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Directionally Specific Paracrine Communication Mediated by Epithelial FGF9 to Stromal FGFR3 in Two-Compartment Premalignant Prostate Tumors

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ABSTRACT

Tissue homeostasis in normal prostate and two-compartment nonmalignant prostate tumors depends on harmonious two-way communications between epithelial and stromal compartments. Within the fibroblast growth factor (FGF) family, signaling to an epithelial cell-specific FGF receptor (FGFR) 2IIIb-heparan sulfate complex from stromal-specific FGF7 and FGF10 delivers directionally specific instruction from stroma to epithelium without autocrine interference. Using a two-compartment transplantable prostate tumor model in which survival of stromal cells *in vivo* depends on epithelial cells, we show that signaling from epithelial FGF9 to stromal FGFR3 potentially mediates epithelial-to-stromal communication that also is directionally specific. FGF9 mRNA was expressed exclusively in the epithelial cells derived from well-differentiated, two-compartment Dunning R3327 rat prostate tumors. In contrast, FGFR3 was expressed at functionally significant levels only in the derived stromal cells. Competition binding and immunoprecipitation assays revealed that FGF9 only bound to an FGFR on the stromal cells. FGF9 also failed to covalently cross-link to clonal lines of stromal cells devoid of FGFR3 that expressed FGFR1 and FGFR2IIIc. Furthermore, FGF9 specifically stimulated DNA synthesis in stromal cells expressing FGFR3. These results demonstrate a directionally specific paracrine signaling from epithelial FGF9 and stromal FGFR3. Similar to the FGF7/FGF10 to FGFR2IIIb signaling from the stroma to the epithelium, the directional specificity from epithelium to stroma appears set by a combination of cell-specific expression of isoforms and cell-context specificity of FGFR isotypes for FGF.

INTRODUCTION

Precise two-way communication between cellular compartments of tissues is essential for homeostasis, whereas lack of proper communication underlies tissue dysfunction and diseases, including cancer. The fibroblast growth factor (FGF) signaling system is a ubiquitous short-range tissue-intrinsic signaling system that mediates cell-to-cell communication during embryonic development and in adult tissues (1, 2). The FGF signaling complex consists of oligomeric structures comprising 1 of 22 signaling polypeptides defined by homology, one of a large number of combinatorial splice variants from four genes that encode an FGF receptor (FGFR) transmembrane tyrosine kinase and one or more of a host of poorly characterized, but FGF- and FGFR-specific, structural motifs within heparan sulfate (1–3). Specificity in FGF signaling lies in the combination of FGFR kinase isotypes, the heparan sulfate that combines with it, and the type of activating FGF polypeptide (4–6). In addition to endocrine signaling, in which signal originates outside the tissue expressing the receptor, two types of intratissue signaling have been described in reference to origin of signal. Autocrine signaling refers to self-stimulation in which signal and receptor originate in the same cell. Paracrine signaling refers to the case in which signal and receptor are partitioned between different cells, usually different cell types. Although autocrine signaling may be of utility in normal processes for transient amplification of endocrine or paracrine signals, autocrine signaling requires stringent feedback control and therefore usually is associated with pathologic processes in which cells are no longer responsive to their neighbors and the external environment. For intercompartmental cellular communication, it is critical that the signaling be paracrine or directional from one cell type to another representative of the compartment without interference with autocrine self-stimulation. In a variety of developing and adult parenchymal organs comprising a well-defined stroma and epithelium, directionally specific signaling from stromal to epithelium within the FGF family has been achieved by partitioning the specific FGF signal and specific receptor complex comprising FGFR kinase and a specific heparan sulfate oligosaccharide motif between different cell types or compartments. In the prostate and several other parenchymal organs with distinct epithelial and stromal compartments, expression of FGF7 and FGF10 is limited to stromal cells (7–9). A specific FGFR complex of heparan sulfate and splice variant FGFR2IIIb that recognizes FGF7 and FGF10 is expressed only in epithelial cells (7–9). Epithelial cells do not express sufficient levels of an FGF that will bind and activate FGFR2IIIb, and stromal cells do not express an FGFR isotype capable of activation by FGF7 and FGF10. Although cell-free complexes of heparin and the FGFR2IIIc splice variant that is expressed in stromal cells have weak affinity for FGF7, any binding to FGFR2IIIc and autocrine activity of FGF7 in stromal cells are prevented by stromal cell-specific heparan sulfate (5, 10, 11). FGFR1 that is expressed specifically in the stroma cannot bind FGF7 or FGF10 under any conditions (1, 6, 12). Whereas isolated cell-free FGFR2IIIb-heparin complexes bind FGF2 significantly, a dependence on stromal FGF7 and FGF10 is set by epithelial cell heparan sulfate that, when complexed to FGFR2IIIb, prevents the binding of FGF2 (5, 10, 11). Stromal to epithelial cell signaling via FGF7/FGF10 and FGFR2IIIb has a net effect of promoting epithelial cell homeostasis that includes growth but limits population growth overall by feedback inhibition mechanisms and induction of differentiation (7, 8, 12–17). Subversion of the strict directional instruction from stroma to epithelium mediated by the FGF7/FGF10 and FGFR2IIIb system at multiple steps and acquisition of an autocrine signaling loop by ectopic activation of FGF family members in epithelium and stroma that are unrestrained in respect to growth are hallmarks of the progression of premalignant prostate tumor epithelial cells to malignancy (1).

Although it is agreed that a dialogue requires directional communication between both parties, less attention has been focused on epithelial to stromal cell communication, which may be equally important in maintenance of overall tissue homeostasis. FGF9 recently has emerged as important for epithelial to mesenchymal cell signaling in embryonic development (9). In testicular and lung development, the defects appear to be deficiencies in development of mesenchyme that

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has a net impact on development of the epithelium that characterizes the tissue. Relative to other FGFR, FGFR3 binds FGF9 with highest affinity in the presence of heparin, either as a purified receptor in solution (18) or when expressed on the surface of leukemia cells (6, 19). Affinity for other FGFR isoforms appears to be cell type, context, and assay dependent, except for FGFR2IIIb, which failed to bind FGF9 in all of the reports to date. Despite the evidence for directional paracrine FGF9 to FGFR3 signaling in several tissues with developing epithelial and stromal compartments, there are conflicting reports on the origin of FGF9 in adult parenchymal organs. On the basis of apparent expression of FGF9 and FGFR isotypes in human prostate tissues and primary cell cultures derived from them, it has been reported that FGF9 is expressed in stroma and has paracrine growth stimulation activity in prostate epithelium and autocrine activity in the stroma (20). FGF9 expression has been reported to be limited to stroma in human endometrium and to exhibit only autocrine activity in stroma in that tissue (21). A stromal or mesenchymal origin of FGF9 with autocrine activity for stroma differs considerably from analyses in developing tissues. In adult parenchymal tissues, this would subvert a comparable intercompartmental communication system between epithelial and stromal compartments. On the basis of reverse transcription-PCR analysis, an epithelial origin of FGF9 in human retinal pigment epithelium has been reported, but an autocrine function was proposed because of the presence of FGFR2IIIc and FGFR3IIIc, which have been reported to bind FGF9 in other contexts (22). It is difficult to generate pure populations of epithelial and stromal cells in primary cell cultures without cross-contamination. Therefore, we tested the FGF9 and FGFR3 pair for the criteria necessary for directional epithelial to stromal communication in a well-defined reconstituted, two-compartment *in vitro*/*in vivo* model of prostate tumor progression in which the FGF7/FGF10 and FGFR2IIIb pair constitutes the directionally specific stromal to epithelial signaling and survival of stroma depends on the epithelium. We found that FGF9 is expressed significantly in only epithelial cells and binds only to FGFR3 that is present only at functionally significant levels in stromal cells. Therefore, the FGF9/FGFR3 pair completes a two-way paracrine communication circuit between prostate epithelium and stroma that may be important for intercompartmental homeostasis in normal nonmalignant two-compartment tumors and subject to subversion during progression to malignancy.

