FGF-8 Isoforms Differ in NIH3T3 Cell Transforming Potential

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Abstract

We previously identified Fgf-8 as a frequently activated gene in tumors from mouse mammary tumor virus-infected Wnt-1 transgenic mice, suggesting that Fgf-8 is a proto-oncogene. We further determined that multiple, secreted protein isoforms that differ at their mature amino termini are encoded by alternatively spliced mRNAs transcribed from the gene. We now present evidence that there are differences in the potency of NIH3T3 cell transformation displayed by three of the FGF (fibroblast growth factor)-8 isoforms. We find that stable transfection of a cDNA for the FGF-8b isoform leads to marked morphological transformation of NIH3T3 cells and rapid tumorigenicity of the transfected cells in nude mice. In contrast, transfection of a cDNA for the FGF-8a or FGF-8c isoform results in moderate morphological changes in the NIH3T3 cells, and the transfected cells are weakly tumorigenic in nude mice. All three transfusions result in cells that express comparable amounts of Fgf-8 mRNA and that produce the FGF-8 protein isoforms. The morphological changes observed in NIH3T3 cells can be reproduced by the addition of recombinant FGF-8 protein isoforms to the culture medium. Therefore, these results indicate that there are differences in the potency of NIH3T3 cells by FGF-8 protein isoforms and suggest that these FGF-8 isoforms may have different in vivo functions.

Introduction

Oncogenesis is a multistep process involving the sequential acquisition of multiple genetic alterations. Because this process is prolonged and complicated in humans (1), animal models to study the process of oncogenesis are desirable. We have used a Wnt-1 transgenic mouse model to study breast cancer development (2). We infected Wnt-1 transgenic mice with mouse mammary tumor virus to accelerate tumorigenesis and to “molecularly tag” proto-oncogenes that are activated in the resulting tumors and that cooperate with Wnt-1 in mammary tumorigenesis (3, 4). Using this approach, we identified Fgf-3 and Fgf-4 as Wnt-1-collaborating proto-oncogenes (3) and subsequently cloned a genomic locus that contained MMTV insertions in the DNA from several mammary tumors of MMTV-infected Wnt-1 transgenic mice (4). We determined that the activated gene in this locus was also a member of the FGF3 family, Fgf-8, previously described as androgen-induced growth factor (5).

FGFs are a family of growth factors, related by amino acid sequence similarity, that are encoded by at least nine genes in mammals (5–7). They are mitogenic for a variety of cell types, although their physiological roles in vivo may be in wound healing and embryonic development (reviewed in Ref. 8). FGFs are thought to elicit their effects by binding to high affinity tyrosine kinase receptors on the cell surface, encoded by a family of at least four genes (Fgfr1–4) in mammals (9–15). Heparan sulfate proteoglycans are low affinity cell surface receptors important for FGF effects (16, 17), although heparin can substitute for these receptors in vitro (18). In addition, a cysteine-rich transmembrane receptor for FGFs exists, but its significance to FGF effects is unknown (19). Most Fgf genes code for single proteins (6), but different isoforms of FGF-2 and FGF-3 exist and have different cellular locations, due to alternative translation initiation (20–23).

Fgf-8 consists of at least six exons and codes for at least seven protein isoforms, due to alternative splicing of the primary transcript (4, 5, 24). RNAs for the FGF-8 isoforms are present during murine embryogenesis (4, 24–26) and are detectable only in gonadal tissue of adult mice at low levels (4). These results and the insertional activation of Fgf-8 described above suggest that Fgf-8 is a normal embryonic gene that is oncogenic when overexpressed in adult mammary tissue. The significance of the different FGF-8 isoforms is not known; however, we hypothesize that their existence suggests that they have different biological properties.

