Abstract

Background: MMTV-Wnt1 transgenic mice develop mammary hyperplasia early in development, followed by the appearance of solitary mammary tumors with a high proportion of cells expressing early lineage markers and many myoepithelial cells. The occurrence of tumors is accelerated in experiments that activate FGF proto-oncogenes or remove the tumor suppressor genes Pten or P53, implying that secondary oncogenic events are required for progression from mammary hyperplasia to carcinoma. It is not known, however, which oncogenic pathways contribute to Wnt1-induced tumorigenesis – further experimental manipulation of these mice is needed. Secondary events also appear to be required for mammary tumorigenesis in MMTV-Neu transgenic mice because the transgene in the tumors usually contains an acquired mutation that activates the Neu protein-tyrosine kinase.

Methods: cDNA or DNA from the mammary glands and mammary tumors from MMTV-Wnt1, MMTV-Wnt1/p53-/-, MMTV-Neu transgenic mice, and newly generated MMTV-Wnt1/MMTV-Neu bitransgenic mice, was sequenced to seek activating mutations in H-Ras, K-Ras, and N-Ras genes, or in the MMTV-Neu transgene. In addition, tumors from bitransgenic animals were examined to determine the cellular phenotype.

Results: We found activating mutations at codons 12, 13, and 61 of H-Ras in just over half of the mammary tumors in MMTV-Wnt1 transgenic mice, and we confirmed the high frequency of activating mutations of Neu in tumors in MMTV-Neu transgenic mice. Tumors appeared earlier in bitransgenic MMTV-Wnt1/MMTV-Neu mice, but no Ras or MMTV-Neu mutations were found in these tumors, which were phenotypically similar to those arising in MMTV-Wnt1 mice. In addition, no Ras mutations were found in the mammary tumors that arise in MMTV-Wnt1 transgenic mice lacking an intact P53 gene.

Conclusions: Tumorigenic properties of cells undergoing functionally significant secondary mutations in H-Ras or the MMTV-Neu transgene allow selection of those cells in MMTV-Wnt1 and MMTV-Neu transgenic mice, respectively. Alternative sources of oncogenic potential, such as a second transgenic oncogene or deficiency of a tumor suppressor gene, can obviate the selective power of those secondary mutations. These observations are consistent with the notion that somatic evolution of mouse mammary tumors is influenced by the specific nature of the inherited cancer-promoting genotype.
Background

Several transgenic mouse models have been generated in efforts to identify or confirm genes that can participate in breast carcinogenesis and to study the pathogenic process (for review, see [1]). In general, these models have revealed that a wide variety of proto-oncogenes can initiate mammary tumorigenesis when expressed under the control of an appropriate promoter (usually the mouse mammary tumor virus long terminal repeat (MMTV LTR) or the whey acidic protein (WAP) transcriptional control domain). They have also revealed that tumorigenesis is a stochastic process, resulting in the appearance of solitary tumors in one or a few of the 10 mammary glands several months after birth, and that secondary mutational events, inherited deficiencies in tumor suppressor genes, or a combination of transgenes can accelerate the onset of tumor growth. In addition, persistent tumor growth generally requires the continued expression of the initiating oncogene, unless certain secondary mutations have occurred to render the tumor independent of the transgenic oncogene [2-4].

Despite these generalizations, the characteristics of mammary tumors arising in transgenic mice differ in a fashion characteristic of the initiating oncogene. For example, the expression profiles of tumors reflect the transgenic oncogene [5]; moreover, those tumors induced by genes that activate the Ras signaling pathway (for example, polyoma middle T antigen, ErbB2/Neu, and H-Ras) are more similar to each other than to tumors induced by components of the Wnt signaling pathway (Wnt1, β-catenin, and c-Myc) with respect both to gene expression patterns ([5]. S. Huang et al., in preparation) and to cellular composition and histology [6-9]. In tumors induced by components of the Wnt signaling pathway, a high proportion of cells produce proteins (Sca-1, Keratin 6) associated with early stages of mammary development, whereas few such cells are observed in tumors induced by activators of Ras signaling [9]. In the presence of many tumor cells with myoepithelial features (for example, expression of smooth muscle actin) and other observations suggest that in the Wnt pathway oncogenes transform cells positioned early in the developmental program for mammary tissue, whereas oncogenes like ErbB2/Neu that activate Ras proteins, either transform cells that are more advanced developmentally or drive cells to a more mature epithelial character during oncogenesis.

Efforts to understand these complex pathogenic events must take into consideration the secondary oncogenic events that are presumed to be required to convert one or a few of the many mammary cells expressing a transgenic oncogene into a frankly tumorigenic cell. Several findings offer clues to the nature of such secondary events. For example, the appearance of mammary tumors in MMTV-Wnt1 transgenic mice can be accelerated by a second transgene (MMTV-Fgf3, [10]), by proviral insertion mutations in Fgf genes [11-13], and by inherited deficiencies of P53 or Pten [14,15]. Similarly, tumors appear earlier in MMTV-c-Myc/MMTV-v-H-Ras bitransgenic mice than in monotransgenic mice [16], and, in c-Myc transgenic mice, tumors that do not require continued expression of c-Myc usually contain somatically mutated K-Ras2 or N-Ras genes [3]. When tumors occur in animals that inherit a transgene encoding normal ErbB2/Neu protein, the tumors usually exhibit a secondary somatic mutation of ErbB2/Neu that stimulates the protein-tyrosine kinase activity of the gene product [17].

