Prostate intraepithelial neoplasia induced by prostate restricted Akt model: The MPAKT model


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To determine whether Akt activation was sufficient for the transformation of normal prostate epithelial cells, murine prostate restricted Akt kinase activity was generated in transgenic mice (MPAKT mice). Akt expression led to p70S6K activation, prostatic intraepithelial neoplasia (PIN), and bladder obstruction. mRNA expression profiles from MPAKT ventral prostate revealed similarities to human cancer and angiogenic signature that included three angiogenin family members, one of which was found elevated in the plasma of men with prostate cancer. Thus, the MPAKT model may be useful in studying the role of Akt in prostate epithelial cell transformation and in the discovery of molecular markers relevant to human disease.

The insulin-like growth factor signaling pathway is implicated in both the initiation and progression of prostate cancer. For example, higher plasma insulin-like growth factor-1 levels are associated with prostate cancer risk, whereas inactivating somatic mutations of PTEN or loss of the PTEN protein are common in prostate cancer cell lines and in primary and metastatic tumor specimens (reviewed in ref. 1). The tumor suppressor activity of the lipid phosphatase PTEN is linked to its ability to antagonize phosphoinositide 3-kinase (PI3K) signaling (2). Thus, mutations in PTEN lead to deregulated PISK signaling, resulting in constitutive activation of downstream targets including the Akt/PKB kinase family (Akt; reviewed in ref. 3). In keeping with these data, Akt kinase activity is frequently elevated in primary prostate tumors (4).

Akt is activated through membrane recruitment to sites of phosphatidylinositol 3,4,5-trisphosphate, through conformational change and through phosphorylation on residues Thr-308 and Ser-473 (3). Recruitment to the membrane as a gag-fusion (as in v-akt) or through the addition of a myristoylation sequence is sufficient both for kinase activation and for transformation of rodent and avian cells (5, 6).

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P-40/5 mM EDTA, pH 8.0/1 mM NaOVal/1 mM DTT/5 mM NaF/5 μg/ml leupeptin/5 μg/ml aprotinin/1 mM PMSF), separated by gel electrophoresis, transferred, and immunoblotted as described (7). Anti-Akt-S473, anti-pan-Akt, anti-GSK3-S9, antipan GSK3 (Cell Signaling Technology) were used at 1:1,000, and anti-hemagglutinin (HA) (Santa Cruz Biotechnology) was used at 1:2,000.

RNA Isolation and Microarray Expression Analysis. Total RNA was prepared from VP by the Trizol method (Invitrogen). The generation of biotinylated target cRNA (using 15 μg of total RNA per sample) fragmentation, hybridization to the Affymetrix Mouse U74Av2 arrays, washing, and scanning were carried out as described (15). Raw expression values were normalized to the array intensity of array 1. Genes whose expression varied <5-fold between any two samples in the experiment were filtered. Genes differentially expressed between the four transgenic and four wild-type VPs were ranked by using a modified signal-to-noise metric calculated as follows: \[ |x_{\text{act}} - x_{\text{wt}}| / (\sigma_{\text{act}} + \sigma_{\text{wt}}) \] (16).

In Situ Hybridization. A 316-bp PCR product for angiogenin-3 was generated by RT-PCR using the oligonucleotides and 5'-TTAACCCTACTAAGGGACCAAGCAGCAGT-2A-3' and 5'-TAAAGCCACTCATAGGGCACA-GCAGCCTGTATGTGG-3', which include 17 and 13 polymerase binding sites. A cRNA probe for prostate stem cell antigen (PSCA) was generated as described (17). Digoxigenin-labeled (PSCA) and radiolabeled (angiogenin-3) riboprobes were generated, and in situ hybridization (ISH) was carried out as described (18).

