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Fibroblast Growth Factor Receptor 1 Phosphotyrosine 766: Molecular Target for Prevention of Progression of Prostate Tumors to Malignancy[1]

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ABSTRACT

Dissection of processes that promote the slow progression to malignancy from those that drive the malignant phenotype, once acquired, is important for identification of molecular targets for rational design of dietary and pharmaceutical intervention to hold premalignant cancer in check. In adult parenchymal organs, fibroblast growth factor receptor (FGFR) kinase isotypes are partitioned between stroma and epithelium, respectively, and mediate communication between the two compartments to maintain organ homeostasis. The ectopic appearance of stromal FGFR1 is a hallmark of epithelial cells from model transplantable rat prostate tumors that progress to malignancy. Here we show that, despite the fact that it is transcriptionally active, the appearance of FGFR1 in nonmalignant prostate tumor epithelial cells at first does not drive cell proliferation or support a malignant phenotype. These properties develop over time with proliferative aging of the cell population coincident with FGFR1-dependent activation of the mitogen-activated protein kinase signaling pathway. Phospholipase Cγ-interactive phosphotyrosine 766 of FGFR1 is required for the age-dependent acquisition of the proliferative response to FGFR1, although it appears not to be required for the mitogenic response. Although of little utility in late-stage therapy, this suggests that pathways linked to FGFR1 tyrosine 766 may be specific targets for prevention of progression of latent nonmalignant tumors to the life-threatening malignant state.

INTRODUCTION

The prevention of progression to life-threatening malignancy by dietary or pharmaceutical intervention that holds relatively benign asymptomatic cancers in check is receiving increasing attention as an alternative to complete prevention of initiation and cure. In addition to early detection, the design of strategies for prevention requires dissection of mechanisms underlying the age-dependent slow progression of asymptomatic cancers to life-threatening malignancies from those that drive the malignant phenotype. The transplantable Dunning R3327 rat prostate (DT3) tumor is nonmalignant, grows slowly, and, like normal prostate, is androgen responsive (1). The gradual progression of the DT3 tumor or its isolated epithelial cells from a manageable, relatively benign dormant phenotype to the life-threatening malignant phenotype resembles that of human prostate tumors and their slow progression to malignancy. Previously, we showed that during the progression, which requires 8–12 months *in vivo*, the resident epithelial cell FGFR2IIIb[3] isoform switches to the FGFR2IIIc isotype, which disrupts directional regulatory instructions from the stroma to the epithelium and possibly back to the stroma (2, 3). Expression of the *FGFR2* gene is lost altogether in 30% of cells concurrent with abnormal activation of the *FGFR1* gene in 100% of cells that comprise the malignant cell population (2). FGFR1 is normally expressed only in stromal cells. Forced ectopic expression of FGFR1 in nonmalignant DTE cells by transfection accelerates the process, but progression to malignancy still requires 6–8 months *in vivo* (2). This suggests that simply the ectopic expression of FGFR1 in the nonmalignant epithelial cells is insufficient to support malignancy and that the connection of abnormally expressed FGFR1 to the reception mechanism, which promotes malignancy, requires time to develop.

Four homologous genes encode an extensive repertoire of alternately spliced isoforms of FGFR transmembrane tyrosine kinase that vary in the extracellular ligand-binding and intracellular tyrosine kinase domains (4). The expression of FGFR isoforms is temporally and spatially specific in embryos and cell specific in adult organs. Abnormal expression and activation of FGFR tyrosine kinase due to mutations or aberrant expression of FGF and pericellular matrix heparan sulfate proteoglycan cause development disorders and are involved in various adult tissue-specific pathologies, including malignancy (2, 4). Currently, the determinants of signal specificity among FGFR subtypes are unknown. The activation of elements of the MAP kinase signal transduction pathway has been implicated in most FGFR1 responses studied to date including mitogenesis (5), mesoderm induction (6), and neuronal differentiation (7). Phosphorylation of one or more membrane-anchored SNT (also called FRS2 for FGFR substrate 2) by the FGFR1 kinase recruits and activates the GRB2/SOS1 complex, which then interacts with *ras* to activate the MAP kinase pathway (8, 9). Derepression of the FGFR1 kinase by autophosphorylation of one or two tyrosines (Tyr^{653} and/or Tyr^{654}) in an active site repressor domain is required (5, 10, 11). Pathways initiated by PLCγ are also activated upon activation of the FGFR1 complex (12, 13). This occurs by autophosphorylation of Tyr^{766} in the COOH terminus, which recruits PLCγ to the FGFR1 kinase for activation and elicits phosphoinositol hydrolysis and metabolism, protein kinase C activation, and Ca^{2+} mobilization (12, 13). However, Tyr^{766}, the activation of PLCγ, phosphatidylinositol turnover, and calcium flux appear dispensable for FGFR1-elicited cellular responses described to date, which include mitogenesis (11–13), neuronal differentiation (7), mesoderm induction (14), induction of urokinase-type plasminogen activator (15), and chemotaxis (16).

Here we show that the ectopic FGFR1 signaling complex is transcriptionally active in nonmalignant prostate tumor epithelial cells, despite the fact that it fails to elicit proliferation of the cell population. A proliferative response coincident with activation of the *ras*-dependent MAP kinase signaling pathway and acceleration of the progression to malignancy only appears with proliferative aging of the cell population expressing FGFR1. PLCγ-interactive phosphotyrosine 766 is required for the age-dependent acquisition of the proliferative response to FGFR1, although it does not appear necessary for FGFR1-mediated mitogenesis once it is acquired. These results suggest that the phosphorylation of FGFR1 tyrosine 766 and the downstream pathways linked to it may be a specific molecular target for prevention

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[3] The abbreviations used are: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; PD, population doubling; SNT, SUC1-associated neurotrophic factor target proteins; PLC, phospholipase C; FiRE, FGF-inducible response element; ERK, extracellular-regulated kinase; MAP, mitogen-activated protein.

of the age-dependant progression of relatively benign dormant cancer to malignancy.

