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Harnessing 3D models of mammary epithelial morphogenesis. An off the beaten path approach to identify candidate biomarkers of early stage breast cancer

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Abstract

Regardless of the etiological factor, an aberrant morphology is the common hallmark of ductal carcinoma in situ (DCIS), which is a highly heterogeneous disease. To test if critical core morphogenetic mechanisms are compromised by different mutations, we performed proteomics analysis of five mammary epithelial HME1 mutant lines that develop a DCIS-like morphology in three dimensional (3D) culture. Here we show first, that all HME1 mutant lines share a common protein signature highlighting an inverse deregulation of two annexins, ANXA2 and ANXA8. Either ANXA2 downregulation or ANXA8 upregulation in the HME1 cell context are *per se* sufficient to confer a 3D DCIS-like morphology. Seemingly, different mutations impinged on a common mechanism that differentially regulates the two annexins. Second, we show that ANXA8 expression is significantly higher in DCIS tissue samples versus normal breast tissue and atypical ductal hyperplasia (ADH). Apparently, ANXA8 expression is significantly more upregulated in ER- negative versus ER-positive cases, and significantly correlates with tumor stage, grade and positive lymph node. Based on our study, 3D mammary morphogenesis models can be an alternate/complementary strategy for unraveling new DCIS mechanisms and biomarkers.

Keywords

3D mammary epithelial morphogenesis; breast cancer; ductal carcinoma in situ (DCIS); biomarkers; annexins

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CONFLICT OF INTEREST

The authors have no conflict of interest.

1. INTRODUCTION

The aberrant morphology of mammary ducts or lobules is a hallmark of *in situ* breast cancer lesions, such as ductal carcinoma *in situ* (DCIS), which may, or not, progress to invasive breast cancer [1, 5, 6, 8]. As first demonstrated by Mina Bissell, tumorigenic mammary epithelial cells can be easily distinguished from non-tumorigenic cells based on the three dimensional (3D) morphology that they acquire once seeded in a basement membrane microenvironment [25]. Typically, non-tumorigenic mammary epithelial cells develop into 3D acinar structures with a lumen-enclosing epithelial monolayer, whereas mammary tumorigenic epithelial cells develop into morphologically aberrant 3D acinar structures with a luminal space filled with proliferating cells. Since 3D amorphous acini resemble early-stage breast cancer lesions, such as DCIS, 3D mammary epithelial morphogenesis systems have been extensively used to identify normal molecular and physical developmental mechanisms (e.g. lumenogenesis and branching morphogenesis) that go awry in breast cancer [10, 14, 25].

By using a human, non-tumorigenic HME1 mammary epithelial model, we previously reported that stable distinct genetic mutations, by interfering with different functions or signaling pathways, affect the 3D HME1 cell morphogenetic potential [2, 7, 28, 29]. Specifically, regardless of the initiating mutation, all HME1 clonal lines when seeded in basement membrane culture, develop into 3D amorphous and proliferative “DCIS-like” acini. Thus, we hypothesized that HME1 cells carrying different genetic mutations, but with the same potential to form 3D DCIS-like acini, could be suitable for identifying new critical core mammary epithelial morphogenetic mechanisms and biomarkers.

To start tackling this hypothesis, we compared the proteomics profile of five HME1 clonal lines carrying distinct genetic mutations (hereafter referred to as HME1 mutant lines) relative to control parental HME1 cells. Remarkably, all the HME1 mutant lines shared a common protein signature, characterized by deregulation of proteins associated with signaling pathways and functions relevant to key morphogenetic processes, such as cytoskeleton organization, cell death, and cell polarity. Within this common protein signature, what mostly attracted our attention, was the inverse deregulation of two members of the annexin family; while Annexin A8 (ANXA8) was upregulated, Annexin 2 (ANXA2) was downregulated. ANXA2 not only is known to be involved in the regulation of membrane dynamics and actin remodeling [17], but also plays a key role in the establishment of epithelial cell polarity and lumen formation [3, 22], two fundamental processes required for normal mammary epithelial morphogenesis. Conversely, apart from evidence of ANXA8 expression in a subpopulation of mammary progenitor cells [19] and its modulation during mouse mammary gland involution [31], less clear is the morphogenetic function of ANXA8.

In the first part of this study we show that either stable ANXA2 downregulation or ANXA8 upregulation in the HME1 context affect 3D morphogenesis, leading to the formation of 3D acinar structures with DCIS-like morphology. After excluding a mutual regulation between ANXA2 and ANXA8, we found preliminary evidence supporting the alternate hypothesis that a common mechanism, compromised in all HME1 mutant lines, must be involved in the

deregulation of the two annexins. In the second part of this study we focused on ANXA8 as a potential DCIS biomarker. We found that this annexin is significantly more expressed in DCIS relative to normal tissue and atypical ductal hyperplasia (ADH) samples. Since ANXA8 expression was significantly higher in ER-negative vs. ER-positive DCIS, and was associated with clinical features of tumor progression, ANXA8 may qualify as a biomarker particularly suitable for the identification of the ER-negative DCIS subgroup.

