Inducible Prostate Intraepithelial Neoplasia with Reversible Hyperplasia in Conditional FGFR1-Expressing Mice

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ABSTRACT

Accurate determination of the contributions of oncogenes toward tumor progression requires their regulation. Herein, we created transgenic mice with prostate-specific expression of ligand-inducible FGFR1 or FGFR2, based on lipid-permeable dimerizing molecules, called chemical inducers of dimerization. Despite extensive homology and equivalent expression by both chimeric receptors in the ventral prostate gland, only FGFR1 triggers detectable nuclear translocation of Erk and progression to prostatic intraepithelial neoplasia (PIN). Induction of PIN grade I-II, indicated by multiple layers of atypical cells, is seen consistently by 12 weeks of chemical inducers of dimerization treatment. By 6 months, more extensive nuclear atypia, thickened “reactive” stroma, and basement membrane herniation occurs, corresponding to PIN IV. By timed removal of FGFR1 signaling, we show that induced hyperplasia is reversible until extensive intraductal vascularization occurs, but continued progression requires prolonged FGFR1 signaling. Additionally, by highlighting differences between the two receptors and creating the foundation for controlling FGFR1 signaling during prostate cancer progression, a model of early stage prostate cancer is established for developing targeted intervention directed toward the FGFR signaling axis.

INTRODUCTION

Despite improvements in the management of organ-confined disease, CaP5 will claim ~30,000 lives in the United States in 2003 (1), largely due to the absence of effective treatment for metastatic disease. More accurate modeling of CaP is required to better understand the molecular signaling events and pathological stages associated with disease progression, and to facilitate development of more effective therapeutics. Several mouse models of CaP have been developed previously (2). These models fall into two broad categories, the more aggressive SV40 T antigen-based models, such as TRAMP (3) or “Lady” (4) that target multiple tumor suppressor genes, and a variety of other less aggressive models that target a single proto-oncogene or tumor suppressor molecule, such as FGF-8 (5) or PTEN (6). Whereas the single-gene models tend to highlight the early stages of progression, TRAMP mice consistently develop metastatic CaP by 28 weeks of age, allowing for therapeutic studies that span a relatively short time (7, 8). Whereas both sets of models can help determine genetic lesions capable of contributing to disease, neither permits temporal ordering of deleterious genetic events or highlights whether those genetic changes only initiate disease or are also required for disease maintenance, and may, therefore, be targeted for therapeutic intervention.

To achieve temporal control of the FGFR axis implicated in early changes associated with CaP (9) we used CID technology, which allows rapid activation of target proteins, such as growth factor receptors, that are naturally activated by oligomerization (10). CID-inducible growth factor receptors are engineered by substituting the ligand-binding extracellular domain of the receptor with a cytoplasm-localized drug-binding domain (e.g., FKBP12 variants), which, on binding of a lipid-permeable dimeric drug (e.g., AP20187), causes receptor oligomerization, transphosphorylation, and activation of downstream signaling cascades (11–14). Because the investigator dictates the schedule of AP20187 administration, temporal control in the activation and inactivation of the receptor is possible, highlighting the pathological features dependent on the growth factor receptor for both initiation and maintenance of phenotype. Additionally, unlike bigenic transcription switch (e.g., tet)–regulatable systems or conditional lesions based on recombination (e.g., Cre/lox), this approach requires only a single transgenic change, greatly simplifying breeding strategies.

In the currently accepted model of FGF signaling in the normal prostate, stromal-derived FGF7/keratinocyte growth factor and FGF10 help maintain epithelial differentiation and survival through glandular epithelium-confined FGFR2-IIIb, contributing to epithelial dependence on the stroma. During progression to malignancy, the observed molecular changes in the FGF signaling axis that occur in the epithelium include loss of normal FGFR2 signaling (by isoform changes that alter ligand binding or by loss of FGFR2 expression), acquisition of FGFR1 expression, and up-regulation of distinct FGF family members. These changes likely allow autocrine signaling via FGFR1 and possibly FGFR2, permitting independence from stromal-derived FGFs and, thus, deregulated growth of the epithelium (9, 15). In addition to observed changes in CaP, these alterations are common in bladder and salivary adenocarcinomas, where up-regulation of FGFR1 is associated with tumor progression, and ectopic expression of FGFR2 inhibits progression and growth of transplanted tumors (16, 17). Despite accumulating data in model tumor systems, the effects of these two receptors have not been investigated before in a normal prostate epithelium, although overexpression of FGFs in the prostate has led to the development of prostatic hyperplasia and PIN (5, 18–20).

