

Wnt-1 is Dominant Over Neu in Specifying Mammary Tumor Expression Profiles

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Wnt-1 and *Neu* collaborate to induce mammary tumors in bitransgenic mice carrying both MMTV-*Wnt-1* and MMTV-*Neu*. In this report, gene expression profiles were determined for tumors from these bitransgenic mice, and compared with expression profiles of tumors from mice singly transgenic for MMTV-*Wnt-1* or MMTV-*Neu*. While very different from tumors arising in MMTV-*Neu* transgenic mice, tumors from these bitransgenic mice were found not to have identifiable differences from tumors from MMTV-*Wnt-1* transgenic mice, using clustering and multidimensional scaling analyses (unsupervised and supervised), One-way Analysis of Variance (ANOVA), and two sample *t* test (the later two of which were combined with false discovery rate computation). These observations suggest that *Wnt-1* is dominant over *Neu* in specifying mammary tumor expression profiles.

Key words: Gene expression profiling; Mouse model; and Breast cancer.

Introduction

Many genetic mutations are implicated in human breast cancers, which are also histologically heterogeneous. Expression profiling of human breast cancers has divided these tumors into several different subgroups, including luminal epithelial/ER+ (luminal subtype A, B, and C), basal-like, Erb-B2+, normal-breast-like, and more recently an additional “molecular apocrine” subgroup, which is androgen receptor (AR)-positive and ER-negative (1-5). Tumors of the ER-positive luminal type have a generally good prognosis and usually respond to tamoxifen or other selective estrogen receptor modulators. The HER2-positive patients usually benefit from anti-HER monoclonal antibody therapies. The basal-like cancers have the worst prognosis, and have been suggested to arise from progenitor cells (1, 6).

There exist many mouse models of breast cancer (7-11). Besides having differences in histopathology and possibly in cell of origin, these models also have distinct gene expression profiles (12-16). For example, distinct gene signatures have been identified for mammary tumors arising in mice transgenic for *Neu*, *c-Myc*, *H-Ras*, *SV40 T antigen*, and *polyoma middle T antigen* (12).

Gene expression profiles are very different in tumors arising in transgenic mice that express *Wnt-1* from the mouse mammary tumor virus promoter (MMTV-*Wnt-1*) as compared with tumors from MMTV-*Neu* transgenic mice (15). However, spontaneous or germline secondary genetic alterations, such as an activating Ras mutation or deletion of either the *Pten* or *p53* tumor suppressor gene, do not much alter the global expression profiles of mammary tumors induced by the MMTV-

Shixia Huang, Ph.D.^{1, 2, 3,*}
Katrina Podsypanina, Ph.D.⁴
Yidong Chen, Ph.D.⁵
Weiyan Cai¹
Anna Tsimelzon, Ph.D.^{1,2}
Susan Hilsenbeck, Ph.D.^{1,2}
Yi Li, Ph.D.^{1,3}

¹Breast Center

²Dan L. Duncan Cancer Center

³Dept. of Molecular and Cellular Biology
Baylor College of Medicine

Houston, TX 77030, USA

⁴Program in Cancer Biology and Genetics
Sloan-Kettering Institute

Memorial Sloan-Kettering Cancer Center
New York, New York, USA

⁵National Human Genome
Research Institute

National Institutes of Health
Bethesda, Maryland, USA

*Corresponding Author:
Shixia Huang, Ph.D.
Email: shixiah@bcm.tmc.edu

Abbreviations: MMTV: Mammary tumor virus; MDS: Multidimensional scaling analysis; GCOS: GeneChip Operation System; ANOVA: Analysis of Variance.

Wnt-1 transgene, though a small subset of genes can be identified to be differentially expressed between tumors with those genetic alterations using the permutation *t* test (15).

Mice bitransgenic for MMTV-*Wnt-1* and MMTV-*Neu* develop mammary tumors much more rapidly than mice harboring either transgene; thus, these two oncogenes collaborate to induce mammary tumors (17). The resulting mammary tumors contain heterogeneous cell types, including epithelial and myoepithelial cells as well as cells that express putative progenitor cell markers (17), similar to tumors arising in MMTV-*Wnt-1* transgenic mice (18). These tumors in bitransgenic mice also contain patches that lack myoepithelial cells, a feature usually associated with tumors arising in MMTV-*Neu* transgenic mice (17). However, it is not known whether the expression profiles of tumors arising in these bitransgenic mice resemble those of tumors arising in MMTV-*Wnt-1* or in MMTV-*Neu* transgenic mice.