Overexpression of Fgf-3 (27), Fgf-4 (28), Fgf-5 (29), Fgf-6 (30), and at least one Fgf-8 isoform, FGF-8b (pSC17) (5, 31), can transform NIH3T3 cells. Given the ability to observe a phenotype in NIH3T3 cells in response to FGFs, we decided to test the hypothesis that the FGF-8 isoforms might possess different biological properties by stably transfecting NIH3T3 cells with cDNAs for the three FGF-8 isoforms we identified previously (4). We confirm previous findings that transfection and expression of the Fgf-8b cDNA in NIH3T3 cells strongly transforms them, such that they become altered morphologically, clonogenic in soft agar, and tumorigenic in nude mice (31). In contrast, trans-
fection and expression of the Fgf-8a or the Fgf-8c cDNA in NIH3T3 cells lead to more subtle morphological changes. The morphological changes in all cases can be produced by adding the rFGF-8 isoform to the culture medium. NIH3T3 cells producing FGF-8a or FGF-8c are not clonogenic in soft agar and are less tumorigenic than FGF-8b-producing cells, despite similar amounts of FGF-8 isoform RNA and protein being made. These results indicate that the FGF-8 isoforms have different potencies for transformation of NIH3T3 cells and suggest that they may have different functions in vivo.

Results

NIH3T3 Cells Transfected with Different Fgf-8 cDNAs Display Different Morphologies. We have identified three FGF-8 isoforms in our prior work (4) and hypothesize that they have different biological properties. To begin to test this hypothesis, we subcloned the cDNAs encoding the three FGF-8 isoform cDNAs into the expression vector pMIRB (Fig. 1). The majority of the amino acids encoded by these three FGF-8 isoform cDNAs are identical, but they differ in the region immediately following the signal peptide, i.e., the amino terminal portions of the mature secreted proteins (4, 5). The resulting vectors were used in transfection experiments of NIH3T3 cells, and stable cell lines were selected with G418. We chose to pool the selected G418 cells rather than individually clone them, so as to preclude inaccuracies due to clonal variation. The expression vector used, pMIRB, allowed this approach, since virtually all selected G418 cells would express the FGF-8 isoform (Fig. 1).

NIH3T3 cells transfected with expression vectors coding for the FGF-8 isoforms and selected for G418 resistance display different morphologies (Fig. 2). In agreement with previous results (31), cells transfected with pMIRB containing the Fgf-8b cDNA (hereafter called 8B cells) displayed marked morphological transformation with an elongated, spindle-like shape (Fig. 2). In contrast, cells transfected with pMIRB containing the Fgf-8a cDNA (hereafter called 8A cells) or with pMIRB containing the Fgf-8c cDNA (hereafter called 8C cells) displayed modest morphological transformation and a flatter morphology (Fig. 2). NIH3T3 cells transfected with the pMIRB vector alone (no Fgf-8 cDNA, called MIRB cells hereafter) were morphologically identical to nontransfected NIH3T3 cells (Fig. 2). We have observed variability in the degree of morphological transformation observed in 8A cells, ranging from morphologies like MIRB cells to morphologies like 8C cells, which we suspect may be due to different levels of FGF-8a (data not shown). These results suggest that the different FGF-8 isoforms have different potencies with respect to morphological transformation of NIH3T3 cells.

Transfected NIH3T3 Cells Express the Fgf-8 cDNAs. To demonstrate expression of the transfected cDNAs, we prepared total RNAs from pools of G418' NIH3T3 cells that were transfected with the Fgf-8 cDNA (and control vectors described above). We analyzed RNAs from the cell lines using an antisense 5' Fgf-8b riboprobe in an RNase protection assay (25). The antisense Fgf-8b riboprobe is 317 nt in length and is digested to 153 nt when protected by Fgf-8a RNA; 222 nt when protected by Fgf-8b RNA, and 157 nt when protected by Fgf-8c RNA. We observed the correct size-protected fragment in each of the 8A, 8B, and 8C cell lines but did not observe any protected fragments in the MIRB line (Fig. 3). These results confirm that the correct FGF-8 isoform mRNA was produced by the 8A, 8B, and 8C cell lines.

