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SHORT REPORT

Preferential activation of *Fgf8* by proviral insertion in mammary tumors of *Wnt1* transgenic mice

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Mouse mammary tumor virus (MMTV) is an insertional mutagen that has been demonstrated to transcriptionally activate flanking cellular proto-oncogenes. Previously we have used MMTV infection to accelerate mammary tumorigenesis in *Wnt1* transgenic mice in order to identify genes that cooperate with the *Wnt1* oncogene. Initial investigations into the resulting tumor collection, screened primarily by Southern analysis, showed that three fibroblast growth factor genes, *Fgf8*, *Fgf3* and *Fgf4*, sustain activating insertion mutations in 10%, 42% and 6% of the tumors, respectively. Here, in an examination of the tumors from MMTV-infected *Wnt1* transgenic mice that emphasizes Northern analysis, we report transcriptional activation of *Fgf8* in 30 additional tumors (increasing the percentage of activations to 50%), while no significant changes in the activation frequency of *Fgf3* or *Fgf4* were found. To determine the frequency of insertional activation in normal mice, we examined tumors from MMTV-infected nontransgenic littermates of the *Wnt1* transgenics and from MMTV-infected BALB/c mice. *Fgf8*, *Fgf3* and *Fgf4* were found to be activated in 11%, 80% and 5%, respectively, of the tumors in the combined nontransgenic groups. Thus, there appears to be an increased predisposition for *Fgf8* activations in *Wnt1* transgenic mice *versus* normal mice, suggesting that cells expressing *Wnt1* are especially sensitized to stimulation by FGF8 compared with FGF3 or FGF4. In contrast, the activation frequency of *Fgf3* in tumors from MMTV-infected *Wnt1* transgenic mice was approximately one-half that of normal mice. Our results show that this *in vivo* model of multistep tumorigenesis reveals significant differences in the activation rates of *Fgf3* and *Fgf8* depending upon the status of *Wnt1* expression in the mammary gland. The differential activation of these *Fgfs* may relate to differences in their signaling pathways.

Keywords: *Fgf8*; *Wnt1*; MMTV; oncogene cooperation; *Fgf3*; *Fgf4*; multistep tumorigenesis; growth factors

Cancer is a multistep process that arises from the accumulation of genetic lesions over time. Therefore, to understand the development and progression of cancer, it is important to identify not only the single mutations involved, but also which oncogenes work together in tumor formation. Here, we report the results of a continuing investigation aimed at identifying and characterizing genes that cooperate with the *Wnt1* oncogene in murine mammary tumorigenesis.

Wnt1 is frequently activated by proviral insertion in tumors induced by mouse mammary tumor virus (MMTV) (Nusse and Varmus, 1982). The oncogenic potential of *Wnt1* was demonstrated *in vivo* by ectopically expressing a *Wnt1* transgene in mouse mammary glands using the MMTV enhancer (Tsukamoto *et al.*, 1988). Male and female *Wnt1* transgenic mice develop hyperplastic mammary glands which often produce isolated carcinomas especially in breeding females. However, *Wnt1* is not sufficient for induction of mammary neoplasias, since tumor development occurs stochastically in these mice, and some transgenic animals remain tumor-free. Thus, additional events must be necessary for tumor induction in these animals.

