AKT-1, -2, and -3 are Expressed in Both Normal and Tumor Tissues of the Lung, Breast, Prostate, and Colon

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ABSTRACT

Purpose: **The AKT/PKB kinase controls many of the intracellular processes that are dysregulated in human cancer, including the suppression of apoptosis and anoikis and the induction of cell cycle progression. Three isoforms of AKT have been identified: AKT-1, -2, and -3. Selective up-regulation of AKT-3 RNA expression has been reported in hormone-independent breast and prostate cancer cell lines suggesting that AKT-3 expression may be increased with breast or prostate tumor progression. To determine whether AKT-3 RNA expression is selectively up-regulated in human cancers and whether the patterns of AKT RNA expression may change with tumor development, we examined AKT isoform expression by RT-PCR in human cancer cell lines, primary human cancers, and normal human tissues.**

Experimental Design: **AKT-1, -2, and -3 RNA expression was examined by RT-PCR. Because up-regulated AKT-3 expression has been implicated in human breast and prostate cancer progression, we also examined AKT-3 expression levels by semiquantitative RT-PCR using matched normal/tumor first-strand cDNA pairs from colon, breast, prostate, and lung cancers.**

Results: **Our data reveal that the overwhelming majority of both normal and tumor tissues express all three of the AKT isoforms. Moreover, semiquantitative RT-PCR of matched normal/tumor pairs confirmed similar AKT-3 RNA expression levels in both normal and tumor tissue.**

Conclusions: **Our data show that both normal and tumor tissues express all three of the AKT isoforms and indicate that tumorigenesis does not involve a dramatic shift in the RNA expression patterns of the three AKT isoforms.**

INTRODUCTION

The AKT/PKB kinase has been implicated in the genesis and/or progression of numerous human tumors, because AKT regulates many of the key effector molecules involved in apoptosis, anoikis, and cell cycle progression (1). AKT is activated by phosphatidylinositol 3 phosphates, the products of PI-3 kinase activity (2). In addition, AKT activity is commonly dysregulated in a variety of human tumors because of frequent inactivation of the *PTEN* tumor suppressor gene, which negatively regulates phosphatidylinositol 3 phosphate levels (1).

To date, three human isoforms of AKT have been identified, AKT-1, -2, and -3 (2–4). Several studies have found AKT-2 to be amplified or overexpressed in primary tumors and cell lines (5–7). A recent report has shown that AKT-1 and -2 are expressed in a wide array of breast and prostate cancer cell lines but that AKT-3 expression is exclusive to the more advanced, hormone-independent breast and prostate cancer cell lines (8). In addition, AKT-3 RNA expression was selectively up-regulated in the more advanced, ER[3]-negative primary human breast cancers (8). These data therefore suggested that AKT RNA expression patterns may be altered in tumors *versus* normal tissue (8). We have recently shown that AKT-1 and -3 RNA are expressed in both normal prostate tissue and primary prostate cancers, suggesting that patterns of AKT isoform RNA may not be dramatically different in tumor *versus* normal tissue (9).

To clarify whether AKT-3 expression is exclusive to tumors and/or if AKT RNA expression patterns are altered in tumor *versus* normal tissue, we have evaluated AKT isoform expression by RT-PCR in human cancer cell lines, primary human cancers, and normal human tissues. Our data reveal that the majority of the cancer cell lines, primary cancers, and normal tissues express AKT-1, -2, and -3. Moreover, semiquantitative RT-PCR analyses for AKT-3 expression in matched normal/tumor pairs confirm that AKT-3 RNA expression levels are not markedly altered in tumors relative to normal tissue of the lung, colon, prostate, or breast.

MATERIALS AND METHODS

RT-PCR Analysis. Total RNA and poly(A) RNA were isolated using the RNEasy kit and the Oligotex mRNA maxi kit from Qiagen (Chatsworth, CA). Normal tissue total RNA panels I, II, III, IV, and V were obtained from CLONTECH Laboratories (Palo Alto, CA). RT-PCR analysis of the RNA samples was performed with the Titan One Tube RT-PCR kit (Roche

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[3] The abbreviations used are: ER, estrogen-receptor; RT-PCR, reverse transcriptase-PCR.