Analysis of Plasma Levels of Angiogenin in Clinical Samples. Plasma samples were collected from consenting individuals with clinically localized prostate cancer (39 patients), metastatic, hormone-refractory (40 patients), or no history of prostate cancer (37 patients). Plasma angiogenin levels were determined after an 800-fold dilution in duplicate by using a QuantiGlo chemiluminescent ELISA kit (R & D Systems). Descriptive statistics were used to characterize each category of subjects. Wilcoxon rank-sum tests were used to assess the pair-wise differences between categories. Because each group was used in two comparisons, a P value of 0.025 was considered statistically significant. To assure that the conclusions were robust to method of analysis, a second method of analysis was applied. Specifically, the data were log transformed, resulting in a more normal distribution. The 95% confidence intervals were then generated for the natural log-transformed angiogenin levels for each group.

Results and Discussion

Expression of Activated Akt Spatially Restricted to the VP. After pronuclear injections of a plasmid insert containing −421 to +28 of the probasin promoter (19) upstream of a cDNA for HA epitope-tagged, myristoylated (myr), human Akt-1 (Fig. 1A), 10 founders harboring at least one copy of the transgene were identified by Southern blotting (data not shown). All founders were backcrossed to the FVB parental strain (Fig. 1B), and representative transgene-bearing F1 males were killed at 8 wk. RNA and protein of ventral, lateral, dorsal, and anterior prostate lobes were isolated, and evidence for transgene expression and protein production was sought by RT-PCR and anti-HA immunoblotting. A single line, MPACKT, was found to have prostate-specific expression of HA-myr-Akt1 mRNA and protein (Fig. 1C and data not shown). The prostate lobes of all of the F1 offspring from the nonexpressing lines were normal when evaluated histologically and were not evaluated further (data not shown).

Next, protein extracts from tissues of the genitourinary tract were harvested from 8-wk-old MPACKT and littermate control males and probed with anti-HA antibody. HA-myr-Akt1 protein was uniformly detected in the VP, and, in 10% of MPACKT mice, low-level expression was detected in the lateral prostate (LP; see also Fig. 2 E and F). Expression was not detected in any other tissue (Fig. 1D Upper).

To determine the activation state of the Myr-HA-Akt1, immunoblots were probed with antisera against phospho-S473 of Akt and with pan-Akt antisera. Endogenous Akt and Myr-HA-Akt1 were found in equivalent amounts; however, only Myr-HA-Akt1 was phosphorylated (Figs. 1D Lower and 2A). Similarly, genitourinary tract tissues of MPACKT and control males were fixed, embedded, and subject to immunohistochemical analysis (IHC) by using anti-pS473 antisera. Marked plasma membrane and cytoplasmatic staining was seen in the VP of MPACKT, but not in littermate controls (Fig. 2 C and D). In addition, focal pS473 staining of the LP was observed in a minority of transgenic males (Fig. 2 E and F). To determine the phosphorylation state of a downstream substrate of Akt, proteins and tissues isolated from both MPACKT and littermate control mice were immunoblotted or studied by IHC with antisera to phospho-S9 of GSK3 or total GSK3. Phosphorylation of GSK3 was detected by both methods in the VP of MPACKT males (Figs. 2 B, G, and H) and in tissue sections staining of phospho-S9 colocalized with anti-S473 staining. Finally, protein extracts
Akt Expression and Activation Is Sufficient for the Induction of PIN. Histologic study of the VP of the MPAKT line revealed a striking phenotype characterized by a hyperplastic and dysplastic epithelium with disorganized multicell layers, intraepithelial lumen formation, loss of cell polarity, nuclear atypia, and apoptotic bodies (Fig. 3 A–D; and Figs. 7 and 8, which are published as supporting information on the PNAS web site) (20, 21). These histopathologic features are consistent with PIN and were confirmed in independent review by three expert pathologists.

No abnormalities were seen in the dorsal or anterior prostate of the MPAKT mice. However, in the instances where phosphorylated Akt was focally expressed in the LP, coincident focal phosphorylation of a known Akt substrate have been achieved in a predominantly VP-restricted pattern.