MATERIALS AND METHODS

Construction, Expression, and Purification of Radiolabel Grade Recombinant FGF9. Total RNA was isolated from normal Copenhagen rat prostate using the Ultraspec RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX). First-strand cDNA was synthesized by reverse transcription with SuperScript II RNase H^- Reverse Transcriptase (Life Technologies, Rockville, MD) and random primers. The following PCRs were carried out for 30 cycles at 94°C, 60°C, and 72°C for 1 min with PfuTurbo DNA Polymerase (Stratagene, La Jolla, CA).

Rat FGF9 cDNA (encoding amino acid residues 34–208) was generated from the template with sense primer F9P5b (5′-GT*GGATCC*AGTGACCACCTGGGTCAGTCC-3′) containing a *Bam*HI restriction site (italicized) and antisense primer F9P3 (5′-TC*GAATTC*AACTTTGGCTTAGAATATCCTTA-3′) containing an *Eco*RI restriction site (italicized). The PCR product was digested by *Bam*HI and *Eco*RI, then fractionated on a 1.5% agarose gel, and recovered with QIAEX II Agarose Gel Extraction Protocol (Qiagen, Valencia, CA). Active bacterial-derived FGF9 was expressed and recovered by a described generic procedure first applied to obtain sufficient yields of high specific activity radiolabel grade FGF7 for structural analysis with modifications described in the text (23). The cDNA was cloned in-frame into the pGEX-2T expression vector at *Bam*HI and *Eco*RI sites, verified by nucleotide sequencing, and expressed in BL21 (DE3) cells (Novagen, Madison, WI), resulting in a fusion protein with glutathione *S*-transferase (GST) at the NH_2 terminus. *Escherichia coli* BL21 (DE3) was transformed and incubated overnight at 37°C in Luria-Bertani medium containing ampicillin (100 μg/ml) until cell density reached an OD_{600} of 1.2–1.5. The cells then were transferred to 30°C, and 0.1 M isopropyl-1-thio-β-D-galactopyranoside was added to induce expression. After 3–4 h, the bacteria were collected by centrifugation, and the resulting pellet was frozen at −80°C. The cell pellet then was resuspended in PBS; the cells were lysed by sonication and clarified by centrifugation. The lysate was applied to a column of glutathione Sepharose 4B affinity chromatography; the column was washed extensively with PBS and then treated with 1 μg/ml thrombin at room temperature. The eluate containing FGF9 was further purified by fast protein liquid chromatography with a HiTrap heparin affinity column (Amersham Pharmacia Biotech, Piscataway, NJ). Preparations were subjected to analysis by SDS-PAGE immunoblot using goat polyclonal anti-FGF9 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and microsequence analysis (Protein Chemistry Laboratory, Texas A&M University, College Station, TX).

Because efficient production and recovery of FGF7 depended on the length of the NH_2 terminus upstream of the minimal active core, we tested three constructions fused to GST at the NH_2 terminus (GST-Leu4-FGF9, GST-Val13-FGF9, and GST-Ser34-FGF9) for highest yield and recovery of full-length active product after removal of the fusion partner (10, 11, 24). GST-Ser34-FGF9 (residues 34–208) was superior and used throughout the study. Initial purification, recovery, and immobilization of product from bacterial lysates onto heparin-agarose, followed by controlled trypsin treatment to remove the GST part resulted in highest recovery of total FGF9, but purity was unsatisfactory and the product was unstable on storage (24). Therefore, we immobilized the GST-Ser34-FGF9 from the lysate, removed the GST part by treatment of the column with thrombin, and subsequently purified the eluate on fast protein liquid chromatography heparin-agarose. The product of Ser34-FGF9 exhibited a single band of M_r 21,000 on SDS-PAGE and the single N-terminal sequence ser-asp-his-leu-gly expected of mature homogenous Ser34-FGF9. The yield of high specific activity radiolabel grade FGF9 by this procedure was 5–10% and 25% of that reported for FGF1 and FGF7, respectively (24).

Expression of FGF9 and FGFR3 mRNA. FGF9 mRNA was analyzed by reverse transcription-PCR and RNase protection. For reverse transcription-PCR, FGF9 mRNA was analyzed using sense primer F9P5 (5′-GTGGATCCATGGCTCCCTTAGGTGAAGTTG-3′) and antisense primer F9P3 (5′-TCGAATTCAACTTTGGCTTAGAATATCCTTA-3′), flanking the full-length coding sequence of rat FGF9, which yielded a product of 641 bp. Oligonucleotide primers to analyze FGFR3 mRNA were sense primer 667rR3P5 (5′-GTGGTTGAGAACAAGTTTGGCAGC-3′) and antisense primer 1091rR3P3 (5′-CCAGCCTCGTCAACTTCCATCAGC-3′) that flanked the 431-bp coding sequence of rat FGFR3IIIb or the 425-bp coding sequence of rat FGFR3IIIc. Five μg RNA were isolated from the tissues and cells indicated in the text and converted to cDNA, and 10% of the template was used in the PCR. The PCR was carried out for 30 cycles at 94°C, 60°C, and 72°C for 1 min each using TaqDNA Polymerase (Promega, Madison, WI). The reverse transcription-PCR products were analyzed on a 1.5% agarose gel.

For RNase protection, total RNA was isolated as described previously and assessed by RNase protection with the Hybspeed RPA kit (Ambion, Austin, TX) using the following probes transcribed with the Maxiscript kit (Ambion) according to the manufacturer's recommended procedures. All of the probes were radiolabeled with α-[^{32}P]UTP during transcription by either T3 or T7 RNA polymerase. The FGF9 probe was a 528-bp fragment flanked by *Bam*HI and *Eco*RI sites from the FGF9 expression vector described previously and ligated into pBluescript SK. Following digestion by *Eco*RI, T3 RNA polymerase transcribed the probe. The FGFR3IIIc probe construction was a 453-bp PCR-amplified fragment from rat brain cDNA using sense primer rR3P5 (5′-CACCGCATTGGGGGCATTAAGCTC-3′) and antisense primer rR3P3c (5′-ATGGGAAAACCCGATAGAATTGCC-3′) that flanked the 336-bp coding sequence of rat FGFR3IIIb or the 453-bp coding sequence of rat FGFR3IIIc. PCR was carried out for 30 cycles at 94°C, 60°C, and 72°C for 1 min/cycle using PfuTurbo DNA Polymerase (Stratagene). The fragments were cloned into pBluescript SK at the *Sma*I site. Following linearization by *Bam*HI, T7 RNA polymerase transcribed the constructs. The rat β-actin probe was transcribed with T7 RNA polymerase of pTRI-β-actin-125-rat antisense control template (Ambion) containing a 126-bp cDNA fragment of the rat β-actin. Each probe was hybridized with the corresponding RNA sample for 15 min

and incubated in the presence of Ambion RNase A/T1 for 30 min. Protected fragments were separated on 5% polyacrylamide sequencing gels and subjected to autoradiography. Experiments were reproduced at least three times.

