

EMT tumorigenesis in the mouse mammary gland

Patrizia Damonte¹, Jeffrey P Gregg², Alexander D Borowsky^{1,2}, Blaine A Keister³ and Robert D Cardiff^{1,2}

The term EMT (epithelial–mesenchymal transition) is used in many settings. This term is used to describe the mechanisms facilitating cellular repositioning and redeployment during embryonic development and tissue reconstruction after injury. Recently, EMT has also been applied to potential mechanisms for malignant progression and has appeared as a specific diagnostic category of tumors. In mice, most ‘EMT’ tumors have a spindle cell phenotype. The definition of EMT is controversial because spindle cell tumors are not common in humans, especially in human breast cancers. Spindle cell tumors of the mouse mammary gland have been observed for many years where they are usually classified as sarcomas or carcinosarcomas. Genetically engineered mice develop mammary spindle cell tumors that appear to arise in the epithelium and undergo EMT. To better understand the origin and evolution of these spindle cell tumors in progression and metastases, seven cohorts of spindle cell tumors from the archives of the University of California, Davis Mutant Mouse Pathology Laboratory were studied. This study provides experimental and immunohistochemical evidence of EMT showing that dual epithelial and mesenchymal staining of tumor spindle cells identifies some, but not all, EMT-type tumors in the mouse. This suggests that potential EMT tumors are best designated EMT-phenotype tumors.

Laboratory Investigation (2007) **87**, 1218–1226; doi:10.1038/labinvest.3700683; published online 8 October 2007

KEYWORDS: epithelial–mesenchymal transition; cancer; mice; immunohistochemistry

Epithelial–mesenchymal transition (EMT) breast cancers have become a topic of intense interest and of some controversy.^{1–4} Some experiments suggest that EMT is an intrinsic component of neoplastic progression to invasion and metastases.^{5,6} The controversy focuses on whether EMT tumors occur in human breast carcinoma.² However, in mouse tumorigenesis, probable EMT-type phenotypes have been recognized and were described in the early 1900s as carcinosarcomas.^{7,8} The early investigators primarily associated these spindle cell carcinosarcomas with transplantation experiments performed before the advent of the inbred mouse strains.⁹ Some have suggested that these tumors were artifacts of transplantation.^{8,10} With the development of inbred mouse strains and the recognition of the mouse mammary tumor virus, these murine mammary carcinosarcomas remained in the tumor nomenclature but were viewed as an artifact of tissue culture.¹⁰ In 1989, Thiery^{4,11} recognized the similarity of some features of *in vitro* transformation with developmental EMT. The term ‘EMT tumor’ has found increasing use in the subsequent literature.¹² The EMT-phenotype tumors in mice have a spindle-cell pattern.³ Since spindle cell tumors with identical morphology but different

origins frequently occur in mice, the challenge for the pathologist is distinguishing between the different types of spindle cell tumors.

Recently, EMT tumors have been reported in primary mammary tumors of certain genetically engineered mice that have lost expression of the initiating oncogenes and in tumors that escaped oncogene addiction.^{12–15} These tumors were initially categorized as ‘spindle-cell’ tumors but immunohistochemical analysis demonstrated the presence of cytokeratins (CK), vimentin and smooth muscle actin (SMA), suggesting a mixed lineage or a myoepithelial cell origin.¹⁵ Subsequent analysis revealed upregulation of the Snail transcription factor associated with other types of EMT.¹⁴ These characteristics justified the application of the term ‘EMT’ to certain tumors. Since mice develop spindle cell tumors apparently originating from epithelial and mesenchymal tissues, the term cannot be applied to all such tumors. Reports of EMT in human tumors have emphasized the loss of expression of ‘epithelial’ genes such as *E-Cadherin* and upregulation of genes associated with mesenchymal differentiation. But examination of the histopathology demonstrates readily identifiable disorganized epithelial tumors

¹Center for Comparative Medicine, University of California at Davis, Davis, CA, USA; ²Department of Pathology and Laboratory Medicine, School of Medicine, University of California at Davis, Sacramento, CA, USA and ³Department of Cancer Biology, Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA, USA
Correspondence: Dr RD Cardiff, MD, PhD, Center for Comparative Medicine, University of California, Davis, County Road 98 and Hutchison Drive, CA 95616, USA.
E-mail: rdcardiff@ucdavis.edu

Received 7 June 2007; revised 4 September 2007; accepted 4 September 2007

which do not have the spindle cell differentiation found in the mouse tumors.¹⁶ These applications of the term, EMT, raise the question of criteria used for 'EMT'.

Given that the full range of histological and immunological characteristics of the EMT tumor phenotype in mice has not been systematically characterized, we undertook a study of several experimental examples from our archives. Here, we record our observations on spindle cell tumor cohorts from the mouse mammary gland from the archives of the University of California, Davis Mutant Mouse Pathology Laboratory. We studied at least 11 examples of spindle cell tumors from each of seven cohorts submitted from five laboratories since 1990. Our intention is to help define the range of possible phenotypes for spindle cell tumors derived from mouse mammary epithelium and help establish reasonable criteria for the diagnosis of EMT tumors.

MATERIALS AND METHODS

Tumor Cohorts

All the tumors material used in this study were provided by University of California, Davis (UCD) Mutant Mouse Pathology Laboratory Archive of the Center for Comparative Medicine.

Cohort 1: 'Met-1' and 'Met-1AS-OPN' tumors described previously.¹⁷

Cohort 2: tumors from FVB/N (*Trp53* +/−) × FVB/N (*Hus* +/−) mice.¹⁸

Cohort 3: primary and recurrent tumors originated from the doxycycline-induced FVB/N Tg(*MMTV-rtTA*) × FVB/N Tg(*Teto-Wnt1*) × Cg FVB/N.129svj.B6(*Trp53* (+/−) mice.¹⁹

Cohort 4: tumors arise from FVB/N Tg(*MMTV-Ilk*) mice.¹³

Cohort 5: tumors from FVB/N (*MMTV-mGSK*) mice.²⁰

Cohort 6: tumors arise in FVB/N Tg(*MMP-3/stromelysin-1*) mice.²¹

Cohort 7: tumors arise in FVB/N Tac-Tg(*Hba-x-v-Ha-ras*)^{TG-ACLed 22}.

