[CANCER RESEARCH 63, 8784–8790, December 15, 2003]

Cooperation between Ectopic FGFR1 and Depression of FGFR2 in Induction of Prostatic Intraepithelial Neoplasia in the Mouse Prostate

Chengliu Jin,[1] Kerstin McKeehan,[1] Wei Guo,[1] Scot Jauma,[1] Michael M. Ittmann,[2] Barbara Foster,[4] Norman M. Greenberg,[3] Wallace L. McKeehan,[1] and Fen Wang[1]

[1]Center for Cancer Biology and Nutrition, Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, Texas; [2]Departments of Pathology, and [3]Cell and Molecular Biology, Baylor College of Medicine, Houston, Texas; and [4]Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York

ABSTRACT

Disruption of the regulatory communication from the stroma to the epithelium mediated by the FGF7/10-FGFR2 signaling axis in the prostate and expression of ectopic FGFR1 in prostatic epithelial cells often correlate with prostate cancer progression both in human and in experimental animals. Ectopic expression of constitutively active FGFR1 mutant (caFGFR1) at low levels in prostate epithelial cells induces low- to intermediate-grade prostatic intraepithelial neoplasia (PIN) within 6–8 months and high-grade PIN in 20–25 months. Depression of the FGFR2 signaling in the prostate also disturbs homeostasis in the prostate and induces prostate hyperplasia. To study whether PIN lesions induced by the caFGFR1 were expression-level dependent, and whether expression of the caFGFR1 and depression of the FGFR2 signaling in the prostate synergistically disturbed prostate homeostasis, we generated two new strains of ARR2PBi-caFGFR1 transgenic mice, which highly expressed caFGFR1 in prostatic epithelial cells. The mice were crossed with KDNR mice to generate ARR2PBi-caFGFR1/KDNR bigenic mice. The ARR2PBi-caFGFR1 mice developed high-grade PIN within 8 months, which was significantly faster than the mice expressing caFGFR1 at low levels. In addition, depression of the FGFR2 signaling clearly promoted perturbation of cellular homeostasis induced by the caFGFR1. The results demonstrated that the PIN development in caFGFR1 transgenic mice was caFGFR1 dosage-dependent, and indicated that the ectopic FGFR1 and the resident FGFR2 in epithelial cells had opposite impacts on intercompartmental homeostasis in the prostate. The bigenic mice provide a model with cooperative aberrations in the fibroblast growth factor signaling axis for evaluation of tumor-initiating events in prostate tumorigenesis.

INTRODUCTION

Prostate cancer is the most frequently diagnosed and the second leading cause of cancer deaths in men in the United States. It is largely unclear how the life-threatening malignant prostate cancer arises as a consequence of changes in relationships between epithelial and stromal compartments cells. These changes allow epithelial cells to escape the homeostatic restraints imposed by the local environment. The fibroblast growth factor (FGF) signaling complex has long been implicated in mediating stromal/epithelial communication and homeostasis, which consists of 23 single polypeptide ligands, 4 transmembrane tyrosine kinase FGF receptors (FGFR), and a pool of highly heterogenic heparan sulfate proteoglycans (HSPGs) in the extracellular matrix (1, 2). Among them, the FGF7/FGF10 and their cognate receptor FGFR2IIIb are partitioned in the stromal and epithelial compartments, respectively, which together with epithelial cell-specific heparan sulfates underlie a directionally specific stromal to epithelial cell signaling that promotes epithelial cell homeostasis by a net restriction on proliferation and promotion of differentiation (1, 3). Recent studies reveal that stromal cells express FGFR1 and FGFR3 in a cell-specific mode, and that the FGF9 is expressed only in epithelial cells. The FGF9 specifically acts on clonal stromal lines expressing FGFR3, which underlies another directionally specific system within the FGF family from the epithelium to stroma of which the consequences are currently under investigation (4, 5).

Many components of the FGF signaling system are often found aberrantly expressed in prostate tumors (6–8). Particularly, the FGF7/10-FGFR2IIIb and the FGF9-FGFR3 signaling axes, which are common in tissues consisting of epithelial and stroma compartments, are frequently lost during progression to malignancy by splice switching or loss of expression of the gene altogether in human prostate cancers and animal tumor models, including the Dunning and TRAMP tumor models, and other tumor epithelial cells from a variety of parenchymal tissues (4, 9–11). These changes free the premalignant cells from homeostasis-promoting instructive signals elicited by the stroma. In addition to loss of the homeostasis promoting FGF7/10-FGFR2 paracrine signaling axis, fully malignant tumors often ectopically express the FGFR1 kinase that is normally restricted to the stromal compartment, and promotes autonomous growth of tumor cells by establishing an autocrine loop with abnormally expressed autocrinal FGFs (8, 10, 12, 13).

Despite high homology in amino acid sequence of the FGFR1 and the FGFR2, especially in the kinase domain, accumulating data indicate that signals elicited by the two FGFRs are different in many tissues, including the prostate (10, 13–16). Depression of the FGFR2 signaling axis by expression of the KDNR, a truncated FGFR2 construct disturbs normal interaction between stromal and epithelial cells, resulting in dysplastic and hyperplastic changes in both compartments, and an increase in neuroendocrine (NE) cell population (17). Forced expression of FGF3 and FGF8 leads to abnormal prostate development and induces prostatic intraepithelial neoplasia (PIN) lesions (18, 19). We reported previously that expression of the constitutively active caFGFR1 mutant at low levels in the prostate-induced prostate hyperplasia accompanied intermediate grade PIN at age 6–8 months and high-grade PIN at age 20–25 months, although no signs of overt carcinoma were observed (20). These suggest that chronic activation of ectopic FGFR1 kinase in prostate epithelial cells upsets homeostasis in the prostate and induces PIN.

To further study the function of FGF signaling in the prostate, we generated two new strains of ARR2PBi-caFGFR1 transgenic mice that highly expressed caFGFR1 in the prostate. The mice were then crossed with KDNR mice to generate ARR2PBi-caFGFR1/KDNR bigenic mice that expressed the ectopic FGFR1 kinase in a background of repressed resident FGFR2 signaling. Histological examination indicated that the ARR2PBi-caFGFR1 mice developed high-grade PIN within 8 months. Disruption of the basement membrane and microinvasion of epithelial foci in space between the basement membrane and the stromal layer were readily seen in the ARR2PBi-caFGFR1 prostate. This indicates that the PIN development and

Received 7/22/03; revised 9/30/03; accepted 10/10/03.

Grant support: Public Health Service Grants DMAD17-03-0014 from the United States Department of Defense, and CA096824 (F. W.), CA59971 (W. L. M.), and CA84296 and CA64851 (N. M. G.) from the National Cancer Institute.

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.

Requests for reprints: Fen Wang, Institute of Biosciences and Technology, Texas A&M University System Health Science Center, 2121 West Holcombe Boulevard, Houston, TX 77030-3303. Phone: (713) 677-7520; Fax: (713) 677-7512; E-mail: fwang@IBT.tamu.edu.

homeostasis perturbation in the prostate of caFGFR1 transgenic mice are caFGFR1 expression level dependent. Repression of the FGFR2 signaling potentiated perturbation of prostate homeostasis induced by the ectopic caFGFR1 kinase. Together, these data additionally suggest that aberrant FGF signaling in the prostate is a strong factor in disruption of tissue homeostasis, which, in turn, contributes to prostate tumor development and progression. In addition, the ARR2PBi-caFGFR1/KDNR mouse model provides a useful tool for evaluating other tumor-initiating factors including genetic instability and other oncogenic lesions in prostate tumorigenesis.