In this study, we sought to answer this question and attempted to identify genes that are uniquely expressed in tumors arising in the bitransgenic mice, in hope of elucidating the mechanism of collaboration between *Wnt-1* and *Neu* in mammary tumorigenesis.

Materials and Methods

Animals and Tumors

MMTV-*Wnt-1* (FVB/NJ-Tg[Wnt-1] 1 Jev/J) and MMTV-*Neu* (FVB/N-Tg [MMTVNeu] 202 Mul/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained on the FVB background. All mice were housed in a specific pathogen-free facility on a standard diet. Tumors were collected fresh, snap-frozen in liquid nitrogen, and kept at -80 °C until RNA extraction. We chose five animals in each group based on the guidelines set by The Tumor Analysis Best Practices Working Group (19), which require three or four inbred mice per group.

RNA Extraction and

Affymetrix GeneChip Array Hybridization

Frozen samples were ground into fine powder in liquid nitrogen, and their RNAs were extracted by Trizol (Lift Technologies) following the manufacturer's suggestions with an additional step of isopropanol precipitation to reduce potential residual chemical contamination in the Trizol reagent. Double-stranded cDNA was synthesized from 5 µg of total RNA for each sample. This cDNA served as the template for producing biotin-labeled cRNA through an *in vitro* transcription reaction. The One-Cycle Target Labeling and Control Reagents kit (Part #900493; Affymetrix, Santa Clara, CA, USA) was used for both cDNA synthesis and cRNA biotin-labeling.

Fragmented cRNA (6.5 µg) was hybridized onto the Affymetrix GeneChip® M430A 2.0 in the Affymetrix Hybridization Oven 640 for 16-19 hours. Prehybridization, hybridization, washing, and staining with streptavidin-phycoerythrin (SAPE) were performed following manufacturer's recommended protocols. Antibody amplification was employed using anti-streptavidin antibodies conjugated to biotin (Vector Laboratories, Burlingame, CA). After automated staining and washing using Affymetrix GeneChip® Fluidics Station 450 (Protocol: Midi_euk2v3 450), the arrays were scanned using the Affymetrix GeneChip® scanner 3000, which was controlled using the GeneChip® Operating System (GCOS) v1.2. Image analysis and probe level quantification were carried out using GCOS1.2, and the probe level data (CEL file) were saved for each array for subsequent analyses.

Data Normalization and Modeling

Both GCOS and dChip (<http://dchip.org>) (20, 21) were used for quality assessment. From GCOS analysis, the percent present calls for all the 15 arrays were between 61-69% and the GAPDH ratio was between 0.61-0.77. No obvious variation for these two measurements was observed among these samples. The CEL files for all 15 arrays were then imported into dChip, and normalized using the invariant set method (the baseline was set using the WntT H11 tumor sample). The perfect match only (PM-only) modeling algorithm was used to estimate expression. Expression values of probe sets, after rounding up to one for any value that was less than one, were Log₂-transformed for further analyses. dChip analysis showed that all the arrays had percentages of array outliers <5% and percentage of single outliers <0.6%, indicating acceptable quality for all the arrays (19).

Statistical Analyses

Expression patterns among samples were visualized using Hierarchical clustering and multidimensional scaling (MDS), both of which use Pearson correlation as a similarity measure. The clustering analysis was implemented in dChip, with the distances between samples represented on a dendrogram. MDS was developed in Matlab environment (Natick, MA, USA) (22). After filtering out those that had nearly identical expressing values, 7,082 (of 22,690) probe sets were selected for both unsupervised clustering and MDS analyses. To identify genes differentially expressed between samples, we used two-sample *t* test for comparing two groups of samples and One-way Analysis of Variance (ANOVA) for three groups. QVALUE (implemented in R) (23) was used to determine false discovery rates (FDRs), and the estimation of FDRs is based on the entire list of *p* values obtained by ANOVA or *t* test.

