Conditional Deletion of Insulin-like Growth Factor-I Receptor in Prostate Epithelium

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Abstract
Insulin-like growth factor-I (IGF-I) is a polypeptide hormone that can influence growth, differentiation, and survival of cells expressing the cognate type 1 receptor (IGF-IR). To better understand cell autonomous IGF-IR signaling in the epithelial compartment of the prostate gland, we generated a conditional (Cre/loxP) prostate-specific IGF-IR knockout mouse model. In contrast to epidemiologic studies that established a correlation between elevated serum IGF-I and the risk of developing prostate cancer, we show that abrogation of IGF-IR expression in the dorsal and lateral prostate could accelerate extracellular signal-regulated kinase 1/2 signaling and cause cell autonomous proliferation and hyperplasia. Moreover, persistent loss of IGF-IR expression in dorsal and ventral lobes induced p53-regulated apoptosis and cellular senescence rescue programs, predicting that titration of IGF-IR signaling might facilitate growth of tumors with compromised p53 activity. Therefore, we crossed the mice carrying the prostate-specific IGF-IR knockout alleles into the transgenic adenocarcinoma of the mouse prostate model that is driven, in part, by T antigen–mediated functional inactivation of p53. Consistent with our prediction, prostate epithelial–specific deletion of IGF-IR accelerated the emergence of aggressive prostate cancer when p53 activity was compromised. Collectively, these data support a critical role for IGF-IR signaling in prostate tumorigenesis and identify an important IGF-IR–dependent growth control mechanism. [Cancer Res 2008;68(9):3495–504]

Introduction
Prostate cancer remains the most common malignancy diagnosed in men in the United States with mortality second only to lung and bronchus cancers. These statistics underscore the need to elucidate and characterize the molecular mechanisms that predispose and promote spontaneous disease and the development of efficacious targeted therapeutic strategies for prevention and intervention. Because the insulin-like growth factor (IGF) signaling pathway has been implicated by clinical, epidemiologic, and experimental data as a contributing factor in the natural history of prostate cancer, strategies to target the IGF-I axis are being developed. Unfortunately, efforts to thoroughly investigate the causal relationship between IGF-I signaling and prostate cancer or validate the efficacy of anti–IGF-I-based therapies for autochthonous spontaneous disease have been hindered by the complexity of the IGF-I axis, the heterogeneous nature of clinical prostate cancer, and the paucity of appropriate models.

The IGF signaling axis is composed of two highly homologous ligands, IGF-I and IGF-II, and their cognate tyrosine kinase receptor [IGF type I receptor (IGF-IR)]. A structurally distinct second receptor (IGF-IR) binds and internalizes IGF-II but does not directly contribute to transmembrane signaling. A conserved family of six homologous IGF-binding proteins (IGFBP-1 to IGFBP-6) with high affinity for IGF-I and IGF-II, and two IGFBP-related proteins (IGFBP-rP1 and IGFBP-rP2) have been described (see refs. 1–6 for recent reviews). The circulating IGF-I ligand is mainly synthesized and secreted by the liver under control of growth hormone (GH). Production and pulsatile secretion of GH in the juvenile and adult pituitary gland are regulated by antagonistic actions of hypothalamic GH-releasing hormone (GHRH), somatostatin, and their corresponding receptors. The entire somatotropic neuroendocrine function is modulated by multiple hypothalamic and pituitary responses to such factors as stress, exercise, nutrition, and the hormone ghrelin. IGF-I functions primarily as a polypeptide hormone regulator of growth but is known to exert additional metabolic effects. Although IGF-I is also produced in other tissues and organs such as the prostate, the regulation and consequence of localized IGF-I production remain the subject of investigation.

The IGF-IR transmembrane tyrosine kinase receptor is composed of two extracellular α-subunits that constitute the ligand-binding domain and two intracellular β-subunits that compose the kinase domain. Ligand-dependent activation of the IGF-IR kinase can initiate multiple signaling events in part through phosphorylation of insulin receptor substrate (IRS) proteins, a family of which IRS-1 is the predominant member. Other notable downstream molecules that can transduce IGF-IR signals include SHC, Grb2, SHP2, phosphatidylinositol-3-kinase (PI3Ks), Akt, and mitogen-activated protein kinase (MAPK; refs. 7–9).

Increased lifetime prostate cancer risk has been associated with levels of serum IGF-I in the high reference range (10). Elevated levels of IGFBP-2 and reduced levels of IGFBP-3 have also been found in serum and prostate tissues of prostate cancer patients. Because IGFBP-3 is a primary carrier of IGF in serum, decreased functional expression of IGFBP-3 could influence the level of bioavailable IGF. This is of particular interest in prostate cancer because prostate-specific antigen, a kallikrein serine protease often found elevated in patients with prostate cancer, can cleave IGFBP-3 (11). In contrast to serum IGF-I, there is limited and conflicting data about the role of IGF-IR in clinical cancers. For example,
enforced expression of IGF-IR can cause fibroblasts to display a malignant phenotype in xenographs (12), whereas blockade of IGF-IR cellular activity impeded neoplastic progression in other model systems (see refs. 1, 13 for recent reviews). Reduced levels of both IGF-I and IGF-IR have been noted in disseminated androgen depletion-independent specimens from human (14) and mouse (15) prostate cancer, whereas elevated levels of IGF-IR have been correlated with reduced metastasis, local recurrence, and disease-free survival for patients with malignant soft tissue sarcomas (16). Recent reports have also shown that selective suppression of the IGF-IR signaling substrate IRS-1, favoring the function of IRS-2, can promote metastatic breast cancer in transgenic mice (17).

