Partial Mesenchymal to Epithelial Reverting Transition in Breast and Prostate Cancer Metastases

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Abstract Epithelial to mesenchymal transition (EMT) is an oft-studied mechanism for the initiation of metastasis. We have recently shown that once cancer cells disseminate to a secondary organ, a mesenchymal to epithelial reverting transition (MErT) may occur, which we postulate is to enable metastatic colonization. Despite a wealth of in vitro and in vivo studies, evidence supportive of MErT in human specimens is rare and difficult to document because clinically detectable metastases are typically past the micrometastatic stage at which this transition is most likely evident. We obtained paired primary and metastatic tumors from breast and prostate cancer patients and evaluated expression of various epithelial and mesenchymal markers by immunohistochemistry. The metastases exhibited increased expression of membranous E-cadherin compared to primary tumors, consistent with EMT at the primary site and MErT at the metastatic site. However, the re-emergence of the epithelial phenotype was only partial or incomplete. Expression of epithelial markers connexins 26 and/or 43 was also increased on the majority of metastases, particularly those to the brain. Despite the upregulation of epithelial markers in metastases, expression of mesenchymal markers vimentin and FSP1 was mostly unchanged. We also examined prostate carcinoma metastases of varied sizes and found that while E-cadherin expression was increased compared to the primary lesion, the expression inversely correlated with size of the metastasis. This not only suggests that a second EMT may occur in the ectopic site for tumor growth or to seed further metastases, but also provides a basis for the failure to discern epithelial phenotypes in clinically examined macrometastases. In summary, we report increased expression of epithelial markers and persistence of mesenchymal markers consistent with a partial MErT that readily allows for a second EMT at the metastatic site. Our results suggest that cancer cells continue to display phenotypic plasticity beyond the EMT that initiates metastasis.

Introduction

Recapitulation of the developmental process of epithelial to mesenchymal transition (EMT) has been proposed as a mechanism for enabling cancer cell invasion and dissemination. During cancer-associated EMT, loss of cell-cell adhesions via downregulation of E-cadherin allows for both physical detachment from the tumor mass and for external autocrine growth factor and internal signaling that activates cell migration [1]. EMT in cancer progression and metastasis has been widely studied through in vitro cell culture and in vivo animal models of cancer progression. In addition, EMT has been visualized at the invasive front of primary carcinomas as individual cells or a group of cells migrating into the surrounding tissue [2]. However, the true extent of EMT in human cancer specimens is still open to debate as is the role of EMT in metastatic seeding [1, 3, 4].

Despite the strong clinical association between decreased expression of adhesion molecules and invasion and poor prognosis, metastases can present a well-differentiated,

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epithelial phenotype, bringing into question whether EMT is reversible. We and others have proposed that a reverse EMT, or mesenchymal to epithelial reverting transition (MErT), occurs to enable metastatic colonization [4–7]. Therefore, while induction of EMT through loss of E-cadherin may promote tumor invasion and dissemination, MErT through re-expression of epithelial genes and downregulation of mesenchymal genes may allow the metastatic cancer cell to complete the last steps of the metastatic process and to survive in the secondary organ. However, just as it has been difficult to capture EMT in vivo, there is also a dearth of histological evidence for MErT.

Opponents of cancer-associated EMT argue that there is a lack of convincing evidence in clinical samples that support the in vitro findings [3]. However, lack of evidence in clinical samples does not mean that an EMT or MErT has not occurred at some point in time, as pathological specimens are often end-stage observations. Unless clinically indicated, only a small percentage of metastases undergo surgical resection or biopsy, as systemic adjuvant endocrine, chemotherapy, or palliative radiation is more commonly used as therapy. Furthermore, specimens of metastases that are resected or that undergo biopsy originate from tumors of various stage and size (and ER/PR/HER2/neu status for breast cancer), making direct comparisons between patients difficult. Tumors often exhibit areas of poor differentiation and morphological changes, with cell scattering and spindle-shaped cells that are distinct from the bulk of the tumor, but pathologists do not routinely stain for markers of epithelial or mesenchymal phenotype as diagnostic and prognostic value is absent. Despite these shortcomings, histological examination of epithelial and mesenchymal markers in primary tumors and their corresponding metastases is important to determine whether EMT and MErT occurs clinically, with implications for the development of new approaches to cancer.

Recently, we have reported that breast and prostate cancer metastases express increased levels of E-cadherin when compared to the matched primary tumor [8, 9]. In addition, E-cadherin-negative MDA-MB-231 breast cancer cells were induced to re-express E-cadherin by in vitro coculture with liver parenchymal cells or following spontaneous metastasis to the lung in a mouse model [8]. However, despite the findings of E-cadherin re-expression and an accompanying morphological change, it remained to be seen whether a full or partial mesenchymal to epithelial transition had occurred. Thus, for the present study we evaluated the expression of mesenchymal and epithelial markers in a larger set of matched primary and metastatic tumor samples from breast cancer patients. We also focused on membranous expression of epithelial markers E-cadherin, β-catenin, connexin 26, and connexin43 as an indicator of normal function. Expression of epithelial markers was increased in metastases while expression of mesenchymal markers FSP1 and vimentin was variably changed, suggesting a partial MErT. In addition, we corroborated our results in a set of unmatched primary and metastatic prostate cancer samples and found that E-cadherin expression decreased with increasing metastatic tumor size, an observation that suggests that MErT is also reversible and helps to answer the question of whether metastases likely generate other metastases or if all metastases arise from the primary tumor.