Immunohistochemistry and Immunofluorescence

Four-micrometer-thick paraffin sections were stained with Mayer's H&E or immunostained as described previously.²³ The following primary antibodies were used with the VECTASTAIN ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA): guinea-pig anti-CK 8/18 (1:1000 RDI, Concord, MA, USA), sheep anti-CK 14 (1:400 Binding site Inc., San Diego, CA, USA) sheep anti-vimentin (1:2000 Binding site Inc.), rat anti-PyVmT (1:1000 Borowsky lab, Davis, CA, USA) and rat anti-F4/80 (1:100 AbD Serotec, Raleigh, NC, USA). Dako Ark kit (Dako, Carpinteria, CA, USA) was used for immunohistochemistry with mouse anti-E-cadherin (1:100 BD transduction lab, San Jose, CA, USA) and mouse anti-SMA (1:1000 Sigma, St Louis, MO, USA) antibody. Immunofluorescence technique was performed using an anti guinea pig FITCH conjugate secondary antibody (Abcam Inc., Cambridge, MA, USA) for CK8/18 detection and a

TEXAS RED conjugated Streptavidin (Vector Laboratories, Burlingame, CA, USA) for Vimentin detection. Images of slides were captured using ×20 and ×40 objectives using filters for FITC and Rhodamine on a Carl Zeiss (Thornwood, NY, USA) Axioscop fluorescence microscope with AxioCam camera and processed using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA) software.

Polymerase Chain Reaction

Total genomic DNA was isolated by DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) from MET-1, MET-1 AS-OPN cell lines and FVB/N tg (*MMTV-PyVT*)634Mul mouse tails and tested by PCR analysis for the presence of PyVmT oncogene using PYMT-F 5' TGTGCACAGCGTGATAATCC-3' PYMT-R2 5'-TGTTCCCTCCGGTAGGATGTC-3' primers. PCR products were revealed by staining with ethidium bromide (PCR).

Gene Expression

Total RNA extraction and biotin-labeled probe synthesis for gene expression analysis has been described previously.^{23,24} Frozen tissues from three different Met-1 and Met-1 AS-OPN tumor were used for hybridization onto Murine Genome U74Av2 GeneChips (Affymetrix, Santa Clara, CA, USA). The scanned images were processed using GCOS 1.4, and then the data was analyzed with GC-RMA in the ArrayAssist software package (Stratagene). GCRMA derived expression values were log-transformed and analyzed in ArrayAssist (*t*-test, unequal variance). Expression comparisons of Met-1 and Met-1 AS-OPN were carried out in ArrayAssist using *t*-test with FDR correction.

RESULTS

The most detailed information come from a transgenic mouse model and derivative transplantable tumor cell cohorts developed and studied in our laboratory. This permitted in-depth comparisons of individual experimental cohorts. Other cohorts included in this study come from samples developed in other institutions and submitted to the UCD Mutant Mouse Pathology Laboratory for interpretation (see Supplementary Table 1).

Cohort 1: Met-1 and Met-1 As-OPN Tumors

The primary experimental cohort is represented by a total of 15 tumors from transplantation of 'Met-1' (*n* = 4) and 'Met-1 AS-OPN' (*n* = 11) cells. Met1 cells are derived from a FVB/N Tg(*MMTV-PyVT*)634Mul mammary tumor and were in their seventh passage when transplanted into syngeneic mice.²⁵ The Met-1 AS-OPN cells are a subset of Met-1 cells that were stably transfected with a full-length osteopontin (*Spp1*) anti-sense RNA plasmid expression construct.¹⁷ The molecular and biological properties of both lines have been described.¹⁷ The Met-1 tumors, as described previously, can be classified as adenocarcinomas with cohesive nodules of cells with patchy glandular development.²⁵ In contrast, the

Met-1 AS-OPN-derived tumors have all the morphological features of spindle cell tumors and are typically arranged in interlacing bundles (Figure 1). Each Met-1 AS-OPN tumor was heterogeneous with variations of spindle cell morphology and organization (Figure 1). The cells were mainly fusiform, with large pleomorphic, hyperchromatic nuclei with multiple, prominent nucleoli and abundant deeply eosinophilic

polar cytoplasm. Significant numbers of tumor giant cells were also present. The tumors derived as explants of Met-1 AS-OPN cells rarely contained glandular structures.

Immunohistochemical (IHC) analyses confirmed the differences in tumor types. As seen in Figure 1, the Met-1 tumors are positive for epithelial markers like CK8/18 and E-cadherin but do not show myoepithelial or mesenchymal