Coupling *in vitro* 3D models of human mammary epithelial morphogenesis (e.g. the HME1 model) with global analyses (e.g. proteomics) not only can be harnessed for the identification of new morphogenetic pathways, but can also provide an alternate strategy for identifying new candidate DCIS biomarkers.

2. MATERIALS AND METHODS

2.1. Cells and cell culture

For standard 2D culture on plastic, h-TERT-HME1 human mammary epithelial cells (here referred to as HME1) (Clontech, Mountain View, CA) and derived clonal lines were grown in Mammary Epithelial Growth Medium (MEGM) (Lonza, Walkersville, MD). 3D culture on growth-factor reduced Matrigel (BD Biosciences, San Jose, CA) was performed as we previously described [29]. Briefly, 3×10^3 single cells/well were seeded in 8-well chamber slides on a layer of Matrigel covered with MEGM + 2% Matrigel and grown 10–12 days until they developed into mature 3D acini. Medium was refreshed every 2–3 days.

HME1-derived stable clonal lines HME1-DNC4, HME1-shERA, HME1-shPER2, HME1-shMTG16, and HME1-MYC (in this study collectively referred to as HME1 mutant lines) were previously described [2, 28, 29]. HME1 clones with stable ANXA2 knock down were generated by stable transfection with pSUPER-shANXA2 sequence B or pSUPER-shANXA2 sequence C targeting both ANXA2 transcript variant 1 and 2. To generate pSUPER-shANXA2 constructs, two independent 19 bp sequences (sequence B: 5'-CGGGATGCTTTGAACATTG-3' and sequence C: 5'-GGAAGTTGCATCAGCACTG-3') were cloned into pSUPER-puro from OligoEngine, Seattle, WA, as per manufacturer's instructions. Control HME1 clones were stably transfected with pSUPER-shSCR containing a scrambled sequence that does not target any human gene as previously described [2]. After transfection with Lipofectamine LTX (Thermo Fisher, Waltham, MA) cells were selected with 1 µg/ml puromycin. Single clones isolated with cloning rings, were expanded and analyzed. HME1 clones stably overexpressing ANXA8 and relative control cells were generated by stable transfection with either pLNCX2-ANXA8 or empty pLNCX2. To generate pLNCX2-ANXA8, human ANXA8 CDS (splice variant 2, NM_001040084.2) was amplified from HME1 cDNA by PCR with a forward primer introducing an Xho I restriction site (5'-CTCAGATCTCGAGATGGCCTGGTGGAAATC-3') and a reverse primer introducing a Sal I restriction site (5'-TAAGGCCTGTCGACTTGTCTTCTGTGCCTCAG-3'), and cloned into the same restriction sites of pLNCX2 (Clontech). After transfection, cells were selected with 1 mg/ml G418, and single clones were isolated, expanded, and analyzed. All HME1-derived clonal lines used in this study were authenticated by amplification of the transfected construct by PCR.

2.2. Immunostaining and analysis of 3D acini

Mature 3D HME1 acini (10–12 days) were stained as described [29]. Briefly, after fixation with 4% paraformaldehyde for 15 minutes, 3D acini were incubated with PBS + 0.2% Triton X100 for 10 minutes, followed by blocking with PBS + 1% BSA, 1% FBS and 0.05% Tween 20 for 1 hour, and incubation with the primary antibody overnight at 4 °C. Cells were rinsed with PBS, and incubated with appropriate secondary antibodies for 2h, rinsed with PBS, and stained with DAPI (Sigma, St Luis, MO). Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Golgi apparatus (marker of apicobasal polarity) and integrin (marker of basolateral polarity) were detected with anti-GM130 antibody (BD Biosciences) and anti-CD49f antibody (EMD Millipore, Billerica, MA), respectively. Acini were analyzed with a confocal microscope (SP2 Spectral Confocal Microscope, Leica).

2.3. EdU incorporation assay

EdU incorporation assay (Click-iT EdU imaging kit, Thermo Fischer), used to assess cell proliferation within the 3D acini, was performed according to the manufacturer's protocol. Briefly, 3D mature acini, previously incubated for 2 hours in the presence of 40 µM EdU under standard growth conditions, were fixed with 4% paraformaldehyde for 20 minutes, incubated with PBS + 0.5 % Triton X100 for 20 minutes, washed twice with PBS + 3% BSA, incubated with Click-iT reaction cocktail for 45 minutes, and stained with DAPI. Slides were mounted with Vectashield (Vector Laboratories) and analyzed by confocal microscopy (SP2 Spectral Confocal Microscope, Leica).