MATERIALS AND METHODS

Reagents. The mouse monoclonal antiphosphotyrosine antibody 4G10, antiphosphorylated ERK1/2, and anti-PLCγ antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). All oligonucleotides were customer ordered from Integrated DNA Technology, Inc. (Coralville, IA). All radioactive isotopes were purchased from DuPont NEN Life Science Products (Boston, MA). Luciferase assay reagents were from Promega (Madison, WI). Male Copenhagen rats were from Harlan Sprague Dawley (Houston, TX).

FGFR Constructions. Tyrosines 653/654 or 766 were replaced with phenylalanine to yield mutant constructs FGFR1Y653/654F and FGFR1Y766F, respectively. A mutant FGFR1K425A in which the essential lysine 425 in the ATP binding site was replaced with alanine was also prepared. Mutants were generated by PCR-mediated site-directed mutagenesis with the FGFR1β1 template, a pair of complementary mutation primers, and both forward and reverse flanking primers (17, 18). The forward primer p766-1 (AACCAGGAGTTCCTGGACCTG) and reverse primer p766-2 (CAGGTCCAGGAACTCCTGGTT) were used for FGFR1Y766F, p653-1 (CACCACATCGACTTCTTTAAAAAGACAACC) and p653-2 (GGTTGTCTTTTTAAAGAAGTCGATGTGGTG) were used for FGFR1Y653–4/F, and p425-1 (GTGGCTGTGGCGATGTTGAAG) and p425-2 (CTTCAACATCGCCACAGCCAC) were used for FGFR1K425A. The PCR fragments were ligated with the FGFR1 flanking sequences and cloned in SK vector. The sequence of PCR fragments and the sequences across the ligation were determined. The full-length coding sequences were then digested with *Bam*HI and cloned into mammalian expression vector pcDNA1neo. Full-length cDNA for human SNT1 was amplified by RT-PCR from a human HepG2 RNA pool with forward primer snt1a (TCACATGGGCAGATCAGTACTATT) and reverse primer snt1b (AAGCCATGGGTAGCTGTTGTAGCT). The cDNA coding for mutant SNT1-his was PCR-amplified with forward primer snt1a, reverse primer snt1hisb (CCCTTAGTGATGGTGGTGATGATGCATGGGCAGATCAGTACTATT), and SNT1 cDNA template. The PCR fragment was cloned in SK vector for sequence verification and then transferred to pcDNAneo1 for expression in mammalian cells.

Prostate Cells and Tumors. Lines of DTE cells were maintained, transfected, and selected for the desired levels of transfected FGFR variants as described previously (2, 3). Cell lines that exhibited reduced levels of FGFR2 were used to reduce the opposing effects of FGFR2 (2, 19) and to accelerate the impact of ectopic FGFR1 on progression to malignancy. After selection with Geneticin, the indicated DTE cell lines were screened for the expression of ectopic FGFR1 by interaction with ^{125}I-labeled FGF-2. The bound FGF was cross-linked to FGFR with disuccinimidyl suberate (DSS), and the Triton X-100 extract was separated on SDS-PAGE and detected by autoradiography. The cells were then serially cultured for the indicated number of population doublings. R1-2, R1-3, and so forth (the number after the hyphen indicates DTE cells that were serially cultured for 2, 3, etc. PDs after selection). R1-100 indicates cells that had undergone at least 100 PDs, after which the precise number was not tracked. The DTE cells were implanted s.c. in syngenic male rats, and the rats were examined for tumors biweekly.

FiRE Reporter and Luciferase Activity. The 1.1-kb mouse syndecan I promoter (20) was inserted into the pGL2-Basic vector (Promega) at the *Hin*dIII and *Bgl*II sites, and the 280-bp far upstream FiRE enhancer element from the mouse syndecan 1 gene (20) was inserted at the *Bam*HI and *Xho*I/*Sal*I sites. The DTE cells were transfected with pGL3-SFiRE DNA, incubated at 37°C for 4 h, and distributed into 24-well plates in RD medium containing 10 μg/ml heparin at 37°C overnight. The medium was then changed to RD medium containing 2 μg/ml heparin and 2 ng/ml of the indicated FGF. After incubation overnight at 37°C, the cells were lysed, the cell lysates were transferred to 96-well plates and mixed with 25 μl of luciferase substrate, and the light intensity was measured with a microplate scintillation counter (Packard, Meriden, CT).

Substrate Phosphorylation. Cells (5×10^5) were incubated overnight in 6-well plates with serum-free medium and 10 μg/ml heparin. Then, FGF (50 ng/ml) was added for 30 min at 37°C, and the cells were lysed with 1% Triton X-100-PBS. Phosphorylated SNT1 was affinity-purified with ^{Suc13}P-Agarose beads and detected with antiphosphotyrosine antibody 4G10. Phosphorylated ERK1 (M_r 42,000) and ERK2 (M_r 44,000) in the lysates were detected with anti-phosphorylated ERK antibody.

RESULTS

Mitogenically Inactive Ectopic FGFR1 Is Transcriptionally Active Independent of Tyr^{766} in Premalignant Prostate Tumor Cells (DTE). Epithelial cells (DTE) from nonmalignant rat prostate tumors, which are devoid of detectable FGFR1 (2, 3), were forced by transfection to express 20–30 times more wild-type or mutant ectopic FGFR1 than the low level of resident FGFR2IIIb (Ref. 2; Fig. 1*A*). FGF-dependent autophosphorylation of the ectopic wild-type FGFR1 was apparent as soon as the transfected population was available for analysis at about 3 (R1-3) to 4 (R1-4) doublings after transfection (Fig. 1*B*). FGF-dependent activation of the FiRE from the mouse syndecan 1 gene (20) was similarly observed (Fig. 1*C*; Fig. 2*A*). FGF2 specifically activates the ectopic FGFR1, but not endogenous FGFR2IIIb. This transcriptional activity of transfected FGFR1 required the active kinase and intact tyrosines 653/654, but not tyrosine 766 (Figs. 1*C* and 2*A*). The FGFR1-mediated, Tyr^{766}-independent activation of FiRE was also evident in DTE cells only 3–4 PDs after transfection with FGFR1 and persisted indefinitely through >100 PDs of the transfected cell populations (Fig. 2*A*). These results indicated that ectopic FGFR1 in the epithelial cells was capable of high affinity FGF binding, FGF-activated autophosphorylation, and was transcriptionally active. The transcriptional activity was independent on FGFR1 Tyr^{766}.

