Geometric Tumor Embolic Budding Characterizes Inflammatory Breast Cancer

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Abstract

Background

The *sina qua non* of IBC is numerous tumor emboli especially within dermal lymphatics. The explanation remains a mystery.

Methods

In observational human studies comparing IBC with non-IBC, although common tumor parameters such as Ki-67 index, mitotic count and nuclear size showed a reasonable overlap, there was a dramatic exponential or geometric difference in embolic density and a significant difference in embolic size distribution in IBC v non-IBC. In companion experimental studies using contrasting properties of two xenografts, Mary-X and Karen-X, both derived from IBC patients, this study offers a novel explanation and mechanism to explain the high tumor embolic numbers in IBC.

Results

The explanation is geometric embolic budding. Mary-X exhibits florid lymphovascular tumor emboli *in vivo* which give rise to high numbers of CTCs and pulmonary metastases whereas Karen-X lacks these features. Mary-X also gives rise to tight spheroids *in vitro* which exhibit dynamic budding whereas Karen-X exhibits only loose non-budding aggregates. Furthermore Mary-X emboli also bud dramatically into daughter emboli *in vivo*. The mechanism that regulates the compactness of the spheroids as well as the emboli involves the generation of E-cad/NFT1, a calpain-mediated cleavage product of membrane E-cadherin. Inhibiting the generation of E-cad/NFT1 by blocking either the calpain site of cleavage (SC) or the site of binding (SB) with specific decapeptides both reduces spheroid compactness and decreases budding.

Conclusions

Since E-cad/NFT1 retains the p120ctn binding site but loses both the β -catenin and α -binding sites, promoting its 360° distribution around the cell's membrane, the varying levels of expression of this truncated molecule may trigger budding of both the spheroids as well as the emboli. Recurrent and geometric budding of parental emboli into daughter emboli then would account for the plethora of tumor emboli seen in IBC patients.

Background

The *sina qua non* of inflammatory breast cancer (IBC) is numerous lymphovascular tumor emboli within the breast and especially within overlying dermal lymphatics where they are thought responsible for the

clinical signs of IBC including rubor, calor, dolor and swelling.¹⁻³ All molecular subtypes of IBC exhibit this unifying phenotype whose mechanism remains unknown.⁴

Previous studies by us and others using human xenograft models of inflammatory breast cancer and related cell lines have shed some light on the mechanisms responsible for the genesis of tumor clumps and derived emboli in the metastatic cascade of IBC.⁵⁻¹⁰ In these studies membrane E-cadherin is an important adhesion molecule and its overexpression responsible for the tumor clumping that occurs.¹⁰⁻¹² Although E-cadherin generally is also considered a tumor suppressor gene / protein in tumor invasion and metastasis whose loss contributes to epithelial-mesenchymal transition (EMT),¹³⁻¹⁵ in the setting of the tumor cell clump or the lymphovascular embolus of IBC, E-cadherin acts more like a dominant oncogene.¹⁶⁻¹⁸

However these previous observations do not explain the plethora of emboli that occur in IBC or offer an explanation of how so many emboli are observed within lymphovascular channels of this disease, especially within dermal lymphatics. Certainly E-cadherin overexpression while contributing to the tumor cell clump and the tumor cell embolus certainly can not alone account for the plethora of lymphovascular emboli observed in IBC.

For these reasons we sought explanations that might be responsible for this unique phenotype of IBC. We had collected a number of cases of human breast cancer of all types including IBC and non-IBC and their microdissected lymphovascular emboli that were available to study through TMA digital algorithmic image analysis for detailed human observational studies. We had also established two contrasting xenograft models of IBC, Mary-X and Karen-X, to use in experimental studies to explain the unique phenotype of florid lymphovascular emboli observed in IBC.

Methods

Institutional Approvals

Collection and use of human tissues from patients with breast cancer including non-IBC as well as IBC cases, completely anonymized, was approved by The Ohio State University Cancer Institutional Review Board (IRB) under protocol 2006C0042.

Both Mary-X and Karen-X were derived from patients with a biopsy proven diagnosis of IBC in the 1990's and made into transplantable xenografts. Patient consents were obtained and studies were conducted under the UCLA's Human Subject Protection Committee and the Chancellor's Animal Research Committee (Certification 95-127-11). Mary-X was derived from a biopsy of primary IBC whereas Karen-X was derived from a biopsy of a hepatic metastases. Mary-X exhibited triple (ER, PR and Her-2/neu) negativity whereas Karen-X was weakly estrogen receptor (ER) positive. Both xenografts have been phenotypically stable for over 25 years of passage. Continuing animal studies were approved by The Ohio State University's Animal Care and Use Committee (IACUC), protocol 2007A0218 and by The Ohio State University's

Institutional Biosafety Committee, protocol 2007R0057. Additional animal studies were approved by the University of Nevada's School of Medicine and the Nevada Cancer Institute's IACUC, protocols 00439 and 00440 when the corresponding author of this study was affiliated with these previous institutions. Final animal studies were conducted under an Interinstitutional Agreement between the California University of Science and Medicine and Anticancer, Inc. using the latter's IACUC protocol D16-00503 and OLAW A3873-01.

ATCC Patent Deposits and Cell Identification

Mary-X and its *in vitro* derived spheroids were deposited in the ATCC cell repository (Manassas, VA) as PTA-2737 and PTA-27376 respectfully and recently verified and re-verified to be both novel and human in origin (STRA4993). Likewise Karen-X and its *in vitro* derived spheroidal aggregates were deposited in the ATCC cell repository as PTA-126676 and PTA-126677 respectfully and recently verified to be both novel and human in origin (STRA4980). Mary-X is a well-known transplantable model of inflammatory breast cancer and has been used in numerous previous studies.¹⁹⁻²¹ Karen-X has not previously been reported, although it has been ATCC deposited.

Observational Studies

Cases of IBC

2000 cases of non-IBC and 100 cases of IBC were randomly selected from a database and The Ohio State University's Information Warehouse and anonymized.

TMA Construction

Multiple 2-millimeter tissue cores of tumor from each paraffin-embedded donor block (average of 10 cores / block) were arrayed into recipient TMA blocks. Our specific TMA algorithms carried out virtual alignment, image processing, and the application of the epithelial recognition algorithms (ERAs) and specific recognition algorithms (SRAs), the latter based upon immunocytochemical studies.²²⁻²⁵ Additional specific morphological algorithms included a mitosis algorithm²⁶, a nuclear grade (size) algorithm²⁷, and epithelial clustering algorithms measuring shape (elongation ratio: short/long axis) and size (perimeter) utilizing ImageJ software.²⁸ Image acquisition by either the Aperio ScanScope T2 System (Aperio, Vista, CA) or the iSCAN System (BioImagene, Inc, Cupertino, CA.) produced equivalent results.

Histological and Immunohistochemistry Studies

Primary antibodies used included rat anti-mouse Ki-67 (DakoCytomation, Carpinteria, CA), D2-40 anti-podoplanin (clone D2-40, Dako, catalog number M3619) and anti-CD31 (rabbit polyclonal, Spring Bioscience, catalog number E11114). The appropriate secondary antibodies were linked to an identifying chromogen.

ERAs applied to each TMA core were successful in recognizing tumor emboli based on the Gaussian kernel and other imaging techniques. SRAs imaged and quantitated nuclear Ki-67 immunoreactivity.²² Mitotic algorithms recognized and identified the various stages of mitosis including prophase, metaphase and telophase.²⁶ Specific circumferential lymphovascular immunoreactivities were detected and analyzed by the appropriate SRAs which included membrane recognition and cytoplasmic recognition algorithms whose efficacy had been demonstrated previously.²⁵ ImageJ was used to measure both the shape (elongation ratio) as well as the size (perimeter) of the emboli.²⁸

Experimental Studies

Initial Xenograft Studies

Athymic (nude) mice as well as Scids on BALB/c backgrounds, 4 week old females, purchased from Anticancer, Inc. (San Diego, CA) were derived from their respective breeding colonies. Mary-X and Karen-X were grown as transplantable xenografts. At selected intervals ranging up to 4 months, the mice were euthanized with 25 mg/ml ketamine, 2 mg/ml xylazine and acepromazine and subjected to necropsy. Routine histological and immunocytochemical studies were carried out on extirpated tumors. Antibodies used included biotinylated E-cadherin rabbit mAb (24E10) (Santa Cruz Biotechnology, Santa Cruz, CA) which recognized both full length E-cadherin (E-cad/FL) and its E-cad/NTF1 fragment, followed by a labeled Streptavidin-Biotin Complex (Santa Cruz Biotechnology, Santa Cruz, CA). DAB chromogen was used to develop the brown color.

In Vitro Studies

Both Mary-X and Karen-X were placed in culture and 2 mm thick slices of each tumor were minced in RPMI media with 10% fetal calf serum giving rise to liberated loose aggregates in suspension culture. These aggregates were then passed through cell strainers whose pore sizes ranged from 40–500 µm. The morphology of the aggregates were observed over the next 24–72 hr by phase contrast microscopy using the CytoSMART Lux2 (CytoSmart Technologies B.V. The Netherlands).

