A Transgenic Mouse Model for Early Prostate Metastasis to Lymph Nodes

Hyun-Kyung Ko¹, Shin Akakura¹, Jennifer Peresie¹, David W. Goodrich², Barbara A. Foster², and Irwin H. Gelman¹

Abstract

The emergence of recurrent, metastatic prostate cancer following the failure of androgen-deprivation therapy represents the lethal phenotype of this disease. However, little is known regarding the genes and pathways that regulate this metastatic process, and moreover, it is unclear whether metastasis is an early or late event. The individual genetic loss of the metastasis suppressor, SSeCKS/Gravin/AKAP12 or Rb, genes that are downregulated or deleted in human prostate cancer, results in prostatic intraepithelial neoplasia. Here, we show that the combined loss of Akap12 and Rb results in prostatic intraepithelial neoplasia (PIN) that fails to progress to malignancy after 18 months. Strikingly, 83% of mice with PIN lesions exhibited metastases to draining lymph nodes, marked by relatively differentiated tumor cells expressing markers of basal (p63, cytokeratin 14) and luminal (cytokeratin 8 and androgen receptor) epithelial cells, although none expressed the basal marker, cytokeratin 5. The finding that PIN lesions contain increased numbers of p63/AR-positive, cytokeratin 5-negative basal cells compared with WT or Akap12/-/- prostate lobes suggests that these transitional cells may be the source of the lymph node metastases. Taken together, these data suggest that in the context of Rb loss, Akap12 suppresses the oncogenic proliferation and early metastatic spread of basal-luminal prostate tumor cells. Cancer Res; 74(3); 945–53. ©2014 AACR.

Introduction

Prostate cancer is the most commonly diagnosed noncutaneous cancer and the second leading cause of cancer mortality in men in the United States (http://seer.cancer.gov/statfacts/html/prost.html). During prostate carcinogenesis in humans, tumors progress from clinically undetectable precursor lesions, termed prostatic intraepithelial neoplasia (PIN), to locally invasive carcinomas often associated with metastatic disease to local lymph nodes and/or bones. Androgens act as prosurvival and proliferation factors in prostate cancer cells such that chemical or surgical therapeutic castration to deplete serum androgen levels results in a rapid and in most cases, durable tumor regression. However, failure of androgen ablation therapy leads to so-called castration-recurrent prostate cancer, marked by more aggressive, metastatic disease that progresses in the presence of very low, intracellular androgen levels (1).

Genetic comparisons between primary site and prostate cancer metastases within the same patient have been unable to resolve whether metastatic spread is an early or late event (2–4). Comparative array-CGH analyses by Holcomb and colleagues (5) offer evidence of both possibilities: cases of strong relationship between a patient’s primary-site and metastatic lesions, suggesting late metastatic dissemination versus cases in which there is more relatedness between metastases of different patients than their own primary-sites, suggesting early dissemination. Transgenic mouse prostate cancer models have shown that at least two, and more typically, three genetic lesions are required to induce spontaneous metastasis (6). Yet, these models may be influenced by whether the oncogenic mutations are built into basal (7) or luminal prostate epithelial cells (8), or both (9). For example, building on evidence that Rb and p53 are often mutated or deleted in prostate cancer (10), a conditional Cre-loxP deletion of both Rb and p53 driven by a truncated probasin promoter (expressed in prostate and seminal vesicle epithelial cells) was produced (11). This model exhibits prostatic adenocarcinoma and lymph node metastases, whereas the loss of Rb alone causes prostatic hyperplasia (12).

SSeCKS/Gravin/AKAP12 (Akap12) has been shown to function as a metastasis suppressor in prostate cancer, likely through its ability to scaffold key signaling and cytoskeletal proteins such as Src, protein kinase (PK)C, PKA, cyclin D, and F-actin in a spatiotemporal manner (13). Akap12 re-expression suppressed macroscopic metastasis formation by inhibiting VEGF-mediated neovascularization at distal colonization sites (14). Akap12 is downregulated by Src family kinases (15), known to be activated in castration-recurrent prostate cancer progression (16, 17), and indeed, the forced re-expression of Akap12 suppresses Src-induced oncogenic proliferation, invasiveness, and cell motility in prostate cancer cells by

