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FOREWORD

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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 *Wnt1* gene under the control of an MMTV enhancer were generated. Both male and female transgenics 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* transgenics 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 *Wnt1* transgenic 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 activation of *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 insertional activation 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 activation 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 insertionally 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.

Summary of previous results (report-1995)

In my previous progress report, I described the identification and cloning of a proviral-cellular junction fragment from tumor #76 from infected *Wnt1* transgenic mice. 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 (Figure 1).

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 (23). 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 results from this analysis were sent to Jackson lab and, thorough analysis of the database mapped the locus very nicely to the distal region of mouse chromosome-7 (Figure 2). Unfortunately, this locus was linked to the *int2/Fgf3* locus suggesting that this was a likely candidate to be activated by MMTV proviral insertions. However, previous analysis of these tumors did not detect MMTV insertions within the *int2/Fgf3* locus (15). This suggests that either these insertions are activating other genes that are linked to this locus, or that these insertions are long-range activations of *int2/Fgf3*. I decided to address both possibilities.

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

Several genes (*cyclinD1*, *Igf2*, *H-19*, *hst/Fgf4*) are located near *int2/Fgf3* on mouse chromosome-7 and seem to be good candidates for MMTV activations (23). *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 (25). Since *H-19* is a maternally imprinted gene (26), MMTV insertions into this locus could possibly knock out the functional copy of the gene and thereby contribute to mammary tumorigenesis. *Hst/Fgf4* is a previously described target for MMTV activations (7,15). To test if there were activations in these other genes, I performed Southern analysis using DNAs from

tumors with MMTV insertions 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*. 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 has been previously described. Peters et al showed long range activations of *int2/Fgf3* by MMTV insertions ~20kb upstream of the gene (10). 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 *int2/Fgf3* probe showed activation of the gene in the tumors and no expression in mammary gland controls, proving that *int2/Fgf3* is the gene activated by MMTV in these tumors.

Changes

The oncogenic potential of *int2/Fgf3* has already been demonstrated, we and others have shown *int2/Fgf3* to be a strong oncogenic collaborator of *Wnt1* in promoting mammary carcinogenesis (11,15,16). As a consequence of this fact, I am slightly altering 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 will be that further characterization of the identified gene *int2/Fgf3* will not be performed, instead I will pursue the characterization of a gene (*Fgf-8*) that I had previously identified to be frequently activated by MMTV proviral insertions in these infected *Wnt1* transgenic mice (17).

Prior to cloning the tumor 76 insertion, I had cloned another junction fragment from tumor 111 and identified the gene *Fgf-8*, to be activated by MMTV proviral insertions in 10% of tumors from infected *Wnt1* transgenic mice (17). *Fgf8* was the third member of the FGF family found to be activated in these tumors further supporting the evidence that Wnt and FGF factors are strong oncogenic collaborators. I published these results as a co-first 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).

In the last half year I have been analyzing the biological effects of the different isoforms of *Fgf8* on mammary epithelial cells, since we originally cloned *Fgf8* from mammary tumors. These studies resulted in a serendipitous observation that I will describe in the following paragraphs. In addition, I have also been collaborating with Dr.

P.Roy-Burman's group at the University of Southern California to study and elucidate the oncogenic potential of the human *FGF8* gene.

Biological effects of murine Fgf8 isoforms on mammary epithelial cells

In order to determine the oncogenic potential of Fgf8 isoforms in mammary epithelial cells, the cDNAs encoding Fgf-8a, Fgf8b and Fgf8c isoforms of the protein were cloned into the expression vector mirb and transfected into a normal mammary epithelial cell line C57MG as previously described (22). The transfected cells were observed for the formation of discrete foci. These cells transfected with the *Fgf-8b* cDNA started to form foci on reaching confluence indicating that the cells expressing the *Fgf-8b* protein were not contact inhibited and hence continued to divide. Surprisingly, however the cells within the focus showed classical signs of apoptotic death (27): the cells were rounding up and floating in the medium, and eventually all the cells in the focus detached from the plastic leaving open spaces in the monolayer (Figure 4).

