mRNA expression. PNCK expression was not detected in NIH 3T3 fibroblasts, consistent with its epithelial-specific pattern of expression in the mammary gland in vivo. Interestingly, PNCK was expressed in all seven cell lines derived from mammary tumors or hyperplasias arising in MMTV-c-myc and MMTV-int-2/Fgf3 transgenic mice. In contrast, PNCK expression was undetectable in the eight cell lines derived from mammary tumors arising in MMTV-neu and MMTV-H-ras transgenic mice, despite the fact that RNase protection analysis was performed using poly(A)^+ RNA. Similarly, PNCK expression was not detected in any of the three nontransformed mammary epithelial cell lines examined including confluent or differentiating HC11 cells (Fig. 4 and data not shown). Analysis of the expression of 40 other protein kinases identified in our screen indicated that this particular oncogene-associated pattern of expression is unique to PNCK (7, 20). Of note, PNCK expression did not appear to correlate with absolute levels of either endogenous c-myc or c-myc transgene expression (Fig. 4). This observation suggests that the oncogene-restricted pattern of PNCK expression may not be the result of c-myc-induced activation of PNCK transcription.

PNCK Expression in Human Breast Tumor Cell Lines and Primary Breast Tumors. To further investigate the potential involvement of PNCK, or a cell type in which PNCK is expressed, in mammary carcinogenesis, we determined PNCK expression levels in a panel of human breast cancer cell lines (Fig. 5). Similar to the wide range of PNCK expression observed in the murine mammary epithelium and in murine mammary tumor cell lines, PNCK expression was detected in only a subset of human breast tumor cell lines. High levels of PNCK expression were observed in 3 of 18 breast tumor cell lines. Eight cell lines expressed low but detectable levels of PNCK, whereas no PNCK expression was detected in the remaining seven cell lines. As in murine mammary tumor cell lines, PNCK expression levels did not correlate with c-MYC expression (data not shown).

The heterogeneous pattern of PNCK expression observed in vitro in both murine and human breast tumor cell lines suggested the possibility that PNCK-expressing and PNCK-nonexpressing breast tumor types might exist. To test this hypothesis directly, we used RNase protection analysis to quantitate PNCK mRNA expression levels in a panel of 23 primary human breast tumors. The resulting expression levels were compared with PNCK expression levels in 12 benign breast tissue samples (Fig. 6A). This analysis revealed two interesting aspects of the pattern of PNCK expression in breast tumors compared with benign tissue: (a) PNCK is expressed at significantly higher levels in breast tumors compared with benign tissue; and (b) PNCK expression in human tumors is markedly heterogeneous.

Statistical analysis of the above PNCK expression levels indicated that when normalized to β-actin expression, PNCK expression in human primary breast cancers is ~5-fold higher than in benign breast tissue (Student’s t test, P = 0.01; Fig. 6B). However, because PNCK expression in the mammary gland is epithelial specific and because tumors typically have a higher epithelial content than benign breast tissue, we also normalized PNCK expression to expression of the epithelial-specific marker, cytokeratin 18, (CK18), to control for the increased epithelial cell content of tumors (Fig. 6B). Strikingly, even after normalization to CK18 expression, PNCK expression levels were...

Fig. 3. Proliferation-dependent expression of PNCK. A, RNase protection analysis of PNCK expression in actively growing versus confluent cells. β-actin, 16MB9a, M158, and HBL2 were hybridized with 32P-labeled antisense riboprobes specific for PNCK or β-actin.

Fig. 4. PNCK expression in nontransformed and transformed murine mammary epithelial cell lines. Transformed cell lines were derived from murine adenocarcinomas arising in MMTV transgenic mice expressing the int-2/Fgf3, c-myc, neu, or H-ras oncogenes in the mammary gland (17). RNase protection analysis was performed on 6 µg of poly(A)^+ RNA isolated from actively growing murine cell lines hybridized with a 32P-labeled antisense riboprobe specific for the 3^ untranslational region of PNCK (top panel). Northern analysis was performed on 6 µg of poly(A)^+ RNA using 32P-labeled cDNA probes specific for c-myc (middle panel) or cyclin D3 (bottom panel). Note, the upper band observed in MMTV-c-myc-derived cell lines correspond to c-myc transgene expression. The poly(A)^+ RNA beneath the 28S rRNA band is shown as a loading control. Cell lines are: NIH 3T3 fibroblast, nontransformed (Non-Tx); Lane 1, NMuMG; Lane 2, HC11, and Lane 3, CL-S1; MMTV-int-2/Fgf3; Lane 4, HBL2; and Lane 5, 1128, MMTV-c-myc; Lane 6, 8MA1a; Lane 7, MBP6; Lane 8, M1011; Lane 9, M158; and Lane 10, 16MB9a; MMTV-neu; Lane 11, SMF; Lane 12, NaF; Lane 13, NF639; Lane 14, NF11005; and Lane 15, NK-2. MMTV-H-ras; Lane 16, AC816; Lane 17, AC711; and Lane 18, AC236.