Results

Breast Cancer Metastases Exhibit Increased Levels of Localization of Adherens Junction Components to the Membrane

A few studies have compared E-cadherin expression in the primary tumor and distant metastases [3, 9–11]. We recently reported on a small set of matched primary breast carcinomas and their metastases to the lung, liver, and brain [8]; besides bone, these comprise the most common sites of breast cancer metastases. In that study, we quantified both cytosolic and membranous staining as positive E-cadherin expression because E-cadherin expression was not always localized to the membrane. We have re-analyzed the data to include only positive membranous staining, as functional E-cadherin that both participates in intercellular adhesion and sequestration of the catenins is only localized at the membrane. In addition, we expanded the sample set to include additional pairs of matched specimens. Percentage of membrane expression was calculated as the number of cells positive for E-cadherin expression localized to the membrane over the total number of cancer cells in each field. Overall, 17/20 (85%) cases showed increased membranous E-cadherin expression in the metastases compared to the primary tumors (Fig. 1a), with this being consistent across the various sites; 2/2 (100%) of liver metastases, 5/6 (83%) of brain metastases, and 10/12 (83%) of lung metastases exhibited increased E-cadherin expression. The increase in E-cadherin levels across all tumor types was significant ($p < 0.05$ by Wilcoxon paired analyses), as it was within brain and lung metastases (the liver numbers were insufficient for assessment).

Localization of β-catenin at the cell membrane has been shown to be a critical suppressor of cancer cell migration and invasion as it forms part of a stable adherens junction [12, 13]. We therefore evaluated primary and metastatic tumors for membranous β-catenin expression (Fig. 1b). Overall, 9/20 (45%) of metastases exhibited increased expression of membranous β-catenin; 7/12 (58%) of lung metastases, 1/2 (50%) of liver metastases, and 1/6 (17%) in brain metastases. When positive β-catenin expression is
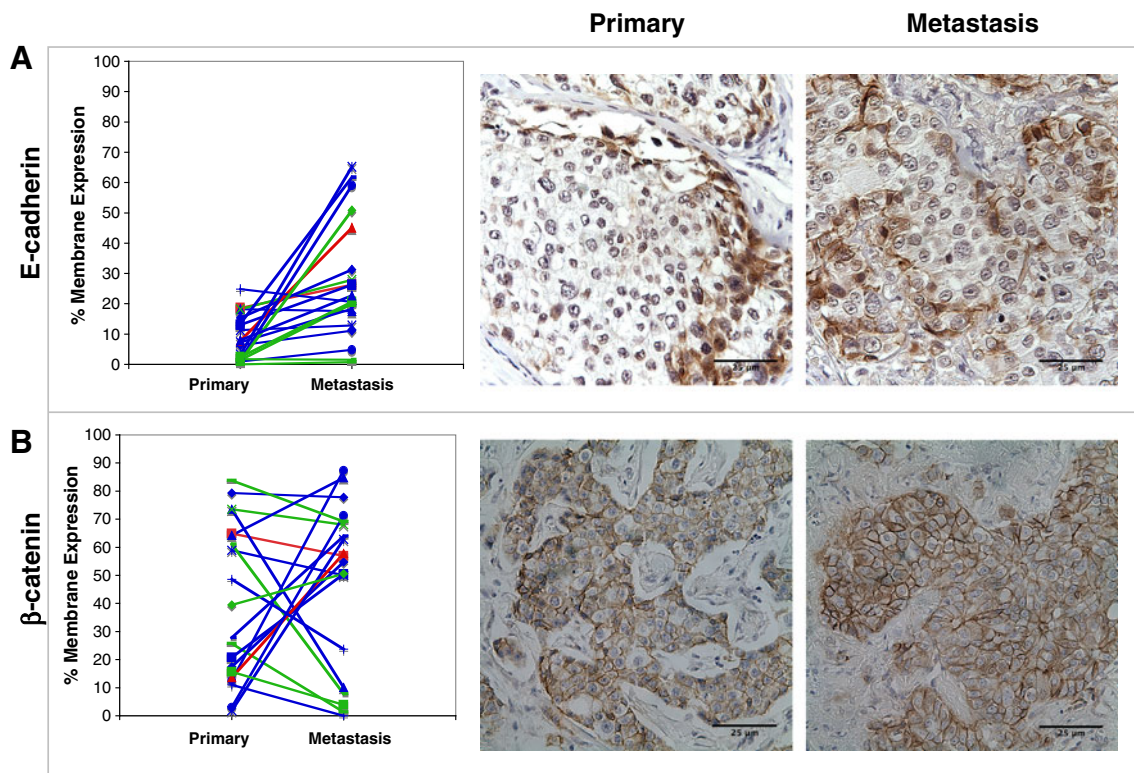
Primary **Metastasis**