Cell Culture, Competition Binding, Covalent Affinity Cross-linking, and DNA Synthesis. Methods for selective isolation, maintenance, validation, and characterization of the properties of pure clonal cultures of epithelial and stromal cells from transplantable nonmalignant Dunning R3327PAP rat prostate tumors and the single cell type from their malignant variant AT3 have been described in detail (7, 12, 25–27). In brief, cultures enriched in epithelial (DTE) cells and stromal (DTS) cells from two-compartment nonmalignant tumors were generated in selective low or serum-free and high serum culture medium for the respective cell type (28). They then were classified based on morphology and absence or presence of cytoskeletal markers, and cell lines were developed with continuing passage in culture. DTE epithelial and DTS stromal cultures were further distinguished by survival and proliferative behavior when reimplanted alone in syngenic animals (7, 12). Purity of DTE cell cultures from nonmalignant tumors was monitored by the exclusive expression of FGFR2IIIb and absence of trace levels of FGFR1 determined in the PCR (7, 12). Purity of DTS cultures was monitored by the absence of trace levels of FGFR2IIIb. AT3C cell lines, the single cell type that emerges from malignant AT3 tumors, were established in conditions similar to DTS cultures but were distinguished by relative independence on culture medium, epithelial cell-like morphology, the absence of FGFR2IIIb, and malignant behavior when implanted *in vivo*. After establishment, cell lines were maintained in RD medium containing 2%, 10%, or 5% fetal bovine serum for DTE cells, stromal (NPS) cells from normal prostate tissue, and DTS cells, respectively (27). Clonal analysis, establishment of clonal cultures, and systematic aging in respect to population doublings of DTS cell populations have been described in detail (27).

Binding was performed under conditions optimized to yield maximum temperature-dependent binding to cell surface receptors with minimal loss by temperature-induced internalization (29). Recombinant FGF1, FGF7, and FGF9 were iodinated (10), reactivated by reduction, and purified to a specific activity of 2–5 $\times$ 10^5 cpm/ng (4). For competition binding at the cell surface, 1×10^5 cells were cultured in 24-well plates overnight and then incubated with 0.5 ml serum-free medium containing 2 ng/ml ^{125}I-FGF1 and the indicated amount of unlabeled FGF1, FGF7, or FGF9 in the presence of 2 μg/ml heparin. After incubation at 37°C for 1 h, the cells were washed with PBS, followed by PBS containing 250 μg/ml heparin, and then PBS again. The bound radioactivity was extracted with 0.5% NaOH and counted.

For covalent affinity cross-linking, cells (5×10^5) were cultured in six-well plates overnight. The medium then was replaced with 1 ml serum-free medium containing 2 ng/ml of iodinated FGF. After incubation at 37°C for 1 h, the cells were washed as described previously and incubated with 1 ml of PBS containing 1 mM disuccinimidyl suberate for 15 min under gentle shaking and lysed in Triton X-100. The lysate was clarified by centrifugation and mixed with sample buffer for SDS-PAGE, followed by autoradiography (30). Immunoanalysis of FGFR3 covalently radiolabeled with FGF1 was performed as described with rabbit anti-FGFR3 serum (31). Serum was prepared against synthetic peptide sequences DAVELSCPPPGGGPMGPTVWVKD (D55-D77) and SGDDSVFAHDLLPPAPPSSGGSRT (S783-T806) from N- and COOH-terminal human FGFR3 sequences that were unique to FGFR3 among the four FGFR isotypes (Cocalico Biologicals, Inc., Reamstown, PA). Antisera exhibiting ELISA titers of ~1:25,000 and 1:50,000 against D55-D77 and S783-T806, respectively, reacted strongly with 10 ng insect cell-derived recombinant FGFR3 at 1:100 dilution applied to immunoblots. Serum from the same animal before immunization was used as the control.

DNA synthesis was measured by [^{3}H]thymidine incorporation. Cells at a density of 5×10^4/well were plated on 24-well plates containing 1 ml RD medium with 2% fetal bovine serum and incubated overnight. The medium in DTS cultures then was replaced with serum-free medium containing 2 μg/ml heparin and that for DTE cultures with medium containing 0.5% fetal bovine serum and 2 μg/ml heparin and incubated for 48 h. The individual FGF indicated in the text was added for 16 h, and 0.5 μCi/ml [^{3}H]thymidine (NEN, Boston, MA) then was added. After 6 h, the cells were solubilized in 0.5 M NaOH after washing with PBS, 10% trichloroacetic acid, and then PBS. The radioactivity was counted by liquid scintillation.

Construction, Expression, and Specificity of Recombinant FGFR Isoforms. Specificity analyses with purified FGFR isoforms were carried out with recombinant isoforms tagged with GST at the COOH-terminus from baculoviral-infected insect cells as described previously (4, 10, 11, 32). Preparation and expression of recombinant FGFR1, FGFR2, and FGFR4 have been described previously (10, 11, 32). Recombinant FGFR3IIIb and FGFR3IIIc were prepared and expressed as described below. Full-length cDNAs of FGFR3IIIb and FGFR3IIIc were amplified from cDNAs of mouse liver and brain, with sense primer R3P1 (5′-GCT*GGATCC*GGACGCCGCGGC-CCCCGC-3′) and antisense primer mR3LO4 (5′-CG*GGATCC*TCACGTC-CGAGGTCCCCCGTTACT-3′) containing a *Hind*III restriction site (italicized). The PCR was carried out for 25 cycles (each cycle: 94°C for 45 s, 65°C for 45 s, and 72°C for 3 min) using Pfu DNA Polymerase (Stratagene). The PCR product was digested with *Hind*III and cloned into pBluescript SK at the *Hind*III site and then verified by nucleotide sequencing. Preparation of recombinant baculovirus encoding FGFR-GST constructs and their expression in Sf9 was performed as described previously (4). Fragments of FGFR3IIIb and FGFR3IIIc in the GST fusion constructs were prepared by PCR using sense primer 428mR3P5 (5′-ATAAGAAACTGCTGGCTGTGCCA-3′) and antisense primer mR3LO3 (5′-TGGGGTACCGCGCAGGCGGCAGAGTAT-CAC-3′), followed by digestion with *Pvu*I and *Kpn*I. Infected Sf9 cells were lysed with 1% Triton X-100 in PBS containing 10 mM $MgCl_2$. After clarification of the lysate by centrifugation at 15,000 $\times$ *g* for 30 min, the supernatant was incubated at room temperature for 30 min with GSH Sepharose beads (Amersham Pharmacia Biotech) in PBS containing 2.5 M NaCl and 10 mM $MgCl_2$. After preincubation with 10 μg/ml heparin and subsequent removal of soluble heparin, the beads were incubated for 30 min with 1 ng/ml ^{125}I-FGF for binding assay or 1 ng/ml ^{125}I-FGF1 and the indicated amount of unlabeled FGF9 or FGF1 for competition binding assays. The bound FGF radioactivity was extracted with SDS-sample buffer and counted after washing three times with PBS. For competition binding, 5 μl of packed beads representing lysate from ~1×10^5 infected Sf9 cells were used per assay. For cross-linking, 25 μl beads were used per assay. After binding, the immobilized complexes were incubated with 100 μl PBS containing 1 mM disuccinimidyl suberate for 15 min. The beads then were mixed with 25 μl sample buffer for SDS-PAGE analysis, followed by autoradiography.