To control for differences in RNA loading and to confirm the integrity of the RNAs, we performed a RNase protection assay with an antisense β-actin riboprobe. This riboprobe is 300 nt in length and is digested to 250 nt when protected by β-actin mRNA. All four cell lines protected a 250-nt fragment (Fig. 3). The resulting gels were quantitatively imaged and analyzed as described in "Materials and Methods." The cell lines make comparable amounts of Fgf-8 RNA (Fig. 3). The 8B cells, which have the most dramatic morphology, actually produce less FGF-8 RNA than the 8A and 8C cell lines, indicating that the weaker biological responses of 8A and 8C cells cannot be explained by low expression levels.

The Transfected NIH3T3 Cells Produce FGF-8 Protein. Although the RNase protection assays in Fig. 3 show that the 8A, 8B, and 8C cell lines appropriately expressed the transfected Fgf-8 cDNAs, it is formally possible that one or more of the FGF-8 isoforms might not be produced or may be rapidly degraded. To address this question, we prepared an affinity-purified polyclonal antibody to FGF-8a (anti-mouse FGF-8) that should theoretically bind the three FGF-8 isoforms. The affinity-purified anti-mouse FGF-8 was tested in Western blot analysis of rFGF-8 isoform proteins and bound the three rFGF-8 isoforms (Fig. 4). FGF-8 isoforms were not readily detected in the conditioned medium from these cell lines by immunoblotting, possibly due to FGF-8 binding to cell surface heparan-sulfated proteoglycans (16, 17).

To demonstrate the production of FGF-8 isoforms in these cells, we performed immunohistochemical staining of the
fixed cell lines using a biotin-avidin-labeled secondary antibody system with HRP staining. The 8A, 8B, and 8C cell lines show positive staining using the affinity-purified, anti-FGF-8 polyclonal antibody described above, although the control MIRB cells do not (Fig. 5). These results confirm that the 8A, 8B, and 8C cell lines produce detectable amounts of FGF-8 isoform protein and suggest that the morphological differences in the transfected cells are not due to absence of the protein but rather are due to differences in the potency of FGF-8 isoforms to transform NIH3T3 cells.

**NIH3T3 Cells Producing Different FGF-8 Protein Isoforms Display Different in Vitro and in Vivo Properties.**

The pooled G418' cells from each isoform transfection were compared in several biological assays. The doubling times of the cells lines were examined, and the 8A, 8B, and 8C cell lines displayed slightly shorter doubling times when compared to the MIRB cells (16 h versus 18 h), but the difference was not statistically significant (Table 1). In conditions of lower serum (2% FCS and 8% newborn serum), the MIRB and 8A cells died, while the 8C cells stopped growing but did not die (Table 1). 8B cells continued to grow and displayed the same transformed morphology (Table 1 and data not shown), suggesting that it was the more potent FGF-8 transforming protein.

We examined the saturation densities of the FGF-8-expressing cell lines and found clear differences. At confluence, the number of 8A, 8B, and 8C cells was two, five, and four times the number of MIRB cells, respectively (Table 1). In soft agar clonogenicity assays, only 8B cells were able to form soft agar colonies at an average frequency of 5% (Table 1). These in vitro assays of proliferation and transformation all suggest that FGF-8b is the more potent transforming protein isoform.

In nude mice tumorigenicity assays (Table 1), tumors formed rapidly when 10⁶ 8B cells were injected into nude mice, with all 10 animals possessing fibrosarcomas 2 cm or larger after 1 week. No tumors were seen in animals injected with 10⁶ MIRB cells, even after 4 months of observation. Tumors were observed in two of four animals injected with 10⁶ 8A cells and three of four animals injected with 10⁶ 8C cells, but the tumors in both groups of animals took 4–6 weeks to attain a size of 2 cm and were not detected in the first 3 weeks after the injection of the cells.

Tumors were isolated from all animals, and G418' cells from tumors from each of the FGF-8 isoform groups were reselected in culture. The morphology of the cells after passage as tumor in the animals was identical to the morphology of the cells prior to passage as tumor, i.e., 8B cells were the most transformed morphologically, and the 8A and 8C cells were less transformed (data not shown). Furthermore, Northern blot analysis of RNA from 8A, 8B, and 8C cell lines after passage as tumors in nude mice show...
that RNA for the FGF-8 isoforms was present (data not shown). These results indicate that the morphology and expression of the transfected Fgf-8 cDNAs are stable and not altered by passage as tumors in nude mice and suggest that the production of the FGF-8 protein isoform is responsible for the observed phenotypes.