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-13-1157
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directly scaffolding Src from growth factor receptor and FAK complexes to lipid rafts, without altering Src’s intrinsic kinase activity (18). Consistent with its function as a tumor suppressor in the prostate, there is significant Akap12 loss in prostate cancer progression, due to either transcriptional downregulation or allelic deletion (6q24-25.3; ref. 19). In addition, Akap12-null mice exhibit prostate hyperplasia marked by increased levels of cell senescence (20). Akap12-null mouse embryonic fibroblasts (MEF) suffer an RB-dependent premature senescence marked by multinucleation or polyploidy, suggesting a role for Akap12 in the regulation of cytokinesis and cell aging (21). The relative ease of isolating immortalized Akap12-null MEF in which components of the RB pathway were downregulated suggested that the combined loss of Akap12 and RB might be sufficient for oncogenic transformation. Here, we show that male Akap12-null mice with concomitant prostate-specific RB deletion readily formed low- and high-grade PIN lesions by 8 months of age. Unexpectedly, 83% of these mice exhibit metastases in local draining lymph nodes, marked by relatively well-differentiated tumor cells expressing markers of a transitional basal-luminal phenotype. Our data strongly suggest that prostate cancer metastasis to the lymph node is an early event facilitated by the combined loss of Akap12 and RB in the prostate.

Materials and Methods

Generation of mutant mice

Akap12<sup>−/−</sup>;Pb-Cre/Rb<sup>fl/fl</sup> males were generated by first producing Akap12<sup>−/−</sup>;Rb<sup>fl/fl</sup> mice from crossing C57BL/6 Akap12<sup>−/−</sup> mice (20) with FVB/N;129 Rb<sup>fl/fl</sup> males (11), and then intercrossing Akap12<sup>−/−</sup>;Rb<sup>fl/fl</sup> parents. Akap12<sup>−/−</sup>;Rb<sup>fl/fl</sup> females were then mated to C57BL/6 xDBA2 Pb-Cre males (11), and the Akap12<sup>−/−</sup>;Pb-Cre/Rb<sup>fl/fl</sup> males mated to Akap12<sup>−/−</sup>;Rb<sup>fl/fl</sup> females over several generations to yield Akap12<sup>−/−</sup>;Pb-Cre/Rb<sup>fl/fl</sup> mice. Note that the Pb-Cre allele was only passed through males in the intermediate lineages because of oocyte expression of the probasin promoter (22). All experiments were carried out in compliance with NIH guidelines and under the supervision of the Roswell Park Cancer Institute (RPCI, Buffalo, NY) Institutional Animal Care and Use Committee.

Genotyping

Rb<sup>fl/fl</sup>H alleles were identified by PCR of tail-snip DNA using primers Rb183M3′ (5′GGCGTGTGGCCATCATG3′) and Rb19EM5′ (5′CTCAAGACTGCACTGAGTGG3′); amplification of wild-type and floxed Rb gene sequences resulted in 235 bp and 283 bp DNA fragments, respectively, whereas Cre-excisected alleles resulted in 260 bp PCR products using primers Rb212M5′ (5′CGAAGAAAGTGACGGACATGGGC3′) and Rb183M3′. Wild-type and Akap12<sup>−/−</sup> genotypes were identified as described previously (20).

Immunohistochemistry and immunofluorescence analyses

Tissues were fixed in 10% buffered formalin (VWR) for 24 hours at room temperature before embedding in paraffin and sectioning (5 μm). Serial tissue sections each containing separated prostate lobes, seminal vesicle, lymph nodes (inguinal and pelvic), lung, liver, and kidney were stained first with hematoxylin and eosin (H&E) and then stained for Ki67 (Leica Biosystems; cat. #NCL-Ki67p; dilution 1:500), TUNEL, cytokeratin (CK) 5 (Covance; cat. #PRB-160P; 1:200), CKs (Covance; cat. #MMS-162P; 1:1,000), CK14 (Abcam; cat. #Ab7800; 1:100), E-cadherin (Becton Dickinson Biosciences; cat. #610181; 1:500), androgen receptor (AR: Upstate Biotechnology; cat. #06-680; 1:200), vimentin (Sigma; cat. #A5691; 1:400), or p63 (Santa Cruz Biotechnology; cat. #sc-8431; 1:1,000) as described previously (20) using a DAKO Autostainer Plus system. Slides were dehydrated with graded alcohols and xylenes, then coverslipped, imaged, and analyzed using an Aperio digital pathology system (Spectrum software v.11.0.0.725). Double immunofluorescence analyses were performed on paraffin-embedded tissues with p63 (1:1,000), CK5 (1:1,000), and AR (Santa Cruz, cat. #sc-816; 1:100). Secondary antibodies include 1:1,000 dilutions of Alexa Fluor 568- or 488-goat-anti-mouse IgG or goat anti-rabbit IgG (Invitrogen-Molecular Probes). Fluorescent images were acquired on a Nikon TE 2000-E inverted microscope using Metavue v.7.7.6.0 software (Molecular Devices). Histologic assessment was based on published guidelines (23). p63/CK5/AR immunohistochemical (IHC) staining was quantified on an Aperio Imaging System. Two-hundred to 300 total epithelial cell nuclei were counted in at least 6 independent prostate regions and the numbers of p63<sup>+</sup>/CK5<sup>−</sup>, p63<sup>−</sup>/CK5<sup>+</sup>, or p63<sup>−</sup>/AR<sup>−</sup> cells were reported as a percentage of total epithelial cells in a lobe.