Confocal Single and Double Label Immunofluorescence Experiments on Mary-X Spheroids, Mary-X, Karen-X Aggregates and Karen-X

Mary-X spheroids and Karen-X loose aggregates were subjected to single label immunofluorescence studies using Alexa Fluor 488 conjugated 24E10 (#3199) (Cell Signaling Technology, Inc.) which recognized both E-cad/FL as well as E-cad/NTF1. In order to immobilize the spheroids and loose aggregates, glass-bottom dishes were coated with Cell-TEK adhesive. After washing with PBS 4–5 times, each for 10 min, the spheroids and aggregates were incubated with 24E10. The dishes were finally

mounted with Vectorshield mounting medium with DAPI (#H-1200) (Vector Laboratories) and viewed with a Olympus Fluoview-1000 confocal scanning system.

Mary-X and Karen-X were also subjected to double label immunofluorescence studies. Double label immunofluorescence experiments were carried out using the following combinations of antibodies: Alexa Fluor 488-conjugated 24E10 which recognized both E-cad/NTF1 as well as E-cad/FL and goat polyclonal antibody to mouse podoplanin or CD31 (R&D Systems, Inc.) which recognized murine lymphatics or blood vessels followed by Alexa Fluor 594-conjugated donkey anti-goat (#A11058) (Invitrogen, Inc.). Tumor emboli were recognized by E-cadherin positivity surrounded by circumferential podoplanin (lymphatics) or CD31 positivity (positivity).

Isolation of CTCs Using EpCAM Microbeads

CTCs were isolated from 10 mice each harboring the Mary-X and Karen-X xenografts. Blood from mice harboring at least 2 cm in diameter tumors were obtained by cardiac puncture and CTCs isolated with EpCAM microbeads.²⁹

Laser Capture Microdissection

Frozen sections (8 μ m) of Mary-X and human IBC cases were obtained, fixed in 70% ethanol, stained with hematoxylin and progressively dehydrated. Tumor emboli and non-embolic solid areas of Mary-X and human IBC cases were microdissected using a Pixcell II Laser Capture Microdissection 788 Laboratory System (Arcturus, Inc., Mountain View, CA) and stored at -80°C.

Preparation of Protein Lysates and Western Blot Analysis

The xenografts and laser-captured microdissected and pooled frozen IBC emboli were lysed using ice-cold RIPA lysis buffer (Pierce Biotechnology, Inc, Rockford, IL). For western blot analysis, boiled protein was loaded onto a 4–12% precast gradient gel (Invitrogen, Inc.), transferred to nitrocellulose membranes (Bio-rad, Hercules, CA) and incubated with either E-cadherin rabbit mAb (24E10) or rabbit anti-human ectodomain E-cadherin (H108) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology Inc., Danvers, MA). Rabbit mAb (13E5) was used to a housekeeping protein, ACTB. Bound antibodies were detected by a chemiluminescent detection system (West Femto) (Pierce Biotechnology, Inc.) according to the manufacturer's instructions.

Synthesis, Labelling and Use of SC and SB Decapeptides

The calpain cleavage and binding sites on full length E-cadherin were used as a template to design decapeptides to block calpain cleavage and binding. Two decapeptides: the site of cleavage (SC) peptide: DARPEVTRND and the site of binding (SB) peptide: GGEEDQDFDL were purchased and used in concentration ranges of 50nM to 50 μ M (ThermoFisher Scientific, Inc., Waltham, MA). The same decapeptides were also obtained labelled respectively with Alexa Fluor 488 and Alexa Fluor 594 at their NH₂-terminal ends and used in the same concentration ranges. Both the labelled and unlabelled decapeptides were studied *in vitro* for their abilities to penetrate the spheroidal aggregates, block E-

cad/NTF1 generation and affect spheroidogenesis. Specifically effects on both spheroid disadherence and budding were observed and recorded. Decapeptides with random amino acid sequences in the same concentration ranges of 50nM to 50μM served as negative controls in both the studies of E-cad/NTF1 generation as well as spheroidogenesis.

Statistical analysis

For Ki-67 index, mitotic count and nuclear grade (size) and measurements of embolic density, shape (elongation ratio) and size, both modes and means $\pm$ SD values were determined. All experiments were performed with a minimum of five replicates and representative results depicted showing either means or means $\pm$ standard deviations. All stated or calculated differences imply differences of statistical significance, assessed by the two tailed students *t* test as well as ANOVA.

Results

Observational studies

In observational human studies comparing IBC with non-IBC, although common tumor parameters such as Ki-67 index, mitotic count and nuclear grade (size) showed a similar overlapping distribution of modes and mean values, there was a dramatic exponential or geometric difference in both mode and mean embolic density and a significant difference in both mode and mean embolic size distribution in IBC *v* non-IBC (Fig. 1). IBC cases compared to non-IBC cases showed a greater Ki-67 mean index ($p = .05$) (Fig. 1A), a higher mean mitotic count ($p = 0.1$) (Fig. 1B) and an increased mean nuclear grade ($p = .05$) (Fig. 1C) but the distribution of these tumor features showed considerable overlap. In contrast, mean embolic density in IBC dwarfed non-IBC by a factor of 100 fold ($p = .0001$) (Fig. 1D). Furthermore although the overall mode and mean shape of the emboli in IBC *v* non-IBC did not differ ($p = .5$) (Fig. 1E), the modal and mean size of the IBC emboli were significantly smaller ($p = .01$) (Fig. 1F).

Experimental studies

Initial Xenograft Studies

Mary-X grew equally well in both athymic (nude) mice as well as Scids as a transplantable xenograft (Fig. 2; Fig. 2A). Mary-X exhibited a stable phenotype for over 25 years. Mary-X grew in a nodular fashion with islands of tumor cells embedded within a murine fibrovascular matrix (Fig. 2B). The nodules of Mary-X express strong membrane E-cadherin immunoreactivity (Fig. 2D). Mary-X gives rise to large numbers of CTC's (Fig. 2F: inset) which measured out at 10–15 cells / 100 μl blood as well as distal metastases including pulmonary metastases (Fig. 2F). These metastases also strongly express E-cadherin.

Karen-X, in contrast, grew only in Scids as a transplantable xenograft (Fig. 2). Karen-X also exhibited a stable phenotype for over 25 years. Karen-X exhibited a significantly longer latency than Mary-X but once a tumor emerged exhibited a similar rate of growth (Fig. 2A). Karen-X was similar to Mary-X histologically

(Fig. 2C). Karen-X also expressed E-cadherin membrane immunoreactivity which overall was less intense and less circumferential (Fig. 2E) than that expressed in Mary-X. In contrast to Mary-X, Karen-X did not generate CTC's (Fig. 2G: inset) or metastases (Fig. 2G).

In Vitro Studies

Mary-X and Karen-X exhibited markedly different *in vitro* properties when grown in suspension culture (Fig. 3). When their respective xenografts were extirpated and minced in culture, both initially gave rise to loose tumor aggregates. After 24–48 hr, the Mary-X aggregates tightened to form high density spheroids (Fig. 3A) whereas the Karen-X aggregates remained loosely associated (100%) and did not tighten (Fig. 3B). Over the next 24 hr – 48 hr, the Mary-X spheroids exhibited both partial budding (12–15%) (Fig. 3A,3C) as well as complete budding (1–3%) (Fig. 4A, 4B). When observed with time-lapsed photography, this budding was rapid and quite dynamic (Supplement 1). In this time-lapsed photography, one parent spheroid begeted another which then begeted two more. The strong E-cadherin immunoreactivity was expressed equally in the budding spheroidogenesis (Fig. 4B). In contrast, Karen-X did not bud over time in culture (Fig. 3D) but remained as loose aggregates (Supplement 2). Neither Mary-X nor Karen-X ever attached and grew as monolayers even when grown on fibronectin or vitronectin-coated dishes (data not shown).

Additional xenograft studies

Double label immunofluorescence studies of the extirpated Mary-X xenograft showed florid tumor embolic lymphovascular budding (Fig. 4C). In contrast the Karen-X xenograft showed only rare lymphovascular invasion but no budding (data not shown). The parental tumor embolic budding of Mary-X gave rise to a dramatic geometric increase in the number of tumor emboli within lymphovascular channels (Fig. 4D). Both parental and daughter tumor emboli retained strong membrane E-cadherin immunofluorescence (Fig. 4C, 4D). Non-embolic tumoral areas of Mary-X showed no evidence of budding.

Studies of Mary-X and Karen-X E-cadherin Proteolysis.