Fig. 1 Breast cancer metastases exhibit increased localization of adherens junctions components to the membrane. a Quantification of membrane-bound E-cadherin in breast cancer primary tumors and metastases. Representative images of a primary tumor exhibiting cytoplasmic or absent E-cadherin and the paired lung metastasis with membranous E-cadherin expression. *$p<0.05$ for all cases and in lung and brain metastases subsets. b Quantification of membranous β-catenin in primary and metastatic tumors. Images from a case that exhibited increased membranous β-catenin staining in a metastasis to the lung. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns

quantified as including both membranous and cytoplasmic expression, increased β-catenin is evident in metastases compared to primary tumors, in 9/12 (75%) of lung metastatases, 2/2 (100%) of liver metastases, and 1/6 (17%) of brain metastases (data not shown). None of these associations were statistically significant ($p<0.05$ for all cases; $p<0.20$ for lung; $p<0.10$ for brain), likely due to the high β-catenin in the primary site coupled with both EMT and MErT occurring on a spectrum, rather than absolute phenotype switches. Due to the activation of the downstream Wnt pathway, nuclear localization of β-catenin is most commonly associated with the invasive phenotype; therefore β-catenin involvement in an epithelial phentype maybe best be quantified by membranous and cytoplasmic localization.

Expression of Gap Junction Proteins is Increased in Breast Cancer Metastases to the Brain

While adherens junctions facilitate intercellular adhesion, gap junctions mediate intercellular communication by mediating the exchange of small molecules and ions through a membrane-spanning pore composed of con-

nexins. In the breast, connexin 26 (Cx26) is expressed by luminal cells while connexin 43 (Cx43) is expressed by myoepithelial cells [14]. Loss of Cx26 and Cx43 has been shown to correlate with tumor progression in breast and colorectal cancer and over-expression of Cx43 reduces breast cancer metastasis [15–17]. Furthermore, just as re-expression of E-cadherin has been observed in metastases, increased expression of Cx26, Cx43, and Cx32 has been found in breast cancer lymph node metastases, suggesting that re-expression of gap junctions could also contribute to a MErT [18, 19]. We therefore surveyed the expression of membranous Cx26 and Cx43 in primary and metastatic tumors. For Cx26, 10/19 (53%) metastases showed increased membranous expression: 5/11 (45%) of lung metastases, 1/2 (50%) of liver metastases, and 4/6 (66%) of brain metastases (Fig. 2a). Increased expression of membranous Cx43 expression was observed in 55% (11/20) of all metastases, specifically in 4/12 (33%) of lung metastases, 1/2 (50%) of liver metastases, and 6/6 (100%) of brain metastases (Fig. 2b). For the most part, the two connexins changed, or stayed similar in parallel fashion within each metastasis. While there was
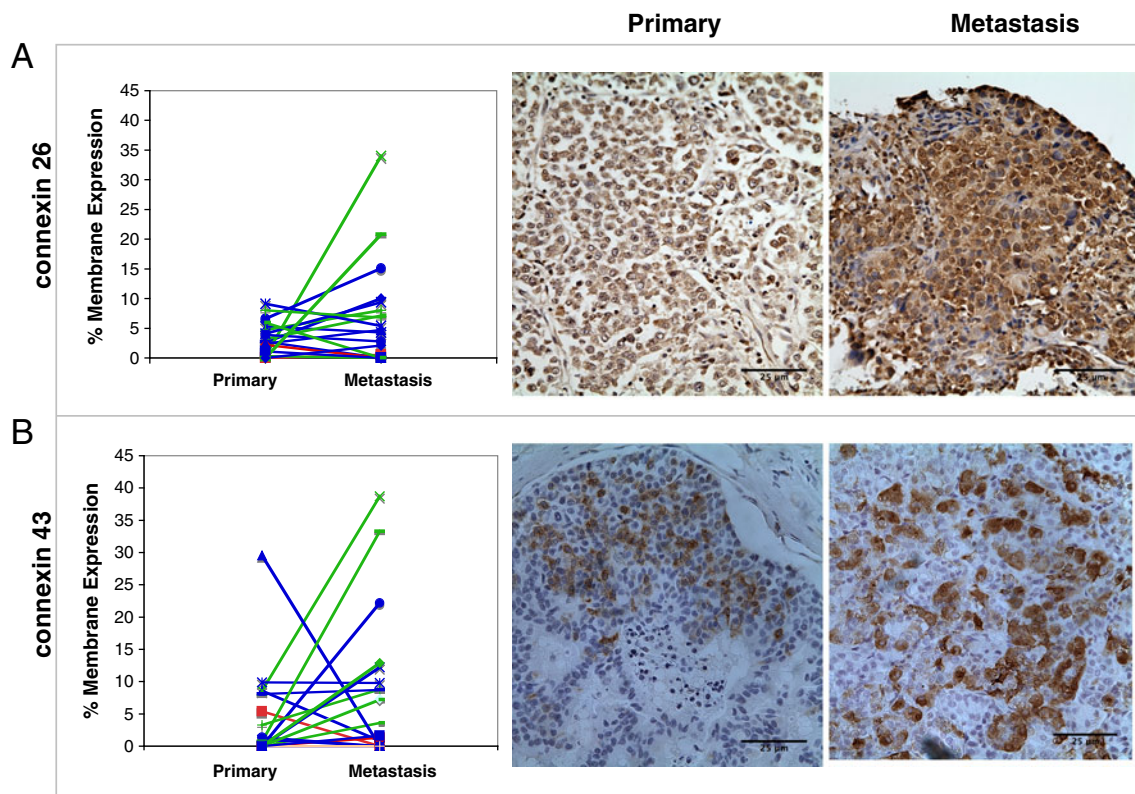
Fig. 2 Expression of gap junctional proteins is increased in breast cancer metastases to the brain. Quantification of membranous Cx26 (**a**) and Cx43 (**b**) staining in primary and metastatic breast cancer tumors. Shown are representative images of connexin staining in primary tumors and brain metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns. *$p<0.05$ for brain subsets of Cx26 and Cx43