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found to be three times higher in human primary breast tumors than in benign tissue (t test, P = 0.039).

Formally, the increase in PNCK expression levels in breast tumors compared with benign tissue could result either from increased expression among all tumors or from increased expression in a subset of tumors. In this regard, analysis of the distribution of PNCK expression among the 23 ductal carcinomas studied revealed a wide range of PNCK expression levels, in contrast to the relatively similar levels of PNCK expression observed among benign breast tissue samples. Notably, the mode for the benign and tumor distributions was the same (Fig. 6, A and C). Indeed, examination of the histogram representing CK18-normalized PNCK expression levels revealed that 8 of the 23 primary breast tumors analyzed express PNCK at levels greater than 3 SDs above the mean observed for benign samples (Fig. 6C). This difference is highly significant because no tumors would have been predicted to express PNCK at these levels if the distribution of PNCK expression in tumors was similar to that observed in benign tissues. Even more strikingly, four breast tumors were found to express PNCK at levels >10 SDs above the mean observed for benign tissues. Together, these data indicate that PNCK is overexpressed in human primary breast cancers compared with benign tissue, and that this observed increase is attributable to high levels of PNCK expression in a subset of breast tumors.

DISCUSSION

We have demonstrated that expression of the CaM kinase, PNCK, is temporally and spatially regulated in the murine mammary gland during postnatal development and that PNCK expression is restricted to a subset of mammary epithelial cells both in vivo and in vitro. Furthermore, our findings that PNCK is up-regulated in serum-starved and confluent cells suggest that the up-regulation of PNCK expression in the mammary gland late in pregnancy may be related to the decreased proliferation of mammary epithelial cells during this stage of development. We have shown that PNCK is expressed in an oncogene-associated manner in murine mammary tumor cell lines with defined genetic initiating events, and that PNCK expression is restricted to a subset of human breast tumor cell lines. Finally, we have demonstrated that PNCK is overexpressed in human primary breast cancers compared with benign breast tissue and that this overexpression is restricted to a subset of human breast tumors. In aggregate, our findings are consistent with the hypothesis that PNCK expression is restricted to a subset of ductal carcinomas in humans and suggest a role for PNCK, or a cell type that expresses PNCK, in mammary carcinogenesis. Our findings represent the first data implicating a CaM kinase in mammary development or carcinogenesis.

Both calmodulin and CaM-dependent kinases have been reported previously to be involved in cell cycle progression (21–26). Our data demonstrate that PNCK expression in vitro is inversely correlated with cellular proliferation. Specifically, decreasing the proliferative status of mammary epithelial cells in vitro resulted in increased PNCK expression. Interestingly, both the up-regulation of PNCK observed in confluent cells and the down-regulation of PNCK observed as serum-starved cells reenter the cell cycle are consistent with PNCK expression patterns in the mammary gland during late pregnancy. Although the up-regulation of PNCK observed during late pregnancy could simply be an effect of decreased epithelial proliferation, PNCK up-regulation could also be directly involved in inhibiting cellular proliferation or contributing to the exit of epithelial cells from the cell cycle prior to their terminal differentiation. Nevertheless, although a role for PNCK in cell cycle regulation is plausible, further work will be required to establish this relationship.

The markedly heterogeneous spatial expression pattern that we have observed for PNCK in the mammary gland is unusual compared with other genes that we have investigated (7, 6). Moreover, the observation that PNCK expression peaks late in pregnancy and remains heterogeneous throughout pregnancy and lactation distinguishes PNCK from milk protein genes and other markers of mammary epithelial differentiation (27). Although the expression patterns of milk protein genes such as β-casein, WAP, and α-lactalbumin are spatially heterogeneous during the developmental stages at which they are initially induced, each of these genes is expressed homogeneously throughout the mammary epithelium when their expression peaks during lactation. These data suggest that PNCK expression is not simply a marker for terminally differentiated mammary epithelial cells. Indeed, the down-regulation of PNCK expression in the mammary gland during lactation is consistent with a model in which this kinase plays a role in the process of differentiation but not in the maintenance of the differentiated state per se.

At least two hypotheses could account for the heterogeneous pattern of PNCK expression in mammary epithelial cells in vivo and in vitro: (a) only a small percentage of mammary epithelial cells may express PNCK at any given time, but all cells may express PNCK at some time. Such a model is consistent with genes whose expression is cell cycle regulated but is inconsistent with our finding that multiple mammary epithelial cell lines do not express PNCK; (b) alternatively, we favor the hypothesis that PNCK expression may identify an as yet undefined mammary epithelial cell type. This hypothesis is consistent with our findings that PNCK expression is detected within only a subset of cells in the mammary epithelium in vivo, as well as within a subset of murine breast cancer cell lines, human breast cancer cell lines, and primary human breast cancers. Nevertheless, this hypothesis does not rule out the possibility that PNCK expression may be modulated within expressing cell types or that PNCK-expressing cell types may only express PNCK during certain physiological states. In aggregate, the expression patterns for PNCK described in this report suggest that a PNCK-expressing cell type exists that may have unique properties with respect to mammary development and mammary epithelial transformation.