MATERIALS AND METHODS

Animals and Reagents. Young adult FVB mice (6 weeks old) were purchased from Charles River (Wilmington, MA), and young adult Swiss/Webster mice were from Harlan Sprague Dawley (Houston, TX). The animals were housed in the Institute of Biosciences and Technology Program for Animal Resources facility, and all of the experiments in which animals were involved were performed in compliance with the procedures approved by the Institute of Biosciences and Technology Animal Care Committee. Oligonucleotides were from Integrated DNA Technology, Inc. (Coralville, IA). PCR reagents and restriction enzymes were from Promega (Madison, WI). Pooled mouse monoclonal anti-pan cytokeratin, α-actin, and mouse anti- proliferating cell nuclear antigen (PCNA) antibodies from Sigma-Aldrich (St. Louis, MO). Rabbit antisynaptophysin polyclonal antibody was from Zymed Laboratories, Inc. (San Francisco, CA).

Generation of ARR2PBi-caFGFR1 Transgenic Mice. The cDNA encoding for FLAG epitope-tagged caFGFR1 was constructed and inserted in the SSI vector as described (20, 21). A transgene-specific sense primer pFLAG1 and a common FGFR1 antisense primer p240 were used to verify correct insertion and expression of the transgene by PCR (Fig. 1). The transgene was excised and purified for pronuclear microinjection as described (20).

Genomic DNAs were purified from the tails of founder mice at day 7 after birth and screened by PCR. Total RNA was isolated from the mice, and analyzed by reverse transcription-PCR with pFLAG1 and p240 primers. For more quantitative analyses of mRNA by RNase protection assay (RPA), the cDNA template for the 231-bp RNA probe was PCR amplified from human FGFR1 cDNA with forward primer pRPA1 (GCTCTCCCCTCCTCGCAGGAT) and reverse primer pRPA2 (CCTCCAATTCTGTGGTCAGGT), and cloned into the pBluescript SK vector. The RNA probe was transcribed and radiolabeled with α-[^{32}P]UTP from the template with the Maxiscript kit (Ambion, San Antonio, TX; Ref. 4). The labeled antisense RNA probe was hybridized with 25 μg of total RNA samples, and the protected fragments analyzed as described (4). The luciferase coding sequence was excised from the pGL3 vector (Promega) and inserted to the SSI vectors as illustrated in Fig. 1*B* for construction of the ARR2PBi-luciferase reporter.

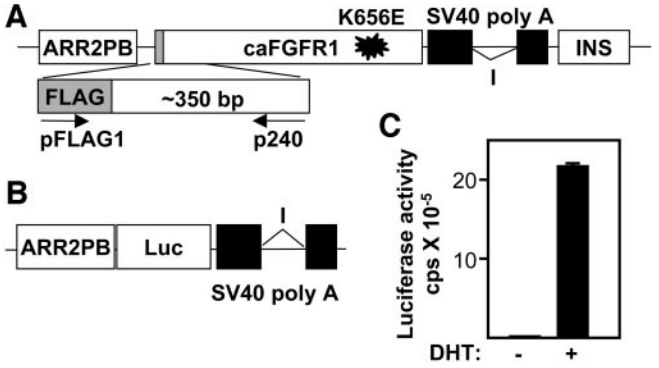

Fig. 1. Construction the ARR2PBi-caFGFR1 transgene. *A*, schematic of the caFGFR1 transgene. The human two immunoglobulin-loop isoform of FGFR1 bearing the K656E mutation and an artificial FLAG epitope was inserted downstream of the ARR2PB composite promoter. The *arrows* indicate transgene specific primer pFLAG1 and p240 used in PCR screening and reverse transcription-PCR analysis. *B*, schematic of the ARR2PB-luciferase reporter gene. *C*, androgen-dependent expression of the ARR2PB-luc reporter. The DTE-AR cells were transiently transfected with the ARR2PB-luc reporter DNA and cultured in the presence or absence of DHT. The expressed luciferase was assessed. Data were means of duplicated samples. *Luc*, luciferase; *ARR2PB*, the composite probasin promoter; *SV40 polyA*, SV40 splice site and polyA addition site; *I*, intronic sequence of SV40 T antigens; *INS*, insulator.

Histology. The urogenital complex was excised, fixed, and embedded as described (20). One of every five slides was rehydrated and stained with H&E for scanning of general tissue structures of the prostate. Prostatic intraepithelial lesions were classified using the system described recently by Park *et al.* (22), and were additionally accessed by a pathologist (M. M. I.). Immunohistochemistry analyses with anti-pancytokeratin, anti-α-actin, anti-PCNA, and anti-synaptophysin antibodies were performed on 5-μm paraffin sections. All of the samples, excluding those for anti-synaptophysin staining, were incubated at 95°C in 10 mM citric acid buffer (pH 6.0) for 20 min for antigen retrieval. No antigen retrieval was carried out for anti-synaptophysin staining per the manufacturer's suggestion. The sections were then incubated with 5% BSA for 30 min, followed by primary antibodies (1:200) for 1 h. The ExtrAvidin Peroxidase Staining kit (Sigma-Aldrich) was used as an amplification system according to the manufacturer's instructions where indicated. Otherwise, the specific bound primary antibodies were detected and visualized with alkaline phosphatase-conjugated secondary antibodies.

For periodic acid Schiff's staining, the sections were rehydrated and treated with 0.5% periodic acid at 37°C for 30 min. After being rinsed with water, the slides were stained with Schiff's reagent (Sigma Co.) for 15 min and counterstained with hematoxylin according to the manufacturer's instructions.

Luciferase Assay. The ARR2PB-luciferase report construct was transiently introduced to DTE-AR prostate epithelial cells that are stably transfected with androgen receptor. Four h after the transfection, the cells were harvested and replated in a 24-well plate in the medium containing 5% charcoal-stripped fetal bovine serum in the presence or absence of 5 mM 5α-dihydrotestosterone as indicated. The cells were lysed with the lysis buffer (Promega) after incubation at 37°C overnight; the cell lysates were mixed with 25 μl of luciferase substrate, and the fluorescence light intensity was measured with a microplate scintillation counter (Packard, Meriden, CT).

RESULTS

Generation of ARR2PBi-caFGFR1 Mice. We previously generated PB-caFGFR1 transgenic mice that used the minimal probasin promoter (PB) to target expression of the caFGFR1 specifically to prostate epithelial cells. Ectopic presence and chronic activation of the FGFR1 in mouse prostate epithelial cells, although at low levels, induced age-dependent progressive PIN accompanied by stroma thickening (20). To determine whether expression of the caFGFR1 kinase at high levels induced more profound phenotypes in younger mice, we constructed a new transgene ARR2PBi-caFGFR1 (Fig. 1*A*) with the improved ARR2PB composite promoter to direct expression of the transgene in prostate epithelium. *In vitro* data clearly demonstrated that the ARR2PB promoter exhibited a strong androgen-dependent activity in initiating transcription of the ARR2PB-luciferase reporter (Fig. 1*B*) in DTE epithelial cells derived from the Dunning 3327 prostate tumor (Fig. 1*C*). As in the PB-caFGFR1 transgene, an artificial FLAG epitope was tagged to the NH_2 terminus of FGFR1 to facilitate identification of the transgene products. The ARR2PBi-caFGFR1 transgene was excised for generation of transgenic mice in FVB inbred background. Two ARR2PBi-caFGFR1 transgenic lines were established, both of which stably passed the transgenes to offspring.