PIN was noted in these areas (Fig. 2E and F). In addition, rarely areas of the VP in MPAKT mice failed to express Akt, and in this setting there was no evidence of PIN (data not shown). Thus, there is a direct correlation between transgene expression, activation of Akt, and the development of PIN. These latter data strongly suggest that this phenotype is a direct result of the expression and function of Akt kinase activity and is unlikely to have been caused by a gene activation or inactivation event resulting from the integration of the transgene.

Human PIN lesions are characterized by preservation of the basal cell layer and the basal cell layer. In the murine prostate, basal cells are similarly aligned circumferentially around the secretory epithelium. The p53 family member p63 is a highly specific basal cell marker that, in the mouse, is necessary for the appropriate maintenance of prostate basal cells (22). In keeping with observations made in human PIN, IHC staining for p63 revealed normal numbers and organization of the basal cell...
Akt Activation Leads to Activation of the p70^S6K Pathway, Increases in Prostate Epithelial Cell Size, and Urinary Bladder Obstruction. The PIN lesions observed in the MPAKT line seem to result from both an increase in cell number (a mean of $26.7 \times 10^4$ WT vs. $98.3 \times 10^4$ MPAKT) and an increase in cell size (a mean of $10.7 \pm 1.6$ μm WT vs. $15.74 \pm 3.3$ μm MPAKT) (Fig. 4G). This phenotype seen in D. melanogaster harboring PTEN mutations (23, 24) and in PTEN^+/− mice has been linked to the activation of p70^S6K (8). In keeping with these data, robust cytoplasmic staining for the p70^S6K substrate phospho-RPS6 (ppS6) was seen in the VP of MPAKT but not in WT males and colocalized with anti-Akt pS473 staining (Fig. 4 A and B). Likewise, in the LP ppS6, staining colocalized with areas of focal PIN and with focal staining of phospho-Akt (Fig. 4 C and D). These data suggest that the activation of Akt results in the activation of p70^S6K and is linked to the development of PIN in MPAKT mice.

The MPAKT PIN phenotype was markedly distinct from the pathological changes seen in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice where a cribriform intraepithelial hyperplasia containing smaller cells with hyperchromatic nuclei is detected at 10 wk and invasive adenocarcinoma is detected at 20 wk (19), but was similar, although more uniform and extensive, to that seen in PTEN^−/− mice. In these mice, we found that the prostate histology remained normal until 38 wk whereupon focal loss of PTEN protein was detected by IHC (in VP, LP, and dorsal prostate; Fig. 3G) along with coincident gain of anti-pS473 staining (Fig. 3H). In these areas, focal PIN lesions were found that were similar in histological appearance to those seen with Akt expression. In these lesions, activated endogenous Akt was localized primarily to the plasma membrane with a marked absence of nuclear staining, and, as seen in the MPAKT mice, phosphorylation of S6 was coincident with the activation of Akt (Fig. 4 E and F). These data suggest a marked overlap in the phenotype induced by activation of Akt and that resulting from loss of PTEN, and are consistent with the results of transgenic Akt1 expression in other organs (25–28).