In a parallel experiment, conditioned medium from NIH3T3 cells expressing Fgf8a, Fgf8b, Fgf8c and mirb (vector) was used as source of Fgf8 proteins. These cells have been previously shown to secrete the Fgf8 proteins into the medium, and that these secreted proteins have biological activity (22). NIH3T3 cells show the same morphological transformation when treated with the *8b* conditioned medium as the cells stably expressing Fgf8b(22). The conditioned medium from these cells was collected from actively dividing cells, filtered and transferred on C57MG cells. Mammary epithelial cells treated with the Fgf-8b conditioned medium showed molecular transformation from a flat cuboidal morphology to a more spindle shaped elongated morphology within 48 hrs. By day 3 after treatment, many of the cells rounded up and detached from the plate and showed classical signs of cell death which was complete by day 4 (Figure 5a). C57MG cells transfected with Fgf8a or Fgf8c did not form foci. Also, the C57MG cells treated with Fgf8a and Fgf8c conditioned medium did not show any morphological changes.

Based on these observations of cell death, I decided to test the possibility of apoptosis (programmed cell death), since the morphology of the cells showed characteristic features of apoptotic cell death (27,28). To test for apoptosis, the treated cells and the foci were fixed in carnoyls fixative, stained with Hoescht 3328 a fluochrome that stains the nuclei, and observed through a fluorescent microscope in order to look for chromatin condensation and nuclear fragmentation. In addition, DNA was extracted from the treated cells and tested for DNA laddering by agarose gel electrophoresis. Cells transfected with the vector alone or treated with condition medium from cells expressing the vector alone were used as controls.

Staining of the foci with Bisbenzimidazole/Hoescht 3328 showed the presence of condensed chromatin more intensely fluorescent than the normal uniform nuclei, and fragmented nuclei of the cells in the foci, in contrast to the normal nuclei of the cells in the monolayer. Similarly the nuclei of cells treated with the *8b* conditioned medium showed classical apoptotic nuclear morphology, exhibiting varying degrees of chromatin condensation and nuclear fragmentation, in sharp contrast to the control cells (Figure 5b). DNA laddering experiments showed the cleavage of the DNA from the *8b* treated cells into the classical ~180bp DNA ladders while the DNA from the control cells only showed the presence of high molecular weight genomic DNA (Figure 5c). DNA laddering is very strong evidence for apoptosis. These results suggest that stimulation of

mammary epithelial cells C57MG with Fgf8b protein signals the cells to enter the apoptotic pathway.

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. The main goal of this experiment was to deplete the condition medium of Fgf-8b by binding it to a heparin sulphate column, and partially purifying the Fgf-8b protein. It has been previously shown that Fgf-8b like other Fgfs can bind heparin proteoglycans, and Fgf8 was initially purified by binding to heparin.(20)

NIH3T3 cells were used as a control in this experiment to test the activity of the conditioned medium and elution fractions. Both cell lines were treated with conditioned medium (8b, vector control), flow-through, and fractions 1 through 4 (0.5M, 1M, 1.5M and 2M NaCl concentrations). C57MG cells treated with the conditioned medium went through programmed cell death (tested by Hoescht and laddering). This activity was lost in the flow-through (conditioned medium after passing through the heparin-sepharose column) indicating that the factor inducing apoptosis bound heparin, and was also responsible for transformation of NIH3T3 cells, since the flow-through did not transform the 3T3 cells. Both the transformation activity in NIH 3T3 cells and apoptotic activity in C57MG cells was restored when the cells were treated with the 1.5M NaCl fraction from Fgf8b but not vector control. This fraction is most likely to contain the Fgf8b protein since these results corroborate the previous finding where the Fgf-8b protein was eluted at a concentration of 1.1M NaCl(20).

Oncogenic potential of human *FGF8* isoforms:

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 8c isoforms. The human FGF8a and FGF8b exhibit identical amino acid sequences to their murine counterparts, while FGF8c 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 tumorigenicity 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 FGF8c 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 FGF8c 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 accepted for publication 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. This tumor expresses high levels of *Fgf8*RNA and hence, the transgene is likely to express well. The transgene contains the 3' MMTV LTR, and the entire genomic *Fgf8* gene in a single DNA fragment of ~13 Kb. The DNA was size selected on an agarose gel and cloned into a lambda vector (Lambda Dash) and the resulting library was screened using both MMTV specific and *Fgf8* specific DNA fragments as probes. The 13kb fragment was excised from this vector and used for microinjection of fertilized mouse eggs. The injections were performed at the transgenic core facility of the University of Southern California.