RESULTS

Differential Expression Pattern of FGF9 and FGFR3 in Normal Rat Prostate, Transplantable Prostate Tumors, and Derived Cell Lines. Expression of FGF9 and FGFR3 mRNA was examined in normal rat ventral prostate, the transplantable nonmalignant Dunning R3327PAP (DT), and derived malignant AT tumors, as well as cell lines derived from the latter two tumors by trace analysis using reverse transcription-PCR and a more quantitative analysis by RNase protection (RPA) using antisense probes. Reverse transcription-PCR analysis revealed that FGF9 mRNA was present in normal prostate, the DT tumor, and the malignant AT tumor that emerges from it (Fig. 1*A*). The quantitative analysis of mRNA by RPA revealed that FGF9 expression was relatively low in normal prostate and the DT tumor and high in the AT tumor (Fig. 1*B*). Pure cultures of epithelial (DTE) and stromal (DTS) cells from the two-compartment DT tumors, the single epithelial cell type (AT3) that comprises the AT tumors, and stromal cells (NPS) from normal prostate were derived as described in "Experimental Procedures." Although a strong signal was present in the DTE and AT3 tumor-derived epithelial cells, even trace levels of FGF9 mRNA were undetectable in the DT tumor-derived stromal cells (DTS). These results suggested that FGF9 is expressed in the epithelial compartment and is absent or low in stromal cells in nonmalignant two-compartment tumors.

The epithelial (DTE) and stromal (DTS) cell cultures from nonmalignant DT tumors and the AT3 cells from malignant AT tumors have been characterized extensively in respect to expression of FGFR1 and FGFR2 and FGF2, FGF7, and FGF10 ligands (7, 8, 12, 26, 33). DTE cells exhibit little clonal heterogeneity and express exclusively the

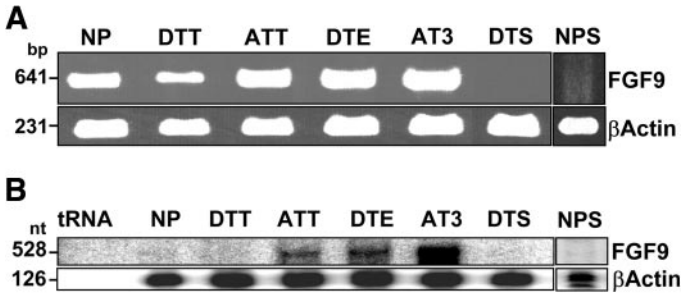

Fig. 1. Expression of fibroblast growth factor (*FGF*) 9 mRNA in rat prostate, prostate tumors, and derived cells. *A*, reverse transcription-PCR was performed on the indicated prostate tissue and cells with FGF9-specific primers described in "Materials and Methods." *B*, total RNA (20 μg) from each sample was analyzed by RNase protection. *NP*, rat normal prostate; *DTT*, Dunning R3327PAP tumor tissue; *ATT*, AT3 tumor tissue; *DTE*, DT tumor-derived epithelial cells; *AT3*, AT3 tumor-derived malignant cells; *DTS*, DT tumor-derived stromal cells; and *NPS*, stromal cells derived from normal prostate.

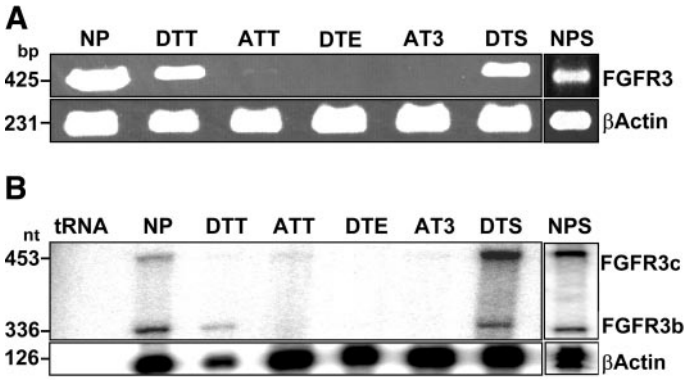

Fig. 2. Expression of fibroblast growth factor receptor (*FGFR*) 3 mRNA in rat prostate, prostate tumors, and derived cells. *A*, reverse transcription-PCR was performed on the indicated tissue and cells with the FGFR3-specific primers described in "Materials and Methods." *B*, total RNA (20 μg) from the indicated sources as described in Fig. 1 was analyzed by RNase protection. *NP*, rat normal prostate; *DTT*, Dunning R3327PAP tumor tissue; *ATT*, AT3 tumor tissue; *DTE*, DT tumor-derived epithelial cells; *AT3*, AT3 tumor-derived malignant cells; *DTS*, DT tumor-derived stromal cells; and *NPS*, stromal cells derived from normal prostate.

IIIb splice variant of FGFR2, whereas AT3 cells exhibit extensive clonal heterogeneity and express FGFR1, FGFR2, FGF2, and low levels of FGF7 and FGF10. DTS cultures express FGF7 and FGF10 at high levels. The complete expression and clonal heterogeneity of FGFR isotypes and FGF7 and FGF10 of DTS cultures only recently have been analyzed (27). Because FGFR3 has received the most attention as the FGFR isotype with highest affinity for FGF9, we tested the expression pattern of FGFR3 mRNA by the same methods (Fig. 2). Reverse transcription-PCR indicated that FGFR3 mRNA was present in normal prostate and the two-compartment DT tumor tissue with only a trace detected in the AT tumors (Fig. 2*A*). However, in notable contrast to FGF9 expression, an FGFR3 signal was completely absent from the DT and AT tumor-derived epithelial cells, whereas the DT tumor-derived stromal cells exhibited the strongest signal. RPA confirmed the presence of the FGFR3IIIb and FGFR3IIIc isoforms, with the FGFR3IIIb form predominating in normal prostate (Fig. 2*B*). The DT tumor also exhibited both isoforms in a similar proportion but at lower levels than normal prostate, whereas FGFR3 was at or near undetectable levels in the AT tumors. FGFR3 was undetectable by RPA in DTE and AT3 cells. The RPA confirmed that FGFR3 expression was highest in the DTS cells, with the FGFR3IIIc predominating (Fig. 2*B*). The combined results suggested that FGF9 mRNA is solely expressed in the DT epithelial cells, whereas FGFR3 mRNA is solely expressed in the DT stromal cells. Lastly, we established primary and short-term serial cultures of normal rat prostate stromal cells to determine whether FGF9 and FGFR3 were partitioned in cells derived from normal tissue (27). In contrast to epithelial cells, primary cultures enriched in stromal cells can be serially subcultured as epithelial cells decline for a sufficient number of population doublings for analysis of relatively pure cultures. The results indicated the absence of FGF9 (Fig. 1) and the presence of FGFR3 (Fig. 2) at similar levels to the DT stromal cells in stromal cultures from normal prostate that had undergone 15 population doublings (15 subcultures at 1:2 dilution).