Kaplan-Meier analysis showed minimal overall differences in survival between transgenic and nontransgenic littermates (Fig. 4H). However, older mice (15/41 transgenic mice vs. 0/23 WT mice) developed a prouteran abdominal as a result of a bladder outlet obstruction and were killed, resulting in a decrease in survival at later time points (Fig. 4 F and J). Sectioning of the urethra revealed an obstruction at the level of the prostate (data not shown). To date, histological examination of mice at 8, 17, 27, 45, 60, and 78 wk has failed to identify evidence of invasive cancer, including 41 transgenic mice aged 78 wk or older (Fig. 6). PTEN heterozygous mice do develop prostate adenocarcinomas (29), and, whereas Akt expression is sufficient to induce PIN, it is not sufficient for the induction of overt tumors in this strain of mice, suggesting that additional oncogenic events are required in vivo for progression to adenocarcinoma. The difference in the prostate phenotype between MPAKT and PTEN^+/− mice (PIN vs. invasive cancer) may arise as a result of growth/survival advantages conferred through loss of PTEN and not simply reproduced by activation of Akt. Alternatively, strain-specific differences between the C57BL/6 background of the PTEN^+/− mice and the FVB background of the MPAKT mice might account, at least in part, for a difference in the phenotype. Expression profiling data from our lab have revealed surprisingly strong strain-specific expression differences in the ventral prostate, strong enough that FVB and C57BL/6 segregate in self-organized maps (P.K.M. and W.R.S., unpublished results). Moreover, initial crosses of MPAKT onto the C57BL/6 background have revealed an increase in BrdUrd incorporation compared with the FVB strain (data not shown). Because

MPAKT mice do not suffer the consequence of an extraprostatic phenotype, this model provides an opportunity to study mutagenic, environmental, dietary, and possible strain-related or other genetic factors that may cooperate with activation of Akt to induce prostate tumorigenesis.
Expression Profiling and Angiogenesis in Transgenic Prostate. To determine whether the epithelial cells expanded within the PIN lesions observed in the MPAKT animals were similar at a molecular level to prostate epithelial cells found in human prostate cancer, the patterns of gene expression in MPAKT vs. nontransgenic animals were analyzed. To this end, total RNA was isolated from the VP of four MPAKT and four littermate control mice at 8 wk. Labeled cRNA was generated and hybridized to the Affymetrix U74Av2 murine expression array. Average difference values generated by using the MAS4 software (Affymetrix, Santa Clara, CA) were scaled (scaling factors ranged from 0.79–1.8). Genes were filtered to eliminate those that had less than 5-fold variation between the maximum and minimum values across the eight samples. The expression differences between FVB and MPAKT mice were sufficiently robust to allow for the unsupervised separation of FVB and MPAKT mice by hierarchical clustering (data not shown). Genes whose expression pattern was strongly correlated with the class distinction MPAKT vs. WT (FVB) were ranked by using a modified signal-to-noise metric and statistical significance assessed by permutation testing. Up-regulated genes (350) and 175 down-regulated genes exceeded the 5% permutation threshold. The top 50 up- and down-regulated genes are shown in Fig. 5A, and the list of the top 100 up- and down-regulated genes is shown in Tables 1 and 2, which are published as supporting information on the PNAS web site. All raw and normalized data can be found in Tables 3 and 4, which are published along with cel files as supporting information on the PNAS web site. All raw and normalized data and cel files also are available at http://research.dfc.earm.edu/sellerslab/datasets/index.html.

Among the most significantly up-regulated genes was PSCA, a gene that is expressed in prostate ductal tips during prostate development (17). In human prostate cancers, PSCA levels both at the mRNA and protein level are increased (30). Moreover, PSCA induction has been reported in PTEN at the mRNA and protein level are increased (30). Moreover, a gene that is expressed in prostate ductal tips during prostate revealed an extensive vasculature in the VP of MPAKT mice suggested that Akt-induced PIN might be associated with neo-membrane (Fig. 5). ISH; however, in contrast to the results for PSCA, expression was (Hig1; 3.3-fold). Angiogenin-3 overexpression was confirmed by

Additional proangiogenic factors or hypoxia-induced genes also overexpressed (10- and 3-fold induction, respectively; Table 3). The gene for osteocalcin (or gla protein), a gene also up-regulated in primary prostate cancer (32) and present in the serum of men with metastatic prostate cancer (33), was also strongly up-regulated (27-fold) in the expression profiles from the MPAKT mice (Fig. 5A and Table 1). ISH using a PSCA cRNA probe revealed robust staining of MPAKT, but not wild-type (Wt) littermate (Fig. 5B and C). These data suggest that multiple markers of human prostate cancer are expressed in this model.