In this report, we have attempted to identify additional somatic events that can promote progression to a tumorigenic phenotype in MMTV-Wnt1 transgenic mice by seeking acquired mutations in Ras. In addition, we have asked whether accelerating factors, such as inheritance of a second transgenic oncogene, MMTV-Neu, or deficiency in a tumor suppressor gene, p53, diminishes selection for cells that have acquired potentially oncogenic mutations. In the course of these studies, we were also able to determine that the phenotypic properties of tumors arising in MMTV-Wnt1/MMTV-Neu bitransgenic mice resemble those of tumors induced by Wnt rather than Ras signaling.

Methods

Mice and tissues

MMTV-Wnt1 (FVB/NJ-Tg [Wnt1] 1 Hev/J), MMTV-Neu (FVB/N-Tg [MMTVNeu] 202 Mul/J), and p53+/− (129-Tp53tm1Tyj/J) mice were purchased from Jackson Labs (Bar Harbor, ME, USA) and maintained or backcrossed for many generations on the FVB background. All mice were maintained in a specific pathogen-free facility on a standard diet.

RNA, DNA, and protein extraction from tumors

Mouse mammary tumors were collected at the time of necropsy or tumor extraction surgery and snap frozen in liquid nitrogen. Frozen tumors were ground in liquid nitrogen and the resulting powder was placed in three tubes containing appropriate buffers. For RNA extraction, about 50 mg of tumor powder was dissolved in Trizol (#15596-018; Invitrogen, Carlsbad, CA, USA), followed by phenol-chloroform extraction and ethanol precipitation. For DNA extraction, about 50 mg of tumor powder was digested overnight with 2 mg/ml Proteinase K (#1 373 200; Roche, Indianapolis, IN, USA) in 20 mM Tris, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, pH 8.5 buffer, followed by phenol-chloroform extraction and ethanol precipitation. For protein extraction, about 50 mg of tumor powder was dissolved in 20 mM Tris, 150 mM NaCl, 1% Triton X, 2 mM EDTA, 1x protease inhibitor cocktail (complete tablets, #1 697 498, Roche), 1 mM Na2VO4, 40
mM NaF, incubated on ice for up to 30 min and cleared by centrifugation.

Some DNA samples from MMTV-Wnt1 tumors with wild type, heterozygous or null p53 allele (generated by L. Donehower [18]) on a mixed genetic background were a gift from Larry Donehower (Baylor College of Medicine).

**Ras and MMTV-Neu cDNA synthesis**

Extracted RNA was copied with gene-specific primers to make cDNA, which was then amplified with the appropriate primers using the SuperScript™ One-Step RT-PCR Kit (#10928-034; Invitrogen) according to manufacturer's instructions. For mouse K-Ras and N-Ras amplification, primer sequences and cycling conditions were kindly provided by Lewis Chodosh and Robert Boxer (University of Pennsylvania School of Medicine). Mouse H-Ras codons 3–99 were amplified either with primers generated based on the sequences provided by Chodosh and Boxer, or with an additional reverse primer spanning exons 2 and 3: HARAS.B2: 5’GATCTGCTCCCTGTACTGATGG3’.

**MMTV-Neu transgene cDNA was amplified with primers AB2913 and AB1310 [17], which amplify the region corresponding to nucleotides 1492–2117 of rat Neu cDNA.**

Amplification products were purified with QIAquick PCR Purification Kit (#28106; Qiagen, Valencia, CA, USA) according to manufacturer's instructions.

**H-Ras DNA synthesis**

Tumor DNA was extracted as described above, and extracted DNA was amplified with Taq polymerase (#N808-0152; Roche) in 1 × PCR Buffer (#N808-0006; Roche), 2.5 mM dNTPs in the presence of the following primers: mouse H-Ras exon 1: HRAS.F1A: 5’-GCTT-GGCTAACGTTGCTTCTC-3’, HRAS.B1A: 5’-CCACCTCTGGCAGGTAAG-3’; mouse H-Ras exon 2: HRAS.F2A: 5’-GGATTTCTCTGGTCTGCAGG3-’, HRAS.B2A: 5’-GGATAT-GAGCCAGCTAGC-3’. Amplification was performed for 30 cycles of 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. Amplification products were purified as above.

**Sequencing**

All sequencing was carried out by the Memorial Sloan-Kettering Cancer Center (MSKCC) Sequencing Core Facility. Every cDNA was sequenced with both forward and reverse primers used for amplification, and some samples were also sequenced with the nested reverse primer. Sequencing peaks were inspected for overall integrity and legibility. The sample was scored as mutant when heterozygous peaks at positions 12, 13, 59, and 61 were at least the same height as the wild type peaks in at least one sequencing direction. Sequences were also downloaded into Vector NTI 5.2.5 (InforMax, Inc., Frederick, MD, USA) program and aligned on the Baylor College of Medicine Search Launcher [19] against the sequences deposited in GenBank (mouse H-Ras: nm008284; mouse K-Ras: xm110615; mouse N-Ras: nm010937, rat Neu: X03362), to detect mutations (or lack of thereof) in the transcripts.