RNA in situ hybridization of paraffin-embedded tissue using digoxigenin-labeled cRNA probes

241 bp cDNA templates for antisense or sense RNA probes were generated by PCR amplification (95°C/3 minutes: 37 cycles of 95°C/30 seconds, 55°C/30 seconds, 72°C/30 seconds) of pBabe<sub>hygro</sub>-Cre plasmid DNA (gift of Eugene Kandel, RPCI) with the following primer pairs containing T7 RNA polymerase promoter sites (underlined):

Sense: forward, 5′-TAATACGACTACTATAGGGCCATTTCT-GGGGATGTGTTA-3′
reverse, 5′-CCCCGGAAGAACAGGTTGATTA-3′

Anti-sense: forward, 5′-GCATCCTGAGGTGTGAGTTA-3′
reverse, 5′-TAATACGACTACTATAGGGCCATTTCT-GGGGATGTGTTA-3′

Digoxigenin (DIG)-UTP–labeled antisense and sense RNA probes were generated by in vitro transcription using a DIG RNA Labeling Kit (Roche Applied Science, cat. #11175025910) according to the manufacturer’s instructions. The concentration of RNA probes was measured using a Thermo Scientific Nanodrop and then adjusted with diethyl pyrocarbonate (DEPC)-treated water to 500 ng/μL. In situ hybridization was carried out as follows: 5 μm sections of the formalin-fixed paraffin-embedded mouse prostate tissue were deparaffinized in three changes of xylene for 7 minutes each, washed twice in 100% ethanol for 5 minutes, followed by rehydration in a graded series of ethanol (95%, 70%, and 50%, 5 minutes each) and two washes in DEPC water for 3 minutes. Endogenous...
peroxidase activity was blocked with 3% hydrogen peroxide in DEPC water for 15 minutes and rinsed twice with DEPC water. The sections were then incubated at 37 °C for 30 minutes in Tris-buffered saline (TBS: 50 mmol/L Tris, pH 7.5, 180 mmol/L NaCl) containing proteinase K (20 μg/mL). After two washes in TBS (TBS plus 1% Triton X-100) for 3 minutes each, the sections were prehybridized for 1 hour at 55 °C in 100 μL hybridization buffer (Chemicon; cat. #S4040). DIG-labeled sense or anti-sense probes were diluted to 1 ng/μL in 150 μL of hybridization buffer, denatured at 65 °C for 2 minutes and put on ice. Tissue sections were covered with the denatured RNA probes and incubated for 16 to 18 hours at 55 °C. Unhybridized probe was removed by washing in 2 × prewarmed SSC (300 mmol/L NaCl, 30 mmol/L trisodium citrate, pH 7.0) at 45 °C for 10 minutes and room temperature for 5 minutes, and subsequently incubated for 30 minutes at 37 °C with 250 μL of RNase Cocktail (Invitrogen-Ambion; cat. #AM2286) diluted 1:30 in 2 × SSC. The slides were washed twice at high-stringency in prewarmed 50% formamide in 2 × SSC for 30 minutes at 55 °C, once with prewarmed 0.08 × SSC for 20 minutes at 55 °C, and then rinsed twice with TBST. Probe hybridization was detected using a DAKO autostainer, as follows: tissue sections were blocked with Super Block (Thermo-Pierce; cat. #37755) for 1 hour, incubated for 30 minutes with anti-DIG-HRP conjugate (Roche; #11207733910; 1:100) in Super Block, three washes with TBST for 5 minutes each, and then for 15 minutes with biotinylated rabbit anti-sheep Ig (Vector Labs; cat. #BA-6000; 1:1,000). Signal enhancement was facilitated by 30-minute incubation with ABC reagent (Vector; cat. #PK 6100), 5-minute incubation with DAB substrate (Dako-Agilent; cat. #K4367) and counterstaining with modified Harris hematoxylin (Thermo-Richard-Allan Scientific; cat. #72704) for 20 seconds. The slides were dehydrated with graded alcohols and then xylenes, coverslipped, and digitally analyzed using an Aperio system.