In this study we focused on the hyperplasia-stimulating effects of ectopic FGFR1 signaling on normal prostatic epithelium, demonstrating diverse effects of iFGFR1 and iFGFR2 in a normal prostate background, and showing dependence of hyperplasia and proliferation on continued iFGFR1 signaling. Whereas the profound hyperplasia that is seen after only 1 month of FGFR1 stimulation is completely reversible, neovascularization, which is well established by 2 months, is associated with hyperplastic cells becoming largely independent of iFGFR1 signaling for their survival. Moreover, moderate-term (i.e., <6 months) iFGFR1 signaling can promote high-grade (i.e., type IV) PIN that is associated with a thickened stroma, fulminant glandular hyperplasia, nuclear atypia, and basement membrane herniation. This

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is the first reported example of a conditionally reversible CaP model, and the first study of the diverse effects of FGFR1 and FGFR2 signaling in the context of a normal mouse prostate.

MATERIALS AND METHODS

Construction of Transgene. The plasmid construct pSH1/M-FGFR2-F\textsubscript{v}F\textsubscript{vls}-E as described previously (21). Briefly, the cytoplasmic signaling domains of FGFR1 and FGFR2 were PCR amplified with Xho\textsubscript{I} and Sal\textsubscript{I} flanking sites, and cloned into parent expression vector pSH1/M-FGFR1-F\textsubscript{v}F\textsubscript{vls}-E as described previously (21). The Xho-Bam\textsubscript{HI} fragment from pSH1/M-FGFR1-F\textsubscript{v}F\textsubscript{vls}-E and pSH1/M-FGFR2-F\textsubscript{v}F\textsubscript{vls}-E were subcloned into the ClaI-Bam\textsubscript{HI} site of expression vector, KBPA, to create ARR\textsubscript{PB}-KBPA and ARR\textsubscript{PB}-KBPA-iFGFR1 and ARR\textsubscript{PB}-KBPA-iFGFR2. Both transgene constructs were cleaved with Not\textsubscript{I} and Kpn\textsubscript{I}, and fragments were isolated by agarose gel and purified by Qiagen (Valencia, CA) spin columns for injection into FVB strain-derived embryonic stem cells.

Mice and Reagents. All of the mice were FVB (bred-in-house) background and kept pathogen free in the Texas Mouse Facility under veterinary supervision. AP20187 was dissolved in 16.7% propanediol, 22.5% PEG400, 1.25% Tween 80 and injected i.p. biweekly at 2 mg/kg. On the basis of an estimated half-life of 7 h\textsuperscript{1} an EC\textsubscript{50} of ~10 nm (data not shown), this level was calculated to evoke signaling for 2–3 days in vivo. Alternatively, carrier alone was injected. Initiation of treatments was staggered so that all of the mice were the same age when prostate were harvested.

Immunoprecipitation and Western Blot Analysis. Organs were harvested, frozen, pulverized, and then lysed in 200–300 μl of radioimmunoprecipitation assay buffer [0.01 M Tris-CL (pH 8.0), 0.14 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS] with 1 mg phenylmethylsulfonyl fluoride and protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) and incubated on ice for 2 h. SDS-PAGE was performed as described previously (22). Membranes were incubated with primary antibody HA.11 (Covance, Richmond, CA) at 1:1500 or anti-CD31/RAM (Ramos, CA) for 10 min at 25 °C, and incubated with either anti-Ki-67 antibody (Vector Laboratories, Burlingame, CA) at 1:2000 or goat antimouse biotin-conjugated antibody (Santa Cruz Biotechnology) at 1:1000, anti-CD31/RAM (Ramos, CA) at 1:1000, or anti-phospho-Erk (Novacastra, Newcastle upon Tyne, United Kingdom) at 1:1000, anti-COX-2 (Cell Signaling Technology, Beverly, MA) at 1:100 in Power Block overnight. Membranes were washed 3X with Tris-Buffered Saline plus 0.1% Tween-20 (TBST) for 5–15 min after each antibody step. Bands were visualized with SuperSignal chemiluminescence substrate (Pierce, Rockford, IL). Immunoprecipitation was carried out using the IMMUNO-catcher kit (Cytosignal Research Products, Irvine, CA) following the manufacturer’s protocol using 2 μg antiphosphotyrosine antibody 4G10 (Upstate, Lake Placid, NY) for immunoprecipitation and HA.11 immunoblotting as above. Phosphatase inhibitor I and II (Sigma) were added to lysates.