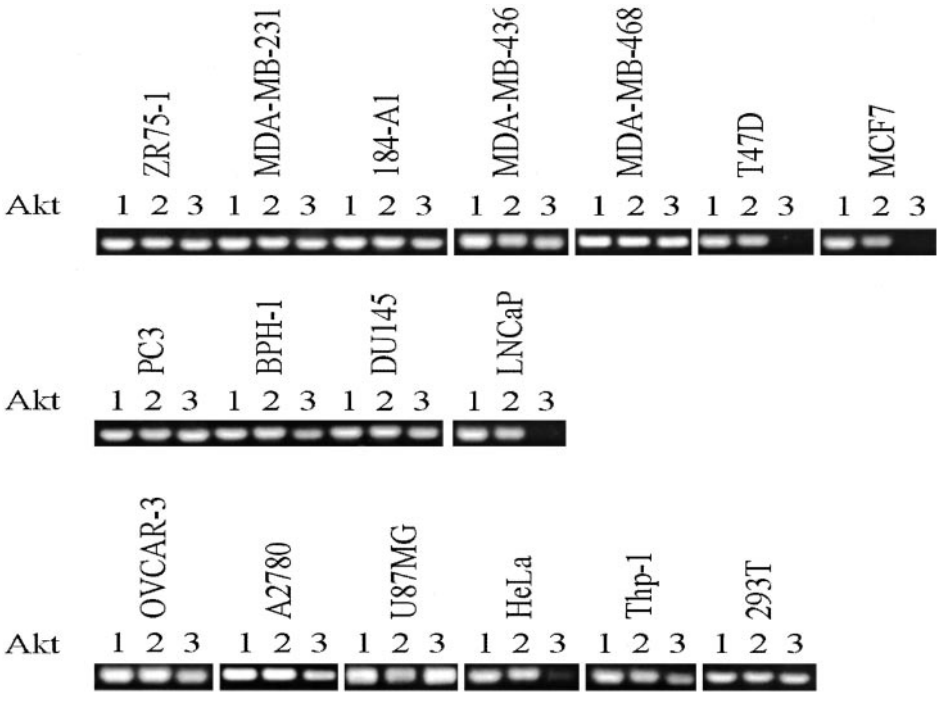

Fig. 1 RT-PCR analyses for AKT-1, -2, and -3 RNA in human cell lines. RT-PCR analysis was performed on 0.25–0.5 μg total or poly(A)+ RNA using primers specific for AKT-1, -2, and -3 (8) for 35 PCR cycles as described (9).

Biochemicals, Indianapolis, IN) as described previously (9) using the specific primer sequences and RT-PCR conditions described for AKT-1, -2, and -3 (8). The SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Life Technologies, Inc.) using 1–5 μg of total or poly(A) RNA with an oligo(dT) primer was used to confirm low abundance or absence of a specific mRNA identified from the One Tube RT-PCR reactions. Controls without RNA and without reverse transcriptase were routinely performed (data not shown). Restriction digestion with *Eag*I or *Pst*I (Life Technologies, Inc.) was used to verify the identity of AKT-1, -2, and -3 RT-PCR products (data not shown). *Eag*I restricts AKT-1 and -3 but not AKT-2, whereas *Pst*I restricts AKT-1 and -2 but not AKT-3.

Semiquantitative RT-PCR. To evaluate semiquantitatively the expression of AKT-3 RNA in tumor *versus* normal tissue, we performed RT-PCR with the High-fidelity PCR kit (Roche Biochemicals) using the aforementioned AKT-3-specific primers on first-strand cDNAs from matched tumor/normal pairs (CLONTECH Laboratories). We specifically evaluated the following tumor/normal pairs: prostate 1 and 3; breast 1 and 2; lung 4 and 5; and colon 2 and 4. To control for differences in template quantity between normal and tumor tissue, we also performed semiquantitative RT-PCR for the ribosomal protein S9 using the specific primers and conditions included with each tumor/normal pair (CLONTECH Laboratories).

