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### Analysis of Multistep Mammary Tumorigenesis in Wnt-1 Transgenic Mice

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### Abstract

Breast cancer like all cancers, is a multistep process involving the sequential acquisition of genetic alterations over a period of time. Studying this process in humans is a prolonged and arduous task; therefore, animal models are a desirable alternative. We have used a Wnt1 transgenic mouse model to study the multiple genetic events in mammary cancer development. We infected these transgenic mice with the mouse mammary tumor virus (MMTV) 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. By examination of the tumors that lack activation of genes that are usual targets of MMTV insertions, we identified a common insertion locus for MMTV and determined the activated gene in this locus to be another member of the FGF family, Fgf8. Fgf8 is transcriptionally activated in 50% of the tumors from infected Wnt1 transgenic mice in comparison to the lack of Fgf8 RNA in other tumors and mammary tissues, suggesting a strong oncogenic cooperation between Fgf8 and Wnt1 in mammary tumorigenesis. Fgf8 induced apoptosis of mammary epithelial cells as evidenced by nuclear condensation and fragmentation and oligonucleosomal laddering. Overexpression of the anti-apoptotic gene bcl2 in these cells transiently rescued or delayed the apoptosis induced by FGFs. These results describe a new property for FGFs and suggest that these growth factors play very important roles in regulating cell growth and death both in normal development as well as in pathological conditions like cancer.
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INTRODUCTION

Breast cancer, like other cancers, results primarily from accumulation of genetic mutations. Many of the identified mutations associated with cancer result in the activation of proto-oncogenes or inactivation of tumor suppressor genes. In most cases, a single chromosomal aberration is insufficient to cause carcinogenesis but rather mutations in two or more genes are required. In order to understand the development and progression to cancer, it is imperative to identify not only the single mutations involved, but also synergistically acting groups of cancer related genes.

The role of retroviruses in viral-induced cancers has been well established in mice. For example, MMTV has proven to be a powerful tool for studying murine mammary tumorigenesis. MMTV is a B type retrovirus that is known to cause mammary adenocarcinomas in certain inbred strains of mice (e.g. C3H, BR6, GR) (1, 2). The tumor inducing property of MMTV is intrinsically related to an obligatory step in its life cycle, the insertion of a proviral copy of its genome into host cellular DNA. The integration is a mutagenic event for the host cells and, as a consequence, may lead to the transcriptional activation of closely linked proto-oncogenes by the mechanism of insertional mutagenesis (3). The activation of the proto-oncogene by MMTV contributes to the transformation of the cell and development of a tumor. A number of proto-oncogenes activated by MMTV in mammary tumors have been identified. They are Wnt1, Wnt3, Wnt10b, Int2/Fgf3, Fgf3, Fgf-4 and Fgf8 (4,5,6,7,8,9,11,17).

While the structure of these genes is known, less is known about their function. A common factor among the MMTV activated genes is that they all appear to play key roles in early embryonic development (12). To prove the oncogenic potential of Wnt1, transgenic mice containing the Wnt1gene under the control of an MMTV enhancer were generated. Both male and female transgensics developed mammary adenocarcinomas following a generalized mammary hyperplasia (13). The median latency of mammary tumor formation was ~5 months in female mice. Males developed tumors less frequently and later in life. The generalized hyperplasia, coupled with the long latency and the sporadic nature of the tumor formation, suggest that Wnt1 contributes to but is not sufficient for mammary tumorigenesis in these mice. Activation of Wnt1 is probably an early event in the process of tumor formation; therefore other events, presumably genetic, are necessary for tumor progression.

In an attempt to identify genes acting in synergy in the multistep process of murine mammary tumorigenesis, these Wnt1 transgenic mice were mutagenized by infection with MMTV (14,15). The strategy was that since MMTV transcriptionally activates proto-oncogenes by insertion of its own DNA near them (2), one could possibly identify additional oncogenes that oncogenically cooperate with Wnt1 by tagging them with viral DNA. Activation of the cooperating oncogene would therefore confer a growth advantage and would presumably produce a tumor composed mainly of cells that are clonally derived from the cell bearing the proviral insertion. Implicit in this hypothesis was the expectation of a reduction in tumor latency. As predicted, in MMTV infected Wnt1 transgensics the median latency of tumor formation decreased from ~5 months to 2.5 months and the number of tumors per mouse increased (15). Southern blot data reveal that most of these tumors contained clonal tumor-specific proviruses in addition to the endogenous proviruses found in laboratory mice (15). The advantage of this approach over other mutagenesis procedures is that tumors arising due to proviral insertions contain proviruses physically linked to the activated proto-oncogenes, forming a molecular tag, which permits easy identification, and cloning of the activated genes (2).

Analysis of the tumor DNAs derived from infected Wnt1transgenic mice by Southern blotting showed that at least 80 of 128 tumors (59%) contained clonal MMTV-specific
proviruses (15). These tumors were examined for the insertional activation of proto-oncogenes known to be activated by MMTV: int2/Fgf3, hst/Fgf-4, int-3 and Wnt-3 (2,5,7,8). Approximately 45% of these tumors contained insertional activated int2/Fgf3 and/or hst (15). These results show the cooperation of int2/Fgf3 and hst with Wnt-1, which strongly corroborates prior findings indicating the same cooperation (16). I (in collaboration with a post-doctoral fellow Craig MacArthur) recently identified another member of the FGF family of growth factors that is insertionally activated by MMTV in 8 of 80 mammary tumors with clonal tumor-specific proviral insertions (17). This gene (Fgf-8) was cloned from one of the tumors that had a single tumor specific proviral insertion as described in the methods section. Fgf8 is transcriptionally activated in the tumors from a silent state (17). This is the third member of the FGF family to be activated in this system, indicating that Fgfs and Wnts are strong collaborators in inducing mammary tumors.

As we have already demonstrated, this infected Wnt1 transgenic system can be used to identify novel or/and unexpected oncogenes that are involved in mammary tumorigenesis, thereby demonstrating oncogenic cooperation with Wnt1 and elucidating the multiple steps involved in murine mammary tumorigenesis. At the time of the original proposal, we still had ~55% of the mammary tumors from infected Wnt1 transgenic mice with new proviral insertions in which the known targets of MMTV mutations are not affected.

My specific aims:

1. Isolation and identification of proto-oncogenes (novel and unexpected) insertional activated by MMTV in tumors of infected Wnt1 transgenic mice.
   - Identification of proviral-cellular junction fragments.
   - Clone cellular sequences flanking the proviral insertion.
   - Locate and isolate the activated gene in the locus using Northern blot and exon trap strategies.
   - Determine the expression pattern of the gene in normal tissues and in tumors.

2. Characterization of the gene and analysis of the oncogenic potential of the identified proto-oncogene.
   - Demonstrate the oncogenic potential of the isolated proto-oncogene in cell culture transfection assays.
   - Demonstrate the gene's oncogenic potential in vivo using transgenic mice.

3. Demonstration of the cooperativity of Wnt1 with the proto-oncogene that is activated by MMTV.
   - Demonstrate cooperativity by cotransfection of C57MG cells.
   - Obtain definite proof of cooperativity by generating bitransgenic mice.

**BODY OF ANNUAL REPORT**

**Specific Aim 1. Isolation and identification of proto-oncogenes insertional activated by MMTV in tumors of infected Wnt1 transgenic mice.**

**Task-1:** Cloning the junction fragment(s), and isolation of the gene(s) activated by MMTV insertions.
A proviral-cellular junction fragment from tumor #76 from infected Wnt-1 transgenic mice was identified and cloned. Screening of the tumor panel by Southern analysis using cellular probes derived from this cloned region detected insertions in 12 of 85 tumors within this locus indicating that this is a new common insertion locus for MMTV. Northern analysis and exon trapping procedures failed to identify any coding sequences within the cloned region.

Mapping of tumor 76 locus to mouse chromosome:

In the process of identifying the gene activated by proviral insertions in this locus, I decided to localize this locus to the mouse chromosome. I reasoned that this process would save time, provide reasonable determination if the cloned fragment was located near a previously identified MMTV insertion site, and/or indicate any probable "candidates" for MMTV activations. The locus was mapped to the chromosome using the Jackson laboratory Backcross DNA Panel Map service.

Briefly, a restriction length polymorphism (RFLP) was identified between the two mouse strains M.spretus and C57BL/6. This RFLP was traced through the entire BSS reciprocal cross panel by Southern analysis. The result from this analysis was sent to Jackson lab and, thorough analysis of the database mapped the locus very nicely to the distal region of mouse chromosome-7. This locus was linked to the int-2/Fgf-3 locus unfortunately suggesting that this was probably the most likely candidate to be activated by MMTV proviral insertions. However, previous analysis of these tumors did not detect MMTV insertions within the int-2 locus (15). This suggests that either these insertions are not activating int-2 but probably other genes that are linked to this locus, or that these insertions are long-range activations of int-2. I decided to address both possibilities.

Identification of the proto-oncogene activated by MMTV insertions in this locus:

To test if there were activations in other genes, I performed Southern analysis using DNAs from these tumors digested with several enzymes. Southern blots were hybridized to probes containing coding sequences of the following genes: Cyclin D1, H-19, IGF-2, and hst. These genes are located near int-2 on mouse chromosome-7 and seemed to be good candidates for MMTV activations. Cyclin D1 has been implicated in tumorigenesis and overexpression of cyclinD1 in the mammary gland using a MMTV promoter results in hyperplasia and tumor formation (24). H-19 has been shown to function as a tumor suppressor and therefore inactivation of the gene could result from MMTV insertions. Hst/Fgf4 is a previously described target for MMTV activations (15). Results from these experiments showed no rearrangements in any of the tumors, when probed with any of the genes.

Long range activations of genes by MMTV have been previously described by many investigators (5,10). Peters et al showed long range activations of Int-2/Fgf-3 by MMTV insertions ~20kb upstream of the gene. Comparison of the restriction maps between my lambda clones and their published map showed that the new cluster of insertions that I identified maps to the same place as that previously published (10). This further supports the idea of long range activations. Northern analysis of some of the tumor RNAs probed with an int-2 probe showed activation of the gene in the tumors and no expression in mammary gland controls, proving that Int-2/Fgf-3 is the gene activated by MMTV in these tumors.

The Oncogenic potential of int-2/Fgf-3 has already been demonstrated, and others & we have shown int-2/Fgf-3 to be a strong oncogenic collaborator of Wnt-1 in promoting mammary carcinogenesis (7,11,15,16). Because of this fact, I slightly altered the focus of my proposal from that originally described. This change does not affect or change my primary goals towards identifying genes involved in the multistep process of mammary tumorigenesis, nor does it alter the specific aims and focus of research described in the original proposal. The only difference is
that further characterization of the identified gene int-2 was not performed, instead I pursued the characterization of a gene (Fgf-8) that I had previously identified to be frequently activated by MMTV proviral insertions in these infected Wnt-1 transgenic mice (17).