Acquisition of the Proliferative Response to FGFR1 Requires Proliferative Aging of the DTE Cell Population in the Presence of FGFR1 Tyr^{766}. Fig. 2*B* confirmed that transcriptionally active ectopic FGFR1 failed to mediate a proliferation response in the nonmalignant R1-3 DTE cell population (Ref. 2; Fig. 2*B*). The population began to exhibit a growth response to FGF1 and FGF2 only after proliferative aging by serial culture for a minimum of 50 PDs. After about 100 doublings of the population (R1-100), the growth response reached a maximum, and the population (and cloned cell populations developed from it) remained responsive indefinitely (Fig. 2*B*). Similar to untransfected cells, the DTE cell population transfected with the mutant FGFR1Y766F devoid of Tyr^{766} (and the Y653/654F and K425A mutants, which are not shown) remained unresponsive indefinitely (Fig. 2*B*). DTE cells that acquired the growth response to ectopic FGFR1 during proliferative aging by serial culture (R1-100) exhibited an accelerated rate of progression to malignancy *in vivo* (Ref. 2; Fig. 2*B*, *inset*). Rats injected with untransfected DTE cells and cells transfected with the FGFR1Y766F and FGFR1K425A after culture in excess of 100 PDs exhibited no palpable tumors in the same time frame. Thus, both the proliferative response and the accompanying increase in malignant potential are an acquired property requiring intact Tyr^{766}, although ectopic FGFR1 is fully active in the epithelial cell context with respect to FGFR1 Tyr^{766}-independent transcriptional activity.

The Acquired Proliferative Response to FGFR1 Kinase Is Independent of Tyr^{766}. We then attempted to distinguish between the structural requirements for the acquisition of the growth response to ectopic FGFR1 and the acquired growth response mediated by the FGFR1 kinase in FGFR1-transfected cells (DTE/R1-100). Chimeric constructions were prepared comprised of the FGFR2IIIb ectodomain, which is specifically recognized and activated by FGF7, and the FGFR1 intracellular kinase domain. In experiments not shown here, the constructs were validated in mouse 3T3 fibroblasts. Mouse fibroblasts, in which FGFR1 is the resident FGFR isotype, expressed no FGFR2IIIb (21). Transfection of 3T3 cells with the FGFR2IIIb/R1 chimera resulted in an immediate activation of FiRE (20), anchorage-

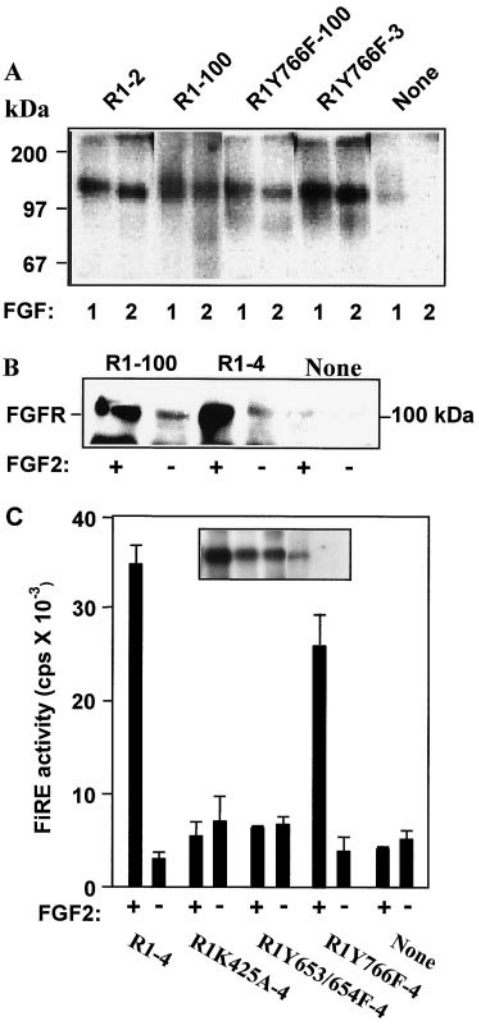

Fig. 1. Ectopic FGFR1 expressed in premalignant prostate tumor epithelial cells binds FGF2, exhibits FGF-stimulated autophosphorylation, and activates transcription but not DNA synthesis. *A*, covalently cross-linked complexes of ^{125}I-labeled FGF bound to DTE cell lines transfected with the indicated construct and subsequently cultured for the indicated number of PDs. For example, R1-3 indicates cells analyzed after 3 PDs, and R1-100 indicates cells analyzed 100 PDs or more after transfection. *R1*, wild-type FGFR1; *R1Y766F*, FGFR1 with Tyr^{766} mutated to phenylalanine; *None*, untransfected DTE cells; *Lanes 1*, FGF1; *Lanes 2*, FGF2. *B*, autophosphorylation of ectopic FGFR1 in DTE cells. DTE cells were transfected with the indicated constructs of FGFR1 and serially cultured for the indicated number of PDs. Cultures were placed in serum-free medium for 2 days, and FGF2 (50 ng/ml) was added, where indicated, for 30 min at 37°C. The cells were then lysed, the FGFR1 was immobilized on protein A-Sepharose beads bearing anti-FGFR1 antiserum A40 (34), and the phosphorylated FGFR1 was detected with antiphosphotyrosine antibody 4G10. *C*, activation of FiRE by FGFR1 requires an active kinase and Tyr^{653}/Tyr^{654}, but not Tyr^{766}. DTE cells (1×10^6) expressing the indicated constructions 4 PDs after selection were transfected with the FiRE-luciferase reporter DNA, and the luciferase activity was analyzed as described in "Materials and Methods" and expressed as counts/s (*cps*). The mean and variance between duplicates are shown. Constructions are those described in *A* and R1K425A-4, a kinase-defective FGFR1 with lysine 425 mutated to alanine, and R1Y653/654F-4, a FGFR1 with both Tyr^{653} and Tyr^{654} mutated to phenylalanine. *Inset*, expression of FGFR1 constructions assessed by covalent affinity cross-linking with ^{125}I-labeled FGF2. The indicated lanes (*left* to *right*) correspond to DTE cells transfected with the four constructs and untransfected cells (*None*) indicated at the *bottom* of *C*.