Histology and Immunohistochemistry. Microdissected prostates were placed overnight in 10% neutral phosphate-buffered formalin, transferred to 50% neutral phosphate-buffered formalin/50% ethanol for 1 h and then 70% ethanol until paraffin embedding. After sectioning (5 μm) and mounting onto ProbeOn-Plus slides (Fisher, Pittsburgh, PA), slides were deparaffinized and hydrated with progressive xylene washed followed by a gradient of ethanol and PBS. Slides were either directly stained with H&E or immunostained. For antigen retrieval, slides were either placed into boiling 10 mM citric acid for 30 min and allowed to cool at 25 °C or were proteinase K-treated. Both sets were quenched in 3% H\textsubscript{2}O\textsubscript{2}, blocked with Power Block (Biogenex Laboratories, San Ramon, CA) for 10 min at 25 °C, and incubated with either anti-Ki-67 antibody (Novacastra, Newcastle upon Tyne, United Kingdom) at 1:1000, or anti-CD31/RAM (Cell Signaling Technology, Beverly, MA) at 1:100 in Power Block overnight at 4 °C, followed by incubation with biotin-conjugated goat antirabbit antibody (Vector Laboratories, Burlingame, CA) at 1:2000 or goat antimouse biotin-conjugated antibody (Santa Cruz Biotechnology) at 1:100 for 1 h. After each incubation, slides were washed with PBS plus 0.1% Tween for 2 h. Immunohistochemistry and immunoprecipitation were performed as above except slides were counterstained with methyl green and dehydrated using a gradient of ethanol to xylene before mounting under coverslips.

RESULTS

Targeting Conditional FGFR1 and FGFR2 to the Prostate. In these studies iFGFR1 and iFGFR2 transgenes were placed under the transcriptional control of the prostate epithelium-targeted composite probasin promoter, ARR\textsubscript{PB} (23). The iFGFR constructs each contain an NH\textsubscript{2}-terminal myristoylation-targeting signal sequence for membrane localization, followed by the intracellular signaling domains of FGFR1 or FGFR2, two-tandem AP20187 drug binding domains (F,2) and a COOH-terminal HA epitope tag introduced into an expression vector, KBPA, backbone (Fig. 1A). FVB blastocysts were injected separately with both constructs, leading to two founder lines of iFGFR1, collectively named JOCK-1, and one founder line expressing iFGFR2, named JOCK-2. All three of the lines expressed detectable transgene by Western blotting of prostate tissue, and the highest expressing JOCK-1 line was chosen for additional study (data not shown). To determine the tissue-specificity of the transgenes, multiple organs from 12-week-old transgenic mice were analyzed and tissue lysates were separated by SDS-PAGE followed by anti-HA immunoblotting. This analysis revealed that ARR\textsubscript{PB}-driven transgene expression was primarily prostate-specific, consistent with previous reports (Refs. 23, 24; Fig. 1B). Furthermore, to determine expression within the different distinct lobes of the murine prostate, microdissected anterior (coagulating gland), ventral, dorsal, and lateral lobes were also analyzed for specific transgene expression. In ventral lobes, relatively high-level transgene expression was comparable between JOCK-1 and JOCK-2, and in lateral and anterior lobes, weak but equivalent expression was also seen. However, significantly higher transgene expression was seen in the dorsal prostate of JOCK-2 (Fig. 1C). In both JOCK-1 and JOCK-2 mice, immunohistochemistry with antibodies to HA shows equivalent localization and transgene expression in the luminal epithelial cells of the ventral prostate (Fig. 1, D and E), as well as higher transgene expression in the luminal epithelial cells of the dorsal prostate of JOCK-2 mice (data not shown), matching the expression levels observed by anti-HA immunoblotting. Thus, due to high-level comparable transgene expression, we focused most comparative studies on the ventral prostate lobes of JOCK-1 and JOCK-2 mice.