2.4. Proteomics analysis

Two-dimensional difference gel electrophoresis (2D DIGE) and Protein identification by mass spectrometry was performed by Applied Biomics, Inc (Hayward, CA) by using GE Healthcare equipment and protocol. Briefly, cells were lysed in 30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS, and 30 µg proteins from paired samples were labeled with Cy3 and Cy5 and run on 2D gel along with an internal standard labeled with Cy2. Gels were scanned immediately following the SDS-PAGE using Typhoon TRIO (GE Healthcare). The scanned images were analyzed by Image Quant software (version 6.0, GE Healthcare), followed by cross-gel analysis using DeCyder software (version 6.5, GE Healthcare), to identify protein spots differentially expressed in HME1 mutant lines vs. HME1-Ctrl. Differentially expressed protein spots were selected for analysis by mass spec based on: 1) absolute fold change > 1.5 in one or more HME1 mutant lines relative to HME1-Ctrl, and 2) concomitant upregulation or downregulation in all HME1 mutant lines. The selected spots were ranked based on average expression in the HME1 mutant lines, and the 25 most upregulated and the 25 most downregulated protein spots (see Table S1) were excised from the gels by using Ettan Spot Picker (GE Healthcare). After in-gel digestion with modified porcine trypsin protease (Trypsin Gold, Promega), the tryptic peptides were desalted by Zip-tip C18 (Millipore), eluted from the Zip-tip with 0.5 µl of matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on an Opti-TOF™ 384 Well Insert. MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Framingham, MA). MALDI-TOF mass spectra were acquired in

reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions). Both of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) and used to search the database of National Center for Biotechnology Information non-redundant (NCBIInr). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Peptides counts, protein MW, PI, scores and confidence interval (CI) for each of the 50 protein spots analyzed by mass spec are shown in Table S2. Protein score CI > 95 % and/or Total ion score CI > 95% were considered significant.

From the 50 proteins identified by mass spec analysis we selected 42 proteins for further analysis after excluding the following: redundant protein spots (i.e. spots associated with the same protein); proteins with inconsistent expression trend; uncharacterized proteins (see Table S2). Gene Ontology annotations for each protein were obtained from DAVID Bioinformatics Resource (<https://david.ncifcrf.gov/>). Function, pathway, and network analysis of the 42 proteins was performed by using Ingenuity Pathway Analysis (IPA) build version: 131235 with default settings. The Ingenuity Knowledge Base reference set was used for all IPA analyses. Functions were selected based on both p-value (< 0.05) and regulation z-score (> 0.5 or < -0.5). Canonical pathways were selected based on p-value (<0.05). For network analysis, network size was limited to 35 nodes, based on direct and indirect relationship. The functions associated with the networks were selected based on p-value (< 0.05).

2.5. Western Blot

Cells were lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, supplemented with Roche Complete protease inhibitor cocktail). Protein concentration was measured by using Coomassie Plus protein assay reagent (Thermo Scientific), and equal amounts of proteins were separated by SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane by standard methods. Membranes were incubated with anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ANXA8 (LifeSpan Biosciences, Seattle, WA) or anti-ANXA2 (BD Biosciences), washed, and incubated with an appropriate HRP-conjugated antibody (GE Healthcare, Piscataway, NJ) followed by ECL detection (GE Healthcare). Protein band intensity was quantified by using Image J (NIH). Statistical significance was calculated by using the Student's t-test.

2.6. In silico RARE search and miRNA prediction

MiRNAs targeting either ANXA2 3'UTR (NM_001002857, nt 1156–1635) or ANXA8 3'UTR (NM_001040084.2, nt 1210–2060) were identified by using both TargetScan 6.2 release (<http://www.targetscan.org/>) and miRanda August 2010 release (<http://www.microrna.org/microrna/home.do>). Only conserved miRNAs predicted by both algorithms were taken into consideration for subsequent analyses. The 10kb region upstream

of the transcription start site of ANXA2, ANXA8, and miRNA genes was searched for DR2 and DR5 RARE sequences PuG(G/A)(T/A)CA N₂₋₅ PuG(G/T)TCA [20] by using SnapGene.

2.7. miRNA quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed essentially as described [11]. Briefly, total RNA isolated with Trizol (Thermo Fisher) was retrotranscribed by using TaqMan miRNA Reverse Transcription kit and RT primers specific for miR-218 or RNU-44 (internal control) as per manufacturer's instructions (Thermo Fisher). miRNA levels were assessed by real time PCR on an iCycler (BioRad, Hercules, CA) by using iQ Supermix (BioRad) and TaqMan assay real-time primers (Thermo Fisher). The delta-delta Ct method was used for miRNA quantification. Statistical significance was calculated by using the Student's t-test.