independent growth in soft agar, and increases in phosphorylation of both SNT and ERK. All activities were independent of an intact Tyr^{766} in the FGFR1 kinase domain. In marked contrast, DTE cells transfected with the chimeric FGFR2IIIb/R1Y766F failed to exhibit a growth response (Fig. 3*A*), just as they failed to exhibit a growth response when transfected with wild-type FGFR1 or FGFR1Y766F (Fig. 2*B*). As illustrated in Fig. 2*B*, DTE/R1-100 cells, which have acquired the growth response to ectopic FGFR1 by serial passage in culture, exhibited a robust response to FGF1 and FGF2 through the transfected FGFR1. The weak response to FGF7 reflects the low level of resident FGFR2IIIb that recognizes only FGF1 and FGF7. However, when the DTE/R1-100 cells were secondarily transfected with the chimeric construct FGFR2IIIb/R1Y766F, the response to FGF7 was clearly increased (Fig. 3*A*). The FGF7-dependent phosphorylation of ERK1/2 and SNT1 was also immediate and robust in the FGFR2IIIb/R1Y766F-transfected DTE/R1-100 cells (Fig. 3*A*, *inset*, *middle* and *right panels*). These results suggest that Tyr^{766} is essential for neither the mitogenic activity of the FGFR1 kinase nor the accompanying activation of ERK1/2 and SNT1 once the cell population has become responsive. We cannot eliminate the possibility that wild-type FGFR1 Tyr^{766} contributes to the response through transactivation by the transfected chimeric FGFR2/R1Y766F. However, Tyr^{766} has been dissociated from the mitogenic response in all other FGFR1-responsive cells to date, and this would be the first exception (11, 16). Current models backed by structural data suggest that the oligomeric FGFR complex is a symmetric dimer with stoichiometry of two FGFR kinase subunits, two heparan sulfate chains, and two FGF ligands (4, 22–27). Each of the two FGF ligands contacts the ectodomain of both FGFR kinase subunits. This argues against symmetry-breaking heterodimerization of different FGFR isotypes with respect to the ectodomain, particularly the FGFR1-FGFR2IIIb heterodimer, in which FGF7 cannot bind to the FGFR1 subunit. Separate experiments not shown here also indicated that transfection of DTE/R1-100 cells with full-length FGFR2IIIb yielded no response to FGF7.

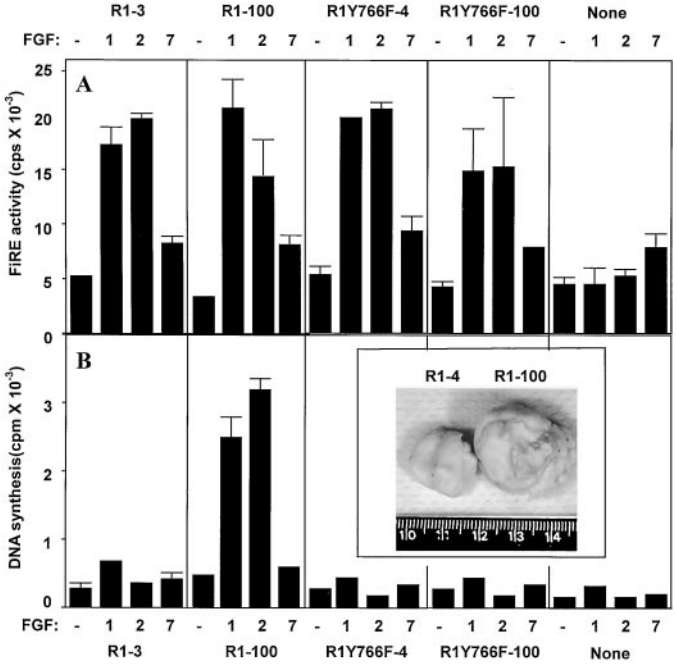

Fig. 2. FGFR1-stimulated DNA synthesis and accelerated tumorigenicity require proliferative aging and Tyr^{766} in DTE cells. *A*, immediate activation of FiRE by FGFR1 independent of Tyr^{766}. The indicated DTE cells were transiently transfected with the FiRE-luciferase reporter DNA. The cells were then treated with the indicated FGF overnight, and the luciferase activity was analyzed as described in Fig. 1*C* and "Materials and Methods" at the indicated number of PDs after transfection. Constructions are those indicated in Fig. 1. *B*, Tyr^{766} is required for acquisition of the proliferative response to ectopic FGFR1 in DTE cells. Incorporation of [^{3}H]thymidine in response to FGF was determined in the indicated types of DTE cells (2, 19). Data are the mean and variance of duplicate samples. *Inset*, accelerated tumorigenesis in FGFR1-expressing DTE cells after proliferative aging *in vitro*. DTE cells (5×10^6 cells/site) expressing ectopic FGFR1 were implanted s.c. into the flank of male Copenhagen rats as described in "Materials and Methods." Tumors were detectable at 3–4 months and excised for analysis after 5 months. The indicated tumors are representative samples from four different animals. *R1-4*, from DTE cells expressing FGFR1 after 4 PDs, average weight = 2.1 ± 0.83 g (n = 4); *R1-100*, from the same cells after >100 doublings, average weight of 7.5 ± 1.9 g (n = 4).