To identify genes involved in these additional events, we previously infected the *Wnt1* transgenic mice with MMTV (Shackleford *et al.*, 1993). Infection accelerated the rate of tumor formation and increased the average number of tumors per mouse, demonstrating that MMTV infection promotes mammary carcinogenesis in *Wnt1* transgenics. Analysis of the tumors from these infected mice for known genetic targets of MMTV revealed insertional activation of *Fgf3* and *Fgf4*, suggesting that these genes cooperate with *Wnt1* in mammary tumorigenesis. Further examination of these tumors by cloning of integration sites and Southern blot screening identified a third fibroblast growth factor gene, *Fgf8*, that is insertionally activated in 10% of the tumors (MacArthur *et al.*, 1995). This was the first report of MMTV activation of the *Fgf8* gene; however, no studies have been reported that test whether *Fgf8* is a target for MMTV insertional mutagenesis in normal (nontransgenic) mice. Since *Wnt1* is normally not expressed in the mouse mammary gland, an analysis of *Fgf8* activation frequencies in tumors of normal and *Wnt1* transgenic mice has the potential to reveal whether the Wnt1 signal affects the mammary gland's sensitivity to FGF8. This is an important question considering the apparent roles of Wnts and FGFs as collaborators in both normal development as well as in tumorigenesis (Peters *et al.*, 1986; Christian *et al.*, 1992; Kwan *et al.*, 1992; Shackleford *et al.*, 1993; Crossley and Martin, 1995; Lee *et al.*, 1995; MacArthur *et al.*, 1995; Parr and McMahon, 1995; Yang and Niswander, 1995; Crossley *et al.*, 1996).

In the current study, we sought to continue our investigation of the collaboration between *Fgf8* and *Wnt1*. Our first aim was to further analyse the

mammary tumor panel generated from MMTV infection of *Wnt1* transgenic mice by extensively testing for transcriptional activation of *Fgf8* using Northern analyses. Secondly, we set out to test whether the *Fgf8* gene is insertionally activated in normal mice by the same method. Additionally, we examined both tumor groups (transgenic and normal) for activation of two previously identified MMTV-activated genes, *Fgf3* and *Fgf4*, to enable a comparison with any differences observed in *Fgf8* activation between these groups.

Activation of Fgf8, Fgf3 *and* Fgf4 *in mammary tumors from MMTV-infected* Wnt1 *transgenic mice*

Northern and Southern analyses (Figures 1a, 2 and 3) were performed in order to test for insertionally activated expression of *Fgf8*, *Fgf3* and *Fgf4* in approximately 70 mammary tumors derived from *Wnt1* transgenic animals that were infected with MMTV. Northern analyses were emphasized in this study to enable detection of gene activations that might not be observed using standard Southern blotting due to the limitations of this technique – for example, in detecting distant insertions. It is widely believed that MMTV insertions activate these *Fgf* genes from the transcriptionally silent state, since expression is generally not observed by Northern analysis in normal mouse mammary glands or in tumors that lack proviruses flanking the gene in question (Dickson *et al.*, 1984; Peters *et al.*, 1989a; Shackleford *et al.*, 1993; MacArthur *et al.*, 1995; Figure 1; data not shown). We found activation of *Fgf8* in 50% (38/76), *Fgf3* in 44% (28/64) and *Fgf4* in 7% (4/60) of the tumors (Figures 1a and 3). Interestingly, *Fgf8* was activated in these mice at least as frequently as *Fgf3*, a common insertion site for MMTV

in nontransgenic mice (Peters, 1990). This indicates that *Fgf8* is a stronger oncogenic collaborator with *Wnt1* than previously suggested (MacArthur *et al.*, 1995).

Using Northern blot analysis 30 new *Fgf8* activations have been found since the initial Southern blot screening of the tumor panel (MacArthur *et al.*, 1995). However, proviral insertions were not detected in 27 of these 30 tumors in a ~36 kb region containing the *Fgf8* gene, suggesting that they may have sustained insertions outside of this region (Figure 2 and data not shown). We mapped the insertions in four of the 27 tumors to within ~2.7 cM of the *Fgf8* gene in the distal region of mouse chromosome 19 using interspecific backcross and Southern analysis (Rowe *et al.*, 1994). A fifth MMTV insertion was also mapped to this chromosomal region, but RNA was not available to test for activation of *Fgf8* in this tumor. It is likely that *Fgf8* expression in the other 23 tumors with unmapped *Fgf8* activations is also induced by long range insertional activation by MMTV. We cannot rule out that expression of *Fgf8* in these cases is an indirect result of MMTV integration near or within a different gene whose product in turn regulates *Fgf8*. However, since long distance activations, apparently direct in nature, by proviral insertions are not uncommon for MMTV and other viruses, the direct scenario seems more likely (Bartholomew *et al.*, 1989; Peters *et al.*, 1989b; Lazo *et al.*, 1990; Fourel *et al.*, 1994).