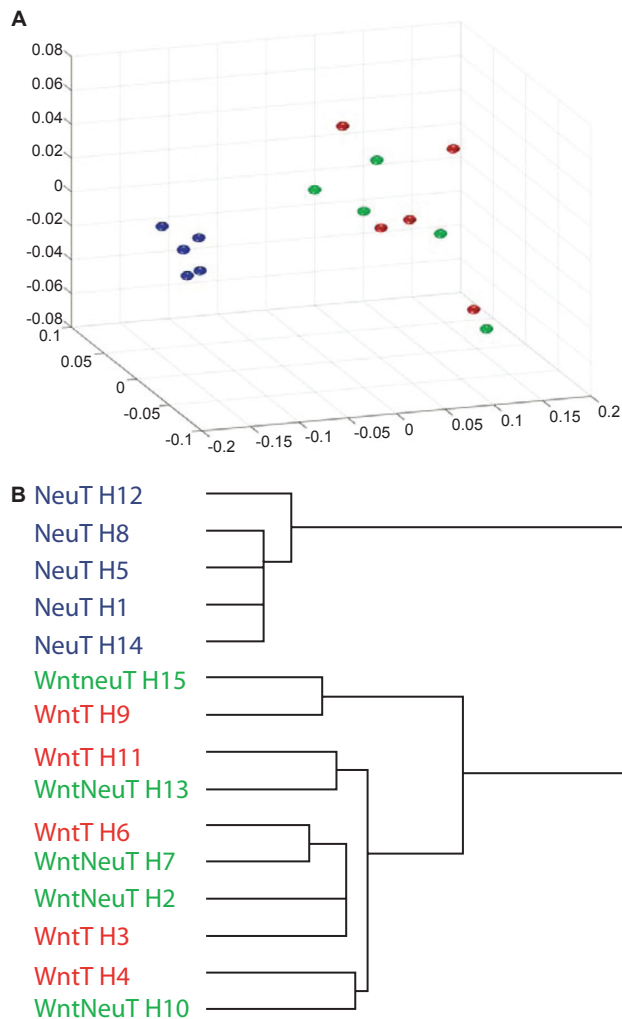


Figure 1: Unsupervised MDS (A) and Clustering (B) analyses. These analyses were carried out using the 7082 (of 22,690) probe sets which satisfied the filtering criteria of variation across samples: $0.04 < SD/Mean < 10.00$. Samples include tumors from MMTV-*Wnt-1* transgenic mice (Red), MMTV-*Neu* transgenic mice (Blue), and MMTV-*Wnt-1*/MMTV-*Neu* bi-transgenic mice (Green).

Results

Gene expression profiles were determined using Affymetrix GeneChip® M430A 2.0 arrays for five tumors from mice bitransgenic for *Wnt-1* and *Neu*, five tumors from MMTV-*Wnt-1* transgenic mice, and five tumors from MMTV-*Neu* transgenic mice. From these 15 arrays, probe sets that had similar expression values were filtered out. The remaining 7,082 (of 22,690) most variable probe sets were used for both unsupervised clustering and MDS analyses. *Neu*-induced tumors were clearly separated from *Wnt-1*-induced tumors (Figure 1), consistent with our previous report using cDNA arrays (15). Tumors from the bitransgenic mice were separable from *Neu*-induced tumors; however, to our surprise, they were not separated from tumors from MMTV-*Wnt-1* transgenic mice, using both analyses (Figure 1).