Our previous Mary-X studies had demonstrated the transcriptome equivalence of xenograft-generated spheroids with the lymphovascular emboli in mice with both structures also demonstrating E-cadherin overexpression and specific proteolytic processing producing a number of specific E-cadherin fragments^{21,30} Only E-cad/NFT1 was present in the well-formed spheroids at the conclusion of initial spheroidogenesis in the present study as well (Fig. 5). Studies by others in other disease processes³¹ had also indicated the proteolytic processing of E-cadherin by a number of different intracellular proteases and mapped their site of binding and cleavage on E-cadherin (Fig. 6A). The well-formed spheroids of Mary-X also expressed high levels of calpains, especially calpain 2 and the use of a calpain inhibitor, calpeptin, blocked the genesis of E-cad/NFT1.^{21,30} Western blots with H108 in the present study confirmed the high levels of both E-cad/FL as well as E-cad/NTF1 in the Mary-X spheroids but decreased E-cad/FL and absent E-cad-NTF1 in the Karen-X aggregates (Fig. 5A). Laser capture microdissected

lymphovascular tumor emboli from patients with IBC (Fig. 5B) showed, on Western blot with 24E10 similarly high levels of both E-cad/FL as well as E-cad/NTF1 (Fig. 5C).

In previous studies we blocked the action of calpain by the use of its inhibitor, calpstatin.^{21,30} This maneuver blocked the generation of E-cad/NTF1 and caused many of the spheroids to disadhere into loose aggregates. Because calpains, as intracellular proteases, have many different actions on many different targets, blocking calpains with calpstatin did not solely block only the cleavage of E-cadherin and the generation of its E-cad/NTF1 fragment. Therefore, it could not be concluded in these previous studies that the effect of calpain inhibition on the disadherence of the spheroids was necessarily mediated by the decrease or absence of E-cad/NTF1. For this reason in the present study, we used specific oligopeptides (decapeptides) that contained respectfully either calpain's site of cleavage (SC) or site of binding (SB) on the full length E-cadherin molecule (Fig. 5D). Both decapeptides by Western blot using H108 and 24E10 respectively, significantly inhibited E-cad/NTF1 (Fig. 5E, 5F).

Using the same SC and SB decapeptides as before but now labelled respectively with Alexa Fluor 488 and Alexa Fluor 594, the decapeptides were administered to the Mary-X spheroids (Fig. 5G, 5H, 5I). Each peptide singly (Fig. 5G, 5H) and in combination (Fig. 5I) penetrated the spheroids. The effects on the Mary-X spheroids were significant in terms of both increased disadherence ($p < .05$) and decreased partial budding ($p < .05$) (Fig. 5J). The effects of the SB peptide were greater than the effects of the SC peptide on disadherence ($p < .05$) but both peptides decreased partial budding equally. The effects of the two peptides in combination were synergistic on increased disadherence and decreased partial budding ($p < .05$). Because the level of complete budding was small in untreated Mary-X spheroids, the effects of these peptides singly or in combination on complete budding could not be assessed. There were no measurable effects of these decapeptides on the loose aggregates of Karen-X (Fig. 5J). Decapeptides with random amino acid sequences which served as negative controls in both the E-cad/NTF1 inhibition experiments as well as in the studies of spheroidogenesis exerted no effects.

Discussion

Inflammatory breast cancer (IBC) is the deadliest type of human breast cancer that presents with florid tumor emboli especially involving overlying dermal lymphatics.¹⁻⁵ These lymphovascular tumor emboli also reoccur locally, making skin-sparing and breast-conserving surgery not an option.³¹⁻³⁵ This property of IBC is, in fact, the disease's distinct diagnostic and biological signature yet the explanation for this intriguing phenotype remains unknown and little studied.

Our human observational studies indicate that whereas there is considerable overlap in the common tumor parameters of Ki-67 index, mitotic count and nuclear grade between IBC v non-IBC, the difference between emboli density is striking and geometric and exponential in nature. Furthermore although the emboli do not differ in shape, the IBC emboli differ in size being significantly smaller. These observations raise the hypothesis that the generation of tumor emboli in IBC involves a different pathway of pathogenesis which is independent of primary tumor parameters such as proliferation and nuclear grade

and possibly even initial lymphovascular invasion. One attractive hypothesis to explain both the very high density of tumor emboli as well as their smaller size in IBC is geometric tumor embolic budding. Because our observational studies in humans only raise but not prove this hypothesis, we were fortunate to be able to conduct experimental studies with our two IBC xenografts.

The phenotype exhibited by Mary-X both *in vitro* and *in vivo* shows tumor embolic budding which give rise to numerous daughter spheroids or emboli emboli. These emboli are the source of numerous CTC's and metastases. The phenotype of Karen-X is devoid of all of these features. The budding of Mary-X *in vitro* is dynamic, dramatic and fairly rapid (occurring in minutes). The budding both *in vitro* and *in vivo* gives rise to daughter emboli significantly smaller in size than their parental embolic source, an experimental finding in agreement with our observational studies that emboli in IBC v non-IBC differ in size, being significantly smaller.

In our study we are distinguishing geometric budding of emboli from the phenomenon that triggers initial lymphovascular invasion in the first place, which, in itself remains unexplained. The recognition of the lymphovascular embolus as an entity unto itself has catalyzed larger observations that many cancers invade and metastasize as clumps or clusters.³⁶⁻⁴³ Up until relatively recently, most scientists considered cancers to metastasize as single cells and their increased locomotion and degradation of the extracellular matrix with subsequent lymphovascular invasion as a manifestation of so called epithelial-mesenchymal transition (EMT).¹³⁻¹⁵ Models epitomizing this phenomenon in the breast included infiltrating lobular cancers where cells lose E-cadherin expression and infiltrate as single cells.^{44,45}

The study of tumor cell clumps or clusters became important not only *in vivo* but also *in vitro* because tumor clumps in suspension culture provided 3D models which were uniformly superior to 2D monolayer cultures of cancer cell lines. *In vitro* 3D models were thought to better recapitulate the 3D situation of *in vivo* cancer. *In vitro* 3D models were thought to more faithfully recapitulate *in vivo* gene expression, provide better evaluation of physiological factors such as hypoxia and improved testing of anticancer pharmacological strategies.⁴⁶⁻⁴⁸

Most 3D models take the form of spheroids in suspension culture. The majority of these spheroids or multicellular aggregates (MTCs) need to be induced to grow as spheroids by growing them on agar or other repellent surface that interferes with the cancer cells natural proclivity to grow as monolayers.⁴⁹⁻⁵⁷

Some of these spheroids when injected into immunocompromised mice will develop into tumors but these tumors do not exhibit obvious lymphovascular invasion or tumor emboli formation. Furthermore although one model has established a tumor embolus *in vitro*,⁵⁸ no previous models have demonstrated the equivalence of *in vitro* spheroids with *in vivo* tumor emboli. In contrast, the *in vitro* spheroids of Mary-X when re-injected into mice develop into tumors showing florid tumor emboli,^{5,6} which by Principal Component Analysis not only express the transcriptome equivalence of Mary-X spheroids but also the equivalence of microdissected tumor emboli from cases of IBC.^{21,30} And the emboli of Mary-X and their *in*

vitro equivalent, ie,, the spheroids are what undergo geometric budding. The non-embolic tumoral areas of Mary-X do not demonstrate budding.

Although budding per se of tumor cells has also been observed and studied *in vivo* in other studies, that budding applies to invasion of the primary cancer and not specific budding of the lymphovascular tumor embolus.^{59–61} Budding per se from the lymphovascular tumor embolus has not previously been observed or studied probably because no models of metastasizing cancer including other models of IBC demonstrate florid lymphovascular tumor emboli from which budding can be observed.

It is interesting that the hypothesis of EMT still dominates the thinking of the metastatic process even when applied to metastasizing clumps and budding where E-cadherin overexpression is observed.^{41–43; 62–64} Despite this observation, the evidence suggests that the mechanism of budding responsible for geometric embolic propagation in our model does not involve either complete or partial EMT. Our time-lapsed photography indicates that complete *in vitro* budding occurs in minutes and that both the bud as well as the parent spheroid or embolus overexpress E-cadherin even at the incident point of budding. It makes more sense that budding results not from EMT but rather from pre-existing molecules like E-cad/NTF1. Certainly budding must involve additional steps some of which that could be energy (ATP) dependent, hypoxia triggered, nutrient-related or density regulated. We plan to use our budding model to delineate these steps in future studies.