Results

Combined deletion of Akap12 and Rb leads to PIN within 6 months

To assess a possible cooperation between Akap12 and Rb in prostate carcinogenesis, Akap12-null mice were crossed with Pb-Cre;Rbfl/fl mice (11), resulting in Akap12fl/fl;Pb-Cre;Rbfl/fl mice (hereafter designated Akap12fl/fl;Rbfl/fl). In males, the prostate-specific composite rat probasin promoter, Pb4, is expressed in basal and luminal prostate epithelium in all prostate lobes (22). Prostate-specific deletion of the Rb exon 19 in all lobes was confirmed by PCR (Supplementary Fig. S1A) in 3-week-old mice. Importantly, no Cre-mediated recombination was detected in other organs such as the inguinal lymph nodes and kidney (Supplementary Fig. S1B).

As reported previously, Akap12-null mice exhibit prostatic hyperplasia starting at 4 months of age (Fig. 1A; ref. 20); similar effects were produced in Pb-Cre;Rbfl/fl mice (11, 12). One-year-old Akap12-null mice showed focal dysplasia in less than 30% of cases and one case of low-grade PIN (LGPIN) was detected in an 18-month-old (Fig. 1B). In contrast, Akap12fl/fl;Rbfl/fl-developed LGPIN in all of prostate lobes starting at 5 months (Fig. 1B) marked by a focal loss of columnar polarity, containing cells with slightly hyperchromatic, elongated, and irregular nuclei (Fig. 1A). Twenty-nine percent of Akap12fl/fl;Rbfl/fl mice also exhibited high-grade PIN (HPIN; Table 1) between 6 and 13 months of age marked by foci of highly atypical cells with severe nuclear pleomorphism and hyperchromasia (Fig. 1A). Dorsal (DP) and lateral prostate (LP) lobes exhibited a more severe phenotype, including HPGIN, than ventral (VP) or anterior prostate (AP) lobes.

To better classify the neoplastic phenotype of the prostate lesions in Akap12fl/fl;Rbfl/fl mice according to the criteria set forth by Pienta and colleagues (23), DP lobes from WT, Akap12fl/fl, or Akap12fl/fl;Rbfl/fl mice were analyzed by immunohistochemistry for progression biomarkers. Compared with Akap12fl/fl prostates, Akap12fl/fl;Rbfl/fl prostates displayed increased numbers of Ki67- and p63-positive cells, increased nuclear AR-staining levels and increased cytoplasmic CK8 levels (Fig. 2A, C, D, and F). AR expression is E2F1-inducible, and thus, its upregulation would be expected following the loss of Rb (24). Total E-cadherin staining was increased although heterogeneous, and significantly, there was little of the normal cell–cell demarcation pattern still retained in the hyperplastic Akap12fl/fl lesions, owing to the loss of polar epithelial organization in the PIN lesions (Fig. 2B).

Unexpectedly, the increased number of p63-positive cells in Akap12fl/fl;Rbfl/fl lesions did not correlate with a similar increase in positivity for CK5 (Fig. 2E), another basal epithelial cell marker. The relative slow growth of these PIN lesions even in the presence of increased apparent proliferation markers was likely explained by a concomitant increase in apoptosis, as measured by TUNEL assay (Supplementary Fig. S2A and S2B). It is noteworthy that the Akap12fl/fl;Rbfl/fl lesions displayed increased stromal layers as well as infiltrates of possible inflammatory cells (Fig. 1A) that were strongly positive for Ki67 and AR staining (Fig. 2A and C).