Prior to cloning the tumor 76 insertion, I had cloned another junction fragment from tumor 111 and identified (in collaboration with Craig MacArthur a fellow in the lab) the gene Fgf-8 or Androgen Induced growth factor (Aigf) to be activated by MMTV proviral insertions in 10% of tumors from infected Wnt-1 transgenic mice (17). Fgf-8 was the third member of the fibroblast family of growth factors to be activated in these tumors further supporting the evidence that Wnt and Fgf family of growth factors are strong oncogenic collaborators. I published these results as a co-author in the Journal of virology (17).

Specific Aim 2. Characterization of the gene and analysis of the oncogenic potential of the identified proto-oncogene.

Task-2a: Demonstration of the oncogenic potential of the isolated proto-oncogene in cell culture assays.

Fgf8 consists of at least six exons and codes for at least seven protein isoforms, due to alternative splicing of the primary transcript (17,18). We analyzed the oncogenic potentials and differences in the biological activities of three isoforms (8a, 8b, and 8c) in NIH3T3 cells and showed that the isoform Fgf8b was highly transforming in NIH3T3 cells and highly tumorigenic in nude mice, while the other two isoforms showed moderate to low transforming potentials (22).

Oncogenic potential of human FGF8 isoforms:

Additionally, I also tested the human isoforms of FGF8 for their oncogenic capabilities (cDNAs obtained from Dr. P. Roy-Burman). The human isoforms of FGF8 were isolated by reverse transcription-polymerase chain reaction (RT-PCR) from a human prostate tumor cell line. Three different isoforms were isolated which correspond to the murine Fgf-8a, 8b and 8e isoforms. The human FGF8a and FGF8b exhibit identical amino acid sequences to their murine counterparts, while FGF8e shows a partial variation from the corresponding murine isoform in the additional exon found in both species (17,18).

To address the biological effect of specific isoform expression, the cDNAs corresponding to the different isoforms (FGF8a, 8b, and 8c) were cloned into the eukaryotic expression vector pcDNA (Invitrogen). The corresponding plasmids together with the empty vector control were transfected into NIH3T3 cells by Lipofectamine mediated transfection (GIBCO-BRL). Twenty-four hours after transfection, the cells were selected for stable transfectants with 400 μg/ml of Geneticin (G418, GIBCO-BRL). At least 60-70 colonies were pooled from each transfection to generate stable cell lines that were used for transformation and tumoriginity assays.

The results from these analyses showed that, the cells expressing FGF8b cDNA exhibited marked morphological transformation from a flat uniform organization (vector controls) to a highly elongated, spindle shaped, refractile morphology. The cells expressing FGF8a and FGF8e cDNAs showed moderate degrees of transformation when compared to the normal morphology of the control cells, but much less than FGF8b. The FGF8b cells also showed higher saturation densities at confluence when compared to the controls, indicating loss of contact inhibition (Figure 3). FGF8a and FGF8e cells also displayed loss of contact inhibition at confluence, but their saturation densities were lower than FGF8b. These results correspond closely to those we described previously for mouse Fgf8 isoforms (22). A manuscript describing these findings has been published in the journal Cell, Growth, and Differentiation (29).
Task-2b: Confirmation of the oncogenic potential in vivo using transgenic mice.

The Fgf8 transgene:

The transgene for the Fgf8 transgenic mice was directly cloned from a tumor (tumor 86) that has an MMTV insertion very close to the 5' end of the Fgf-8. This particular insertion is in a "promoter insertion" orientation, i.e., it is upstream from the initiation codon in the same transcriptional orientation. The transgene contains the 3' MMTV LTR, and the entire genomic Fgf8 gene in a single DNA fragment of ~13 Kb (Figure 1).

Fgf8 transgenic mice:

The founder animals generated were screened by Southern blots of the tail DNAs for the presence of the transgene. We had three transgenic founders that contain the MMTV/Fgf8 transgene. These animals were bred to normal BALB/c mice to generate independent lines. Since the transgenic founders were poor mothers we were unable to establish independent lines from the female mice and the one we established from the male founder did not express the transgene. Mammary tumors started developing in the transgenic mice between 5-7 months. The tumors were resected from the animals and processed for histological and northern analyses.

Expression of the transgene in the mammary gland and tumors.

Very high expression of Fgf8b RNA was seen in the tumors. Expression was also detected in the mammary gland RNA and interestingly in the ovaries. Even though normal expression of Fgf8 can be found in adult ovaries. It could be detected only with a northern blot containing 20 μg of Poly(A) RNA (17), whereas I was able to see a signal with 10 μg of total RNA with 3 hrs exposure (Figure 2).

Histology of the tumors

The tumors were formalin fixed, paraffin embedded and sections were stained with Hematoxylin and Eosin. The stained sections were analyzed microscopically. The tumors from the transgenic animals were identified to be ductal adenocarcinomas with varying degrees of pathogenicity, ranging from benign adenomas to clearly invasive papillomas. In addition we also found mammary hyperplasia and hyperplasia of the ovarian stroma. (32) (Figure 3 & 4)

Biological effects of murine Fgf8 isoforms on mammary epithelial cells

In my previous report I had described an interesting property that I had discovered while analyzing the differences in the biological activities of the Fgf8b protein isoforms. I had found that mammary epithelial cells either over expressing Fgf8b or when treated with 8b protein containing conditioned medium, underwent apoptosis. I demonstrated that the cell death was indeed due to apoptosis by Hoescht staining and DNA ladder analyses. Since this was the first observation so far that Fgfs could cause apoptosis of mammary epithelial cells, to better test our results, we partially purified Fgf8b using a heparin sepharose affinity column and demonstrated induction of apoptosis by the partially purified Fgf8b protein.

In the last year I have done more experiments on this property of Fgf8b. I will describe the results in this report. Eventhough these experiments were not originally proposed, they are highly significant to my proposal, and also to the understanding of oncogenic cooperation in this system.
Induction of apoptosis by several members of the FGF family of growth factors.

To test if the phenomenon of apoptosis was specific to only Fgf-8b or was also seen with any other FGF family member(s), I used purified human FGF1,2,4,5,6,7,and 9 (R&Dsystems) to treat C57MG cells. 100ng/ml of the FGF proteins was added to the cells at 40%confluence with 0.1mg/ml of Heparin. NIH 3T3 cells were used as a control to assay the activity of the protein. Cells treated with FGF1, FGF2, FGF4, FGF6 and FGF9 showed morphological transformation and apoptotic cell death similar to Fgf8b. FGF2, FGF4 and FGF9 showed this effect even in the absence of heparin. FGF5 did not show any show any changes at concentrations of 200 ng/ml. Cells treated with 500 ng/ml of FGF5 showed moderate transformation but no apoptosis, and cells treated with 1mg/ml of the growth factor showed both transformation and apoptosis. The only growth factor that was tested but did not show any activity was FGF7. Even 1mg/ml of FGF7 did not show any changes in morphology.

Table 1. Apoptosis of mammary epithelial cells is induced by several members of the FGF family

<table>
<thead>
<tr>
<th>FGF *</th>
<th>Transformation of NIH3T3 Cells</th>
<th>Apoptosis of C57MG cells</th>
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<tbody>
<tr>
<td>FGF1</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>FGF2</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>FGF4</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>FGF5</td>
<td>+++</td>
<td>+/-†</td>
</tr>
<tr>
<td>FGF6</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>FGF7</td>
<td>+++</td>
<td>–‡</td>
</tr>
<tr>
<td>FGF8b</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>FGF9</td>
<td>+++</td>
<td>+++</td>
</tr>
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</table>

* FGFs were added at 100 ng/ml final concentration and cells observed for four days.
† Apoptosis observed only at 1 µg/ml FGF5; transformation of C57MG but no apoptosis at 500 ng/ml; no effects at 200 ng/ml.
‡ No apoptosis or transformation at 1 µg/ml FGF7.

Neutralization experiments using a polyclonal antibody against human FGF2 completely blocked the apoptosis seen on treatment with FGF2 in C57MG cells (Figure 5). Cells treated with FGF2 showed transformation and apoptosis by days 3-4. However, the cells treated with FGF2 that was pre incubated with the antibody did not show any alteration in cellular and nuclear morphology, indicating that the apoptosis seen was indeed a result of treatment with FGF2/FGFs.

To address the possibility that this effect is not something specific to this cell line, we tested several other mammary cell lines (normal and tumor) for induction of apoptosis by FGF2, FGF4, FGF6 and FGF8. Three cell lines (C127I, BMG, GR ) resembled C57MG in all respects; cellular transformation followed by apoptosis. HC11 cells showed some degree of apoptosis but cell death was not complete as seen in the other cell lines. The human breast carcinoma cell line MCF-7 when treated with the different FGFs did not undergo programmed cell death (Table 2). One explanation to this unresponsivenes to FGF signals is that MCF-7 cells are known to express high levels of the anti-apoptotic gene product Bcl-2. Therefore, Bcl-2 may be inhibiting or blocking the apoptotic signal provided by the FGF. Alternatively, MCF-7 being a carcinoma cell
line could have gone through several genetic alterations, some of them may result in providing survival functions for the cells.

