Current Concepts and New Insights from Mouse Models of Mammary Tumors on Epithelial Mesenchymal Transition and its Synergy with Mutant p53

A. Piersigilli[1,2], A. D. Borowsky[3], Q. Chen[3], N.E. Hubbard[3] and R.D. Cardiff[3,*]

[1]*Institute of Animal Pathology, Vetsuisse, University of Bern, Switzerland*

[2]*Ecole Politechnique Fédérale de Lausanne (EPFL), School of Life Sciences, SV-PTECH PTEC GE, Station 19, Switzerland*

[3]*Department of Pathology and Laboratory Medicine and Center for Comparative Medicine, 4115 Primate Drive, University of California, Davis, Davis, CA 95616, USA*

Abstract: Epithelial Mesenchymal Transition (EMT) is the transdifferentiation of epithelial cells into a mesenchymal phenotype. This process occurs during embryogenesis but also in wound healing and in tumors. The neoplastic EMT is characterized by variably complete shedding of epithelial architectural features and acquisition of mesenchymal traits. In immunohistochemistry a variable coexpression of cytokeratins, vimentin or alpha-smooth muscle actin with loss of E-cadherin and other interepithelial adhesion molecules is characteristic. Such transition is associated with mutations both at the genetic (somatic) and epigenetic levels and is believed to confer a more advantageous phenotype for local and distant spread of cancer cells. Mammary carcinoma can exhibit EMT features in humans and mice and it tends to occur more frequently in women with tumors bearing a worse prognosis such as the claudin low subtype within the triple negative cancer. Missense mutation of TP53 is one of the most common mutations in cancer and it is frequently found in EMT tumor types, often with a more aggressive behavior. The current literature and survey of our mouse EMT cases in the Genomic Pathology Center image archives demonstrate a synergy between p53 and EMT that is independent of the initiating oncogene. However, p53 mutation is not sufficient or causal for EMT. Moreover, despite the local malignant behavior, processes such as spontaneous metastases and Mesenchymal Epithelial Transition (MET) appear not to be as frequent and obvious as previously hypothesized.

Keywords: p53, EMT, metastasis, MET, breast cancer, mouse model, triple negative, claudin low.

EMT AND CANCER

Epithelial-to-Mesenchymal Transition, known by the acronym EMT, consists of transdifferentiation of epithelial cells into mesenchymal cells. This program takes place in a physiological (embryo development) as well as a pathological context (tissue repair, neoplasia). A reverse process named Mesenchymal-to-Epithelial Transition (MET) also exists and, upon withdrawal of initial stimuli, is thought to revert the acquired phenotype back to the initial phenotype [1]. In embryonic life, EMT occurs in most metazoans during gastrulation when the epiblast differentiates into the primordial germ layers [1, 2]. EMT and MET are also involved in tissue repair and wound healing. For example, in skin wounds, keratinocytes transdifferentiate into mesenchymal cells, which migrate into the area of tissue loss. Once the epithelial barrier is reconstituted, the cells revert back to the epithelial phenotype [3]. This program, consistent with many biological processes, is finely regulated, dynamic and reversible, implying a high degree of cellular plasticity.

*Address correspondence to this author at the Center for Comparative Medicine, 4115 Primate Drive, University of California, Davis, Davis, CA 95616, USA; Tel: (530) 752-2726; Fax: (530) 752-7914; E-mail: rdcardiff@ucdavis.edu

An EMT-like process has been recognized in neoplasia [4]. It is assumed that carcinomatous cells must shift to a mesenchymal phenotype, in order to infiltrate tissues and spread to distant organs (metastasize) [5]. This process starts with gradual disorganization and shedding of epithelial architectural features, such as polarity, intercellular adhesion, cytokeratin filaments and the acquisition of mesenchymal traits including a spindle shape of the cell, motility, invasiveness and attributes of stem cells [1, 5]. Downregulation with loss of E-cadherin (intercellular adherens junctions, encoded by CDH1 gene) occludins, claudins; increased expression of mesenchymal markers (eg. Vimentin, alpha-smooth muscle actin) and morphologic changes are all hallmarks of this transdifferentiation program [6].

Transition from one phenotype to the other includes intermediate “stages”, in which some epithelial and mesenchymal traits coexist within the same cell (partial EMT) [7]. In the mouse, neoplastic EMT is recognized by a spindle cell morphology containing, most commonly, both epithelial and mesenchymal intermediate filaments that are detected by immunohistochemistry (IHC) [8, 9]. Partial EMT appears to be the most common form [10, 11].

ISSN: 1927-7210 / E-ISSN: 1927-7229/15

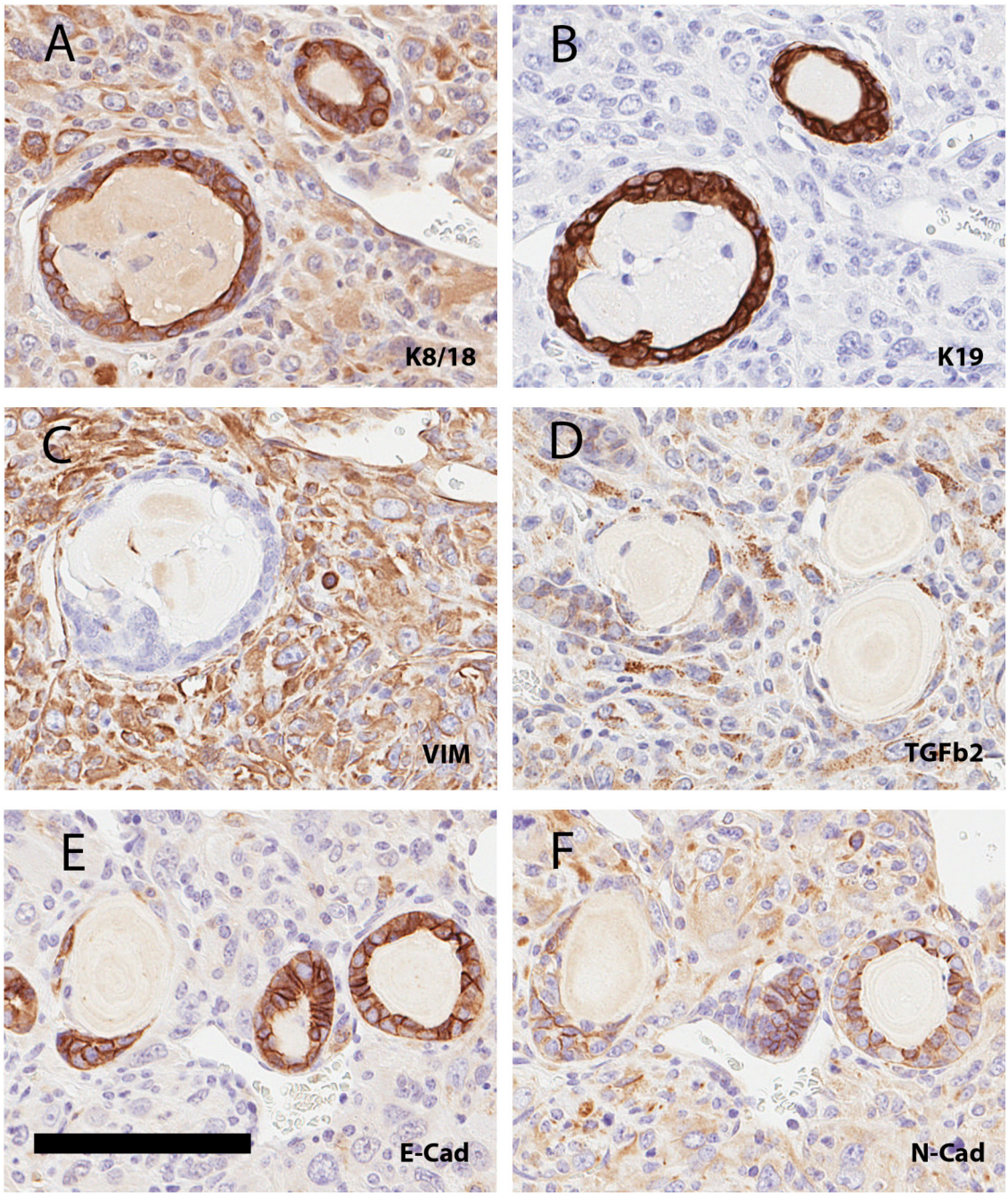

Figure 1: Immunohistochemistry of mouse EMT Phenotype Tumor.

Immunohistochemical (IHC) demonstration of typical mouse mammary EMT phenotype tumor. This Myc-induced tumor from Dr. Eran Andrechek has a clearly delineated epithelial component with oval glands lined by epithelium and surrounded by the spindle cell component with fusiform nuclei. The antigens detected are noted on the panel. Note that anti-keratin 8/18 stains both epithelial and spindle cell components (**A**). Anti-keratin 19 only stains the epithelial cells (**B**). In contrast, anti-vimentin primarily stains the spindle cell component, indicating that these spindle cells have both epithelial and mesenchymal intermediate filaments and, thus, fit the criteria of "dual staining." (**C**) The anti-TGFβ2 stain suggest the activation of a gene regarded as critical to the EMT process (**D**). Finally, the switch of cadehrins from E-Cadherin to N-Cadherin is illustrated (**E**, **F**). The epithelial cells are dual staining. While the spindle cells are primarily positive for N-Cadherin. Scale Bar=100microns.