FGFR1, but not FGFR2, Signaling Leads to Hyperplasia and Dysplasia. To determine the cumulative effects of iFGFR1 and iFGFR2 signaling, 12-week-old male JOCK mice were treated for a period of 1–12 weeks with biweekly i.p. injections of AP20187 (2 mg/kg), which should permit signaling in target prostate tissue for up to 2–3 days after CID administration (see “Materials and Methods”). After drug treatment, prostates were isolated, microdissected, fixed, and processed for histology. In JOCK-1 mice, after 2 weeks of AP20187 treatment, staining with H&E revealed development of low-grade PIN (type I and II; Ref. 25) in the ventral, dorsal-lateral prostate (Fig. 2, C and D), and anterior prostate (data not shown). In contrast, similarly treated JOCK-2 mice showed no obvious phenotypic changes in any lobe, including the ventral and dorsal-lateral prostate (Fig. 2, E and F), suggesting that these homologous receptors have fundamental differences. Furthermore, AP20187 had no effect on wild-type mice treated for 12 weeks (Fig. 2, A and B), consistent with Phase I clinical trials of AP20187 analogue, AP1903 (26).

Because no phenotype was observed in the JOCK-2 mice, tyrosine phosphorylation of the iFGFR2 was analyzed by immunoprecipitation with antiphosphotyrosine-specific antibodies and immunoblotting with antibodies specific to the HA epitope tag of iFGFR2. From

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lysates of total prostate, we observed comparable CID-mediated receptor tyrosine phosphorylation 24 h after CID treatment for both JOCK-1 and JOCK-2 mice, verifying that iFGFR2 was functional (data not shown). Additional previous studies demonstrate that this receptor is functional in mammary epithelium (21) and TRAMP-derived cell lines (30). However, because transgene expression was variable between the different lobes of the prostates of JOCK-1 and JOCK-2 mice, we repeated the above experiment on individual prostate lobes. Although iFGFR1 tyrosine phosphorylation was observed in the ventral lobe of JOCK-1 mice, no receptor tyrosine phosphorylation was localized to the dorsal lobe where JOCK-2 mice show much higher transgene expression than JOCK-1 mice. As predicted, tyrosine phosphorylation of iFGFR2 was detected in the dorsal prostate (Fig. 2H), consistent with grade IV PIN (25). As an additional test of the functional competence of the dorsal prostate to PIN II. This demonstrates for the first time that these two receptors can have broadly different effects on normal prostatic epithelial proliferation (Fig. 2, C and D, versus Fig. 2, E and F).

To determine the progressive effects of continuous (or semicontinuous) iFGFR1 signaling over time, we focused on phenotypic changes in the ventral lobe due to the consistent changes we observed in preliminary experiments. Within the first week of AP20187 treatment, increased vacuolization, a marker for increased metabolic activity, and modest hyperproliferation were already apparent (data not shown). From 2 to 4 weeks of CID treatment, JOCK-1 mice showed pronounced hyperproliferation in all lobes of the prostate. (Fig. 3B) By 8 weeks of AP20187 administration, iFGFR1 activation led to the establishment of extensive intraductal vascularization (Fig. 3F), which was not evident in hyperplastic acini at 4 weeks (Fig. 3E). Additionally, increased nuclear atypia, chromatin condensation, and formation of papillary structures were observed (Fig. 3, C and F). By 12 weeks of activation, mice were determined to have low-grade PIN in all of the lobes examined, characterized by epithelial “piling-up,” elongated and hyperchromatic nuclei, and cribriform glandular structures (Fig. 3D), as well as stromal thickening in the dorsal lobes of some mice. Longer-term treatments of 24 and 30 weeks led to the widespread development of “reactive” thickened stroma (Fig. 3J). Additionally, more extensive dysplastic nuclei and herniated acini with extraglandular extension were observed at 24 (Fig. 3, G and H) and 30 weeks (Fig. 3J), consistent with grade IV PIN (25). As an