An emerging paradox from clinical and experimental studies is that IGF action can help cells survive transformation while simultaneously imposing a strong differentiation signal that may slow tumor progression. We have recently shown that enforced IGF-I signals can cause spontaneous preneoplastic changes in the prostate in a genetically engineered mouse (GEM) model and that selection against this same signal was concomitant with the emergence of more aggressive metastatic states (18), consistent with clinical data (14). Based on these findings, we proposed the "hurdle hypothesis" to explain how the strength of the IGF-I signal can help define the height of a differentiation hurdle that in turn determines how aggressive a cancer must become to achieve malignancy (18).

The role of the IGF-I axis in prostate cancer has been investigated in other GEM models with mixed results relative to clinical observations. In one system, expression of human IGF-I in basal epithelia under control of a bovine keratin 5 promoter (BK5-IGF-I) coordinately elevated systemic levels of IGF-1 and IGFBP-3 (19, 20). These BK5-IGF-I mice developed hyperplasia, prostatic intraepithelial neoplasia (PIN) lesions, and prostatic adenocarcinoma, as well as skin cancer and many other anomalies. Although elevated IGF-I and IGFBP-3 are often observed in the serum of acromegals (21), the patients do not seem specifically predisposed to prostate cancer. Hence, the tumors observed in the BK5-IGF-I mice may actually represent the consequence of the simultaneous activation of a variety of signaling programs in multiple tissue compartments. In another model, enforced systemic expression of rat prolactin under transcriptional control of the metallothionein promoter caused transcriptional control of the metallothionein promoter caused elevated serum levels of prolactin, IGF-1, and testosterone. Although these mice developed enlarged and hyperplastic prostate glands, they did not develop tumors (22, 23). Taken together, the data from GEM models show that the context in which IGF-I signaling is activated seems to be a major determinant of the resulting phenotype.

Using a model of multistage prostate tumorigenesis based on tissue-restricted abrogation of the p53 and Rb tumor suppressor pathways (24), we have previously shown that the prostate can actually be a source of IGF-1 expression (15). In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, serum IGF-I was found to be precociously elevated during prostate cancer progression and serum levels of IGFBP-2 correlated with advanced prostate cancer, in keeping with reports from clinical disease (25, 26). Whereas expression of IGF-IR mRNA was found to increase in the prostate during tumor development in TRAMP, expression was dramatically reduced in metastatic lesions arising in the intact and castrated mice. Using the TRAMP system, we have also shown that a germ-line mutation that disrupts GHRH receptor signaling to reduce circulating levels of both GH and IGF-1 (the \textit{it} mutation; ref. 27) could slow the initiation and progression of primary prostate cancer (28).

In this report, we describe a conditional prostate-specific IGF-IR knockout model to directly investigate how IGF-IR signaling in vivo can influence cellular homeostasis and cancer. Although we expected that selective abrogation of IGF-IR in prostate epithelium would cause widespread cell death and resistance to oncogene-mediated transformation (29), we actually observed a lobe-specific proliferative response in terminally differentiated prostate epithelium and the emergence of prostatic hyperplasia in young mice. In aged mice, we observed that IGF-IR deletion-dependent apoptosis and cellular senescence responses both coincided with the accumulation of stabilized p53, whereas expression of p21 and plasminogen activator inhibitor-1 (PAI-1) specifically correlated with cellular senescence. Although these findings support a critical role for the IGF axis in maintaining tissue homeostasis and differentiation, they also suggest that therapeutics targeting the IGF-I signaling axis might facilitate growth of tumor cells when p53 activity is compromised. To directly test this possibility, we created compound GEM models crossing the conditional IGF-IR alleles into the TRAMP model and showed that conditional deletion of the IGF-IR actually promoted an earlier emergence of more aggressive localized disease. These findings support a very cautious translation of IGF titration strategies to the bedside.

Materials and Methods

Strains and breeding. All animal studies were conducted in accordance with institutional guidelines for humane animal treatment. Mice were maintained at 22°C in a 12-h light and dark cycle with ad libitum access to water and food. Mice hemizygous for the ARRB2P-Cre mice transgene (30) were maintained in a pure FVB/NJ background. The loxP-tagged IGF-IR mice (31) were maintained homozygous (IGF-IR\textsuperscript{loxP/loxP}) in a pure 129/Sv background. TRAMP mice were maintained hemizygous for the PB-Tag transgene in a pure C57BL/6 background. As required, [IGF-IR\textsuperscript{loxP/loxP};ARR2PBi-Cre] F1 animals (50% 129; 50% FVB) were crossed to [IGF-IR\textsuperscript{loxP/loxP};TRAMP] F1 animals (50% 129; 50% B6) to generate all the mice (25% FVB; 50% 129; 25% B6) for the study.