However, intermediate states can be found in which the neoplastic cells are not particularly spindle shaped but lack distinctive cell-to-cell adhesion molecules and may develop marked differences in tumor vascularity and permeability [12]. The intermediate states are particularly prevalent in mouse mammary tumor cell lines carried in serial transplantation or passed through tissue culture [13] and were regarded at one time as an artifact of first, transplantation, and later, of tissue culture [14, 15]. One well-known example is the 4T1

cell line that was derived from a mammary tumor [16] and it is used as a standard for immunotherapy [17-19]. However, 4T1 cells have lost their epithelial phenotype and assumed the EMT phenotype. Discerning investigators are advised to carefully select well-differentiated epithelial tumors before serial transplantation and to monitor the transplant morphology to avoid promulgating inaccurate data.

In addition to changes in structural elements, activation of at least one or more specific transcription factors such as SNAIL, ZEB1, SLUG, TWIST is required for the EMT process. Recently several mechanisms have been elucidated and described [7, 20-21]. Tumor stroma for instance also appears to play a pivotal role in cancer biology by virtue of the paracrine signals delivered to malignant cells which can induce EMT [23]. It has been proposed in fact that when carcinoma cells detach as single cells and invade the surrounding stroma and environment, they are less exposed to epithelial signals and more "sensitive" to EMT signals delivered by mesenchymal cells and leukocytes.

Mechanistically, upon activation of the EMT program, associated transcription factors bind on the CDH1 promoter and repress its transcription [24]. Epigenetic regulators are also involved [5]. Among these, Polycomb Group Proteins (PcG) silence genes by modifying histones and recruiting in the promoter region additional repressors [25]. To this family belong PRC2, which physically interact with transcription factors, followed then by PRC1, which is recruited upon trimethylation of K27 on histone H3 (H3K27me3) in nucleosomes around promoters. BMI1 is an important subunit of PRC1, which is dysregulated in many malignancies [26, 27]. Its contribution to activation of EMT program is exerted *via* transcriptional downregulation of PTEN, followed by activation of PI3K-Akt signaling and post-translational stabilization of SNAIL. Moreover, TWIST enhances BMI1 expression by binding on its promoter. TWIST and BMI1 together are essential for acquisition of a cancer stem cell phenotype [28, 29]. Lastly, EMT requires an increasing demand of energy for the cell to progress through the different stages and reach a full mesenchymal identity. Hence it is especially costly for the economy of the neoplastic cell [5].

As suggested in the preceding discussion, EMT, although a normal physiological process, is quite complex involving numerous possible networks and pathways. Disruption of any or all parts of these network can facilitate an autonomous new growth (neoplasia) within a pre-existing cancer. This disruption can lead to an entirely different tumor phenotype, the EMT tumor phenotype. These tumors acquire new and frequently unique biological and morphological characteristics which are now recognized as EMT tumors. As such, they clearly involve a secondary evolution of the primary neoplasm. Using mouse models, one can document the frequent loss of the inciting oncogene with a change in expression of potential therapeutic targets. This type of autonomous plasticity provides cancer with an "escape route" to avoid the best therapies and may explain the ongoing struggles with therapeutic failures in human cancer.

EMT ASSOCIATED GENOTYPES AND LOSS OF ONCOGENE ADDICTION

In the early 2000s, tumor biologists such as Dean Felsher and Lewis Chodosh, used inducible systems to turn oncogenic transgenes on and off in mice [30, 31]. They demonstrated that the silenced oncogene caused no harm until expressed. When de-induced, the tumors regressed. Such experiments in mice provided proof of principle that tumors were dependent upon the expression of the oncogene (oncogene addiction). Dependency of tumor growth on a single gene appeared as an easy target for molecular medicine. "Oncogene addiction" appeared to justify molecular medicine and targeted therapies. However, when de-induced some the tumors relapsed. The most reasonable hypothesis was that the recurrent tumors harbored a second mutation in either the target gene or within the tumorigenic pathway. Both hypotheses were validated as possibilities [31]. However, the mouse studies revealed yet another phenomenon. Many of the recurrent tumors did not resemble the phenotype of the primary tumor and appeared as completely unique, undifferentiated spindle cell tumors, that is, EMT phenotype tumors [31]. In addition, some studies resulting in spindle cell EMT phenotype tumors were shown to lack expression of the initiating oncogenic transgene. The EMT phenotype tumor and its underlying pathobiology provided renewed interest in the phenomenon described by Boyer and Thiery almost 20 years earlier [32], leading to a flood of papers describing the neoplastic process of EMT.

In contrast to the *physiological* process of EMT, the *neoplastic* process involves a variety of somatic mutations in a number of oncogenes and tumor suppressor genes ("major drivers") which have been associated with the EMT process.

Escape from oncogene addiction is similar to the events observed in women experiencing relapse of breast cancer after initial surgical removal or therapy-induced regression. The recurrent human tumors frequently have a different, more aggressive phenotype [33]. In a like manner, doxycycline-induced tumors in mice can relapse, when "de-induced" by withdrawal of the drug. The recurrent tumors have lost expression of the initiating oncogenic transgene. In most models, the recurrent tumors are no longer purely epithelial but now have an EMT tumor phenotype. Although It may be premature to postulate that the recurrent human tumors use the same EMT mechanisms to "escape" from therapy, it is a biological possibility worthy of consideration. This hypothesis has led to studies designed to reveal some of the genes in the escape mechanism using mouse models.

Debies *et al*. [34] observed, in experiments on tumor escape in a Wnt-1 mouse breast cancer model, that the relapsing tumors showed a p53 heterozygous deficiency and loss of $p19^{Arf}$ after withdrawal of Doxycycline (Dox). Moreover, Dox independent tumors consistently shared morphologic and molecular features of EMT such as spindle cell morphology, SNAIL expression and undetectable E-cadherin [35]. Primary tumors in which p53 and Ink4a/Arf were not mutated and Wnt pathway was intact, exhibited morphology of an adenocarcinoma. Of note, $P19^{Arf}$ relays oncogenic stress signals to p53 [36].

EMT is not solely associated with loss of oncogene addiction. Examination of the process in other models has revealed some examples of primary EMT tumors with maintained expression of the initiating oncogene. For example, Andrechek *et al*. in a study on the role of myc overexpression and ras pathway activation in mouse mammary tumors reported heterogeneity of gene profiles, morphologic phenotypes and metastatic potential. Of note, some primary myc-induced tumors exhibited features of EMT coupled with a dominant ras pathway [37, 38]. Oral administration of 7,12-Dimethylbenz[a]anthracene (DMBA) in FVB mice induces spindle cell tumors [39, 40] (among other histological phenotypes). DMBA is known to cause ras mutation and activation of wnt canonical pathway [41].

Neoplasms with features of EMT are also observed in mice carrying primary mutations in the two most commonly affected genes in human breast cancer: TP53 and PI3KAC. H1047R mutation in Pi3kac allele and monoallelic deletion with loss of function of Trp53 (mouse equivalent of TP53) in these mice is followed by decreased latency, decreased survival, and a predominance of poorly differentiated adeno-carcinomas and EMT tumors [42]. The introduction of Trp53 into a mouse with another oncogene has frequently led to increased nuclear pleomorphism and decreased tumor latency [43].

These data suggest an interplay between mono or biallelic mutation of p53 coding gene and/or its pathway dysfunction and the EMT phenotype.

To further investigate the relationship between EMT and p53 coding gene status we performed a survey of all tumors in our Center of Genomic Pathology (CGP) Image Archives of the Mutant Mouse Pathology Laboratory at University of California, Davis (http://spectrum.ucdavis.edu), which spans the last 25 years and have recorded spindle cell tumors occurring in association with a number of different oncogenes, carcinogens and, occasionally as spontaneous primary tumors (Table **1**) [8]. This, coupled with frequent loss of oncogene expression in these tumors, suggests a critical second event. The second event seemed to be coupled with Trp53 and led to a more thorough analysis of this possibility [44].

Trp53 MUTATIONS AND EMT: EVIDENCE OF A SYNERGY

Trp53 (in mice) or TP53 (in humans) is a tumor suppressor gene that encodes for the protein p53. In its wild type form, p53 functions as a sequence-specific DNA binding transcription factor that regulates a plethora of target genes involved in DNA repair (hence the name "guardian of the genome"), cell cycle control, senescence, apoptosis, angiogenesis, and cell adhesion [45]. Importantly p53 also exerts a negative control on different steps of the neoplastic process from onset to local invasion and metastasis, by a wide range of direct and indirect interactions and effectors. TP53 is often mutated in a variety of malignancies and is the most commonly mutated gene in 30-40% of human breast cancer, with a peak of up to 80% in the triple negative subtype [45].