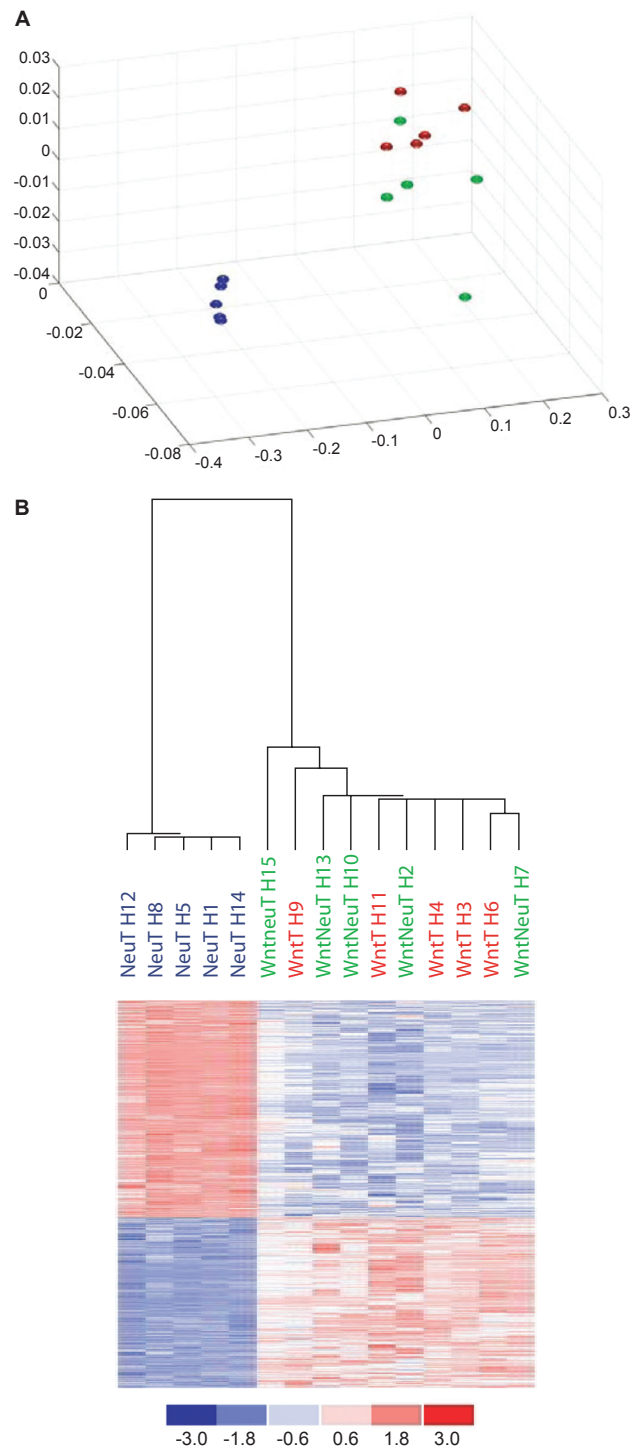


Figure 2: MDS (A) and clustering (B) analyses on genes identified by One-way ANOVA. One-Way ANOVA identified 2537 genes differentially expressed among tumors from MMTV-*Wnt-1* transgenic mice (Red), MMTV-*Neu* transgenic mice (Blue), and MMTV-*Wnt-1*/MMTV-*Neu* bi-transgenic mice (Green).

In an attempt to identify potential differentially expressed genes/patterns among these three groups, One-way ANOVA was performed using all 22,690 probe sets. 2537 genes were

identified ($p \leq 0.001$) (Figure 2). From these 2537 genes, we would expect about $0.001 \times 22,690 = 23$ genes by chance. To better estimate the false discovery rate, we have used a standard method QVALUE to compute the false positives among the identified genes. We observed many more genes with lower p values than with higher p values; a trend indicates more significant genes than by chance (Figure 3A). More importantly, the number of false positives is very low when compared with the identified genes (significant tests) (Figure 3B). Approxi-

mately nine of the 2537 were estimated as false positives by QVALUE (Figure 3B). This data indicated that nearly all of the 2537 genes identified by ANOVA were truly differentially expressed among these three groups of samples.

Next we used the 2537 genes for Hierarchical clustering analysis and MDS. We expected these three groups of samples to be separated from each other, since clustering on selected significant genes usually leads to group differ-