**Immunoblotting and immunohistochemistry**

Proteins were extracted from tumors as described above, and lysates were diluted 1:1 with 2 × sample buffer (#LC2676, Invitrogen) with 10% β-mercaptoethanol (#M-3148, Sigma, St. Louis, MO, USA), boiled, loaded on Novex SDS/PAGE gels (#EC6075BOX, Invitrogen) and separated by electrophoresis for 1.5 hrs at 125 V and then stained with Coomassie Blue to estimate protein concentrations. Equal amounts of tumor lysates were then reloaded on the 10% SDS/PAGE gels and separated by electrophoresis. Separated proteins were transferred onto nitrocellulose membranes according to the gel manufacturer's instructions. Membranes were incubated for 1 hour with 5% solution of non-fat dried milk (Carnation, Nestlé, Solon, OH, USA), followed by 1 hour incubation with rabbit polyclonal antibodies against phospho-ERK, 1:500 dilution, (#9101S; Cell Signaling, Beverly, MA, USA), total ERK, 1:500 dilution, (#9102; Cell Signaling) or mouse monoclonal antibody against γ-tubulin, 1:1,000 dilution, (GTU88; Sigma), followed by appropriate secondary antibodies conjugated with HRP (donkey-anti-rabbit, 1:10,000, #111-035-144, Jackson Immunochemicals, West Grove, PA, USA; or rat-anti-mouse kappa light chain, 1:2,000, #2067 1323, Zymed, San Francisco, CA, USA).

Tissues were fixed in 10% buffered formalin (#SF100-4, Fisher, Fair Lawn, NJ, USA) for 16–24 hrs, transferred to 70% ethanol, and shipped to be paraffin-embedded and sectioned at Histoserv (Germantown, MD, USA). Individual sections were deparaffinized, rehydrated and boiled in 1 mM EDTA for antigen retrieval. Slides were then treated with peroxidase, followed by incubation with the appropriate blocking serum from the Vectastain kits (anti-rat-ABC-peroxidase, #PK6104; anti-rabbit-ABC-peroxidase, #PK6101; MOM, # PK2200, Vector Laboratories, Inc., Burlingame, CA, USA) according to manufacturer's instructions. Primary antibodies against phospho-ERK (rabbit polyclonal, #9101S; Cell Signaling), keratin 8 (TROMA, rat polyclonal, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), smooth muscle actin (SMA, mouse monoclonal, #M0851; Dako, Carpinteria, CA, USA), or mouse keratin 6 (rabbit polyclonal, #PRB-169P; Covance, Princeton, NJ, USA) were applied for 1 hr, followed by an incubation with biotin-conjugated appropriate secondary antibody and signal amplification according to Vectastain kit manufacturer's instructions (Vector Laboratories, Inc.). Color was developed with NovaRed™ substrate kit (#SK-
4800; Vector Laboratories, Inc.) according to manufacturer's instructions.

**Results**

**Activating mutations in H-Ras occur in about half of mammary tumors in MMTV-Wnt1 mice**

Wnt1-induced mammary tumors contain elevated levels of c-Myc RNA and protein, as expected from stimulation of the Wnt signaling pathway [20]. Since an H-Ras transgene can hasten tumorigenesis when combined with a c-Myc transgene [16] and since K-Ras or N-Ras is frequently mutated in c-Myc-induced tumors [3], we decided to look for secondary somatic mutations in mammary tumors arising in MMTV-Wnt1 transgenic mice by sequencing cDNA copies of Ras mRNAs in the tumors. Mutations were scored only if approximately half of the resulting amplified DNA had the same mutation, implying growth selection of an oncogenic cell in which the observed mutation occurred.

**Figure 1**

Mutations in the H-Ras gene. Representative examples of somatic mutations in the H-Ras cDNA from primary mammary tumors of the MMTV-Wnt1 mice. Sequence chromatograms of codons 12, 13, and 61 are shown as they appear in the forward and reverse sequencing directions. Mutant peaks are indicated by arrows. The nucleotide and amino acid alterations are indicated on the right.

**Table 1: Frequency and type of H-Ras mutations in MMTV-Wnt1 tumors**

| Codon mutated | Number (% total) |
|---------------|-----------------|
| CAA61CTA      | 10 (22%)        |
| GGA12GAA      | 5 (11%)         |
| CAA61CGA      | 4 (9%)          |
| CAA61AAA      | 2 (4%)          |
| CAA61CAT      | 1 (2%)          |
| GGC13AGA      | 1 (2%)          |
| GGC13GTT      | 1 (2%)          |
| H-Ras WT      | 22 (48%)        |
| Total         | 46 (100%)       |