Competition and Covalent Affinity Cross-Linking of FGF9 to Specifically DT Stromal Cells Expressing FGFR3. To determine whether the DT epithelial and/or the DT stromal cells exhibited FGFR binding sites for epithelial cell-specific FGF9, we first tested competition of FGF9 with radiolabeled FGF1 that binds to FGFR on the surfaces of DTE and DTS cells (8). On the basis of cell-free binding to recombinant FGFR isoforms in the presence of heparin, FGF1 does not discriminate among FGFR isotypes and is expected to bind to all of the isotypes present on DTE and DTS cells, including FGFR3 (4). The results showed that FGF9 specifically competed with radiolabeled FGF1 bound to DTS cells (Fig. 3). As expected, unlabeled FGF7 competed only with FGF1 bound to the DTE cells that display FGF7-specific FGFR2IIIb (8). Next, to determine whether the FGF1 bound to DTS cells was, in part, caused by cell type-specific display of FGFR3, we covalently cross-linked the radiolabeled FGF1 to cell surface sites and examined the cross-linked complexes for immunoreactivity with anti-FGFR3 antisera. No immunoprecipitable FGF1-labeled FGFR3 could be detected in DTE cells, whereas DTS cultures exhibited positive signals (Fig. 4*A*). Preimmune serum yielded no reaction under any conditions. A recent clonal analysis of DTS cultures revealed that the stromal cells derived from the nonmalignant, two-compartment tumors comprise two clonal cell types that can be distinguished on the absence (DTS1) or presence (DTS2) of smooth muscle cell α-actin (27). Fibroblast-like DTS1 cells are distinguished by the expression of predominantly FGFR1 and FGF10. Smooth muscle cell-like DTS2 cells are distinguished by the absence of FGF10 and presence of FGFR3. Both clonal types express FGF7 and FGFR2IIIc (27). The ratio of DTS1 to DTS2 cells in uncloned DTS cultures increases as a function of population doublings and passage in cell culture (27). Fig. 4*A* indicates that high- and low-passage DTS cultures exhibited FGF1-labeled FGFR3 complexes and that the low-

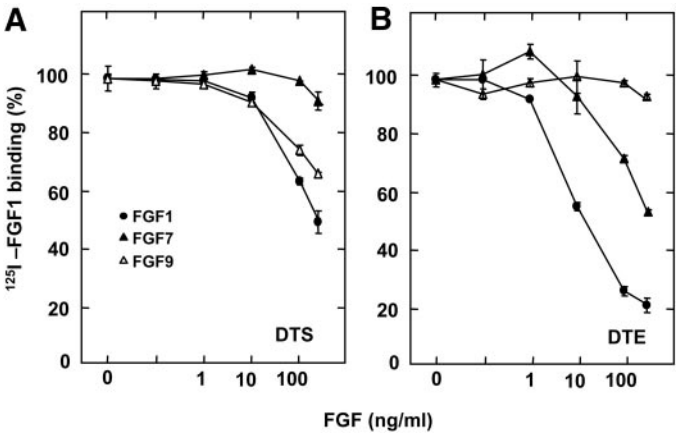

Fig. 3. Competition of fibroblast growth factor (*FGF*) 9 with radiolabeled FGF1 bound to Dunning R3327PAP (*DT*) stromal cells. Cultured DT tumor-derived stromal cells (*DTS*; *A*) and DT tumor-derived epithelial cells (*DTE*; *B*) cells were incubated with 2 ng/ml radiolabeled FGF1 together with the indicated amount of unlabeled FGF1, FGF7, or FGF9 in the presence of 2 μg/ml heparin for 1 h. The bound radioactivity was extracted with 0.5 M NaOH and determined by gamma counter after rinsing the cells with PBS, PBS containing 250 μg/ml heparin, and then PBS to remove nonspecifically bound ligand. Data points are the mean ± SD of triplicate determinations.

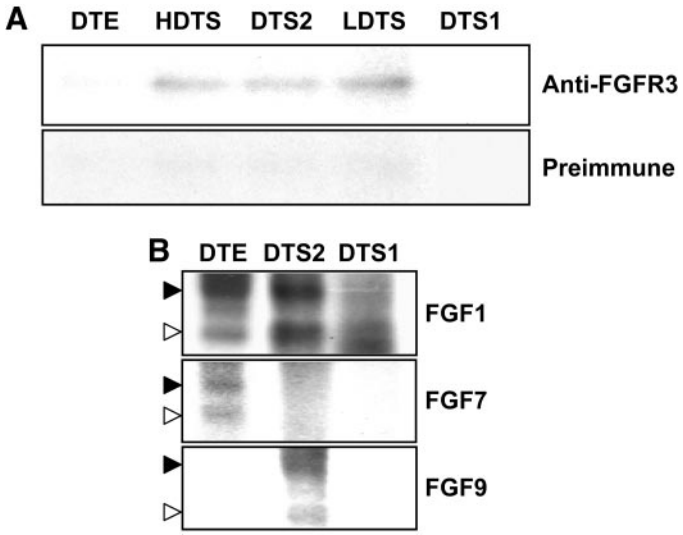

Fig. 4. Binding of fibroblast growth factor (*FGF*) 9 only to stromal cells expressing FGF receptor (*FGFR*) 3. *A*, immunoanalysis of FGF1-labeled complexes. Cultured Dunning R3327PAP (*DT*) tumor-derived epithelial (*DTE*) cells and DT tumor-derived stromal (*DTS*) cells were incubated with 2 ng/ml radiolabeled FGF1 in the presence of 2 μg/ml heparin for 1 h and cross-linked after sequentially washing with PBS, PBS containing 250 μg/ml heparin, and then PBS. The soluble fraction of lysate prepared by extraction with 1% Triton 100-X was incubated with protein A Sepharose beads and anti-FGFR3 serum or preimmune serum for 2 h at 4°C and subjected to SDS-PAGE and autoradiography. *B*, covalently labeled complexes with FGF1, FGF7, and FGF9. Cultured DTE and DTS cells were incubated with 2 ng/ml radiolabeled FGF1, FGF7, and FGF9 in the presence of 2 μg/ml heparin. The high-affinity binding was confirmed by cross-linking, followed by SDS-PAGE and autoradiography. *HDTS*, high-passage DTS; *DTS2*, FGFR3-positive DTS cell clone; *DTS1*, FGFR3-negative DTS cell clone; and *LDTS*, low-passage DTS. Isolation and characterization of clonal cell lines are described in Wu *et al.* (27). FGF1, FGF7, and FGF9 recombinant ligands have molecular weights of M_r 15,900, 16,300, and 21,000, respectively. The molecular weight and heterogeneity of mammalian FGFR isotypes varies depending on the ratio of splice variants expressed that impacts length of product and heterogeneity in glycosylation. The upper bands are in the range of molecular mass expected of full-length FGF-labeled FGFR isoforms. The lower bands result from proteolytic truncation of the intracellular domain (37). The (▲) and (△) symbols indicate a molecular mass of M_r 135,000 and 85,000, respectively, as estimated by molecular weight markers.