RESULTS

Expression of AKT-1, -2, and -3 RNA in Human Cancer Cell Lines. Hormone-dependent breast and prostate cancer cell lines lack expression of AKT-3 (8), whereas many hormone-independent breast cancer cell lines express AKT-3, suggesting that more advanced cancers may selectively up-regulate AKT-3 expression (8). We therefore chose to catalogue expression of each AKT isoform in a large number of cell lines. RT-PCR analysis of 17 different cell lines revealed that only the prostate line LNCaP and the breast cancer cell lines MCF-7 and T47D lacked AKT-3. In the cervical carcinoma line HeLa, AKT3 expression was barely detectable (Fig. 1). All other cell lines, including the immortalized, nontumorigenic breast epithelial cell line 184A1 and the nontumorigenic prostate epithelial cell line BPH-1, expressed AKT-1, -2, and -3. Moreover, the ER-positive breast cancer cell line ZR-75–1 (10) also expressed AKT-3. Therefore, these data suggest that AKT-3 RNA expression is not restricted to cell lines derived from advanced cancers nor to tumorigenic cell lines.

Expression of AKT-1, -2, and -3 RNA in Primary Human Tumor and Normal Tissues. Because the majority of cell lines expressed AKT-1, -2, and -3, we next assessed expression of these isoforms in primary cancer tissues of the most common human solid tumors (colon, lung, breast, and prostate). RT-PCR analyses revealed AKT-1, -2, and -3 expression in each of the 28 tissues from breast, prostate, colon, and non-small cell lung cancers analyzed (Fig. 2). These data from primary human cancer tissues suggest that tumors routinely express all three AKT isoforms, though these tumor samples were not microdissected to separate tumor from normal tissue. As such, it is feasible that these patterns of AKT RNA expression may reflect that from normal tissue, tumor tissue, or both.

We therefore examined RNA expression patterns of the AKT isoforms in normal tissues. Our data revealed that the majority of normal tissues expressed AKT-1, -2, and -3 RNA (Fig. 3*A*). Northern blot analyses verified AKT-3 expression in normal tissues with the highest expression in brain (data not

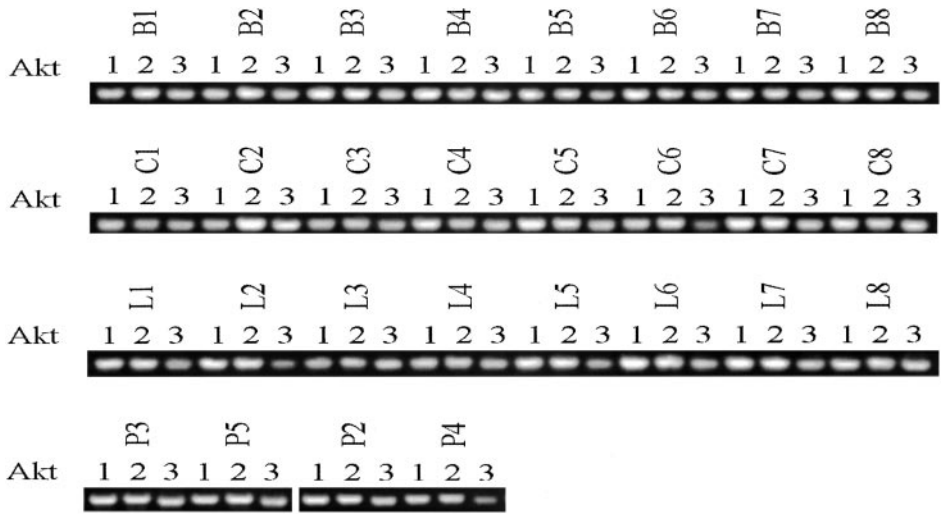

Fig. 2 RT-PCR analysis for AKT-1, -2, and -3 mRNA in primary human cancers. RT-PCR analysis for the AKT isoforms was performed on total RNA from primary human cancer tissues of the breast (*B*), colon(*C*), lung (*L*), and prostate (*P*) using primers specific for AKT-1, -2, and -3 (8) for 35 PCR cycles as described (9).