Genotyping. Isolation of tail DNA and \textit{ex vivo}–harvested tissue DNA for PCR-based screening assays were performed as previously described (24, 32). Alternatively, 5-μm paraffin sections on slides were deparaffinized by standard ethanol/xylene protocols. DNA was extracted from individual prostate lobes using the PicoPure DNA Extraction kit (Arcturus Bioscience) according to the manufacturer's instructions. Identification of ARR2PBi-Cre transgenic mice used primers CF (5′-ATGGGAGATGATGATGACG-3′) and CR (5′-CTACACCCAGAGGGAAATC-3′) to yield a 256-bp fragment. Identification of PB-Tag mice used primers PB1 (5′-CCGGTTGACCGGAGGACTTTCTCACAAGTGCAATTT-3′) and SV40tagRev (5′-CTCCTTTTCAAGGCTTTAGTGCTC-3′) to amplify a 650-bp fragment. Identification of the wild-type (wt) or IoxP-modified IGF-IR loci used primers RLnt (5′-ATCTTGGAGTGGTGGTCTGTGTTC-3′) and RLxt (5′-ATGTAATGCTGGTGAGGTTGTC-3′) to amplify 312-bp IoxP IGF-IR and 256-bp wt IGF-IR fragments. Deletion of IGF-IR exon 3 was confirmed using primers RLxt and RLex (5′-CCATGTTGTTTAAATGTTACG-3′), yielding a 204-bp fragment.

Histology. At necropsy, the entire genitourinary tract consisting of the bladder, urethra, seminal vesicles, ampullary gland, and the prostate was excised, weighed, and dissected under a microscope. The dorsal (DP), lateral (LP), ventral (VP), and anterior (AP) prostate lobes were separated when possible. Tissues were fixed in 4% paraformaldehyde for 36 h at 4°C and processed into paraffin blocks. Multiple 5-μm sections were cut and stained with H&E. Histopathology was determined according to the GEM grading classification scheme (33) and expressed as the percentage of total gland area exhibiting murine PIN (with or without nuclear atypia), well differentiated adenocarcinoma, moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, or carcinoma with a phylloides-like appearance.

Immunohistochemistry. The 5-μm paraffin sections were deparaffinized by standard ethanol/xylene protocols and sequentially placed in boiling 10 mmol/L citric acid for 30 min for antigen retrieval, 3% hydrogen peroxide for 15 min, and incubated with a primary antibody for 1 h. After washing in PBS, sections were incubated with a secondary antibody for 30 min, followed by washing and counterstaining with DAPI for 5 min. Images were acquired using a fluorescence microscope and processed with ImageJ software.
Peroxidase/methanol to block endogenous peroxidase activity, and SuperBlock (Pierce Scientific) for 1 h at room temperature. Primary antibodies and working dilutions were as follows: anti-Cre recombinase (1:1,000; Novagen), anti-IGF-IR (1:200; Santa Cruz Biotechnology), anti-E-cadherin (1:750; BD Biosciences), anti-p63 (1:1,000; NeoMarkers), anti-smooth muscle actin (SMA; 1:500; Sigma), anti-Ki67 antibody (1:1,000; Novocastra Laboratories), anti-active caspase-3 (1:100; Fisher), anti-phosphorylated p44/42 (1:200; Cell Signaling), or anti-phosphorylated jNK (1:200; Cell Signaling). All antibodies were diluted in SuperBlock and samples were incubated overnight at 4°C with gentle rocking. Primary antibodies were detected with biotinylated anti-rabbit IgG (H+L; 1:100; Vector) or the DakoCytomation LSAB+System-HPR universal detection system with 3,3’-diaminobenzidine substrate. Slides were counterstained with 0.1% methyl green or a mild hematoxylin treatment.

**Proliferation rate.** Analysis with an anti-Ki67 antibody identified proliferating cells. Three to six random fields were counted at ×400 magnification to obtain the proliferation index. Averages (±SD) were calculated from data obtained from three mice per cohort and significance was determined using the two-sided, two-tailed Student’s *t* test method.

**Cell death.** Apoptotic cells were identified by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer’s instructions (In Situ Cell Death Detection Kit-AP, Boehringer Mannheim). Because TUNEL staining recognizes fragmented DNA, we confirmed that cells were undergoing apoptosis by assessing abnormal nuclear morphology and by using an antibody to detect active caspase-3 (as described above). The relative amount of TUNEL staining was determined using the NIH ImageJ software analysis package. At least six random fields of view (>400 magnification) were used to calculate the average pixel intensity. Significance was determined using the two-sided Student’s *t* test method.