no correlation in metastases to lung or liver, both Cx26 and Cx43 expression was strikingly increased in metastases to the brain ($p<0.05$) but the differences in connexin 26 or 43 expression between primaries and metastases when all cases were considered together did not reach statistical significance ($p<0.20$ for Cx26 and $p<0.10$ for Cx43). This was because there was no correlation in cases involving metastases to the lung ($p<0.50$ for Cx26 and $p<0.80$ for Cx43).

Persistence of Mesenchymal Markers in Metastases Suggests a Partial Mesenchymal to Epithelial Reverting Transition

To determine if the increase in epithelial markers signified the occurrence of a full MErT, which includes a loss or decrease in expression of mesenchymal markers in metastases, we next evaluated the expression of FSP1 and vimentin. FSP1 is considered one of the few truly fibroblast-specific markers and is commonly used as an early marker of EMT [20, 21]. Vimentin is also a widely accepted marker of the mesenchymal phenotype in EMT. During EMT, cells undergo a shift from using cytokeratin intermediate filaments to vimentin intermediate filaments, which are involved in the changes in adhesion and motility [22, 23]. Immunohistochemistry revealed that overall only 9/19 (47%) of metastases showed decreased expression of FSP1: 4/11 (36%) of lung metastases, 1/2 (50%) of liver metastases, and 4/6 (66%) of brain metastases (Fig. 3a). Similarly, 13/20 (65%) of metastases exhibited decreased expression of vimentin: 7/12 (64%) of lung metastases, 2/2 (100%) of liver metastases and 4/6 (66%) of brain metastases (Fig. 3b). For metastases that did display a decrease in expression of FSP1 or vimentin, the degree of change was small relative to the change observed in E-cadherin. Statistical analyses revealed no statistically significant difference between FSP1 ($p>0.80$ for all cases; $p>0.50$ for cases metastatic to lung; $p>0.50$ for cases metastatic to brain) or vimentin ($p>0.15$ for all cases; $p>0.45$ for cases metastatic to lung; $p<0.60$ for cases metastatic to brain) expression between primary and metastatic tumors. The lack of a dramatic downregulation of mesenchymal markers, along with the variability of epithelial markers noted above, suggest that only a partial MErT occurs during metastatic colonization.
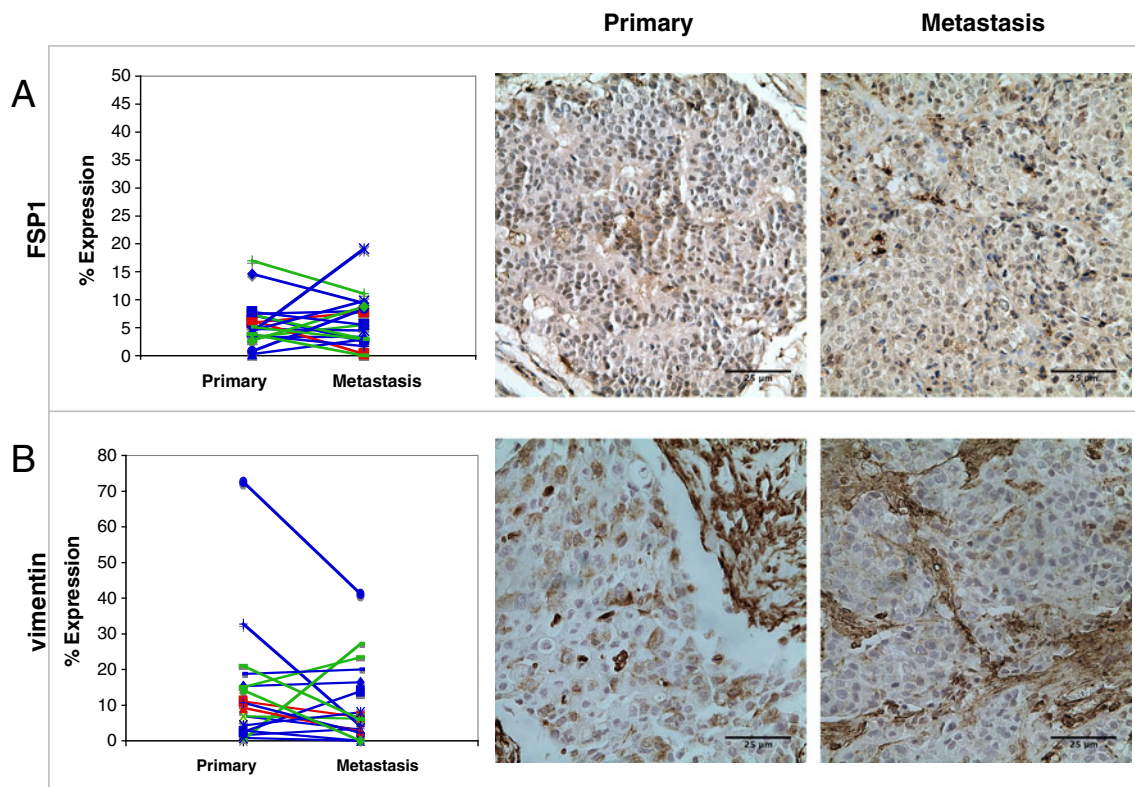
Fig. 3 Mesenchymal markers persist in metastases, suggesting only a partial MErT. Quantification of immunostaining for mesenchymal markers FSP1 (**a**) and vimentin (**b**). Images of FSP1 and vimentin staining in primary tumors and metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns

E-Cadherin Expression is Inversely Correlated with Size of Metastasis

To extend our findings beyond breast cancer metastases, we obtained a number of unmatched prostate carcinoma primary tumors and metastases. Organ sites of metastases included liver, lung, kidney, and thyroid. Primary and metastatic tumors were immunostained for membrane-associated E-cadherin and the percentage of cancer cells staining positive for E-cadherin was quantified in each field. Metastases exhibited increased staining of E-cadherin compared to primary tumors ($p<0.05$), suggesting that E-cadherin re-expression can occur in other cancers besides breast carcinoma (Fig. 4a). Due to a shortage of specimens, staining for other epithelial and mesenchymal markers was not performed.

Several of the metastatic specimens from individual patients contained multiple foci of different sizes. The metastatic foci within one patient sample were divided into three categories based on size: less than 50 μm in diameter (small), between 50 μm and 100 μm in diameter (medium), and larger than 100 μm in diameter (large) (Fig. 4b). It is recognized that sizing of tumor nodules depends on selection, but as our earlier study found an inverse correlation between E-cadherin levels and distance from normal parenchyma, the cross-sectional area was considered more critical than the absolute volume. The staining intensity of E-cadherin was quantified for each individual focus. Interestingly, E-cadherin expression inversely correlated with tumor size, with increased E-cadherin expression in small metastases compared to large ($p<0.01$) (Fig. 4c), suggesting that the partial MErT that allows for metastatic colonization is transient and reversible like the EMT that enables metastatic dissemination.