**Recombinant FGF-8 Isoforms Morphologically Alter NIH3T3 Cells.** To begin to understand the mechanism of the differences observed in the preceding transfection experiments, we added rFGF-8 isoforms, carboxy-terminal histidine-tagged (10 nM), and heparin (3 μg/ml), to the culture medium of NIH3T3 cells. After 3 days in culture, the cells developed the same morphological changes observed with transfection of the cDNAs (Fig. 6). Specifically, the NIH3T3 cells cultured with rFGF-8b were elongated and spindle shaped. The NIH3T3 cells cultured with rFGF-8a or rFGF-8c grew to a higher density and were less contact inhibited than untreated NIH3T3 cells. The cells treated with rFGF-8a or rFGF-8c did not develop the elongated spindle shape of cells treated with rFGF-8b, even when 100 nM concentrations were tried (Fig. 6 and data not shown). These results indicate that the morphological differences observed in the stably transfected NIH3T3 cells are likely due to biological differences in the secreted forms of these FGF-8 proteins.

**Discussion**

The existence of several FGF-8 isoforms suggests the possibility that they possess different biological functions. We now show that three FGF-8 isoforms have different potencies for transformation of NIH3T3 cells. In agreement with prior results (31), 8B cells are morphologically transformed (Fig. 2), clonogenic in soft agar (Table 1), and rapidly form tumors in nude mice (Table 1). 8A and 8C cells show modest morphological transformation (Fig. 2), are not clonogenic in soft agar (Table 1), and are weakly tumorigenic in nude mice (Table 1). RNase protection analyses indicate that the cell lines produce the correct FGF-8 isoform RNA (Fig. 3) and suggest that the correct FGF-8 isoform protein is produced by the cell lines. The observed differences in potency of NIH3T3 cell transformation shown here are not due to differences in amounts of FGF-8 isoform RNA in the cell lines (Fig. 3) or to the absence of FGF-8 isoform protein (Fig. 5). We show that recombinant FGF-8 isoforms added to the culture medium of NIH3T3 cells leads to the same morphological changes observed when the cells are transfected with the appropriate cDNA (Fig. 6). Although subtle quantitative differences in the production of FGF-8 isoform proteins cannot be excluded, these results suggest that these FGF-8 isoforms possess different potencies for transformation of NIH3T3 cells.

Alternative isoforms of FGF-2 and FGF-3 exist due to different translation initiation sites (20–23), which results in the targeting of these isoforms to different cellular locations (extracellular versus nuclear). In contrast, the three FGF-8 isoforms examined in this work have identical signal peptides, are presumably secreted, and differ only at the amino termini of the mature secreted isoform (4). Assuming that the three FGF-8 isoforms are secreted, we would predict that their biological effects would relate to binding of FGFRs. Hence, the differences observed in NIH3T3 cell transformation potency between the FGF-8 isoforms suggest that the amino terminal differences in the FGF-8 isoforms result in the differential ability of FGF-8 isoforms to bind to, or induce signals through, the FGFRs present on NIH3T3 cells.

The FGF-8b isoform has been shown to bind to a mutated FGFR1 that was isolated from the same SC-3 mammary carcinoma cell line from which FGF-8 was originally purified (31). Whether FGF-8b binds normal FGFR1, or other FGFRs, is unknown. No information is available concerning the ability of the other FGF-8 isoforms to bind the various FGFRs. This information is important in order to understand the role of FGF-8 in mammalian development, as well as the potential tissue specificity of Fgt-8-induced oncogenesis.