Table 2. FGFs induce apoptosis of several mammary epithelial, but not fibroblast, cell lines *

<table>
<thead>
<tr>
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<th>Apoptosis</th>
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<tr>
<td><strong>Mammary epithelial cells</strong></td>
<td></td>
</tr>
<tr>
<td>C127I</td>
<td>+++</td>
</tr>
<tr>
<td>BMG</td>
<td>+++</td>
</tr>
<tr>
<td>GR</td>
<td>+++</td>
</tr>
<tr>
<td>HC11</td>
<td>+++/- †</td>
</tr>
<tr>
<td>MCF-7</td>
<td>-</td>
</tr>
<tr>
<td>S115 (negative control)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Fibroblasts</strong></td>
<td></td>
</tr>
<tr>
<td>NIH3T3</td>
<td>-</td>
</tr>
<tr>
<td>Rat-1</td>
<td>-</td>
</tr>
</tbody>
</table>

* Apoptosis was assessed using FGF2, FGF4, FGF6 and FGF8b at 100 ng/ml. All caused similar effects on an individual cell line.
† HC11 cells appeared to be composed of two cell populations, one that responded to FGFs with apoptosis and one which was nonresponsive

Inhibition of FGF induced programmed cell death by Bcl2

The Bcl2 protein is known to block apoptotic cell death induced by various agents (31). However, Bcl-2- independent apoptotic pathways are also known. We tested to see if Bcl-2 could inhibit FGF induced apoptosis of mammary epithelial cells. C57MG cells were transfected with an expression vector containing the Bcl-2 cDNA. Stable clones expressing the Bcl-2 gene product were generated and treated with FGF2, FGF4 and FGF6. Stable clones expressing the vector alone was used as controls. The treated cells were observed for a period of 4 days. The control cells (clones transfected with vector alone) showed morphological changes within 12-16 hrs after treatment. By 24 hr's many cells started dying and coming off the plate. By day 3 after treatment approximately 80% of the cells were apoptotic. In contrast, the Bcl-2 clones remained viable and were morphologically transformed from a flat cuboidal morphology to a more elongated spindle shaped appearance (Figure 6). No apoptosis was observed suggesting that Bcl-2 can block the apoptotic signal from FGF. However, on days 4 and 5, when the control cells were completely dead, the Bcl-2 clones started showing signs of apoptosis. This result could be explained by the possibility of transient expression of the protein from the vector or poor stability of the protein. It could also be possible that Bcl-2 does not completely inhibit apoptosis but delays the onset. This effect has been previously described for Bcl2 (31).

To test for deregulation of members of the Bcl2 family, FGF-treated mammary epithelial cells was assayed by northern and western analysis for upregulation of the proapoptotic genes like Bax and Bik and down regulation of anti-apoptotic genes like Bcl2 and BclxL. RNA and protein have been extracted from C57MG cells treated with FGF2, FGF4, FGF8 and controls, and analyzed in the process of generating northern and western blots which will be probed with Bcl2, BclxL Bax, and Bik cDNAs and antibodies (Calbiochem).

If down regulation of Bcl2 or BclxL is observed, then these genes will be overexpressed in C57MG cells using a retroviral (LNCX) or plasmid (pMIRB) expression vectors. Clones of
cells expressing the gene at high levels will be generated and then treated with FGFs to see if this can inhibit or suppress FGF mediated apoptosis thereby indicating that Bcl2 or BclXL is involved in this pathway.

Specific Aim 3: Demonstration of the cooperativity of Wnt1 with the proto-oncogene that is activated by MMTV.

Task 3a: Demonstrate cooperativity by cotransfection of C57MG cells.

Since Fgf8b by itself is highly transforming in NIH 3T3 cells, and induces apoptosis of C57MG mammary epithelial cells, it would not be possible to demonstrate oncogenic cooperativity as defined in the classical experiments. However, we could speculate that the cooperation may not be the synergistic action of two oncogenes, but maybe Wnt 1 provides survival signals to the cells, that have received the death signal from Fgf8b and hence cooperation towards cellular proliferation leading to transformation. To test this hypothesis, Wnt1 was expressed in C57MG cells by transfection of a retroviral expression vector carrying the Wnt1 cDNA epitope tagged with HA (heme agglutinin) (LNXC-Wnt1HA). Stable clones were generated by selection in G418 (Life technologies). Cells transfected with the vector containing the HA tag alone will serve as the controls. The clones generated are being tested for expression of Wnt1 at the RNA and protein levels. The Wnt1 clones with stable and high expression of the protein will be treated with FGF proteins (FGF2, FGF-4 and FGF-8) as described previously. The cells will be observed for transformation and apoptosis. Suppression of apoptosis if present will also be quantitatively determined in flow cytometry assays using Annexin V and propidium iodide staining. If Wnt1 can suppress apoptosis even partially it can be determined using this assay. An alternate approach would be to use soluble wingless (drosophila) as the source of Wnt protein and treat C57MG cells with wg and FGF, wg alone and FGF alone. If soluble wingless protein can be produced, we will perform this experiment to corroborate the Wnt1 cell clone approach.

Task 3b: Obtain definite proof of cooperativity by generating bitransgenic mice.

Unfortunately we have had a severe set back in our experiments, because of our inability to generate Fgf8 transgenic lines. In addition since we discovered FGF induced apoptosis, we have concentrated on characterization of this finding and hence this specific aim could not be accomplished in the specified time frame.

CONCLUSIONS

A proviral-cellular junction fragment was identified and cloned form a tumor from infected Wnt1 transgenic mice. Twelve of eighty-five tumors tested had MMTV insertions within this locus. Analyses of these tumors showed that this newly identified cluster of MMTV insertions activate the previously characterized proto-oncogene Fgf3, over a long range (~20kb upstream of the gene). Since this gene has already been identified as an oncogenic collaborator of Wnt-1, I changed my focus to characterization of another gene Fgf8 that I had previously cloned from a tumor from infected Wnt1 transgenic mice, and identified to be activated from a silent state in 10% if the tumors. This gene encodes at least seven different protein isoforms, three (Fgf8 a, b and c) of which were isolated in our lab.

Characterization of the biological activities of the different isoforms in mammary epithelial cells identified a new property (apoptosis) for the isoform Fgf8b. Stimulation of
C57MG cells by Fgf8b containing conditioned medium resulted in apoptotic cell death as shown by characteristic nuclear changes and DNA laddering. This finding was further proved by demonstration of apoptosis using partially purified Fgf8b protein (heparin sepharose affinity chromatography). This property was not confined to Fgf8b alone, but several other members of the FGF family of growth factors show the same effect. Apart from C57MG cells, other mammary epithelial cells also undergo apoptosis when induced by FGFs. Overexpression of Bcl2 in C57MG cells delayed the onset of apoptosis induced by FGFs.

To demonstrate the oncogenicity of Fgf8 in vivo, the mouse Fgf8 transgene was cloned from a tumor with insertion in this locus (tumor 86). Three founders were obtained and unfortunately we were unable to establish any transgenic lines. We are currently injecting mouse embryos to generate more transgenic mice. The female founders developed tumors in 5-7 months. The tumors and surrounding mammary tissue highly expressed the transgene. The tumors were found to be ductal adenocarcinomas and stromal hyperplasia of the ovaries was also observed. In Summary, the results from this study has led to the discovery of a new property for fibroblast growth factors; induction of programmed cell death. Further characterization of this property and the signal transduction pathway could be of great help in the design of rational therapeutic interventions.

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ABSTRACTS AND PRESENTATIONS


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Personnel. Deepa B. Shankar, 100% effort.
Figure 1. Expression of the Fgf8 transgene in mammary tumors and some adult mouse tissues. Northern blot of total cellular RNA (10 mg) from three normal tissues (salivary gland (Sg), mammary gland (Mg), and ovary (Ov)), and three tumors from Fgf8 transgenic mice (1, 2, & 3). The blot was probed with a full length Fgf8b cDNA (upper panel). Ethidium bromide stained photograph of the RNAs (lower panel).
MMTV-Fgf8 Transgene

Figure 2
Figure 3. Histopathology of the MMTV-Fgf8 mammary tumors. Histological section through fixed mammary gland tumors from MMTV-Fgf8 transgenic mice. A. benign adenoma (100x), B. invasive adenocarcinoma (100x). The sections were stained with hematoxylin and eosin.
Figure 1. Pathology of the ovaries from MMTV-Fgf8 transgenic mice. Histological section of an ovary from Fgf8 transgenic mouse showing extensive stromal hyperplasia (100x magnification). The sections were stained with hematoxylin and eosin.
Figure 5. Neutralization of FGF2 induced apoptosis of mammary epithelial cells by an anti-human FGF2 antibody. C57MG cells were treated with FGF2 protein, anti-FGF2 antibody, or FGF2 protein preincubated with the FGF2 antibody. The cells treated with the neutralized protein showed no evidence of transformation or apoptosis in contrast to the cells treated with the FGF2 protein alone.
MMTV-Fgf8 transgenic mice develop mammary and salivary gland neoplasia and ovarian stromal hyperplasia

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Prior studies have identified Fibroblast Growth Factor-8 (Fgf8) as a possible proto-oncogene in mouse mammary tumorogenesis. We now report on the generation of two types of Fgf8 transgenic mice that each utilize the mouse mammary tumor virus (MMTV) promoter. The first transgene (MMTV-Fgf8/h) results in the overexpression of the Fgf8b isoform exclusively. Male and female MMTV-Fgf8/h transgenic mice are viable and fertile. RNA for Fgf8b is detected in mammary gland and salivary gland tissues of transgenic mice by Northern blot analysis. Nearly 85% of breeding transgenic female mice developed mammary lobular adenocarcinomas by 12 months of age, while no tumors developed in non-transgenic littermates. Salivary gland tumors occurred in some animals, always in association with mammary tumors. Several MMTV-Fgf8b transgenic mice had lung metastases at necropsy. The second transgene (MMTV-Fgf8) uses the entire Fgf8 gene and potentially encodes all Fgf8 isoforms. Fgf8 is expressed by this transgene in several tissues in addition to those described above, notably the ovaries. The two MMTV-Fgf8 founders developed mammary ductal adenocarcinomas at five and eight months of age, and both displayed ovarian stromal hyperplasia. The founders expressing either transgene did not successfully nurse their pups. These results demonstrate that production of Fgf8b, and possibly other Fgf8 isoforms, in the mammary and salivary glands contributes to oncogenesis, and that ovarian expression results in stromal hyperplasia.

Keywords: Fibroblast Growth Factor; FGF8; Fgf8; mammary tumorogenesis; oncogenesis; breast cancer

Introduction

Most carcinogenic processes are multi-step, and understanding the multiple genetic events involved in mammalian carcinoma may lead to rational interventional strategies (Harris et al., 1992a,b). As one of these potential steps, alterations and/or over-expression of members of the Fibroblast Growth Factor (FGF) family and their receptors have been described in human breast cancers (Jaakkola et al., 1993; Jacobson et al., 1994; Penault-Llorca et al., 1995; Payson et al., 1996; Bansal et al., 1997; Yangou et al., 1997).

FGFs are secreted peptide growth factors that are thought to have most of their biological effect by binding to membrane receptor tyrosine kinases that transmit intracellular signals that ultimately affect cellular growth and/or differentiation programs (Basilico and Moscatelli, 1992; Johnson and Williams, 1993; Ornitz et al., 1996).