passage cultures displayed the strongest intensity. Clonal cultures of DTS2 cells that express FGFR3 exhibited a strong band, whereas the DTS1 clones that did not express FGFR3 mRNA even at trace levels probed by reverse transcription-PCR exhibited no immunoreactive material as expected. These results show that the FGF1 bound to DT stromal cells, with which FGF9 specifically competes, was, at least in part, bound to FGFR3. Moreover, it binds to a specific subfraction of stromal cells in which FGFR3 is expressed and fails to bind to cells devoid of FGFR3 despite the presence of FGFR1 and FGFR2 (27).

FGF ligands vary in affinity for abundant isolated pericellular heparan sulfate sites that vary from low-affinity electrostatic interactions to high-affinity interactions approaching that of the binary complex of rare heparan sulfate motifs and the ectodomain of the FGFR transmembrane kinase (1, 4, 34). To confirm that the FGF1 competition reflected FGF9 binding to specific high-affinity sites containing the FGFR transmembrane kinase, we radiolabeled purified recombinant FGF9 and compared resultant covalent affinity cross-linked complexes with those generated by radiolabeled FGF1 and FGF7 (Fig. 4*B*). As expected, radiolabeled FGF1 covalently cross-linked to DTE cells and both clonal types of DTS cells. FGF7 cross-linked only to DTE cells bearing FGFR2IIIb, whereas radiolabeled FGF9 only cross-linked to clonal DTS2 cells exhibiting expression of FGFR3 mRNA. Together, the results show that epithelial cell-specific FGF9 binds only to stromal cells exhibiting expression of FGFR3 mRNA and FGFR3 antigen that can cross-link to FGF1. This is despite the fact that DTS cell clones (DTS1) that do not express FGFR3 but express FGFR2IIIc and high levels of FGFR1 (27) that have been suggested to bind FGF9 in other contexts (6).

Stromal Cell Context Limits Specificity of FGF9 to FGFR3 Relative to Cell-Free Complexes of FGFR3 and Heparin. We have shown that the specificity of FGF isoforms for isotypes of the FGFR kinase ectodomain is cell-type specific and, in part, caused by the cell type-specific repertoire of heparan sulfate (4, 5, 10, 11). The specificity of FGF7 for the two isotypes of FGFR2 (IIIb and IIIc) that are distributed among prostate epithelial and stromal cells is cell-type specific and differs considerably from that of isolated complexes of the two recombinant isoforms in a binary complex with highly sulfated heparin *in vitro* (5, 10, 11). To determine whether stromal cell context limited the binding of FGF9 to FGFR3 in the presence of the other FGFR isotypes, we compared the whole cell binding results described previously with cell-free complexes of heparin and recombinant FGFR isoforms. FGFR isoforms with GST substituted for the intracellular domain were prepared in baculoviral-infected insect cells, extracted, and immobilized to GSH affinity matrix, and binary complexes then were prepared by absorption of FGFR-specific heparin to the immobilized FGFR-GST before introduction of radiolabeled FGF (4, 10, 11, 34). Similar to the whole cell experiments in Fig. 3, we first compared the competition of FGF9 with radiolabeled FGF1 binding with the preformed heparin-FGFR complexes. The depression of FGF1 binding by 50% to heparin-FGFR1, heparin-FGFR2IIIb, and heparin-FGFR4 complexes required >300 ng/ml FGF9, indicating a potentially low affinity of FGF9 for these complexes (Fig. 5, *A*, *B*, and *F*). Competition for complexes of heparin-FGFR2IIIc and heparin-FGFR3IIIb was significant with a half of maximum competition with FGF1 at ~40 ng/ml FGF9 (Fig. 5, *C* and *D*). Competition was strongest and near that of unlabeled FGF1 for complexes of heparin-FGFR3IIIc, indicating a preference of FGF9 for this FGFR isotype (Fig. 5*E*).

Covalent affinity cross-linking analysis generally confirmed the competition analysis, although, in some cases, covalent affinity cross-linking did not correlate precisely with the degree of competition with radiolabeled FGF1. This likely reflects subtle differences in the two assays described in "Experimental Procedures" and the possibility that the binding sites of FGF1 and other FGFs may overlap where there is detectable competition but are not identical in respect to cross-linkable neighbor groups. As expected, the preformed complexes of FGFR-extracted heparin and all of the FGFR isotypes formed detectable complexes with radiolabeled FGF1 (Fig. 6). No covalently cross-linked bands could be observed in binding assays containing GSH beads in which FGFR constructs were omitted or beads were incubated with untransfected cell extracts (not shown). As reported previously, FGF7 bound FGFR2IIIb and FGFR2IIIc but with preference for FGFR2IIIb (5, 10, 11). FGF7 surprisingly cross-linked significantly to complexes of FGFR3IIIb and FGFR3IIIc but, as expected, essentially failed to bind preformed complexes of heparin-FGFR1IIIc and heparin-FGFR4. Fig. 6 confirms that FGF9 binds best to heparin-FGFR2IIIc, heparin-FGFR3IIIc, and, to a lesser extent, heparin-FGFR2IIIb, heparin-FGFR3IIIb, and heparin-FGFR4. These results show that isolated cell-free complexes of FGFR isotypes loaded with FGFR-affinity extracted heparin before introduction of FGF are remarkably less specific than the same FGFR isotype in the context of the prostate epithelial and stromal cells. This strongly suggests that cell type-specific membrane context, most likely the repertoire of cell-specific heparan sulfate, confers ultimate directional specificity of epithelial FGF9 for FGFR3-expressing stromal cells.