**Western blot analysis.** Total protein lysates were prepared from prostate tissues by homogenization in 50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 10 mmol/L NaF, and 1% Triton X-100. Approximately 40 to 50 μg of protein from each sample were denatured in lithium dodecyl sulfate loading buffer (Invitrogen) by boiling for 10 min and loaded onto 8% or 10% SDS-polyacrylamide gels. Proteins separated by electrophoresis were transferred to Immobilon-P nylon membranes (Millipore) and filters were blocked for 1 to 2 h at room temperature in PBS-Tween (0.1%, v/v) and filters were blocked for 1 to 2 h at room temperature in PBS-Tween (0.1%, v/v) and 5% (w/v) nonfat dry milk (Carnation). Primary antibodies (and working dilutions) were as follows: anti-IGF-IR (1:750); anti-phosphorylated IGF-IR (1:1,000; Cell Signaling), anti-phosphorylated p44/42 (1:1,000), anti-phosphorylated Akt (1:1,000; Cell Signaling), anti-Akt (1:1,000; Cell Signaling), anti-phosphorylated Src (1:1,000; Cell Signaling), anti-phosphorylated R-Araf (1:1,000; Cell Signaling), anti-phosphorylated C-Raf (1:1,000; Cell Signaling), anti-phosphorylated MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 (1:1,000; Cell Signaling), anti-E-cadherin (1:2,000), anti-protein phosphatase 2A (PP2A; 1:500; Santa Cruz Biotechnology), anti-MAPK phosphatase 3 (MKP3; 1:500; Santa Cruz Biotechnology), anti-p53 (1:1,000; Santa Cruz Biotechnology), anti-p21 (1:500; Santa Cruz Biotechnology), anti-PIA-1 (1:1,000; Santa Cruz Biotechnology), anti-androgen receptor (AR; 1:2,000; Upstate), or anti-mouse probasin (1:3,000; in-house rabbit polyclonal; ref. 34) diluted in 5% bovine serum albumin in PBS-Tween (0.1%, v/v) and incubated at 4°C overnight with gentle rocking. Membranes were washed thrice for 5 min with PBS-Tween (0.1%, v/v) and incubated with either horseradish peroxidase–conjugated anti-rabbit or anti-mouse (Bio-Rad) IgG antibody diluted 1:2,000 or 1:5,000, respectively, for 1.5 h at room temperature in 5% nonfat dry milk in PBS-Tween (0.1%, v/v). Membranes were washed thrice for 5 min with PBS-Tween and routinely developed using either enhanced chemiluminescence Western blotting detection system (Amersham Biosciences) or SuperSignal West Femto Maximum Sensitivity Substrate (1:5 dilution in water; Pierce Scientific) and exposed to X-ray film (XAR-1, Eastman Kodak). The NIH ImageJ software analysis package was used to determine the level of IGF-IR protein. Background and sample loading variation were determined from negative controls and β-actin intensities, respectively.

**Results**

Conditional deletion of IGF-IR causes hyperplasia and proliferation. To study the role of IGF-IR in prostate epithelium *in vivo*, we crossed the transgenic ARR2PBi-Cre activator mice (30) to mice harboring IGF-IR-loxP-tagged genomic loci (31) generating the IGF-IRloxP/loxP;ARR2PBi-Cre (IGF-IR knockout), IGF-IRloxP/loxP;ARR2PBi-Cre (IGF-IR titrated), and littermate [IGF-IRloxP/loxP or IGF-IRloxP/wt] controls (IGF-IR intact). Hereafter, the presence of ARR2PBi-Cre will simply be denoted as “Cre”.

The consequence of prostate epithelial–specific abrogation of IGF-IR was examined in adult bieginic IGF-IRloxP/loxP;Cre (*n* = 10), IGF-IRloxP/loxP;Cre (*n* = 11), and control IGF-IRloxP/loxP or IGF-IRloxP/wt (*n* = 10) mice at 12 weeks of age. As shown in Table 1, we observed no significant changes in animal weight, genitourinary tract (consisting of bladder, prostate glands, seminal vesicles, urethra, and ampullary gland) weight, the genitourinary/animal weight ratio, or prostate lobe wet weights between the various groups. Prostate-restricted knockout of IGF-IR did not influence overall body weight or prostate mass. Male IGF-IRloxP/loxP;Cre mice remained fertile up to 37 weeks of age (data not shown).

As shown in Fig. 1, expression and localization of Cre protein were restricted to the luminal prostate epithelial cells of mice harboring the Cre transgene consistent with findings from previous studies (34–36). To date, no developmental defects associated with ARR2-Pbi-Cre expression have been reported. Similarly, we were unable to detect any prostate developmental defects associated with Cre expression at 6 weeks in IGF-IRloxP/loxP;Cre mice (data not shown).

The extent of prostate tissue–specific recombination at the IGF-IR loci was determined from DNA of multiple tissues of

