Cancer Letters 380 (2016) 375–383

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

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

Stefano Rossetti [a], Wiam Bshara [b], Johanna A. Reiners [a], Francesca Corlazzoli [a], Austin Miller [c], Nicoletta Sacchi [a,*]

[a] Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY, USA
[b] Department of Pathology, Roswell Park Cancer Institute, Buffalo, NY, USA
[c] Department of Biostatistics & Bioinformatics, Roswell Park Cancer Institute, Buffalo, NY, USA

ARTICLE INFO

Article history:
Received 1 April 2016
Received in revised form 3 July 2016
Accepted 4 July 2016

Keywords:
3D mammary epithelial morphogenesis
Breast cancer
Ductal carcinoma in situ (DCIS)
Biomarkers
Annexins

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.

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 may not progress to invasive breast cancer [1–4]. 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 [5]. 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 [5–7].

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 [8–11]. 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

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.

* Corresponding author. Fax: 716 845 1741.
E-mail address: nicoletta.sacchi@roswellpark.org (N. Sacchi).

http://dx.doi.org/10.1016/j.canlet.2016.07.003
0304-3835/

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 [12], but also plays a key role in the establishment of epithelial cell polarity and lumen formation [13,14], two fundamental processes required for normal mammary epithelial morphogenesis. Conversely, apart from evidence of ANXA8 expression in a subpopulation of mammary progenitor cells [15] and its modulation during mouse mammary gland involution [16], 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.

Materials and Methods

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 [11]. 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 for 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 [8,10,11]. 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′-GGAACTTGCATCAGCACTG-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 [8]. 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′-TAAGGCCTGTCGACTTGTTCTTCTGTGCCTCAG-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.

Immunostaining and analysis of 3D acini

Mature 3D HME1 acini (10–12 days) were stained as described [11]. 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 2 h, rinsed with PBS, and stained with DAPI (Sigma, St Luis, MO). Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Golgi apparatus (marker of apico-basal polarity) and integrin (marker of baso-lateral 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).

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).

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 (NCBInr). 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 Bionformatics 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).

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).