2.8. Patient samples

The tissue specimens, obtained from the Roswell Park Cancer Institute (RPCI) Pathology Network Resource (PNR), were collected under remnant tissue protocol I-115707, which is approved by the RPCI Internal Review Board (IRB) and is in compliance with all legal regulations. For this study we analyzed atypical ductal hyperplasia (ADH) individual tissue sections from 43 patients and a tissue microarray (TMA) with ductal carcinoma in situ (DCIS) specimens from 178 patients developed by the RPCI-PNR. Of the DCIS patients, 24 had only DCIS, while 154 also had infiltrating breast lesions. The slides with ADH tissue were used to evaluate ANXA8 expression both in ADH tissue and in the adjacent normal tissue. After staining, only 41 ADH tissue samples and 39 normal tissue samples could be analyzed. Of the 178 cores in the DCIS TMA, 151 could be examined after sectioning and staining. To identify correlations between ANXA8 expression in DCIS and clinical features, only DCIS samples from patients with a complete clinical history (n=115, see Table S4) were analyzed.

2.9. Tissue staining and analysis

Formalin-fixed, paraffin-embedded samples were cut into 4µm sections, placed on charged slides, and dried for one hour at 60°C. Slides were deparaffinized with xylene, rehydrated with graded alcohols, and microwaved for 10 minutes in citrate buffer pH=6 (BioCare Medical) for antigen retrieval. After quenching endogenous peroxidase with aqueous 0.3% H₂O₂ for 10 minutes, slides were washed with PBS/T, loaded on a Dako autostainer, and blocked with a serum-free protein block (Dako, Carpinteria, CA) for 5 minutes. Slides were incubated with rabbit anti-ANXA8 antibody (Lifespan Biosciences) diluted 1:900 (1.1 µg/ml IgG) for one hour, followed by incubation with HRP anti-rabbit (Dako) for 30 minutes. Finally, slides were incubated with 3,3'-Deaminobenzidine (DAB) (Dako) for 10 minutes for chromogen visualization, counterstained with Hematoxylin, rinsed, and mounted with a cover slip.

Stained slides were digitally scanned using Aperio Scanscope. ANXA8 expression was evaluated independently by two pathologists on three sections based on three different scoring systems. The intensity score evaluated the staining intensity with 0=negative, 1=weak, 2=medium, 3=high. The percentage score evaluated the percentage of positive

cells, with 0=negative, 1= less than 5%, 2= 6–25%, 3=26–50%, 4=more than 50%. The combined score, based on Stein et al. [31], considered both intensity and percentage as follows: negative = all cells negative; strong, 2 in >50% cells or 3 in >25% cells; weak, between negative and strong.

2.10. Statistical analysis of ANXA8 staining in patient samples

For each Score, pairwise comparisons of the observed empirical distribution functions across Methods (ADH vs NORMAL, DCIS vs NORMAL, and DCIS vs ADH) were obtained from two Kolmogorov-Smirnoff tests. This test considers the null hypothesis that the two methods produce the same Score distributions, vs the alternative hypothesis that the Score distributions are different. Associations between categorical factors were considered using Fisher's Exact test. Distributional differences of continuous markers between categorical factors were assessed using the Kruskal-Wallis test, and displayed with Empirical Distribution plots. P values less than 0.05 were considered statistically significant. These p values were not adjusted to correct for multiple testing. All analyses were done using SAS v9.3 (Cary, NC).

3. RESULTS

3.1. HME1 mutant lines share a common protein signature highlighting an inverse deregulation of two annexins

As we reported in previous studies [2, 7, 28, 29], when grown in reconstituted basement membrane 3D culture (Matrigel), HME1 non-tumorigenic human mammary epithelial cells develop into acinar structures that are hollow (see DAPI-stained nuclei lining the lumen), lined by polarized cells (see localization of Golgi and integrin), and growth-arrested (see lack of EdU-positive, proliferating cells) (Figure 1A, left). In contrast, five HME1-derived stable clonal lines carrying distinct genetic factors (collectively referred to as HME1 mutant lines) develop into "DCIS-like" morphologically aberrant 3D acini with a lumen filled with proliferating cells (Figure 1A, right). Specifically, the 3D HME1 aberrant morphology was consequent to stable interference with the following: a) retinoic acid (RA) transcriptional signaling by a dominant negative RA receptor alpha (RARA-403) [2]; b) estrogen signaling by estrogen receptor alpha (ERA) knock down [28]; c) circadian rhythm by Period 2 (PER2) knock down [28]; d) rRNA transcription by MTG16 knock down [29]; and e) MYC signaling by MYC overexpression [29]. The DCIS-like morphology developed by the five HME1 mutant lines let us test if the different genetic factors, by impinging on critical core morphogenetic mechanisms, confer a common protein signature. To this end, we performed cross-comparison analysis of the protein profile of the five HME1 mutant lines relative to parental HME1 control cells. By 2D gel electrophoresis (2D DIGE) it emerged that all five HME1 mutant lines shared a similar protein signature encompassing 212 proteins (Figure 1B, based on Table S1). We identified with high confidence (CI > 95%) 50 proteins differentially expressed in the HME1 mutant lines vs. control HME1 parental cells by mass spectrometry analysis of the 25 most upregulated and 25 most downregulated protein spots (see frames in Figure 1B and Material and Methods). Protein scores, NCBI accession numbers, peptide counts, theoretical molecular weights and isoelectric points of the 50 proteins are shown in Table S2. After discarding a few redundant protein spots,

