Published in final edited form as:

Breast Cancer Res Treat. 2016 June ; 157(2): 241–252. doi:10.1007/s10549-016-3820-1.

Protein disulfide isomerases in the endoplasmic reticulum promote anchorage-independent growth of breast cancer cells

Randi Wise, Sara Duhachek-Muggy, Yue Qi, Michal Zolkiewski, and Anna Zolkiewska

Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, Kansas

Abstract

Purpose—Metastatic breast cancer cells are exposed to stress of detachment from the extracellular matrix (ECM). Cultured breast cancer cells that survive this stress and are capable of anchorage-independent proliferation form mammospheres. The purpose of this study was to explore a link between mammosphere growth, ECM gene expression, and the protein quality control system in the endoplasmic reticulum (ER).

Methods—We compared the mRNA and protein levels of ER folding factors in SUM159PT and MCF10DCIS.com breast cancer cells grown as mammospheres *versus* adherent conditions. Publicly available gene expression data for mammospheres formed by primary breast cancer cells and for circulating tumor cells (CTCs) were analyzed to assess the status of ECM/ER folding factor genes in clinically relevant samples. Knock-down of selected protein disulfide isomerase (PDI) family members was performed to examine their roles in SUM159PT mammosphere growth.

Results—Cells grown as mammospheres had elevated expression of ECM genes and ER folding quality control genes. CTC gene expression data for an index patient indicated that upregulation of ECM and ER folding factor genes occurred at the time of acquired therapy resistance and disease progression. Knock-down of PDI, ERp44, or ERp57, three members of the PDI family with elevated protein levels in mammospheres, in SUM159PT cells partially inhibited the mammosphere growth.

Conclusion—Breast cancer cell survival and growth under detachment conditions require enhanced assistance of the ER protein folding machinery. Targeting ER folding factors, in particular members of the PDI family, may improve the therapeutic outcomes in metastatic breast cancer.

Keywords

Breast cancer; anoikis; mammospheres; endoplasmic reticulum; extracellular matrix

Corresponding author: Anna Zolkiewska, Department of Biochemistry and Molecular Biophysics, Kansas State University, 141 Chalmers Hall, Manhattan, KS 66506, zolkiea@ksu.edu.

Disclosure of potential conflict of interest

The authors declare no conflicts of interest.

Introduction

Metastatic breast cancer is initiated by circulating tumor cells (CTCs) that originate from the primary tumor and spread in the body through the blood circulatory system [1,2]. Early detection and potential targeting of CTCs are of great importance in the effective treatment of metastatic breast cancer [3–5]. CTCs exist as single cells with increased features of epithelial-to-mesenchymal transition (EMT) or as small clusters of 2–50 cells with partial EMT [6,7]. CTCs are detached from the extracellular matrix (ECM) and, in the case of non-clustered CTCs, they are also devoid of cell-cell interactions typical for epithelial cells. Loss of cell-ECM or cell-cell interactions among a majority of cancer cells induces anoikis, a form of apoptotic cell death [8–10]. To acquire a metastatic potential, CTCs must develop resistance to anoikis [11–13]. The adaptive processes that help CTCs evade anoikis include EMT [14,15], metabolic changes and antioxidant activity [16–18], activation of receptor tyrosine kinases [19–22], activation of the NF- κ B pathway [23,24], activation of YAP/TAZ transcription coactivators [25,26], or increased autophagy [27–30].

ECM is a key component of the local tumor microenvironment and has a major impact on each of the classical hallmarks of cancer [31,32]. Since ECM deprivation is the primary trigger of anoikis, stimulated secretion of ECM protein components by detached cells, followed by engagement of their integrin receptors should provide pro-survival signals and promote anchorage-independent growth. However, whether increased expression of ECM genes is a general feature of anoikis-resistant breast cancer cells is currently unknown. Also, a specific role of the secretory protein folding machinery during anchorage-independent growth of breast cancer cells has not been established.

Efficient folding of secreted proteins within the endoplasmic reticulum (ER) depends on the function of a complex protein folding machinery that consists of three classes of proteins: molecular chaperones (e.g., the HSP70-class chaperones BiP and GRP-170, and the HSP90-class chaperone GRP-94), foldases (e.g., disulfide isomerases PDI, ERp57, ERp72, and ERp44), and lectins (e.g., calreticulin and calnexin) [33,34]. ER stress associated with the accumulation of misfolded proteins leads to activation of the unfolded protein response (UPR), which comprises three parallel signaling pathways: PERK, IRE1, and ATF6 [34]. In solid tumors, including breast cancers, an altered metabolism, hypoxic conditions, or signals from the tumor microenvironment, have been shown to induce ER stress, activate the UPR, and lead to increased expression of the ER chaperones [35–38]. Significantly less is known about the status of the UPR and the role of the ER folding machinery during the stage of cancer dissemination via CTCs and the detachment from ECM.