As human carcinogenic processes are temporally long and have limited potential for experimental exploitation, animal models of mammalian gland carcinogenesis are necessary to rapidly advance our knowledge and our potential for intervention (Harris et al., 1992a,b). Few mouse models of FGF in mammary gland cancer exist. Insertional activation of Fgfr3/int-2 (Dickson et al., 1984), Fgfr4/int2 (Peters et al., 1989), and Fgfr8 (MacArthur et al., 1995c; Kapoun and Shackleford, 1997) by mouse mammary tumor virus (MMTV) in murine mammary cancers have been described. Female MMTV-Fgf3 transgenic mice develop mammary gland cancer after several months of epithelial hyperplasia, while male MMTV-Fgf3 mice develop prostatic hypertrophy (Muller et al., 1990; Ornitz et al., 1991) Similarly, MMTV-Fgfr7 transgenic mice develop the same phenotype as MMTV-Fgf3 transgenic mice (Kitsberg and Leder, 1996). Both Fgf3 and Fgfr7 proteins activate the KGF receptor (Fgfr2b) (Miki et al., 1991; Mathieu et al., 1995), so it is not surprising that these FGFs, when over-expressed in the mammary gland as transgenes, result in similar mammary cancer phenotypes. Cooperativity of FGFs with Wnt genes in mammalian gland cancer development has been demonstrated by two approaches: (1) more rapid tumorogenesis by insertional activation following MMTV infection of transgenic mice (Shackleford et al., 1993; Lee et al., 1995; MacArthur et al., 1995c; Kapoun and Shackleford, 1997); and (2) more rapid tumorogenesis in bitransgenic Fgf3/Wnt1 mice compared to the single transgenic mouse (Kwan et al., 1992).

Fgf8 was originally identified as a gene encoding an androgen inducible growth factor (Tanaka et al., 1992). We identified Fgf8 as a gene that is frequently activated by MMTV insertion in MMTV-Wnt1 transgenic mice (MacArthur et al., 1995c; Kapoun and Shackleford, 1997). Fgf8 encodes multiple protein isoforms by alternative splicing at the 5' end of the gene (Crossley and Martin, 1995; MacArthur et al., 1995c), and these protein isoforms activate Fgfr2c, Fgfr3c, and Fgfr4 (MacArthur et al., 1995b; Blunt et al., 1997). Normal functions of Fgf8 are thought to include paracrine signaling during gastrulation, limb and central nervous system development (Crossley and Martin, 1995; Crossley et al., 1996a,b; Meyers et al., 1996).
FGF8b protein can transform NIH3T3 cells, demonstrating an oncogenic potential for at least one of the FGF8 isoforms (Kouhara et al., 1994; MacArthur et al., 1995a). FGF8 expression has been described in human breast cancer samples (Tanaka et al., 1995; Payson et al., 1996). Here, we demonstrate that overexpression of FGF8b, and possibly other isoforms, in the mammary gland results in mammary tumorigenesis, confirming that overexpression of one or more isoforms of Fg8 is oncogenic for the mammary gland.

**Results**

**Generation of MMTV-Fg8 transgenic mice**

To determine the mammary oncogenicity of Fg8, we produced two transgenes under the control of the MMTV LTR (Figure 1). The MMTV-Fg8b transgene contains the Fg8b cDNA and encodes only the FGF8b protein isoform (Figure 1a). The MMTV-Fg8 transgene (Figure 1c) was cloned from genomic DNA from a previously described mammary tumor possessing a MMTV insertion into the Fg8 gene (tumor 86, MacArthur et al., 1995c). PCR analysis of tail DNA from potential MMTV-Fg8b founder transgenic animals revealed three founder animals that possessed the 179 bp amplified fragment (Figure 1b). The lower molecular weight fragment in Figure 1b likely represents a 'primer-dimer' artifact, since it is observed when the primers are added and independent of whether template DNA is present (data not shown). Mouse #4 was a male that never transmitted the transgene to any of his 70 offspring (data not shown). Mouse #14 was a female that transmitted the transgene to her offspring, but none of the offspring expressed the transgene (data not shown). Mouse #16 (8b-16) was a female whose first two litters died within 48 h of birth; all offspring appeared normal at birth. Subsequent litters were rescued with foster mothers, and the transgene was found to be transmitted and expressed. Although the offspring from 8b-16 required rescue by foster mothers, subsequent generations of this transgenic line did not require foster mother rescue. Southern blot analysis of potential founders of the MMTV-Fg8 transgene revealed two female founders (Mouse #3 and #17, Figure 1d). Both MMTV-Fg8 transgenic animals produced pups that died within 48 h of birth, possibly due to an inability to deliver milk.

**Expression of MMTV-Fg8 transgenes**

Northern blot analysis of tissue RNAs from the 8b-16 transgenic line demonstrated strong expression of the transgene in normal parous mammary tissue and in mammary tumors with weaker expression in salivary glands (Figure 2a).

The MMTV-Fg8b transgenic founder #17 expressed the transgene in a somewhat different pattern, with higher levels of Fg8 RNA in mammary tumor, parous mammary glands, lung and ovary, and lower level in kidneys, salivary gland and spleen (Figure 2b). The MMTV-Fg8b transgenic founder #3 also expressed high levels of Fg8 RNA in mammary tumor and parous mammary glands (Figure 2b). A direct comparison showed that the extent of expression of the MMTV-Fg8b transgene was very similar to that of the MMTV-Fg8 transgene in mammary glands and mammary tumors (Figure 2b).

To further identify the cells responsible for transgene expression, we employed in situ hybridization of frozen tissue sections, using an anti-sense Fg8b riboprobe (Heikinheimo et al., 1994). We observed weak expression of the MMTV-Fg8 transgene in the ductal epithelium of the mammary gland (Figure 1. MMTV-Fg8 transgene constructs. Graphical representation of the MMTV-FGF8b (a) and MMTV-Fg8 (c) transgenes. Both transgenes are under the transcriptional control of the MMTV LTR (SSS) in the same orientation as the Fg8 construct. In MMTV-Fg8b (a), the Fg8b cDNA is indicated by ( ), and as arc rabbit β-globin exon ( ) and intron ( ) sequences of the MMTV-Fg8 transgene. In the MMTV-Fg8b transgene (a) translation starts and stops with the authentic Fg8b sites. Animals derived from the MMTV-Fg8 transgene were screened by PCR methods with primers shown in (a). The expected amplified band was 179 bp ( )). In (b), ( ) is the positive control, ( ) is the negative control, the numbers over the lanes represent potential MMTV-Fg8b transgenic mice, and numbers at left indicate the molecular weight markers in basepairs (bp). Animals #4, 14, and 16 were positive for the MMTV-Fg8b transgene (b). In the MMTV-Fg8 transgene (c), the entire Fg8 gene is present. The 15.8 kb transgene contains 12 kb of Fg8 sequence with 3.5 kb of the 3' end of an inserted MMTV provirus. In (c), Fg8 exons that encode protein in all isoforms are designated by ( ), alternatively spliced exons that encode protein in some isoforms are depicted by ( ), and non-coding exons are depicted by ( ). The XE 2.0 probe ( ) used for Southern blot screening of BglII (Bg) digested tail DNA is indicated below the transgene. In (d), a Southern blot of tail DNA digested with BglII and hybridized to probe XE 2.0 is shown. The transgene fragment is a 6 kb band with these conditions (c and d), while the wild type Fg8 gene gives a 22 kb band under the same conditions (c and d). Note that the numbers in (b) and (d) refer to different animals and transgenes.
Figure 2. Northern blot analysis of MMTV-Fgfb and MMTV-Fgfb transgene expression. (a) Upper panel: hybridized to murine 
Fgfb cDNA. Lower panel: hybridized with rat GAPDH cDNA. RNAs were prepared from brain (Br), heart (H), kidney (K), liver 
(Li), lung (Lu), mammary tumors (MT), mammary glands (MG), ovary (O), salivary glands (SG), spleen (Sp), seminal vesicle/ 
prostate (GU), and testis (Te). The 28S and 18S markers are indicated to the left of the blots. FO-1 and FO-2 refer to two female 
offspring of the founder female (FF #16) of the MMTV-Fgfb transgenic line. MO-1 refers to a male offspring of FF #16. (b) Upper 
and middle panels: hybridized to murine Fgfb cDNA. Middle exposure is approximately 10 times longer than upper exposure. 
Lower panel: hybridized to rat GAPDH cDNA. Fgfb refers to the FO-1 animal in (a), the MMTV-Fgfb transgenic line. Fgfb #3 
and #17 are the founder transgenic mice for MMTV-Fgfb transgene.

3a,b). In contrast, expression of the MMTV-Fgfb transgene in lobular carcinomas from breeding animals was much higher (Figure 3a,b). No expression of the MMTV-Fgfb transgene was detected in kidney (Figure 3c,d) or normal lung (Figure 3e,f). These in situ results (Figure 3) confirm the expression of the MMTV-Fgfb transgene in the 8b-16 transgenic line observed by Northern blot analysis (Figure 2) and localize its expression to the epithelial component of the hyperplastic and malignant mammary glands, as in MMTV-Fgfb and MMTV-Fgfb transgenic mice (Muller et al., 1990; Ornitz et al., 1991; Kitsberg and Leder, 1996).

Tumor incidence in MMTV-Fgfb transgenic mice.

Forty-two transgenic females, 20 transgenic males, and 20 non-transgenic littermates (10 males and 10 females) from the MMTV-Fgfb transgenic line 8b-16 were followed for the development of tumors (Figure 4). The transgenic females were separated into two groups: one group remained virgins (23 mice), and the other group was allowed to breed (19 mice). None of the 20 non-transgenic control littermates developed any type of tumor in the one year they were followed (Figure 4). Breeding transgenic females developed mammary gland tumors with a several month latency (Figure 4). The majority of tumors in this breeding cohort developed after six months of age and after multiple pregnancies (average number of pregnancies was six). Four virgin transgenic females developed fairly rapid tumors (all less than 4 months of age), but the remainder of the virgin transgenic mice did not develop tumors after one year (Figure 4). One transgenic male developed a mammary gland tumor (Figure 4). Salivary gland tumors were seen in three mice, all of which had
coincidental mammary gland tumors. The two actively breeding MMTV-Fgfb transgenic founder animals (Mice #3 and #17, Figure 1d) developed mammary tumors at a similar rate, showing tumors at five and eight months of age.

**Histopathology of MMTV-Fgfb transgenic mice**

Histopathological analysis of tissues from MMTV-Fgfb transgenic mice (line 8b-16) demonstrated ductal hyperplasia in the breeding females (Figure 5a), which preceded tumor development. The mammary tumors in this MMTV-Fgfb transgenic line were lobular carcinomas in all cases (including the transgenic male) (Figure 5b,c, and data not shown). In eight of 20 of these MMTV-Fgfb transgenic mice with mammary tumors, pleural-based metastases in the lungs were observed both grossly (data not shown) and microscopically (Figure 5d). Three animals with mammary tumors also had coincidental salivary gland tumors. These salivary gland tumors were adenocarcinomas that were well-circumscribed in all cases (Figure 5e).