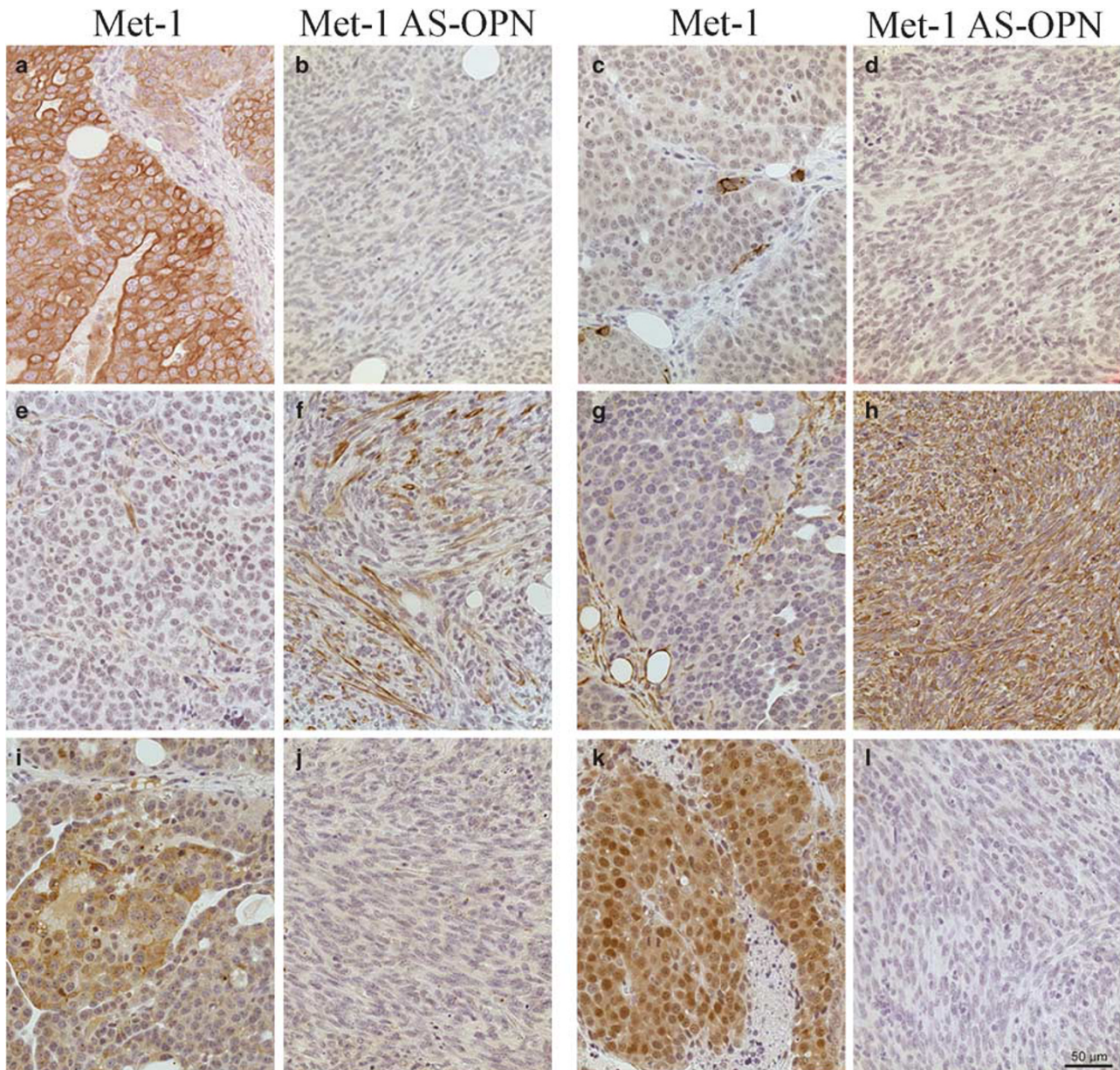


Figure 1 Met-1 and Met-1 AS-OPN tumor cohorts. Immunohistochemical analyses of CK8/18 (a, b), CK14 (c, d), smooth muscle actin (SMA) (e, f), vimentin (g, h), E-cadherin (i, j) and PyVmt (k, l) of tumors derived from Met-1 (a, c, e, g, i, k) and Met-1 AS-OPN (b, d, f, h, j, l) cells. Met-1 tumors show an epithelial pattern characteristic of adenocarcinomas with glandular structures and polygonal cells, which are positive for CK8/18 (a) and E-cadherin (i). CK14 (c) and SMA (e) are present only surrounding the glandular structures and vimentin is expressed only in the stromal cells (g). In contrast, Met-1 AS-OPN tumors present morphological characteristics of spindle cells, which are positive only for mesenchymal markers such as SMA (f) and vimentin (h). The Met-1 AS-OPN tumors also show loss of expression of E-cadherin (j) and of the oncogene PyVmt (l). All images (a–l) have identical magnifications with a 50 μ m scale bar shown in (l).

markers as judged by staining for vimentin, CK14 or SMA. In contrast, tumor cells from transplants of Met-1 AS-OPN cells were positive for mesenchymal markers such as SMA and vimentin but none of the epithelial and myoepithelial markers were detectable (Figure 1).

The Met-1 AS-OPN tumors also have a reduced expression of the transgene, *PyVmT*, as compared to the Met-1 tumor cells or the native FVB/N Tg(MMTV-*PyVmT*)634Mul primary tumors.²⁵ Nonetheless, it still contained the *PyVmT* gene (see Supplementary Data). The *PyVmT* antigen and RNA were not detectable in the Met-1 AS-OPN-derived tumors (data not shown). The expression microassays of Met-1 AS-OPN tumors also show upregulation of EMT-related genes such as Snail, transforming growth factor, beta1 (TGF β 1), S100 calcium binding protein, syndecan 1 (SDC1), Syndecan 2 (SDC2) and stress-induced protein-1 (SIP-1) (see Supplementary Table 2).

Cohort 2: *Trp53*^{+/-} X *Hus1*^{+/-} Tumors

Eleven FVB/N(*Trp53*^{+/-}) × FVB/N(*Hus1*^{+/-}) tumor samples from various locations such as mammary and salivary glands, neck and genitalia had poorly differentiated patterns of interlacing spindle cells.¹⁸ Each tumor was heterogeneous with variations of spindle cell morphology and organization (Figure 2). The cells were primarily fusiform, with large pleomorphic, hyperchromatic nuclei with multiple and prominent nucleoli and abundant deeply eosinophilic polar cytoplasm. Tumor giant cells were also present.

The immunohistochemistry demonstrated tumor cells had both basal or myoepithelial keratins (CK14) and luminal keratins (CK8/18) with SMA but lacked E-cadherin staining (data not shown). All tumors stained with the mesenchymal marker vimentin. The spindle cells generally displayed overlapping populations containing two or more of the intermediate filaments and SMA. However, the tumors were morphologically and immunologically heterogeneous. Some areas stained for only one of the intermediate filaments or had foci with more intense staining for one marker. Many tumors had scattered and poorly formed gland-like epithelial structures that recapitulated the luminal-basal organization of the normal mammary gland.