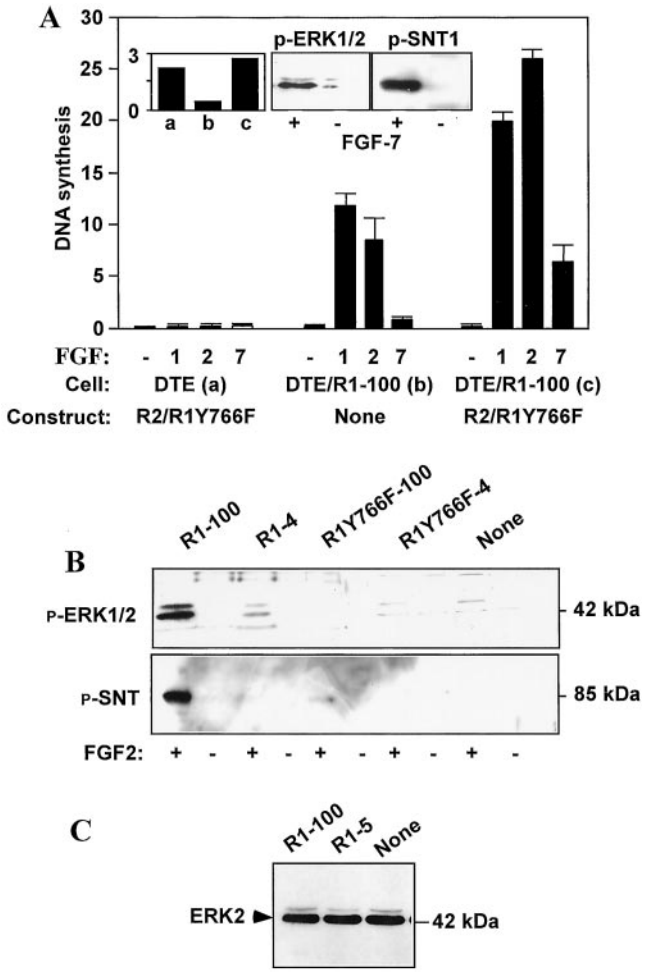

Fig. 3. Similar requirements for acquisition of FGFR1-dependent activation of DNA synthesis, ERK, and SNT in DTE cells. *A*, lack of requirement for Tyr^{766} for the acquired response to FGFR1 in DTE cells after proliferative aging. Untransfected DTE cells or DTE cells expressing ectopic full-length FGFR1 that had been serially cultured for >100 PDs were transfected with cDNA coding for the chimeric receptor FGFR2IIIb/R1Y766F, where indicated, and selected for an 8–10-fold increase in ability to bind FGF7 specifically (*inset, left panel*). After serial culture for 4 PDs after selection, DNA synthesis was determined as described previously (2, 19) and expressed as the fold stimulation elicited by the addition of FGF1, FGF2, or FGF7, as indicated. Data are the mean and variance of duplicates. *Inset: left panel*, expression of chimeric receptors R2/R1Y766F in the indicated DTE cells (*a–c*) assessed by binding of ^{125}I-FGF7. Data are expressed in cpm/10 cells and are the mean of duplicates. *Inset, middle* and *right panels*, immediate activation of SNT and ERK in R1-100 DTE cells. FGF7 was added as indicated to the DTE/R1-100 cells transfected with the R2/R1Y766F chimeric receptor. Phosphorylated ERK1, ERK2, and SNT1 were analyzed as described in "Materials and Methods." *B*, requirement for Tyr^{766} for acquisition of FGFR1-dependent activation of SNT1 and ERK1/2 in DTE cells. DTE cells expressing the indicated constructions for the indicated number of PDs after selection were analyzed as described in "Materials and Methods." The indicated bands are the phosphorylated (activated) forms of ERK1/2 and SNT1. *None* indicates untransfected cells. FGF2 was added where indicated. *C*, constitutive expression of ERK2 in DTE cells during proliferative aging *in vitro*. The lysates of the indicated cell lines were electroblotted on nylon membranes, and then ERK2 was detected with anti-ERK2 antibody (Upstate Biotechnology, Inc.). The lower band at about M_r 42,000 is unphosphorylated ERK2, and the upper band at about M_r 44,000 is the phosphorylated form.

Activation of the MAP Kinase Signaling Pathway is Coincident with Tyr^{766}-dependent Acquisition of the Proliferative Response to FGFR1. We then determined whether phosphorylation of SNT1, ERK, and PLCγ correlated with the mitogenically silent transcriptional activity of the ectopic FGFR1 or the acquired proliferative response to it. In fibroblast-like 3T3 cells that were used as a stromal cell control prototype (data not shown) and in DTE/R1-100 cells that have undergone more than 100 PDs since transfection with wild-type FGFR1 (Fig. 3*A*), the increased phosphorylation of both SNT and ERK was observed immediately after transfection of a FGFR construct bearing the FGFR1 intracellular kinase domain. Phosphorylation of SNT and ERK was independent on Tyr^{766} in both cases. However, in the DTE cell population that was transfected with the FGFR1 kinase, the phosphorylation of both SNT and ERK was an acquired property, coincident with the acquisition of the proliferative response (Fig. 3*B*). Similar to the proliferative response, proliferative aging of the population in the presence of FGFR1 with an intact Tyr^{766} was required for acquisition of the response of SNT and ERK in the premalignant epithelial cells. Analysis of total ERK2 antigen confirmed that ERK2 and presumably ERK1, as reported by others (28), are expressed at a relatively constant level and that the FGF- and FGFR-dependent differences reported by anti-phospho-ERK1/2 are due to changes in phosphorylation (Fig. 3*C*). The anti-ERK2 antibody also indicated that the majority of ERK2 protein is in the unphosphorylated M_r 42,000 form in absence of FGF.