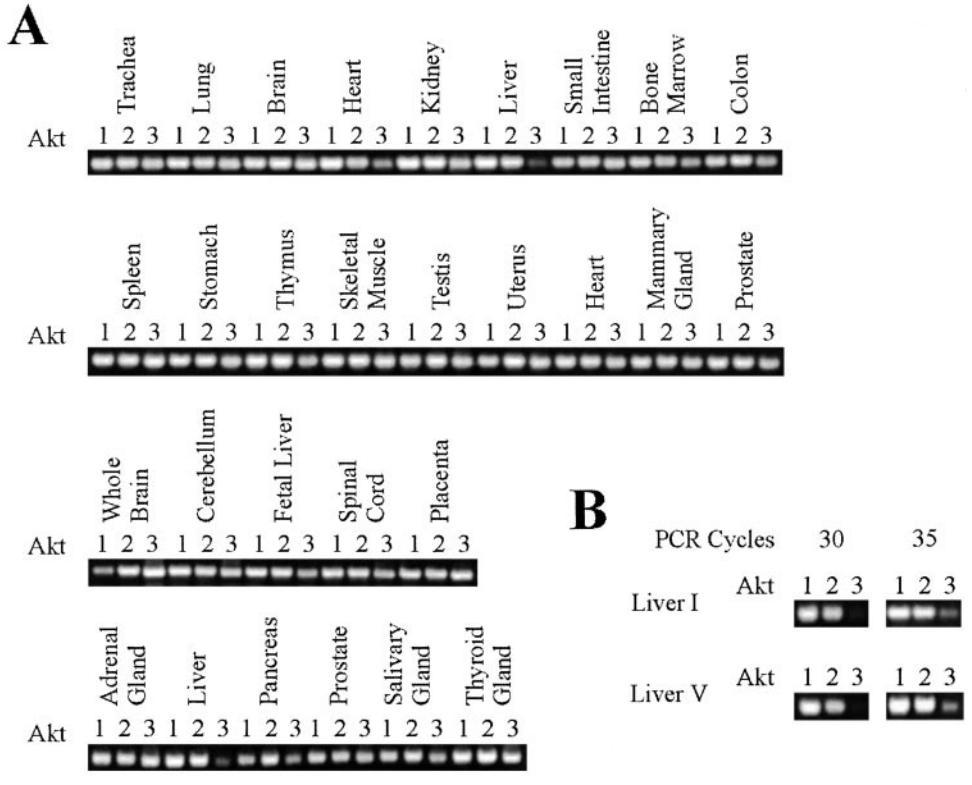

Fig. 3 RT-PCR analysis for AKT-1, -2, and -3 mRNA in normal human tissues. *A*, RT-PCR analysis was performed on CLONTECH total human RNA panels (I–V) using primers specific for AKT-1, -2, and -3 (8) for 35 PCR cycles as described (9). *B*, low level of AKT-3 RNA expression in adult liver was confirmed by performing RT-PCR for 30 or 35 cycles as indicated on first-strand cDNA from two independent adult liver RNA samples (CLONTECH).

shown), as has been published previously (3). Adult liver tissue showed only minimal evidence for AKT-3 RNA expression (Fig. 3*A*), which was subsequently verified in two independent adult liver tissue samples after PCR from first-strand cDNAs for 30 or 35 PCR cycles (Fig. 3*B*). These data are consistent with previously published Northern blot data showing that adult liver had low levels of AKT-3 mRNA (3, 11), whereas fetal liver maintained a relatively high level of AKT-3 mRNA (11). Together, these data indicate that the majority of both normal and tumor tissues express AKT-1, -2, and -3 RNA.

Semiquantitative RT-PCR for AKT-3 Expression in Matched Normal/Tumor Pairs. Our data indicate that normal tissues, with the exception of adult liver, express AKT-1, -2, and -3 RNA. Because up-regulated expression of AKT-3 RNA has been implicated in breast and prostate cancers (8), we wanted to examine AKT-3 RNA expression in tumor *versus* normal tissue in a more quantitative manner. We therefore examined first-strand cDNAs from matched tumor/normal pairs of the breast, prostate, colon, and lung using 30–38 PCR cycles. Our data revealed that both normal and tumor tissues express