Fgf8 transgenic mice:

The founder animals generated were screened by Southern blots of the tail DNAs for the presence of the transgene. We now have at least three transgenic founders that contain the MMTV/*Fgf8* transgene. These animals are currently being bred to normal BALB/c mice to generate independent lines. At present, there are progenies from two of the founders. Once we have enough transgenic mice from each line, I will test, by northern analysis, the mammary and salivary glands of a representative of each line for the expression of the transgene (endogenous Fgf8 is expressed only in adult testis and ovaries). These mice are being observed for the appearance of mammary tumors and for any other abnormal phenotype.

CONCLUSIONS

A proviral-cellular junction fragment was identified and cloned from a tumor from infected Wnt1 transgenic mice. Twelve of eighty five tumors tested had MMTV insertions within this locus. This locus was mapped to the distal region of the mouse chromosome 7, and was found to be linked to the genes; *Fgf3*, *Fgf4*, *H-19*, *cyclinD1*, and *Igf2*. Analyses for activations in any of these genes 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 % of the tumors. This gene encodes at least seven different protein isoforms, three (*Fgf8a*, *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).

To test the significance of the oncogenic capabilities of murine Fgf8 in humans, cDNAs corresponding to human FGF8 protein isoforms (8a, b and e) were transfected into NIH3T3 cells and their transforming potentials analysed. FGF8b was found to be the most transforming of all three isoforms, while FGF8a and FGF8e showed moderate degrees of transformation.

To demonstrate the oncogenicity of Fgf8 *in vivo*, the mouse *Fgf8* transgene was cloned from a tumor with insertion in this locus (tumor 86). At present there are three transgenic founders that are being crossed with normal BALB/c mice to generate transgenic lines. These animals are being monitored for the appearance of mammary tumors and any other abnormal phenotype.

The oncogenic collaboration between *Fgf8* and *Wnt1* will be tested in cell culture experiments. Once I have fully characterized the singly transgenic animals, I will initiate experiments to cross them with *Wnt* mice to demonstrate oncogenic cooperation *in vivo*.