Primary tumor cDNA was amplified and sequenced with H-Ras-specific primers at least once in each direction as described in Methods. Products containing mutant peaks equal to or greater in height than the wild type peaks in at least one sequencing direction were scored as mutation-positive. Mutations in codons 12, 13, 59, and 61 were scored as activating mutations.
We initially sought activating Ras mutations by sequencing codons 3–99 of H-Ras, 1–98 of N-Ras, and 1–94 of K-Ras. All of the sequences contain codons 12, 13, 59, and 61 – sites of the mutations most commonly associated with oncogenic Ras proteins. We found mutations in codons 12 and 61 of H-Ras, but not in those of N-Ras or K-Ras in cDNA from the first 10 tumor samples (Figure 1). We subsequently focused on the H-Ras cDNA and found activating mutations in codons 12, 13, or 61 in 24 of an additional 36 primary mammary carcinomas (Table 1). We then examined 20 tumors without activating mutations in H-Ras for activating point mutations in K-Ras and N-Ras, but failed to find mutations in these two genes. In addition, we were unable to detect mutant H-Ras cDNA made from RNA extracted from hyperplastic mammary glands from MMTV-Wnt 1 transgenic mice (n = 6) or from virgin or lactating mammary glands from non-transgenic mice (n = 6), implying that, if H-Ras mutations occurred before the appearance of primary tumors, an insufficient number of cells in the glands harbored the mutations to allow detection, and that selection during tumorigenic growth would be required.

We made a preliminary effort to define the selectable trait conferred on mammary cells in MMTV-Wnt 1 transgenic mice by an activating mutation in H-Ras. Tumors in these mice arise after a latency of similar length, have similar growth characteristics, and metastasize to the lungs with similar frequencies regardless of whether they do or do not contain a mutant H-Ras gene (data not shown).

These findings may simply mean that any requirement for activation of the Ras pathway in Wnt1-induced tumors may be satisfied by some other means, such as other oncogenic mutations; this issue is further addressed in a later section.

We have also used biochemical methods to look for the consequences of the H-Ras mutations. For example, the oncogenic activity of Ras proteins in mouse cells is dependent on the Raf kinase pathway, which phosphorylates and activates the ERK1/p44 and ERK2/p42 mitogen-activated protein kinases (MAPKs) [21]. We have compared levels of phospho-ERK in Wnt1-induced tumors with mutant and wild type H-Ras genes by protein blotting and immunohistochemistry (Figure 2). While tumors induced by an MMTV-v-H-Ras transgene contained high levels of phospho-ERK, MMTV-Wnt1 and MMTV-c-Myc-induced tumors had lower levels, independent of H-Ras mutation status (Figure 2A). Similarly, there was no difference in phospho-ERK staining patterns in tumor sections from any of the MMTV-Wnt1-induced tumors, although intense staining was observed in MMTV-v-H-Ras induced tumors (Figure 2B). Thus we do not know which of the several signaling pathways affected by Ras may be responsible for growth selection of tumor cells containing H-Ras mutations.

**Mammary tumorigenesis is accelerated in MMTV-Wnt1/ MMVT-Neu bitransgenic mice**

Her-2/Neu acts in part through the Ras signaling pathway [22-24], a feature that may account for the remarkable similarity between the phenotypes of mammary tumors found in MMTV-Neu and MMTV-v-H-Ras transgenic mice [25]. These observations suggest that an inherited Neu transgene might mimic the effects of somatic mutation of a Ras gene. If so, tumors might arise earlier in MMTV-Wnt1/MMTV-Neu bitransgenic mice than in mice carrying a single transgene, and any cells that acquired H-Ras mutations would not have a selective growth advantage; hence H-Ras mutations would not be detected in our tests of tumor genotypes. In addition, with bitransgenic mice, we could ask whether a co-existing MMTV-Wnt1 transgene eliminated the selective advantage conferred by an activating mutation in the coding domain of the MMTV-Neu transgene, and we could ask whether the phenotype of tumor cells in resulting tumors resembled the phenotype of tumors induced by components of the Wnt pathway, the Neu/Ras pathway, or both.

Therefore we crossed MMTV-Wnt1 transgenic mice to a MMTV-Neu transgenic line that carries a cDNA encoding normal rat ErbB2/Neu protein [26]. In the bitransgenic MMTV-Wnt1/MMTV-Neu progeny, tumors were detected significantly earlier than in sibling females harboring only an MMTV-Neu transgene or in MMTV-Wnt1 transgenic mice (Figure 3). Over fifty percent (8/14) of the female MMTV-Wnt1/MMTV-Neu mice developed tumors by 12 weeks of age, twice as early as a similar cohort of MMTV-Wnt1 mice (t50 = 25 weeks, n = 44), and almost four times as early as the MMTV-Neu congenic mice (t50 = 39 weeks, n = 26), maintained in the same facility.