Discussion

One of the major limitations of studying metastasis in vivo is that studies involving animal models and clinical samples are end-stage time points that can only provide a snapshot of the metastatic cascade at the point of tissue harvest. Although intravital imaging and use of organotypic bioreactors has improved the ability to visualize metastasis at various stages, the phenotypic plasticity exhibited during EMT and MErT is nonetheless difficult to capture [24–26]. Evidence of EMT and MErT in clinical specimens is rare and has been used as an argument that cancer-associated
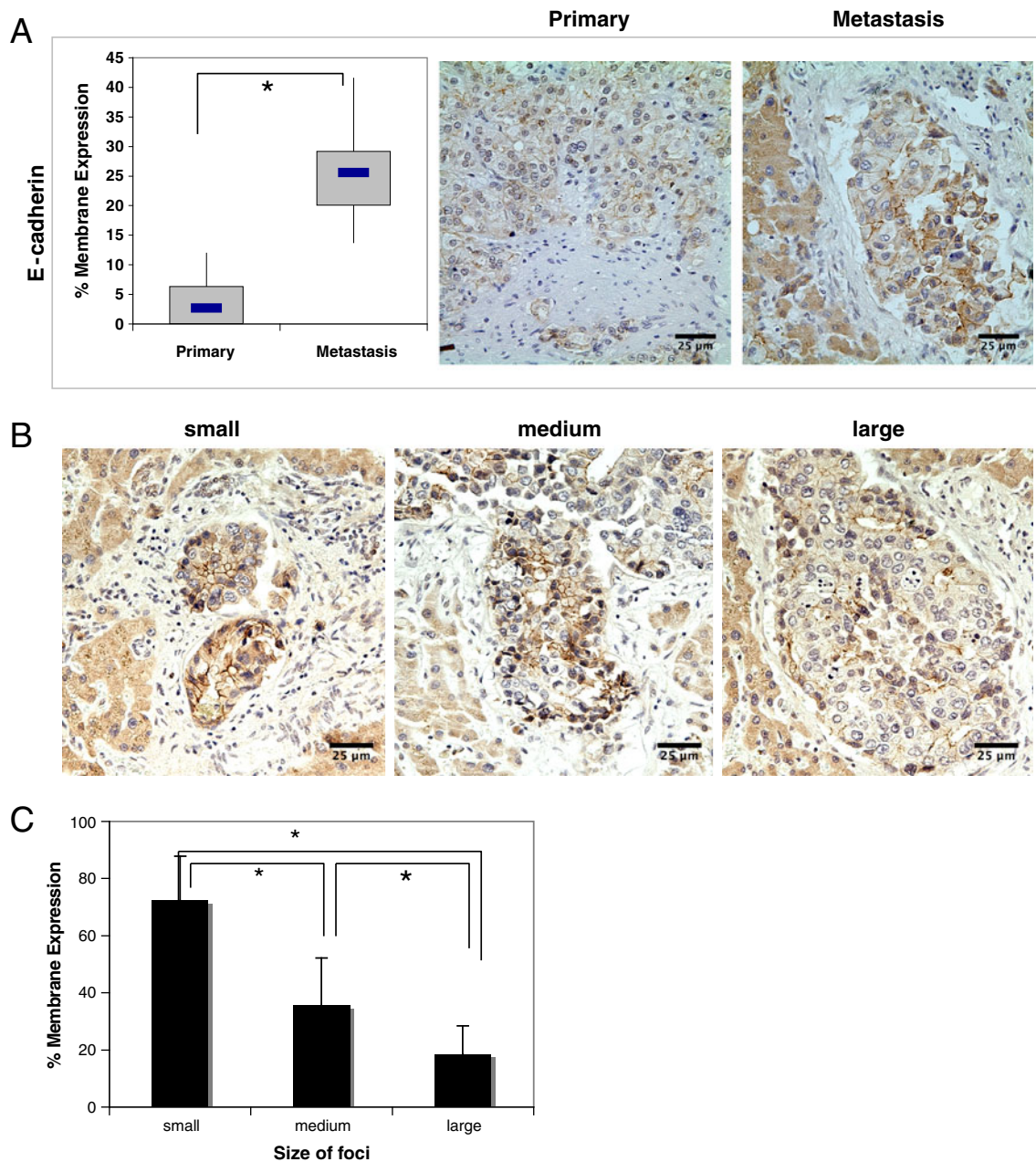
Fig. 4 E-cadherin expression in prostate cancer metastases is inversely correlated with size of metastasis. **a** Quantification and representative images of prostate cancer primary and metastatic tumors immunostained for E-cadherin. *p<0.05 Images of three random fields were quantifie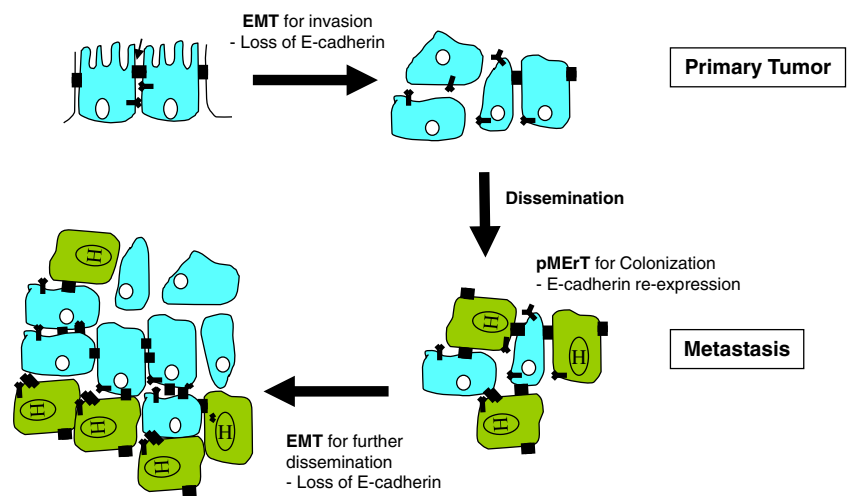d with ImageJ. **b** Images of metastatic tumors stained for E-cadherin as categorized by size: small (less than 50 μm in diameter), medium (between 50 μm and 100 μm) and large (bigger than 100 μm). **c** Quantification of E-cadherin expression in different sized prostate cancer metastases. *p<0.05. Size bar in the photomicrographs is 25 microns

EMT does not occur during the course of disease. Using matched primary and metastatic tumors, we have examined expression of epithelial and mesenchymal markers in specimens obtained from human breast cancer patients. Unfortunately, such paired specimens are few due to advances in imaging obviating the necessity for subsequent surgical biopsies, limiting the ability to accrue sufficient numbers that would allow for rigorous statistical analyses

and subset identifications. However, within this limitation, our results show that the occurrence of cancer-associated EMT and MErT is possible (Fig. 5).

Paget's "seed and soil" hypothesis posits that cancer cells can only survive and grow in appropriate environments; the reversible phenotypic plasticity of cancer cells during EMT and MErT is therefore one way in which cancer cells can adapt to the foreign soil of ectopic organ microenviron-

Fig. 5 Model of reversible phenotypic transitions during metastasis. EMT and loss of E-cadherin enables dissemination, followed by E-cadherin re-expression and a partial MErT that facilitates metastatic colonization at a secondary site. MErT is reversible, and with tumor growth may undergo an additional EMT