Basal cell proliferation in Akap12fl/fl;Rbfl/fl PIN lesions

A hallmark of many transgenic mouse models of PIN or prostatic adenocarcinoma is a concomitant loss of basal cells amidst proliferating epithelial cells expressing luminal markers such as CK8 or 18 (23). However, there is evidence that prostate cancer can arise from basal epithelial cells that transition to a luminal phenotype including induction of AR and CK8 expression (7, 9). We followed up on the observation of increased numbers of p63-positive cells in the Akap12fl/fl;Rbfl/fl PIN lesions by first showing that all four lobes displayed significantly increased numbers of basal cells, especially in the DP and AP, compared with WT or Akap12fl/fl mice (Fig. 3A). Moreover, the normally flat cell morphology of a basal cell layer was replaced by apolar cells with enlarged, atypically shaped nuclei containing segmented chromatin (Fig. 3B). Double staining for p63 and CK5 showed that WT and Akap12fl/fl–basal cells had fully concordant coexpression of both markers, whereas there was a 35% decrease in p63-positive cells in the Akap12fl/fl;Rbfl/fl PIN lesions containing with CK5 compared with controls (Fig. 3C (left) and D; Supplementary Fig. S3). In addition, while nuclear androgen receptor staining was mainly in WT or Akap12fl/fl luminal...
cells (Fig. 3C, right; Supplementary Fig. S3), many Akap12−/−; RbPE−/− cells displayed combined p63 and AR positivity. Taken together, these data suggest that Akap12−/−; RbPE−/− PIN lesions contain both proliferating luminal as well as transitional basal-luminal epithelial cells.

Loss of Akap12 and Rb results in significant prostate metastasis to the lymph node

None of the Akap12−/−; RbPE−/− mice aged to 24 months showed evidence of adenocarcinoma, indicating that the combined loss of Akap12 and Rb was only partially oncogenic in regards to the primary prostate site. However, consistent with Akap12 functioning as a metastasis suppressor in prostate cancer cell lines (14, 18, 25), and with the increased levels of AKAP12 promoter hypermethylation in human prostate cancer lesions with Gleason grades 8–10 (26), 83% of the Akap12−/−; RbPE−/− mice that had PIN lesions also displayed metastases in their pelvic or inguinal lymph nodes (Fig. 4A; Table 2). In contrast, none of the WT or Akap12−/− mice had such lesions. Moreover, this frequency of metastasis is significantly higher than in mice with combined deletions of Rb and p53 in the prostate (83% vs. 68% ± 8; ref. 11). All mice with lymph node metastases had foci of primary site HGPIN, many of which showed clear evidence of localized invasiveness, an example of which is shown in Supplementary Fig. S4A. Notably, macroscopic metastases were not detected in other peripheral organs.

**Table 1. Summary of prostatic epithelial defects in Akap12−/−;RbPE−/− mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Normal</th>
<th>Hyperplasia</th>
<th>LGPIN</th>
<th>HGPIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>10/11</td>
<td>1/11 (9%)</td>
<td>0/11</td>
<td>0/11</td>
</tr>
<tr>
<td>Akap12−/−</td>
<td>0/8</td>
<td>8/8 (100%)</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Akap12−/−;RbPE−/−</td>
<td>0/21</td>
<td>21/21 (100%)</td>
<td>20/21</td>
<td>6/21</td>
</tr>
</tbody>
</table>

aSix to 13-months-old.  
b18-month-old.

Figure 1. The combined loss of Akap12 and Rb results in PIN lesions. A, H&E staining of formalin-fixed paraffin sections of prostatic lobes from 8-month-old WT, Akap12−/−, and Akap12−/−; RbPE−/− mice. The hyperplasia of luminal epithelial cells found in Akap12−/− mice (yellow arrowheads) was replaced by PIN (arrows) in Akap12−/−;RbPE−/− mice. Insets show nuclear atypia with prominent nucleoli. B, graphic analysis of PIN formation in WT, Akap12−/−, and Akap12−/−; RbPE−/− mice over time, based on mouse numbers (n) per genotype.
or non-pelvic lymph nodes, and moreover, the pelvic lymph node lesions (Fig. 4A) were detected as early as 1 month following the earliest appearance of PIN lesions (Fig. 1B). We cannot rule out the possibility that other sites contain micro-metastases. However, to address whether the increased metastasis in Akap12<sup>fl/fl</sup>:Rb<sup>PE/−</sup> mice correlates with changes in circulating tumor cells (CTC), peripheral blood samples obtained by cardiac puncture from Akap12<sup>fl/fl</sup>:Rb<sup>PE/−</sup>, Akap12<sup>fl/fl</sup> or Rb<sup>PE/−</sup> mice (6 mice/group) were analyzed by sensitive PCR for CTCs, either for the floxed form of Rb or for the neo<sup>B</sup> cassette (in Akap12<sup>fl/fl</sup> mice). Although this assay could readily detect 1-CTC in 10<sup>3</sup>–10<sup>6</sup> buffy coat cells (Supplementary Fig. S5A), no increases in CTCs were found in Akap12<sup>fl/fl</sup>:Rb<sup>PE/−</sup> compared with Akap12<sup>fl/fl</sup> mice (Supplementary Fig. S5B). This strongly suggests that the increased lymph node metastasis in the Akap12<sup>fl/fl</sup>:Rb<sup>PE/−</sup> mice results from increased localized trafficking, possibly through the known ability of SSeCKS/Akap12 to regulate chemotaxis (25, 18). Another characteristic of the metastases is that these lesions are primarily within the lymph node sinusoids, with little or no evidence of invasion to the lymphoid follicles (Fig. 4B). This suggests that the disseminating cells are a subset of primary-site PIN cells that have gained trafficking potential yet have failed to redifferentiate to establish a mature metastatic focus.