<table>
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<tr>
<th>Genotype</th>
<th>Age (wk)</th>
<th>n</th>
<th>Animal weight (mg)*</th>
<th>Genitourinary/animal (mg)</th>
<th>Prostate weight (mg)</th>
<th>Prostate/animal average (mg)</th>
<th>Seminal vesicle weight (mg)</th>
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<td>IGF-IRloxP/loxP orloxP/wt</td>
<td>12</td>
<td>10</td>
<td>32.4 ± 3.4</td>
<td>360 ± 90</td>
<td>0.011 ± 0.002</td>
<td>40 ± 11</td>
<td>1.2 ± 0.3</td>
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<td>24</td>
<td>14</td>
<td>36.5 ± 5.2</td>
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<td>0.013 ± 0.003</td>
<td>56 ± 10</td>
<td>1.6 ± 0.3</td>
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<tr>
<td>IGF-IRloxP/loxP;Cre</td>
<td>12</td>
<td>12</td>
<td>32.3 ± 3.9</td>
<td>370 ± 80</td>
<td>0.011 ± 0.003</td>
<td>38 ± 10</td>
<td>1.3 ± 0.3</td>
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<tr>
<td></td>
<td>24</td>
<td>12</td>
<td>36.6 ± 4.3</td>
<td>470 ± 80</td>
<td>0.013 ± 0.002</td>
<td>51 ± 9</td>
<td>1.3 ± 0.3</td>
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<tr>
<td>IGF-IRloxP/loxP;Cre</td>
<td>12</td>
<td>10</td>
<td>32.7 ± 3.5</td>
<td>350 ± 60</td>
<td>0.011 ± 0.002</td>
<td>42 ± 10</td>
<td>1.3 ± 0.3</td>
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<td>24</td>
<td>15</td>
<td>36.8 ± 4.1</td>
<td>450 ± 70</td>
<td>0.012 ± 0.003</td>
<td>51 ± 8</td>
<td>1.4 ± 0.2</td>
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*Mean ± SE.
Figure 1. Conditional deletion of IGF-IR in prostate epithelial cells causes proliferation. A, left, immunohistochemical analysis with anti-Cre antibody to show how expression of transgene-encoded Cre recombinase (brown nuclei) is restricted to epithelium of prostate glands in DLP (top) and VP (bottom) tissues in IGF-IRloxP/Cre at 6 wk of age. No Cre expression was detected in IGF-IRloxP/wt mice. Magnification, ×200. Right, IGF-IR is expressed in IGF-IRloxP/Cre mice but absent in IGF-IRloxP/wt, Cre mice by 12 wk of age. Nuclei were counterstained with methyl green. Magnification, ×400. B, left, immunoblot analysis to show IGF-IR expression in all lobes of IGF-IRloxP/loxP mice (lanes 1 and 2), Cre-specific titration of IGF-IR protein in DP, LP, and VP lobes of IGF-IRloxP/Cre (lanes 3 and 4), and abrogation of IGF-IR protein in IGF-IRloxP/loxP;Cre (lanes 5 and 6) mice. Expression of h-actin verified equal loading. The levels of IGF-IR protein (relative to control; lanes 1 and 2) are indicated in brackets. Right, representative formalin-fixed and paraffin-embedded sections from IGF-IRloxP/loxP and IGF-IRloxP/loxP;Cre mice stained with H&E to show Cre-dependent expansion of epithelium compartment consistent with proliferative response. Magnification, ×200. C, left, robust expression of E-cadherin (E-Cad), AR, and cyclin B1 (Cyc B1) in DLP of 12-wk-old IGF-IRloxP/loxP;Cre mice by immunohistochemistry. Analysis with anti-Ki67 and anti-p63 antibodies shows the proliferative response is restricted to the epithelial and not basal compartment. Magnification, ×400. Right, proliferation index calculated from percentage (%) of Ki67-positive cells in prostate lobes of IGF-IRloxP/loxP, IGF-IRloxP/loxP;Cre, and IGF-IRloxP/loxP;Cre mice at 12 wk of age. Columns, average; bars, SD. P value was calculated using two-sided Student’s t test at 95% confidence.
Figure 2. Abrogated IGF-IR expression in the prostate causes minimally invasive disease with lobe-specific apoptosis or senescence. A, representative formalin-fixed and paraffin-embedded sections of DP and VP from IGF-IRloxP/loxP and IGF-IRloxP/loxP;Cre mice at 24 wk of age were stained with H&E or analyzed by immunohistochemistry with antibodies to E-cadherin, p63, and SMA. Arrows, invasion of epithelial cells into surrounding mesenchyme of DP. Original magnification, ×100 (H&E and SMA) or ×200 (E-cadherin and p63). B, left, apoptotic cells in VP of IGF-IRloxP/loxP;Cre and IGF-IRloxP/loxP mice at 24 wk of age detected by TUNEL. Nuclei were counterstained with DAPI or hematoxylin. Merged images show atypical nuclear morphology associated with apoptosis. Inset, apoptotic cells were confirmed by immunostaining for active caspase-3. Original magnifications, ×100 or ×200 (TUNEL; left and right, respectively) and ×630 (caspase-3). Bottom, senescent cells in DP and VP were identified using a β-galactosidase assay on tissues harvested from IGF-IRloxP/loxP;Cre and IGF-IRloxP/loxP;Cre mice at 24 wk of age. Original magnification, ×200. Right, proliferation index in the prostates of IGF-IRloxP/loxP, IGF-IRloxP/loxP;Cre, and IGF-IRloxP/loxP;Cre at 24 wk of age determined from the percentage (%) of Ki67-positive epithelial cells in at least three 400× random fields of view. Columns, average; bars, SD. No significant differences were evident using two-sided Student’s t test at 95% confidence.
IGF-IR<sup>loxP/wt</sup>;Cre and IGF-IR<sup>loxP/loxP</sup>;Cre mice (Supplementary Fig. S1). At 12 weeks of age, membrane-localized IGF-IR protein was reproducibly detected in VP of IGF-IR<sup>loxP/loxP</sup> but was dramatically absent in IGF-IR<sup>loxP/loxP;Cre</sup> knockout mice (Fig. 1A). A reproducible correlation was also observed between IGF-IR gene dose and level of IGF-IR protein in VP, dorsolateral prostate (DLP), and LP tissues (Fig. 1B). There was no apparent compensatory change in expression of the wt IGF-IR allele in IGF-IR<sup>loxP/loxP;Cre</sup> mice consistent with previous reports (31).