EMT for invasion
- Loss of E-cadherin

Primary Tumor

Dissemination

pMErT for Colonization
- E-cadherin re-expression

Metastasis

EMT for further dissemination
- Loss of E-cadherin

ments. Expression of adhesion molecules has been shown to be necessary to complete the final steps of the metastatic cascade including intravasation and colonization [27]. Based on previous observations of increased E-cadherin expression in metastases compared to primary tumors, we expanded our analysis to include E-cadherin binding partner β-catenin, gap junction molecules Cx26 and Cx43 and mesenchymal markers FSP1 and vimentin to discern whether a full or partial MErT occurs (summarized in Table 1). We limited our quantification of E-cadherin, β-catenin, Cx26, and Cx43 to expression localized to the membrane to account for proteins functioning in the epithelial phenotype, as dysfunctional proteins are commonly dislocated in the cytoplasm or nucleus during tumor progression. Increased expression of membranous E-cadherin was observed in metastases compared to primaries, across all organ sites of metastases. While we expected these results in metastases to lung and to liver where E-cadherin is expressed by pneumocytes and hepatocytes, it was surprising that 83% of metastases to the brain also exhibited increased E-cadherin expression. Of interest, disseminated cells in lymph nodes do not show similar E-cadherin expression but more closely resemble the original primaries [28]. Breast cancer cells that metastasize to bone have been shown to express OB-cadherin, the cadherin expressed by osteocytes, so it was expected that metastases would exhibit increased expression of the adhesion molecule native to the ectopic organ [29, 30]. Thus, increased E-cadherin expression was not expected in metastases to the brain, which primarily expresses N-cadherin. When we queried N-cadherin expression in primay and metastastic tumors, only 2/5 brain metastases exhibited increased N-cadherin expression (data not shown).

It is not surprising that an overall corresponding increase in membranous β-catenin was not observed in metastases,

as in all specimens the percentage of cells expressing β-catenin was higher than the E-cadherin-expressing cells. Thus, there was limited amount of increase that could be noted with β-catenin. This high level could be due to β-catenin binding to other cadherins. E-cadherin is not the only molecule that sequesters β-catenin, as the cytoplasmic domains are conserved among the type I classical cadherins. To test this, samples were also stained for N-cadherin (data not shown). While there was no consistent pattern of N-cadherin expression between primary tumors and metastases, high N-cadherin expression in the primary tumor was observed in many cases that exhibited no change or decreased localized β-catenin expression in metastases.

We also evaluated expression of gap junction molecules as another measure of epithelial gene expression in MErT. Cx26 and Cx43 are disparately expressed in the breast-luminal cells express Cx26 while myoepithelial cells express Cx43 [14]. Although the luminal and basal breast cancer subtypes arise from these two different cell types, there was no association between connexin expression and ER/PR/Her2 status, and therefore breast cancer subtype (Table 1). Overall, metastases exhibited increased expression of Cx26 and Cx43 compared to the primary tumors. This was most striking in brain metastases, where 66% of brain metastases demonstrated increased Cx26 expression and 100% showed increased Cx43 expression. In the brain, Cx26 and Cx43 are expressed by astrocytes, which suggests that gap junctions and not adherens junctions may be the driving force behind brain metastases. We have hypothesized that MErT in metastatic colonization serves to protect the metastatic cancer cell from inflammatory or chemotherapeutic insult [4]. Recent in vitro work by the Fidler group supports both our findings of increased connexin expression in brain metastases and also the theory that this re-expression confers a survival advantage. Melanoma or breast cancer cells

Table 1 Summary of epithelial and mesenchymal marker expression data. Green, cases that exhibited an increased expression in metastases; red, decreased expression in metastases compared to primary tumors; yellow, absent or no change in expression; white, unable to quantify sample

	Case	Subtype		E-cad	β-cat	Cx26	Cx43	FSP1	Vim
LUNG	1	ER-/PR-/HER2-	Basal						
	4	ER+/PR+/HER2-	Luminal						
	8								
	9	ER-/PR-/HER2-	Basal						
	10	ER-/PR-/HER2+	HER2						
	13	ER+/PR-/HER2-	Luminal						
	14								
	16	ER-/PR-/HER2-	Basal						
	18	ER-/PR-/HER2+	HER2						
	19	ER-/PR-/HER2-	Basal						
	20	ER-/PR+/HER2+	HER2						
	21								
	26	ER+/PR-/HER2-	Luminal						
				10/12 (83%)	7/12 (58%)	5/11 (45%)	4/12 (33%)	4/11 (36%)	7/12 (58%)

Legend: UP (green), DOWN (red), NO CHANGE (yellow), NO SAMPLE (white)

	Case	Subtype		E-cad	β-cat	Cx26	Cx43	FSP1	Vim
LIVER	3	ER-/PR-/HER2-	Basal						
	15	ER-/PR-/HER2+	HER2						
				2/2 (100%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	2/2 (100%)

	Case	Subtype		E-cad	β-cat	Cx26	Cx43	FSP1	Vim
BRAIN	4	ER+/PR+/HER2-	Luminal						
	11	ER+/PR-/HER2-	Luminal						
	12	ER-/PR-/HER2+	HER2						
	22								
	24	ER-/PR-/HER2+	HER2						
	25								
				5/6 (83%)	1/6 (17%)	4/6 (66%)	6/6 (100%)	4/6 (66%)	4/6 (66%)

cultured with astrocytes demonstrated reduced chemo-sensitivity, which was mediated by expression of connexins [31–33].

When immunostaining was performed for FSP1 and vimentin, expression of these markers in metastases was either unchanged or slightly decreased, suggesting only a partial MErT. Limitations in tissue prevented us for more examining other markers of mesenchymal phenotype, particularly N-cadherin which promotes interactions during intravasation and extravasation [34]. However, in a limited sampling, we did not find a correlation between N-cadherin levels and metastases, though this may reflect the metachronicity between the carcinoma dissemination and specimen acquisition. In addition, tumors are typically surrounded by reactive fibrosis and normal stromal cells that stain positive for mesenchymal markers so the possibility of false positives is high. Ideally, dual staining for breast cancer-specific and mesenchymal markers would overcome this problem; however, a reliable breast cancer-specific marker does not exist. Cell-cell adhesion and cell motility are usually viewed as opposite sides of the epithelial and mesenchymal phenotypic spectrum. However, partial EMT and MErT in which cells maintain some level of both is not an unusual phenomenon, as many examples can be found throughout cancer progression. During invasion, tumors have been shown to invade the ECM collectively as strands of cancer cells that maintain expression of adhesion molecules [35]. Similarly, during extravasation cancer cells re-express molecules that permit adhesion to endothelial cells yet still maintain the ability for trans-endothelial migration [36, 37].