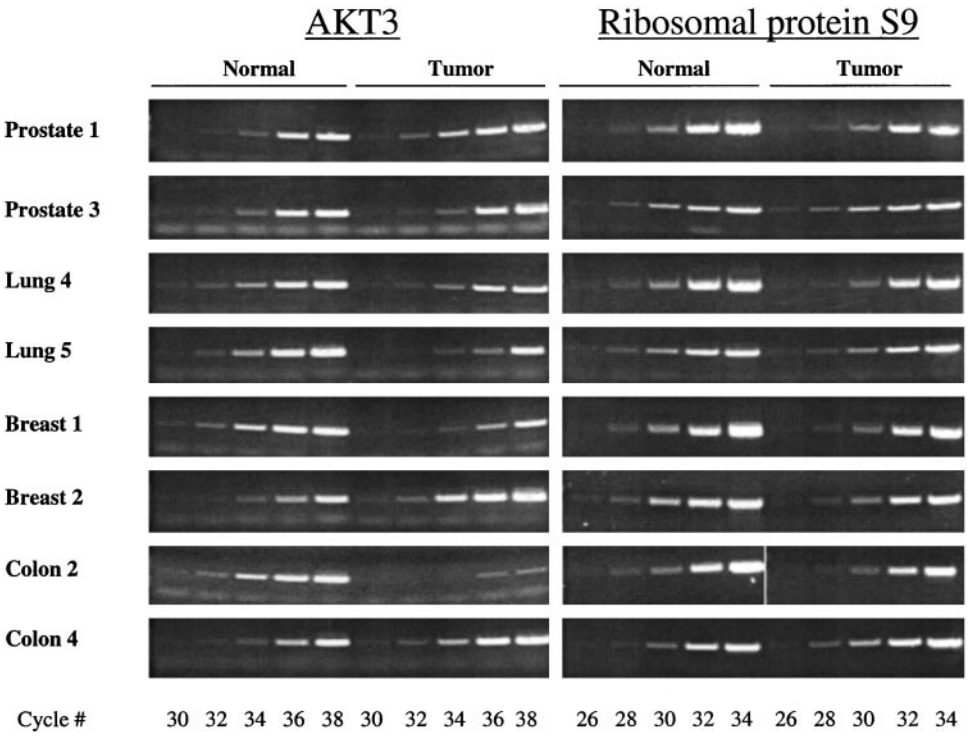

Fig. 4 Semiquantitative RT-PCR for AKT-3 in matched tumor/normal pairs. RT-PCR analysis was performed for AKT-3 on first-strand cDNA from matched tumor/normal pairs (CLONTECH) for the number of PCR cycles indicated. To control for variations in template concentrations, RT-PCR was also performed for a control gene, ribosomal protein S9.

similar levels of AKT-3 RNA (Fig. 4). AKT-3 expression in the samples Prostate 1 and Breast 2 may be elevated in tumor relative to normal but does not appear to be so in the other prostate and breast samples (Fig. 4). Moreover, the colon and lung samples show no apparent differences in AKT-3 levels when controlled for template concentration (Fig. 4). Therefore, these data indicate that there are no consistent differences in AKT-3 expression between normal and tumor tissue. In fact, the differences in AKT-3 signal are largely related to template concentration and are reflected by differences in expression of the control gene, ribosomal protein S9 (Fig. 4).

DISCUSSION

In this report, we have demonstrated that the majority of human tumor cell lines routinely express AKT-1, -2, and -3. As published previously, the hormone-dependent breast cancer cell lines MCF-7 and T47D and the hormone-sensitive prostate cancer cell line LNCaP lack AKT-3 expression (8). Our data now reveal that the hormone-dependent, ER-positive breast cancer cell line ZR-75–1 (10) expresses AKT-1, -2, and -3. Furthermore, the nontumorigenic, immortalized human breast epithelial cell line 184A1 and human prostate epithelial cell line BPH-1 also expressed all three isoforms. These data therefore suggest that AKT-3 expression is not restricted to tumorigenic cell lines or to ER-negative breast cancer cells.

We have also shown that primary human cancers of the breast, colon, prostate, and lung routinely express all three AKT isoforms. Likewise, virtually all normal tissues, with the exception of adult liver tissue (Ref. 3; Fig. 3*B*), express AKT-1, -2, and -3. These data suggest that there may be no differences in the expression of AKT-3 between normal and tumor tissue. However, up-regulated AKT-3 expression has been implicated in the progression of human breast cancers (8). Therefore, we chose to assess AKT-3 expression in normal *versus* tumor tissue more quantitatively. Semiquantitative RT-PCR analysis for AKT-3 RNA expression in matched tumor/normal pairs of the colon, breast, lung, and prostate confirmed that the level of AKT-3 RNA expression in normal tissue is not dramatically different from that of tumor tissue (Fig. 4).