uncharacterized proteins, and proteins with inconsistent expression trend, we narrowed down the list of differentially expressed proteins to 42, of which 22 were upregulated and 20 downregulated (Table S2). Ingenuity Pathway Analysis (IPA) of the 42 differentially expressed proteins shows deregulation of cellular functions (Figure 1C, top), signaling pathways (Figure 1C, bottom), and protein networks (Figure S1) mostly related to cell death, cell movement, cell invasion, and cytoskeleton organization. In particular, analysis of the function regulation Z-score, which indicates whether there is an increase or a decrease of a given function, shows a decrease of cell death functions and an increase of cell movement-related functions (Figure 1C, top). These findings are relevant, because lumen formation requires both spatiotemporal programmed cell death and cell migration to the outer cell layer [10, 26]. Closer inspection of proteins expressed by the five HME1 mutant lines highlighted deregulation of proteins that play important roles in morphogenetic processes, such as VIM, TUBB, and ACTG, which are cytoskeletal proteins; ARHGDIA, which regulates RHO GTPases-mediated cell adhesion and cell motility [12]; and GLO1, which confers resistance to apoptosis [32].

Last, we found of particular interest that all HME1 mutant lines displayed an inverse deregulation of ANXA2 and ANXA8, two members of the annexin family that may be relevant to mammary epithelial morphogenesis. This prompted us to assess if either ANXA2 downregulation or ANXA8 upregulation can affect HME1 3D morphogenesis, and whether their inverse deregulation is, or not, casual.

3.2. ANXA2 downregulation and ANXA8 upregulation affect 3D HME1 morphogenesis

After validating by quantitative western blot analysis the downregulation of ANXA2 protein in the five HME1 mutant lines that form morphologically aberrant DCIS-like 3D acini (Figure 2A), we set out to knock down ANXA2 in HME1 cells with pSUPER constructs carrying either one of two different shRNA sequences targeting ANXA2 (Figure 2B, left). Independent stable HME1-shANXA2 clones showing significantly lower ANXA2 protein level relative to control HME1-shSCR cells stably transfected with a scrambled sequence (Figure 2B, right), developed into morphologically aberrant 3D acini characterized by partially, or completely, filled lumen and presence of EdU-positive proliferating cells (Figure 2C).

Similarly, by quantitative western blot analysis we confirmed ANXA8 upregulation in all five HME1-derived clonal lines relative to control HME1 cells (Figure 3A). By stable transfection of a pLNCX2 construct carrying ANXA8 cDNA cloned from parental HME1 cells (transcript variant 2) (Figure 3B, left), we derived stable HME1 clones expressing a higher level of ANXA8 relative to control HME1 cells stably transfected with an empty pLNCX2 vector (EV). The level of ANXA8 in these clones was similar to the one detected in the five HME1 mutant clones (Figure 3B, right). The HME1 clonal lines overexpressing ANXA8 developed into morphologically aberrant 3D acini (Figure 3C).

Overall these findings indicate that either downregulation of ANXA2 or upregulation of ANXA8 in the HME1 cell context is sufficient to affect the cell morphogenetic potential, thus leading to the formation of DCIS-like amorphous acini resembling the morphology of early-stage breast cancer lesions.

Interestingly, we found that in the HME1 context neither downregulation of ANXA2 *per se* affects ANXA8 expression, nor ANXA8 upregulation *per se* affects ANXA2 expression (data not shown), thus ruling out a potential functional redundancy of these annexins [23]. Next, we asked whether the concomitant inverse deregulation of the two annexins in HME1 mutant lines is consequent to the fact that the diverse genetic factors may compromise a common “bottleneck” mechanism. Based on preliminary studies of our laboratory [27], all five HME1 mutant lines are insensitive to the growth-inhibitory and pro-apoptotic morphogenetic action of physiological retinoic acid (RA), a signal that plays a critical role in mammary epithelial morphogenesis [2, 7, 24, 30, 33]. Thus, deregulation of the two annexins may be consequent to lack of genome-wide transcriptional surveillance by RA, given that by *in silico* analysis we detected several RA responsive elements (RAREs) both in ANXA8 and ANXA2 gene regulatory regions as well as in a few microRNAs predicted to target the 3'UTR of the two annexins (Figure S2A). So far, we found that miR-218, which is predicted to target ANXA8 3'UTR, is downregulated in all HME1 mutant lines (Figure S2B). Consistently, miR-218 was also found downregulated in basal-like DCIS with ANXA8 upregulation (Dr. Zhou, personal communication and [21]), suggesting that ANXA8 upregulation in DCIS could be due, at least in part, to a miRNA-mediated mechanism regulated by RA.