Additional evidence has been reported for the idea that differences in the amino terminal portion of FGF proteins can alter their interactions with FGFRs. A recombinant amino-truncated FGF-4 is more active than the full-length FGF-4 in an in vitro FGFR-binding assay (32). In contrast, a recombinant amino-truncated FGF-7 shows equivalent binding when compared to full-length FGF-7, but the trun-
cated FGF-7 is unable to induce the intracellular tyrosine phosphorylation observed with the full-length FGF-7 (33). Amino-truncated forms of FGF-4 and FGF-7 are not known to exist in vivo. However, their in vitro properties confirm that amino terminal differences in FGFs can alter the ability of FGFs to bind FGFRs or to transduce a signal following FGFR binding. These findings support the hypothesis that the amino terminal differences observed in natural FGF-8 isoforms are involved in differential interactions of FGF-8 isoforms with FGFRs. Whether the observed phenotypic differences are due to differences in FGF-8 isoform affinity for the various FGFRs and/or differences in the ability of FGF-8 isoforms to lead to signal transduction following receptor occupation remains to be determined.

We and others have recently characterized the temporal and spatial expression of Fgf-8 during postgastrulation mouse development (24–26). We observed Fgf-8 expression in the ectoderm of the first branchial arch, nasal pits, and limb buds, as well as in the neuroectoderm of the telencephalon, diencephalon, mesencephalon-metencephalon junction, and infundibulum from days 9 to 13 of development. Isoform-specific localizations were not performed, but preliminary results with an Fgf-Bc-specific probe indicate that Fgf-Bc RNA is present in all of the above locations.4 RNA for the three FGF-8 isoforms was detected by RNase protection assays at days 10–12 of mouse development (25). These results suggest that the splicing of the Fgf-8 transcript is not regulated during development. If this is the case, then the specific interactions of FGF-8 isoforms with FGFRs during development would depend on the FGFRs present in the local environment of Fgf-8 expression. FGF signaling pathways have been implicated in the genetic dysmorphology syndromes of achondroplasia (FGFR3 transmembrane mutation; Refs. 34 and 35), Jackson-Weiss and Crouzon Syndromes (FGFR2 bek exon mutations; Refs. 36 and 37), and Pfeiffer Syndrome (FGFR1 mutations; Ref. 38). Therefore, characterization of FGF-8 isoform/FGFR interactions may provide a clearer molecular understanding of these and other rare craniofacial and/or limb dysmorphogenesis syndromes.

Similarly, whether Fgf-8 has any role in human malignancy will depend on at least three factors: (a) whether Fgf-8 can be transcriptionally activated by a carcinogenic event in a target tissue; (b) whether the target tissue has one or more FGFRs that bind one or more FGF-8 isoforms; and (c) whether the FGF-8 isoform/FGFR interaction leads to an oncogenic (presumably mitogenic) signal. Since Fgf-8 is a developmentally silenced gene, it seems likely that oncogenic events could transcriptionally activate the gene in a target tissue, as was observed for metallothioneins in thymic lymphoma cells (39, 40). Therefore, understanding the tissue distribution of FGFRs, their ability to bind the FGF-8 isoforms, and their ability to transduce a mitogenic signal in the target tissue in response to FGF-8 isoform binding will help elucidate the role of Fgf-8 in cancer.

Materials and Methods

Cell Lines, Vectors, and Transfection. NIH3T3 cells (gift of R. Weinberg, Whitehead Institute, Cambridge, MA) were grown in DMEM-FCS in humidified incubators with 5% CO2 at 37°C. Cells were passaged at confluence to avoid selecting for spontaneous transfectants; any cultures with such variants were discarded.

We used an expression vector, pMIRB (generous gift of D. M. Ornitz, Washington University, St. Louis, MO), that generates a bicistronic mRNA that has an internal ribosome entry site for the downstream neomycin phosphoribosyltransferase gene, allowing both transcription units in the mRNA to be translated (Fig. 1). Previously isolated cDNAs with full coding potential for three FGF-8 protein isoforms (4) were cloned into the upstream position of pMIRB (Fig. 1) using the EcoRI and blunted SpeI sites. The cDNAs were sequenced with Fgf-8 primers to confirm authenticity. The resulting expression vectors were used to transfect NIH3T3 cells using OptiMEM and Lipofectamine (GIBCO-BRL). Briefly, 6 × 105 cells were cultured on 6-well dishes (Falcon). The next day, the cells were washed with OptiMEM and then placed in 1 ml of OptiMEM. Expression vector

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4 M. Heikinheimo and C. A. MacArthur, unpublished results.
FGF-8 Isoform Transformation Differences

Transformation Assays. At least 50 G418' colonies (above) were pooled from each transfection/selection. This pooling of clones was done to minimize the possibility that a single clone that was morphologically transformed by an event unrelated to expression of the FGF-8 isoform would bias our results. The initial transformation assay was a simple morphological examination of the transfected and selected cells. The morphology was documented by photomicroscopy.