This acceleration of tumor formation demonstrates that Wnt1 and Neu transgenes can cooperate in oncogenic transformation of the mouse mammary gland. To determine whether the presence of an inherited Neu transgene obviated the selective advantage of secondary somatic mutations in H-Ras, observed in many of the mammary tumors induced by a single MMTV-Wnt1 transgene (Table 1), we sequenced DNA amplified from H-Ras RNA obtained from 11 tumors arising in the MMTV-Wnt1/ MMTV-Neu bitransgenic animals. None of these samples showed mutations in the first two exons of H-Ras, supporting the notion that the presence of a Neu transgene provided most or all of the growth advantage attributed to Ras mutations in MMTV-Wnt1 or MMTV-c-Myc transgenic mice (Table 1, [3]).
Mammary tumors reported in the MMTV-Neu monotransgenic line used here have usually acquired mutations in the Neu coding domain of the transgene, enhancing the enzymatic activity of its product; about 70% of these tumors exhibit point mutations, small deletions, or insertions in the extracellular portion of the receptor in or near the cysteine-rich domain encoded by nucleotides 1492–2117 of rat Neu cDNA [17]. However, none of 11 mammary tumors derived from MMTV-Wnt1/MMTV-Neu bitransgenic mice had evidence of mutations in the relevant portions of the Neu transgene. This result has two implications: that the presence of an inherited MMTV-Wnt1 transgene strongly diminishes any selective advantage conferred by secondary somatic mutations in the Neu transgene, and that expression of a wild-type version of the Neu transgene is sufficient to provide the growth advantage that is apparently conferred by secondary somatic mutations of H-Ras in MMTV-Wnt1-induced tumors.

**Activating H-Ras mutations are not found in Wnt1-induced tumors arising in p53 null mice**

We have previously shown that deficiency of p53 dramatically accelerates the appearance of mammary tumors in MMTV-Wnt1 transgenic mice [14]. More recently, Gunther et al. showed that tumors induced by Wnt1

**Tumors in the MMTV-Wnt1/MMTV-Neu bitransgenic mice are morphologically similar to Wnt1-induced tumors, despite expression of the Neu transgene**

MMTV-Wnt1-induced tumors contain multiple mammary cell types, and many cells express early lineage markers, such as Sca-1 and keratin-6 [9]. In contrast, MMTV-Neu-induced mammary tumors are composed nearly exclusively of luminal epithelial cells and have a low proportion of Sca-1- and keratin-6-positive cells [9]. Remarkably, all tumors from bitransgenic mice were similar to the Wnt1-induced tumors, and not to the Neu-induced ones (Figure 4A). We detected multiple cell types within these tumors, including smooth muscle actin-positive myoepithelial cells, keratin-8-positive luminal epithelial cells, and keratin 6-positive cells (Figure 4B, panels a-c). Expression of the MMTV-Neu transgene was observed in the luminal epithelial cells from these tumors by immunohistochemistry (Figure 4B, panel d) and by an RT-PCR assay with transgene-specific primers (Figure 4C).

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**Figure 2**

ERK phosphorylation in MMTV-Wnt1 tumors with and without H-Ras mutations. (A) Protein lysates from tumors from a MMTV-v-H-Ras mouse (first lane) and MMTV-Wnt1 mice with and without activating H-Ras mutations were subjected to electrophoresis in 10% polyacrylamide gels, transferred to a nitrocellulose membrane and then exposed to antibodies against phospho-ERK, total ERK and γ-tubulin as described in Methods. (B) Primary tumor sections from (a) MMTV-v-H-Ras mouse and (b) MMTV-Wnt1 mice without activating H-Ras mutations and (c,d) with activating H-Ras mutations were incubated with antibodies against phospho-ERK as described in Methods.
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Wnt1

mice, with or without loss of heterozygosity (LOH). Wnt1-induced tumors arising on a p53 heterozygous background, irrespective of the LOH status, contain H-Ras mutations at a frequency (5/11) similar to that described above for Wnt1-induced tumors on a wild type p53 background. Importantly, however, no H-Ras mutations were found in any of the sixteen Wnt1-induced tumors on a p53+/− background (P < 0.05). We conclude that an inherited deficiency of p53 is likely to reduce or eliminate, by an unknown mechanism, any selective growth advantage that might otherwise be contributed by secondary somatic mutations of H-Ras in Wnt1-producing mammary cells. The finding of H-Ras mutations in the small number of tumors with loss of p53 heterozygosity implies that Ras mutation occurs before p53 LOH, since Ras mutations appear not to confer a selective advantage in p53 null cells. However, a larger cohort of tumors undergoing LOH at the p53 locus will be required to establish the conclusion more firmly.

Discussion

Most contemporary accounts of oncogenesis assume that multiple mutations affecting tumor suppressor genes and proto-oncogenes collaborate to convert a normal cell into a cancer cell. When these genetic events occur sequentially by somatic mutations during tumor development, it is assumed that the functionally significant mutations confer a selective advantage, such as an augmented rate of growth, protection from apoptosis, or promotion of further mutations, which explains why most if not all cells in a tumor exhibit multiple acquired mutations, consistent with repeated clonal selection.

In this report, we have used several features of recent studies of breast carcinogenesis in genetically manipulated mice to explore the contributions made to a multi-step oncogenic process by a variety of inherited and somatic mutations. We have identified mutations in the H-Ras proto-oncogene as a significant feature of Wnt1-induced tumorigenesis (Table 1), and we have shown that an inherited Neu transgene or an inherited deficiency of the p53 tumor suppressor gene can provide a selective advantage to Wnt1-expressing mammary cells that overrides the selective advantage conferred by somatic mutation of H-Ras. This conclusion is based on our failure to find H-Ras mutations in any tumors arising in bitransgenic (MMTV-Wnt1/MMTV-Neu) or p53-deficient (MMTV-Wnt1/p53−/−) mice (Table 2).