3.3. Evidence of increased ANXA8 protein expression level in DCIS

Since ANXA8 upregulation is apparently sufficient to disrupt 3D HME1 morphogenesis, we next investigated if ANXA8 protein expression level was increased in atypical ductal hyperplasia (ADH) and/or DCIS relative to normal breast tissue. To this end, we analyzed by immunocytochemistry ANXA8 protein expression in formalin fixed paraffin embedded (FFPE) samples from normal breast tissue (39 samples), ADH (41 samples) and DCIS (151 samples, embedded in a single tissue microarray). As shown in Figure 4A, left, normal breast tissue samples displayed low ANXA8 staining in both luminal and basal cells, with prevalent cytoplasmic localization. In ADH tissue ANXA8 showed prevalent cytoplasmic localization (Figure 4A, middle), while in DCIS it seemed to be localized both in the cytoplasm and in the nucleus (Figure 4A, right). Next, we evaluated ANXA8 expression in tissue samples by using three different scoring systems based on either overall staining intensity (intensity score), percentage of ANXA8-positive cells (% score), or a combination of the two parameters (combined score) (see Methods and Table S3 for details). As shown in Figure 4B, ANXA8 expression was modestly, but not significantly increased in ADH samples relative to normal tissue. In contrast, in DCIS samples, ANXA8 was expressed significantly more relative to both normal tissue and ADH according to all three scoring systems (Figure 4B).

Finally, we assessed whether ANXA8 staining in either ADH or DCIS correlated with specific clinical features, which we could gather from the patient clinical history associated with the FFPE samples. For DCIS analysis, we only took into consideration samples with complete clinical history (n=115). ADH analysis did not show any significant correlation between ANXA8 staining and progression to breast cancer or other clinical features (data not shown). In contrast DCIS analysis showed a significant correlation between ANXA8 staining and features of breast cancer progression (Figure 5A and Supplementary Table S4).

Since the vast majority of DCIS samples (113 out of 115) were associated with the presence of infiltrating breast lesions, we were not able to determine whether ANXA8 expression in DCIS significantly correlated with the presence of invasive breast cancer. However, we found a significant correlation between ANXA8 expression and positive nodes, tumor stage, tumor grade, estrogen receptor (ER) negativity and progesterone receptor (PR) negativity (Figure 5A). These correlations were most significant when took into consideration ANXA8 intensity score (a few examples are shown as empirical distribution plots in Figure 5B).

Taken together, these findings indicate that ANXA8 upregulation in DCIS significantly correlates with features of breast cancer progression.

4. DISCUSSION

Progress in the identification of molecular and physical morphogenetic mechanisms of mammary gland development, which are affected in breast cancer, has been greatly advanced by *in vitro* 3D mammary epithelial morphogenesis models [10, 14, 25]. Thus, we reasoned that 3D models, like the HME1 3D model, which we extensively used for several mechanistic studies [2, 7, 28, 29], can represent another strategy for discovering new candidate mechanisms and biomarkers of early breast cancer lesions (e.g. DCIS).

In the first part of this study, by proteomics analysis of a panel of five HME1 mutant lines, which form morphologically aberrant 3D acini with DCIS-like features, and their control parental HME1 line, which forms 3D acini with features of normal mammary ducts, we identified a common protein signature comprising forty-two differentially expressed proteins associated with aberrant, DCIS-like, 3D HME1 morphology. Bioinformatics analysis of this protein signature highlighted many signaling pathways and functions relevant to morphogenetic processes, including a decrease in cell death functions coupled with an increase of functions relevant to cytoskeleton organization and cell polarity. Apparently, different mutations causing aberrant 3D HME1 morphogenesis did impinge upon one, or more, critical core morphogenetic mechanism(s).

Within the common protein signature shared by the five HME1 mutant lines, we focused on the inverse deregulation of two members of the annexin superfamily of membrane- and calcium-binding proteins, ANXA2 and ANXA8 [13, 17, 18]. This finding was relevant because deregulation of annexins in DCIS was reported in a pioneering proteomics study by the Steeg lab showing that in addition to proteins relevant to cytoskeletal architecture, apoptosis, and the microenvironment, also proteins regulating the intracellular trafficking of membranes and vesicles, ions and fatty acids were involved in DCIS formation [34]. Indeed, ANXA2 plays a key role in the establishment of epithelial polarity and lumenogenesis by regulating actin cytoskeleton dynamics at the apical surface of the plasma membrane and recruiting to this compartment the Cdc42 Rho-GTPase [3, 22]. Similarly, ANXA8, known to be expressed in a subset of mammary progenitor cells and upregulated during mammary gland remodeling [19, 31], is involved in the formation of endosomes [15], which are implicated in the establishment and maintenance of epithelial cell polarity [16].