We then determined whether the expression of total SNT1 protein limited the proliferative response of DTE cells to FGFR1 before proliferative aging of the cultures (Fig. 4*A*). Overexpression of His-tagged SNT1 had no effect on FGF-stimulated DNA synthesis in DTE cells expressing ectopic FGFR1 before prolonged expansion of the cell population in culture. In sum, these results indicate that the bridge to the MAP kinase pathway, SNT, is intact in the initially unresponsive DTE cell population. The ability of FGFR1 to activate SNT and ERK1/2 is acquired during proliferative aging of the DTE population along with the proliferative response to FGFR1.

Lastly, we attempted to determine steady-state levels of phosphorylated PLCγ in DTE cells. With currently available reagents, the level is low and at the limits of detection relative to NIH3T3 cells (Fig. 4*B*). However, in contrast to phosphorylated SNT and ERK,

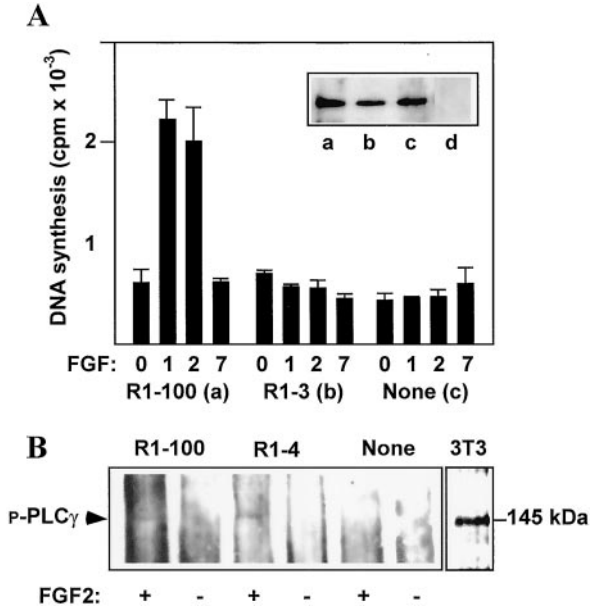

Fig. 4. *A*, levels of SNT1 do not limit the proliferative response to FGFR1 in nonmalignant DTE epithelial cells. DTE cells expressing ectopic FGFR1 that had been serially cultured for the indicated number of PDs or untransfected DTE cells (*None*) were stably transfected with cDNA coding for His-tagged SNT1. After serial culture for 4 PDs after selection, DNA synthesis was determined in the presence of the indicated FGF. Data are the mean and variance of duplicates. *Inset*, the three types of transfected cells (*a–c*) and nontransfected DTE cells (*d*; 1×10^6) in 6-well plates were lysed, and the overexpressed SNT1-His was captured on Ni-agarose beads. The bound SNT1-His was extracted from the beads, electroblotted on nylon membranes, and detected with antibody against the His tag (Santa Cruz Biotechnology, Santa Cruz, CA). *B*, constant low level of FGFR1-stimulated phosphorylated PLCγ during proliferative aging of DTE cells *in vitro*. Cell lysates were treated with protein A-agarose beads bearing anti-PLCγ antibody to concentrate the PLCγ, immunocomplexes were extracted and electroblotted on nylon membranes, and then the phosphorylated PLCγ was detected with antiphosphotyrosine antibody 4G10. *3T3*, a lysate from the same number of control NIH3T3 cells.

phosphorylated PLCγ in response to FGF2 in the FGFR1-transfected DTE cells was detectable as soon as cells could be selected for analysis (4 PDs) and was unchanged during proliferative aging of the population. Separate experiments not shown here using the kinase-defective construct R1K425A and the R1Y653/654F construct confirmed that the intact FGFR1 kinase and intact tyrosines 653 and 654 were required for phosphorylation of SNT, ERK, and PLCγ, as reported by others (5).

DISCUSSION

The development of life-threatening malignant cancer from asymptomatic, relatively benign premalignant states occurs with age. The time dependence is thought to result from gradual acquisition of genetic changes at the single cell level and expansion of genetically altered clones that overcome homeostatic restraints in the microenvironment. To date, the majority of molecular targets associated with cancer are those identified for therapy of symptomatic cancers rather than those that promote the progression of occult, often undetectable premalignant cancers to the life-threatening state. Rational design of strategies for prevention requires identification of the molecular targets involved in the progression to malignancy, independent of whether they are involved in support of the malignant state *per se*. Here we used an *in vitro-in vivo* shuttle model of prostate tumor progression to dissect such molecular targets within the FGF signal transduction axis. Members of the FGF signal transduction family mediate communication back and forth between the epithelial and stromal compartments of numerous adult parenchymal organs (4). This maintains homeostasis between the two compartments with respect to proliferation and differentiation. Specific members at the signaling end (FGF polypeptides) and reception end (FGFR kinase and heparan sulfate) are partitioned between the two compartments to result in directional specificity of the communication via paracrine mechanisms (4). The loss of resident FGFR2 and abnormal expression of stromal cell-derived FGFR1 characterize epithelial cells of rat prostate tumors that progress from nonmalignant to the malignant state after castration of hosts bearing tumors or implantation of cloned epithelial cells in absence of homologous stroma (2, 3, 29). The progression to malignancy with the accompanying loss of FGFR2 and gain of FGFR1 occurs over a period of 8 months to 1 year (2, 3, 30). Transfection of the premalignant epithelial cells with ectopic FGFR1 accelerated the time frame, but development of tumors still takes months. This suggested that acquisition of malignant properties takes time to develop even after ectopic FGFR1 has appeared. Here we show that in contrast to stromal cells where it is resident, ectopic FGFR1 in nonmalignant epithelial cells does not support proliferation of the premalignant epithelial cell population. The ability of FGFR1 to drive proliferation *in vitro* and the malignant phenotype *in vivo* is an acquired property that develops during extended proliferation of the FGFR1-expressing cells. This suggests that the contribution of FGFR1 to malignancy occurs in two stages. The first stage is the appearance of epithelial cells expressing FGFR1, which is a benign event. The second stage is the acquisition of the proliferative response to ectopic FGFR1 that occurs during proliferative aging of the FGFR1-expressing cell population. This acquired ability to activate pathways required for uncontrolled proliferation supports the malignant phenotype.