Conclusions

Our study therefore is the first study that observes and quantitates actual budding of tumor emboli in an IBC model both *in vivo* and *in vitro*. Our study then offers a novel explanation to explain the plethora of tumor emboli observed in IBC and begins to address the mechanism behind the budding. Blocking the calpain-mediated proteolysis of membrane E-cadherin by SC and SB blocking decapeptides suggests that E-cad/NTF1 participates in both tight spheroidogenesis as well as budding. Reduction in E-cad/NTF1 promotes decreased partial budding. Decreased partial budding is due specifically to the decreased availability of E-cad/NTF1. Given that previous studies⁶⁵ had mapped the different E-cadherin cleavage and binding sites for the different intracellular proteases, especially calpain, mediating the generation of the different E-cadherin fragments (Fig. 6A) and that our present studies have demonstrated that specific decapeptides containing the SC or SB on E-cadherin are able to block E-cadherin cleavage and decrease partial budding, the explanation for all this is that because E-cad/NTF1 retains the p120ctn binding site but loses both the β -catenin and α -binding sites, facilitating its disassembly from traditional CAJs, it can exhibit 360° mobility around the membrane which would promote budding (Fig. 6B). This insight could lead to therapies at inhibiting budding and embolic propagation, thereby decreasing skin recurrences in IBC.⁶⁶

Abbreviations

IBC, inflammatory breast cancer; EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition; ERAs, epithelial recognition algorithms; SRAs, specific recognition algorithms; TMA, tissue microarray; DAPI, 4', 6 – diamidino-2-phenylindole; CTCs, circulating tumor cells; MTCs, multicellular aggregates; SC, site of cleavage; SB, site of binding; ctn, catenin; CAJs, cadherin-based adherens junctions; NTF, N-terminal fragment; CTF, C-terminal fragment; ER, estrogen receptor; PR, progesterone receptor; ANOVA, analysis of variance.

Declarations

Ethics Approval and Consent to Participate

Initial xenograft studies were conducted under the UCLA's Human Subject Protection Committee and the Chancellor's Animal Research Committee (Certification 95-127-11). Continuing animal studies were approved by The Ohio State University's Animal Care and Use Committee (IACUC), protocol 2007A0218 and by The Ohio State University's Institutional Biosafety Committee, protocol 2007R0057. Additional animal studies were approved by the University of Nevada's School of Medicine and the Nevada Cancer Institute's IACUC, protocols 00439 and 00440 when the corresponding author of this study was affiliated with these previous institutions. Final animal studies were conducted under an Interinstitutional Agreement between the California University of Science and Medicine and Anticancer, Inc. using the latter's IACUC protocol D16-00503 and OLAWA3873-01. Collection and use of human tissues from patients with breast cancer including non-IBC as well as IBC cases, completely anonymized, was approved by The Ohio State University Cancer Institutional Review Board (IRB) under protocol 2006C0042.

Consent for Publication

Not applicable.

Availability of Data and Materials

Both Mary-X and Karen-X are available to any investigator upon request. Imaging algorithms used in the study are also available. All data sets generated and used in the study are available upon request.

Competing Interests

The authors declare that they, at the present time, have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. None of the sources of support listed influenced the collection, analysis and interpretation of data, the generation of the hypothesis, the writing of the manuscript or the decision to submit the manuscript for publication.

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Author Contributions

All authors made an intellectual contribution to the work. Arnav P. Modi, carried out the vast majority of the *in vitro* spheroidogenesis and budding experiments. Julie P.T. Nguyen conducted a critical interpretation of the data and further characterized the nature of the budding. Justin Wang supervised the xenograft experiments. Jonathan S. Ahn analyzed the immunofluorescence and immunocytochemical experiments and conducted additional *in vitro* budding experiments. William A. Libling troubleshooted additional experiments designed to test the significance of the hypothesis. Jacob M. Klein and Preeanka Mazumder conducted a review of the relevant literature and suggested additional experiments. Sanford H. Barsky supervised all of the experiments and wrote the manuscript which was reviewed by all the authors.

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References

1. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA: Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin Cancer Res* 2007, 13:4429–4434.
2. Brenton JD, Carey LA, Ahmed A, Caldas C: Molecular classification and molecular forecasting of breast cancer: Ready for clinical application? *J Clin Oncol* 2005, 23:7350–7360.
3. Cristofanilli M, Buzdar AU, Hortobágyi GN: Update on the Management of Inflammatory Breast Cancer; Update on the Management of Inflammatory Breast Cancer. *Oncologist* [Internet] 2003 [cited 2021 Jul 19], 8:141–148. Available from: www.TheOncologist.com
4. Kertmen N, Babacan T, Keskin O, Solak M, Sarici F, Akin S, Arik Z, Asian A, Ates O, Aksoy S, Ozisik Y, Altundag K: Molecular subtypes in patients with inflammatory breast cancer; A single center experience. *J BUON* 2015, 20:35–39.
5. Alpaugh ML, Tomlinson JS, Shao ZM, Barsky SH: A novel human xenograft model of inflammatory breast cancer. *Cancer Res* 1999, 59:5079–5084.
6. Tomlinson JS, Alpaugh ML, Barsky SH: An intact overexpressed E-cadherin/ α , β -catenin axis characterizes the lymphovascular emboli of inflammatory breast carcinoma. *Cancer Res* 2001, 61:5231–5241.

7. Alpaugh ML, Tomlinson JS, Ye Y, Barsky SH: Relationship of sialyl-Lewisx/a underexpression and E-cadherin overexpression in the lymphovascular embolus of inflammatory breast carcinoma. *Am J Pathol* [Internet], American Society for Investigative Pathology, 2002, 161:619–628. Available from: [http://dx.doi.org/10.1016/S0002-9440\(10\)64217-4](http://dx.doi.org/10.1016/S0002-9440(10)64217-4)
8. Alpaugh ML, Tomlinson JS, Kasraeian S, Barsky SH: Cooperative role of E-cadherin and sialyl-Lewis X/A-deficient MUC1 in the passive dissemination of tumor emboli in inflammatory breast carcinoma. *Oncogene* 2002, 21:3631–3643.
9. Eckhardt BL, Gagliardi M, Iles LK, Evans K, Ivan C, Liu X, Liu CG, Souza G, Rao A, Meric-Bernstam F, Ueno NT, Bartholomeusz GA: Clinically relevant inflammatory breast cancer patient-derived xenograft–derived ex vivo model for evaluation of tumor-specific therapies. *PLoS One* 2018, 13:1–23.
10. Colpaert CG, Vermeulen PB, Benoy I, Soubry A, Van Roy F, Van Beest P, Goovaerts G, Dirix LY, Van Dam P, Fox SB, Harris AL, Van Marck EA: Inflammatory breast cancer shows angiogenesis with high endothelial proliferation rate and strong E-cadherin expression. *Br J Cancer* 2003, 88:718–725.
11. Ye Y, Tellez JD, Durazo M, Belcher M, Yearsley K, Barsky SH: E-cadherin accumulation within the lymphovascular embolus of inflammatory breast cancer is due to altered trafficking. *Anticancer Res* 2010, 30:3903–3910.
12. Alpaugh ML, Barsky SH: Reversible model of spheroid formation allows for high efficiency of gene delivery Ex Vivo and accurate gene assessment In Vivo. *Hum Gene Ther* 2002, 13:1245–1258.
13. Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. *J Clin Invest* [Internet] 2009, 119:1420–1428. Available from: <http://www.jci.org/articles/view/39104>
14. Thiery JP, Acloque H, Huang RYJ, Nieto MA: Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* 2009, 139:871–890.
15. Zheng X, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, Wu C, Lebleu VS, Kalluri R, Biology C: EMT Program is Dispensable for Metastasis but Induces Chemoresistance in Pancreatic Cancer. *Nature* 2015, 527:525–530.
16. Fernandez S V., Robertson FM, Pei J, Aburto-Chumpitaz L, Mu Z, Chu K, Alpaugh RK, Huang Y, Cao Y, Ye Z, Cai KQ, Boley KM, Klein-Szanto AJ, Devarajan K, Addya S, Cristofanilli M: Inflammatory breast cancer (IBC): Clues for targeted therapies. *Breast Cancer Res Treat* 2013, 140:23–33.
17. Robertson FM, Bondy M, Yang W, Yamauchi H, Wiggins S, Kamrudin S, Krishnamurthy S, Le-Petross H, Bidaut L, Player AN, Barsky SH, Woodward WA, Buchholz T, Lucci A, Ueno N, Cristofanilli M: Inflammatory Breast Cancer: The Disease, the Biology, the Treatment. *CA Cancer J Clin* [Internet] 2010, 60:351–375. Available from: <http://doi.wiley.com/10.3322/caac.20082>
18. Cristofanilli M, Valero V, Buzdar AU, Kau SW, Broglio KR, Gonzalez-Angulo AM, Sneige N, Islam R, Ueno NT, Buchholz TA, Singletary SE, Hortobagyi GN: Inflammatory breast cancer (IBC) and patterns of recurrence: Understanding the biology of a unique disease. *Cancer* 2007, 110:1436–1444.
19. Xiao Y, Ye Y, Yearsley K, Jones S, Barsky SH: The lymphovascular embolus of inflammatory breast cancer expresses a stem cell-like phenotype. *Am J Pathol* 2008, 173:561–574.