The lymph node metastases contained tumor cells with highly epithelioid morphologies, lacking mesenchymal morphologies typical of aggressiveness. Moreover, they expressed both basal and luminal epithelial markers: strong staining for p63 (96% of mice with positive staining), CK8, and 14 (78% and 67% positivity, respectively), E-cadherin (82% positivity), and vimentin (60% positivity) but not for CK5 (9% positivity; Fig. 4B; Supplementary Fig. S6). It is noteworthy that the same CK5 antibody stained basal epithelial cells in prostate lobes (Fig. 2E) on the same slide as the lymph node samples, ruling out the possibility of a technical artifact. Staining for nuclear AR and E-cadherin was heterogeneous among cells within lymph node lesions (Fig. 4B), although there was a significant increase in TUNEL-positive apoptotic cells compared with controls lymph nodes (Supplementary Fig. 2C). Less than 5% of the lymph node lesions homogeneously expressed the neuroendocrine marker, synaptophysin (Supplementary Fig. S4B), and taken with the expression of cytokeratins in the remaining 95%, this indicates that the vast majority were likely adenocarcinoma precursors. We excluded the possibility that the lymph node lesions were CD68-positive histiocytes (Supplementary Fig. S6), that is, cells that might be recruited in cases of chronic inflammation.

Similar to the mild oncogenic penetrance of the primary PIN lesions, the lymph node metastases did not grow aggressively although the inguinal lymph nodes of 10-month-old Akap12<sup>fl/fl</sup>; Rb<sup>PE/−</sup> mice typically weighed 3 to 4 times more than Akap12<sup>fl/fl</sup> controls. Notably, foci could be detected within Akap12<sup>fl/fl</sup>:Rb<sup>PE/−</sup> lymph nodes that contained cells with severe nuclear pleomorphism and abnormal nucleus to cytoplasm ratios (Fig. 4B, inset arrow) typical of HGPIN or carcinoma. PCR analysis of DNA isolated from laser microdissected lymph node or prostate tumor tissue indicated that the lymph node metastases were of prostatic origin in that they, but not tail DNA, encode Cre-mediated deletions of the Rb<sup>fl/fl</sup> locus (Fig. 4C).

Figure 2. IHC analysis of Akap12<sup>fl/fl</sup>;Rb<sup>PE/−</sup> PIN lesions. AP lobes from 8-month-old WT, Akap12<sup>fl/fl</sup>, and Akap12<sup>fl/fl</sup>;Rb<sup>PE/−</sup> mice were stained for Ki67 (A), E-cadherin (B), AR (C), p63 (D), CK5 (E), or CK8 (F). Cell-cell junctional E-cadherin staining in B (arrow) is contrasted with cytoplasmic staining (arrowhead). A focus of increased nuclear AR staining in Akap12<sup>fl/fl</sup>;Rb<sup>PE/−</sup> versus WT is shown in C (insets; arrows).
Importantly, lymph node DNA from 3-week-old Akap12−/−; RbPE−/− newborns were negative for the Rbfl/fl locus deletion (Supplementary Fig. S1B), strongly suggesting that the Rb-deleted cells in the adult lymph node originated from prostates.

To strengthen the notion that most, if not all, the lymph node lesion cells were of prostate origin, we performed RNA in situ hybridization using single-stranded Cre probes. The Cre probe showed no hybridization signals in primary VP lobes or lymph nodes from WT, RbPE−/− or Akap12−/− mice (Fig. 4D; Supplementary Fig. S7), whereas it induced strong cytoplasmic signals in both the PIN and lymph node lesions from Akap12−/−; RbPE−/− mice (Fig. 4D). Moreover, young Akap12−/−; RbPE−/− mice (<4 months), which show no evidence primary-site PIN disease (Fig. 1B), showed no Cre expression in lymph node (Supplementary Fig. S7). These data strongly suggest that the loss of Akap12 can potentiate an early metastatic colonization of Rb-negative prostate cells to local lymph nodes, although oncogenic progression in the prostate and lymph node is limited to non-malignant HGPIN.