Most mutations in humans occur in exons 4-9: the region which encodes for the DNA binding domain of the protein and are missense. Within this region several "hot spots" have been identified corresponding to the residues: R175, R248, R249, R273, R282, G245 [46], which code for amino acids responsible for binding of the protein to the nucleic acid. TP53 mutants in R175H or 273 result in increased incidence of

Table 1:

	Genomic	Tumor Type	IHC	Comment	PMID
1	PyVmT B/6:Tg(PvVmT)	Transplant	P53+	Ellies cell lines with mets	26467658
2	PyVmT FVB:Tg(PvVmT)	Transplant	P53+	MET 1 cell lines with mets	23049838
3	Stat 1 KO	Transplant	P53+	MET 1 cell lines with mets	23399853
4	4T1	Transplant	P53-	BALB/c mammary cell line	1540948
5	cNeu N7639 cells	Transplant	n.a.	N7639 cells (MMTV-Her-2/neu	18253935
6	Myc	Primary	p53+/-	Mutant MYC	19805309
7	CK2	Primary	p53+	CK2 Overexpression	11423974
8	cNeu R-CAS	Primary	p53+	PABC with RCAS	24317513
9	cNeu TOM	Primary	p53+	Inducible NEU-recurrent tumor	16169465
10	FVB WT with Prolactinomas	Primary	p53+	spontaneous spindle cell tumor	19276050
11	Met1	Primary	p53+	$MET^{mt\ over\ expression}$	1552940
12	Trp53×PI3KKO wnt and arf	Primary	p53+	p53+/-, pTEN +/-	19717424
13	Wnt 10b	Primary	p53+	WNT10B	23307470
14	Wnt TOM	Primary	p53+	Inducible WNT-recurrent tumor	18060046
15	cNeu Delta16E	Primary	p53-	Inducible ErbB2 mutant X MTB recurrent tumor	Submitted
16	DMBA+wt MICE	Primary	p53-	Carcinogen Treated	16263698
17	Trp53	Primary	p53-	Bigenic FVB/N ($Trp53^{+/-}$)×FVB/N ($Hus^{+/-}$)	11704830
18	Trp53ΔP	Primary	p53-	Radiation induced tumors	26310697
19	Ha-ras FVB/N Tac-Tg(Hba-x-v-Ha-ras)	Primary	n.d.	Harvey-Ras	8475993
20	GSK FVB/N (MMTV-mGSK)	Primary	n.d.	Wnt pathway	15994955
21	ILK1 transgenic	Primary	n.d.	ILK mouse that started EMT search	19276050
22	Met1 MMTV:Met^{mt}1 X Trp53f1/+ cre	Primary	n.d.	Bigenic METx p53 KO with metastases	23509284
23	MMP-3 FVB/N Tg(MMP-3/stromelysin-1)	Primary	n.d.	Bigenic MMP3xp53	10713697
24	Scr MMTV0ScrP305L	Primary	n.d.	MMTV-Scribble p305L mammary tumors	24662921
25	Trp53 and PI3KKO and Hunk KO	Primary	n.d.	Conditional KO p53+/x pTEN+/-x hunk	21393859
26	Trp53 F1/+ cre	Primary	n.d.	No $MET^{mt\ control\ Trp53}$	23509284
27	Cyclin D1	Primary	n.a.	Subgroup of tumors	25940700
28	Lunatic Fringe	Primary	n.a.	Lunatic Fringe	22624713
29	PIK3CA mutant E545K	Primary	n.a.	One of a PIK3CA series	24080956
30	Stat 5 KO	Primary	n.a.	STAT5 (delta)	15382041
31	Trp53	Primary	n.a.	Original-53 mutant “sarcomas”	1552940
32	Trp53 and PI3K	Primary	n.a.	pi3k x p53flox:MMTV-Cre	21324922
33	Trp53 Beclin Autophagy P53 and Beclin	Primary	n.a.	Beclin+/-/Tp53F/+/WapCre	23509284
34	Trp53 Cre-Lox deleted Trp53	Primary	n.a.	Targeted Deletion	15150107
35	Trp53 Mutant and KO p53	Primary	n.a.	Mutant p53 (R172H or R270H)	25512531
	Human BrCa of P53 and EMT Phenotype				
36	DKAT	explant	P53+	Breast Cancer, Human cell line	23049838
37	TMA with Claudin lo tumors	Primary	P53+	Breast Cancer, Human TMA	23509284
	Mouse Prostate Models with EMT				
1	PTEN/p53 Martin	Primary	n.a	Prostate	3644021
2	PTEN/p53 KO in prostate	Primary	n.a	Prostate	3644021
3	Pb-Cre;PtenL/W;KrasG12D/W prostate	Primary	p53+	Prostate and lung mets	3644021
4	SV40Tag/p53/Rb	Primary	ND	Prostate	3644021
5	2B PTEN/AKT	Primary	p53+	Prostate	3644021
6	PTEN WT/p53+/-/Nkx+/-	Primary	p53+	Prostate	3644021
7	TMPRSS2-ERG	Primary	p53+	Prostate	3644021
8	PTEN;p53;telomerase reactivation	Primary	n.a.	Prostate	3644021

highly metastatic carcinoma in mouse models [47, 48]. Moreover, a polymorphism which occurs commonly at codon 72, within the self-regulatory domain of p53: the N-terminal proline-rich domain, has been associated with altered cancer risk in humans [48].

In heterozygosity, the mutant p53 can antagonize the residual WT allele functioning as a tumor suppressor, exerting a dominant negative effect (haploinsufficiency). This effect is produced through interference with the formation of p53 tetramers and their binding to DNA, ultimately impeding transcription of the WT residual allele [49-51].

Mutations in TP53 do not only cause loss of the canonical tumor suppressor functions, but can even, and apparently more frequently, result in gain of function with biological effects similar to oncogenes, as observed in human neoplasia [52, 53]. The mutated form regulates genes and transcription factors expression by localizing on their promoters, leading to activation of further oncogenic pathways. Such interaction could be mostly indirect, through other proteins and intact transactivation domains, as most of the mutations occurring in p53 hot spots impair or even ablate the DNA binding domain.

In normal conditions, wild type p53 prevents occurrence of EMT by repressing ZEB1 and ZEB2 (transcription factors also associated with such transdifferentiation program) *via* miRNAs [54]. Recent experimental works highlighted a link between loss of p53 function and EMT induction in neoplastic cell lines [55]. Moreover, missense mutated p53 also leads to expression of EMT-inducing transcription factors such as SLUG and SNAIL [46, 51, 55].

The synergy between EMT and p53 was also observed in our immunohistochemical survey as it documented one or more examples of EMT tumorigenesis arising in 36 separate cohorts with many different genotypes and experimental conditions. Interestingly, 12 of 36 or 30% of those cohorts with spindle cell tumors (EMT) surveyed were directly related to a genetically modified mutant Trp53. Three were monogenic TP53 and nine were associated with an additional genetically modified engineered gene. In addition, eight other cohorts containing examples of EMT tumorigenesis arose in mice with monogenic mutated Receptor Tyrosine Kinase receptors and five from WNT-associated cohorts. Of the remaining examples, two are from the STAT pathway. Of particular note, is the EMT tumors among the spontaneous primary tumors that arose in a colony of "wild type" FVB mice with prolactinomas [56].

Since dysfunctional and, presumably, mutant forms of p53 can be detected with immunohistochemistry (IHC), a survey of 18 cohorts with available paraffin blocks was performed. Thirteen of the available 18 cohorts (72%) had IHC detectable p53 positive EMT tumors. Five were IHC negative and one, TOM/MBT-Myc, had five examples of completely negative EMT tumors and one p53 IHC positive EMT tumor. Some of the p53 positive tumors also had areas of negative p53 staining, introducing the specter of heterogeneity. Over all, this survey indicates that the majority of genetic cohorts in the collection with EMT tumorigenesis (25/35=71%) have direct evidence of association with dysfunctional p53.

This survey cannot be construed as a scientific random sampling of all possibilities but a limited sampling that was submitted directly or indirectly to our archives by interested investigators. Further, we do not have accurate records of the proportion of EMT tumors in the native colony in each submitted cohort. With these restrictions, the survey is about as comprehensive as that available elsewhere. As such, it provides insight into the extent of the process in colonies with mammary tumorigenesis.

Is dysfunctional p53 required for EMT mouse mammary tumorigenesis? While the association between EMT and Trp53 is striking, EMT tumorigenesis clearly can occur also without an intentional or detectable genetic modification of the gene or with IHC-based evidence of dysfunctional Trp53. This can be interpreted as evidence of other potential pathways playing a role, or with dysfunctional Trp53 without accumulation of IHC detectable p53. We know that the STAT1 EMT tumorigenesis is associated with mutations in exons 5, 6 and 8 (unpublished data) and have begun a sequencing project to determine whether some or all of the negative examples may have silent mutations. Further research is required to address this issue.

Can mouse epithelial differentiation of tumors occur in the presence of dysfunctional Trp53? The answer to this is yes. Most mouse models featuring genetically modified Trp53 produce a variety of tumor phenotypes [43]. In fact, most bigenic mice with a mutant Trp53 produce tumors that are similar to the signature phenotype for oncogenic transgene [43]. Figure **1** illustrates an example of explants with the same p53

positive cell line in the same animal resulting in epithelial and spindle cell phenotypes.

Can EMT occur in the absence of mutant Trp53? This is difficult to ascertain with the current data. By implication, the five IHC negative tumors lack detectable mutations. However, as intimated by Jones *et al.* IHC fails to detect all p53 mutations [60, 61]. We have begun Trp53 sequencing. Thus far p53 mutations have been detected in the IHC positive tumors but not the negative tumors. However, this data needs to be extended and verified by deeper sequencing.

Although, the majority of our studies are based upon mouse mammary tumorigenesis, we have observed the process in mouse models of prostate cancer and in a limited number of human cases. In each case, IHC for p53 was positive, indicating that the phenomenon discussed here is not limited to the mammary gland or the genus *Mus*. The human carcinomatous cell line DKAT resulting from the culturing of the pleural fluid, proved to be p53 positive with a mutation proved by sequencing [6].

The current literature and the survey of the CGP Image Archives provide evidence of a synergistic relationship between Tpr53 mutation and EMT tumor phenotype in mouse mammary tumorigenesis [36, 42 57, 58, 59]. However, they also suggest that Trp53 is not sufficient or causal but does have a key role in the phenomenon of EMT tumorigenesis.