20. Xiao Y, Ye Y, Zou X, Jones S, Yearsley K, Shetuni B, Tellez J, Barsky SH: The lymphovascular embolus of inflammatory breast cancer exhibits a Notch 3 addiction. *Oncogene* 2011, 30:287–300.
21. Ye Y, Tian H, Lange AR, Yearsley K, Robertson FM, Barsky SH: The genesis and unique properties of the lymphovascular tumor embolus are because of calpain-regulated proteolysis of E-cadherin. *Oncogene* 2013, 32:1702–1713.
22. Sharangpani GM, Joshi AS, Porter K, Deshpande AS, Keyhani S, Naik GA, Gholap AS, Barsky SH: Semi-automated imaging system to quantitate estrogen and progesterone receptor immunoreactivity in human breast cancer. *J Microsc* 2007, 226:244–255.
23. Joshi AS, Sharangpani GM, Porter K, Keyhani S, Morrison C, Basu AS, Gholap GA, Gholap AS, Barsky SH: Semi-automated imaging system to quantitate Her-2/neu membrane receptor immunoreactivity in human breast cancer. *Cytom Part A [Internet]* 2007, 71A:273–285. Available from: <https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.20374>
24. Barsky SH, Gentchev L, Basu AS, Jimenez RE, Boussaid K, Gholap AS: Use and validation of epithelial recognition and fields of view algorithms on virtual slides to guide TMA construction. *Biotechniques* 2009, 47:927–938.
25. Mahooti S, Porter K, Alpaugh ML, Ye Y, Xiao Y, Jones S, Tellez JD, Barsky SH: Breast carcinomatous tumoral emboli can result from encircling lymphovasculogenesis rather than lymphovascular invasion. *Oncotarget* 2010, 1:131–147.
26. Gholap AS, Gholap GA, Rao CVK, Barsky SH, Vipra M, Patil SM, Jadhav P, Abhyankar J. Method and system for morphology based mitoses identification and classification of digital images. U.S. Patent 7,979,212; July 12, 2011.
27. Petrova SC, Ahmad I, Nguyen C, Ferrell SD Jr, Wilhelm SR, Ye Y, Barsky SH. Regulation of breast cancer oncogenesis by the cell of origin's differentiation state. *Oncotarget*. 2020 Oct 27;11(43):3832–3848. doi: 10.18632/oncotarget.27783. PMID: 33196707; PMCID: PMC7597414.
28. <https://ij.imjoy.io/>
29. Grover PK, Cummins AG, Price TJ, Roberts-Thomson IC, Hardingham JE: Circulating tumour cells: The evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research. *Ann Oncol* 2014, 25:1506–1516.
30. Ye Y, Gao JX, Tian H, Yearsley K, Lange AR, Robertson FM, Barsky SH: Early to intermediate steps of tumor embolic formation involve specific proteolytic processing of E-cadherin regulated by Rab7. *Mol Cancer Res* 2012, 10:713–726.
31. Yamauchi H, Woodward WA, Valero V, Alvarez RH, Lucci A, Buchholz TA, Iwamoto T, Krishnamurthy S, Yang W, Reuben JM, Hortobágyi GN, Ueno NT: Inflammatory Breast Cancer: What We Know and What We Need to Learn. *Oncologist* 2012, 17:891–899.
32. Patani N, Mokbel K: Oncological and aesthetic considerations of skin-sparing mastectomy. *Breast Cancer Res Treat* 2008, 111:391–403.
33. Hoffman DI, Santos PMG, Goldbach M, Keele LJ, Taunk NK, Bogen HS, Burkbauer L, Jankowitz RC, Fosnot J, Wu LC, Freedman GM, Tchou JC: Immediate Breast Reconstruction for Inflammatory Breast

- Cancer: Trends in Use and Clinical Outcomes 2004–2016. *Ann Surg Oncol* [Internet] 2021, . Available from: <https://link.springer.com/10.1245/s10434-021-10404-4>
34. Norhisham NF, Chong CY, Safuan S: Peritumoral lymphatic vessel density and invasion detected with immunohistochemical marker D240 is strongly associated with distant metastasis in breast carcinoma. *BMC Clin Pathol, BMC Clinical Pathology*, 2017, 17:1–7.
 35. Kong L-L, Yang N-Z, Shi L-H, Zhao G-H, Zhou W, Ding Q, Wang M-H, Zhang Y-S: The optimum marker for the detection of lymphatic vessels. *Mol Clin Oncol* 2017, 7:515–520.
 36. Chernofsky MR, Felix JC, Muderspach LI, Morrow CP, Ye W, Groshen SG, Roman LD: Influence of quantity of lymph vascular space invasion on time to recurrence in women with early-stage squamous cancer of the cervix. *Gynecol Oncol* 2006, 100:288–293.
 37. Sykes P, Allen D, Cohen C, Scurry J, Yeo D: Does the density of lymphatic vascular space invasion affect the prognosis of stage Ib and IIA node negative carcinoma of the cervix? *Int J Gynecol Cancer* 2003, 13:313–316.
 38. Roman LD, Felix JC, Muderspach LI, Varkey T, Burnett AF, Qian D, Morrow CP: Influence of quantity of lymph-vascular space invasion on the risk of nodal metastases in women with early-stage squamous cancer of the cervix. *Gynecol Oncol* 1998, 68:220–225.
 39. Scurry J, Hacker NF, Barlow E, Friedlander M, Jackson M: Is quantification of lymphovascular space invasion useful in stage 1B2 cervical carcinomas? *J Obstet Gynaecol (Lahore)* 2015, 35:377–381.
 40. Jolly MK, Boareto M, Debeb BG, Aceto N, Farach-Carson MC, Woodward WA, Levine H: Inflammatory breast cancer: A model for investigating cluster-based dissemination. *npj Breast Cancer* [Internet], Springer US, 2017, 3:1–7. Available from: <http://dx.doi.org/10.1038/s41523-017-0023-9>
 41. Somarelli JA, Schaeffer D, Marengo MS, Bepler T, Rouse D, Ware KE, Hish AJ, Zhao Y, Buckley AF, Epstein JI, Armstrong AJ, Virshup DM, Garcia-Blanco MA: Distinct routes to metastasis: Plasticity-dependent and plasticity-independent pathways. *Oncogene* [Internet], Nature Publishing Group, 2016, 35:4302–4311. Available from: <http://dx.doi.org/10.1038/onc.2015.497>
 42. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, Yu M, Pely A, Engstrom A, Zhu H, Brannigan BW, Kapur R, Stott SL, Shioda T, Ramaswamy S, Ting DT, Lin CP, Toner M, Haber DA, Maheswaran S: Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell* [Internet], Elsevier Inc., 2014, 158:1110–1122. Available from: <http://dx.doi.org/10.1016/j.cell.2014.07.013>
 43. Cheung KJ, Padmanaban V, Silvestri V, Schipper K, Cohen JD, Fairchild AN, Gorin MA, Verdone JE, Pienta KJ, Bader JS, Ewald AJ: Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. *Proc Natl Acad Sci U S A* 2016, 113:E854–E863.
 44. Choi YJ, Pinto MM, Hao L, Riba AK: Interobserver variability and aberrant E-cadherin immunostaining of lobular neoplasia and infiltrating lobular carcinoma. *Mod Pathol* 2008, 21:1224–1237.
 45. Palacios J, Sarrió D, García-Macias MC, Bryant B, Sobel ME, Merino MJ: Frequent E-cadherin gene inactivation by loss of heterozygosity in pleomorphic lobular carcinoma of the breast. *Mod Pathol*

2003, 16:674–678.

46. Weiswald LB, Bellet D, Dangles-Marie V: Spherical Cancer Models in Tumor Biology. Neoplasia (United States) [Internet], Neoplasia Press, Inc., 2015, 17:1–15. Available from: <http://dx.doi.org/10.1016/j.neo.2014.12.004>
47. Zanoni M, Piccinini F, Arienti C, Zamagni A, Santi S, Polico R, Bevilacqua A, Tesei A: 3D tumor spheroid models for in vitro therapeutic screening: A systematic approach to enhance the biological relevance of data obtained. Sci Rep [Internet], Nature Publishing Group, 2016, 6:1–11. Available from: <http://dx.doi.org/10.1038/srep19103>
48. Nath S, Devi GR: Three-dimensional culture systems in cancer research: Focus on tumor spheroid model. Pharmacol Ther [Internet], Elsevier B.V., 2016, 163:94–108. Available from: <http://dx.doi.org/10.1016/j.pharmthera.2016.03.013>
49. Young SR, Saar M, Santos J, Nguyen HM, Vessella RL, Peehl DM: Establishment and serial passage of cell cultures derived from LuCaP xenografts. Prostate 2013, 73:1251–1262.
50. Oktem G, Sercan O, Guven U, Uslu R, Uysal A, Goksel G, Ayla S, Bilir A: Cancer stem cell differentiation: TGF β 1 and versican may trigger molecules for the organization of tumor spheroids. Oncol Rep 2014, 32:641–649.
51. Froehlich K, Haeger JD, Heger J, Pastuschek J, Photini SM, Yan Y, Lupp A, Pfarrer C, Mrowka R, Schleußner E, Markert UR, Schmidt A: Generation of Multicellular Breast Cancer Tumor Spheroids: Comparison of Different Protocols. J Mammary Gland Biol Neoplasia [Internet], Journal of Mammary Gland Biology and Neoplasia, 2016, 21:89–98. Available from: <http://dx.doi.org/10.1007/s10911-016-9359-2>
52. Arora J, Sauer SJ, Tarpley M, Vermeulen P, Rypens C, Laere S Van, Williams KP, Devi GR, Dewhirst MW: Inflammatory breast cancer tumor emboli express high levels of anti-apoptotic proteins: Use of a quantitative high content and high-throughput 3D IBC spheroid assay to identify targeting strategies. Oncotarget 2017, 8:25226–25241.
53. Kang HG, Jenabi JM, Zhang J, Keshelava N, Shimada H, May WA, Ng T, Reynolds CP, Triche TJ, Sorensen PHB: E-cadherin cell-cell adhesion in Ewing tumor cells mediates suppression of anoikis through activation of the ErbB4 tyrosine kinase. Cancer Res 2007, 67:3094–3105.
54. Van Golen KL, Wu ZF, Xiao Tan Qiao, Li Wei Bao, Merajver SD: RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Res 2000, 60:5832–5838.
55. Weiswald LB, Richon S, Massonnet G, Guinebretière JM, Vacher S, Laurendeau I, Cottu P, Marangoni E, Nemati F, Validire P, Bellet D, Bièche I, Dangles-Marie V: A short-term colorectal cancer sphere culture as a relevant tool for human cancer biology investigation. Br J Cancer 2013, 108:1720–1731.
56. Weiswald LB, Richon S, Validire P, Briffod M, Lai-Kuen R, Cordelières FP, Bertrand F, Dargere D, Massonnet G, Marangoni E, Gayet B, Pocard M, Bieche I, Poupon MF, Bellet D, Dangles-Marie V: Newly characterised ex vivo colospheres as a three-dimensional colon cancer cell model of tumour aggressiveness. Br J Cancer 2009, 101:473–482.