Discussion

Building on our observation that the loss of Akap12 induces Rb-dependent senescence associated with the hyperplasia of luminal prostate epithelial cells (21), we now show that the crossing a prostate-specific Rb loss into an Akap12-null background induces PIN lesions that do not readily progress to adenocarcinomas. Indeed, while spontaneous death was rare even in 16 to 20-month-old Akap12−/−; RbPE−/− mice, the median survival for the combined prostate-specific loss of p53 and Rb was only 200 days (11). Although the prostate lesions in Akap12−/−; RbPE−/− mice display increased markers of proliferation, such as Ki67, it is likely that a concomitant increase in apoptosis in all four prostate lobes (as measured by TUNEL staining) antagonizes further progression to malignancy. This suggests that the combined loss of Rb and Akap12 fails to fully defeat all senescence pathways. Examples of parallel, tertiary senescence control mediators include p21, which mediates senescence of PC-3 prostate cancer cells following activation of the androgen receptor (27), or SMAD4, which facilitates prostate cancer malignancy in the background of PTEN loss (28).

Given the lack of malignant progression of the primary prostate lesions in Akap12−/−; RbPE−/− mice, the high incidence of lymph node metastasis was notable and suggests that prostate cancer metastasis to the lymph node can be an early event. The lymph node lesions, all of which derived from mice with focal HGPIN, contained cells with epithelioid, nonaggressive morphologies and expressed both basal and luminal prostate epithelial markers, such as CK14, p63, CK8, E-cadherin and AR, but not the basal marker, CK5. This correlated in the primary-site PIN lesions with a significant increase in total p63+ cells yet a relative decrease in coincident CK5 staining. The p63+ CK5+ cells in the primary PIN lesion lack typical basal cell morphologies and polarization, and the increase in this population correlates with increased nuclear AR expression. Although further experiments are required, it is interesting to speculate that the lymph node metastases arise from basal cell
populations that are transitioning to more luminal phenotypes. Interestingly, more than 50% of human prostate cancer cases with minimal primary site disease show evidence of prostate-specific antigen positivity in the bone marrow (2) or disseminated tumor cells in local lymph nodes (29), strongly suggesting early metastatic dissemination. Given that the

Figure 4. Lymph node metastases in Akap12−/−;RbPE−/− mice. A, incidence of lymph node metastasis in WT, Akap12−/−, and Akap12−/−;RbPE−/− mice based on numbers of mice (n) per genotype. B, characterization of cell markers by immunohistochemistry. Pelvic lymph nodes from 13-month-old Akap12−/−;RbPE−/− mice were analyzed for basal (p63, CK5, and CK14) or luminal (AR, E-cadherin) epithelial cells markers, as well as for a marker of mesenchymal transition (vimentin). Note the lack of CK5 staining. Arrow, examples of HGPIN-like cells with pleiomorphic nuclei and abnormal nuclear:cytoplasmic ratios. C, PCR-based confirmation of the prostatic origin of the lymph node tumor. PIN or lymph node metastasis lesions were isolated using laser capture microscopy (RPCI Pathology Resource Network, Carl Morrison, Director), and DNA isolated using a Qiagen QIAamp DNA Micro Kit was subjected to PCR using primers specific for the deleted Rbfl/fl allele (Rb212M5, Rb18M3; Supplementary Fig. S1A) and then analyzed by agarose gel electrophoresis. DNA isolated from the tail snip of the same Akap12−/−;RbPE−/− mouse (PCR-positive for the undeleted Rbfl/fl allele) was used as a negative control. D, RNA in situ hybridization for Cre expression in primary prostate (DP) versus lymph node in 9-month-old WT, Akap12−/− or Akap12−/−;RbPE−/− mice. Scale bars, 100 μm. E, Oncomine analysis (http://www.oncomine.org) of Lapointe prostate cancer expression data (38) showing AKAP12 downregulation in human prostate cancer metastasis compared with levels in primary prostate cancer (1# prostate cancer) or normal prostate tissue. F, Kaplan–Meier plot analysis (http://www.cbioportal.org/public-portal/) of metastasis occurrence vs. time to onset in 37 prostate cancer metastasis cases from Taylor and colleagues (37) in which 11 cases (29.7%) displayed AKAP12 downregulation compared with levels in primary prostate cancer lesions versus 26 cases with no change in AKAP12 levels.