P53, EMT AND METASTASES

Activation of the EMT program endows neoplastic cells within the primary tumor to become more invasive and execute most steps of the metastasis cascade [46,60]. Beside the "canonical" metastatic cascade other mechanism of spread to distant organs have been described. Condeelis has directly viewed and recorded these types of phenomena using *in vivo* microscopy in which the macrophages play an essential role [61]. Tumor cells can also be disseminated *via* the circulatory system to distant sites through a distinct mechanism, which is invasion-independent [62, 63]. This form of metastasis can be observed, especially in filter organs such as the lung, within vessels as nests of neoplastic cells surrounded by two layers of endothelium. Angiogenesis within neoplasia clearly plays a critical and active role both in mice and humans in this mechanism, as thin vessels proliferate around, envelope and isolate small groups of tumor cells that are pinched off [62]. Therefore, they form a neoplastic embolus [62, 63]. Once the embolus lodges in the target organ, tumor cells may grow without infiltrating the vessels' wall or, break through the (now) two layers of endothelium to invade into the surrounding parenchyma [63]. Non invasive vascular neoplastic spread is still rather unknown although it was already illustrated between the end of the 19th and beginning of the 20th centuries [64]. Even when clear active invasion of tumor associated vasculature is not observed, metastases should still be searched or expected (also at later time points) when sinusoidal vessels proliferate, surround and even dissect the tumor into small "blood floating islets". Immunohistochemical staining with antibodies against CD31 or VEGF will be helpful in identifying the thin endothelial layer wrapping around those tumor nests. Their occurrence is not infrequent in mice mammary, hepatocellular and endocrine pancreas tumors. Alternatively, nests of tumor cells can be found floating on "rafts" of fibrin clots. In either circumstance, the metastatic cells bring their own microenvironment.

Trp53 has numerous functional connections with EMT [45]. P53 is known to regulate components of the adhesion machinery that contribute to cell motility and invasion through the stroma by repression of the transcription of plasminogen activator by inducing expression of plasminogen activator inhibitor-1 (PAI-1). Plasminogen activator and conversion to plasmin leads to degradation of a variety of ECM (extracellular matrix) proteins such as laminin, fibronectin and fibrin [65].

Wild type p53 induce anoikis [66] and increases cell adhesion of epithelial cells to ECM (collagen type I and III) by means of maspin. Overexpression of maspin inhibited invasion and metastasis of TM40D cells after syngeneic orthotopic transplantation [65].

Another role of mutated p53 includes activation of podosome formation in neoplastic cells (invadopodia), which triggers degradation of basal membrane and ECM to move across stroma [67]. Upregulation of MMP1, MMP2 and MMP9 and basement membrane dissociation occurs upon p53 loss [68].

However, despite abundant evidence of the suppressor effect of p53 on the metastatic process, bi-allelic loss of p53 in knock out mice do not display increased frequency of metastases nor a more invasive phenotype of the tumor suggesting that p53 loss of function alone is not sufficient to drive tumor cell migration [46, 69].

Our original survey of mouse mammary EMT tumorigenesis did not find evidence of metastatic disease in association with the EMT tumor phenotype [8]. Rather, EMT phenotype tumors exhibited extensive local invasion and rapid growth that led to early regulatory sacrifice of the animals due to tumor size.

However, Andrechek *et al*. and others have found that the EMT tumors subgroup in mice exhibited a strongly predictive metastatic signature compared to other subtypes, such as papillary and microacinar [37]. None of the metastasis showed overt EMT differentiation but had features of poorly differentiated carcinomas. Only primary epithelial tumors were found to be associated with metastasis. These observations are consistent with the hypothesis that the EMT tumor phenotype and spontaneous metastasis are not tightly related.

The current survey of the CGP Image Archives does contain records of pulmonary metastases with EMT phenotypes from three genotypes: MMTV-Wnt10b-IRES-LacZ [58], B6:TG(PyVmT) [70] and 129:Tm(Stat1-/-) (unpublished, manuscript submitted). Tail vein injection of normal C57BL/6 mice with EMT neoplastic cells harvested from B6:TG(PyVmT) resulted in metastasis [13]. The SSM2 cell line developed from a 129:Tm(Stat1-/-) tumor did undergo EMT when transplanted into an intact 129 WT mammary fat pad (manuscript being written, unpublished data). These SSM2 tumors resulted in metastases to the lungs. The B6:TG(PyVmT) metastases also came from a primary EMT phenotype transplant tumor.

In these animals, the pulmonary lobes were infiltrated with marked effacement of the parenchyma by a mixed population of oval to spindle neoplastic, organized in bundles, solid nodules and whorls. In both of these examples, the hematogenous route of spread was confirmed by the angiocentric pattern which filled the pulmonary branches of the veins from hilus, and by frequent obliteration of other vascular lumena by neoplastic emboli. Of particular note, neoplastic cells in vascular emboli and infiltrating the lung had the same morphology and immunohistochemical profiles as the primary tumor and were p53 positive. Invasion of organs in some animals such as lymph nodes, kidneys and liver also document the highly malignant behavior. Again, p53 loss of function can actually increase the metastatic potential of tumors (EMT or non-EMT type). Deep sequencing performed on primary, metastatic tumors and xenografts showed in several studies that p53 mutation precedes metastasis as the frameshift mutation was a genetic aberration shared by all 3 specimens [13].

THE EMT-MET SWITCH

Claiming EMT as the most common mechanism for metastatic spread of tumors requires the invoking of a second phenomenon. Pathologists have been identifying the source of metastases by their similarity of morphological structure to the primary tumor, if the differentiation grade is maintained. A metastasis from the prostate resembles the primary tumor and is distinct from a colonic metastasis. If the escape from the primary tumor requires EMT, one has to postulate that reverse is true once the tumor reaches the metastatic site. Therefore, a reverse mesenchymal-to-epithelial transition (MET) process must occur [1]. The EMT is well documented by Condeelis's *in vivo* imaging [61]. In fact, many mice with pulmonary metastases contain small clusters of cells entrapped in the small peripheral vessel. These could be evidence of spread of solitary tumor cells that become entrapped in the smallest vessels. However, the MET process requires plasticity [1] that has rarely been documented in human or mouse models.

Plasticity, in our experience, was best exemplified by the DKAT cells from the human claudin low breast cancer [6]. These cells readily switched from vimentin positive mesenchymal cells in stromal growth media to keratin positive cells in epithelial growth media [6]. In the mouse, most EMT tumors have nests of adherent epithelial cells, indicating the ability to maintain both phenotypes and, possibly, plasticity.

In the examples we observed in the mouse, the metastases from animals bearing EMT phenotype tumors uniformly have the EMT phenotype and metastases from epithelial phenotype tumors have the epithelial phenotype. Metastases might lack the EMT morphology because 1) a switch from EMT to MET might have occurred before metastatic growth; 2) EMT is a later event in the primary tumor compared to metastases; 3) Mice bearing EMT tumors either die or are sacrificed before metastatic spread occurs or before metastases are of detectable size; 4) EMT and metastases are not related, 5) tumor cells from primary tumors lack the plasticity to undergo MET.

As previously stated, EMT is a dynamic process, encompassing intermediate stages in the process of acquisition or shedding of mesenchymal-like features,

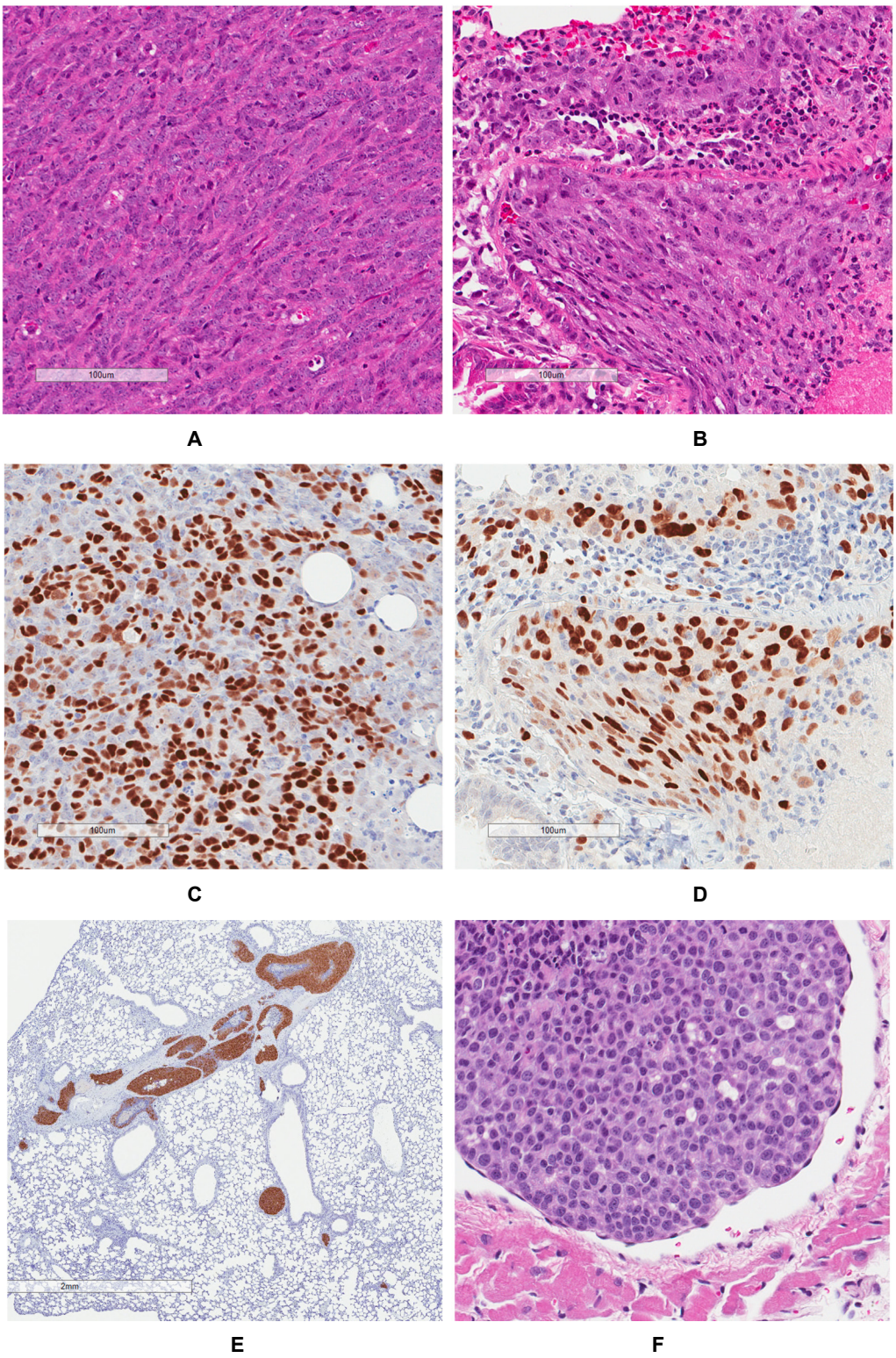

Figure 2: Metastatic Phenotypes of EMT and Adenocarcinomas.