In this study, we found that breast cancer cells grown as mammospheres had elevated expression of a number of ECM genes and ER folding quality control genes. Analysis of a publicly available gene expression data for CTCs in an index patient revealed that upregulation of ECM genes, as well as genes encoding ER folding factors, but not cytoplasmic chaperones, occurred at the time of acquired therapy resistance and disease progression. Despite the elevated expression of ECM and ER folding quality genes during anchorage-independent growth, we did not detect a robust activation of UPR, as only the PERK branch of the UPR, but not IRE1 or ATF6, was activated in mammospheres. An

inducible knock-down of PDI, ERp44, or ERp57, three different members of the protein disulfide isomerase family, resulted in reduced mammosphere formation by SUM159PT cells. Collectively, these results suggest that the anchorage-independent growth of breast cancer cells *in vitro* and anoikis-resistance of CTCs *in vivo* may be associated with an increased flux of secretory proteins through the ER and require an enhanced assistance of the ER folding machinery.

Methods

Data mining

mRNA expression data for ECM and ER quality control genes in attached cells and mammospheres formed by primary tumor cells, described in [39], were retrieved from Gene Expression Omnibus (GEO) using the accession number GSE7515. mRNA levels of ECM genes and chaperones in CTCs from a breast cancer patient, described in [6], were retrieved from GEO using the accession number GSE41245.

Additional methods are described in Electronic Supplementary Material.

Results

To investigate the significance of the ECM and ER protein folding machinery during anchorage-independent growth of breast cancer cells, we compared expression levels of several representative ECM genes, ER chaperones, PDI family members, and lectins in cells grown under two different conditions: without cell-ECM or cell-cell interactions and under normal adherent conditions. For the former conditions, single cell suspensions were plated into ultra-low attachment plates and were incubated in serum-free media (to eliminate the effect of ECM proteins present in the serum). The media were supplemented with essential growth factors and methylcellulose (to prevent cell aggregation). Under these conditions, cells that are anoikis-resistant and survive the initial stress associated with depletion of ECM and cell-cell contacts may proliferate and, over the course of 8–12 days, form mammospheres. Typical mammospheres are composed of ~100–200 cells and reach ~50–100 μm in size [40]. For adherent conditions, cells were plated in tissue culture-treated plates and were incubated with full media containing serum. We utilized two different breast cancer cell lines, SUM159PT and MCF10DCIS.com. The SUM159PT cell line was originally developed from a primary tumor in a patient with estrogen receptor-negative, progesterone receptor-negative, and HER2-negative anaplastic carcinoma of the breast [41]. SUM159PT cells are highly invasive and tumorigenic [42]. The MCF10DCIS.com cell line was derived from a tumor originating from xenografting premalignant MCF10AT cells into severe combined immunodeficient mice [43]. MCF10DCIS.com cells are estrogen receptor/progesterone receptor/HER2-negative, and they form comedo-type ductal carcinoma *in situ* after injection into mouse mammary glands [42]. Both SUM159PT cells and MCF10DCIS.com cells form mammospheres with a relatively high efficiency (Fig. 1a and ref. [44]).

First, using qRT-PCR, we determined that expression levels of several ECM genes: collagen alpha-1(III) chain (*COL3A1*), collagen alpha-2(VI) chain (*COL6A2*), laminin subunit β -1

(*LAMB1*), and fibronectin (*FNI*) were ~5–1,600-fold higher in mammospheres than in adherent SUM159PT and MCF10DCIS.com cells (Fig. 1b), although the extent of upregulation of the ECM transcripts differed between the two cell lines. Second, our analysis of a publicly available gene expression dataset obtained for primary cells isolated from breast tumors (GSE7515, available at <http://www.ncbi.nlm.nih.gov/geo/>) [39] revealed that expression of *COL3A1*, *COL6A2*, *LAMB1*, *FNI* (Fig. 1c) and many other ECM genes (Supplementary Table 3) was significantly higher in mammospheres grown in suspension than in bulk tumors. Among 87 genes encoding collagens, common glycoproteins, and proteoglycans [45] that were differentially expressed between mammospheres and adherent conditions, expression of 57 genes was significantly upregulated in mammospheres (Supplementary Table 3). These results suggested that an increased expression of ECM genes might be a general feature of breast cancer cells grown as mammospheres, which in turn might lead to an increased demand for upregulation of the ER folding machinery.