We mechanistically determined that stable downregulation of ANXA2, as well as stable upregulation of ANXA8, in the HME1 cell context are both sufficient to affect HME1 3D morphogenesis. These initial findings raise many questions about the causes and consequences of annexin deregulation in breast cancer initiation and progression. Focusing on the potential causes, we first hypothesized that the inverse deregulation of the two annexins in HME1 mutant lines could be due to annexins functional redundancy [23]. However, we discounted this hypothesis because stable ANXA8 upregulation *per se* did not induce ANXA2 downregulation as well as stable ANXA2 downregulation *per se* did not induce ANXA8 upregulation (Reiners, unpublished observations). Over twenty years ago ANXA8 upregulation was detected in acute promyelocytic leukemia (APL) characterized by PML-RARA [4]. This leukemia fusion protein differentially affects the transcription of retinoic acid (RA) receptor alpha (RARA)-target genes by physiological RA [9]. Consistently, the five HME1 mutant lines with inverse deregulation of the two annexins are all insensitive to the growth-inhibitory and pro-apoptotic morphogenetic action of physiological RA because we found that, directly (e.g. the dominant negative RARA mutant) or indirectly, the different mutations hamper the activation of the transcriptional RA-RARA signaling during 3D morphogenesis [2, 27]. Thus, the concomitant occurrence of ANXA2 and ANXA8 deregulation could be explained by lack of transcriptional surveillance by physiological RA-responsive genes. Indeed, by *in silico* analysis we detected RA responsive elements (RAREs) both in ANXA8 and ANXA2 gene regulatory regions as well as in a few microRNAs predicted to target the 3'UTR of the two ANXA proteins, indicating that the expression of the two annexins can be regulated by RA directly and/or indirectly via regulatory miRNAs. As shown in this study, one of the RA-regulated microRNAs targeting ANXA8, miR-218, is downregulated in all five HME1-mutant lines, suggesting that ANXA8 upregulation in DCIS could be due, at least in part, by interference with a RA-mediated miRNA mechanism. This supposition is supported by the fact that miR-218 was also found downregulated in basal-like DCIS with ANXA8 upregulation (Dr. Zhou, personal communication and [21]). Ongoing mechanistic studies should let us determine whether RA signaling plays (directly, or indirectly via miRNAs) a role in the concomitant regulation of ANXA2 and ANXA8 expression.

In the second part of this study we assessed the potential value of ANXA8 as a biomarker in normal and breast tissue samples of patients with atypical ductal hyperplasia (ADH) as well as in a tissue microarray including both ER- positive and ER- negative DCIS with known clinical history. We found that ANXA8 was increased in all DCIS tissue samples relative to both normal breast tissue and ADH tissue. Notably, ANXA8 expression level was significantly higher in ER-negative versus ER-positive DCIS and correlated with features of cancer progression, such as tumor grade, stage and lymph node positivity. Since ER-negative, basal-like DCIS seem to have particularly high ANXA8 transcript level relative to normal breast tissue (Dr. Zhou personal communication), as also reported previously in invasive breast cancer [31], ANXA8 could be a suitable biomarker for this DCIS subgroup.

In summary, our study suggests that 3D mammary epithelial morphogenesis models, which can be genetically manipulated and subjected to global analyses, not only can be relevant for the identification of novel morphogenetic mechanisms (e.g. annexin-mediated

morphogenetic mechanisms), but can also be harnessed for the discovery of new candidate protein and miRNA biomarkers useful for further unraveling the heterogeneity of DCIS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2D	Two dimensional
2D DIGE	Two dimensional difference gel electrophoresis
3' UTR	3' untranslated region
3D	Three dimensional
ADH	atypical ductal hyperplasia
ANXA2	Annexin A2
ANXA8	Annexin A8
C.I	confidence interval
DCIS	Ductal carcinoma in situ
ERA	estrogen receptor alpha
EV	empty vector
HME1	Human mammary epithelial cell line
HME1-MYC	HME1 cells overexpressing MYC
HME1-shERA	ERA knock down HME1 cells
HME1-shMTG16	MTG16 knock down HME1 cells
HME1-shPER2	PER2 knock down HME1 cells
HME1-shANXA2	ANXA2 knock down cells
IPA	Ingenuity pathway analysis

miRNA	microRNA
MW	molecular weight
PI	isoelectric point
PER2	Period 2
RA	retinoic acid
RARA403	dominant negative RA receptor alpha
SCR	scrambled
TMA	tissue microarray

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Highlights

- HME1 cells with distinct genetic mutations develop similar 3D DCIS-like morphology
- HME1 cells developing a 3D DCIS-like morphology share a core protein signature
- Inverse deregulation of ANXA2 and ANXA8 is a feature of DCIS-like HME1 precursors
- ANXA8 expression is significantly higher in DCIS vs normal breast tissue and ADH