Expression of the ARR2PBi-caFGFR1 Transgene in the Prostate. To assess expression of caFGFR1 in the prostate, both ARR2PBi-caFGFR1 transgenic mice and wild-type (WT) littermates of 6–8 weeks were sacrificed, and total RNAs were extracted from the prostates. Expression of caFGFR1 was evaluated with reverse transcription-PCR with the primers showed in Fig. 1*A*. The results clearly showed that the ARR2PBi-caFGFR1 was expressed only in the prostate of transgenic mice. In addition, the 350-bp fragment representing caFGFR1 was not present in samples without reverse transcription, indicating that the fragment was amplified from the first

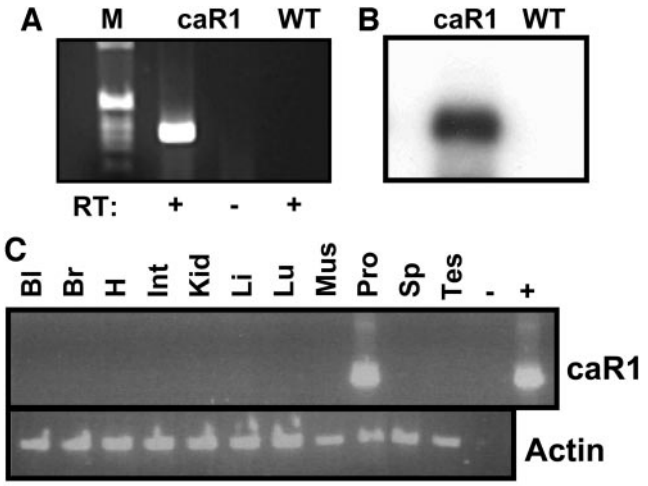

Fig. 2. Expression of the caFGFR1 in the prostate. Total RNA samples purified from the indicated tissues were analyzed with reverse transcription-PCR (*A* and *C*) or RPA (*B*). The 230-bp actin fragments in *C* were amplified from the same first-strand cDNAs as loading controls. *Bl*, bladder; *Br*, brain; *Int*, small intestine; *H*, heart; *Kid*, kidney; *Li*, liver; *Lu*, lung; *M*, molecular weight ladders; *Mus*, skeletal muscle; *Pro*, prostate; *Sp*, spleen; *Tes*, testis; −, negative control without the first-strand cDNA; +, positive control with FGFR1 cDNA; *RT*, reverse transcription.

strand cDNAs specifically (Fig. 2*A*). Furthermore, RPA results demonstrated that the caFGFR1 was expressed strongly in the prostate of ARR2PBi-caFGFR1 transgenic mice (Fig. 2*B*). Under similar conditions, expression of caFGFR1 was undetectable in the prostate of PB-caFGFR1 mice with RPA (data not shown), and the 350-bp fragment representing caFGFR1 can only be weakly detected in oligodeoxythymidylic acid column purified mRNA samples from the PB-caFGFR1 prostate (20), which suggests that expression of caFGFR1 was significantly improved in the prostate of ARR2PBi-caFGFR1 mice. To additionally determine whether the ARR2PBi-caFGFR1 transgene was expressed only in the prostate, total RNA samples were extracted from different tissues including bladder, brain, heart, intestine, kidney, liver, lung, muscle, spleen, prostate, and testis. PCR analysis clearly indicated the 350-bp fragment was only amplified from the RNA extracted from the prostate (Fig. 2*C*). The 230-bp actin fragments were amplified from the same first-strand cDNA pools for loading controls as described (4).

ARR2PBi-caFGFR1 Mice Developed High-Grade PIN within 8 Months. In general, ARR2PBi-caFGFR1 mice exhibited no significant phenotype and were reproductively active before the age of 3–4 months, which were similar to PB-caFGFR1 mice that expressed caFGFR1 kinase at low levels in the prostate. ARR2PBi-caFGFR1 males frequently lost reproductive ability after the mice had reached the age of 6 months. The average wet tissue weight of the ARR2PBi-caFGFR1 prostate was 131 ± 20 mg (n = 13), and the average prostate tissue weight of WT littermates was 78 ± 14 mg (n = 30). Clearly, the prostate of ARR2PBi-caFGFR1 mice was significantly larger than that of WT littermates ($t < 0.001$). To study the time-dependent developments of prostatic lesions induced by the caFGFR1 kinase at high dosages, prostate tissues were collected from ARR2PBi-caFGFR1 mice and WT littermates at 3–12 months of age. The prostate tissues were completely sectioned; one of every five slides was selected, stained with H&E, and examined histologically. No significant difference between the prostate tissues of ARR2PBi-caFGFR1 mice and WT littermates at the age of 3–4 months was observed (data not shown). However, most ARR2PBi-caFGFR1 prostates developed PIN 3 to PIN 4 lesions in every lobe when the mice were older than 8 months (Table 1). In the foci with high-grade PIN, the lumens were filled with disorganized, atypical epithelial cells (Fig. 3). The nuclear to cytoplasmic ratio was significantly increased. Hyperchromatic nuclei with clearly visible nucleoli were present. Areas of cells with a cribriform pattern were observed in both dorsal-lateral and anterior lobes. Frequently, epithelial cells were observed pushing into the stromal layers and formed microinvasion foci in the stromal layer. Mitotic figures were also apparent in the foci with high-grade PIN. Cellular and nuclear atypia were clearly more pronounced compared with that of PB-caFGFR1 prostates that only expressed the caFGFR1 at low-levels and developed intermediate PIN at similar ages.

Table 1 *Pathology of ARR2PBi-caFGFR1 mice*

Age (mo)	Tissue no	Line no[a]	PIN[b] Lesions			Epithelial cell push through	Cribriform structures
			AP	DLP	VP		
6	373	31	3	3	3	Yes	Yes
7	311	31	3	3	2	Yes	Yes
7	314	31	3	3	2	Yes	No
7	315	31	3	3	2	No	No
8	289	21	4	4	4	Yes	Yes
9	250	21	3	3	4	Yes	Yes
10	370	21	3	4	4	Yes	Yes
10	371	31	4	4	4	Yes	Yes
11	365	21	4	4	3	Yes	Yes
11	367	21	3	3	2	No	No
11	466	21	3	3	4	Yes	Yes
12	430	21	3	4	4	Yes	Yes
12	433	21	4	3	3	Yes	Yes
15	426	21	4	4	3	Yes	Yes

[a] Line 21 and 31 are independent ARR2PBi-caFGFR1 transgenic lines generated through microinjections.

[b] PIN, prostatic intraepithelial neoplasia; AP, anterior prostate; DLP, dorsal lateral prostate; VP, ventral prostate.