Functional Interaction of Specific Binding of FGF9 to Stromal Cells Expressing FGFR3. To confirm that the specific binding of FGF9 to stromal cells correlated with function, we compared the ability of FGF1, FGF7, and FGF9 to stimulate DNA synthesis in DTS

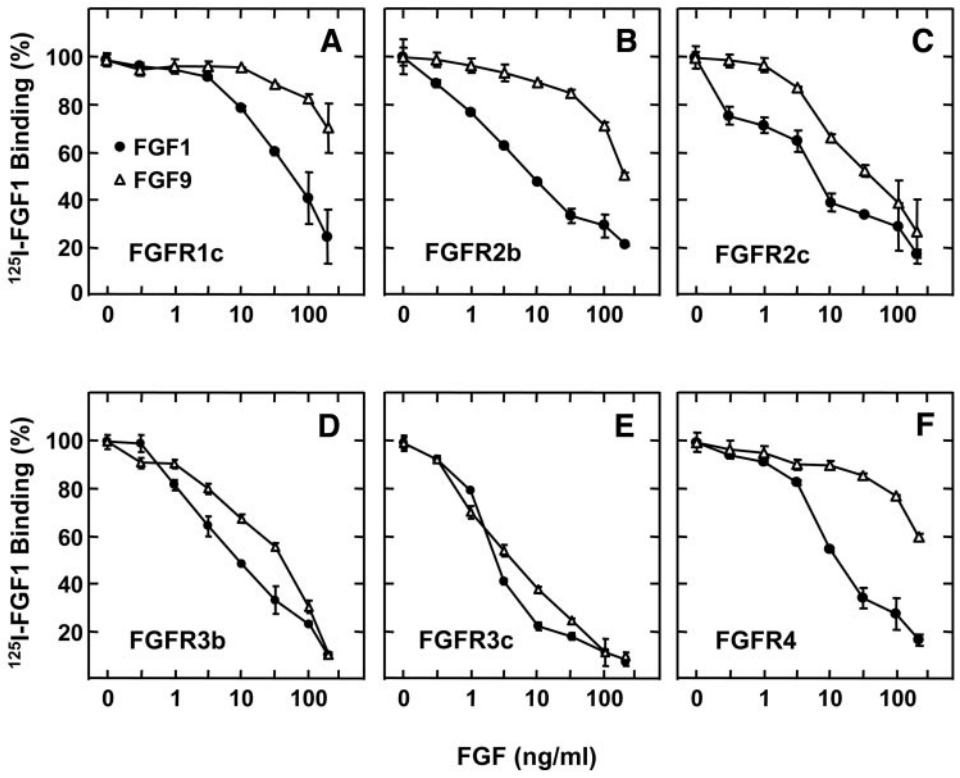

Fig. 5. Competition binding of fibroblast growth factor (*FGF*) 9 to isolated binary complexes of heparin and the four recombinant isotypes of FGF receptors. The indicated recombinant FGF receptors (*FGFR*s) were expressed as glutathione *S*-transferase fusion proteins in baculoviral-infected Sf9 insect cells. The FGFRs were immobilized on GSH Sepharose beads (Amersham Pharmacia Biotech) and specific unliganded binary heparin-FGFR complexes prepared by incubation with 10 μg/ml heparin. After removal of unbound heparin by extensive washing, the immobilized FGFRs were further incubated with 1 ng/ml ^{125}I-FGF1, and the indicated amount of unlabeled FGF1 or FGF9 was added. The bound FGF radioactivity was extracted with SDS-sample buffer and counted. Data points are the mean ± SD of triplicate determinations.

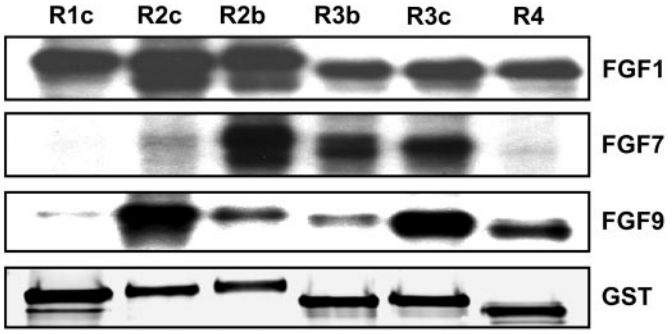

Fig. 6. Covalent affinity cross-linking of radiolabeled fibroblast growth factor (*FGF*) 1, FGF7, and FGF9 to heparin-FGF receptor (*FGFR*) complexes. The indicated recombinant FGFR isotype was expressed as the glutathione *S*-transferase (*GST*) fusion protein in Sf9 insect cells. FGFR was isolated, and binding of the indicated radiolabeled FGF to immobilized binary complexes heparin-FGFR was analyzed as in Fig. 5. Amount of different GST-FGFR was standardized by indirect immunoanalysis of electrophoretic blots with anti-GST antibody (*bottom*).

cells that exhibit FGFR3IIIc (Fig. 7*A*) and DTE cells that express only FGFR2IIIb (Fig. 7*B*). Consistent with the specificity of cross-linkable binding sites, FGF1 and epithelial FGF9 stimulated DNA synthesis of DTS cells, whereas stromal FGF7, which fails to cross-link to FGFR3 and acts specifically on epithelial cells, had no effect on DTS cells. FGF1 and stromal FGF7 stimulated DNA synthesis of DTE cells, whereas epithelial FGF9 had no effect on DTE cells. We reported previously that FGF9 stimulates DNA synthesis of only the subset of cells in the DTS cultures that express FGFR3 (DTS2 cells), whereas DTS1 cells devoid of FGFR3 but expressing FGFR1 failed to respond (27). Moreover, the functional interaction of FGF9 with FGFR3 on DTS2 cells is not limited to short-term stimulation of DNA synthesis but sustains an increase in cell number in serum-free medium for >1 week (27). Together, these results confirm that the specific interaction of FGF9 with authentic FGFR kinase sites on specifically DTS cells expressing FGFR3 strongly correlates with a proliferative response.

DISCUSSION

Directionally Specific Communication from Epithelium to Stroma via FGF9 and FGFR3. We used pure cloned cultures of epithelial and stromal cells from the well-characterized transplantable two-compartment Dunning R3327 model of differentiated premalignant prostate cancer (7, 8, 25, 26, 33) to show that the FGF9 and FGFR3IIIc pair fulfills the criteria for a directionally specific paracrine signaling system from epithelial to stromal cells. This bypassed complications caused by cellular cross-contamination and heterogeneity of intact prostate tissues and primary cultures. This model, in which cloned cell types can be identified then mixed and two-compartment tumors observed and recultured, is unique for study of stromal-epithelial interactions because stromal cells prevent progression of epithelial cells to malignancy *in vivo*. Moreover, although stromal cells do not have malignant potential, they depend on epithe-

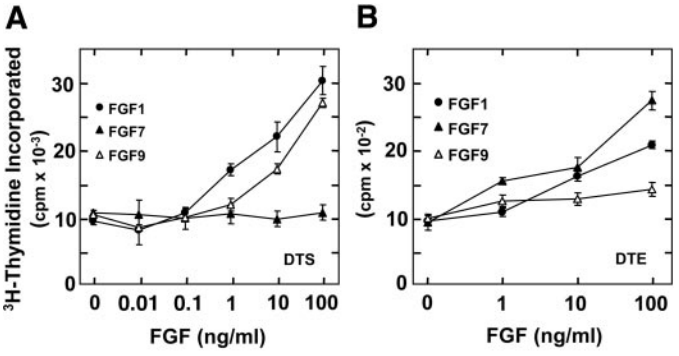

Fig. 7. Effect of fibroblast growth factor (*FGF*) 9 on DNA synthesis of specifically stromal cells. Analysis of DNA synthesis was determined in uncloned Dunning R3327PAP (*DT*) tumor-derived stromal (*DTS*; *A*) and DT tumor-derived epithelial (*DTE*; *B*) cell cultures as described in "Experimental Procedures." The indicated amount of recombinant FGF1, FGF7, and FGF9 was added to medium containing 2 μg/ml heparin. Data points are the mean ± SD of triplicate determinations.