Loss of functional IGF-IR caused reproducible gross histologic and epithelial cell–specific changes in the prostates of IGF-IR<sup>loxP/loxP;Cre</sup> mice. As shown in Fig. 1B, we observed atypical epithelial tufting.
Increase in the proliferation index in all prostate lobes of IGF-IR
expressed mice relative to IGF-IRloxP/loxP mice (P = 0.007, two-sided Student’s t test at 95% confidence). Analysis with an anti-activating caspase-3 antibody confirmed the apoptotic response in the epithelial cell compartment (Fig. 2).

Using an antibody specific to senescence-associated β-galactosidase (SA-β-gal), we also observed the senescence response in DP tissues of IGF-IRloxP/loxP Cre mice relative to controls (Fig. 2B). Consistent with an accumulation of lysosomal content, the SA-β-gal signal localized to the cytoplasm in discrete pockets. In general, these data support a lobe-specific “switch” in response to abrogated IGF-IR signaling. Our inability to show apoptosis or senescence in LP tissues may indicate that a nonclassic form of cell cycle arrest is induced in these epithelial cells following IGF-IR ablation.

The proliferation index for IGF-IRloxP/loxP Cre mice at 24 weeks was not significantly different than in age- and lobe-matched IGF-IRloxP/loxP and IGF-IRloxP/loxP Cre mice (Fig. 2B). Given our observations that (a) the epithelial cells in the VP were increasingly apoptotic and (b) there was increased evidence of senescing cells in the DP phenotype at 12 weeks of age, the abrogation of IGF-IR seemed to have triggered a somewhat self-limiting cellular response.

**Conditional deletion of IGF-IR causes age-dependent apoptosis and senescence.** Remarkably, the focal regions of hyperplasia evident at 12 weeks of age did not progress to carcinoma over time. Although IGF-IRloxP/loxP Cre mice displayed significant pathology in DP at 24 weeks (Fig. 2), the integrity of this compartment remained mostly intact based on pattern of p63 and SMA expression, although there was evidence of local invasion in the DP (Fig. 2A). Here, the expression of E-cadherin was punctuate and nonuniform and the incidence of abnormal epithelium remained significantly higher relative to that of age-matched control IGF-IRloxP/loxP mice [50.7 ± 9.0% (n = 14) and 16.9 ± 16.2% (n = 17), respectively; P < 0.0001, Student’s t test]. We were unable to detect significant pathology or evidence of invasive disease in the VP and LP lobes.

Because oncogene-induced proliferation can cause cellular apoptosis or senescence (37), we performed TUNEL analysis and observed a significantly higher apoptotic response restricted to the VP of IGF-IRloxP/loxP Cre mice (Fig. 2B) relative to IGF-IRloxP/loxP mice (P = 0.007, two-sided Student’s t test at 95% confidence). Analysis with an anti-activating caspase-3 antibody confirmed the apoptotic response in the epithelial cell compartment (Fig. 2).

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**Conditional deletion of IGF-IR can increase ERK1/2 activation in DP and LP.** Following ligand binding, the intracellular activation of IGF-IR β-subunit kinase domain can initiate signaling through the PI3k/Akt and Ras/MAPK cascades (7–9). As expected, levels of phosphorylated Akt and phosphorylated p44/42 (ERK1/2) levels were consistently reduced in VP of IGF-IRloxP/loxP Cre mice relative to controls (Fig. 3). Surprisingly, in contrast, the steady-state levels of phosphorylated ERK1/2 in DP and LP were increased at 12 weeks of age relative to controls. Moreover, the increased levels of phosphorylated ERK1/2 in DP and LP did not correspond to appreciable changes in phosphorylated Akt levels or phosphorylation of Src kinase, A-Raf, B-Raf, or C-Raf that are known to directly or indirectly activate MEK1/2. Similarly, we were unable to detect changes in steady-state levels of the PP2A

### Table 2. Consequence of IGF-IR titration and ablation on prostate cancer

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (wk)</th>
<th>n</th>
<th>Genitourinary range</th>
<th>Genitourinary average (g)*</th>
<th>Genitourinary/animal (g)</th>
<th>Macroscopic lesion (%)</th>
<th>Advanced tumors (%)</th>
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<td>Max (g)</td>
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<td></td>
</tr>
<tr>
<td>IGF-IRloxP/loxP or IGF-IRloxP/loxP;TRAMP</td>
<td>18</td>
<td>18</td>
<td>0.31</td>
<td>1.16</td>
<td>0.65 ± 0.20</td>
<td>0.020 ± 0.006</td>
<td>1/18 (6)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>20</td>
<td>0.43</td>
<td>12.5</td>
<td>2.7 ± 3.35</td>
<td>0.065 ± 0.079</td>
<td>10/20 (50)</td>
</tr>
<tr>
<td>IGF-IRloxP/loxP CreTRAMP</td>
<td>18</td>
<td>8</td>
<td>0.41</td>
<td>5.0</td>
<td>1.69 ± 1.57</td>
<td>0.046 ± 0.047</td>
<td>3/8 (38)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>6</td>
<td>0.69</td>
<td>1.92</td>
<td>1.18 ± 0.29</td>
<td>0.032 ± 0.014</td>
<td>2/24 (33)</td>
</tr>
<tr>
<td>IGF-IRloxP/loxP CreTRAMP</td>
<td>18</td>
<td>7</td>
<td>0.44</td>
<td>10.5</td>
<td>1.99 ± 3.47</td>
<td>0.059 ± 0.008</td>
<td>1/18 (14)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7</td>
<td>0.61</td>
<td>8.68</td>
<td>2.2 ± 2.74</td>
<td>0.052 ± 0.065</td>
<td>6/24 (86)</td>
</tr>
</tbody>
</table>