It is not difficult to reconcile the lack of H-Ras mutations in tumors from bitransgenic mice based on the overlapping signaling pathways in which Neu and Ras participate [22,23,27,28] and on the evidence for similarities in gene expression profiles and cell-type composition of mouse mammary tumors induced by these two genes [5,9,24,25]. However, the absence of H-Ras mutations in Wnt1-induced tumors from p53 null mice is surprising, since it is well known that an activated Ras gene can collaborate with p53 deficiency to promote both transformation of cultured cells [29] and tumor formation in vivo. For example, a mutant K-Ras gene induces lung adenocarcinomas more rapidly in p53-deficient than in p53-wild type mice [30,31], and K-Ras mutations are often accompanied by loss of p53 function in human cancers of the colon.

acquire Wnt1 independence in a p53-deficient background [2]. We therefore asked whether we could detect H-Ras mutations in mammary tumors induced by an MMTV-Wnt1 transgene in a p53-null background; a failure to find such mutations would imply either that p53 is somehow required for such mutations to occur or, more likely, that an inherited deficiency of p53 conferred a growth advantage to Wnt1-expressing mammary cells that would outweigh any selective advantage provided by a secondary somatic mutation in H-Ras.

To explore this issue, we examined cDNA or DNA from 16 tumors from MMTV-Wnt1 transgenic, p53+/− mice and from 11 tumors from MMTV-Wnt1 transgenic, p53−/− mice, with or without loss of heterozygosity (LOH). Wnt1-induced tumors arising on a p53 heterozygous background, irrespective of the LOH status, contain H-Ras mutations at a frequency (5/11) similar to that described above for Wnt1-induced tumors on a wild type p53 background. Importantly, however, no H-Ras mutations were found in any of the sixteen Wnt1-induced tumors on a p53+/− background (P < 0.05). We conclude that an inherited deficiency of p53 is likely to reduce or eliminate, by an unknown mechanism, any selective growth advantage that might otherwise be contributed by secondary somatic mutations of H-Ras in Wnt1-producing mammary cells. The finding of H-Ras mutations in the small number of tumors with loss of p53 heterozygosity implies that Ras mutation occurs before p53 LOH, since Ras mutations appear not to confer a selective advantage in p53 null cells. However, a larger cohort of tumors undergoing LOH at the p53 locus will be required to establish the conclusion more firmly.

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In this report, we have used several features of recent studies of breast carcinogenesis in genetically manipulated mice to explore the contributions made to a multi-step oncogenic process by a variety of inherited and somatic mutations. We have identified mutations in the H-Ras proto-oncogene as a significant feature of Wnt1-induced tumorigenesis (Table 1), and we have shown that an inherited Neu transgene or an inherited deficiency of the p53 tumor suppressor gene can provide a selective advantage to Wnt1-expressing mammary cells that overrides the selective advantage conferred by somatic mutation of H-Ras. This conclusion is based on our failure to find H-Ras mutations in any tumors arising in bitransgenic (MMTV-Wnt1/MMTV-Neu) or p53-deficient (MMTV-Wnt1/p53−/−) mice (Table 2).

It is not difficult to reconcile the lack of H-Ras mutations in tumors from bitransgenic mice based on the overlapping signaling pathways in which Neu and Ras participate [22,23,27,28] and on the evidence for similarities in gene expression profiles and cell-type composition of mouse mammary tumors induced by these two genes [5,9,24,25]. However, the absence of H-Ras mutations in Wnt1-induced tumors from p53 null mice is surprising, since it is well known that an activated Ras gene can collaborate with p53 deficiency to promote both transformation of cultured cells [29] and tumor formation in vivo. For example, a mutant K-Ras gene induces lung adenocarcinomas more rapidly in p53-deficient than in p53-wild type mice [30,31], and K-Ras mutations are often accompanied by loss of p53 function in human cancers of the colon,
pancreas, and other organs [32-34]. However, effects of inherited mutations on the detection of somatic mutations were previously reported in other mouse models. For example, mammary tumors from MMTV-TGF-α/ MMTV-Neu and from MMTV-Neu/ p53R172H bitransgenic mice do not contain somatic mutations in the Neu transgene [35,36].

One curious aspect of our findings is the observation of Ras mutations affecting only H-Ras and never K- or N-Ras in Wnt1-induced mammary tumors. This contrasts sharply with the report by D’Cruz et al. [3] of mutations affecting mostly K-Ras and also N-Ras, but never H-Ras, in mouse mammary tumors induced by an MMTV-c-Myc transgene. However, three tumors from the small cohort of the MMTV-c-Myc mice maintained in our lab were found to carry mutations in H-Ras, and one had a mutation in K-Ras (KP, unpublished data). We do not have an explanation for these differences in mutation spectrum and do not know whether they reflect differences in mutational rates at Ras loci (for example, as a consequence of environmental exposures), differences in the selective advantage conferred by different mutant Ras proteins in cells expressing different oncogenes, or differences in the genetic backgrounds of the mouse lines used.