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APPENDIX

A NEW COMMON INSERTION LOCUS FOR MMTV

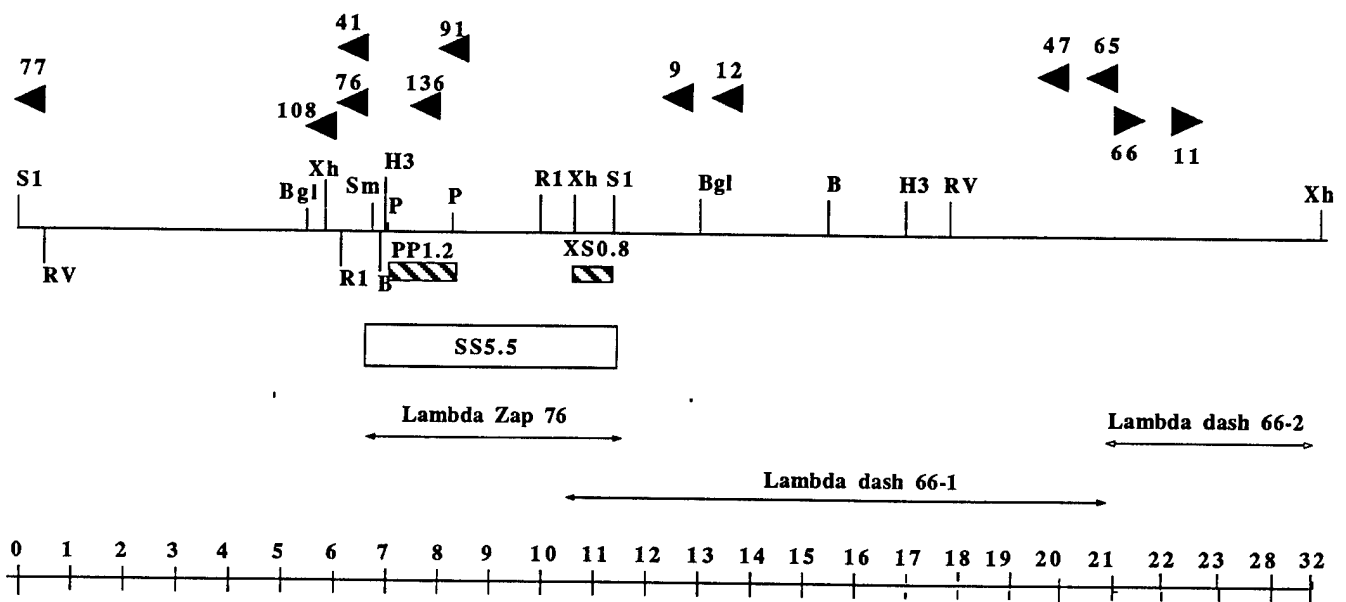
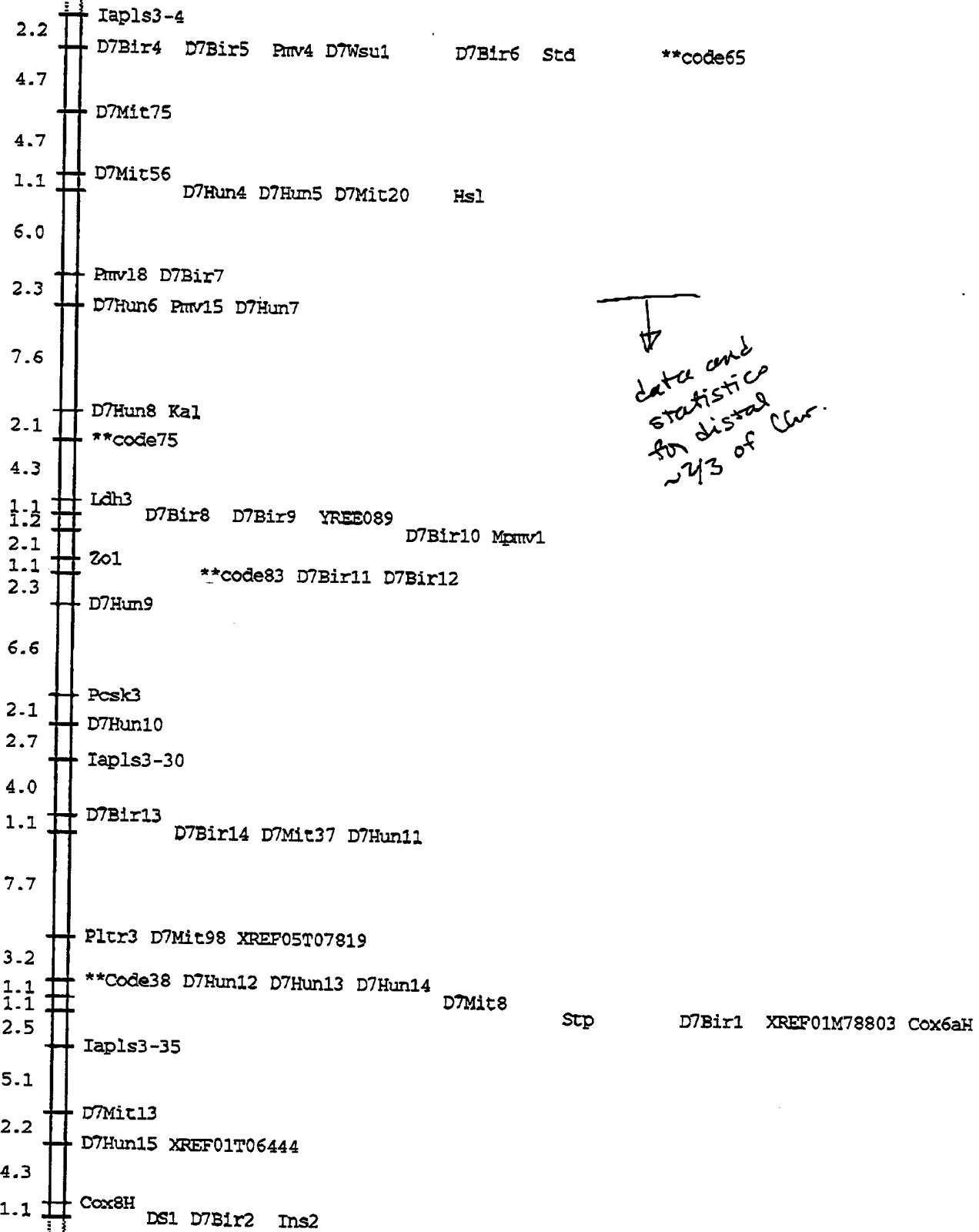


Figure-1: Map of the Tumor 76 locus . Arrowheads represent MMTV insertion sites and the orientation. R1 represent EcoR1, Bgl: Bgl II, B: Bam HI, P: Pst I, H3: Hind III, RV: EcoRV, S1: Sst I, Sm: Sma I, Xh: Xho I. Filled in boxes represent repeat free probes. Lines with arrowheads represent the Lambda clones.