This Panel compares the phenotypes of primary and metastatic tumors with EMT phenotypes or adenocarcinoma phenotypes. Images **A** and **B** are from Dr. Gustavo Miranda-Carboni's Wnt 10b induced animals. The primary tumor has the EMT phenotype spindle cells (**A**) as does the pulmonary metastasis (**B**). Both the primary (**C**) and the metastasis (**D**) have prominent nuclear staining for Trp53, illustrating an accumulation of a mutant antigen. In contrast, a typical cNeu solid, nodular carcinoma from Dr. Alexander Borowsky, metastasize with solid nests of ErbB2 positive tumor cells as tumor emboli (**E**). When viewed at low power (**E**), they clearly follow the vascular supply. When viewed at high power (**F**), the tumor cells are clearly well organized, solid clusters of adherent epithelial cells. Note that the tumor embolus is covered by the layer of endothelium frequently observed in these types of metastasis.

depending on epigenetic conditions [1, 6]. This requires genomic plasticity and enough energy for the cell. One well documented example in the CGP image archives is the EMT phenotype tumor from a human breast cancer cell line that co-expressed vimentin and keratin in the primary tumors and some of their metastases [6]. The poorly differentiated primary and metastases with dual staining tumor fits the operational definition of an EMT phenotype [8] but was clinically diagnosed as a pleomorphic, triple negative, breast cancer. The tumor epithelial cell culture derived from the pleural effusion of this tumor (DKAT) underwent EMT when placed in culture with a medium specific for stromal cells (SCGM), enriched with insulin and b-Fibroblast Growth Factor [6]. The spindle cell EMT phenotype cells could be reverted by passing the same DKAT cells back into a culture composed of a mammary epithelial growth media (MEGM). This observation provided *in vitro* evidence of plasticity with a MET or an EMT type transition under appropriate growth conditions. These cells not only retained the tumorigenic potential but also recapitulated the same phenotypes observed in the human cancer of origin as orthotopic xenografts in host mice.

Interestingly, other cell lines derived from different breast cancer types failed to switch to EMT or MET, depending on the histotype of origin, under the same experimental conditions as for DKAT [6]. The DKAT cells with an EMT phenotype had other EMT characteristics such as increased migratory phenotype, suggestive of local invasiveness and metastatic potential. Transplantation into ectopic sites in immunologically impaired mice results in different tumor phenotypes suggesting extensive plasticity in response to different extrinsic environmental signals.

In the mouse cohorts thus far examined, no evidence of MET in metastases has been found. When originating from epithelial tumors, the metastases were also epithelial. When originating from EMT phenotype tumors, the metastases exhibited a spindloid cell phenotype. In conclusion, MET has been proven to occur *in vitro* but there is scarce evidence *in vivo*.

One possible interpretation of the observation of plasticity in human but not mouse would be that the process in humans is a provisional, relatively early process. In contrast, the EMT phenotype tumor in mouse appears to be in a terminally differentiated stage which lacks the plasticity to undergo MET reversion. Since the epithelial differentiation program is a default pathway, persistence of cells in a mesenchymal state apparently requires a “tonic signaling”. TGF-β is the best known activator of MET that responds to epigenetic signals [71]. If the signals are not sufficient to sustain MET in the metastatic environment, mesenchymal neoplastic cells could promptly revert to the epithelial state or to an intermediate identity. Based also on what was previously mentioned and that the switch to the epithelial identity is also a more favorable state in terms of energy for cancer cells, MET might take place faster than EMT, making its detection more challenging time-wise.

Although EMT in primary and/or associated metastases and MET has not been documented in our studies [9], the scarcity of convincing examples or reports could have been due to methodological limitations. Many of the archived projects used assays and available technologies with end points that produce one or few static pictures representing events suspended in time. This experimental flaw does not represent an otherwise dynamic process such as the EMT-MET biology. Many early investigations, eager for proof-of-principle, ignored the biology and did not include searches for metastasis or even complete necropsies that included lung. Further, those of us that served as their pathologists did not understand the significance of spindle cell morphology in mice and, in part, claudin low in human [58].

As biological systems are much more complex than originally envisioned and with increasing and multiple variables, the challenge is to spot and document the key moment in such a dynamic system. Considering that events like expression of genes, synthesis or degradation of mRNA and proteins can be complete within seconds to a few minutes, the interpretation requires a complete understanding of structure and function. The pathologist must be privy to the full range of phenomenon in order to interpret change in the structure of disease.

P53 AS A DIAGNOSTIC AND PROGNOSTIC CLINICAL MARKER

Immunohistochemical detection of p53 mutation is a common tool widely used in clinical oncology. In patients with breast cancer, p53 status has shown prognostic significance [72].

This has some merit because constitutive levels of wild type forms of p53 are generally low and

undetectable with IHC [73]. Conversely, missense mutations in TP53 lead to accumulation within the cells with increased frequency and intensity of immunohistochemical staining. However, accumulation does not necessarily mean that a mutation occurred in TP53.

Other factors involved in the p53 cycle can be deregulated, such as decreased activity of MDM2 with impaired degradation of an otherwise normal p53 [73]. On the other hand, non-missense mutations usually do not lead to any accumulation of p53. As such, both situations would appear, respectively, as false positive (about 30%) and false negative when only immunohistochemistry is used for screening [74].

Gene sequencing provides more reliable information on p53 mutational status compared to Southern blot and even more to immunohistochemistry, thanks to its higher sensitivity and specificity. Costs, however, are a current deterrent to application of this testing procedure on a large scale.

A humoral response against p53 protein has also been detected in several human malignancies (lung, esophagus, liver, colon-rectum). A strong correlation between the antibodies and poor differentiation of the neoplasm has been reported [75]. Antibody titers also mirrored effects of antineoplastic treatment, with decrease of concentration occurring in parallel with tumor mass reduction. Moreover, titers increased prior to detection of disease relapse. Despite such encouraging data, only 30% of patients have detectable antibodies. The current assays relying on anti-p53 antibodies appear to lack enough sensitivity, required for diagnostic and/or prognostic markers [75].

CLINICAL IMPACT OF EMT AND P53 IN HUMAN (BREAST) CANCER: MORE TO ADD TO TRIPLE NEGATIVE SUBTYPE?

As previously mentioned, p53 is mutated in about 40% of all breast tumors, with the highest frequency (80%) in the "basal-like" subtype [45]. These tumors are primarily triple negative phenotype (TNP) and, as such, lack targets for specific therapy. Clinically these tumors are highly aggressive, invasive and metastatic. They do not respond to chemotherapy or radiation or if they initially do, they often acquire resistance and relapse [76, 77].

One subset, the claudin low TNP somewhat resembles the mouse EMT phenotype which lacks traditional epithelial junctions such as E-cadherin. However, more recent literature has recognized a subset of tumors with "mesenchymal like" properties in human and mouse mammary tumors [78]. Now, Pietenpol, Lehmann and colleagues have subdivided triple negative into seven categories two of which are, by their criteria, EMT [76, 77]. The mesenchymal subtype, designated, "M" is characterized by expression of Wnt, TGFβ, IG1FR, Notch and cell proliferation. The second subtype, Mesenchymal Stem-Like, designated "MSL" also has Wnt, TGFβ but has MAPK, Rac PI3K and PDGF expression but has a decreased expression of genes involved in cell proliferation [77]. Moreover, a link between these tumor subtypes, EMT and stem cell gene signatures is found in residual neoplastic cells after chemotherapy [79, 80]. Although other evidence supporting the link between a subset of triple negative human breast cancers, and mouse equivalents with EMT can be found in several experimental studies [6, 37, 81], the reader needs to realize that the vast majority of spontaneous, induced and engineered mouse mammary tumor models do not express ER, PR or Her2 and can, thus, be mis-classified as "triple negative". Further, tumors virus-induced or Wnt-induced frequently contain a well-organized and extensive myoepithelium which then places them into the molecular category of "Basal Cell Tumor". Another difference was found in our survey, where the different EMT type tumors expressed more frequently luminal cytokines, while the basal signature was more often loss or weak. These molecular classifications, developed without knowledge of the biology or morphology of mouse mammary tumorigenesis, have created many papers with faulty claims and great confusion in the field of comparative genomic pathology. As an example, triple negative tumors are highly metastatic while EMT associated metastasis are rather uncommon, as already mentioned previously. Accurate modeling requires matching attributes of mouse tumors with those of human. Unwarranted application of such broad terms does a disservice to science by hiding the true biological value of the mouse models.

Given the dramatic impact of triple negative subtype breast cancer and the lack of adequate therapy, the p53 pathway may represent a possible strategic target to cure such disease. In this effort, mouse models have great value in that they can provide the experimental insight that can lead to therapeutic advances. Restoring p53 normal function can be achieved experimentally by adenoviral delivery of WTp53 cDNA and by administration of small molecules. In case of

heterozygous loss of wild type allele, the normal pathway can be rescued by binding to mutated p53 isoforms, inducing a conformational change converting them into the original WT form again [82]. Experimental restoration of the function of p53 pathways in a Kras;trp53 mouse model of lung cancer leads to tumor regression but not complete eradication. However, since mutation in the p53 gene appears to be a late, secondary event in primary tumor progression, target therapies at this stage could potentially impede metastatic spread [82-84].