![Fig. 1. Generation of inducible FGFR1 and FGFR2 mice. Schematic of iFGFR transgene construct showing the intracellular domain of either FGFR1 or FGFR2 (FGFRc), downstream of two tandem AP20187-binding proteins (F, 2) and the HA epitope tag (A). Expression is regulated by the prostate-targeted promoter ARR2PB, the KCR “intron,” and the “poly(A)” sequence from the bovine growth hormone gene. B and C Western blot analysis, using antibodies specific for the HA epitope of the iFGFRs (B and C) or α-tubulin (B), of lysates from various organs harvested from JOCK-1 and JOCK-2 mice (B), or of lysates from the anterior (AP), ventral (VP), dorsal (DP), or lateral (LP) lobes of the prostate (i) indicating full-length protein and (ii) indicating a degradation product (C). Both JOCK-1 and JOCK-2 mice display prostate-specific expression of the transgenes (B), with equivalent strong expression in the ventral prostate, equivalent weak expression in anterior and lateral lobes, and stronger dorsal expression in JOCK-2 mice (C). Paraffin sections from the ventral prostate of JOCK-1 and JOCK-2 mice immunostained with anti-HA for transgene expression (D and E). Panels originally at ×200 (D and E) and insets at ×400.](image-url)
additional indication of PIN, we observed increases in cytokeratin-8 as early as 12 weeks of age with more profound increases appearing by 24 weeks (data not shown; Ref. 25). These results demonstrate iFGFR1-driven progression of normal epithelial cells to high-grade PIN and show a strong oncogenic effect of FGFR1 on normal prostate epithelium that is not shared by FGFR2.

Hyperplasia Is Reversible Until Neovascularization. In contrast to conditional genetics based on tissue-specific activation of dominant-negative or constitutive alleles (e.g., using Cre/lox technology), a distinct feature of the JOCK model is the ability to remove signaling by withholding CID. After demonstrating the degree to which iFGFR1 signaling could alter normal prostate epithelial cells in <6 months, we wanted to determine at which stages of progression, if any, changes would become irreversible. Therefore, we first investigated the effects of CID withdrawal on the prostate after ~4 weeks of AP20187 treatment, which is before initiation of obvious intraepithelial vascularization. Mice were treated biweekly with CID for 4 weeks, and then AP20187 administration was terminated for various times up to 8 weeks. Although dramatic changes in proliferation were apparent within days (Fig. 5A), loss of supernumerary epithelial cells was gradual over the 8-week period, resulting in a prostate almost indistinguishable from untreated tissue (Fig. 4A versus B; data not shown). Thus, before extensive neovascularization, iFGFR1 signaling is required for maintenance of hyperplasia.

Because newly vascularized tissue might be able to support greater cellularity even in the absence of ectopic signaling, we wanted to determine whether later stages of hyperplasia associated with intraglandular vascularization were also reversible. Therefore, JOCK-1 mice were treated for 8 weeks with CID, when extensive intraglandular vascularization is consistently observed, and treatment was stopped for an additional 8 weeks before histological examination. In contrast to animals treated for only 4 weeks, we observed no loss of hyperplasia in the ventral prostate. (Fig. 4, C versus D) Although regression was not observed in the ventral prostate, there was a decrease in proliferation, and additional progression to low-grade PIN was not observed (Fig. 4, E versus F; data not shown). Thus, hyperplasia is reversible until established neovascularization, whereas prolonged iFGFR1 activation is required for continued proliferation and additional progression.