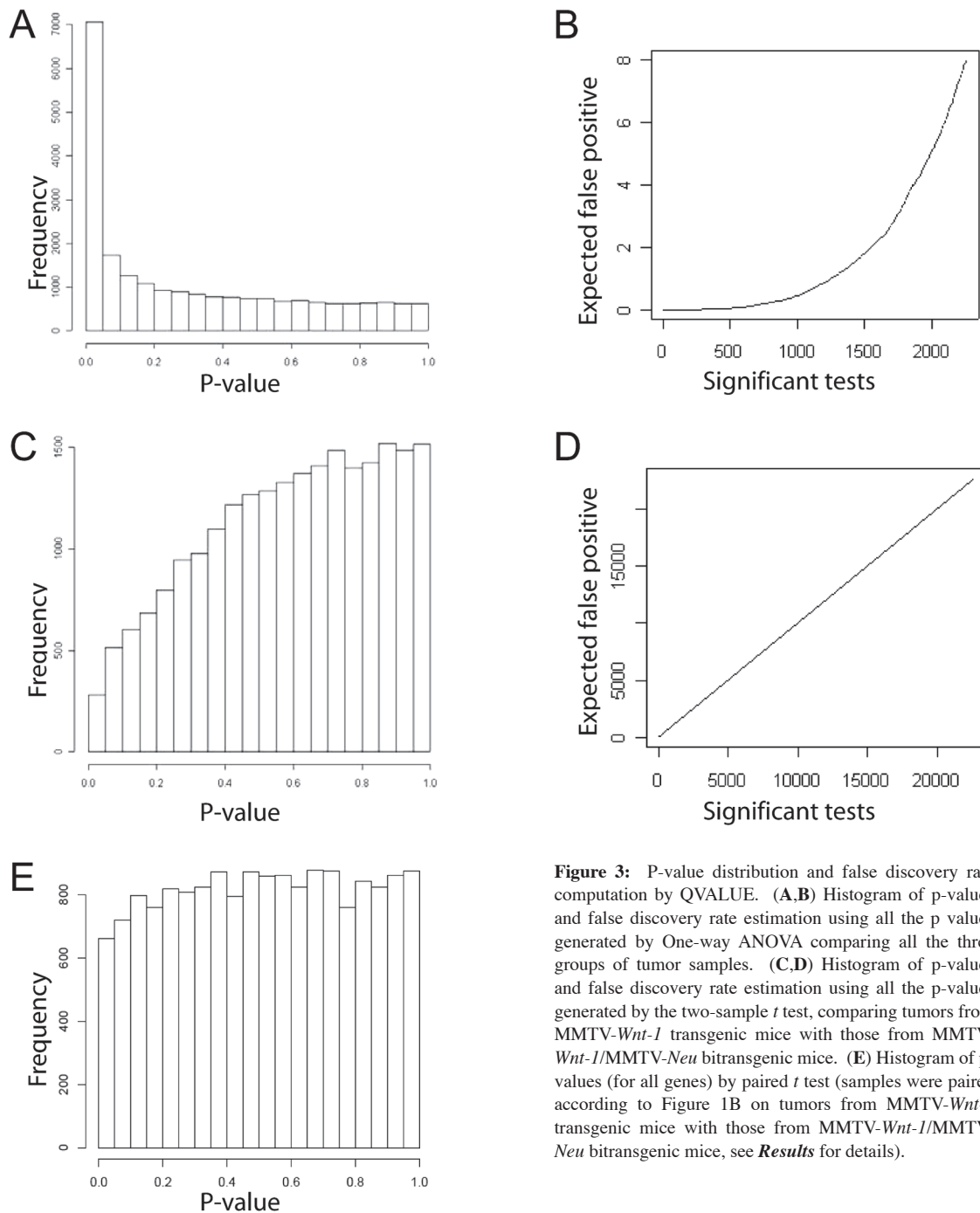


Figure 3: P-value distribution and false discovery rate computation by QVALUE. (A,B) Histogram of p-values and false discovery rate estimation using all the p values generated by One-way ANOVA comparing all the three groups of tumor samples. (C,D) Histogram of p-values and false discovery rate estimation using all the p-values generated by the two-sample *t* test, comparing tumors from MMTV-*Wnt-1* transgenic mice with those from MMTV-*Wnt-1*/MMTV-*Neu* bitransgenic mice. (E) Histogram of p-values (for all genes) by paired *t* test (samples were paired according to Figure 1B on tumors from MMTV-*Wnt-1* transgenic mice with those from MMTV-*Wnt-1*/MMTV-*Neu* bitransgenic mice, see **Results** for details).

entiation. We also hoped to identify genes/patterns specifically regulated in tumors induced jointly by *Wnt-1* and *Neu*. These 2573 genes clearly separated tumors induced by *Neu* from tumors induced by either *Wnt-1* alone or a combination of *Wnt-1* and *Neu* (Figure 2). However, tumors induced jointly by *Wnt-1* and *Neu* could not be differentiated from tumors induced by *Wnt-1* alone, by either analysis (Figure 2). These data suggest that these two groups of tumors are very similar in their gene expression profiles.