![Figure 4](image)  
**Figure 4** Tumor incidence versus age in MMTV-Fgfb transgenic mice. The percentage of animals that remained tumor-free at each age in months is plotted. Female transgenic mice were split into 2 groups: breeding females (■) and virgin females (□). Non-transgenic littermates (10 male, 10 female) are indicated by (○), and transgenic males are indicated by (□). The total number of animals followed in each group are indicated in parentheses. The animals were followed for a maximum of 12 months.

![Figure 3](image)  
**Figure 3** *In situ* hybridization analysis of MMTV-Fgfb transgene expression. Bright field (a, c, e) and dark field (b, d, f) views of sections of frozen tissues derived from MMTV-Fgfb transgenic mice (100×), hybridized to anti-sense FGFβ cDNA and stained with hematoxylin and eosin. (a,b), mammary gland containing normal (top) and tumor (lower) tissue, with expression of the transgene being higher in the tumor tissue; (c,d), kidney, with no transgene expression; and (e,f), lung/bronchus, with no transgene expression.

![Figure 5](image)  
**Figure 5** Histopathology of MMTV-Fgfb transgenic mice. (a) Ductal hyperplasia in a mammary gland from a breeding female (400×). (b) Invasive lobular carcinoma in a mammary gland from a virgin female (100×). (c) Invasive lobular carcinoma in a mammary gland from a breeding female (400×). (d) Lung metastasis in a breeding female with an invasive lobular carcinoma (100×). (e) Salivary gland tumor (right) with adjacent normal salivary gland (left) (200×).
In the MMTV-Fgf8 founder animals, ductal hyperplasia (Figure 6a) was seen in most non-tumorous mammary glands, and an adenoma (Figure 6b) was present in one of the mammary glands of these animals at the time of the development of infiltrating ductal adenocarcinomas (Figure 6c). Metastases were not observed in these two animals. Interestingly, these two animals were observed to have ovarian enlargement at necropsy, and histological examination of ovarian sections revealed stromal hyperplasia (Figure 6d), presumably as a direct result of transgene expression in this tissue (Figure 2b).

Discussion

Prior studies suggested that activation and inappropriate expression of Fgf8 by MMTV insertion might be involved in mammary gland cancer development in mice (MacArthur et al., 1995a; Kapoun and Shackleford, 1997). FGF8 expression has been observed in human breast cancer cell lines (Payson et al., 1996; Wu et al., 1997), and the FGF8b protein isoform has been shown to transform NIH3T3 cells (Kouhara et al., 1994; MacArthur et al., 1995a). We now demonstrate that overexpression of the FGF8b isoform in the mammary and salivary glands (Figures 2 and 3), under the control of the MMTV LTR, results in the development of carcinomas of both tissues (Figure 5). Similarly, a transgene capable of overexpressing all of the FGF8 protein isoforms in several tissues results in the development of carcinomas of the mammary gland and ovarian stromal hyperplasia (Figure 6). Mammary ductal hyperplasia was seen in both transgenic lines, preceding the development of mammary adenocarcinomas in these transgenic lines (Figures 5 and 6). Although only one line of MMTV-Fgf8b transgenic mice was obtained, the expression patterns of the transgene, the corresponding phenotypes in the expressing tissues, and the similarity to the MMTV-

Fgf8 transgenic mice all strongly suggest that the observed phenotypes are due to the MMTV-Fgf8b transgene and not to insertion site effects.

In both MMTV-Fgf8 transgenic lines, the development of most of the mammary adenocarcinomas was prolonged (Figure 4) and preceded by mammary ductal hyperplasia in the female mice (Figures 5 and 6). Only one tumor was seen in a male animal, and only four tumors in virgin females (Figure 4). The increased incidence of tumors in breeding females is likely due to higher expression of the transgene in the mammary glands with hormonal effects on the MMTV LTR (Haraguchi et al., 1992; Le Ricoussel et al., 1996; Haraguchi et al., 1997), together with the cell proliferation induced by cycles of pregnancy. The prolonged tumor latency indicates that additional genetic events are necessary for tumor development. Activated Wnt genes are likely candidates for one of these events in these tumors, given the strong oncogenic cooperation between FGFs and Wnts (Kwan et al., 1992; Shackleford et al., 1993; Lee et al., 1995; Kapoun and Shackleford, 1997).

Although mammary adenocarcinomas developed in both MMTV-Fgf8 transgenic lines, the histology was different in the two transgenic lines. MMTV-Fgf8b mice developed lobular adenocarcinomas (Figure 5) and MMTV-Fgf8 mice developed ductal carcinomas (Figure 6). Possible explanations for this difference in mammary gland cancer histology between these two Fgf8 transgenes include: (1) different levels of transgene expression; (2) different spatial or temporal expression of the transgenes in the mammary gland; and (3) effects of heterodimerization of FGF8 isoforms. Different quantitative levels of expression are possible, due to differences in transgene copy number, or insertion site. However, we did not detect significant differences in expression of the transgenes, as detected by Northern blot (Figure 2b). Different spatial or temporal expression patterns in the mammary gland of the two transgenes might be due to differences in the transgene insertion sites, or perhaps more likely to enhancers present in the MMTV-Fgf8 transgene that are not present in the MMTV-Fgf8b transgene. We have previously demonstrated that the different FGF8 isoforms interact with the different FGFRs in a redundant fashion, with FGF8b activating FGFR2c, FGFR3c, and FGFR4 (MacArthur et al., 1995b; Blunt et al., 1997). Our work did not address the possibility that different FGF8 isoforms might heterodimerize and interact in a quantitatively or qualitatively different fashion with the known FGFRs (McKeehan and Kan, 1994). Thus it is possible that quantitatively or qualitatively different interactions due to potential heterodimerization of FGF8 isoforms present in the MMTV-Fgf8 transgenic mammary gland might be responsible for the ductal tumor histology observed in this line.

Another intriguing difference between the two transgenic lines produced here is the ovarian stromal hyperplasia of the MMTV-Fgf8 transgenic mice (Figure 6). This phenotype is rare, if not unique, among mice generated using MMTV LTR-driven transgenes. Since both MMTV-Fgf8 transgenic founders of this transgene displayed the ovarian hyperplasia, it seems probable that the transgene, which consists of the entire Fgf8 gene and at least 6 kb of downstream

Figure 6 Histopathology of MMTV-Fgf8 transgenic mice. (a) Non-neoplastic mammary gland (100 x). (b) Adenoma of mammary gland (100 x). (c) Invasive ductal carcinoma of the mammary gland (100 x). (d) Stromal hyperplasia of ovary (100 x)
genomic sequences, contains enhancers that promote expression in ovarian tissue. Indeed, the ovary is one of the few sites of normal Fgf8 expression in the adult mouse, suggesting that the Fgf8 gene contains such ovary-specific enhancers (MacArthur et al., 1995c).

Fgf8b is known to be produced by epithelial cells, and to activate in a paracrine manner Fgfr2c, Fgfr3c, and Fgfr4, which are primarily located in mesenchymal locations (MacArthur et al., 1995b; Ornitz et al., 1996; Blunt et al., 1997). We demonstrate expression of the MMTV-Fgf8b transgene in the epithelial compartment of the mammary tumors (Figure 3). Given the observed Fgf8b interactions with Fgfrs, we would not expect Fgf8b to act in an autocrine manner to stimulate mammary epithelial growth and tumorigenesis directly. Fgf7 is known to be produced by mesenchymal cells and to interact with epithelially localized Fgfr2b, in a paracrine fashion (Rubin et al., 1995; Ornitz et al., 1996). Fgf7 is a physiological stimulus for mammary epithelial growth (Coleman-Knacak and Rosen, 1994; Imagawa et al., 1994; Ulrich et al., 1994). Furthermore, MMTV-Fg7 transgenic mice develop mammary adenocarcinomas with lung metastases (Kitsberg and Leder, 1996), similar to our MMTV-Fgf8b transgenic mice. On the basis of these findings, we speculate that overexpression of Fgf8b in the epithelial compartment results in the up-regulation of Fgf7, or another epithelial growth factor(s), in the mesenchymal compartment of the mammary gland, which then provides the stimulus for mammary epithelial growth, and ultimately ductal hyperplasia and adenocarcinomas. This coupled paracrine stimulation occurs in vertebrate limb development, where Fgf10 (an FGF closely related to Fgf7) in limb mesenchyme induces expression of Fgf8 in the apical ectodermal ridge, and Fgf8 expression in the apical ectodermal ridge maintains Fgf10 expression in the mesenchyme (Obuchi et al., 1997).

The MMTV-Fgf8b transgenic line displayed a metastatic phenotype, similar to MMTV-Fg7 transgenic mice (Kitsberg and Leder, 1996), with several of our animals becoming ill-appearing during the development of the tumors. This is an uncommon phenotype for mouse models of mammary gland cancer. The similarities in the MMTV-Fgf7 and MMTV-Fgf8b transgenic mouse phenotypes, and the possible cross-paracrine interactions between Fgf7 and Fgf8b make it difficult to sort out which FGF, if either, might be primarily responsible for the metastatic phenotype. Future work looking at the expression differences in hyperplastic mammary glands, primary tumors, and metastatic lesions from these mice might elucidate unique metastatic genes.

Materials and methods

Preparation of MMTV-Fgf8 transgenes

The MMTV-Fgf8 transgene was prepared by inserting the murine Fgf8b cDNA (MacArthur et al., 1995c) into the EcoRI site of pMMTV-TGFα (Matsumura et al., 1990), thereby replacing TGFα with Fgf8b. The pMMTV-Fgf8b plasmid was digested with AffIII (New England Biolabs) and XhoI (Promega), and the 3.4 kb MMTV-Fgf8b transgene (Figure 1a) was purified by low melting agarose gel electrophoresis (Life Sciences Tech.), followed by treatment with β-Agarase (New England Biolabs), and ethanol precipitation.

The MMTV-Fgf8 transgene was obtained by cloning the 15.5 kb BamHI fragment, containing the env gene 3' of the BamHI site in MMTV, the 3' LTR, and 12 kb of genomic sequence containing Fgf8, from mammary tumor 86 DNA (Figure 1c) (MacArthur et al., 1995c). This cloning was accomplished by digesting tumor 86 DNA with BamHI, size selecting the DNA on a low melting agarose gel and purifying the DNA with β-Agarase. The BamHI-digested DNA was ligated into pCI-3 (Stratagene), packaged into phage, and the resulting phage library screened for the correct insert with the genomic fragment, XE 2.0 (Figure 1c), and with a MMTV-env fragment, EB 0.9 (data not shown). The 15.5 kb MMTV-Fgf8 insert from the correct phage was isolated by digestion with BamHI, size selection in a 0.5% agarose gel, and purified using beads from the Lambda Quick Kit (BIO 101, Inc.) and ethanol precipitation.