DISCUSSION

The EMT tumor phenotype is associated with a more aggressive phenotype, with increased local invasiveness and a molecular stem cell-like signature in both mouse and humans, and possibly with higher metastatic potential.

Homo- or heterozygous mutations of the TP53 (or Trp53) gene occur at the highest frequency in more malignant breast cancers, and are correlated with poor prognosis [46]. This is confirmed in genetically engineered mouse models of cancers in which the knockout of at least one of the Trp53 alleles accelerates tumor onset and induces a more malignant phenotype [85, 86].

Hence, the common denominator of EMT and p53 (mutated or deleted) is a more aggressive biologic behavior in the neoplasm. Moreover, the two phenomena are commonly found in association with each other, especially in the claudin low tumor subtype [58].

Based on the clinical observations and dissection of molecular pathways, which suggest a role of mutated p53 in inducing EMT, a synergy seems to exist. A thorough review of the literature and our immunohistochemical survey of more than 30 mouse cohorts exhibiting EMT tumor phenotype were performed to test the synergy hypothesis. Our survey of the murine engineered genotypes suggests that p53 mutation is not essential for EMT tumorigenesis. However, a mono or biallelic mutation of the tumor suppressor gene was found in 70% of the cohorts examined. In other words, EMT is more often found when Trp53 is mutated. However, the absence of stainable Trp53 suggests that a mutation is not necessarily required for the neoplastic EMT phenotype (Table **1**). The weakness in this conclusion is the lack of sequence data. Coradini *et al.* investigated the association between the mutational status of TP53 and the expression of genes associated with EMT (TGFβ, polarity loss and stemness related genes) in a microarray dataset of 251 primary breast cancers. Surprisingly, overall the correlation between TP53 mutation and the EMT-related genetic signature was statistically weaker in the primary tumor *in vivo* samples compared to cell culture samples [45, 54, 87, 88].

Possible explanations for this phenomenon could be that the 1) initial mutation of Trp53 induces EMT and later “stochastic” restoration of TP53 mutated allele/s occur; 2) existence and activation of p53 independent pathways leading to transformation or selection of an EMT tumor type clone.

While the first hypothesis could be debatable from a probabilistic point of view because “spontaneous” restoration of the wild type allele is rather infrequent, though a possible, event [89]. The second hypothesis is based on the well-known redundancy of biological systems, in physiologic and even more in a neoplastic context and, thus, seems more likely.

If the proposed synergy is correct, p53 could be a druggable target for treatment of some subtypes of breast cancer that do not respond to standard therapies. Restoration of p53 pathway would logically also impact EMT tumor homeostasis, possibly leading to regression or even complete eradication of the cancer. However this scenario would only be possible if two essential conditions are met: 1) there is interdependence between the 2 main genomic players and 2) EMT does not lose mutated p53 “addiction” at any time point.

To draw more definitive conclusions, time-wise modulation of p53 activity and impact on EMT (at morphologic and molecular levels) would be required in preclinical and clinical settings. Since synergy implies a greater effect when 2 or more components act together, compared to the effect of any one gene. In order to examine this issue, the incidence of metastases associated with a primary EMT tumor type was examined. Surprisingly, very few of the mice with EMT neoplasms bearing a mutated p53 exhibited metastases. As such, this could suggest that the mutation of Trp53 in mice EMT tumor phenotype does not necessarily lead to metastatic spread.

Several observations could explain this unexpected “paradox”. First, a more thorough understanding of the

EMT biology and synergy with p53 has been impaired by the variability in data collection and in the experimental settings in the published reports. Despite the plethora of reports of EMT with or without p53 mutation, the documentation of time and end-points, search for metastases, diagnostic criteria and methodology are drastically different making any comparative analysis challenging.

As a result, the spontaneous occurrence of metastases in mice, including transgenics, is inconsistently and rarely recorded. While the leading cause of death associated with cancer in humans is metastatic disease, mice tend to succumb to large primary tumors (requiring euthanasia) and are sacrificed before metastases have occurred. Mutation of p53 in the untreated mice might require a longer time for metastases to arise or to be detected. Metastases often go undetected when the investigator only samples the tumor and ignores possible metastatic sites such as the lungs. Transplantation experiments of the primary tumor in immunocompetent, syngenic mice also support the notion of time-dependency. When the "lifetime" of the tumor is increased by serial transplantation, the "incidence" of metastasis increases. A second explanation could be that the specific type of mutation(s) can actually produce different effects, with variable impact on distant colonization capabilities.

Finally, the most likely explanation, supported by the experimental and empirical evidence, is the demonstrable differences in tumor biology of mice and humans. The basis of the difference remains the major challenge to comparative analytical oncology.

Given these restrictions, we can still conclude that a synergy between mutated p53 and EMT does exist but we cannot find evidence to support interdependence. Given the coexistence of mutated p53 and EMT-like signature in the claudin low, mesenchymal subtype human breast cancer, further investigation on the relationship between the proposed synergy are sure to offer new insights and new therapeutic targets.

REFERENCES

[1] Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009; 119(6): 1420-8. http://dx.doi.org/10.1172/JCI39104

[2] Radaelli E, Damonte P, Cardiff RD. Epithelial-mesenchymal transition in mouse mammary tumorigenesis. Future Oncol 2009; 5(8): 1113-27. http://dx.doi.org/10.2217/fon.09.93

[3] Arnoux V, Nassour M, L'Helgoualc'h A, Hipskind RA, Savagner P. Erk5 controls Slug expression and keratinocyte activation during wound healing. Mol Biol Cell 2008; 19(11): 4738-49. http://dx.doi.org/10.1091/mbc.E07-10-1078

[4] Boyer B, Vallés AM, Edme N. Induction and regulation of epithelial-mesenchymal transitions. Biochem Pharmacol. 2000; 60(8): 1091-9. http://dx.doi.org/10.1016/S0006-2952(00)00427-5

[5] Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. Nat Med 2013; 19(11): 1438-49. http://dx.doi.org/10.1038/nm.3336

[6] D'Amato NC, Ostrander JH, Bowie ML, Sistrunk C, Borowsky A, Cardiff RD, Bell K, Young LJ, Simin K, Bachelder RE, Delrow J, Dawson A, Yee LD, Mrozek K, Clay TM, Osada T, Seewaldt VL. Evidence for phenotypic plasticity in aggressive triple-negative breast cancer: human biology is recapitulated by a novel model system. PLoS One 2012; 7(9): e45684. http://dx.doi.org/10.1371/journal.pone.0045684

[7] Leroy P, Mostov KE. Slug is required for cell survival during partial epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia 2010; 15: 117-134. http://dx.doi.org/10.1007/s10911-010-9178-9

[8] Damonte P, Gregg JP, Borowsky AD, Keister BA, Cardiff RD. EMT tumorigenesis in the mouse mammary gland. Lab Invest 2007; 87(12): 1218-26. http://dx.doi.org/10.1038/labinvest.3700683

[9] Cardiff RD. The pathology of EMT in mouse mammary tumorigenesis. J Mammary Gland Biol Neoplasia 2010; 15(2): 225-33. http://dx.doi.org/10.1007/s10911-010-9184-y

[10] Bednarz-Knoll N, Alix-Panabières C, Pantel K. Plasticity of disseminating cancer cells in patients with epithelial malignancies. Cancer Metastasis Rev 2012; 31(3-4): 673-87. http://dx.doi.org/10.1007/s10555-012-9370-z

[11] Chao Y, Wu Q, Acquafondata M, Dhir R, Wells A. Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases. Cancer Microenviron 2012; 5(1): 19-28. http://dx.doi.org/10.1007/s12307-011-0085-4

[12] Watson KD, Lai CY, Qin S, Kruse DE, Lin YC, Seo JW, Cardiff RD, Mahakian LM, Beegle J, Ingham ES, Curry FR, Reed RK, Ferrara KW. Ultrasound increases nanoparticle delivery by reducing intratumoral pressure and increasing transport in epithelial and epithelial-mesenchymal transition tumors. Cancer Res 2012; 72(6): 1485-93. http://dx.doi.org/10.1158/0008-5472.CAN-11-3232

[13] Bao L, Cardiff RD, Steinbach P, Messer KS, Ellies LG. Multipotent luminal mammary cancer stem cells model tumor heterogeneity. Breast Cancer Res 2015; 17(1): 137. http://dx.doi.org/10.1186/s13058-015-0615-y

[14] Dunn TB Morphology of mammary tumors in mice with and without the agent Acta Unio Int Contra Cancrorum 1951; 7 (2): 234-7

[15] Dunn TB Morphology and histogenesis of mammary tumors. In Moulton FRe., editor. Symposium on mammary tumors in mice. Washington: Am Assoc Adv Sci 1945: 13-38.

[16] Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. Curr Protoc Immunol 2001; Chapter 20: Unit 20.2.