To ensure that the above ANOVA on the three groups did not mask small but consistent differences between tumors arising in MMTV-*Wnt-1*/MMTV-*Neu* bitransgenic mice and in MMTV-*Wnt-1* transgenic mice, the two-sample *t* test was used to uncover genes that might differ between these sets of tumors. Among all the 22,690 probe sets, two genes were identified ($p < 0.001$). Unlike results from ANOVA analysis (Figure 3A), we did not observe more genes with lower *p* values than with higher *p* values with the two sample *t* test (Figure 3C); more importantly, the estimation of the false discovery rate by QVALUE, using all the *p* values from this two sample *t* test, indicated that the same number of genes that would be identified as significant would also be identified by chance (FDR 100%) (Figure 3D). Such a null hypothesis predicted a uniform *p* value distribution. Unexpectedly, the *p* value distribution was not uniform across all the genes, with a higher prevalence of very large *p* values (Figure 3C), suggesting that, for any particular gene, the difference between the means of the two groups is smaller than would be expected by chance, given the within-group variability. Small experimental/biological variations from pairing individual samples between the compared groups during sample collection, cDNA/cRNA synthesis, and array hybridization, may have been the reason for such a non-uniform distribution of *p* values, though these experiments were designed to minimize introduction of experimental and biological variations between groups. Noticeably, unsupervised clustering analysis clustered the ten tumors into five pairs (Figure 1B), four of which reflected these commonalities (two pairs were littermates of the same age and two pairs were assayed at the same time), while the remaining pair (*WntNeuT H10 vs. WntT H4*) had no identifiable commonality. (These subtle differences between pairs of samples were noticeable on clustering analysis, apparently, because these two groups of samples were very similar. In contrast, such pairing did not affect the comparison between *Neu*-induced tumors and tumors arising in mice either bitransgenic for *Neu* and *Wnt-1* or monotransgenic for *Wnt-1*, presumably due to the large differences in expression profiles among them.) Therefore, we next performed a paired *t* test for samples between tumors arising in MMTV-*Wnt-1*/MMTV-*Neu* bitransgenic mice and in MMTV-*Wnt-1* transgenic mice. As expected, only a small number of genes were identified (11 genes, $p \leq 0.001$), and the estimated FDR

was 100% (data not shown). Importantly, the distribution of *p* values was nearly uniform across all the genes (Figure 3E), confirming the null hypothesis. Hence, we conclude that gene expression profiles of tumors from MMTV-*Wnt-1*/MMTV-*Neu* bitransgenic mice are highly similar to those of tumors from MMTV-*Wnt-1* transgenic mice.

Discussion

Wnt-1 and *Neu* oncogenes cooperate in mammary tumorigenesis (17). Tumors in mice bitransgenic for *Wnt-1* and *Neu* are histologically similar to tumors arising in *Wnt-1* transgenic mice, though myoepithelial tumor cells appear to be less prevalent in these bitransgenic tumors (17). Based on Affymetrix oligo arrays, tumors arising in MMTV-*Wnt-1* were very different from tumors from MMTV-*Neu* transgenic mice. This observation is in agreement with our previous comparison of these two groups of tumors using cDNA arrays (15), and this list of differentially expressed genes will complement our previous list of genes in future studies of these two tumor models, since the current list was generated from inbred mice, unlike in our previous study (15) where the expression differences could be partially attributed to differences in the genetic background, as has been reported in the MMTV-*PyMT* model (24). Furthermore, expression profiles were different between tumors arising in mice transgenic for both *Neu* and *Wnt* vs. for *Neu* alone; however, no expression differences could be detected between tumors arising in these bitransgenic mice and those induced by the *Wnt-1* transgene alone. This observation establishes that *Wnt-1* is a dominant oncogene over *Neu* in specifying tumor gene expression profiles.