Backcross Stats, 95% Limit



↓
 data and
 statistics
 for distal
 ~2/3 of Chr.

Figure 3.

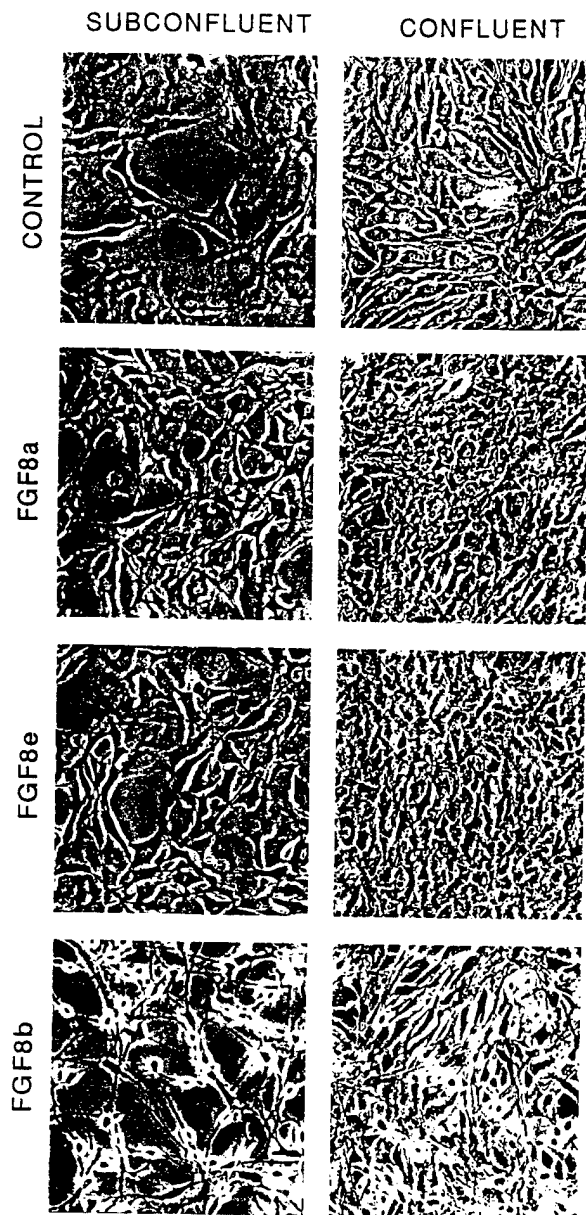


Fig. 7. Representative illustrations of morphological transformation of NIH3T3 cells by FGF8 isoforms. In this experiment, NIH3T3 cells were transfected with expression plasmids containing cDNAs of *FGF8a*, *FGF8b*, *FGF8e*, or a control plasmid with no insert. G418-resistant colonies were pooled and grown for examination of induced changes in morphology and contact inhibition. *FGF8b* showed strong morphological transforming ability, and *FGF8a* and *FGF8e* showed moderate transforming activity. All three isoforms induced a loss of contact inhibition at confluence.