Activation of Fgf8, Fgf3 and Fgf4 *in mammary tumors from MMTV-infected nontransgenic mice*

Although proviral activation of *Fgf8* occurs frequently in mammary tumors of MMTV-infected *Wnt1* trans-
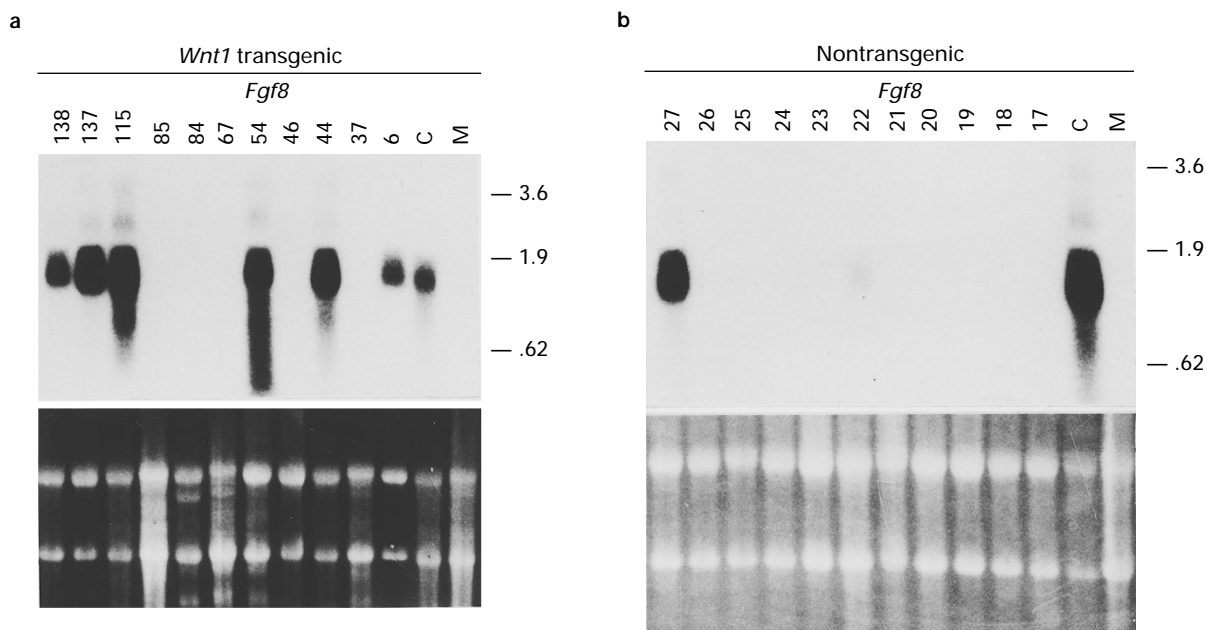