*Mean ± SE.*

† Compound tumors (defined as those where the individual lobes were indistinguishable) and lobe confined tumors grossly visible at necropsy.

‡ Poorly differentiated primary carcinomas with no glandular features.

§ Significantly increased (P = 0.02, two-sided Student’s t test at 95% confidence) compared with IGF-IRloxP/loxP or IGF-IRloxP/loxP CreTRAMP at 18 wk of age.
and MKP3 phosphatases that negatively regulate both MEK and p44/42. These data clearly show that IGF-IR depletion-dependent activation of p44/42 likely occurs through a nonclassic and as of yet uncharacterized signaling mechanism.

**Role of p53 following conditional IGF-IR knockout.** The activation, stabilization, and accumulation of the p53 tumor suppressor gene can induce apoptosis, cell cycle arrest, or cellular senescence in response to cellular stress and declining p53 function has been recently associated with increased tumor incidence as a function of aging (38). To explore the possibility that differential activation of p53-dependent signaling cascades was responsible for the VP-specific apoptosis and DP-specific senescence observed following IGF-IR loss, we examined the steady-state levels of p53 in DP and VP tissues harvested from 12- or 24-week-old IGF-IRloxP/loxP;Cre and IGF-IRloxP/loxP mice. Although we did not detect appreciable levels of p53 in any prostate lobe at 12 weeks of age (data not shown), increased steady-state levels of p53 were readily observed in DP and VP from IGF-IRloxP/loxP;Cre mice by 24 weeks of age (Fig. 3). These data support a p53-dependent cell-specific link between loss of IGF-IR and the repression of phosphorylated Akt and phosphorylated p44/42 in the VP and activation of p44/42 in the DP.
The p53 protein is a transcription factor that can regulate expression of a variety of target genes (39). As shown in Fig. 3, we observed increased levels of p21 [involved in p53-mediated cell cycle arrest (40)] and PAI-1 [involved in p53-mediated cellular senescence (41)], concomitant with increased steady-state levels of p53 as a function of abrogated IGF-IR expression in DP tissues. This was consistent with the increased levels of SA-βgal previously noted in the DP of IGF-IR knockout mice (Fig. 2). However, reduced levels of PAI-1 expression seemed concomitant with decreased IGF-IR expression in VP despite only very modest changes in the level of p21 protein. Taken together, these data support a differential role for p53-mediated responses in VP and DP epithelium in response to ablated IGF-IR signaling.

Conditional deletion of IGF-IR does not hinder progressive prostate cancer in a p53 depletion-dependent model of multistage tumorigenesis. To investigate the possibility that targeted inhibition of IGF-IR signaling could possibly exacerbate tumor progression in prostate cancer with compromised p53 function, we generated cohorts of 18- and 24-week-old IGF-IRloxP/loxP;Cre;TRAMP, IGF-IRloxP/loxP;wt;Cre;TRAMP, and IGF-IRloxP/loxP;wt;Cre;TRAMP mice (24). As shown in Table 2, the incidence of lobe confined and compound lesions were not reduced by titration [IGF-IRoxP/loxP;Cre;TRAMP] or ablation [IGF-IRloxP/loxP;Cre;TRAMP] of IGF-IR at any age. Indeed, at 18 weeks of age, although 38% (n = 8) of IGF-IR titration and 14% (n = 7) of IGF-IR knockout mice had confined or compound lesions, we only observed these lesions in 6% (n = 18) of control mice. Although we did not find a significant difference in the tumor weights between IGF-IRloxP/loxP;Cre;TRAMP (1.99 ± 3.47 g and 2.2 ± 2.74 g) and IGF-IRloxP/loxP;wt;Cre;TRAMP mice (0.65 ± 0.20 g and 2.36 ± 3.12 g) at 18 or 24 weeks of age, respectively, we did determine a significant increase (1.69 ± 1.57 g; P = 0.02; n = 8) in tumor weights at 18 weeks of age in the IGF-IRloxP/loxP;Cre;TRAMP mice compared with IGF-IRloxP/loxP or IGF-IRloxP/loxP;wt;Cre;TRAMP mice (0.65 ± 0.20 g; n = 18). Remarkably, 43% and 67% of titration [IGF-IRoxP/loxP;Cre;TRAMP] and 29% and 71% of knockout [IGF-IRloxP/loxP;Cre;TRAMP] mice contained aggressive high-grade poorly differentiated and phyllodes-like adenocarcinomas (Fig. 4) compared with 13% and 41% of control [IGF-IRoxP/loxP;TRAMP] animals at 18 and 24 weeks of age, respectively (Table 2). We confirmed that prostate tissue-specific recombination at the IGF-IR loxP loci had occurred (Supplementary Fig. S3) and that expression of SV40 Tag was not influenced by loss of IGF-IR in our system (data not shown). Remarkably, all advanced tumors had essentially lost IGF-IR protein expression, irrespective of IGF-IR loxP status but consistent with previous reports (Supplementary Fig. S3; ref. 15).