Potentially related to the expression of PNCK in a mammary epithelial cell subtype, PNCK expression was found to be restricted to cell lines derived from murine mammary tumors initiated by the oncogenes c-myc or int-2/Fgf3 as compared with those initiated by an activated form of neu or by H-ras. Although such oncogene-associated patterns of expression are unusual, we and others have reported genes whose patterns of expression are the inverse of that observed for PNCK (7, 17, 28). Previous reports have demonstrated that murine mammary tumors induced by the expression of H-ras, c-myc, neu, or int-2/Fgf3 each have histological patterns that are highly specific for...
the inducing oncogene (29, 30). These morphological differences have been hypothesized to result either from the activation of unique downstream pathways or from the preferential transformation of different epithelial cell types by these oncogenes (17). Although c-myc or int-2/Fgf3 could directly up-regulate Pnck expression, the lack of correlation between Pnck expression and c-myc expression in mammary tumor cell lines, along with the punctate expression of Pnck in vivo, raises the possibility that the oncogene-associated expression of Pnck may be more likely to result from the preferential transformation of a Pnck-expressing cell type by c-myc. Experiments are currently under way to directly address these and other potential explanations.

Fig. 6. PNCK is overexpressed in a subset of human primary breast tumors. RNA was isolated from 12 benign breast tissue samples and from 23 primary breast tumors obtained after surgery as indicated. A, RNase protection analysis was performed using 10 μg of total RNA hybridized with a 32P-labeled antisense riboprobe specific for PNCK or for β-actin as indicated. Northern hybridization analysis was performed on the same RNA samples using 3 μg of total RNA hybridized with a 32P-labeled cDNA probe specific for cytokeratin 18 (CK18). The 28S RNA band is shown as a control for equal RNA loading. PNCK, β-actin, and CK18 expression levels were quantitated by phosphorimager analysis. PNCK expression levels normalized to CK18 are shown for each sample. B, PNCK expression levels in breast tumors compared with benign tissue. PNCK expression levels for the samples shown in A were normalized either to β-actin or to CK18, as indicated. Normalized PNCK expression levels in benign tissues was set equal to 1.0. The means of each distribution are shown. Bars, SE. *, P = 0.01 for PNCK/β-actin expression in tumors compared with benign tissue. ‡, P = 0.039 for PNCK/CK18 expression in tumors compared with benign tissue. C, histogram of individual PNCK expression levels normalized to CK18 for primary breast tumors and benign breast tissue samples shown in A. PNCK and cytokeratin 18 expression levels were quantitated by phosphorimager analysis. PNCK expression for each sample was normalized to CK18 expression and the average expression in benign samples was set equal to 1.0. Values represent fold changes relative to the mean PNCK/CK18 expression level observed for benign breast tissue. Bin sizes are 0.5 unit. Note that the mode for both the tumor and the benign samples is the same.
Superficially, our finding that one-third of human primary breast tumors overexpress Pnck compared with benign tissue seems inconsistent given the data presented in this report demonstrating an inverse correlation between Pnck expression and cellular proliferation. However, because negative regulators of the cell cycle are commonly up-regulated in tumors, it is possible that the observed up-regulation of Pnck in human tumors is a consequence of intact cell cycle checkpoints functioning to retard tumor growth. That is, if Pnck plays a negative role in cell cycle progression, the up-regulation of Pnck in breast tumors may be a result of the transformation process rather than an indication that Pnck plays a causal role in tumorigenesis.

We have hypothesized that Pnck expression is restricted to a mammary epithelial cell type that is transformed in a subset of breast cancers. This hypothesis is consistent with our findings that expression of the human homologue of Pnck is restricted to a subset of human breast tumor cell lines and is highly overexpressed in a subset of human breast tumors since these results could reflect the selection and enrichment of a particular epithelial cell type. Alternately, it is possible that Pnck expression in these cell lines and primary tumors does not correlate with a particular epithelial cell type or with a particular initiating genetic event. However, given the restricted pattern of Pnck expression in the mammary epithelium in vivo, along with the oncogene-associated pattern of Pnck expression in murine mammary tumor cell lines, we favor the hypothesis that selective expression of Pnck in human tumors is not random but rather reflects differences in the events that led to their transformation. Because tailoring specific therapeutic regimens to individual cancers bearing distinct molecular profiles may enhance the efficacy of breast cancer treatments, it will be important to evaluate the biological significance of the differential expression of Pnck in human breast cancers. For instance, it is possible that Pnck-overexpressing tumors may behave differently than other breast tumors and may thereby be associated with a different prognosis. Ultimately, the identification of either additional genes involved in breast cancer or genetic markers that identify different molecular subtypes of breast cancers will be invaluable both in improving our understanding of the pathogenesis of breast cancer and in promoting more effective treatments.

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Heather Perry Gardner, Seung I. Ha, Carol Reynolds, et al.

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