Figure 1 Representative Northern blot analysis of *Fgf8* expression in mammary tumors from MMTV-infected mice. Total cellular RNAs from mammary tumors from (**a**) *Wnt1* transgenic mice and (**b**) nontransgenic littermates were electrophoresed, blotted to nylon membranes, and hybridized with a ^{32}P-labeled *Fgf8* cDNA probe as described previously (Shackleford and Varmus, 1987). RNAs from normal mammary tissue of breeding mice, M, and a tumor known to be positive for *Fgf8* expression, C, are indicated. The upper panels show the hybridization signals from the membranes after Northern transfer, and the lower panels display ethidium bromide stained RNA gels before blotting. Numbers to the right of the panels indicate the positions of RNA size markers in kilobases
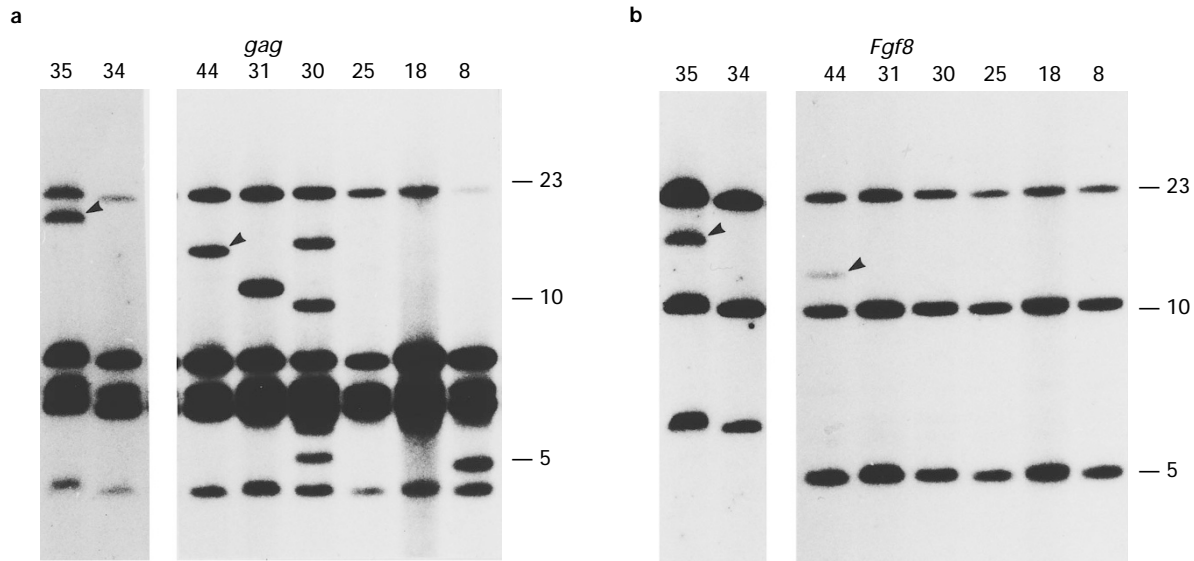
a

gag

35 34   44 31 30 25 18 8

— 23

— 10

— 5

b

Fgf8

35 34   44 31 30 25 18 8

— 23

— 10

— 5

Figure 2 Representative Southern blot analysis of tumor DNAs from infected *Wnt1* transgenic mice. Two independent Southern blots are shown in each panel containing DNAs isolated from 8 mammary tumors. DNAs were digested with *Bgl*II, electrophoresed, blotted to nylon membranes, and hybridized with ^{32}P-labeled probes stated below as described previously (Shackleford and Varmus, 1988). (**a**) The blots were first hybridized with an MMTV *gag* probe. In addition to the endogenous MMTV proviruses, exogenous proviruses are detected as tumor-specific fragments of unique size, indicating independent clonal or quasi-clonal populations of infected cells. (**b**) Subsequently, the blots were stripped of the *gag* probe and hybridized with a mixture of *Fgf8* probes. An *Fgf8a* cDNA probe was used in combination with probes called XP1200 (left blot) or BB2.4 (right blot) to detect regions surrounding the *Fgf8* gene of ∼30 kb or ∼36 kb, respectively. The XP1200 probe detects a DNA fragment of ∼6 kb containing a *Nub1* pseudogene (MacArthur *et al.*, 1995), now called *Npm3-ps1* (MacArthur and Shackleford, 1997), in addition to a ∼21 kb fragment at the *Fgf8* locus. Only two tumors (35 and 44) showed rearrangements in the *Fgf8* locus despite the fact that *Fgf8* is transcriptionally activated in all of the tumors. Arrowheads indicate fragments that hybridize to both *gag* and *Fgf8* probes. Numbers to the right of the blots indicate the positions of DNA size markers in kilobases
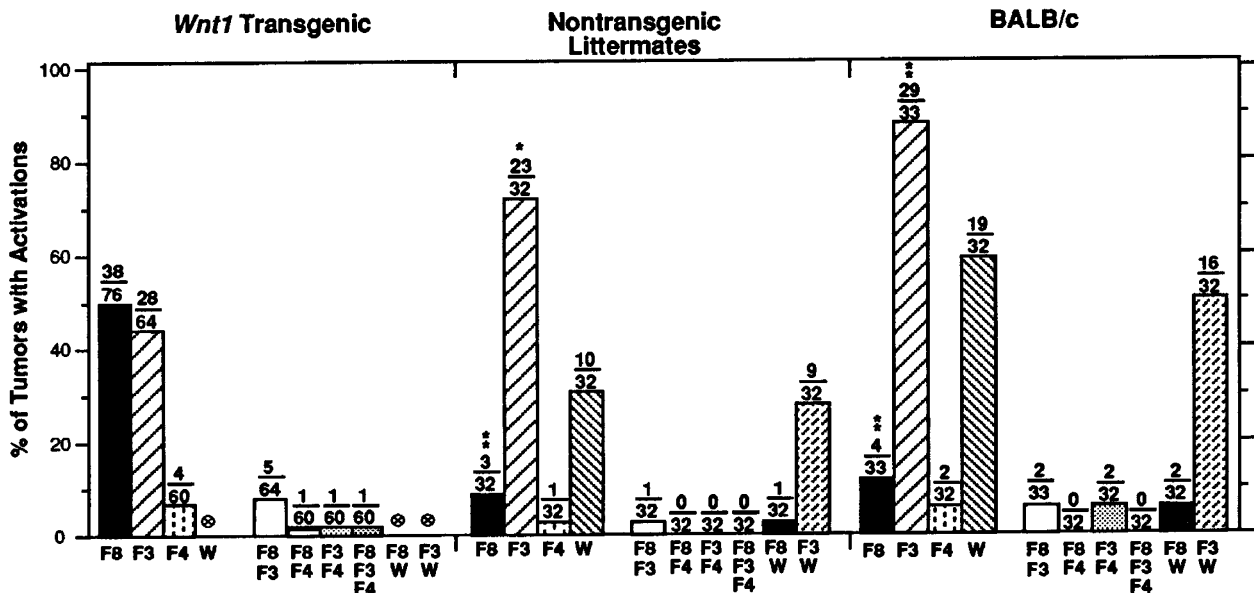