Cohort 3: Tet-inducible *Wnt-1* X *p53*^{+/-} Tumors

Eleven primary and recurrent mammary tumors from doxycycline-induced, FVB/N Tg(MMTV-*rtTA*) × FVB/N Tg(*Teto-Wnt1*) × CgFVB/N.129B6(*Trp53*(+/-)) female mouse were studied. In this model, primary tumors arise after induction of the oncogene with doxycycline in the drinking water. Removal of the doxycycline results in dramatic regression of the tumors.¹⁹ Some tumors, however, either persist or reappear despite the loss of oncogene expression¹⁹ The histologic examination of primary tumors showed classical Wnt-type tumors that have microacinar, cord-like or cystic patterns surrounded by a dense collagenous stroma.²⁶ These tumors have epithelial populations with various

organizational types that characteristically include well-organized myoepithelium. The tumors have expansile margins. In contrast, the recurrent tumors frequently have infiltrative margins. Most cells are spindle cells with large, oval normochromatic nuclei and indistinct, polar cytoplasm. These cells are arranged in interwoven patterns and are discohesive. Some recurrent tumors have focal clusters of cells with epithelial phenotype and form glands identical to the primary tumors. These epithelial foci are in direct continuity with tumor spindle cell areas (Figure 3).

IHC analyses show CK8/18 expression in all the epithelial cells identified on routine H&E stains in both primary and recurrent neoplasms. In addition, some fusiform cells present in the spindle cell tumor are also CK8/18 positive. On the other hand, CK14 was only expressed in myoepithelial cells surrounding the glandular structures in both adenocarcinoma and spindle cell tumor. SMA, also a marker for myoepithelium, was expressed, not only in its usual basal location in the epithelium, but also in some spindle cells within the recurrent tumor mass. In contrast, vimentin was found only in the stroma and not in the neoplastic cells of the primary adenocarcinoma. In the recurrent tumors, however, a variety of proportions of the neoplastic spindle cells were vimentin positive. The spindle cells did not express E-cadherin and most epithelial cells did express E-cadherin.

An example of a recurrent tumor (Figure 3) appears as a spindle cell tumor with well-differentiated glandular clusters at one end that merge and transit into the spindle cell population. This area of tumor shows a mixture of glandular (CK8/18) and myoepithelial cells (CK14/SMA) but the spindle cell population is not stained by epithelial markers or by E-cadherin, but is positive for vimentin.

Cohorts 4, 5, 6 and 7

Additional tumor cohorts from our archives had mammary tumors with spindle cell phenotypes. They have similar characteristic and have been described in previous publications but were not called 'EMT' tumors.^{13,20,21} As reported previously the phenotypes of the MMTV/*Ilk*-induced tumors in FVB/N Tg(MMTV-*Ilk*) were variable, in a spectrum from papillary adenocarcinomas to undifferentiated spindle cell tumors. Most of the spindle-cell tumors showed evidence of an epithelial to mesenchymal transition with reduced transgene expression. The FVB/N Tg(MMTV-*Ilk*) cohort is the prototypic example of EMT in transgenic animals that stimulated the study of EMT in mouse tumors.¹³ In these tumors, the well-differentiated adenocarcinomas express the epithelial marker CK8/18 and E-cadherin, the mixed tumors exhibited dual staining with vimentin and cytokeratin and the spindle cell tumors were positive for mesenchymal markers but negative for E-cadherin and CK8/18. Similarly, tumors from a FVB/N (MMTV-*mGSK*) cohort show a range of histologic subtypes as glandular, squamous and spindle cell tumors. Cells from the spindle tumors exhibit dual stain for epithelial and mesenchymal marker.²⁰ Tumors arising in