In the course of these experiments we have also found that tumors from MMTV-Wnt1/MMTV-Neu bitransgenic

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Table 2: Frequency of H-Ras mutations in MMTV-Wnt1/p53+/-, MMTV-Wnt1/p53-/-, and MMTV-Wnt1/MMTV-Neu tumors

|                      | MMTV-Wnt1/p53+/- | MMTV-Wnt1/p53-/- | MMTV-Wnt1/MMTV-Neu |
|----------------------|------------------|------------------|------------------|
| With H-Ras mutations | 5 (2 with p53 LOH) | 0                | 0                |
| Without H-Ras mutants| 6 (3 with p53 LOH) | 16               | 11               |
| Total                | 11 (5 with p53 LOH) | 16               | 11               |

Primary tumor cDNAs (11 MMTV-Wnt1/p53+/- samples) or DNAs (5 MMTV-Wnt1/p53-/- samples and all of the MMTV-Wnt1/p53-/- samples) were amplified and sequenced with H-Ras-specific primers at least once in each direction, as described in Methods. Mutations were scored by the same criteria described in Table 1.
animals were remarkably similar to those induced in MMTV-Wnt1 mice, although they appeared much earlier than tumors in monotransgenic animals, indicating cooperative effect of the two transgenes. The dominant effect of Wnt1 expression in these tumors closely resembles the dominant effect of the Myc transgene in tumors from the MMTV-c-Myc/MMTV-Neu and MMTV-c-Myc/MMTV-v-Ha-Ras bitransgenic mice [6]. This observation suggests that the Wnt signaling pathway has a dominant effect over the Ras signaling pathway in transformation of mammary epithelial cells. This might be related to the recently described effects of Wnts on stem cell maintenance during normal development [37-40]. Dominance of the Wnt phenotype in this cross might also be explained by a difference in time and level of transgene expression in the mammary tissue.

Conclusions
Selection of somatic oncogenic mutations in mouse mammary tumors depends on the nature of inherited factors: transgenic oncogenes and loss of function mutations in tumor suppressor genes.

Competing interests
None declared.

Authors' contributions
KP planned, analyzed, and presented the experiments described in this paper. KP carried out the MMTV-Wnt1/MMTV-Neu cross and YL carried out the MMTV-Wnt/p53-/- cross. YL helped to analyze the data and contributed to editing the manuscript. HV helped to plan and oversee the experiments and contributed to the writing and editing of the manuscript.