Figure 3 Activation frequencies of *Fgf8*, *Fgf3*, *Fgf4* and *Wnt1* in mammary tumors of MMTV-infected mice. Data is compiled from Northern blots containing total cellular RNAs from tumors of three groups of infected mice: *Wnt1* transgenics, nontransgenic littermates, and BALB/c. Genes tested for activation include *Fgf8* (F8), *Fgf3* (F3), *Fgf4* (F4), and *Wnt1* (W); some tumors had multiple genes activated in the same tumor as indicated. The four bars labeled 'F8', 'F3', 'F4' and 'W' include all tumors with activations in these genes, including those tumors with double or triple activations. The bars labeled for multiple activations include only those tumors that have the indicated double or triple activations. The statistical significance of the difference in gene activation frequencies between the nontransgenic littermate tumor group and the *Wnt1* transgenics, and between the BALB/c group and the *Wnt1* transgenics was determined by Chi Square analysis. Activation frequencies that are statistically significant between the transgenic and the normal groups are indicated by * ($P < 0.01$) or ** ($P < 0.001$). Numbers above the bars indicate the fractions of tumors with activations. The presence of the *Wnt1* transgene precludes testing for activation of *Wnt1* in the transgenic group (indicated by '⊗'). The probes used for hybridization were *Fgf8a* cDNA (MacArthur *et al.*, 1995), an *Fgf3* cDNA called c.28 (Mansour and Martin, 1988), an *Fgf4* clone called HH1 (Peters *et al.*, 1989a), and *Wnt1* cDNA clone #26 (Fung *et al.*, 1985)

genic mice, such events have not been reported in infected nontransgenic mice. We therefore examined this issue by Northern analysis using tumor RNAs from MMTV-infected nontransgenic littermates of the *Wnt1* transgenics and from MMTV-infected BALB/c mice. Blots were hybridized with a probe for *Fgf8* (Figures 1b and 3) as well as probes for *Fgf3*, *Fgf4* and *Wnt1* to allow a comparison to the transgenic results above.