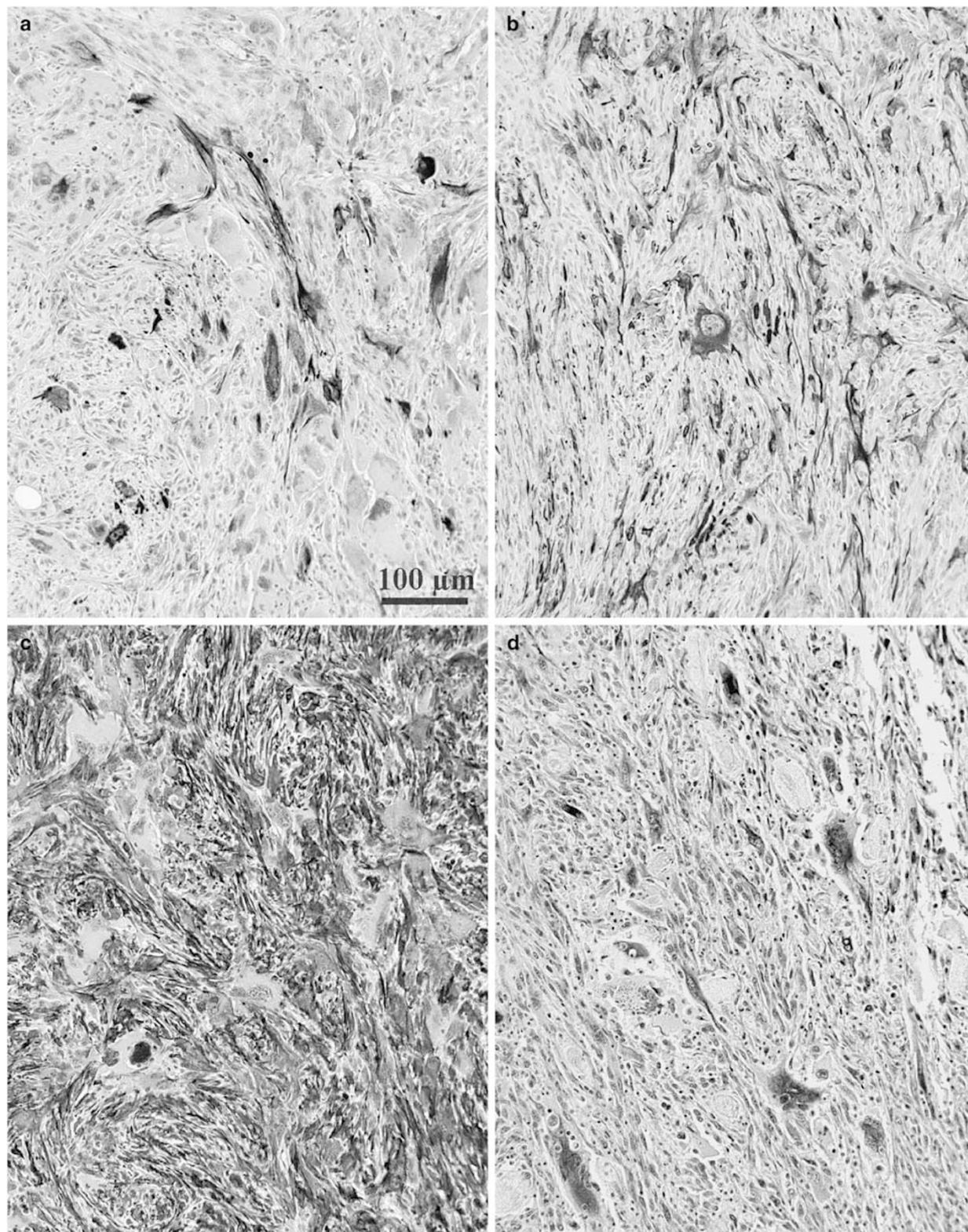


Figure 2 Mammary tumor of a FVB/N (*Trp53*^{+/-}) × FVB/N (*Hus*^{+/-}) female mouse showing a combination of spindle cells, giant cells and dysplastic epithelial gland-like structures. The immunohistochemistry shows the distribution of CK8/18 (a), CK14 (b), smooth muscle actin (SMA), (c) and vimentin (d). The tumor cells show dual staining of mesenchymal and epithelial markers, with some overlapping of intermediate filaments and SMA. All images have identical magnifications with a 100 μm scale bar in (a). Color whole slide images are available at: <http://imagearchive.compmc.ucdavis.edu/publications/emt>.

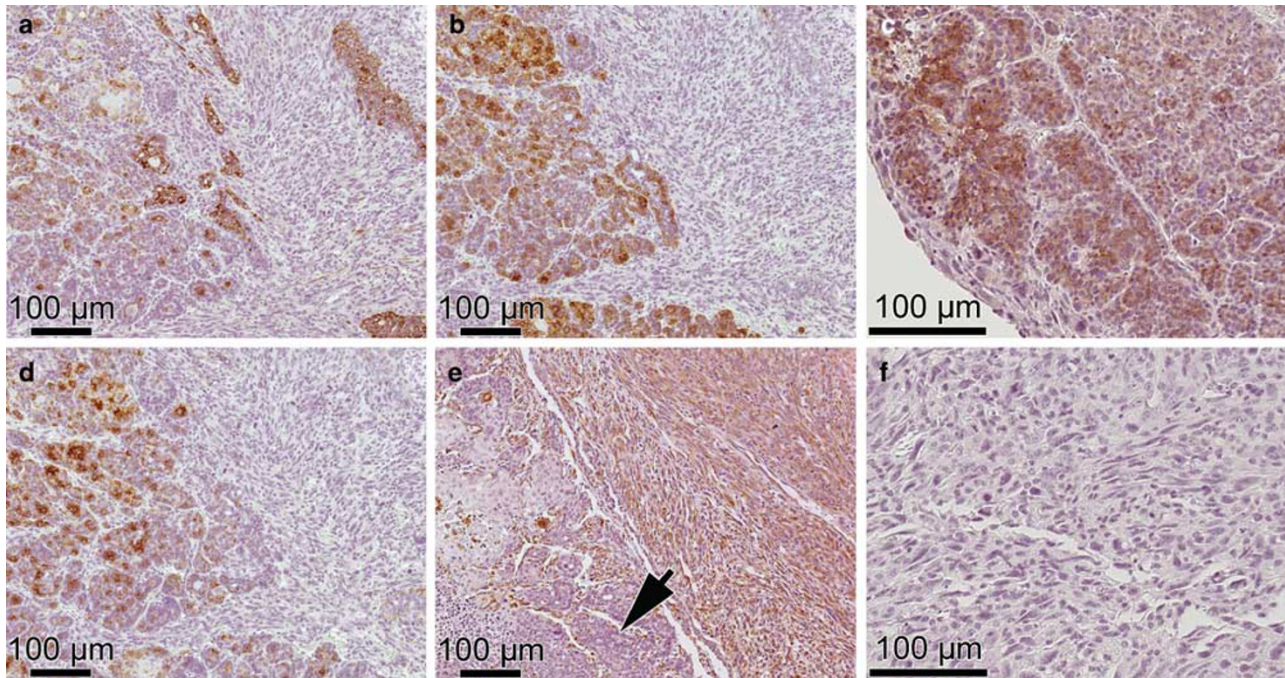


Figure 3 Immunohistochemical analysis of one example of a recurrent tumor from doxycycline-induced-Tg(TV-rtTA) × Tg(TetO-*Wnt-1* × *p53*^{+/-}) cohort. This tumor presents as well differentiated glandular clusters which are positive for epithelial and myoepithelial markers such as CK8/18 (a), CK14 (b), E-cadherin (c), and negative for the mesenchymal marker vimentin (e arrow). The glandular clusters transition into a spindle-cell population, which is positive for vimentin (e) but negative for epithelial markers, CK8/18 (a), CK14 (b) and E-cadherin (f). The image magnifications are shown by the corresponding 100 μm scale bar.