To begin testing this hypothesis, we compared expression levels of selected ER chaperones and folding factors in cells grown as mammospheres *versus* adherent conditions. The investigated ER chaperones included BiP, GRP-94, and GRP-170; the foldase/PDI family was represented by PDI, ERp44, ERp57, and ERp72; and the tested lectins were calnexin and calreticulin. Using qRT-PCR, we determined that mRNA levels of *PDI*, *ERp44*, and *ERp57* were significantly higher in mammospheres than in adherent SUM159PT and MCF10DCIS.com cells (Fig. 1d). In the lectin family, *Calnexin* mRNA was significantly higher in mammospheres than in adherent SUM159PT cells, but this effect was not replicated in MCF10DCIS.com cells. Among molecular chaperones, *GRP-94* mRNA was elevated in mammospheres formed by MCF10DCIS.com cells, but not in SUM159PT cells. Furthermore, mRNA expression levels of several ER folding factors were significantly elevated in mammospheres formed by primary breast cancer cells, as compared to bulk tumors (Fig. 1e) [39].

To extend our *in vitro* analysis into breast tumors *in vivo* and to determine whether our findings might have a translational relevance, we examined a publicly available dataset containing gene expression profiles of CTCs from an index breast cancer patient undergoing anti-cancer treatment (GSE41245, available at <http://www.ncbi.nlm.nih.gov/geo/>) [6]. This patient was diagnosed with estrogen receptor-positive/progesterone receptor-positive lobular carcinoma and was treated for seven months with inhibitors targeting phosphatidylinositol 3-kinase (PI3K) and MAP kinase kinase (MEK). Initially, the patient responded well to the treatment regimen. Three serial blood specimens were obtained during this time period, CTCs were captured from blood using a microfluidic chip, and RNA sequencing (RNA-seq) was performed to analyze the global gene expression during this initial treatment (Fig. 1f, columns 1–3, designated as “R”). After seven months, the patient showed disease progression (Fig. 1f, column 4, “P”), which was accompanied by an increase in CTCs and the appearance of multicellular CTC clusters. After the treatment regimen was switched to adriamycin chemotherapy, the patient showed a transient response (Fig. 1f, column 5, “R”). Blood samples from 10 healthy donors were used as controls (Fig. 1f, columns 6–15).

As noted previously [6], the expression levels of many ECM genes, including *COL3A1*, *COL6A2*, *LAMB1*, and *FNI*, were strongly upregulated in CTCs at the time of disease

progression and an acquired resistance to PI3K- and MEK-targeted therapy (Fig. 1f). Strikingly, the expression levels of several ER folding factors, including *ERp57* (gene name *PDIA3*), *PDI* (*P4HB*), *BiP* (*HSPA5*), *GRP-94* (*HSP90B1*), and *GRP-170* (*HYOU1*) also surged at the same time point (Fig. 1f). In contrast, the expression levels of cytoplasmic chaperones such as *HSP70* (*HSPA1A* and *HSPA1B*), *HSP90* (*HSP90AA1*), and *HSP110* (*HSPH1*) did not increase during the time of disease progression (Fig. 1f). These data underscore essential differences between the expression patterns of the ER protein folding factors *versus* those in the cytoplasm in CTCs, corroborate our results obtained for mammospheres, and highlight an important role of the ER folding machinery in CTCs during breast cancer progression.

Importantly, a trend of increased expression of the ER folding machinery during anchorage-independent growth of breast cancer cells was also detected at the protein level. We observed that the abundance of PDI, ERp44, ERp57, calnexin, and calreticulin was higher in mammospheres than in adherent cells for both cell lines (Fig. 2). We concluded that the members of the ER folding machinery that were the most consistently upregulated at both the mRNA and protein levels upon the anchorage-independent growth of SUM159PT and MCF10DCIS.com cells were PDI, ERp44, and ERp57, and these three proteins became the focus of our subsequent investigations.

We asked whether elevated mRNA and protein levels of PDI, ERp44, and ERp57 might be associated with the activation of the UPR in mammospheres. Using Western blot analysis of total cell lysates, we observed that the mobility of PERK was reduced in lysates prepared from SUM159PT or MCF10DCIS.com mammospheres, as compared to adherent cells (Fig. 3a), suggesting that PERK might have been phosphorylated and activated during anchorage-independent growth. Moreover, Western blot analysis of phosphorylated eIF2 α , a downstream effector of PERK, revealed an increase of p-eIF2 α in mammospheres *versus* adherent cells in both SUM159PT and MCF10DCIS.com cell lines (Fig. 3a). In contrast, we did not detect *XBPI* mRNA splicing (a downstream effector of IRE1; Fig. 3b) or activation of an ATF6 reporter (Fig. 3c) in mammospheres. Both *XBPI* mRNA splicing and the ATF6 reporter were strongly upregulated by tunicamycin, a well-known UPR inducer, validating our assays (Fig. 3b and c). These results indicated a lack of robust UPR during anchorage-independent growth of SUM159PT and MCF10DCIS.com cells.