Figure 1. HME1 mutant lines share a common protein signature highlighting an inverse deregulation of two annexins

A. Confocal analysis shows that parental control HME1 cells form hollow acini lined by polarized, non-proliferating (EdU-negative) cells in 3D culture (left), while five HME1 mutant lines, due to different genetic factors, develop into aberrant “DCIS-like” 3D amorphous acini containing proliferating (EdU-positive) cells (right). **B.** DIGE proteomics analysis shows that, relative to control parental HME1 cells, the HME1 mutant lines share a common protein signature, which includes downregulation of ANXA2 and upregulation of

ANXA8. The frames identify the protein spots analyzed by mass spec. **C.** Ingenuity Pathway analysis (IPA) shows that the proteins differentially expressed in the five HME1 mutant lines are associated with cellular functions ($p < 0.05$, z-score > 0.5 or < -0.5) (top) and pathways ($p < 0.05$) (bottom) relevant to mammary epithelial morphogenesis. Functions and pathways known to be particularly relevant to mammary epithelial morphogenesis are underlined.

Figure 2. Stable ANXA2 knock down affects 3D HME1 morphogenesis

A. Quantitative western blot analysis confirms ANXA2 downregulation in the five HME1 mutant lines relative to control HME1 cells. **B.** HME1 clones stably transfected with pSUPER-shANXA2 (sequence B or C) (left) express significantly less ANXA2 protein relative to HME1 control cells stably transfected with pSUPER carrying a scrambled (SCR) sequence (right). **C.** Confocal analysis shows that HME1-shSCR control cells grown in 3D culture develop into mature 3D acini with the lumen lined by polarized (see Golgi/integrin staining), growth-arrested (EdU-negative) cells, while HME1-shANXA2 clones form morphologically aberrant 3D acini. The chart on the left shows the quantification of normal and aberrant 3D acini formed by control HME1-shSCR cells and ANXA2 knock down clones; the micrographs on the right show the 3D morphology of representative HME1-shANXA2 and control HME1-shSCR acini.

Figure 3. Stable ANXA8 ectopic expression affects 3D HME1 morphogenesis

A. Quantitative western blot analysis confirms ANXA8 upregulation in the five HME1 mutant lines relative to control HME1 cells. **B.** HME1 clones stably transfected with pLNCX2-ANXA8 (left) express significantly more ANXA8 protein relative to HME1 control cells stably transfected with the empty pLNCX2 vector (EV) (right). **C.** Confocal analysis shows that HME1-ANXA8 cells overexpressing ANXA8 form aberrant 3D acini, while HME1-EV control cells form normal 3D acini. The chart on the left shows the quantification of normal and aberrant 3D acini formed by control HME1-EV and HME1-ANXA8 cells; the micrographs on the right show the 3D morphology of representative control HME1-EV and HME1-ANXA8 3D acini.

Figure 4. Evidence of increased ANXA8 protein expression level in DCIS

A. ANXA8 immunostaining of formalin fixed paraffin-embedded (FFPE) samples shows ANXA8 expression in representative sections of normal breast tissue, ADH tissue, and DCIS tissue. Normal tissue: intensity score = 1; percentage score = 3; combined score = weak; ADH: intensity score = 2, % score = 3; combined score = weak; DCIS: intensity score = 3, % score = 4, combined score = strong. **B.** Quantification of ANXA8 staining by intensity score (left), % score (middle) or combined score (right) in all tissue samples shows that ANXA8, in average, is significantly more expressed in DCIS relative to normal tissue and ADH (see Methods for more details).

A

		ANXA8 Intensity		ANXA8 percentage		Combined score		
		mean	p-value	mean	p-value	Strong	Neg/weak	p-value
Positive nodes	No	1.34	0.0384	3.38	0.0100			0.3530
	Yes	1.56		3.82				
Stage	Stage 1	1.38	0.0142	3.50	0.3460			0.0430
	Stage 2	1.37		3.60				
	Stage 3/4	1.86		3.83				
Grade	Grade 1/2	1.25	0.0161	3.57	0.1570			0.0700
	Grade 3	1.50		3.60				
ER status	Positive	1.29	0.0001	3.52	0.0657			0.0010
	Negative	1.67		3.70				
PR status	Positive	1.30	0.0120	3.58	0.1100			0.0240
	Negative	1.56		3.61				

B

Figure 5. ANXA8 upregulation in DCIS is associated with clinical features of breast cancer progression

A. Analysis of ANXA8 expression in DCIS tissue from patients with known clinical history shows that ANXA8 is significantly associated with features of breast cancer progression (positive nodes, stages, and grade) and is expressed significantly more in ER-negative vs ER-positive and PR-negative vs PR-positive DCIS. **B.** Empirical distribution plots showing significant correlation between ANXA8 intensity in DCIS and presence of positive nodes, tumor stage, tumor grade and ER status.