57. Kondo J, Endo H, Okuyama H, Ishikawa O, Iishi H, Tsujii M, Ohue M, Inoue M: Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer. *Proc Natl Acad Sci U S A* 2011, 108:6235–6240.
58. Lehman HL, Dashner EJ, Lucey M, Vermeulen P, Dirix L, Laere S Van, Van Golen KL: Modeling and characterization of inflammatory breast cancer emboli grown in vitro. *Int J Cancer* 2013, 132:2283–2294.
59. Dawson H, Lugli A: Molecular and pathogenetic aspects of tumor budding in colorectal cancer. *Front Med* 2015, 2:11.
60. Man Y gao: Tumor cell budding from focally disrupted tumor capsules: A common pathway for all breast cancer subtype derived invasion. *J Cancer* 2010, 1:32–37.
61. Pease JC, Brewer M, Tirnauer JS: Spontaneous spheroid budding from monolayers: A potential contribution to ovarian cancer dissemination. *Biol Open* 2012, 1:622–628.
62. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA: Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin Cancer Res* 2007, 13:4429–4434.
63. Brenton JD, Carey LA, Ahmed A, Caldas C: Molecular classification and molecular forecasting of breast cancer: Ready for clinical application? *J Clin Oncol* 2005, 23:7350–7360.
64. Cristofanilli M, Buzdar AU, Hortobágyi GN: Update on the Management of Inflammatory Breast Cancer; Update on the Management of Inflammatory Breast Cancer. *Oncologist* [Internet] 2003 [cited 2021 Jul 19], 8:141–148. Available from: www.TheOncologist.com
65. Kertmen N, Babacan T, Keskin O, Solak M, Sarici F, Akin S, Arik Z, Asian A, Ates O, Aksoy S, Ozisik Y, Altundag K: Molecular subtypes in patients with inflammatory breast cancer; A single center experience. *J BUON* 2015, 20:35–39.
66. Alpaugh ML, Tomlinson JS, Shao ZM, Barsky SH: A novel human xenograft model of inflammatory breast cancer. *Cancer Res* 1999, 59:5079–5084.
67. Tomlinson JS, Alpaugh ML, Barsky SH: An intact overexpressed E-cadherin/ α , β -catenin axis characterizes the lymphovascular emboli of inflammatory breast carcinoma. *Cancer Res* 2001, 61:5231–5241.
68. Alpaugh ML, Tomlinson JS, Ye Y, Barsky SH: Relationship of sialyl-Lewisx/a underexpression and E-cadherin overexpression in the lymphovascular embolus of inflammatory breast carcinoma. *Am J Pathol* [Internet], American Society for Investigative Pathology, 2002, 161:619–628. Available from: [http://dx.doi.org/10.1016/S0002-9440\(10\)64217-4](http://dx.doi.org/10.1016/S0002-9440(10)64217-4)
69. Alpaugh ML, Tomlinson JS, Kasraeian S, Barsky SH: Cooperative role of E-cadherin and sialyl-Lewis X/A-deficient MUC1 in the passive dissemination of tumor emboli in inflammatory breast carcinoma. *Oncogene* 2002, 21:3631–3643.
70. Eckhardt BL, Gagliardi M, Iles LK, Evans K, Ivan C, Liu X, Liu CG, Souza G, Rao A, Meric-Bernstam F, Ueno NT, Bartholomeusz GA: Clinically relevant inflammatory breast cancer patient-derived

- xenograft–derived ex vivo model for evaluation of tumor-specific therapies. PLoS One 2018, 13:1–23.
71. Colpaert CG, Vermeulen PB, Benoy I, Soubry A, Van Roy F, Van Beest P, Goovaerts G, Dirix LY, Van Dam P, Fox SB, Harris AL, Van Marck EA: Inflammatory breast cancer shows angiogenesis with high endothelial proliferation rate and strong E-cadherin expression. Br J Cancer 2003, 88:718–725.
 72. Ye Y, Tellez JD, Durazo M, Belcher M, Yearsley K, Barsky SH: E-cadherin accumulation within the lymphovascular embolus of inflammatory breast cancer is due to altered trafficking. Anticancer Res 2010, 30:3903–3910.
 73. Alpaugh ML, Barsky SH: Reversible model of spheroid formation allows for high efficiency of gene delivery Ex Vivo and accurate gene assessment In Vivo. Hum Gene Ther 2002, 13:1245–1258.
 74. Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest [Internet] 2009, 119:1420–1428. Available from: <http://www.jci.org/articles/view/39104>
 75. Thiery JP, Acloque H, Huang RYJ, Nieto MA: Epithelial-Mesenchymal Transitions in Development and Disease. Cell 2009, 139:871–890.
 76. Zheng X, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, Wu C, Lebleu VS, Kalluri R, Biology C: EMT Program is Dispensable for Metastasis but Induces Chemoresistance in Pancreatic Cancer. Nature 2015, 527:525–530.
 77. Fernandez S V., Robertson FM, Pei J, Aburto-Chumpitaz L, Mu Z, Chu K, Alpaugh RK, Huang Y, Cao Y, Ye Z, Cai KQ, Boley KM, Klein-Szanto AJ, Devarajan K, Addya S, Cristofanilli M: Inflammatory breast cancer (IBC): Clues for targeted therapies. Breast Cancer Res Treat 2013, 140:23–33.
 78. Robertson FM, Bondy M, Yang W, Yamauchi H, Wiggins S, Kamrudin S, Krishnamurthy S, Le-Petross H, Bidaut L, Player AN, Barsky SH, Woodward WA, Buchholz T, Lucci A, Ueno N, Cristofanilli M: Inflammatory Breast Cancer: The Disease, the Biology, the Treatment. CA Cancer J Clin [Internet] 2010, 60:351–375. Available from: <http://doi.wiley.com/10.3322/caac.20082>
 79. Cristofanilli M, Valero V, Buzdar AU, Kau SW, Broglio KR, Gonzalez-Angulo AM, Sneige N, Islam R, Ueno NT, Buchholz TA, Singletary SE, Hortobagyi GN: Inflammatory breast cancer (IBC) and patterns of recurrence: Understanding the biology of a unique disease. Cancer 2007, 110:1436–1444.
 80. Xiao Y, Ye Y, Yearsley K, Jones S, Barsky SH: The lymphovascular embolus of inflammatory breast cancer expresses a stem cell-like phenotype. Am J Pathol 2008, 173:561–574.
 81. Xiao Y, Ye Y, Zou X, Jones S, Yearsley K, Shetuni B, Tellez J, Barsky SH: The lymphovascular embolus of inflammatory breast cancer exhibits a Notch 3 addiction. Oncogene 2011, 30:287–300.
 82. Ye Y, Tian H, Lange AR, Yearsley K, Robertson FM, Barsky SH: The genesis and unique properties of the lymphovascular tumor embolus are because of calpain-regulated proteolysis of E-cadherin. Oncogene 2013, 32:1702–1713.
 83. Sharangpani GM, Joshi AS, Porter K, Deshpande AS, Keyhani S, Naik GA, Gholap AS, Barsky SH: Semi-automated imaging system to quantitate estrogen and progesterone receptor immunoreactivity in human breast cancer. J Microsc 2007, 226:244–255.