It is a surprise that expression profiles did not have detectable differences between tumors arising in the bitransgenic mice and tumors induced by the MMTV-*Wnt-1* transgene. In this report, five tumors of each genotype of inbred mice were analyzed. According to the guidelines recommended by The Tumor Analysis Best Practices Working Group (19), three or four inbred mice per group are usually adequate to provide statistically robust results; though five or six per group are recommended for outbred animals, and considerably more samples per group are necessary for microarray studies of human samples due to greater variation including tissue heterogeneity, stage of disease, and variation in genetic background (19). Nevertheless, it is possible that a small number of genes might be found to be differentially expressed if a larger sample size was analyzed on tumors from the bitransgenic mice and/or MMTV-*Wnt-1* transgenic mice.

The impact of cancer cell origin on expression profiles has not been established, but the cell of origin may be partly responsible for the close similarity between tumors arising in the bitransgenic mice and in MMTV-*Wnt-1* transgenic mice, and for the great differences between MMTV-*Neu*-

induced tumors and the other two groups. Progenitor cells have been proposed to be the origin of cancer in MMTV-*Wnt-1* transgenic mice; since hyperplastic mammary glands in these mice contain an expanded population of progenitor cells as determined by staining for progenitor cell markers and transplantation of either total mammary cells or isolated progenitor cells by limiting dilution, and since the resulting tumors harbor heterogeneous cell types including putative transformed progenitor cells (6, 18, 25-29). Progenitor cells may also be the origin of tumors in the bitransgenic mice, since heterogeneous cell types are present in these tumors (17). On the other hand, more differentiated cells have been suggested to be the origin of cancer in MMTV-*Neu* transgenic mice; since cellular heterogeneity appears to be lacking in tumors arising in MMTV-*Neu* transgenic mice, and since Cre recombinase-mediated cell tracking experiments suggested that these tumors arise from more differentiated cells that express the gene encoding a mammary cell differentiation marker, whey acidic protein (18, 30).

The undetectable influence of *Neu* on expression profiles of tumors in these bitransgenic mice may also be caused by high levels of overlap in oncogenic signaling between tumors in the bitransgenic mice and MMTV-*Wnt-1* transgenic mice. *H-Ras* is mutated to more active forms in 50% of tumors arising in MMTV-*Wnt-1* transgenic mice (17). This observation suggests that the Ras signaling pathway is very important in mammary tumorigenesis initiated by *Wnt-1*, though it remains to be determined whether Ras signaling is activated by other means in the other 50% of tumors that are wild-type at the *H-Ras* locus. Ras is one of the principal downstream targets of activated *Neu*. Indeed, transgenic overexpression of *Neu* negates the need of *H-Ras* mutations in mice that are transgenic for *Wnt-1* (17). Therefore, the Ras signaling pathway may be activated in tumors arising both in bitransgenic mice and in mice transgenic for *Wnt-1*. Consequently, levels of transcriptional targets of Ras signaling may not be expected to differ between these two groups of tumors, though it remains to be determined whether other signaling pathways (such as phospholipase C) activated by *Neu* are selectively activated in tumors arising in mice bitransgenic for both *Wnt-1* and *Neu*.

The lack of detectable expression differences between tumors arising in these bitransgenic mice and mice transgenic for *Wnt-1* does not imply that premalignant lesions in these two sets of mice are also identical or similar in expression profiles. In fact, it would be interesting to analyze and compare gene expression profiles of different stages of the tumor development in these mice. Those analyses might identify differences of gene expression that are not apparent in samples of full malignant transformation. For example, premalignant lesions in MMTV-*Wnt-1* transgenic mice appear to lack activating *Ras* mutations (17), while the Ras pathway

is presumably activated in premalignant lesions in mice bitransgenic for both *Wnt-1* and *Neu*.

Proteomics and microRNA expression profiles are increasingly being used to supplement expression arrays in detecting differences between tumors or classifying cancers (31, 32). These newer methods may identify differences in proteins and microRNAs between tumors (and premalignant lesions) arising in these bitransgenic mice vs. those arising in MMTV-*Wnt-1* transgenic mice.

In conclusion, *Neu* has very little influence on expression patterns of tumors after the MMTV-*Neu* transgene is crossed into the MMTV-*Wnt-1* transgenic line, so that *Wnt-1* is dominant over *Neu* in specifying the expression profiles of mammary tumors.

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