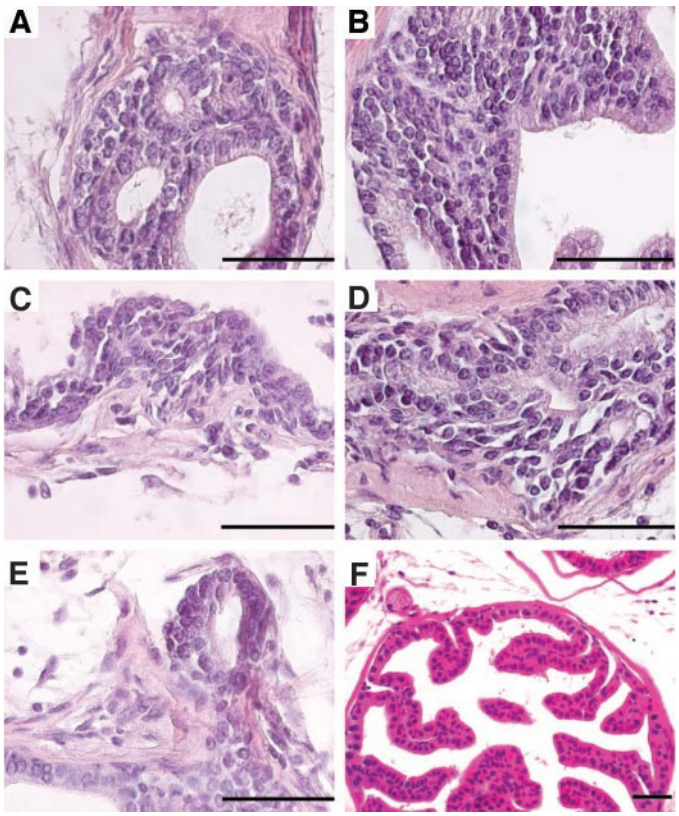

Fig. 3. High-grade PIN in the prostate of ARR2PBi-caFGFR1 transgenic mice. Sections were prepared from prostates of 30-week-old ARR2PBi-caFGFR1 transgenic mice (*A–E*) and WT FVB mouse (*F*) as described in "Materials and Methods," and representative fields are shown. Note the cribriform structures are illustrated in *A*; hyperplasia of epithelial cells in *B*; discontinued stromal-epithelial boundaries in *C* and *D*; and bulging of epithelial cells into the stroma is shown in *E*. Bar = 50 μm.

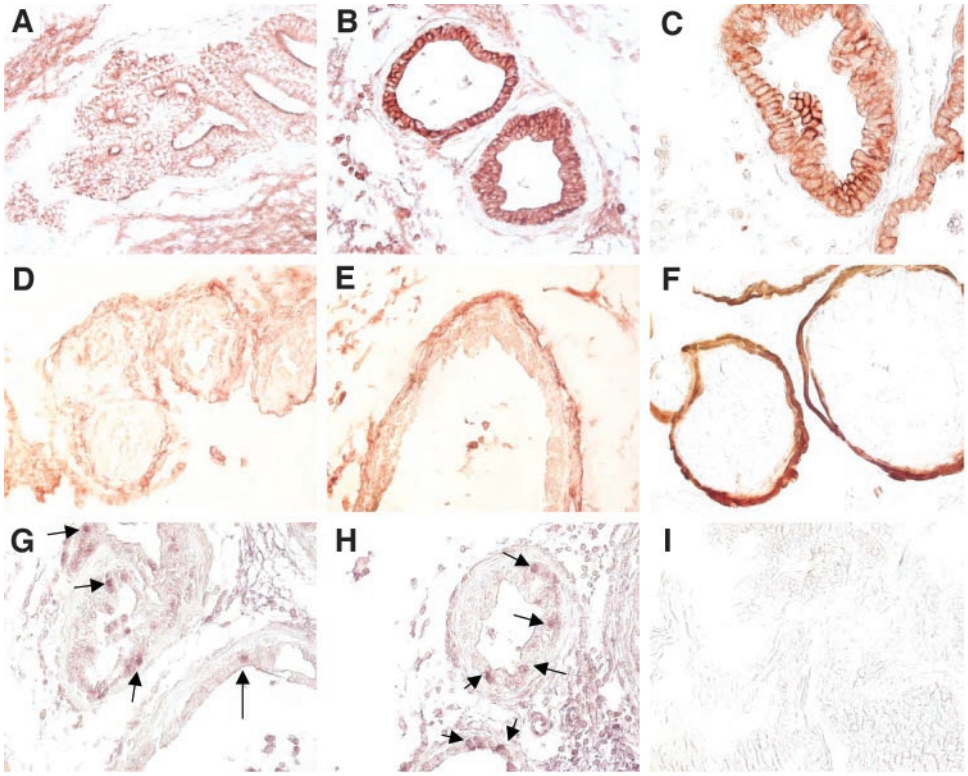

Fig. 4. Immunohistochemical analysis of cytokeratins, α-actin, and PCNA. Sections from the prostates of 8–10 months old mice were stained with anti-pan-cytokeratin (*A–C*), anti-α-actin (*D–F*), and anti-PCNA (*G–I*) as described in "Materials and Methods." Typical positive staining of the PCNA was indicated with *arrows*. *A, B, D, E, G,* and *H,* prostate sections of ARR2PBi-caFGFR1 mice; *C, F,* and *I,* WT FVB mice.

Histochemical Characterization of the Prostate of ARR2PBi-caFGFR1 Mice. To determine whether cells within the PIN foci in the ARR2PBi-caFGFR1 prostate expressed cytokeratins that are characteristic markers of well-differentiated prostate epithelial cells, the prostate sections of the ARR2PBi-caFGFR1 and WT littermates were stained with anti-pancytokeratins as described (20). The results clearly revealed that the expression of cytokeratins was reduced significantly in epithelial foci with high-grade PIN lesions, especially those with cribriform structures. It was necessary to extend the developing time to demonstrate some faint staining in these slides, which increased the background staining (Fig. 4*A*), whereas the cells in relatively normal areas, even those adjacent to the PIN foci, still highly expressed cytokeratins (Fig. 4*B*) as the cells in WT prostates (Fig. *4C*). In addition, the high-grade PIN foci, particularly those with cribriform structures, often had a disrupted stromal layer indicated by discontinuous and faint staining of smooth muscle cell characteristic α-actin (Fig. 4, *D* and *E*). Similarly, it was necessary to extend the development time for these slides to demonstrate weak staining of α-actin, which was almost indistinguishable from the background staining. In contrast, the epithelial compartment of WT prostates was usually surrounded by well-organized stromal cells readily to be demonstrated with α-actin staining (Fig. 4*F*). To determine whether the cells in the ARR2PBi-caFGFR1 prostate were actively engaged in proliferation, the prostate sections from both ARR2PBi-caFGFR1 and WT littermates were stained with anti-PCNA antibody. The results indicated that the PCNA was readily detected in the ARR2PBi-caFGFR1 prostate (Fig. 4, *G* and *H*) but not in WT prostate (Fig. 4*I*). Periodic acid Schiff's staining, which stained polysaccharides in the basement membrane that separated the epithelial and stromal compartments, revealed that the basement membrane in the ARR2PBi-caFGFR1 prostate was discontinuous, especially around the high-grade PIN foci (Fig. 5, *A–C*). Frequently, the overproliferating epithelial cells disrupted the basement membrane, invaded to the stromal compartment, and formed small epithelial foci in the space between the basement