[17] Nakamori M, Fu X, Rousseau R, Chen SY, Zhang X. Destruction of nonimmunogenic mammary tumor cells by a fusogenic oncolytic herpes simplex virus induces potent antitumor immunity Molecular Therapy 2004; 9(5): 658-665. http://dx.doi.org/10.1016/j.ymthe.2004.02.019

[18] Li H, Dutuor A, Fu X, Zhang X Induction of strong antitumor immunity by an HSV-2-based oncolytic virus in a murine mammary tumor model J Gene Med 2007; 9(3): 161–169. http://dx.doi.org/10.1002/jgm.1005

[19] Tao K, Fang M, Alroy J, Gary GG. Imageable 4T1 model for the study of late stage breast cancer. BMC Cancer 2008; 8, article 228. http://dx.doi.org/10.1186/1471-2407-8-228

[20] Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcription pathways. Cancer Res 2008; 68: 3645-54. http://dx.doi.org/10.1158/0008-5472.CAN-07-2938

[21] Herranz N, Pasini D, Diaz VM, Franci C, Gutierrez A, Dave N, Escrivà M, Hernandez-Munoz, Di Croce L, Helin K, Garcia de Herreros A, Peiro' S. Polycomb complex 2 is requiredfor E-cadherin repression by the Snail1 transcription factor Mol Cell Biol 2008; 28(15): 4772-81. http://dx.doi.org/10.1128/MCB.00323-08

[22] Cao Q, Yu J, Dhanasekaran SM, KimJH, Mani RS, Tomlins SA, Mehra R, Laxman B, Cao X, Yu J, Kleer CG, Varambally S, Chinnaiyan AM. Repression of E-cadherin by the polcycomb group protein EZH2 in cancer. Oncogene 2008; 27(58): 7274-84. http://dx.doi.org/10.1038/onc.2008.333

[23] Scheel C, Eaton EN, Li SH, Chaffer CL, Reinhardt F, Kah KJ, Bell G, Guo W, Rubin J, Richardson AL, Weinberg RA. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell 2011; 145(6): 926-40. http://dx.doi.org/10.1016/j.cell.2011.04.029

[24] Peinhado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumor progression: an alliance against the epithelial phenotype? Nat Rev Cancer 2007; 7: 415-428. http://dx.doi.org/10.1038/nrc2131

[25] Cedar H. & Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 2009; 10: 295-304. http://dx.doi.org/10.1038/nrg2540

[26] Molofsky AV. *et al*. Bmi-1 dependence distinguishes neural stem cells self-renewal from progenitor proliferation. Nature 2003; 425: 962-67. http://dx.doi.org/10.1038/nature02060

[27] Sangiorgi E, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet 2008; 40: 915-920. http://dx.doi.org/10.1038/ng.165

[28] Martin A, Cano A. Tumorigenesis: Twist1 links EMT to self renewal. Nat Cell Biol 2010; 12: 924-25. http://dx.doi.org/10.1038/ncb1010-924

[29] Yang MH. *et al*. Bmi1 is essential in Twist1- induced epithelial mesenchymal transition. Nat Cell Biol 2010; 12: 982-92. http://dx.doi.org/10.1038/ncb2099

[30] Felsher DW, Bishop JM. Reversible tumorigenesis by MYC in hematopoietic lineages. Mol Cell 1999; 4(2): 199-207. http://dx.doi.org/10.1016/S1097-2765(00)80367-6

[31] 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(2): 235-9. http://dx.doi.org/10.1038/84691

[32] Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002; 2(6): 442-54. http://dx.doi.org/10.1038/nrc822

[33] Kimbung S, Kovács A, Danielsson A, Bendahl PO, Lövgren K, Stolt MF, Tobin NP, Lindström L, Bergh J, Einbeigi Z, Fernö M, Hatschek T, Hedenfalk I. Contrasting breast cancer molecular subtypes across serial tumor progression stages: biological and prognostic implications. Oncotarget 2015.

[34] Debies MT, Gestl SA, Mathers JL, Mikse OR, Leonard TL, Moody SE, Chodosh LA, Cardiff RD, Gunther EJ. Tumor escape in a Wnt1-dependent mouse breast cancer model is enabled by p19/p53 pathway lesions but not p16 loss. J Clin Invest 2008; 118(1): 51-63.

[35] Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, Notorfrancesco KL, Cardiff RD, Chodosh LA. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell 2005; 8(3): 197-209. http://dx.doi.org/10.1016/j.ccr.2005.07.009

[36] Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev 2003; 13(1): 77-83. http://dx.doi.org/10.1016/S0959-437X(02)00013-8

[37] Andrechek ER, Cardiff RD, Chang JT, Gatza ML, Acharya CR, Potti A, Nevins JR. Genetic heterogeneity of Myc-induced mammary tumors reflecting diverse phenotypes including metastatic potential. Proc Natl Acad Sci USA 2009; 106(38): 16387-92. http://dx.doi.org/10.1073/pnas.0901250106

[38] Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, Rehm S, Russo J, Tavassoli FA, Wakefield LM, Ward JM, Green JE. The mammary pathology of genetically engineered mice: The consensus report and recommendations from the Annapolis meeting. Oncogene 2000; 19: 968-988. http://dx.doi.org/10.1038/sj.onc.1203277

[39] Dandekar S, Sukumar S, Zarbl H, Young LJ, Cardiff RD. Specific activation of the cellular Harvey-ras oncogene in dimethylbenzanthracene-induced mouse mammary tumors. Mol Cell Biol 1986; 6(11): 4104-8. http://dx.doi.org/10.1128/MCB.6.11.4104

[40] Strange R, Aguilar-Cordova E, Young LJ, Billy HT, Dandekar S, Cardiff RD. Harvey-ras mediated neoplastic development in the mouse mammary gland. Oncogene 1989; 4(3): 309-15.

[41] Currier N, Solomon SE, Demicco EG, Chang DL, Farago M, Ying H, Dominguez I, Sonenshein GE, Cardiff RD, Xiao ZX, Sherr DH, Seldin DC. Oncogenic signaling pathways activated in DMBA-induced mouse mammary tumors. Toxicol Pathol 2005; 33(6): 726-37. http://dx.doi.org/10.1080/01926230500352226

[42] Adams JR, Xu K, Liu JC, Agamez NM, Loch AJ, Wong RG, Wang W, Wright KL, Lane TF, Zacksenhaus E, Egan SE.Cooperation between Pik3ca and p53 mutations in mouse mammary tumor formation. Cancer Res 2011; 71(7): 2706-17. http://dx.doi.org/10.1158/0008-5472.CAN-10-0738

[43] Cardiff RD, Munn RJ, Galvez JJ. The Tumor Pathology of Genetically Engineered Mice: A New Approach to Molecular Pathology. In: Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL, editors. The Mouse in Biomedical Research: Experimental Biology and Oncology Second ed. New York: Elsevier, Inc; 2006; p. 581-622.

[44] Couto SS, Bolon B, Cardiff RD. Morphologic manifestations of gene-specific molecular alterations ("genetic addictions") in mouse models of disease. Vet Pathol 2012; 49(1): 116-29. http://dx.doi.org/10.1177/0300985811430962

[45] Coradini D, Fornili M, Ambrogi F, Boracchi P, Biganzoli E.TP53 mutation, epithelial-mesenchymal transition, and stemlike features in breast cancer subtypes. J Biomed Biotechnol 2012; 2012: 254085. http://dx.doi.org/10.1155/2012/254085

[46] Powell E, Piwnica-Worms D, Piwnica-Worms H.Contribution of p53 to metastasis. Cancer Discov 2014; 4(4): 405-14. http://dx.doi.org/10.1158/2159-8290.CD-13-0136

[47] Lang GA, Iwakuma T, Suh YA, Liu G, Rao VA, Parant JM, Valentin-Vega YA, Terzian T, Caldwell LC, Strong LC, El-Naggar AK, Lozano G. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell 2004; 119(6): 861-72. http://dx.doi.org/10.1016/j.cell.2004.11.006

[48] Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, Crowley D, Jacks T. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell 2004; 119(6): 847-60. http://dx.doi.org/10.1016/j.cell.2004.11.004

[49] Milner J, Medcalf EA. Cotranslation of activated mutant p53 with wild type drives the wild-type p53 protein into the mutant conformation. Cell 1991; 65(5): 765-74. http://dx.doi.org/10.1016/0092-8674(91)90384-B

[50] Milner J, Medcalf EA, Cook AC. Tumor suppressor p53: analysis of wild-type and mutant p53 complexes. Mol Cell Biol 1991; 11(1): 12-9. http://dx.doi.org/10.1128/MCB.11.1.12

[51] Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res 2000; 60(24): 6788-93.

[52] Bertheau P, Lehmann-Che J, Varna M, Dumay A, Poirot B, Porcher R, Turpin E, Plassa LF, de Roquancourt A, Bourstyn E, de Cremoux P, Janin A, Giacchetti S, Espié M, de Thé H. p53 in breast cancer subtypes and new insights into response to chemotherapy. Breast 2013; 22(Suppl 2): S27-9. http://dx.doi.org/10.1016/j.breast.2013.07.005

[53] Zhu J, Sammons MA, Donahue G, Dou Z, Vedadi M, Getlik M, Barsyte-Lovejoy D, Al-awar R, Katona BW, Shilatifard A, Huang J, Hua X, Arrowsmith CH, Berger SL. Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. Nature 2015; 525(7568): 206-11. http://dx.doi.org/10.1038/nature15251

[54] Kim T, Veronese A, Pichiorri F, Lee TJ, Jeon YJ, Volinia S, Pineau P, Marchio A, Palatini J, Suh SS, Alder H, Liu CG, Dejean A, Croce CM. p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. J Exp Med 2011; 208(5): 875-83. http://dx.doi.org/10.1084/jem.20110235

[55] Rivlin N, Brosh R, Oren M, Rotter V. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer 2011; 2(4): 466-74. http://dx.doi.org/10.1177/1947601911408889

[56] Radaelli E, Arnold A, Papanikolaou A, Garcia-Fernandez RA, Mattiello S, Scanziani E, Cardiff RD. Mammary tumor phenotypes in wild-type aging female FVB/N mice with pituitary prolactinomas. Vet Pathol 2009; 46(4): 736-45.