FOCUS OF C57MG CELLS TRANSFECTED
WITH *Fgf8b* cDNA

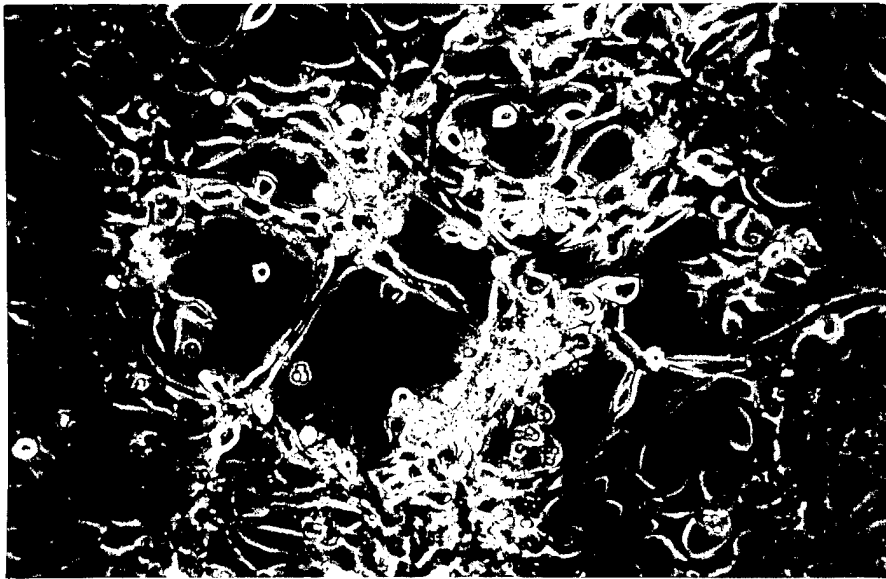


Figure4. C57MG cells transfected with an expression vector (MIRB) containing the *Fgf8b* cDNA. The cells were grown to confluence and observed for focus formation. Arrows indicate the rounded and disintegrated cells.

CONDITIONED MEDIUM TRANSFER

VECTOR



FGF-8b

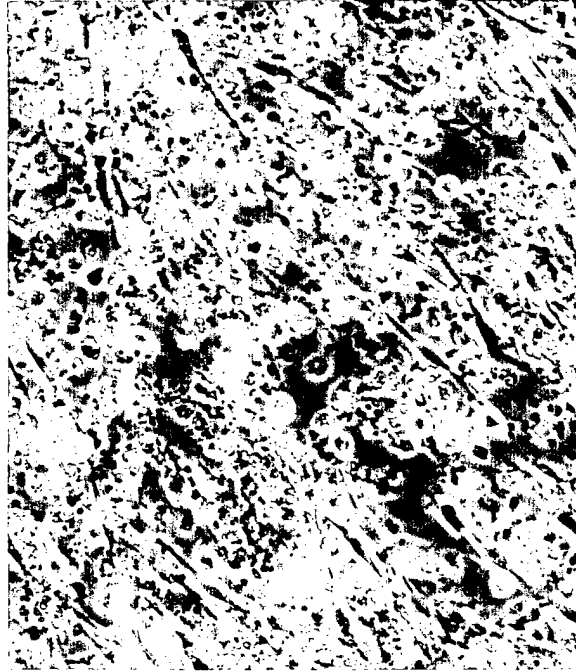
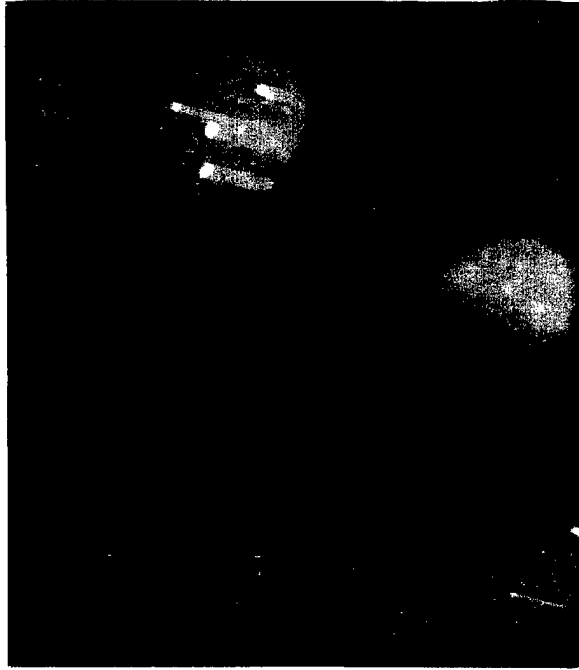


Figure 5a. C57MG cells treated with either *Fg8b* containing conditioned medium or the control (vector) conditioned medium. The cells were photographed 3 days after treatment. Apoptotic and fragmented cells are observed only in the cells treated with 8b conditioned medium and not in the control cells.

CONDITIONED MEDIUM TRANSFER

(Hoechst Stain)

VECTOR



FGF-8b



Figure 5b. C57MG cells treated with conditioned medium (8b and Vector) were fixed in carnoyl's fixative and stained with Hoescht 3328. Arrows denote the condensed chromatin and fragmented nuclei: characteristic features of cells undergoing apoptosis.

APOPTOTIC DNA FRAGMENTATION



Figure 5c. DNA Laddering; DNA from C57MG cells treated with conditioned medium was extracted. Approximately 1 μg of DNA from the control and *8b* treated cells was electrophoresed through a 1.5% agarose gel. Lanes 1 and 2 are the control lanes, lanes 3 and 4 are the *8b* lanes. The cells treated with *Fgf8b* show the DNA laddering typical of cells undergoing apoptosis.