Generation of MMTV-FG8 transgenic mice

Pronuclear injection of the MMTV-Fgf8b transgene into C57BL/6 x SJL F1 hybrid embryos, followed by insertion of the embryos into pseudo-pregnant females, was performed at the NICHD Transgenic Development Facility at the University of Alabama, Birmingham, USA. Potential founder animals were analyzed by PCR of tail DNA (1 µg), using a forward primer in the rabbit β-globin intron 2 (5'-GGCAACGTGCTGTATTTGTTG-3') and a reverse primer in the Fgf8b cDNA (5'-TCTGCTCCCCCCATGCTGTG-3'). PCR conditions were as follows: 95°C for 3 min, then 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Three of 18 potential founders were positive for the 179 bp amplified fragment, signifying possession of the MMTV-Fgf8b transgene (Figure 1b). The founder mice were bred to FVB mice, and offspring were analyzed by the PCR methods above.

MMTV-Fgf8 transgenic mice were prepared by pronuclear injection of the transgene into C57BL/6 x SJL F1 hybrid fertilized eggs, followed by placement of the embryos into pseudo-pregnant females at the Norris Cancer Center Transgenic Mouse Core Facility of the University of Southern California. Potential founder animals were screened by Southern blot analysis using Bgl II digestion of the DNA and the XE 2.0 probe. Southern blotting was performed as described (MacArthur et al., 1995c) on 10 µg of tail DNA. The normal Fgf8 genomic fragment is 22 kb in size with Bgl II digestion and XE 2.0 as the probe, the MMTV-Fgf8 transgene fragment is 6 kb in size (Figures 1c and 4d).

Analysis of transgene expression

Total RNA was prepared from tissues from transgenic mice by the guanidium isothiocyanate/acid phenol method (Chomczynski and Sacchi, 1987). RNAs (10-20 µg) were denatured in formaldehyde/HEPES-Acetate-EDTA with 50% formamide, and electrophoresed in 1% agarose/formaldehyde/HEPES-Acetate-EDTA gels as described (MacArthur et al., 1995c). Capillary blotting, UV-cross-linking, hybridization to random primed labeled DNA probes, washing and exposure to x-ray film was as described (MacArthur et al., 1995c).

Histological sections of MMTV-Fgf8b transgenic animals were prepared from tissues either fixed in 4% parafomaldehyde, or snap frozen in liquid nitrogen. Following fixation or freezing, the sections were cut by the Washington University Department of Molecular Biology and Pharmacology Histology Core Facility. Fixed sections were stained with
hematoxylin and eosin, and photographed with an Olympus BX60 microscope and a Olympus PM-30 automatic photomicrographic system. Frozen sections were subjected to in situ hybridization with 1 x 10^6 counts/min. of 3P-labeled antisense or sense riboprobes to the FGF8b cDNA, as described (Wilkinson, 1992). Bright-field and dark-field photomicroscopy was performed with an Olympus BX60 microscope and a Olympus PM-30 automatic photomicrographic system.

MTTV-Fgf8b transgenic tissues were prepared for histology by fixing in 10% formalin. Paraffin-embedded sections were cut, stained in hematoxylin and eosin, and photographed by the Children's Hospital Los Angeles Pathology Core Facility.

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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. For the FGF-8a isoform, 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 isoform results in moderate morphological changes in the NIH3T3 cells, and the transfected cells are weakly tumorigenic in nude mice. All three transfusion results 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 transformation 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 FGF 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 insertion 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), and FGF-5 (29), SF-6 (30), and at least one FGF-8 isoform, FGF-8b (pS17) (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-

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2 To whom requests for reprints should be addressed.
fection and expression of the Fgf-8a or the Fgf-8c cDNA in NH3T3 cells lead to more subtle morphological changes. The morphological changes in all cases can be produced by adding the Fgf-8 isoform to the culture medium. NH3T3 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 NH3T3 cells and suggest that they may have different functions in vivo.

Results

NH3T3 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 NH3T3 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).

NH3T3 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). NH3T3 cells transfected with the pMIRB vector alone (no Fgf-8 cDNA, called MIRB cells hereafter) were morphologically identical to nontransfected NH3T3 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 NH3T3 cells.

Transfected NH3T3 Cells Express the Fgf-8 cDNAs. To demonstrate expression of the transfected cDNAs, we prepared total RNAs from pools of G418® NH3T3 cells that were transfected with the Fgf-8 cDNA (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 NH3T3 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 proteins 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 Fgf-8 isoform proteins and bound the three Fgf-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 cloning 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 10⁵ 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 10⁷ 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 resected 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 pg/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 FGF receptors present on NIH3T3 cells.

The FGF-8b isoform has been shown to bind to a mutated FGFRI 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 FGFRI, or other FGF receptors, is unknown. No information is available concerning the ability of the other FGF-8 isoforms to bind the various FGF receptors. This information is important in order to understand the role of FGF-8 in mammalian development, as well as the potential tissue specificity of FGF-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 FGF receptors. A recombinant amino-truncated FGF-4 is more active than the full-length FGF-4 in an in vitro FGF-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-8c-specific probe indicate that Fgf-8c RNA is present in all of the above locations. 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 dysmorphogenesis syndromes of achondroplasia (FGFR3 transmembrane mutation; Refs. 34 and 35), Jackson-Weiss and Crouzon Syndromes (FGFR2b 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 subconfluence to avoid selecting for spontaneous transformants; 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 blunt 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 x 104 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. Helkinholmo and C. A. MacArthur, unpublished results.
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 x 10⁶ G418² 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 NIH Guide to animal welfare. Following injection, the animals were observed every 2 days for the development of tumors. Animals were sacrificed when the tumor became 2 cm in its largest diameter or when the tumor interfered with the animals’ daily tasks. 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.
Table 1. Properties of G418® NIH3T3 cell lines expressing Fgf-8 isoform cDNAs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transfected plasmid</th>
<th>Reduced serum growtha</th>
<th>Doubling time (h)b</th>
<th>Saturation density (10^5 cells/cm^2)c</th>
<th>Colony-forming units (%)d</th>
<th>Tumorogenicitye</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>pMIRB</td>
<td>No</td>
<td>18 ± 2</td>
<td>12 ± 2</td>
<td>0</td>
<td>0/4</td>
</tr>
<tr>
<td>B1</td>
<td>pMIRB+Fgf-8a cDNA</td>
<td>No</td>
<td>16 ± 2</td>
<td>27 ± 2</td>
<td>0</td>
<td>2/4</td>
</tr>
<tr>
<td>B2</td>
<td>pMIRB-Fgf-8b cDNA</td>
<td>Yes</td>
<td>16 ± 2</td>
<td>67 ± 3</td>
<td>5 ± 2</td>
<td>10/10e</td>
</tr>
<tr>
<td>C1</td>
<td>pMIRB-Fgf-8c cDNA</td>
<td>No</td>
<td>16 ± 2</td>
<td>48 ± 3</td>
<td>0</td>
<td>3/4</td>
</tr>
</tbody>
</table>

a Ability of cells to grow in 2% FCS and 8% newborn serum.
b Doubling time, measured while the cells were in log phase in 10% FCS, and presented as the mean of four experiments ± the SE.
c 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.
d Colony-forming units in soft agar, expressed as a percentage of the plated cells, presented as the mean of four experiments ± the SE.
e Presented as the number of animals with tumors divided by the number of animals receiving injections.

Tumor latency was 6 weeks.

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 nm rFGF-8 (N), 10 nm rFGF-8a (A), 10 nm rFGF-8b (B), or 10 nm rFGF-8c (C).

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 riboprobes 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-
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-tailed isoforms) or pQE16 (for carboxy-tailed isoforms) bacterial expression vectors (Qiagen). The histidine-tagged recombinant FGF-8 (rFGF-8) isoforms were purified using the denaturing protocol [6 m 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-tailed 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 nM 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 antisera production. The antisera 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, rFG-8b, and rFGF-8c. Chemiluminescence methods, including a secondary clonary 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 antibody against 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).

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**References**


Fgf-8, Activated by Proviral Insertion, Cooperates with the Wnt-1 Transgene in Murine Mammary Tumorigenesis

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We have used mouse mammary tumor virus (MMTV) infection of Wnt-1 transgenic mice to accelerate mammary tumorigenesis and to molecularly tag insertionally activated proto-oncogenes that cooperate oncogenically with Wnt-1 (G. M. Shackelford, C. A. MacArthur, H. C. Kwan, and H. E. Varmus, Proc. Natl. Acad. Sci. USA 90:740–744, 1993). Here we report the identification and characterization of a 31-kb genomic locus that contains clonal MMTV integrations in 8 of 80 mammary tumors from MMTV-infected Wnt-1 transgenic mice. Two genes were identified within this locus, one of which was transcriptionally activated by MMTV insertions. This activated gene is identical to androgen-induced growth factor (AIGF/Fgf-8) (A. Tanaka, K. Miyamoto, N. Minamino, M. Takeda, B. Sato, H. Matsuo, and K. Matsumoto, Proc. Natl. Acad. Sci. USA 89:8928–8932, 1992), the eighth member of the fibroblast growth factor (FGF) family. Transcriptional activation of Fgf-8 was found in all tumors with MMTV insertions in this locus. Fgf-8 mRNA was absent in normal mammary glands and was detected only in adult testis and ovary and in midgestational embryos. The sequences of Fgf-8 genomic and cDNA clones revealed five coding exons, in contrast to the three coding exons found in other FGF genes. cDNAs encoding three isoforms of the FGF-8 protein were isolated. The 3' corresponding mRNAs resulted from the alternative use of two 5' splice sites and two 3' splice sites for the second and third exons, respectively. These results implicate Fgf-8 as the third FGF gene found to cooperate with Wnt-1 in MMTV-induced murine mammary tumorigenesis, suggesting that FGFs and Wnts are strong collaborators in this process.

Ectopic expression of the Wnt-1 proto-oncogene in the mammary glands of transgenic mice causes a diffuse mammary gland hyperplasia, followed by the stochastic development of adenocarcinomas in both males and females after a minimum latency of several months (44). These findings indicate that activation of Wnt-1 is but one step in the multistep process of mammary tumorigenesis. Early evidence that fibroblast growth factors (FGFs) may be involved in cooperating steps comes from the finding that mouse mammary tumor virus (MMTV) insertionaly activates both Wnt-1 and Fgf-3 in some tumors of infected nontransgenic mice (35). The cooperation of Wnt-1 and Fgf-3 was confirmed with the generation of bitransgenic Wnt-1/Fgf-3 mice, which develop tumors earlier than either of the monotransgenic mice (26).