Discussion

Studies in GEM models have generally supported a relationship between IGF-I action and prostate disease. Recently, we reported that enforced expression of IGF-I in prostate epithelium could promote neoplastic growth but was insufficient to cause carcinoma (18). As well, crossing the lit mice with TRAMP (28) showed that chronically reduced systemic IGF-I and GH expression could significantly delay tumor-related death, the direct role of IGF-I signaling at the level of the prostate epithelium was not specifically addressed nor was the relative contribution of reduced GH levels to prolonged survival.

To more rigorously address the cell autonomous role of IGF-IR in prostate gland biology and cancer, we generated a conditional knockout mouse model and showed that IGF-IR signaling is absolutely required to maintain normal epithelial cell homeostasis. Although substantial evidence predicted that loss of functional IGF-IR expression would lead to prominent cell death, we were surprised to find that abrogation of IGF-IR in the luminal epithelial compartment of DP, LP, and VP lobes consistently correlated with epithelial cell proliferation and a development of focal hyperplasia in young mice. Remarkably, proliferation in DP and LP was found to be concomitant with activation of phosphorylated p44/42, thereby implicating a negative relationship between IGF-IR expression and ERK1/2 activation in these tissues. It was curious that loss of IGF-IR did not seem to directly influence the expression or activation of known positive regulators of ERK1/2 (i.e., Akt, Src, and Raf) or the negative regulators PP2A and MKP3. Efforts are currently under way to more comprehensively study the molecular mechanism whereby loss of IGF-IR expression positively influences ERK1/2 activation and whether this represents a direct or indirect relationship.

Although the loss of IGF-IR in young mice was strongly correlated with proliferation, the hyperplastic lesions did not seem to progress with age. Indeed, we observed a strong correlation with increased cellular senescence in the older cohorts. Because sustained activation of Ras-MAPK signaling is able to promote oncogene-induced cellular senescence (42, 43), it is tempting to speculate that IGF-IR suppresses cellular senescence via the inhibition of phosphorylated p44/42 accumulation and activity, although additional mechanisms may be involved.

It should be noted that tissue-specific ablation in the IGF-IRloxP/loxP;Cre or IGF-IRloxP/loxP;Cre mice could not have influenced overall GH or IGF signaling compared with the controls. Indeed, any significant increase (or decrease) in secretion of systemic GHRH, GH, or IGF-I during the growth period of the animal would have resulted in a significant change in overall body size, and the data presented in Table 1 clearly show that animal body size was similar in all three groups at 12 and 24 months. Moreover, no significant alterations in the size of adipose tissue deposits were noted in the adults and no alterations in genitourinary or prostate wet weights were found to support a possible influence of any increased local IGF-I production on the microenvironment.

The implications of our study are 2-fold. First, our data suggest that IGF-IR activity may relate to the incidence of prostate cancer by suppressing cellular senescence and apoptosis to allow epithelial cells to survive spontaneous transformation while enforcing a strong differentiation block. Second, our data indicate that any decrease in circulating IGF-I in combination with changes to p53 that would physiologically accompany normal aging would provide selective advantage to transformed epithelial cells that could overcome the IGF-IR-mediated differentiation block and progress toward more invasive and disseminated forms of carcinoma. We have previously proposed the hurdle hypothesis to explain how factors that contribute to the height of the differentiation “hurdle” in essence will determine how aggressive a cancer must become to overcome the differentiation barrier/blockade to achieve malignant transformation (18). Because abrogation of functional IGF-IR expression actually promoted earlier emergence of more aggressive, less-differentiated carcinomas in a p53-compromised model of prostate cancer, the current data support the hypothesis that titration of IGF-IR signaling reduces the prodifferentiation block. Importantly, these findings are in remarkable agreement with a prospective study showing a strong association between
lower-grade (Gleason score <7) clinical prostate cancer and elevated serum IGF-I levels (44) and an elevated ratio of IGF-I to IGFBP-3 and the risk of developing extraprostatic and distant metastasis (44, 45).

In summary, GEM models are helping us to define a new paradigm for IGF-I action in the prostate that should further help guide translational initiatives to target the IGF-I axis in prostate and other cancers using small molecule inhibitors and other therapeutic strategies (46). Although we have focused on the enforced expression of IGF-I or abrogation of IGF-IR directly in the prostate, it should be very interesting to determine the effect on tumor initiation and progression in GEM models using the conditional liver-specific IGF-I knockout model (47) that reduces circulating IGF-I without concomitant decrease in GH. As well, it will be interesting to further elucidate the role of p53 and aging on the incidence and nature of prostate cancer.

Acknowledgments

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References

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