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This document is a part of the Cancer Research journal. It discusses the transition of populations to more luminal phenotypes, with a focus on prostate cancer cases showing early metastatic dissemination. The text includes a figure illustrating lymph node metastases in Akap12−/−;RbPE−/− mice, along with methods and results involving immunohistochemistry, PCR-based confirmation, RNA in situ hybridization, and oncomine analysis.
majority of initial prostate cancer metastases in the clinical setting are found in local draining lymph nodes and bones.

In regards to the CK5-negative lesions in our model’s PIN and lymph node lesions, although human CK5-positive basal prostate epithelial cells can serve as progenitors of luminal prostate cancers (7, 30), and CK5/18-positive transitional cells have been described in human prostate cancer (31), small populations of CK5-negative basal cells have been described (32), which our genetic alterations may have selected. Moreover, CK14-expressing prostatic basal cells can serve as prostate cancer progenitors in transgenic models irrespective of their ability to express CK5 (9).

Previous data from our group indicated that Akap12 can suppress metastatic motility, chemotaxis, and invasiveness by inhibiting PKC-mediated pathways through direct scaffolding of FAK adhesion complexes, thereby attenuating MEK/ERK pathways controlling oncogenic motility (18). Thus, the ability of Akap12 to attenuate Src-induced oncogenic pathways (18, 25, 33, 34) likely contributes to its metastasis-suppressing activity, especially given the renewed focus on the role of Src-family kinases in mediating several parameters of metastatic and castration-recurrent prostate cancer (35, 36). Our findings are consistent with a role for Akap12 in suppressing metastatic progression such that loss of Akap12 in specific tissues, such as the prostate epithelium, results in a metastasis-prone condition. This is exemplified by the study of Taylor and colleagues (38), which showed further decreases in AKAP12 mRNA levels in prostate cancer metastases compared with the already downregulated levels in primary-site lesions (Fig. 4E). Indeed, of the 37 cases of metastatic prostate cancer from Taylor and colleagues (37) analyzed in the cbio website (http://www.cbioportal.org/public-portal/), the 11 cases that displayed AKAP12 down-regulation correlated with a more rapid appearance of metastases compared with the 26 cases that showed no changes in AKAP12 expression levels (Fig. 4F). Taken together with our findings, these data argue strongly for a role for AKAP12 in suppressing specific parameters of metastatic growth of prostate cancer.

Disclosure of Potential Conflicts of Interest

L.H. Gelman is a consultant/advisory board member of Kines Pharmaceuticals, LLC. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Acknowledgments

The authors thank Bryan Gillard and Ellen Karasik for excellent technical assistance and Eugene Kandel for sharing reagents and Gisusa Azabulaffari for advice on pathologic analyses.

Grant Support

This work was supported by NIH grants CA70292 (D.W. Goodrich), CA94108 and CA116430 (I.H. Gelman), and DoD grants PC074228 and PC101210 (I.H. Gelman), and in part, through NCI Comprehensive Cancer funds (P30-CA105665).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 25, 2013; revised November 4, 2013; accepted November 18, 2013; published online February 3, 2014.

References


Table 2. Frequency of lymph node metastases

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Lymph node metastasesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0/11</td>
</tr>
<tr>
<td>Akap12+/−</td>
<td>0/8</td>
</tr>
<tr>
<td>Akap12−/−;Rb1PE−/−</td>
<td>15/18 (83%)</td>
</tr>
</tbody>
</table>

aSix to 13-months-old.
bMetastases were identified minimally by H&E staining and by immunohistochemistry for p63, AR, or E-cadherin.


Correction: A Transgenic Mouse Model for Early Prostate Metastasis to Lymph Nodes

In this article (Cancer Res 2014;74:945–53), which was published in the February 1, 2014, issue of Cancer Research (1), the current address note was assigned to the wrong author. The corrected line is below. The publisher regrets this error.

The online version has been corrected and no longer matches the print.

Current address of H.-K. Ko: Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut.

Reference


Published OnlineFirst March 31, 2014.
doi: 10.1158/0008-5472.CAN-14-0603
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A Transgenic Mouse Model for Early Prostate Metastasis to Lymph Nodes