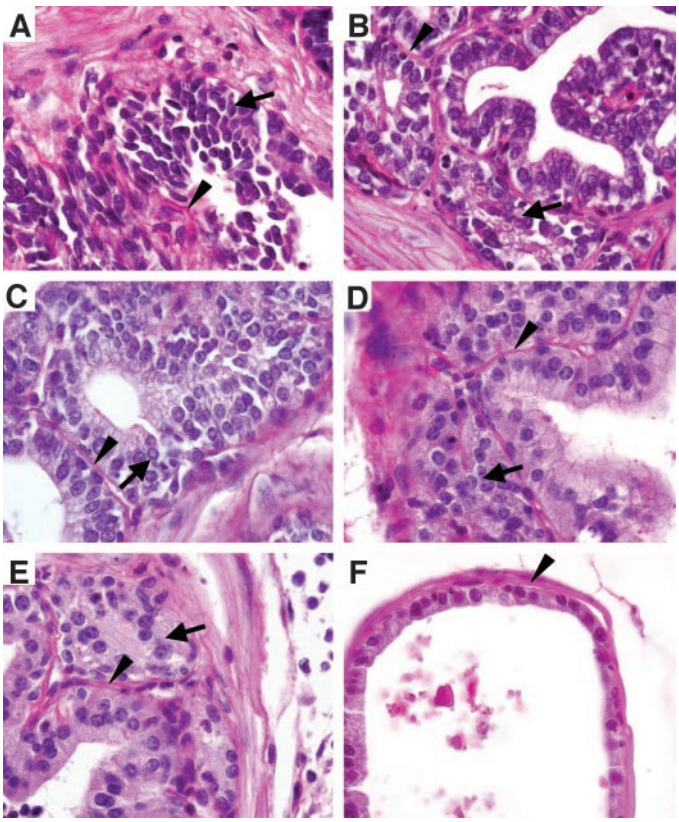

Fig. 5. Disruption of the basement membrane in high-grade PIN foci. Sections from the ARR2PBi-caFGFR1 (*A–C*), PB-caFGFR1/KDNR bigenic (*D* and *E*), and WT (*F*) prostate were stained with periodic acid Schiff's and counterstained with hematoxylin. Note the thin pink-stained basement membrane indicated with *arrowheads* separating the epithelial-stromal compartmentalization was often disrupted in the ARR2PBi-caFGFR1 and the PB-caFGFR1/KDNR prostate. *Arrows* indicated microinvasion of dysplastic epithelial cells into the underlying stroma.

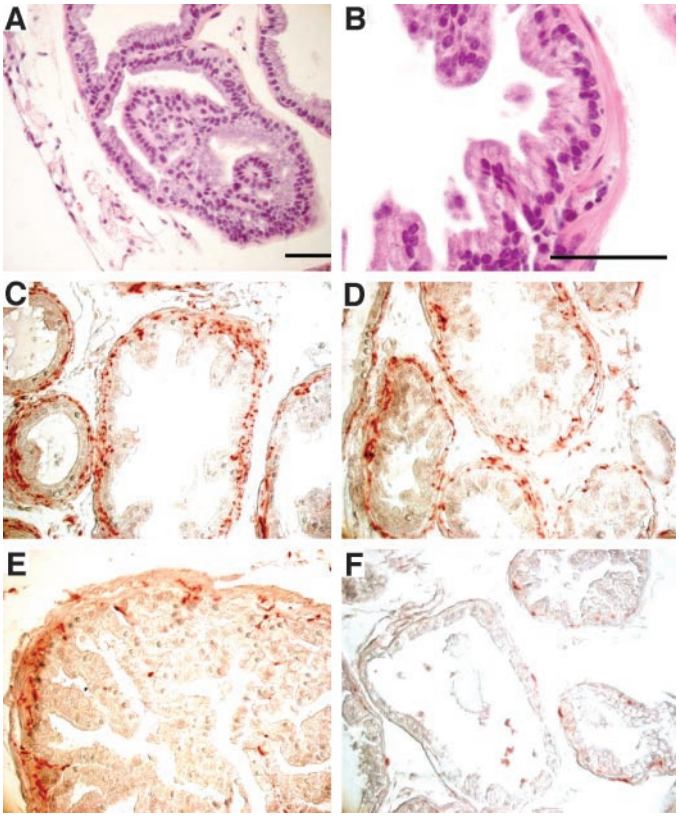

Fig. 6. The increased NE cell population in the prostate of ARR2PBi-caFGFR1/KDNR bigenic mice. (*A* and *B*), sections from the ARR2PBi-caFGFR1 prostate were H&E stained for histological characterization. Sections from the prostate of the ARR2PBi-caFGFR1/KDNR (*C*), KDNR (*D*), ARR2PBi-caFGFR1 (*E*), and WT mice (*F*) were stained with antisynaptophysin antibody as described in "Materials and Methods." *Bar* in *A* and *B* represents 50 μm.

membrane and stromal cells as indicated with arrows in Fig. 5, *A–C*. This indicated that the epithelial cells in these foci were not just pushed through the stromal layer and formed glandule-like structures. Instead, these epithelial cells formed microinvasion foci in the stromal compartment, which were more advanced lesions than high-grade PIN.

Cooperation between Ectopic Expression of FGFR1 and Depression of FGFR2 Signaling in Perturbation of Prostate Homeostasis. Both *in vivo* and *in vitro* analyses of the Dunning and the TRAMP tumor models reveal that FGFR2 promotes homeostasis in the prostate (9, 13, 14, 23). Expression of the KDNR that inhibits endogenous FGFR2 kinase through dimerization in prostate epithelial cells disrupts association between epithelial and stromal cells, causes prostate hyperplasia, and increases the NE cell population in the prostate (17). To determine whether disruption of endogenous FGFR2 signaling axis and ectopic appearance of FGFR1 in prostate epithelial cells had synergistic effects in disturbing prostatic homeostasis, we generated ARR2PBi-caFGFR1/KDNR bigenic mice by crossing. Like the parental strains, young adult bigenic mice did not exhibit significant abnormality in the prostate. Yet, the bigenic prostate gradually developed high-grade PIN foci when the mice were older than 6 months (Fig. 6, *A* and *B*), which was similar to the mice only expressing high levels of caFGFR1. To determine whether ectopically expressed FGFR1 kinase additionally increased the NE cell population, tissue sections of prostates from the ARR2PBi-caFGFR1/KDNR, ARR2PBi-caFGFR1, KDNR, and WT mice were immunohistochemically stained with anti-synaptophysin antibody. The results clearly demonstrated that the NE cell population in the bigenic mice (Fig. 6*C*) was significantly higher than in both KDNR (Fig. 6*D*) and ARR2PBi-caFGFR1 (Fig. 6*E*) counterparts. As reported earlier (17), the KDNR prostate had an increased NE cell population in the prostate (Fig. 6*D*). In addition, the NE cell population in the ARR2PBi-caFGFR1 prostate was also somewhat increased (Fig. 6*E*) compared with that of WT littermates Fig. 6*F*).