lial cells for survival *in vivo*. Our results show that first, FGF9 expression appears limited to epithelial cells from the premalignant tumor and its derived malignant tumor cells. Assessed by competition binding or direct covalent affinity cross-linking, epithelial cell-derived FGF9 exhibits no potential autocrine activity for FGFR on epithelial cells. Second, FGF9 binds only to stromal cells derived from the premalignant tumor. Third, FGF9 binds only to stromal cell subpopulations that express FGFR3, even though stromal cells express other isotypes of FGFR (27). FGF9 appeared specific for binding to FGFR3 on stromal cells even in cells that express FGFR1 and FGFR2IIIc mRNA at significant levels assessed by RPA (27). This was despite the fact that FGF9 binds FGFR2IIIc with high affinity in transfected leukemia cells (6) or to soluble, isolated FGFR2IIIc ectodomain either in the presence of soluble heparin (18) or as a binary complex of FGFR and FGFR-affinity purified heparin (our current results). Either the specific stromal cell context prevents the binding of FGF9 to FGFR2IIIc because of cell- and FGFR-specific heparan sulfate or the indicated level of FGFR2IIIc mRNA is insufficient to produce adequate levels of cell surface FGFR2IIIc (4, 5). The fact that stromal cell clones devoid of FGFR3 expressing FGFR2IIIc and low levels of FGFR1 bind FGF1 and FGF2 argues against the latter (27). We propose that stromal cell context, potentially stromal cell-specific heparan sulfate, may prevent the binding of FGF9 to FGFR2IIIc. Stromal cell-specific heparan sulfate prevents binding of FGF7 to FGFR2IIIc, thus preventing autocrine activity from stromal-derived FGF7 (5). Our results concur with those of Hecht *et al.* (18), who found that FGFR1 has little, if any, affinity for FGF9 under cell-free conditions and in the context of the stromal cells examined here. Despite the presence of trace amounts, levels of FGFR3 mRNA predicted to be necessary for significant expression of cell surface FGFR3 could not be demonstrated in epithelial cells. These combined features suggest that epithelial cell-derived FGF9 may not only be directionally specific toward stromal cells but also that FGFR3 and specific stromal cell subtypes that express FGFR3 receive and mediate the epithelial-derived FGF9 signal to stroma.

Role of the FGF9-FGFR3 Axis in Stromal-Epithelial Homeostasis and Progression to Malignancy. The partitioning of FGF9 and FGFR3 between epithelium and specific stromal cell types, respectively, and the specificity of FGF9 for interaction with only FGFR3 in tissue context complete a two-way directionally specific communication loop between stroma and epithelium within the FGF family (Fig. 8). Both FGF/FGFR pairs constitute specific paracrine signals in opposite directions for which there is no subversion by autocrine loops. The stromal to epithelial part of the loop mediated by stromal FGF7/FGF10 and epithelial FGFR2IIIb and the contribution by the FGF7 or FGF10 isotype, FGFR2, and heparan sulfate to one arm of the directional specificity have been well documented (5, 7, 8, 11, 12, 24, 33). Although the cellular and molecular mechanistic details remain to be worked out, the net effect of FGF7/FGF10 to FGFR2IIIb signaling in epithelial cells in the prostate and other parenchymal epithelium is maintenance of homeostasis. This includes balanced population growth, morphogenesis, differentiation, and function. Loss of FGFR2 in epithelial cells concurrent with the ectopic activation of stromal resident FGFR1 abrogates homeostasis and correlates with stromal independence and progression to malignancy of epithelial cells (12, 33, 35). The net role of the FGF9 to FGFR3 axis on the character of the stromal and overall tissue homeostasis and its abrogation leading to malignancy remains to be established. Clearly, we show here that the interaction of epithelial FGF9 with stromal cells expressing FGFR3 correlates strongly with DNA synthesis and cell proliferation *in vitro*. We showed that it is stromal cells exhibiting smooth muscle cell-like character that bear FGFR3 and exhibit a proliferative response to FGF9 concurrent with further depression of α-smooth muscle cell actin (27). Whether FGF9 plays a net homeostatic role in promoting growth limits, apoptosis, and differentiation similar to FGF7/FGF10 on epithelial cells in addition to expansion of cell number is unclear. FGF9-responsive clonal lines exhibiting FGFR3 have properties described by Rowley *et al.* (36) for "activated stromal cells" that are thought to be related to smooth muscle cells as a common precursor or a dedifferentiated product (27). Chronically activated stroma has been proposed to contribute to the breakdown in tissue homeostasis leading to epithelial cell malignancy (36). Our results show that FGF9 expression is highest in malignant epithelial cell tumors deficient in differentiated stroma and in tumor-derived epithelial cells freed of tissue context in culture. The apparently low level of FGF9 expression in two-compartment normal and nonmalignant tumor tissues may, in part, reflect dilution of the FGF9 mRNA signal by presence of extensive stroma. However, epithelial cell FGF9 expression may be repressed in the well-differentiated, two-compartment tissues (normal and nonmalignant tumors) potentially via intercommunication between stroma and epithelium. The mutual regulation of activity of cell-specific FGF in one compartment and the cell-specific FGFR response in the other, as well as the role of androgen on each, are under investigation.

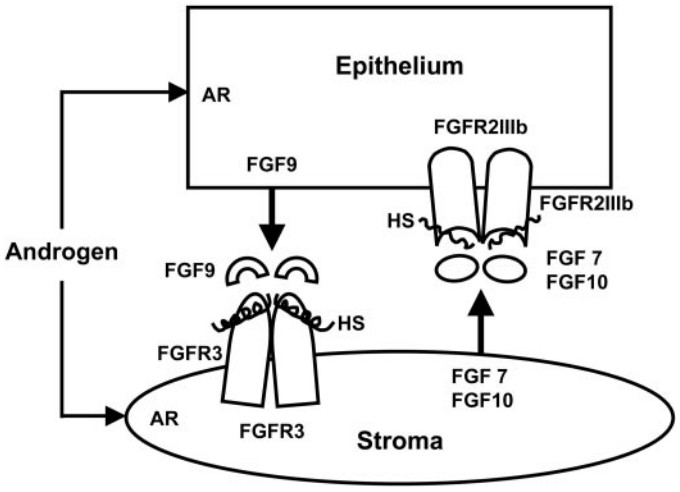

Fig. 8. Two-way directional communication between stroma and epithelium within the fibroblast growth factor (*FGF*) family. Directionally specific communication is achieved by partitioning of the FGF signal, a specific FGF receptor (*FGFR*), and FGF/FGFR-specific heparan sulfate oligosaccharide motif between epithelial and stromal cells. Stromal FGF7/FGF10 and epithelial FGFR2IIIb mediate stromal to epithelial signaling, whereas epithelial FGF9 signaling to stromal FGFR3 completes the two-way conversation. Disruption of the two-way communication may occur at the signal or reception level in either compartment to upset tissue homeostasis. Androgen may differentially modify either the signal or reception in either compartment. Either partitioned FGFR signal may affect reciprocal cross-talk back to the other compartment. *AR*, androgen receptor; *HS*, cell- and FGFR-specific heparan sulfate (*wavy line*).

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