We have studied multistep murine mammary tumorigenesis by infecting Wnt-1 transgenic mice with MMTV, expecting that infection would hasten tumor formation in these mice by insertion activation of proto-oncogenes that oncogenically cooperate with Wnt-1 (37). As a result, activated proto-oncogenes are “tagged” by the nearby provirus, allowing their identification or isolation. Previous studies using murine leukemia virus infection of Eμ-myc transgenic mice have uncovered at least five distinct genetic loci that appear to collaborate with c-myc in lymphomagenesis (20, 45). MMTV infection of Wnt-1 transgenic mice results in accelerated mammary tumorigenesis and increased numbers of tumors per animal (37).

Approximately 45% of the resulting tumors with clonal, tumor-specific MMTV integrations contain insertionally activated Fgf-3 or Fgf-4, suggesting that the Wnt-1 gene product can cooperate with at least two members of the FGF family (37).

Despite being able to detect clonal, tumor-specific MMTV proviruses in the remaining 55% of the tumors, we did not detect insertional activation of any previously identified targets for MMTV insertion mutations (Fgf-3, Fgf-4, int-3, or Wnt-3) in this group (37). To determine the identity of proto-oncogenes that may be activated in these tumors, we have analyzed the DNAs surrounding proviral integration sites. We report here on the cloning of a new common integration locus for MMTV that contains insertions in 10% of our mammary tumors that have clonal, tumor-specific MMTV proviruses. Of the two genes located in this region, only one is activated by MMTV insertion mutations. This activated gene was found to encode androgen-induced growth factor (42), the eighth member of the FGF family. Thus, the selective activation in this system of a third FGF gene by MMTV proviral insertion suggests that FGFs and Wnts are potent collaborators in murine mammary tumorigenesis.

MATERIALS AND METHODS

Tumor samples. All mammary tumors were derived from a previous study (37) in which we infected female Wnt-1 transgenic mice (44) at 3 to 4 weeks of age with MMTV produced from EH-2 cells (39).

Preparation of nucleic acids. Tumor DNAs were isolated as described before (39) except that serum separation tubes were used in the extractions (43). The majority of tumor RNAs were isolated by the urea-lithium chloride method (3) as described before (38). The normal tissue RNAs and some tumor RNAs were isolated by the guanidinium isothiocyanate-phenol-chloroform method (9). Polyadenylated [poly(A)+] RNAs were purified by oligo(dT) chromatography (4). RNAs were stored as isopropanol precipitates at −20 or −80°C.

Southern and Northern (RNA) blot analyses. DNAs (10 μg) were digested with restriction endonucleases, electrophoresed, and capillary blotted to nylon membranes (Amersham Hybond-N) as previously described (59). The blots were

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UV cross-linked (11), prehybridized for 1 h to 2 h at 65°C, and hybridized overnight at 65°C with 2-3P-labeled DNA probes. The hybridization buffer contained 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% bovine serum albumin (fraction V), 7% sodium dodecyl sulfate (SDS), and 15% (v/v) formamide. Blots were washed in 40 mM sodium phosphate (pH 7.2)–1% SDS–1 mM EDTA at 65°C. Blots were exposed to Kodak XAR-5 film with intensifying screens at −80°C.

For Northern blots, RNAs were resuspended in a solution containing 50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 0.25 μg of ethidium bromide per ml, 0.066 M formamide, and 50% (v/v) formamide, denatured at 65°C for 5 min, and kept on ice. The blots were hybridized with radiolabeled cDNA probes, transferred to nitrocellulose paper, washed, exposed to film, and scanned with a densitometer (model GS-700; Bio-Rad Laboratories, Hercules, CA).

Preparation and screening of genomic libraries. Following identification of a proviral-cellular junction fragment by Southern blotting, the fragment was size-selected by agarose electrophoresis and purified by using either glass beads (Gene Clean II; Bio 101) or agarose digestion (B-Agarase; New England Biolabs) and isopropanol precipitation. For this, the probe was ligated into appropriate vector and packaged in vitro, depending on the size of the insert (3’DhII for inserts of 10 to 20 kb, and XZap II for inserts of less than 10 kb [both from Stratagene]). Plasmid DNA was prepared by using plasmid extraction kits (Qiagen). The purified recombinant plasmids were then labeled and screened on appropriate Escherichia coli hosts (Blue Biotin libraries and P3202 for XZap libraries). Plaque lifts were performed as described before (36). UV cross-linked, hybridized, washed, and exposed as above for Southern blots. Putative positive clones were subjected to two rounds of screening to isolate clones. The inserts were obtained in Blue Biotin (Stratagene) plasmids, either by subcloning or by in vivo excision of XZap clones.

Nucleotide sequence accession number. The GenBank accession number of the cDNA encoding Fgf-6 is U18673.

RESULTS

Detection and cloning of a novel tumor-specific common insertion site for MMTV. In previous work, we showed that MMTV infection of Wnt-1 transgenic mouse accelerates mammary tumorigenesis by insertional activation of Fgf-3 and Fgf-4 (37). However, approximately half of the tumors that we examined did not exhibit activation of any of the proto-oncogenes known to be affected by MMTV in tumors of normal mice (including int-3 and Wnt-3), despite the presence of clonal junctional insertions in these tumors (37). We reasoned that these tumors were likely to harbor unpredicted or novel proto-oncogenes activated by proviral insertion. To isolate such genes, we first sought to clone the proviral-cellular junction fragments from tumors that contained only one or two clonally integrated proviruses. Southern blot analysis of tumor DNAs with an MMTV gag probe identified several candidate junction fragments (Fig. 1 and data not shown).

The XhoI-cleaved junction fragments from tumors 95 and 111 (Fig. 1) were cloned into lambda vectors and isolated by screening with an MMTV gag probe. Unique cellular DNAs flanking the MMTV proviruses in these clones were isolated and used as probes on Southern blots of DNAs of 50 tumors with clonal insertions to determine if other tumors had rearrangements due to proviral insertions in these loci. Data from these experiments suggested that the insertions in tumors 95 and 111 were actually in the same locus and, furthermore, that six other tumors also contained proviruses in this region (Fig. 2 and data not shown). Hybridization of these blasts with MMTV probes confirmed that the rearrangements of this locus in affected tumors were due to proviral integrations (Fig. 2 and data not shown). The apparent presence of two insertions in tumor 135 is discussed below.

Detection of expressed genes in the common insertion region. In a search for active genes in this locus, we made probes from the cloned cellular DNAs described above as well as from adjacent regions cloned subsequently for use in Northern blot analyses of tumor RNAs. Two genes were identified. A genomic clone that detected RNA from one of these genes was sequenced and found to be identical with the cDNA of aromatase, a known growth factor (42), the eighth member of the FGF family. We will hereafter refer to this gene as Fgf-8. The second
FIG. 1. MMTV proviruses in tumors of Wnt-1 transgenic mice. Genomic DNAs were isolated from normal mammary gland (lane C) or from mammary tumors (designated by tumor number) of MMTV-infected Wnt-1 transgenic mice. DNAs were digested with XhoI, separated by agarose gel electrophoresis, capillary blotted to nylon membranes, and hybridized with a 32P-labeled MMTV gag probe. All DNAs have large gag fragments, indicative of endogenous retroviruses in these animals. Most of the tumor samples have an additional fragment(s), indicating the presence of clonal, tumor-specific MMTV proviruses. The positions of DNA size markers (in kilobases) are indicated at the right. A diagram showing the origin of the gag probe used and the 5' proviral-cellular junction fragment it detects is shown below the gel. MMTV DNA is represented with bold lines. Open boxes, MMTV LTRs; Xh, XhoI.

gene in this locus, which we have designated Nub-1, is novel. The deduced Nub-1 product is unrelated to any oncoproteins, but it has sequence similarities to two nuclear/enchaparen proteins, nucleoplasmin and B23/nucleophasmin (6, 16). A description of Nub-1, beyond the RNA data below, will be presented elsewhere.

When small amounts of total cellular RNAs available from tumors were examined by Northern blotting for expression of these genes, we found evidence for transcriptional activation of Fgf-8 in all tumors with insertions in this locus except tumor 104; Fgf-8 RNA was undetectable in tumors without insertions and in normal mammary gland (Fig. 4 and data not shown). RT-PCR experiments confirmed the presence of Fgf-8 RNA in tumor 104 and its absence in normal mammary gland (data not shown). In contrast, the RNA levels of the constitutively active Nub-1 gene do not appear to be significantly affected by the surrounding MMTV insertions (Fig. 4). Thus, the activated expression of Fgf-8 in multiple tumors by clonal proviral insertions suggests that this gene is causally related to tumor formation.

Fgf-8 exon structure and alternative splicing. To determine the physical relationship of the proviral insertions to the Fgf-8 gene, we established its exon structure by sequencing portions of our genomic clones and comparing these with the Fgf-8 cDNA sequences reported earlier (42). This revealed that Fgf-8, as represented by these cDNAs, is approximately 6.5 kb in length and has at least five coding exons (Fig. 5A), unlike other analyzed FGF genes, which have only three coding exons (1, 14, 19, 24, 28, 49, 50). All of the insertions detected were mapped upstream of Fgf-8, and all, except those in tumors 61 and 86, were in the opposite transcriptional orientation to...

FIG. 2. MMTV proviral insertions into a new common integration locus. (A) Southern blots of DNAs from kidney as a control (lanes C) and from mammary tumors 86, 104, 111, and 135. Rearrangements of this locus in tumors were detected following digestion of the DNAs with either BglII or XhoI (which cut within MMTV) and hybridization of the resulting blots with the cellular probe PH0.8 (see Fig. 3). Arrowheads identify fragments that anneal to both cellular (left panels) and MMTV gag (right panels) probes, demonstrating the location and orientation of the inserted provirus. (B) Southern blots of DNAs from normal mammary gland as a control (lanes C) and from mammary tumors 63, 94, and 95. Rearrangements of this locus in tumors were detected following digestion of the DNAs with XhoI and hybridization of the resulting blots with the cellular probe XP1200 (see Fig. 3). The orientation of the MMTV provirus was determined as for panel A but with XP1200, gag, and env probes.

Fgf-8 (Fig. 3 and 5A and data not shown). Two of the insertions (tumors 63 and 95) disrupted the Nub-1 gene, which is located approximately 5 kb upstream of Fgf-8 in the same orientation, and two insertions (tumors 61 and 94) were upstream of both genes (Fig. 3 and data not shown).