FGFR1 activates elements of both the MAP kinase and PLCγ-linked signaling pathways in numerous experimental models (31, 32). Activation of the MAP kinase pathway correlates closely with mitogenesis and other responses stimulated by FGF (32). However, PLCγ-linked responses appear dispensable in experimental systems tested to date (1, 16). Consistent with these reports, we show here that FGFR1 failed to activate the MAP kinase pathway monitored by phosphorylation of ERK1/2. This was coincident with the failure to support a mitogenic response in nonmalignant prostate tumor epithelial cells. However, the foreign FGFR1 kinase was capable of binding FGF, FGF-induced receptor autophosphorylation, and activation of transcription. FGF binding to the foreign FGFR1 is supported by a rare heparan sulfate that forms a binary complex with FGFR1 that is competent to bind FGF1 and FGF2 (24). Activation of the syndecan 1 enhancer element FiRE indicated that the ectopic FGFR1 was transcriptionally active. However, neither the activation of ERK1/2 nor FGFR1 Tyr^{766} that links to the PLCγ pathway was essential for the transcriptional activity of the mitogenically silent FGFR1. The appearance of FGFR1-activated ERK1/2 coincident with acquisition of the proliferative response and increased tumorigenicity further suggests that the MAP kinase pathway closely correlates with the mitogenic response and may be essential to it. Consistent with previous reports, FGFR1 Tyr^{766} appears dispensable for the mitogenic response acquired by the premalignant cell population during proliferative aging. However, FGFR1 Tyr^{766} and presumably PLCγ-linked pathways are essential for the time-dependent development of the proliferative response to FGFR1 and activation of the MAP kinase pathway in the premalignant epithelial cell population. We propose that it is the chronic activity of ectopic FGFR1 through specifically Tyr^{766}-dependent and PLCγ-linked pathways in premalignant epithelial cells that underlies the slow acquisition of the proliferative response to FGFR1 and progression to malignancy. The low level of PLCγ in the nonmalignant prostate epithelial cells may also contribute to the time dependence. The age-dependent progression to malignancy as a consequence of chronic perturbation of the ectopic FGFR1 signaling system in premalignant epithelial cells is consistent with the evolution of malignancies in humans that occurs slowly over many years. These findings suggest the FGFR1-linked activation of PLCγ-linked pathways as a distinct molecular target for prevention of the gradual, age-dependent progression to malignancy, although it may be of little utility for late-stage therapy.

How does the chronic activity of ectopic FGFR1 Tyr^{766}-linked pathways result in the acquisition of the response of the MAP kinase pathway and mitogenesis? At the molecular level, a major candidate for the bridge between FGFR1 and the pathway is one or more membrane-anchored proteins called SNT or FRS2 (8, 9). SNT1 is phosphorylated in response to the FGFR1 kinase and recruits the GRB2/SOS1 complex, which then interacts with *ras* to activate the MAP kinase pathway. Here we demonstrated that, similar to ERK1/2, phosphorylation of SNT1 also requires extended proliferation of the premalignant cell population in the presence of FGFR1 with an intact Tyr^{766}. The fact that proliferative aging of the population is required for observation of phosphorylated SNT1 even when both membrane-bound FGFR1 kinase and SNT1 are overexpressed suggests that the enzyme-substrate relationship in cell membranes between the two may be restricted rather than the total concentrations. Such results are consistent with the view that subunits of the FGFR complex (FGF/heparan sulfate/FGFR kinase) and its external substrates exist in proximity, but that the enzyme-substrate relationships are normally restricted at the molecular level until conformational perturbation activates the complex (4, 22, 33). Results to be published elsewhere[4] show that SNT1 is strongly phosphorylated by FGFR1 in intact insect cell membranes coexpressing SNT1 and FGFR1. This was in sharp contrast to mixtures of lysates from separately infected insect cells after extraction by detergent and mixing *in vitro*. This suggests that colocalization and orientation in intact cell membranes may be im-

[4] F. Wang. Cell- and receptor isotype-specific phosphorylation of SNT1 by the FGFR tyrosine kinases, submitted for publication.

portant for the phosphorylation of SNT1 by FGFR1. Both oligomerization of individual FGFR through a specific homeotypic interaction domain in the ectodomain (33) and conformational arrangements among FGFR subunits (25) also require expression of FGFR into intact cell membranes before extraction and analysis. From these observations, we propose that it is the chronic activity of ectopic FGFR1 through Tyr^{766}-dependent and PLCγ-linked pathways that causes alterations in membrane or cytoskeletal structure that overcome restrictions in access of SNT1 to the FGFR1 kinase to open a bridge to the MAP kinase pathway, mitogenesis, and the malignant phenotype. Whether chronic FGFR1 activity promotes emergence of the malignant epithelial cell population primarily through clonal selection or is a cause of additional genetic plasticity favorable for clonal selection is a topic of future study.