To determine whether repression of FGFR2 signaling promoted PIN lesion development in PB-caFGFR1 mice that only expressed caFGFR1 kinase at low levels in the prostate, we then generated PB-caFGFR1/KDNR bigenic mice by crossing. The prostate tissues of the bigenic and both monogenic parental strains were collected for pathological examination at different ages. The PB-caFGFR1 and KDNR parental strains usually developed high-grade PIN foci only when the mice were older than 18 months (20). In contrast, the prostate of PB-caFGFR1/KDNR bigenic mice frequently developed high-grade PIN within 8 months (Fig. 7, *A–D*), which was significantly earlier than both parental strains. Most of the lumen of the gland was filled with multiple layers of atypical epithelial cells with hyperchromatic nuclei and prominent nucleoli. Cellular and nuclear atypia was clearly more prominent compared with the intermediate PIN in the PB-caFGFR1 and the KDNR mice at similar ages (17, 20). The nuclear to cytoplasmic ratio was significantly increased in the cells within the PIN foci as well (Fig. 7, *B–D*). Furthermore, the NE cell population in the PB-caFGFR1/KDNR bigenic prostate also increased significantly (Fig. 7*F*). Periodic acid Schiff's staining indicated disruption of basement membrane and microinvasion of epithelial foci in the stromal compartment (Fig. 5, *D–E*). Trichrome staining also revealed that hyperplasic stroma did not exhibit excessive collagen staining in either ARR2PBi-caFGFR1/KDNR or PB-caFGFR1/KDNR bigenic mice (data not shown), as reported earlier (17).

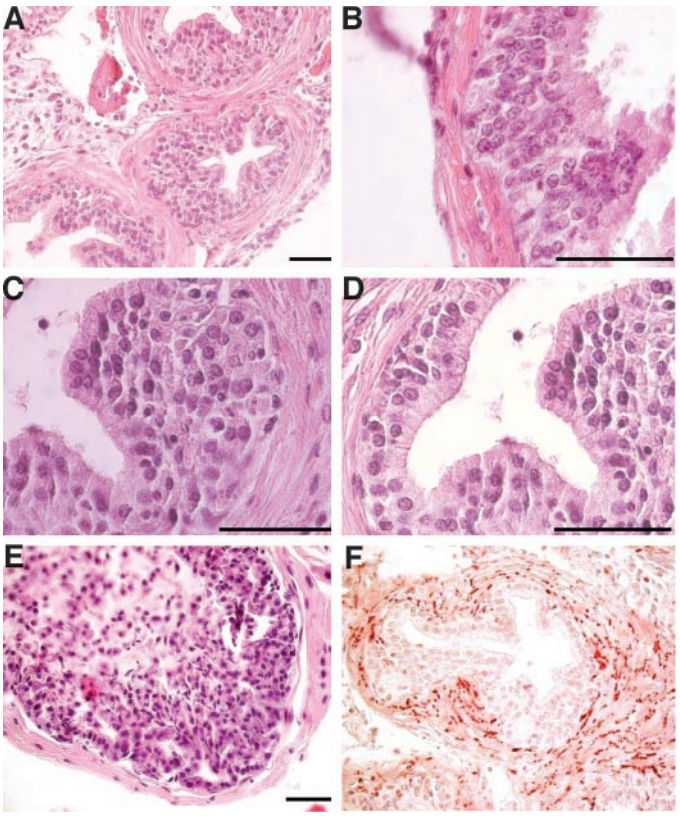

Fig. 7. Disruption of the FGFR2 signaling axis promoted development of PIN induced by the ectopic FGFR1 kinase in prostate epithelial cells. Sections from the prostates of 8-month-old ARR2PBi-caFGFR1/KDNR bigenic (*A–D*) and KDNR (*E*) mice were stained with H&E as described. *F*, anti-synaptophysin immunohistochemical staining of the same bigenic prostate tissue section as described in Fig. 6.

DISCUSSION

Expression and activation of the FGF signaling axis are highly spatial and temporal specific. We reported previously that ectopic expression of low levels of constitutively active FGFR1 mutant in prostate epithelial cells perturbed homeostasis in the prostate and induced development of PIN in the prostate over time (20). Here we additionally demonstrated that an increase in expression of the caFGFR1 kinase dramatically accelerated development of PIN in the prostate. Most ARR2PBi-caFGFR1 transgenic mice developed high-grade PIN within 8 months. Foci with cribriform structures in lumen of the prostatic acini and epithelial cells pushing through stromal compartments were readily seen throughout the prostate. These features are consistent with recently defined PIN 4 (22), which is considered the highest grade of PIN preceding development of overt carcinoma. Epithelial cells in most high-grade PIN foci failed to express cytokeratins that were characteristically expressed in highly differentiated epithelial cells. Furthermore, the stromal cells surrounding the epithelial cells in high-grade PIN foci also failed to express α-actin, a characteristic marker associated with differentiated smooth muscle cells or lineages related to them. The basement membrane surrounding the epithelial compartment was often disrupted, especially in the foci with high-grade PIN. These results indicated that development of PIN induced by the ectopic caFGFR1 kinase was positively correlated with the expression level. Secondly, depression of the FGFR2 signaling axis by expression of KDNR, a dominant kinase inactive construct of FGFR2, in the same prostate epithelial cells contributed to development of PIN induced by low levels of caFGFR1 kinase. Expression of KDNR did not significantly accelerate PIN development induced by high-level caFGFR1 kinase, but expression of caFGFR1 and depression of FGFR2 synergistically increased the population of NE cells in the prostate. The results additionally demonstrated that on the one hand the FGF signaling axis was important in regulating homeostasis in the prostate. On the other hand, aberrant FGF signaling in the prostate perturbed homeostasis in the prostate and induced PIN lesions.

Normally, it takes years to decades for development of full-blown, life-threatening malignant prostate cancers from dormant prostate tumor cells. It is believed that prostate cancers arise from the epithelial cells with progressive and collective changes that allow them to escape homeostatic restraints imposed by the local environment. Escape from the FGF7/10-FGFR2 paracrine regulation from stroma to epithelium, which has a net effect of homeostasis and promotion of differentiation, and expression of ectopic FGFR1 kinase that promotes autonomous growth of tumor cells are the hallmark changes in progression to malignancy of tumors originating from two-compartment tissues, including human prostates, and from experimental animal models (1). Yet, no overt tumor was found in the mice with the aberrant FGF signaling axis even in prostates that developed high-grade PIN and foci indicative of microinvasion rapidly after maturation. This may be due to the androgen dependence and the dependence of high activity of the PBARR2 composite promoter on highly differentiated prostate epithelial cells. Once the caFGFR1-expressing cells dedifferentiate or relax dependence on androgen passed certain stages during the progression to malignancy, potentially expression of the ARR2PBi-caFGFR1 transgene is diminished as the T antigens in the TRAMP mice (23). These cells may be frozen at the particular stage of malignancy, revert to relatively "normal" stages, or enter apoptosis due to lack of sustained high levels of the supporting ectopic FGFR1 kinase. Currently, we are establishing new lines of mice that constantly express caFGFR1 specifically in the prostate independent of androgen and differentiated state. These mice may provide new information on whether overexpression of the ectopic FGFR1 kinase alone or in cooperation with other lesions as FGFR2 depression will induce fully malignant tumors in the prostate.