[57] Ponzo MG, Lesurf R, Petkiewicz S, O'Malley FP, Pinnaduwage D, Andrulis IL, Bull SB, Chughtai N, Zuo D, Souleimanova M, Germain D, Omeroglu A, Cardiff RD, Hallett M, Park M. Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. Proc Natl Acad Sci USA 2009; 106(31):

[58] Knight JF, Lesurf R, Zhao H, Pinnaduwage D, Davis RR, Saleh SM, Zuo D, Naujokas MA, Chughtai N, Herschkowitz JI, Prat A, Mulligan AM, Muller WJ, Cardiff RD, Gregg JP, Andrulis IL, Hallett MT, Park M. Met synergizes with p53 loss to induce mammary tumors that possess features of claudin-low breast cancer. Proc Natl Acad Sci USA 2013; 110(14): E1301-10.

[59] Liu JC, Voisin V, Wang S, Wang DY, Jones RA, Datti A, Uehling D, Al-awar R, Egan SE, Bader GD, Tsao M, Mak TW, Zacksenhaus E. Combined deletion of Pten and p53 in mammary epithelium accelerates triple-negative breast cancer with dependency on eEF2K. EMBO Mol Med 2014; 6(12): 1542-60. http://dx.doi.org/10.15252/emmm.201404402

[60] Nieto MA, Cano A. The epithelial-mesenchymal transition under control: global programs to regulate epithelial plasticity. Semin Cancer Biol 2012; 22(5-6): 361-8. http://dx.doi.org/10.1016/j.semcancer.2012.05.003

[61] Dovas A, Patsialou A, Harney AS, Condeelis J, Cox D. Imaging interactions between macrophages and tumour cells that are involved in metastasis *in vivo* and *in vitro*. J Microsc 2013; 251(3): 261-9. http://dx.doi.org/10.1111/j.1365-2818.2012.03667.x

[62] Sugino T, Kusakabe T, Hoshi N, Yamaguchi T, Kawaguchi T, Goodison S, Sekimata M, Homma Y, Suzuki T. An invasion-independent pathway of blood-borne metastasis: a new murine mammary tumor model. Am J Pathol 2002; 160(6): 1973-80. http://dx.doi.org/10.1016/S0002-9440(10)61147-9

[63] Sugino T, Yamaguchi T, Ogura G, Saito A, Hashimoto T, Hoshi N, et al. Morphological evidence for an invasion-independent metastasis pathway exists in multiple human cancers. BMC Med 2004; 2: 9. http://dx.doi.org/10.1186/1741-7015-2-9

[64] Apolant H. Die epitheliale Geschwülste der Maus. Arbeiten ad Königlchn Inst FExptTher zu Frankfurt am M 1906; 1: 7-68.

[65] Shi HY, Zhang W, Liang R, Abraham S, Kittrel FS, Medina D,Zhang M. Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. Cancer Res 2001; 61: 6945-51.

[66] Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994; 124(4): 619-26. http://dx.doi.org/10.1083/jcb.124.4.619

[67] Mukhopadhyay UK, Eves R, Jia L, Mooney P, Mak AS p53 suppresses Src-induced podosome and rosette formation and cellular invasiveness through the upregulation of caldesmon. Mol Cell Biol 2009; 29: 3088-98. http://dx.doi.org/10.1128/MCB.01816-08

[68] Ozdemir E, Kakehi Y, Okuno H, Habuchi T, Okada Y, Yoshida O. Strong correlation of basement membrane degradation with p53 inactivation and/or MDM2 overexpression in superficial urothelial carcinomas. J Urol 1997; 158: 206-11. http://dx.doi.org/10.1097/00005392-199707000-00072

[69] Attardi LD, Jacks T, The role of p53 in tumor suppression: lessons from mouse models. Cell Mol Life Sci 1999; 55: 48-63. http://dx.doi.org/10.1007/s000180050269

[70] Wend P, Runke S, Wend K, Anchondo B, Yesayan M, Jardon M, Hardie N, Loddenkemper C, Ulasov I, Lesniak MS, Wolsky R, Bentolila LA, Grant SG, Elashoff D, Lehr S, Latimer JJ, Bose S, Sattar H, Krum SA, Miranda-Carboni GA. WNT10B/β-catenin signalling induces HMGA2 and proliferation in metastatic triple-negative breast cancer. EMBO Mol Med 2013; 5(2): 264-79. http://dx.doi.org/10.1002/emmm.201201320

[71] Frisch SM. The epithelial cell default-phenotype hypothesis and its implications for cancer. Bioessays 1997; 19(8): 705-9. http://dx.doi.org/10.1002/bies.950190811

[72] Di Leo A, Tanner M, Desmedt C, Paesmans M, Cardoso F, Durbecq V, Chan S, Perren T, Aapro M, Sotiriou C, Piccart MJ, Larsimont D, Isola J; TAX 303 translational study team. p-53 gene mutations as a predictive marker in a population of advanced breast cancer patients randomly treated with doxorubicin or docetaxel in the context of a phase III clinical trial. Ann Oncol 2007; 18(6): 997-1003. http://dx.doi.org/10.1093/annonc/mdm075

[73] Taylor NJ, Nikolaishvili-Feinberg N, Midkiff BR, Conway K , Millikan RC, Geradts J. Rational Manual and Automated Scoring Thresholds for the Immunohistochemical Detection of TP53 Missense Mutations in Human Breast Carcinomas. Appl Immunohistochem Mol Morphol 2015. http://dx.doi.org/10.1097/PAI.0000000000000207

[74] Sjögren S, Inganäs M, Norberg T, Lindgren A, Nordgren H, Holmberg L, Bergh J. The p53 gene in breast cancer: prognostic value of complementary DNA sequencing versus immunohistochemistry. J Natl Cancer Inst 1996; 88(3-4): 173-82. http://dx.doi.org/10.1093/jnci/88.3-4.173

[75] Soussi T. p53 Antibodies in the sera of patients with various types of cancer: a review, Cancer Res 2000; 60(7): 1777-88.

[76] Lehmann BD, Pietenpol JA, Clinical implications of molecular heterogeneity in triple negative breast cancer, The Breast 2015. http://dx.doi.org/10.1016/j.breast.2015.07.009

[77] Abramson VG, Lehmann BD, Ballinger TJ, Pietenpol JA. Subtyping of Triple-Negative Breast Cancer: Implications for Therapy Cancer 2015; 121(1): 8-16.

[78] Hollern DP, Andrechek ER. A genomic analysis of mouse models of breast cancer reveals molecular features of mouse models and relationship to human breast cancer. Breast Cancer Res 2014; 16(3): R59. http://dx.doi.org/10.1186/bcr3672

[79] Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, Rimm DL, Wong H, Rodriguez A, Herschkowitz JI, Fan C, Zhang X, He X, Pavlick A, Gutierrez MC, Renshaw L, Larionov AA, Faratian D, Hilsenbeck SG, Perou CM, Lewis MT, Rosen JM, Chang JC. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci USA 2009; 106(33): 13820-5. http://dx.doi.org/10.1073/pnas.0905718106

[80] Mani SA Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133(4): 704-15. http://dx.doi.org/10.1016/j.cell.2008.03.027

[81] Leung JY, Andrechek ER, Cardiff RD, Nevins JR. Heterogeneity in MYC induced mammary tumors contributes to escape from oncogene dependence. Oncogene 2012; 31 (20): 2545-54. http://dx.doi.org/10.1038/onc.2011.433

[82] Feldser DM, Kostova KK, Winslow MM, Taylor SE, Cashman C, Whittaker CA, Sanchez-Rivera FJ, Resnick R, Bronson R, Hemann MT, Jacks T. Stage-specific sensitivity to p53 restoration during lung cancer progression. Nature 2010; 468(7323): 572-5. http://dx.doi.org/10.1038/nature09535

[83] Thompson EW, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? Cancer Res 2005; 65(14): 5991-5; discussion 5995. http://dx.doi.org/10.1158/0008-5472.CAN-05-0616

[84] Selivanova G. Therapeutic targeting of p53 by small molecules. Semin Cancer Biol 2010; 20(1): 46-56. http://dx.doi.org/10.1016/j.semcancer.2010.02.006

[85] Maroulaku IG, Shibata MA, Jorcyk CL, Chen XX, Green JE. Reduced p53 dosage associated with mammary tumor metastases in C3(1)/TAG transgenic mice. Mol Carcinog 1997; 20(2): 168-74. http://dx.doi.org/10.1002/(SICI)1098-2744(199710)20:2<168::AID-MC3>3.0.CO;2-J

[86] Parant JM, Lozano G. Disrupting TP53 in mouse models of human cancers. Hum Mutat 2003; 21: 321-6. http://dx.doi.org/10.1002/humu.10186

[87] Chang CJ Chao CH, Xia W, Yang JY, Y Xiong, Li CW, Yu WH, Rehman SH, Hsu JL, Lee HH, Liu M, Chen CT, Yu D, Hung MC. Nat Cell Biol 2011; 13(3): 317-23. http://dx.doi.org/10.1038/ncb2173

[88] Pinho AV, Rooman I, Real FXs. P53 dependent regulation of growth, epithelial mesenchymal transition and stemness in normal pancreatic epithelial cells. Cell Cycle 2011; 10(8): 1312-21. http://dx.doi.org/10.4161/cc.10.8.15363

[89] Selivanova G, Wiman KG. Reactivation of mutant p53: molecular mechanisms and therapeutic potential. Oncogene 2007; 26: 2243-54. http://dx.doi.org/10.1038/sj.onc.1210295

Received on 11-09-2015 Accepted on 30-11-2015 Published on 18-12-2015

http://dx.doi.org/10.6000/1927-7229.2015.04.04.8