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REFERENCES

1. Coffey, D. S., Smolev, J., Heston, W. D., and Scott, W. W. Growth characteristics and immunogenicity of the R-3327 rat prostate carcinoma. Natl. Cancer Inst. Monogr., *49:* 289–291, 1978.
2. 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.
3. 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:* 4513–4519, 1993.
4. 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.
5. Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W. H., Jaye, M., and Schlessinger, J. Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol. Cell. Biol., *16:* 977–989, 1996.
6. MacNicol, A. M., Muslin, A. J., and Williams, L. T. Raf-1 kinase is essential for early *Xenopus* development and mediates the induction of mesoderm by FGF. Cell, *73:* 571–583, 1993.
7. Spivak-Kroizman, T., Mohammadi, M., Hu, P., Jaye, M., Schlessinger, J., and Lax, I. Point mutation in the fibroblast growth factor receptor eliminates phosphatidylinositol hydrolysis without affecting neuronal differentiation of PC12 cells. J. Biol. Chem., *269:* 14419–14423, 1994.
8. Ong, S. H., Goh, K. C., Lim, Y. P., Low, B. C., Klint, P., Claesson-Welsh, L., Cao, X., Tan, Y. H., and Guy, G. R. Suc1-associated neurotrophic factor target (SNT) protein is a major FGF-stimulated tyrosine phosphorylated 90-kDa protein which binds to the SH2 domain of GRB2. Biochem. Biophys. Res. Commun., *225:* 1021–1026, 1996.
9. Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the *Ras*/MAPK signaling pathway. Cell, *89:* 693–702, 1997.
10. Hou, J., McKeehan, K., Kan, M., Carr, S. A., Huddleston, M. J., Crabb, J. W., and McKeehan, W. L. Identification of tyrosines 154 and 307 in the extracellular domain and 653 and 766 in the intracellular domain as phosphorylation sites in the heparin-binding fibroblast growth factor receptor tyrosine kinase (flg). Protein Sci., *2:* 86–92, 1993.
11. Mohammadi, M., Schlessinger, J., and Hubbard, S. R. Structure of the FGF receptor tyrosine kinase domain reveals a novel autoinhibitory mechanism. Cell, *86:* 577–587, 1996.
12. Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Del Rosario, M., Mirda, D., and Williams, L. T. Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca^{2+} flux but not mitogenesis. Nature (Lond.), *358:* 678–681, 1992.
13. Mohammadi, M., Dionne, C. A., Li, W., Li, N., Spivak, T., Honegger, A. M., Jaye, M., and Schlessinger, J. Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature (Lond.), *358:* 681–684, 1992.
14. Muslin, J., Peters, K. G., and Williams, L. T. Direct activation of phospholipase C-γ by fibroblast growth factor receptor is not required for mesoderm induction in *Xenopus* animal caps. Mol. Cell. Biol., *14:* 3006–3012, 1994.
15. Roghani, M., Mohammadi, M., Schlessinger, J., and Moscatelli, D. Induction of urokinase-type plasminogen activator by fibroblast growth factor (FGF)-2 is dependent on expression of FGF receptors and does not require activation of phospholipase Cγ1. J. Biol. Chem., *271:* 31154–31159, 1996.
16. Landgren, E., Klint, P., Yokote, K., and Claesson-Welsh, L. Fibroblast growth factor receptor-1 mediates chemotaxis independently of direct SH2-domain protein binding. Oncogene, *17:* 283–291, 1998.
17. Wang, F., Kan, M., Xu, J., Yan, G., and McKeehan, W. L. Ligand-specific structural domains in the fibroblast growth factor receptor. J. Biol. Chem., *270:* 10222–10230, 1995.
18. Wang, F., Kan, M., and McKeehan, W. L. Multiple mutant cDNAs from one reaction mixture using asymmetric primers in PCR. Biotechniques, *19:* 556–559, 1995.
19. 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.
20. Jaakkola, P., Vihinen, T., Maatta, A., and Jalkanen, M. Activation of an enhancer on the syndecan-1 gene is restricted to fibroblast growth factor family members in mesenchymal cells. Mol. Cell. Biol., *17:* 3210–3219, 1997.
21. Maher, P. p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. J. Biol. Chem., *274:* 17491–17498, 1999.
22. Kan, M., Wang, F., To, B., Gabriel, J. L., and McKeehan, W. L. Divalent cations and heparin/heparan sulfate cooperate to control assembly and activity of the fibroblast growth factor receptor complex. J. Biol. Chem., *271:* 26143–26148, 1996.
23. 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.
24. 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. Cancer Res., *61:* 5295–5302, 2001.
25. 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.
26. Stauber, D. J., DiGabriele, A. D., and Hendrickson, W. A. Structural interactions of fibroblast growth factor receptor with its ligands. Proc. Natl. Acad. Sci. USA, *97:* 49–54, 2000.
27. Plotnikov, A. N., Hubbard, S. R., Schlessinger, J., and Mohammadi, M. Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specificity. Cell, *101:* 413–424, 2000.
28. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev., *22:* 153–183, 2001.
29. Isaacs, J. T., Heston, W. D., Weissman, R. M., and Coffey, D. S. Animal models of the hormone-sensitive and -insensitive prostatic adenocarcinomas, Dunning R-3327-H, R-3327-HI, and R-3327-AT. Cancer Res., *38:* 4353–4359, 1978.
30. McKeehan, W. L., Adams, P. S., and Fast, D. Different hormonal requirements for androgen-independent growth of normal and tumor epithelial cells from rat prostate. In Vitro Cell. Dev. Biol., *23*: 147–152, 1987.
31. Klint, P., and Claesson-Welsh, L. Signal transduction by fibroblast growth factor receptors. Front. Biosci., *15:* D165–D277, 1999.
32. Xu, H., Lee, K. W., and Goldfarb, M. Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. J. Biol. Chem., *273:* 17987–17980, 1998.
33. 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.
34. Xu, J., Nakahara, M., Crabb, J. W., Shi, E., Matuo, Y., Fraser, M., Kan, M., Hou, J., and McKeehan, W. L. Expression and immunochemical analysis of rat and human fibroblast growth factor receptor (flg) isoforms. J. Biol. Chem., *267:* 17792–17803, 1992.

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