Increased Hyperplasia in JOCK-1 Is Driven Largely by Hyperproliferation. Because hyperplasia can be caused by increased proliferation or decreased apoptosis, we wanted to determine the contributions of both to hyperplasia, as well as the mechanism(s) responsible for reversion to a normal phenotype after AP20187 re-
DISCUSSION

Although the importance of FGFR1 and FGFR2 in CaP progression has been claimed frequently, published reports of the direct effects of these receptors within the context of a normal prostate gland are only beginning to be reported (20, 27). As an essential step toward better understanding the role of FGFRs in CaP progression, we have developed the first conditionally inducible and reversible CaP model, based on CID and FGFR1 signaling, called JOCK-1. The ability to control growth factor receptor signaling both spatially and temporally has both confirmed previous ideas that FGFR1 promotes CaP progression (28) and has led to new insights into how FGFR1 signaling affects the normal prostate epithelium. The JOCK-1 model demonstrates the dependence of CaP progression on continuous iFGFR1 signaling. This is shown by the reversibility of hyperplasia after early (4-week) CID removal and the termination of progression after later (8-week) CID removal.

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removal. Additionally, FGFR1 signaling is necessary for both the initiation and maintenance of proliferation. In contrast, because detectable neovascularization trails hyperproliferation by several weeks, FGFR1 does not appear to directly stimulate angiogenesis, nor does CID removal lead to its reversal. Overall, in our model angiogenesis and stromal thickening appear to be secondary responses to increased epithelial cell numbers. This likely occurs via paracrine signaling from the epithelial cells and is not apparently due to the direct continuous action of iFGFR1 signaling, implying that the response of normal tissue (e.g., increased vasculature) to pathological proliferation is not as reversible as the hyperproliferation itself.

The JOCK-1 model is reflective of what occurs during carcinogenesis when FGFR1, which is not expressed in adult epithelial cells, becomes expressed in the epithelial compartment. In contrast to FGFR1, FGFR2 signaling did not elicit observable changes in our model even after 12 weeks of CID treatment, consistent with a distinct role for FGFR2 in the normal prostate gland. Previously reported models overexpressing FGFR2-IIIb-binding FGFs (i.e., FGF-3 or FGF-7) slowly develop mild hyperplasia. For example, overexpression of FGF-7 in the prostate, under the control of either mouse mammary tumor virus (18) or minimal probasin promoter (20), leads to hyperplasia after 9 months to a year. Whereas low transgene expression could account for the mild phenotype, an alternative explanation supported by our work is that FGFR2 signaling is insufficient for prostate transformation.

A similar hypothesis may explain the observed hyperproliferative effects of prostate-targeted FGF-3 also under the transcriptional control of the mouse mammary tumor virus promoter. In these mice, FGF-3 expression within the urogenital track led to extensive hyperplasia in all of the prostate glands (19), seemingly at odds with the inability of iFGFR2 to trigger hyperproliferation. However, unlike FGF-7, FGF-3 can also signal through other FGFRs, such as FGFR1-IIIb, which has been found in the tumor vasculature of TRAMP mice (29). Overexpression of FGF-8b, which signals through FGFR2-IIIc, FGFR3-IIIc, and FGFR4, also leads to the development of PIN (5), suggesting that activation of other FGF receptors besides FGFR1 can participate in CaP progression.

Unlike in our model where FGFR2 signaling is targeted to the epithelium, ectopic FGFs likely act as paracrine factors of which the direct effects are harder to pinpoint. Thus, our models help to clarify the unique roles for distinct FGFRs. In this regard, we observed differences previously in osteopontin production and Erk phosphorylation between iFGFR1 and iFGFR2 in TRAMP cell lines (30). Differential phosphorylation of FRS2, the major downstream substrate of FGFR, has also been observed between these two receptors (31). Together, this suggests that dissection of the signaling differences between iFGFR1 and iFGFR2 should illuminate new targets for therapeutic intervention.

Fig. 4. JOCK-1 mice show reversible hyperplasia until established neovascularization. Paraffin sections of ventral prostates from JOCK-1 mice were H&E stained (A-D) or immunostained for proliferation with antibodies specific to Ki-67 (E and F) after treatment with AP20187 for 4 weeks (A and B) followed by AP20187 removal for 8 weeks (B) or treated with AP20187 for 8 weeks (C–F) followed by CID removal for 8 weeks (D and F).
stages of CaP and for testing early intervention strategies. Crossing JOCK-1 mice with other CaP susceptibility models may lead to metastatic CaP, providing novel metastatic CaP models as well. Finally, this approach can be easily adapted to virtually any cell surface receptor that is activated by cross-linking, a growing list that may even include G protein-coupled receptors (32).

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