FVB/N Tg(*MMP-3/stromelysin-1*) mice also exhibit some degree of epithelial to mesenchymal conversion showing vimentin immunoreactivity in addition to epithelial cytokeratin staining.²¹

In contrast, the spindle cell tumors from FVB/N Tac-Tg(*Hba-x-v-Ha-ras*)^{TG-ACLe^d} mice, which have enlarged spleens with myeloid hyperplasia and activation of *HRas*,²² were vimentin positive and F4/80 (CD44) positive but do not stain with anti-cytokeratins (see Supplementary Data). This cohort of spindle cell tumors arising in the dermis has a spindle cell phenotype very similar to the mammary described above. Despite this morphologic similarity, the immunophenotype is distinct and is associated with a probable histiocyte origin.

Co-Localization of Epithelial and Mesenchymal Intermediate Filaments

Representative tumors of EMT tumor cohorts show simultaneous expression of epithelial and mesenchymal marker in the same fusiform cells (Figure 4 and Supplementary Figure 3). The green and red fluorescence (Figure 4a and c) illustrate the expression of CK8/18 and vimentin visualized with separate filters. When both intermediate filaments are present in the same cells, the merged images have a yellow fluorescence (Figure 4b) Arrowheads in each panel point to dual staining cells. The tumor also has two well-differentiated glandular clusters positive for epithelial cytokeratin but

negative for vimentin and fusiform cells that express only the mesenchymal marker vimentin.

DISCUSSION

This study describes the morphology and immunohistochemistry of spindle-cell tumors of the mammary gland from six cohorts published previously. Since most of these tumors arose in association with the expression of epithelial-specific transgenes or silencing of tumor suppressor genes, the tumors, in all probability, arose from the mammary epithelium. Experimental Cohort #1 was derived directly from an epithelial cell line following experimental manipulation. Cohort #2 arose in tumor suppressor gene knockouts. Cohort #3 tumors are recurrent tumors following oncogene de-induction with doxycycline. The other cohorts demonstrate that spindle-cell tumors arise in mammary glands in a variety of genetically engineered mice and, in spite of the diverse oncogenic stimuli, are a relatively common endpoint.

The immunohistochemistry demonstrates that dual staining for the two intermediate filaments, vimentin and cytokeratin, staining for smooth muscle actin and a loss of E-Cadherin staining are common features in the spindle-cell tumors of a number of GEM cohorts. The simultaneous immunohistochemical staining of representative samples demonstrates the two intermediate filaments in the same fusiform cells (Figure 4). Therefore, these mammary tumor

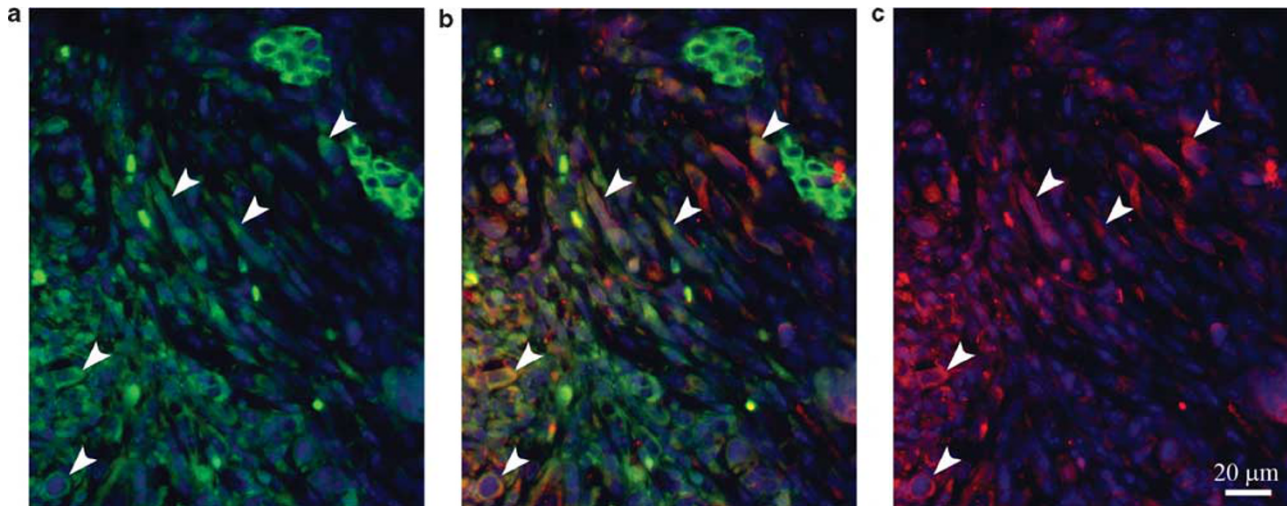


Figure 4 Immunofluorescence analysis of a representative sample from an EMT tumor. This tumor exhibits simultaneous staining for both CK8/18 (a, green) and vimentin (c, red), showing co-localization of epithelial and mesenchymal markers in fusiform cells as revealed by the yellow color (arrowheads) in the merged image from the combination of green and red (b). All images in the panel have identical magnifications with a 20 μm scale bar in (c).

cohorts with spindle cell phenotypes have features that can be regarded as consistent with epithelial–mesenchymal transition (EMT) tumors. This concept is reinforced by the demonstration that transfection with Snail induces a spindle cell phenotype in cultured epithelial *Neu*-induced tumor cells.¹⁴ Identification of upregulation of Snail and Snail-related genes in these and other cohorts reinforces the connection between Snail and EMT in mouse mammary tumorigenesis.^{14,18}

The term EMT was first used in developmental biology to describe a number of reversible transition events occurring during embryogenesis.² It has been applied to tumorigenesis to describe the progressive loss of epithelial traits in some tumors with the acquisition of mesenchymal traits.⁴ The application of the term EMT to tumor biology has resulted in considerable debate.^{1–4} Many investigators have applied the term EMT to the subpopulation of tumor cells that migrate through the stroma to metastasize. To result in recognizable epithelial metastases, reversal of the EMT would be required (MET?) at the implantation/colonization site. Others, such as Tarin, dispute this possibility.² Since mice frequently have spindle-cell tumors, it was important to examine multiple tumors from multiple types of cohorts to begin defining histological and immunological criteria for the diagnosis of an EMT tumor.