Changes in HSPG in the extracellular matrix may also be important for ectopic FGFR1 kinase to abrogate growth restraints on prostate epithelial cells imposed through interaction with the stromal compartment and to induce prostatic lesions beyond PIN. Accumulating data indicate that HSPGs contribute to the FGF signaling axis, possibly through the following mechanisms: (*a*) interaction with HSPG restrains unliganded FGFR dimers in an inactive conformation that is abrogated by docking of the FGF to ligand-binding pockets, and confines ligand-specificity of the binary complex (2, 24–29); and (*b*) intracellular domain of HSPG core proteins may be involved in organizing downstream substrate complexes around the FGFR kinase (25, 30). Hence, changes in structure of heparan sulfate side chains and core proteins of HSPGs may contribute to the signaling specificity of FGFR kinases in the prostate. Although the four FGFRs share high homology in amino acid sequence and form heterodimers in the absence of heparan sulfate cofactors (31), FGFR1 and FGFR2 appear to be able to elicit receptor-specific regulatory activities when expressed in mammalian host cells, including prostate epithelial cells, suggesting no formation of FGFR1/FGFR2 heterodimers on the surface of these cells where FGFR-specific HSPGs are present (10, 14, 15). Hence, it is very unlikely that products of the caFGFR1 and KDNR transgenes would form heterodimers in epithelial cells of the transgenic prostate, because FGFR2-specific HSPGs are highly abundant in the extracellular matrix of prostate epithelial cells (25). It is conceivable that the two transgenes synergistically perturb prostate homeostasis through independent mechanisms.

The NE cells, which are present in normal, hyperplasic, and dysplastic prostate tissues, and are located in all regions of the human prostate, represent a minor epithelial cell population. The function of NE cells in the prostate, as well as the clinical significance of the NE cell in prostate cancer, is unclear, although several studies reveal an increase of NE cell population in high Gleason score prostate lesions. Currently, it is believed that NE cells may be important in regulating the growth, differentiation, and function of the prostate through secreting autocrine and paracrine peptides (23, 32). Normally, NE cells in the prostate show no proliferative activity, do not express androgen receptor, and are not androgen dependent (33). Hence, it is more likely that expression of the ARR2PBi-caFGFR1 and KDNR transgenes in prostate epithelial cells induces paracrine factors that, in turn, induce overproliferation of the NE compartment in the prostate. It should be noted that the appearance of a significantly elevated NE cell population in the ARR2PBi-caFGFR1 and KDNR transgenic mice differed from the situation observed in the TRAMP model, in which advanced prostate cancers were observed to take on a NE phenotype as a stochastic event related to progression in this animal model system (23). Elevation of the NE cells in the ARR2PBi-caFGFR1 and KDNR transgenics resulted from the selective expansion of the non-NE epithelial cells of the prostate, as a function of the deregulated FGF signaling axis, is similar to stromal population changes in rat prostate tumor (Dunning R3327PAP) as a function of changes in the FGF signaling axis (4, 5). Although the NE cells in WT prostate were mainly restricted to the basement membrane, both caFGFR1/KDNR bigenic and KDNR prostate showed a significant amount of NE cells in the hyperplasic stromal layer (Fig. 7*F*). These data additionally suggest that an aberrant FGF signaling axis in prostate epithelial cells induces abnormal expression of regulatory paracrine factors that directly or indirectly disturb homeostasis in the prostate to increase the population of NE and alter the stromal cell population in the prostate.

The KDNR was targeted for expression in prostate epithelial cells with the minimal PB promoter (17). Due to relatively weak transcription initiation activity of the promoter, expression of the KDNR in

prostate epithelial cells was not high. Probably only a fraction of resident FGFR2 kinase in KDNR prostate epithelial cells is suppressed because the degree of dominant-negative inhibition through heterodimerization requires high levels of the dominant species. It is not surprising that expression of the KDNR alone only induces a mild phenotype in the prostate as reported (17). Yet, even at low levels, the kinase inactive KDNR and ectopic FGFR1 kinase cooperatively perturbed homeostasis in the prostate and induced advanced PIN. This additionally demonstrated the homeostasis-promoting role of FGFR2 in the prostate, and that FGFR1 and FGFR2 elicit different receptor-specific regulatory activity in the prostate. It is conceivable that full disruption of the FGFR2 signaling axis in the prostate may induce a more severe perturbation of prostate homeostasis, and this is under investigation. Furthermore, expression of high-level caFGFR1 in a background completely deficient in FGFR2 will rule out the possibility that caFGFR1 perturbs FGFR2-mediated homeostasis in the prostate through heterodimerization and inhibition of FGFR2 signaling in the prostate.

ACKNOWLEDGMENTS

We thank Trinh Nguyen and Sarah Weissenberg for excellent technical assistance, Xinchen Wang for helpful suggestions and critical reading of the manuscript, and Dr. Robert Cardiff for pathological review of some of the prostate sections.