Finally, we also found that E-cadherin expression decreases with increasing metastatic tumor cross-sectional area, if not actual size, suggesting that just as EMT is reversible, so is MErT. These data support earlier experimental evidence that the EMT that allows for escape from the primary lesion is not fixed but can be reverted during metastatic seeding [1, 8, 9]. However, often pathological examination of large metastases removed for palliative or diagnostic needs present de-differentiated cells reminiscent of the original EMT, which superficially appears at odds with our model of MErT. These data can be reconciled by our analysis of the prostate carcinoma micrometastases. In evaluating expression of E-cadherin based on metastasis size, we found the larger metastases (all still microscopic clinically) were less likely to express E-cadherin at the membrane, implying a re-emergence of EMT as with tumor growth. Thus, the phenotypic plasticity of carcinomas allows for continual repositioning of the tumor cell to provide a survival or dissemination advantage.

Evident in the stainings is a heterogeneity of markers within a given tumor at both the primary and metastatic sites. This has always been taken as evidence of the well-accepted concept of carcinoma progression, and often goes unremarked. However, this phenotypic diversity does allow for the possibility that the more epithelial cells in the primary actually seed and give rise to metastases [38, 39]; this is a postulate that cannot be tested by human observational reports even in the absence of markers in a

noted primary due to the metachronosity of the dissemination from the specimen acquisition. We suggest that this does not represent the majority of the epithelial-like metastases based on our experimental findings that demonstrate that a xenograft derived from clonal human breast or prostate (or colorectal, data not published) cancer cells lacking E-cadherin expression can form E-cadherin-expressing metastases [8]. Additionally, we reported that primary human carcinoma cells can be 'educated' to re-express epithelial markers by hepatocytes. Thus, we undertook this study to determine whether the human situation was consistent with such a EMT/MErT hypothesis. The correlations herein support this possibility. Additionally, the seeming evolution of the metastases towards a more mesenchymal-like state with increasing size (Fig. 5) and distance of the carcinoma cells from normal parenchymal [8], suggests the metastable nature of the phenotype displayed and supports the model.

The reversibility of MErT at the secondary site alludes to the question of whether all metastases necessarily arise from the primary tumor or whether metastases can give rise to metastases. An autopsy study of breast cancer patients found that the frequency of metastases to non-common sites was lower when metastases to the lung, liver, or bone were not already present [40]. It has been shown in a mouse model that systemic metastases arise in mice with large lung metastases in the absence of the primary tumor and also shown in melanoma dissemination to lung [41, 42]. One explanation is dormant cells were already seeded in the lung prior to primary tumor removal, but parabiosis experiments showed that the non-tumor bearing partner could develop metastases [43]. Despite these observations, the mechanism by which these secondary metastases occur is unknown. Here we suggest that EMT may occur following MErT in the metastatic site to engender these secondary metastases. Ultimately, the persistence of mesenchymal characteristics in MErT, despite the re-expression of epithelial genes and adhesion molecules, enables metastatic cancer cells to adeptly adapt to changing environments—from primary tumor to secondary organ and beyond.

Materials and Methods

Immunohistochemistry

All studies were performed on de-identified specimens obtained during clinically-indicated procedures; these were deemed to be exempted (4e) from human studies by the University of Pittsburgh Institutional Review Board.

Paraffin-embedded patient samples, excess to clinical need, were obtained from the University of Pittsburgh Tissue Banks, primarily coming from Magee Womens Hospital of UPMC and UPMC Shadyside Hospital, under informed consent of patients undergoing diagnostic and therapeutic procedures. Sections underwent antigen retrieval in citrate solution (Dako) and were incubated with primary antibodies: E-cadherin (Cell Signaling), β-catenin (abcam), connexin 26 (abcam), connexin 43 (abcam), FSP1/S100A4 (abcam), and vimentin (abcam) followed by biotin-conjugated secondary antibody (Jackson Laboratories). Antigen staining was performed using DAB (Vector Laboratories) then counterstained with Mayer's hematoxylin. Secondary antibody alone served as a negative control and adjacent normal tissue served as an internal positive control. Images of three randomly-selected microscope fields of each sample were taken and the percentage of cancer cells with positive staining was quantified as the number of positive cells over the total number of cancer cells in that image. Microscope fields shown were selected to account for the heterogeneity of each sample.

Statistical Analysis

For the matched breast cancer primary and metastatic tumor samples, statistical significance was calculated using the two-tailed Wilcoxon matched pairs test. P values were calculated for all cases combined as well as for individual organ sites. For the unmatched prostate cancer samples the Mann–Whitney test was used. For comparing E-cadherin expression between foci of different sizes, the Kruskal-Wallis ANOVA test was used.

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