Our studies demonstrate that immunohistochemistry provides reliable and specific confirmation of EMT-phenotype in mouse mammary tumors. They also demonstrate that, even within the same cohort, these spindle-cell tumors exhibit a tremendous variation in staining patterns (Supplementary Table 1). At one extreme, tumors known to have originated from epithelium (Cohort #1), lack any evidence of dual staining (Figure 1). At the other extreme, virtually all of the cells in some tumors stain with both epithelial and mesenchymal markers (Figure 2). The observation of great

heterogeneity, even within the same cohort, raises the possibility that not all of the spindle-cell tumors are the result of EMT. For example, they could arise from mesenchymal subpopulations, be mixed cell populations or bi-phasic tumors. However, the demonstration of the two intermediate filaments in the same cells supports the hypothesis that they arose from the same lineage but does not exclude the possibility of fusion of two cell types. These questions of origins or mechanisms cannot be satisfactorily addressed with evidence based on retrospective analysis of paraffin-embedded archives. As with most pathologists faced with this dilemma, we are limited to recognition of the end result and can only speculate about the mechanism.

The study with Met-1 cells provides a well-documented, prototypic example of EMT transformation using tumor cells that arise from a common epithelial origin initiated by the same oncogene. These cells maintained their clonal karyotype and epithelial phenotype during multiple generations of serial transplantation¹⁷ until they were transfected with anti-sense OPN RNA.¹⁸ Their metastatic potential was lost and they underwent a morphological transformation from distinctly epithelial to spindle-cell tumors. This, we believe, is an experimentally based example of EMT tumorigenesis. The biological and morphological observations of this transition should inform future interpretations of EMT tumorigenesis in the mouse by demonstrating one extreme of the EMT phenotype tumor, that is non-dual staining.

Further, genes associated with EMT, Snail and Snail-related genes, are found upregulated in expression microarrays of the spindle cell Met-1 AS-OPN tumors. Upregulation TGF β 1 and SDC1, as well as the transcription factor SIP-1, have been directly implicated in the regulation of EMT target genes and have been found related to increase of progression and invasion in mouse models.^{25–28} These observations all support the idea that the Met-1 AS-OPN spindle cell tumors arose as

a result of EMT transformation of Met-1 cells. If this interpretation is correct, the observations also support the notion that EMT can be associated with a complete loss of detectable epithelial markers, including the initiating oncogene. Perhaps, because this stably transfected cell line was clonally selected *in vitro* before explantation, the characteristic heterogeneity observed in the other cohorts was eliminated. Nevertheless, the model of an isolated epithelial cell, giving rise to a uniform spindle cell population serves as evidence of the potential for complete epithelial transition to mesenchymal phenotype.

The tumors of the other cohorts, which were not clonally selected, frequently demonstrate less complete loss of epithelial traits with frequent coexpression of mesenchymal and epithelial markers and loss of E-cadherin. In contrast, some cases, as illustrated with the doxycycline-induced tumors (Figure 3), retain very distinct epithelial populations in recurrent tumors after transformation from the original epithelial tumor. The tumor illustrated in Figure 3 also raises the possibility that the spindle cell population arises as a distinct subpopulation with unique molecular and phenotypic properties. These cohorts illustrate another possible extreme of the EMT phenotype.

Overexpression of Snail, a transcriptional repressor, has been associated with an increased incidence of metastases in human literature.²⁹ In contrast, the mouse Met-1 AS-OPN EMT tumors, with increased expression of Snail and its downstream pathway (Supplementary Table 2), were associated with decreased metastases.¹⁸ Further, the spindle-cell tumors from our other cohorts were not associated with documented metastases. The hypothesis that EMT is associated with more aggressive types of neoplasia does not support our studies in mice.

Dual epithelial and mesenchymal staining of spindle-cell tumors identifies some, but not all, associated with EMT in the mouse. In the context of the origins of the animal's tumor, the dual staining appears to be specific but not sensitive criteria. Overall, dual staining in spindle-cell tumors is a reliable indicator of EMT, but a lack of epithelial staining in a spindle-cell tumor population does not exclude EMT. As illustrated by FVB/N-Tac-Tg(Hba-x-v-Ha-ras)TG-ACLED, some mouse spindle-cell tumors have morphological features that are identical to EMT tumors but are derived from myeloid elements.²⁴

The cohorts illustrated herein exemplify the interpretative problems in assessing spindle-cell tumors in the mouse mammary gland and may shed light on human cancers. The cohorts illustrate that the emergence of spindle-cell populations might be associated with multiple possible molecular events. They also demonstrate that mouse mammary tumors, which are clearly EMT, have a heterogeneous range of microscopic and immunological phenotypes (Supplementary Table 1). Tumors with a mixture of glands and spindle cells with dual epithelial and mesenchymal markers are at one end of the range. The spindle cell tumors without detectable

immunohistochemical markers are at the other end. The challenge for the practicing pathologist is to recognize the possibilities.

The interpretation of the tumors within each cohort is facilitated by their identical genetic backgrounds and initiation by the same genetic manipulation. Regardless of these factors, the spindle-cell tumors exhibit heterogeneity within each cohort. Based on these observations, we suggest that EMT is not a single tumor 'type' but is a process encompassing a number of phenotypes and, quite possibly, arise from a variety of mechanisms. If this interpretation is correct, more subtle molecular changes suggesting increased expression of mesenchymal-type genes or loss of epithelial genes are within the EMT process. Even some human breast cancers formerly classified as 'metaplastic' tumors may be EMT phenotype tumors.^{30,31-33} We suggest that, until all of the mechanisms are understood, 'EMT phenotype tumor' is a more appropriate term than 'EMT tumor'.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

ACKNOWLEDGEMENT

We thank Drs William Muller, Philip Leder and Lewis Chodosh for permission to use their mouse models from the Mutant Mouse Pathology Laboratory Archives for our study and Lisa Dillard-Telm and her staff for the technical assistance. The work was conducted within the core laboratories of the UCD Mouse Biology Program, including the Mutant Mouse Pathology Laboratory. This work was supported, in part, by Grant U01CA84294/06 from the National Cancer Institute and W81XWH/05/1/405 from the Congressionally Directed Medical Research Program's Breast Cancer Centers of Excellence. Processing and analysis of gene expression was done by the Gene Expression Shared Resource (2 P30 CA93373). Work supported by: Grants U01CA84294/06 from the National Cancer Institute and W81XWH/05/1/405 from the Congressionally Directed Medical Research Program's Breast Cancer Centers of Excellence.