Angiogenin-3 had the highest signal-to-noise score and was induced 32-fold (Table 1). Angiogenin-3 is a member of a family of secreted proteins that induce angiogenesis, and that includes angiogenin-related protein and angiogenin-1, both of which were also overexpressed (10- and 3-fold induction, respectively, Table 1). Additional proangiogenic factors or hypoxia-induced genes that were strongly induced included FGF-BP1 (12.5 fold), en-thelin-1 (gray). The rows representing the genes for osteocalcin (gray) and PSCA (blue) are indicated by arrows. (B–G) Validation of expression differences. PSCA (B and C) and angiogenin3 (Ang3; D and E) were visualized by ISH. Black arrows indicate representative positively stained cells. (F and G) Angiogenesis in the VP of MPAKT. Tissue sections of the VP from MPAKT (Tg-Akt) and wild-type (WT) littermate animals were stained with anti-CD31 antibody. The black arrows indicate representative stained vessels. (H) Plasma angiogenin level in clinical samples. Plasma level of angiogenin (pg/ml) in controls, untreated prostate cancer patients, and hormone refractory prostate cancer patients as indicated (B–G, >200).

The Angiogenins in Human Prostate Cancer Patients. The angiogenic signature found in these early PIN lesions raised the possibility that a number of these secreted factors might be elevated in patients with prostate cancer. In the human genome, there is only one angiogenin (Ang1). Angiogenin-3 (Mm.24663) is 80% homologous to human angiogenin-1, and there is no other apparent homolog. Therefore, angiogenin-1 levels were determined in plasma collected from patients with hormone-refractory prostate cancer (40 patients), newly diagnosed, untreated prostate cancer (39 patients), and from control patients with no evidence of prostate cancer (37 patients). Whereas there was significant variation in angiogenin levels, there was a statistically significant difference in angiogenin levels between the cancer groups and the non-cancer groups. Specifically, there was a statistically significant difference between controls and untreated, hormone-naive prostate cancer patients (P = 0.01) and between controls and hormone refractory prostate cancer patients (P = 0.0009). There was a trend toward higher levels of angiogenin in hormone refractory patients compared with un-
treated patients that did not reach statistical significance ($P = 0.27$). Therefore, angiogenin levels are higher in prostate cancer patients than in men without prostate cancer (Fig. 5H).

The question remains as to whether this signature reflects a direct activity of Akt. There have been a number of connections made between phosphoinositide 3-kinase signaling, PTEN loss, Akt activation, and the regulation of hypoxia-induced factor 1 (HIF-1) (34, 35). On the other hand, the marked segregation of angiogenin-3 expression to cells juxtaposing the basement membrane raises the possibility that this angiogenic signature may reflect a generalized transcriptional response to the proliferative influence of angiogenin-3 expression to cells juxtaposing the basement membrane raises the possibility that this angiogenic signature may reflect a generalized transcriptional response to the proliferative mechanism.

Endothelin-1 is elevated in PIN lesions and in the plasma of men with prostate cancer (36, 37). The finding that angiogenin is likewise elevated in the plasma of prostate cancer patients suggests that the MPAKT model recapitulates a number of elements commonly seen in human disease. Indeed, three independent secreted proteins (osteocalcin, endothelin-1, and angiogenin) are all overexpressed in this model and are found in the serum of patients with prostate cancer. These observations support the idea that the MPAKT model recapitulates certain elements of the prostate cancer disease phenotype, and, consequently, this mouse model may prove useful in the discovery of novel prostate cancer markers.

The robust nature and uniform penetration of the PIN phenotype and of the transcriptional profile, together with the lack of an extraprostatic phenotype and the maintenance of the phenotype in the heterozygous state, combine to make this a useful model in which to test both novel small molecular inhibitors of Akt, of TOR signaling, and of proteins (such as angiogenin) involved in the generation of an angiogenic response.

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