REFERENCES

1. McKeehan, W. L., Wang, F., and Kan, M. The heparan sulfate-fibroblast growth factor family: diversity of structure and function. Prog. Nucleic Acid Res. Mol. Biol., *59:* 135–176, 1998.
2. Wang, F., and McKeehan, W. L. The fibroblast growth factor (FGF) signaling complex. *In:* R. Bradshaw and E. Dennis (eds.), Handbook of Cell Signaling, Ed. 1, Vol. 1, pp. 265–270. St. Louis: Elsevier (USA), 2003.
3. Thomson, A. A. Role of androgens and fibroblast growth factors in prostatic development. Reproduction, *121:* 187–195, 2001.
4. Wu, X., Jin, C., Wang, F., Yu, C., Chan, W. K., and McKeehan, W. L. Stromal cell heterogeneity in FGF-mediated stromal-epithelial cell crosstalk in premalignant prostate tumors*. Cancer Res., *63:* 4936–4944, 2003.
5. Jin, C., Wang, F., Wu, X., Yu, C., Luo, Y., Chan, W. K., and McKeehan, W. L. FGF9 and FGFR3 form a directionally-specific paracrine communication system from rat prostate epithelium to stroma. Cancer Res., submitted, 2003.
6. Dorkin, T. J., Robinson, M. C., Marsh, C., Bjartell, A., Neal, D. E., and Leung, H. Y. FGF8 over-expression in prostate cancer is associated with decreased patient survival and persists in androgen independent disease. Oncogene, *18:* 2755–2761, 1999.
7. Hanada, K., Perry-Lalley, D. M., Ohnmacht, G. A., Bettinotti, M. P., and Yang, J. C. Identification of fibroblast growth factor-5 as an overexpressed antigen in multiple human adenocarcinomas. Cancer Res., *61:* 5511–5516, 2001.
8. Giri, D., Ropiquet, F., and Ittmann, M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. Clin. Cancer Res., *5:* 1063–1071, 1999.
9. Naimi, B., Latil, A., Fournier, G., Mangin, P., Cussenot, O., and Berthon, P. Down-regulation of (IIIb) and (IIIc) isoforms of fibroblast growth factor receptor 2 (FGFR2) is associated with malignant progression in human prostate. Prostate, *52:* 245–252, 2002.
10. Wang, F., McKeehan, K., Yu, C., and McKeehan, W. L. Fibroblast growth factor receptor 1 phosphotyrosine 766: molecular target for prevention of progression of prostate tumors to malignancy. Cancer Res., *62:* 1898–1903, 2002.
11. Kwabi-Addo, B., Ropiquet, F., Giri, D., and Ittmann, M. Alternative splicing of fibroblast growth factor receptors in human prostate cancer. Prostate, *46:* 163–172, 2001.
12. Yan, G., Fukabori, Y., McBride, G., Nikolaropolous, S., and McKeehan, W. L. Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptor genes in prostate epithelial cells accompany stromal independence and malignancy. Mol. Cell. Biol., *13:* 4513–4522, 1993.
13. Feng, S., Wang, F., Matsubara, A., Kan, M., and McKeehan, W. L. Fibroblast growth factor receptor 2 limits and receptor 1 accelerates tumorigenicity of prostate epithelial cells. Cancer Res., *57:* 5369–5378, 1997.
14. Matsubara, A., Kan, M., Feng, S., and McKeehan, W. L. Inhibition of growth of malignant rat prostate tumor cells by restoration of fibroblast growth factor receptor 2. Cancer Res., *58:* 1509–1514, 1998.
15. Zhang, Y., Wang, H., Toratani, S., Sato, J. D., Kan, M., McKeehan, W. L., and Okamoto, T. Growth inhibition by keratinocyte growth factor receptor of human salivary adenocarcinoma cells through induction of differentiation and apoptosis. Proc. Natl. Acad. Sci. USA, *98:* 11336–11340, 2001.
16. Wang, F. Cell- and receptor isotype-specific phosphorylation of SNT1 by fibroblast growth factor receptor tyrosine kinases. In Vitro Cell Dev. Biol. Anim., *38:* 178–183, 2002.
17. Foster, B. A., Evangelou, A., Gingrich, J. R., Kaplan, P. J., DeMayo, F., and Greenberg, N. M. Enforced expression of FGF-7 promotes epithelial hyperplasia whereas a dominant negative FGFR2iiib promotes the emergence of neuroendocrine phenotype in prostate glands of transgenic mice. Differentiation, *70:* 624–632, 2002.
18. Chua, S. S., Ma, Z. Q., Gong, L., Lin, S. H., DeMayo, F. J., and Tsai, S. Y. Ectopic expression of FGF-3 results in abnormal prostate and Wolffian duct development. Oncogene, *21:* 1899–1908, 2002.
19. Song, Z., Wu, X., Powell, W. C., Cardiff, R. D., Cohen, M. B., Tin, R. T., Matusik, R. J., Miller, G. J., and Roy-Burman, P. Fibroblast growth factor 8 isoform b overexpression in prostate epithelium: a new mouse model for prostatic intraepithelial neoplasia. Cancer Res., *62:* 5096–5105, 2002.
20. Wang, F., McKeehan, K., Yu, C., Ittmann, M., and McKeehan, W. L. Chronic activity of ectopic type 1 fibroblast growth factor receptor tyrosine kinase in prostate epithelium results in hyperplasia accompanied by intraepithelial neoplasia. Prostate, in press, 2003.
21. Jin, C., McKeehan, K., and Wang, F. Transgenic mouse with high cre recombinase activity in all prostate lobes, seminal vesicle, and ductus deferens. Prostate, *57:* 160–164, 2003.
22. Park, J. H., Walls, J. E., Galvez, J. J., Kim, M., Abate-Shen, C., Shen, M. M., and Cardiff, R. D. Prostatic intraepithelial neoplasia in genetically engineered mice. Am. J. Pathol., *161:* 727–735, 2002.
23. Kaplan-Lefko, P. J., Chen, T. M., Ittmann, M. M., Barrios, R. J., Ayala, G. E., Huss, W. J., Maddison, L. A., Foster, B. A., and Greenberg, N. M. Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. Prostate, *55:* 219–237, 2003.
24. Kan, M., Uematsu, F., Wu, X., and Wang, F. Directional specificity of prostate stromal to epithelial cell communication via FGF7/FGFR2 is set by cell- and FGFR2 isoform-specific heparan sulfate. In Vitro Cell Dev. Biol. Anim., *37:* 575–577, 2001.
25. Wu, X., Kan, M., Wang, F., Jin, C., Yu, C., and McKeehan, W. L. A rare premalignant prostate tumor epithelial cell syndecan-1 forms a fibroblast growth factor-binding complex with progression-promoting ectopic fibroblast growth factor receptor 1. Cancer Res., *61:* 5295–5302, 2001.
26. Kan, M., Wu, X., Wang, F., and McKeehan, W. L. Specificity for fibroblast growth factors determined by heparan sulfate in a binary complex with the receptor kinase. J. Biol. Chem., *274:* 15947–15952, 1999.
27. Knox, S., Merry, C., Stringer, S., Melrose, J., and Whitelock, J. Not all perlecans are created equal: interactions with fibroblast growth factor (FGF) 2 and FGF receptors. J. Biol. Chem., *277:* 14657–14665, 2002.
28. Ye, S., Luo, Y., Lu, W., Jones, R. B., Linhardt, R. J., Capila, I., Toida, T., Kan, M., Pelletier, H., and McKeehan, W. L. Structural basis for interaction of FGF-1, FGF-2, and FGF-7 with different heparan sulfate motifs. Biochemistry, *40:* 14429–14439, 2001.
29. Pye, D. A., Vives, R. R., Hyde, P., and Gallagher, J. T. Regulation of FGF-1 mitogenic activity by heparan sulfate oligosaccharides is dependent on specific structural features: differential requirements for the modulation of FGF-1 and FGF-2. Glycobiology, *10:* 1183–1192, 2000.
30. Uematsu, F., Jang, J. H., Kan, M., Wang, F., Luo, Y., and McKeehan, W. L. Evidence that the intracellular domain of FGF receptor 2IIIb affects contact of the ectodomain with two FGF7 ligands. Biochem. Biophys. Res. Commun., *283:* 791–797, 2001.
31. Wang, F., Kan, M., McKeehan, K., Jang, J. H., Feng, S., and McKeehan, W. L. A homeo-interaction sequence in the ectodomain of the fibroblast growth factor receptor. J. Biol. Chem., *272:* 23887–23895, 1997.
32. Sciarra, A., Mariotti, G., Gentile, V., Voria, G., Pastore, A., Monti, S., and Di Silverio, F. Neuroendocrine differentiation in human prostate tissue: is it detectable and treatable? BJU Int., *91:* 438–445, 2003.
33. Ismail, A. H., Landry, F., Aprikian, A. G., and Chevalier, S. Androgen ablation promotes neuroendocrine cell differentiation in dog and human prostate. Prostate, *51:* 117–125, 2002.

Cooperation between Ectopic FGFR1 and Depression of FGFR2 in Induction of Prostatic Intraepithelial Neoplasia in the Mouse Prostate

Chengliu Jin, Kerstin McKeehan, Wei Guo, et al.

Cancer Res 2003;63:8784-8790.