The comparison of Fgf-8 genomic and cDNA sequences also
showed the existence of at least two 5' splice sites in exon 2 and two 3' splice sites in exon 3. Alternative splicing between these sites could potentially produce RNAs encoding four isoforms of the protein (Fig. 5A to C). In exon trap experiments with an SrlII genomic fragment containing most of Fgf-8 (see Materials and Methods) and in nested RT-PCR experiments with RNA from adult murine testis and from tumor 61, we amplified and cloned cDNAs of alternatively spliced mRNAs encoding three different isoforms: FGF-8a, FGF-8b, and FGF-8c (Fig. 5A to C). We did not obtain a cDNA containing both exons 2B and 3B, which would encode the fourth potential isoform (data not shown). All three cloned versions have the same predicted signal peptide sequence and code for the same protein downstream of the alternative splice, but differ in the amino-terminal regions of the putative secreted products (Fig. 5C).

Expression of Fgf-8 in murine tissues. We performed Northern blots of poly(A)+ RNA from normal murine tissues to determine the normal expression patterns of Fgf-8 (Fig. 6). Weak expression was detected in ovary and testis, but we did not detect Fgf-8 RNA in normal mammary gland or in any of 10 other tissues (Fig. 6). These data suggest that MMTV insertions in tumors activate this gene from a transcriptionally silent state.

Since FGFs are frequently expressed during embryogenesis (5), we looked for expression of Fgf-8 during murine development by Northern blot analysis of total cellular RNA from whole embryos staged by gestational age. We found high expression of Fgf-8 at 10.5 days postconception, the earliest time tested, which decreased to nearly undetectable levels by 14.5 days (Fig. 6). We did not observe Fgf-8 expression at 16.5 or 17.5 days postconception (data not shown).

**DISCUSSION**

We previously showed that infection of Wnt-1 transgenic mice with MMTV accelerates mammary tumorogenesis by inserting inactivating Fgf-3 and/or Fgf-4, indicating that these two members of the FGF family can cooperate with Wnt-1 in murine mammary tumorogenesis (37). By cloning and analyzing proviral insertion sites from several tumors that lack these activations, we show here that 8 of 80 tumors with clonal, tumor-specific MMTV proviruses had MMTV insertions in a genomic region that contains Fgf-8 (Fig. 2 and 3). Elevated levels of Fgf-8 RNA were detected in affected tumors but not in other tumors or in normal mammary gland (Fig. 4). Analysis of the normal gene and its expression showed that Fgf-8 contains at least five coding exons and can encode at least three different protein isoforms by alternative splicing and that RNA was present in the testes and ovaries of adults and in midgestational embryos. Together, these findings suggest that the overexpression of Fgf-8 which accompanies MMTV insertion into this locus contributes to the formation of these tumors.

**Proviral insertions.** Studies of MMTV insertion sites in mammary tumors indicate that MMTV can transcriptionally activate an adjacent proto-oncogene from upstream or down-stream locations in either transcriptional orientation relative to the gene, although the vast majority are classical "enhancer insertions," in which the 5' long terminal repeat (LTR) of the provirus is situated closest to the activated gene (12, 30, 33). All of the insertions described here are located upstream of Fgf-8; two of these (in tumors 61 and 86) were in the same orientation as Fgf-8 (and Nub-1). The location of the provirus in tumor 61 might predict a large fusion transcript with Nub-1, starting from an MMTV LTR, but no aberrant Nub-1 (or Fgf-8) RNAs were observed in this tumor. Thus, it appears that the Fgf-8 gene is activated by an enhancer insertion in tumor 61, despite the "promoter insertion" orientation of the provirus. The identities of the additional sequences in the Fgf-8 transcripts larger than 1.9 kb observed in several tumors (e.g., tumors 63, 86, and 135 in Fig. 4) are unknown.

Tumors 61 and 94 are unusual in that a second gene, Nub-1,
is found between the proviral insertions and the activated Fgf-8 gene. The basal activity of Nub-1 does not seem to be significantly affected by the insertions, while Fgf-8 is activated from an apparently quiescent state. Although proviral enhancers are known to activate genes from significant distances, to our knowledge, MMTV activation of the distal, but not the proximal, gene of a linked pair has previously not been observed. The apparent selective mechanism involved is unclear but may be explained in part by the relatively high basal level of Nub-1 expression, by possible toxicity of higher levels of Nub-1 protein, or by a discriminating compatibility of MMTV enhancers with the Fgf-8 promoter.

Tumor 135 appears to contain two separate MMTV proviral insertions at similar locations in this locus, but not both insertions on a single chromosome (Fig. 2B). It seems unlikely that a selective advantage for tumor cell growth would result from both copies of the gene being activated by proviral insertion, since, for example, we have never seen insertional activation of the endogenous Wnt-1 gene in MMTV-infected Wnt-1 trans-

FIG. 6. Fgf-8 is normally expressed during embryogenesis and in adult ovariies and testes. Northern blot was made of normal adult tissue poly(A)⁺ RNAs (Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; MG, mammary gland; Mu, muscle; Os, ovaries; SG, salivary gland; Sp, spleen; Tu, testis; Th, thymus; Ut, uterus) and murine embryonic total cellular RNAs (embryo lanes labeled in days postconception). The Fgf-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes are the same as in Fig. 4. The positions of RNA size markers (in kilobases) are shown at the right of each blot.
genic mice (37). Although a requirement for a threshold level of Fgf-8 expression might explain the two insertions, alternative interpretations are that tumor 135 is oligoclonal, with tumor cells possessing one or the other insertion, or that a single insertion exists with a rearranged form present in some tumor cells. In addition, tumor 135 has an insertion in the Fgf-3/Fgf-4 locus (37). This finding reintroduces the notion of possible cooperation among FGF family members (37), but oligoclonality could also explain the data.

Gene activation by MMTV insertions. Almost all tumors with insertions in this locus displayed readily discernable activation of Fgf-8 with small amounts of total RNA by Northern analysis. Only tumor 104 required RT-PCR for detection. The cause for the wide variation in MMTV-activated Fgf-8 gene expression is unknown, but such variation is common for genes activated by MMTV insertions (15, 31, 34, 37). Because of the lack of obvious changes in Nab-1 RNA levels in affected tumors and the interruption of its coding domain in two tumors, alterations in Nab-1 expression by proviral insertions do not appear to contribute to tumorigenesis. The relationship of Nab-1 to the nucleolar proteins nucleoplasmin and B23/nucleophosmin, two abundant and well-studied molecular chaperones with no known oncogenic potential (6, 16), also does not immediately point to a role for Nab-1 in tumorigenesis.

Although the recently described fusion of nucleoplasmin to the cytoplasmic tyrosine kinase domain of a transmembrane receptor in chromosomal translocations in some lymphomas is intriguing in this regard, nucleoplasmin’s proposed role is to provide an active promoter for the kinase (7, 29). Together with the activated expression of Fgf-8 shown here and the reported transforming potential of Fgf-8b cDNA in NIH 3T3 cells (25, 26a), it is likely that Fgf-8, and not Nab-1, is the gene pertinent to oncogenesis in this locus.

Alternative splicing and gene expression. The transcribed portion of the Fgf-8 gene is divided into at least five coding exons (Fig. 5A), two more than are found in the other FGF genes examined (1, 14, 19, 24, 28, 49, 50). The first three exons of Fgf-8 described here correspond to the first exon of other FGF genes. Fgf-8 is also unusual in that it can be alternatively spliced between exons 2 and 3 to yield at least three protein isoforms which differ only in the amino-terminal portion of the secreted mature protein. We isolated the two previously described cDNAs (42), which code for the isoforms Fgf-8b and Fgf-8c, and a third cDNA that encodes the previously undescribed isoform Fgf-8a (Fig. 5A to C). All three mRNA species were detected in both testis and tumor 61. We have not detected a fourth possible splice variant which would contain exons 2B and 3B in either testis or mammary tumor RNA (data not shown). These cDNA clones will allow a comparison of the three isoforms for possible differences in, for example, their transforming potentials, tissue expression patterns, half-lives, or affinities for the various FGF receptors.

We found expression of Fgf-8 in adult testis and ovary (Fig. 6), but this expression is much lower than in the tumors with MMTV insertions near Fgf-8 (Fig. 4). The expression of Fgf-8 during murine embryogenesis (Fig. 6) suggests that this gene may be important in embryonic development. Recent studies using in situ analysis of whole-mount embryos and embryo sections confirm the expression of Fgf-8 during murine embryogenesis (22). Definitive proof that Fgf-8 expression is important in murine development will require the creation and analysis of mice lacking a functional Fgf-8 gene.

The MMTV LTR contains androgen-responsive enhancer elements which can place nearby promoters under androgen regulation (8, 13, 32). In light of our finding that Fgf-8 is active only in mammary tumors with MMTV proviruses in this locus, and given that androgen induction of Fgf-8 has only been demonstrated in cells from a mouse mammary tumor (Shionogi carcinoma 115) and a derivative cell line (SC-3) (42), it seems possible that the androgen-inducible nature of Fgf-8 in these cells might be attributed to MMTV insertional activation of Fgf-8 rather than to any inherent feature of the gene. Consistent with this idea is the finding that glucocorticoids—well known for their stimulatory effects on transcription from the MMTV LTR—can also induce Fgf-8 activity in these cells (47).

Wnt-1 and Fgf-8 cooperation. Fgf-8 is the third member of the FGF family to be identified as a Wnt-1 collaborator in mammary carcinogenesis. Surprisingly, only FGF genes have been found to be activated by MMTV in this transgenic model system (37). Assuming that many proto-oncogenes are potential targets for activating insertion mutations, this selectively suggests that FGFs cooperate oncogenically with Wnt-1 in an especially potent manner. It is possible that the signaling pathways of these two families function in a highly complementary fashion to deregulate mammary epithelial cell growth and proliferation. Alternatively, activated FGFs could contribute to neoplasia in a less direct manner, for example, by stimulating angiogenesis (17, 23). However, it is notable that genes of the Wnt and FGF families can evidently collaborate in an experimental model of mesoderm induction (10), suggesting that cooperation in tumorigenesis may derive from the normal interactions of these genes.

The detection of a third activated FGF gene in these tumors prompted us to survey all other known FGF loci for insertions by Southern blot analysis, but no rearrangements in other FGF genes have been found thus far (40). Given the propensity of this model system to activate FGF genes, we speculate that analyses of other common insertion loci in the remaining tumors from this collection may reveal new members of this gene family or identify important downstream components in the FGF signal transduction pathway.

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C.A.M. and D.B.S. contributed equally to this work; the order of these authors is arbitrary.

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