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References
1. Hutchinson JN, Muller WJ: Transgenic mouse models of human breast cancer. Oncogene 2000, 19:6130-6137.
2. Gunther EJ, Moody SE, Belka GK, Hahn KT, Innocent N, Dugan KD, Cardiff RD, Chodosh LA: Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. Genes Dev 2003, 17:488-501.
3. D’Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, Moody SE, Cox JD, Ha SI, Belka GK, Golant A, Cardiff RD, Chodosh LA: c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. Nat Med 2001, 7:235-239.
4. Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD, Innocent N, Cardiff RD, Schnall MD, Chodosh LA: Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell 2002, 2:451-461.
5. Desai KV, Xiao N, Wang W, Gangi L, Greene J, Powell JH, Dickson R, Furst P, Hunter K, Kucherlapati R, Simon R, Liu ET, Green JE: Initiating oncogenic event determines gene-expression patterns of human breast cancer models. Proc Natl Acad Sci U S A 2002, 99:6967-6972.
6. Cardiff RD, Muller WJ: Transgenic mouse models of mammary tumorigenesis. Cancer Surv 1993, 16:97-113.
7. Cardiff RD, Sinn E, Muller W, Leder P: Transgenic oncogene mice. Tumor phenotype predicts genotype. Am J Pathol 1991, 139:495-501.
8. Cardiff RD, Munn RJ: Comparative pathology of mammary tumorigenesis in transgenic mice. Cancer Lett 1995, 90:13-19.
9. Li Y, Welm B, Podosypina K, Huang S, Chamorro M, Zhang X, Rowlands T, Eggelard M, Cowin P, Werb Z, Tan LK, Rosen JM, Varmus HE: Evidence that transgenes encoding components of the Wnt signaling pathway provide a selective advantage for mammary cancers from progenitor cells. Proc Natl Acad Sci U S A 2003, 100:15853-15858.
10. Kwan H, Pecenka V, Tsukamoto A, Parslow TG, Guzman R, Lin TP, Muller WJ, Lee FS, Leder P, Varmus HE: Transgenes expressing the Wnt-1 and int-2 proto-oncogenes cooperatively during mammary carcinogenesis in doubly transgenic mice. Mol Cell Biol 1992, 12:147-154.
11. Kapoun AM, Shackelford GM: Preferential activation of Fgf8 by proviral insertion in mammary tumors of Wnt1 transgenic mice. Oncogene 1997, 14:2985-2989.
12. Shackelford GM, MacArthur CA, Kwan HC, Varmus HE: Mouse mammary tumor virus infection accelerates mammary carcinogenesis in Wnt1 transgenic mice by insertional activation of int-2/Fgf3 and int/Fgf4. Proc Natl Acad Sci U S A 1993, 90:740-744.
13. MacArthur CA, Shankar DB, Shackelford GM: Fgf-8, activated by proviral insertion, cooperates with the Wnt-1 transgene in murine mammary tumorigenesis. J Virol 1995, 69:2501-2507.
14. Donehower LA, Godley LA, Ailzad CM, Kyle R, Shi YP, Pinkel D, Gray J, Bradley A, Medina D, Varmus HE: Deficiency of p33 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev 1995, 9:882-895.
15. Li Y, Podosypina K, Liu X, Crane A, Tan LK, Parsons R, Varmus HE: Deficiency of p53 accelerates mammary oncogenesis in MMTV-Wnt-1 transgenic mice. BMC Mol Biol 2001, 2:7.
16. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P: Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 1987, 49:465-475.
17. Siegel PM, Dankort DL, Hardy WR, Muller WJ: Novel activating mutations in the neu proto-oncogene in induced in induction of mammary tumors. Mol Cell Biol 1994, 14:7068-7077.
18. Donehower LA, Harvey M, Slagle BL, MacArthur MJ, Montgomery CA, Jr, Butel JS, Bradley A: Mice deficient for p33 are developmentally normal but susceptible to spontaneous tumours. Nature 1992, 356:215-221.
19. BCM Search Launcher: Multiple Alignments [http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html]
20. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-MYC as a target of the APC pathway. Science 1998, 281:1509-1512.
21. Hamad NM, Ebinon JH, Karnoue AB, Bai W, Rich JN, Abraham RT, Der CJ, Counter CM: Distinct requirements for Ras oncogenes in human versus mouse cells. Genes Dev 2002, 16:2046-2057.
22. Dankort D, Maliskowski B, Warner N, Kanno N, Kim H, Wang Z, Morin MF, Oshima RG, Cardiff RD, Muller WJ: Grb2 and Shc adapter proteins play distinct roles in Neu (Erbb-2)-induced mammary tumorigenesis: implications for human breast cancer. Mol Cell Biol 2001, 21:1540-1551.
23. Dankort D, Jeyabalan N, Jones N, Dumont DJ, Muller WJ: Multiple Erbb-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. J Biol Chem 2001, 276:38921-38928.
24. Janes PW, Daly RJ, deFazio A, Sutherland RL: Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. Oncogene 1994, 9:3601-3608.
25. Rosner A, Miyoshi K, Landesman-Bollag E, Xu X, Seldin DC, Moser AR, MacLeod CL, Shiyama G, Gillgrass AE, Cardiff RD: Pathway
pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors. Am J Pathol 2002, 161:1087-1097.

26. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ: Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci USA 1992, 89:10578-10582.

27. Janda E, Litos G, Grunert S, Downward J, Beug H: Oncogenic Ras/Her-2 mediate hyperproliferation of polarized epithelial cells in 3D cultures and rapid tumor growth via the PI3K pathway. Oncogene 2002, 21:5148-5159.

28. Daub H, Weiss FU, Wallasch C, Ullrich A: Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 1996, 379:557-560.

29. Elengaas B, Sprio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Popescu NC, Hahn WC, Weinberg RA: Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev 2001, 15:50-65.

30. Johnson L, Mercer K, Greenbaum D, Bronson RT, Crowley D, Tuveson DA, Jacks T: Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature 2001, 410:1111-1116.

31. Fisher GH, Weller SL, Klimstra D, Lenczowski JM, Tichelaar JW, Lizak MJ, Whitsett JA, Koresky A, Varmus HE: Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. Genes Dev 2001, 15:3249-3262.

32. Wistuba II, Gazdar AF, Minna JD: Molecular genetics of small cell lung carcinoma. Semin Oncol 2001, 28:3-13.

33. Li D: Molecular epidemiology of pancreatic cancer. Cancer J 2001, 7:259-265.

34. Cho KR, Vogelstein B: Genetic alterations in the adenoma – carcinoma sequence. Cancer 1992, 70:1727-1731.

35. Li B, Rosen JM, McMenamin-Balano J, Muller WJ, Perkins AS: neu/ERBB2 cooperates with p53-172H during mammary tumorigenesis in transgenic mice. Mol Cell Biol 1997, 17:3155-3163.

36. Muller WJ, Arteaga CL, Muthuswamy SK, Siegel PM, Webster MA, Cardiff RD, Meise KS, Li F, Halter SA, Coffey RJ: Synergistic interaction of the Neu proto-oncogene product and transforming growth factor alpha in the mammary epithelium of transgenic mice. Mol Cell Biol 1996, 16:5726-5736.

37. Jamora C, DasGupta R, Kocieniewski P, Fuchs E: Links between signal transduction, transcription and adhesion in epithelial bud development. Nature 2003, 422:317-322.

38. Reya T, Duncan AW, Alles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL: A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003, 423:409-414.

39. Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR 3rd, Nusse R: Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 2003, 423:448-452.

40. Sancho E, Battle E, Clevers H: Live and let die in the intestinal epithelium. Cell 2003, 113:763-770.

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