84. Joshi AS, Sharangpani GM, Porter K, Keyhani S, Morrison C, Basu AS, Gholap GA, Gholap AS, Barsky SH: Semi-automated imaging system to quantitate Her-2/neu membrane receptor immunoreactivity in human breast cancer. *Cytom Part A* [Internet] 2007, 71A:273–285. Available from: <https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.20374>
85. Barsky SH, Gentchev L, Basu AS, Jimenez RE, Boussaid K, Gholap AS: Use and validation of epithelial recognition and fields of view algorithms on virtual slides to guide TMA construction. *Biotechniques* 2009, 47:927–938.
86. Mahooti S, Porter K, Alpaugh ML, Ye Y, Xiao Y, Jones S, Tellez JD, Barsky SH: Breast carcinomatous tumoral emboli can result from encircling lymphovasculogenesis rather than lymphovascular invasion. *Oncotarget* 2010, 1:131–147.
87. Gholap AS, Gholap GA, Rao CVK, Barsky SH, Vipra M, Patil SM, Jadhav P, Abhyankar J. Method and system for morphology based mitoses identification and classification of digital images. U.S. Patent 7,979,212; July 12, 2011.
88. Petrova SC, Ahmad I, Nguyen C, Ferrell SD Jr, Wilhelm SR, Ye Y, Barsky SH. Regulation of breast cancer oncogenesis by the cell of origin's differentiation state. *Oncotarget*. 2020 Oct 27;11(43):3832-3848. doi: 10.18632/oncotarget.27783. PMID: 33196707; PMCID: PMC7597414.
89. <https://ij.imjoy.io/>
90. Grover PK, Cummins AG, Price TJ, Roberts-Thomson IC, Hardingham JE: Circulating tumour cells: The evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research. *Ann Oncol* 2014, 25:1506–1516.
91. Ye Y, Gao JX, Tian H, Yearsley K, Lange AR, Robertson FM, Barsky SH: Early to intermediate steps of tumor embolic formation involve specific proteolytic processing of E-cadherin regulated by Rab7. *Mol Cancer Res* 2012, 10:713–726.
92. Yamauchi H, Woodward WA, Valero V, Alvarez RH, Lucci A, Buchholz TA, Iwamoto T, Krishnamurthy S, Yang W, Reuben JM, Hortobágyi GN, Ueno NT: Inflammatory Breast Cancer: What We Know and What We Need to Learn. *Oncologist* 2012, 17:891–899.
93. Patani N, Mokbel K: Oncological and aesthetic considerations of skin-sparing mastectomy. *Breast Cancer Res Treat* 2008, 111:391–403.
94. Hoffman DI, Santos PMG, Goldbach M, Keele LJ, Taunk NK, Bogen HS, Burkbauer L, Jankowitz RC, Fosnot J, Wu LC, Freedman GM, Tchou JC: Immediate Breast Reconstruction for Inflammatory Breast Cancer: Trends in Use and Clinical Outcomes 2004–2016. *Ann Surg Oncol* [Internet] 2021, . Available from: <https://link.springer.com/10.1245/s10434-021-10404-4>
95. Norhisham NF, Chong CY, Safuan S: Peritumoral lymphatic vessel density and invasion detected with immunohistochemical marker D240 is strongly associated with distant metastasis in breast carcinoma. *BMC Clin Pathol, BMC Clinical Pathology*, 2017, 17:1–7.
96. Kong L-L, Yang N-Z, Shi L-H, Zhao G-H, Zhou W, Ding Q, Wang M-H, Zhang Y-S: The optimum marker for the detection of lymphatic vessels. *Mol Clin Oncol* 2017, 7:515–520.

97. Chernofsky MR, Felix JC, Muderspach LI, Morrow CP, Ye W, Groshen SG, Roman LD: Influence of quantity of lymph vascular space invasion on time to recurrence in women with early-stage squamous cancer of the cervix. *Gynecol Oncol* 2006, 100:288–293.
98. Sykes P, Allen D, Cohen C, Scurry J, Yeo D: Does the density of lymphatic vascular space invasion affect the prognosis of stage Ib and IIA node negative carcinoma of the cervix? *Int J Gynecol Cancer* 2003, 13:313–316.
99. Roman LD, Felix JC, Muderspach LI, Varkey T, Burnett AF, Qian D, Morrow CP: Influence of quantity of lymph-vascular space invasion on the risk of nodal metastases in women with early-stage squamous cancer of the cervix. *Gynecol Oncol* 1998, 68:220–225.
100. Scurry J, Hacker NF, Barlow E, Friedlander M, Jackson M: Is quantification of lymphovascular space invasion useful in stage 1B2 cervical carcinomas? *J ObstetGynaecol (Lahore)* 2015, 35:377–381.
101. Jolly MK, Boareto M, Debeb BG, Aceto N, Farach-Carson MC, Woodward WA, Levine H: Inflammatory breast cancer: A model for investigating cluster-based dissemination. *npj Breast Cancer* [Internet], Springer US, 2017, 3:1–7. Available from: <http://dx.doi.org/10.1038/s41523-017-0023-9>
102. Somarelli JA, Schaeffer D, Marengo MS, Bepler T, Rouse D, Ware KE, Hish AJ, Zhao Y, Buckley AF, Epstein JI, Armstrong AJ, Virshup DM, Garcia-Blanco MA: Distinct routes to metastasis: Plasticity-dependent and plasticity-independent pathways. *Oncogene* [Internet], Nature Publishing Group, 2016, 35:4302–4311. Available from: <http://dx.doi.org/10.1038/onc.2015.497>
103. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, Yu M, Pely A, Engstrom A, Zhu H, Brannigan BW, Kapur R, Stott SL, Shioda T, Ramaswamy S, Ting DT, Lin CP, Toner M, Haber DA, Maheswaran S: Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell* [Internet], Elsevier Inc., 2014, 158:1110–1122. Available from: <http://dx.doi.org/10.1016/j.cell.2014.07.013>
104. Cheung KJ, Padmanaban V, Silvestri V, Schipper K, Cohen JD, Fairchild AN, Gorin MA, Verdone JE, Pienta KJ, Bader JS, Ewald AJ: Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. *Proc Natl Acad Sci U S A* 2016, 113:E854–E863.
105. Choi YJ, Pinto MM, Hao L, Riba AK: Interobserver variability and aberrant E-cadherin immunostaining of lobular neoplasia and infiltrating lobular carcinoma. *Mod Pathol* 2008, 21:1224–1237.
106. Palacios J, Sarrió D, García-Macias MC, Bryant B, Sobel ME, Merino MJ: Frequent E-cadherin gene inactivation by loss of heterozygosity in pleomorphic lobular carcinoma of the breast. *Mod Pathol* 2003, 16:674–678.
107. Weiswald LB, Bellet D, Dangles-Marie V: Spherical Cancer Models in Tumor Biology. *Neoplasia (United States)* [Internet], Neoplasia Press, Inc., 2015, 17:1–15. Available from: <http://dx.doi.org/10.1016/j.neo.2014.12.004>
108. Zandoni M, Piccinini F, Arienti C, Zamagni A, Santi S, Polico R, Bevilacqua A, Tesei A: 3D tumor spheroid models for in vitro therapeutic screening: A systematic approach to enhance the biological

- relevance of data obtained. *Sci Rep* [Internet], Nature Publishing Group, 2016, 6:1–11. Available from: <http://dx.doi.org/10.1038/srep19103>
109. Nath S, Devi GR: Three-dimensional culture systems in cancer research: Focus on tumor spheroid model. *PharmacolTher* [Internet], Elsevier B.V., 2016, 163:94–108. Available from: <http://dx.doi.org/10.1016/j.pharmthera.2016.03.013>
 110. Young SR, Saar M, Santos J, Nguyen HM, Vessella RL, Peehl DM: Establishment and serial passage of cell cultures derived from LuCaP xenografts. *Prostate* 2013, 73:1251–1262.
 111. Oktem G, Sercan O, Guven U, Uslu R, Uysal A, Goksel G, Ayla S, Bilir A: Cancer stem cell differentiation: TGF β 1 and versican may trigger molecules for the organization of tumor spheroids. *Oncol Rep* 2014, 32:641–649.
 112. Froehlich K, Haeger JD, Heger J, Pastuschek J, Photini SM, Yan Y, Lupp A, Pfarrer C, Mrowka R, Schleußner E, Markert UR, Schmidt A: Generation of Multicellular Breast Cancer Tumor Spheroids: Comparison of Different Protocols. *J Mammary Gland Biol Neoplasia* [Internet], Journal of Mammary Gland Biology and Neoplasia, 2016, 21:89–98. Available from: <http://dx.doi.org/10.1007/s10911-016-9359-2>
 113. Arora J, Sauer SJ, Tarpley M, Vermeulen P, Rypens C, Laere S Van, Williams KP, Devi GR, Dewhirst MW: Inflammatory breast cancer tumor emboli express high levels of anti-apoptotic proteins: Use of a quantitative high content and high-throughput 3D IBC spheroid assay to identify targeting strategies. *Oncotarget* 2017, 8:25226–25241.
 114. Kang HG, Jenabi JM, Zhang J, Keshelava N, Shimada H, May WA, Ng T, Reynolds CP, Triche TJ, Sorensen PHB: E-cadherin cell-cell adhesion in Ewing tumor cells mediates suppression of anoikis through activation of the ErbB4 tyrosine kinase. *Cancer Res* 2007, 67:3094–3105.
 115. Van Golen KL, Wu ZF, Xiao Tan Qiao, Li Wei Bao, Merajver SD: RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. *Cancer Res* 2000, 60:5832–5838.
 116. Weiswald LB, Richon S, Massonnet G, Guinebretière JM, Vacher S, Laurendeau I, Cottu P, Marangoni E, Nemati F, Validire P, Bellet D, Bièche I, Dangles-Marie V: A short-term colorectal cancer sphere culture as a relevant tool for human cancer biology investigation. *Br J Cancer* 2013, 108:1720–1731.
 117. Weiswald LB, Richon S, Validire P, Briffod M, Lai-Kuen R, Cordelières FP, Bertrand F, Dargere D, Massonnet G, Marangoni E, Gayet B, Pocard M, Bieche I, Poupon MF, Bellet D, Dangles-Marie V: Newly characterised ex vivo colospheres as a three-dimensional colon cancer cell model of tumour aggressiveness. *Br J Cancer* 2009, 101:473–482.
 118. Kondo J, Endo H, Okuyama H, Ishikawa O, Iishi H, Tsujii M, Ohue M, Inoue M: Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer. *Proc Natl Acad Sci U S A* 2011, 108:6235–6240.
 119. Lehman HL, Dashner EJ, Lucey M, Vermeulen P, Dirix L, Laere S Van, Van Golen KL: Modeling and characterization of inflammatory breast cancer emboli grown in vitro. *Int J Cancer* 2013, 132:2283–2294.