Together, these data indicate that the RNA expression patterns of the AKT isoforms are not specifically related to the neoplastic process. Virtually every normal tissue examined, including prostate and breast tissue, showed AKT-3 RNA expression. Moreover, our data would suggest there may be no particular relationship between hormone receptor status and AKT-3 RNA expression. Normal breast and prostate tissues, which routinely express the estrogen and androgen receptors (12), express AKT-3 RNA as does the ER-positive breast cancer cell line ZR-75–1 (10). In fact, because some tumor cell lines lack AKT-3 expression (*e.g.*, LNCaP, MCF-7, and T47D), though the corresponding normal tissues and nontumorigenic epithelial cell lines express AKT-3, our data suggest that a small fraction of tumors may actually lose AKT-3 expression. Indeed, in our previous study, a small minority of prostate cancer metastases showed no evidence of AKT-3 expression (9).

Because the majority of normal tissues examined expressed RNA for all three AKT isoforms, our data suggest that an AKT isoform-specific inhibitor may not be tumor-specific. However, our data cannot exclude the possibility that there may be differences in the activity of AKT-1, -2, and -3 proteins that are not reflected by the patterns of AKT RNA expression. It is feasible that the different AKT proteins may be differentially activated

or that these activated proteins may have different substrate preferences. Such differences at the protein level may provide a therapeutic opportunity to target tumors preferentially by selectively targeting a particular AKT isoform. However, the data in this report suggest that AKT RNA patterns are not dramatically altered in tumors relative to normal tissue. As such, a broad-spectrum AKT inhibitor may be as effective, or perhaps more effective, as an anticancer therapy than an isoform-specific AKT inhibitor.

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REFERENCES

1. Cantley, L. C., and Neel, B. G. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc. Natl. Acad. Sci. USA, *96:* 4240–4245, 1999.

2. Coffer, P. J., Jin, J., and Woodgett, J. R. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem. J., *335:* 1–13, 1998.

3. Masure, S., Haefner, B., Wesselink, J. J., Hoefnagel, E., Mortier, E., Verhasselt, P., Tuytelaars, A., Gordon, R., and Richardson, A. Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. Eur. J. Biochem., *265:* 353–360, 1999.

4. Nakatani, K., Sakaue, H., Thompson, D. A., Weigel, R. J., and Roth, R. A. Identification of a human Akt3 (protein kinase B γ) which contains the regulatory serine phosphorylation site. Biochem. Biophys. Res. Commun., *257:* 906–910, 1999.

5. Miwa, W., Yasuda, J., Murakami, Y., Yashima, K., Sugano, K., Sekine, T., Kono, A., Egawa, S., Yamaguchi, K., Hayashizaki, Y., and Sekiya, T. Isolation of DNA sequences amplified at chromosome 19q13.1-q13.2 including the *AKT2* locus in human pancreatic cancer. Biochem. Biophys. Res. Commun., *225:* 968–974, 1996.

6. Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., and Masciullo, V., *et al*. Molecular alterations of the *AKT2* oncogene in ovarian and breast carcinomas. Int. J. Cancer, *64:* 280–285, 1995.

7. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. *AKT2*, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc. Natl. Acad. Sci. USA, *89:* 9267–9271, 1992.

8. Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J., and Roth, R. A. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. J. Biol. Chem., *274:* 21528–21532, 1999.

9. Graff, J. R., Konicek, B. W., McNulty, A. M., Wang, Z., Houck, K., Allen, S., Paul, J. D., Hbaiu, A., Goode, R. G., Sandusky, G. E., Vessella, R. L., and Neubauer, B. L. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing $p27^{Kip1}$ expression. J. Biol. Chem., *275:* 24500–24505, 2000.

10. Sommers, C. L., Thompson, E. W., Torri, J. A., Kemler, R., Gelmann, E. P., and Byers, S. W. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. Cell Growth Differ., *8:* 365–372, 1991.

11. Brodbeck, D., Cron, P., and Hemmings, B. A. A human protein kinase B γ with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. J. Biol. Chem., *274:* 9133–9136, 1999.

12. Denmeade, S. R., McCloskey, D. E., Joseph, I. B., Hahm, H. A., Isaacs, J. T., and Davidson, N. E. Apoptosis in hormone-responsive malignancies. Adv. Pharmacol., *41:* 553–583, 1997.