Fgf8 was found to be activated in 11% (7/65) of these tumors, with similar frequencies in both subgroups: 9% (3/32) of nontransgenic littermates and 12% (4/33) of BALB/c tumors (Figure 3). Activations of *Fgf3*, *Fgf4* and *Wnt1* were seen in 72% (23/32), 3% (1/32) and 31% (10/32) of the tumors from the nontransgenic littermates compared to 88% (29/33), 6% (2/32) and 59% (19/32) in the BALB/c group, respectively. The sum of the percentages for each group is greater than 100%, since some tumors showed activations of more than one gene. The disparity in *Wnt1* activation frequencies between the two nontransgenic groups (Chi square test; $P < 0.001$) is most likely due to the differences in the genetic backgrounds (Marchetti *et al.*, 1991). Proviral insertions were not detected in the ~ 36 kb region containing the *Fgf8* gene in tumors from the nontransgenic littermate group that showed *Fgf8* expression (data not shown), suggesting long distance activation by MMTV in these tumors.

In summary, there is an apparent selection for *Fgf8* activations in MMTV-infected *Wnt1* transgenic mice. Our results show that the activation of *Fgf8* was significantly more frequent, 4.5-fold higher, in tumors from transgenic mice (50%) than from both groups of nontransgenic mice (littermates and BALB/c; average 11%), demonstrating that *Wnt1* and *Fgf8* are powerful collaborators in mammary carcinogenesis. In contrast, the activation frequency of *Fgf3* in tumors from transgenic mice (44%) was approximately one-half that of both groups of nontransgenic mice (average 80%). The differences in activation frequencies between the transgenic and nontransgenic tumor groups for both *Fgf8* and *Fgf3* were statistically significant (Figure 3). Moreover, a striking difference is apparent in the ratio of *Fgf8* to *Fgf3* activations in the transgenic group (50%:44%) compared to the ratio in the combined nontransgenic group (11%:80%). These results indicate that there is a strong preference for the activation of *Fgf8* when *Wnt1* expression is present. The selection for *Fgf8* activations apparently requires the preexisting expression of *Wnt1* in cells, since there are only a few tumors in the nontransgenic group with double activations of both *Fgf8* and *Wnt1*. It is possible that the selection of *Fgf8* activations in

Wnt1-expressing cells is uncovering an important cooperative event that occurs normally between these two pathways, especially considering the mounting evidence that FGFs and Wnts collaborate in normal development (Christian *et al.*, 1992; Crossley and Martin, 1995; Parr and McMahon, 1995; Yang and Niswander, 1995; Crossley *et al.*, 1996).

At least two possibilities may be proposed to explain the observed differences in activation frequencies between the transgenic *versus* normal tumor groups, in particular the elevated frequency of *Fgf8* activations in *Wnt1* transgenic animals. One might propose that the absence of *Wnt1* as an activation target in the *Wnt1* transgenics (since *Wnt1* is already activated in these mice) would result in an increase in the activation frequencies of other genes in these animals. However, since we observe fewer, not more, *Fgf3* activations in transgenic *versus* nontransgenic tumors, the explanation is not likely to be as simple as target competition.

More likely, this system may be detecting differences in the signaling pathways of the two ligands, FGF3 and FGF8. For example, it is possible that Wnt1 stimulation modulates the expression or activity of one or more components in the FGF8 signaling pathway, such as an FGF8 receptor or a protein(s) farther downstream in the pathway, resulting in enhanced signal transduction. Consistent with this hypothesis is the observation that FGF3 and FGF8 preferentially utilize different FGF receptors (Ornitz *et al.*, 1996). Further investigation will be necessary to determine the exact mechanism of the increased oncogenicity of FGF8 in the presence of Wnt1.

In conclusion, the proviral activation frequency of *Fgf8*, but not *Fgf3* or *Fgf4*, in mammary tumors increased dramatically in the context of *Wnt1* transgene expression. Cells expressing *Wnt1* appear to be particularly sensitized to signaling by FGF8, suggesting that Wnt1 signaling may modulate some aspects of the FGF8 signal transduction pathway.

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