120. Dawson H, Lugli A: Molecular and pathogenetic aspects of tumor budding in colorectal cancer. *Front Med* 2015, 2:11.
121. Man Y gao: Tumor cell budding from focally disrupted tumor capsules: A common pathway for all breast cancer subtype derived invasion. *J Cancer* 2010, 1:32–37.
122. Pease JC, Brewer M, Tirnauer JS: Spontaneous spheroid budding from monolayers: A potential contribution to ovarian cancer dissemination. *Biol Open* 2012, 1:622–628.
123. Bronsert P, Enderle-Ammour K, Bader M, Timme S, Kuehs M, Csanadi A, Kayser G, Kohler I, Bausch D, Hoepfner J, Hopt UT, Keck T, Stickeler E, Passlick B, Schilling O, Reiss CP, Vashist Y, Brabletz T, Berger J, Lotz J, Olesch J, Werner M, Wellner UF: Cancer cell invasion and EMT marker expression: a three-dimensional study of the human cancer-host interface. *J Pathol* 2014, 234:410–422.
124. Grigore A, Jolly M, Jia D, Farach-Carson M, Levine H: Tumor Budding: The Name is EMT. Partial EMT. *J Clin Med* 2016, 5:51.
125. Boareto M, Jolly MK, Goldman A, Pietilä M, Mani SA, Sengupta S, Ben-Jacob E, Levine H, Onuchic JN: Notch-Jagged signalling can give rise to clusters of cells exhibiting a hybrid epithelial/mesenchymal phenotype. *J R Soc Interface* 2016, 13.
126. Marambaud P, Shioi J, Serban G, Georgakopoulos A, Sarner S, Nagy V, Baki L, Wen P, Efthimiopoulos S, Shao Z, Wisniewski T, Robakis NK. A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J*. 2002 Apr 15;21(8):1948-56. doi: 10.1093/emboj/21.8.1948. PMID: 11953314; PMCID: PMC125968.
127. Dobiasova B, Mego M: Biomarkers for inflammatory breast cancer: Diagnostic and therapeutic utility. *Breast Cancer Targets Ther* 2020, 12:153–163.

Figures

Figure 1

Image and algorithmic analyses of multiple histological and immunocytochemical parameters in IBC v non-IBC human cases. (A) Ki-67 immunoreactivity (left), specific algorithmic recognition (middle) and Ki-67 index quantitation (right) in IBC v non-IBC are illustrated. (B) Mitosis histology (left), specific algorithmic recognition (middle) and mitotic count determination (right) in IBC v non-IBC are illustrated. (C) Nuclear histology (left), specific algorithmic quantitation (middle) and nuclear grade (size) distribution (right) in IBC v non-IBC are illustrated: yellow, <20 μm ; green, 20-30 μm ; red, 31-50 μm . (D) Tumor embolic histology (left) and density determinations (right) in IBC v non-IBC are illustrated. (E) Tumor embolic histology (left) and algorithmic imaging shape determinations (right) in IBC v non-IBC are illustrated. (F) Tumor embolic imaging (left) and algorithmic perimeter determinations (right) in IBC v non-IBC are illustrated. Scale bars are provided. For each of these parameters, the graph depicts both modal values (plotted) and calculated means $\pm$ SD. Differences of significance are depicted.

Figure 2

Xenograft Properties.(A) The growth of Mary-X and Karen-X are depicted. Each time point displays the mean $\pm$ standard deviation of 10 mice. Routine histology of Mary-X (B) and Karen-X (C) shows similar nodular islands of tumor cells. E-cadherin immunocytochemistry shows more intense and more circumferential membrane immunoreactivity in Mary-X (D) compared to Karen-X (E). Mary-X gives rise to E-cadherin positive pulmonary metastases (F) as well as CTCs (F: inset) whereas Karen-X lacks both (G, G: inset). Scale bars are provided.

Figure 3

***In Vitro* Clump Properties.** Phase contrast microscopy depicts Mary-X growing in suspension culture as tight aggregates termed spheroids (A) in which there is partial spontaneous budding (PB) which can be quite prominent (C). Karen-X, in contrast, exists only as loose aggregates (B) which remain as such in suspension culture and do not bud (D). Scale bars are provided.

Figure 4

Complete Budding both *In Vitro* and *In Vivo*. Complete budding of Mary-X with phase contrast (A) and single label E-cadherin immunofluorescence (B). E-cadherin immunoreactivity remains intense even at the points of the budding (B). DAPI was used as a nuclear counterstain. White arrows indicate budding points. Time-lapse photography confirms the dynamics of the budding process (Supplement 1). Double label immunofluorescent studies demonstrate tumor emboli exhibiting green immunofluorescence within podoplanin-positive lymphovascular channels exhibiting red immunofluorescence with DAPI used as a nuclear counterstain. Dramatic complete embolic budding is observed *in vivo* (C). E-cadherin

immunoreactivity remains intense even at the points of the budding. White arrows indicate budding points. As a result of this geometric budding, large numbers of tumor emboli are propagated (D). Scale bars are provided.

Figure 5

Effects of E-cad/NTF1 and its Inhibition. Western blot using H108 reveals both full length E-cadherin and E-cad/NTF1 in Mary-X but essential absence of this fragment in Karen-X (A). Laser capture microdissection of tumor emboli (B) from cases of IBC also confirm the presence of E-cad/NTF1 by Western blot using 24E10 (C). Amino acid sequences of site of cleavage (SC) and site of binding (SB) on E-cadherin contained within their respective decapeptides are depicted (D). Each decapeptide singly was partially effective at blocking calpain generation of E-cad/NTF1 illustrated by Western blots using H108 (E) and 24E10 (F). *In vitro* delivery and action of decapeptides on tumor clumps are depicted. SC peptide labelled with Alexa Fluor 488 (green fluorescence) (G) and the SB peptide labelled with Alexa Fluor 594 (red fluorescence) (H) both singly and in combination (yellow fluorescence) (I), both penetrated the Mary-X spheroids and caused increased disadherence and decreased partial budding (J). Effects of both decapeptides were synergistic. Scale bars are provided.

A

B

Figure 6

Generation of E-cadherin and its fragments by different proteases. (A) Schematic (adapted by permission from Macmillan Publishers Ltd., The EMBO Journal 2000; 21: 1948-1956, ref 65) depicts cleavage sites of calpain, caspase-3, MMP and γ-secretase including its obligate PS1 binding domain (highlighted) which is also thought to be the site of calpain binding as well as 8 of the E-cadherin fragments (E-cad/NTF1-4; E-cad/CTF1-4) generated by these specific cellular proteases. EC1-5 denote the extracellular E-cadherin

repeats. TM indicates transmembrane domain (also highlighted). The sites of binding of two anti-E-cadherin antibodies, H108 and 24E10 are also depicted. Schematic adapted from Figure 3, ref 65.

(B) Schematic of E-cad/NTF1 membrane mobility depicts the structure of E-cad/NTF1 bound to p120ctn but not to β - or α -catenin nor the actin cytoskeleton. Therefore while E-cad/FL is tethered to the membrane in traditional CAJs, E-cad/NTF1 becomes more mobile, redistributing itself along adjacent areas of the membrane, contributing to increased budding.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplement1MaryXbudding.avi](#)
- [Supplement2KarenXnonbudding.mp4](#)