1. Thompson EW, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Res* 2005;65:5991-5995; discussion 5995.
2. Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* 2005;65:5996-6000; discussion 6000-5991.
3. Cardiff RD. Epithelial to mesenchymal transition tumors: fallacious or snail's pace? *Clin Cancer Res* 2005;11(Part 1):8534-8537.
4. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;15:740-746.
5. Chaffer CL, Brennan JP, Slavov JL, et al. Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. *Cancer Res* 2006;66:11271-11278.
6. Gotzmann J, Mikula M, Eger A, et al. Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. *Mutat Res* 2004;566:9-20.
7. Apolant H. Die epithelialen Geschwülste der Maus. *Arbeiten ad Koniglchn Inst FExptTher zu Frankfurt aM* 1906;1:7-68.
8. Woglotz WH. *The Study of Experimental Cancer Research. A Review.* Columbia University Press: New York, 1913.
9. Haaland M. *Spontaneous Tumours in Mice.* Imperial Cancer Research Fund: London, 1911, Report No.: 4.
10. Dunn TB. Morphology and histogenesis of mammary tumors. In: Moulton FRe (ed). *Symposium on Mammary Tumors in Mice.* Am.Assoc.Adv.Sci: Washington, DC, 1945, pp 13-38.

11. Boyer B, Tucker GC, Valles AM, *et al*. Reversible transition towards a fibroblastic phenotype in a rat carcinoma cell line. *Int J Cancer Suppl* 1989;4:69–75.
12. Strizzi L, Bianco C, Normanno N, *et al*. Epithelial mesenchymal transition is a characteristic of hyperplasias and tumors in mammary gland from MMTV-Cripto-1 transgenic mice. *J Cell Physiol* 2004;201:266–276.
13. White DE, Cardiff RD, Dedhar S, *et al*. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. *Oncogene* 2001;20:7064–7072.
14. Moody SE, Perez D, Pan TC, *et al*. The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell* 2005;8:197–209.
15. Landesman-Bollag E, Romieu-Mourez R, Song DH, *et al*. Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* 2001;20:3247–3257.
16. Yauch RL, Januario T, Eberhard DA, *et al*. Epithelial versus mesenchymal phenotype determines *in vitro* sensitivity and predicts clinical activity of erlotinib in lung cancer patients. *Clin Cancer Res* 2005;11(Part 1): 8686–8698.
17. Jessen KA, Liu SY, Tepper CG, *et al*. Molecular analysis of metastasis in a polyomavirus middle T mouse model: the role of osteopontin. *Breast Cancer Res* 2004;6:R157–R169.
18. Weiss RS, Matsuoka S, Elledge SJ, *et al*. Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway. *Curr Biol* 2002;12:73–77.
19. Gunther EJ, Moody SE, Belka GK, *et al*. Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. *Genes Dev* 2003;17:488–501.
20. Farago M, Dominguez I, Landesman-Bollag E, *et al*. Kinase-inactive glycogen synthase kinase 3beta promotes Wnt signaling and mammary tumorigenesis. *Cancer Res* 2005;65:5792–5801.
21. Sternlicht MD, Bissell MJ, Werb Z. The matrix metalloproteinase stromelysin-1 acts as a natural mammary tumor promoter. *Oncogene* 2000;19:1102–1113.
22. Cardiff RD, Leder A, Kuo A, *et al*. Multiple tumor types appear in a transgenic mouse with the ras oncogene. *Am J Pathol* 1993;142: 1199–1207.
23. Maglione JE, McGoldrick ET, Young LJ, *et al*. Polyomavirus middle T-induced mammary intraepithelial neoplasia outgrowths: single origin, divergent evolution, and multiple outcomes. *Mol Cancer Ther* 2004;3:941–953.
24. Namba R, Maglione JE, Young LJ, *et al*. Molecular characterization of the transition to malignancy in a genetically engineered mouse-based model of ductal carcinoma *in situ*. *Mol Cancer Res* 2004;2:453–463.
25. Borowsky AD, Namba R, Young LJ, *et al*. Syngeneic mouse mammary carcinoma cell lines: two closely related cell lines with divergent metastatic behavior. *Clin Exp Metastasis* 2005;22:47–59.
26. Rosner A, Miyoshi K, Landesman-Bollag E, *et al*. Pathway pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors. *Am J Pathol* 2002;161: 1087–1097.
27. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8: 1243–1252.
28. Siegel PM, Shu W, Cardiff RD, *et al*. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100:8430–8435.
29. Bindels S, Mestdagt M, Vandewalle C, *et al*. Regulation of vimentin by SIP1 in human epithelial breast tumor cells. *Oncogene* 2006;25: 4975–4985.
30. Lee JM, Dedhar S, Kalluri R, *et al*. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006;172:973–981.
31. Blanco MJ, Moreno-Bueno G, Sarrío D, *et al*. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* 2002;21:3241–3246.
32. Gobbi H, Simpson JF, Borowsky A, *et al*. Metaplastic breast tumors with a dominant fibromatosis-like phenotype have a high risk of local recurrence. *Cancer* 1999;85:2170–2182.
33. Gobbi H, Simpson JF, Jensen RA, *et al*. Metaplastic spindle cell breast tumors arising within papillomas, complex sclerosing lesions, and nipple adenomas. *Mod Pathol* 2003;16:893–901.