The second transformation assay was soft agar clonogenicity. Five thousand G418' cells from each transfection/selection were placed in 2 ml of DMEM-FCS with 0.5% Low Melt Agarose (GIBCO-BRL). They were overlaid on 100-mm plates containing 10 ml of bottom agar (DMEM-FCS with 0.5% Bacto Agar; Difco) and incubated at 37°C and 5% CO₂ for 14 days. The visible colonies were counted at 14 days, and the colony-forming unit was calculated by dividing the number of soft agar colonies observed at 14 days by the number of cells plated (5000) and multiplying by 100 to convert to percentages.

The third transformation assay was in vivo tumorigenesis in nude mice. Five-week-old nude mice (nu/nu; The Jackson Laboratory) were injected with 1 × 10⁶ G418' cells in 0.1 ml of PBS s.c. into the flank. The animals were maintained in laminar cages (4–5 mice/cage) and provided rodent chow (Purina) and water ad libitum in the Pediatric Animal Facility at Washington University School of Medicine, in accordance with the NIH Guide to animal welfare. Following injection, the animals were observed every 2 days for the development of tumors. Animals were euthanized in accordance with NIH and Washington University School of Medicine Animal Study Committee guidelines. The tumors were used to establish posttumor cell lines by mincing tumor tissue in DMEM-FCS.
with 500 µg/ml Geneticin. The resulting suspensions were incubated at 37°C and 5% CO₂.

**Cell Proliferation Assays.** Multiple platings of G418'' Cells (1000) from each transfection were plated in wells of 6-well dishes and grown at 37°C and 5% CO₂ in DMEM-FCS. The cells from each transfected/selected cell line were removed from the wells with trypsin and counted using a hemocytometer each day. The doubling time was determined for cells in log phase growth. The saturation density for each cell line was determined when the cells reached confluence (8–10 days). The data was collected on four occasions, both at the initial development of the cell lines and after several months in cryopreservation. Results are presented as the mean ± SE.

**Isolation of RNA, Northern Blot, and RNase Protection Assays.** Total RNA from cells and from tumors was isolated by the guanidinium isothiocyanate/acid phenol method (41). RNase protection assays (Ambion; RPA II kit) were performed on 10 µg of total RNA using antisense riboprobe derived from the Fgf-8b 5' cDNA and β-actin cDNA, as described previously (25). The radioactive signals in the gels were quantitated by phosphor imaging (Molecular Dynamics). The raw data was corrected for differences in Fgf-8 fragment size (29 UTP residues for Fgf-8a protected fragment, 46 UTP residues for Fgf-8b protected fragment, and 30 UTP residues for Fgf-8c protected fragment) and RNA loading (by dividing by the β-actin signal). The corrected data, expressed as a Fgf-8:β-actin signal ratio, was normal-

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<th>Saturation density (10⁶ cells/cm²)*</th>
<th>Colony-forming units (%)**</th>
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* Ability of cells to grow in 2% FCS and 8% newborn serum.

* Doubling time, measured while the cells were in log phase in 10% FCS, and presented as the mean of four experiments ± the SE.

** Saturation density, measured as the number of cells on the plate at confluence divided by the surface area of the plate and presented as the mean of four experiments ± the SE.

*** Colony-forming units in soft agar, expressed as a percentage of the plated cells, presented as the mean of four experiments ± the SE.

** Presented as the number of animals with tumors divided by the number of animals receiving injections.

*** Tumor latency was 1 week.

** Tumor latency was 4–6 weeks.

*Fig. 6. Recombinant FGF-8 isoforms alter the morphology of NIH3T3 cells. Cells were grown for 3 days in DMEM-FCS, supplemented with 3 µg/ml of heparin and 0 µg/ml rFGF-8 (N), 10 µg/ml rFGF-8a (A), 10 µg/ml rFGF-8b (B), or 10 µg/ml rFGF-8c (C).*
ized so that the value of the least expressed isoform was one. 

Production of Recombinant FGF-8 Isoforms. The cDNAs for the mature forms (i.e., signal peptide removed) of the FGF-8 isoforms were obtained by PCR methods and cloned into the pQE30 (for amino-tagged isoforms) or pQE16 (for carboxy-tagged isoforms) bacterial expression vectors (Qiagen). The histidine-tagged recombinant FGF-8 (rFGF-8) isoforms were purified using the denaturing protocol [6 mM guanidinium hydrochloride, 100 mM sodium phosphate, and 10 mM Tris-chloride (pH 8.0)] of Qiagen and Ni-NTA agarose chromatography. The denatured purified rFGF-8 isoforms were eluted with 8 M urea, 100 mM sodium phosphate, and 10 mM Tris-chloride (pH 5.9). The purified rFGF-8 isoforms were renatured by successive dialysis, first against 1 M urea, 100 mM sodium phosphate, 10 mM Tris-chloride, and 5 mM reduced glutathione (pH 8.0), and then against PBS with 5 mM reduced glutathione. The rFGF-8 isoforms were obtained as a powder by lyophilization and quantitated by amino acid analysis.

Since the differences in the FGF-8 isoforms is at the mature amino terminus, the carboxy-tagged rFGF-8 isoforms were used in NIH3T3 culture experiments. NIH3T3 cells were split 1:20 and allowed to attach for 4 h. The medium was replaced with fresh medium containing heparin (final concentration, 3 μg/ml). The rFGF-8 isoform proteins were reconstituted in sterile PBS and added to the cells at various final concentrations (1 mM to 1 μM). The cells were grown as usual for 3 days and photographed.

Generation of FGF-8 Antibody, Western Blot, and Immunohistochemical Analyses. The purified rFGF-8a (amino-tagged) was submitted to Cocalico Biologicals (Reamstown, PA) for immunization of rabbits and antiserum production. The antiserum produced was further purified, initially by protein A chromatography (42) and subsequently by FGF-8 affinity chromatography, using cyanogen bromide-charged Sepharose and rFGF-8a (42). The affinity-purified, anti-mouse FGF-8 antibody, at a concentration of 0.2–0.5 μg/ml, was used as the primary antibody in Western blots of rFGF-8a, rFGF-8b, and rFGF-8c. Chemiluminescence methods, including a secondary donkey anti-rabbit IgG antibody conjugated with HRP, were used in Western blots (Amersham ECL). The recombinant proteins were separated by SDS-PAGE and transferred by electrophoretic methods to nitrocellulose (Amersham ECL) as described (42). Blocking, incubating the blots with antibodies, washing the blots, and development of the chemiluminescent signal were done according to manufacturer’s instructions. The blots were exposed to film (Amersham’s Hyperfilm-ECL) for appropriate times.

Immunohistochemical analyses of FGF-8 protein expression were performed on the transfected/selected G418' cell lines. The cells were grown as above, washed in PBS, fixed with 4% paraformaldehyde in PBS-Ca for 20 min, bleached with 0.3% v/v H2O2, and permeabilized in 1% Triton X-100 as described (42, 43). The fixed and permeabilized cells were blocked with 10% goat serum in PBS-Ca and then incubated with the affinity-purified, rabbit anti-mouse FGF-8 antibody described above, at a concentration of 0.2 μg/ml in PBS-Ca for 30 min at 37°C. The secondary antibody (biotinylated anti-rabbit IgG) and avidin-HRP detection reagents were part of the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA), and the manufacturer’s procedure was used. HRP staining with diaminobenzidine tetrahydrochloride/nickel chloride and H2O2 were performed as described (42).

Acknowledgments
We thank David M. Ornitz for the gift of the pMR8 expression vector and David B. Wilson for assistance with photography.

References