Next, we asked whether PDI, ERp44, and ERp57 might actively support the anchorage-independent cell growth and mammosphere formation. We established a stable knock-down of PDI, ERp44, or ERp57 in SUM159PT cells using a doxycycline-inducible TRIPZ lentiviral shRNA vector [46]. This vector produces tightly regulated induction of shRNA expression together with turbo red fluorescent protein (tRFP) in the presence of doxycycline (Fig. 4a). SUM159PT cells stably transduced with the vector containing one of two different shRNAs (#1 and #2) targeting PDI, ERp44, or ERp57, or a non-targeting shControl, were selected with puromycin. Upon treatment of cells for 4 days with 1 μ g/ml of doxycycline, the levels of PDI, ERp44, or ERp57 were reduced by at least 90% in adherent SUM159PT cells (Fig. 4b). Induction of shRNA expression in mammospheres could be readily monitored by visualizing the induction of tRFP (Fig. 4c and Supplementary Fig. 1). Western blotting confirmed that PDI, ERp44, and ERp57 knock-downs were maintained in

SUM159PT cells grown as mammospheres (Fig. 4d). Importantly, a loss of expression of any one of these three members of the PDI family was not compensated for by an increased expression of the other two investigated family members (Fig. 4d).

We determined that PDI, ERp44, or ERp57 knock-down did not have significant effects on the growth of SUM159PT cells under adherent conditions (Fig. 5a). However, in contrast to adherent cells, the established knock-downs significantly impaired the growth of SUM159PT mammospheres. The total volume of mammospheres formed by shPDI, shERp44, or shERp57 cells after 10 days of culture in the presence of doxycycline was significantly lower than the volume of mammospheres formed by the same cells in the absence of doxycycline (Fig. 5b). Doxycycline treatment did not affect the volume of mammospheres formed by shControl cells. In our calculations, we included all spheres with diameter $>15\text{ }\mu\text{m}$. This size cut-off was lower than typically used during mammosphere counting ($50\text{ }\mu\text{m}$, [47]) to account for a broad range of spheres, containing both rapidly and slowly proliferating cells.

We next determined whether the reduction in total mammosphere volumes after PDI, ERp44, or ERp57 knock-down was due to a decreased likelihood of sphere formation (i.e., mammosphere numbers) or to a slower sphere growth (i.e., mammosphere size). First, we observed that while the total numbers of mammospheres were not significantly changed after shPDI, shERp44, or shERp57 knock-down, there was a statistically significant decrease in the numbers of large spheres ($>50\text{ }\mu\text{m}$) formed by shPDI, shERp44, or shERp57 cells in the presence of doxycycline (Fig. 6a). Second, when shRNA-expressing, doxycycline-treated mammospheres were directly visualized by tRFP fluorescence, the mean diameter of shPDI-, shERp44-, or shERp57-expressing spheres was significantly lower than the mean diameter of shControl spheres, indicating an impact on the growth of these spheres (Fig. 6b). Altogether, these results suggest that the three tested PDI family members do not impact the formation of mammospheres, but they play important roles in promoting anchorage-independent growth of SUM159PT cells.

Discussion

The propensity for anchorage-independent growth is a common feature of transformed cells in solid tumors. Anchorage-independent growth requires that cells not only survive under detachment conditions, but that they also actively proliferate under the stress associated with matrix deprivation. The mammosphere assay, which measures anchorage-independent proliferation at clonal cell densities in serum-free media in the presence of well-defined growth factors, is well suited for evaluating the capacity of cells to survive and divide without exogenous ECM support. Mammosphere formation has been also associated with the presence of mammary stem cells and cancer stem-like cells, as these cell populations have increased anoikis-resistance [40]. Our results obtained for two different breast cancer cell lines, as well as the results obtained by others for primary breast tumor cells [39], indicate that mammosphere formation and growth are associated with upregulation of ECM gene expression, and this effect correlates with an increased expression of several ER folding factors, in particular those from the PDI family (see Fig. 1d, 1e, and Fig. 2).

The human PDI family consists of more than 20 members, which differ in the domain arrangement and their abilities to oxidize, reduce, or isomerize disulfide bonds in proteins [33,48]. While there is a large degree of functional redundancy between PDIs, some family members play more specialized functions [49–52]. Our results indicate that among the oxidoreductases, PDI, ERp44, and ERp57 are consistently elevated in mammospheres, suggesting a broad but specialized activation of the PDI family during anchorage-independent growth of breast cancer cells. Furthermore, downregulation of expression of PDI, ERp44, or ERp57 in SUM159PT cells partially inhibits mammosphere growth (see Figs. 5 and 6), suggesting that each of these PDI family members plays an important and non-redundant role during anchorage-independent cell proliferation.

What mechanisms are responsible for the observed elevated levels of selected ER folding factors during mammosphere growth (see Fig. 1d, 1e, and Fig. 2)? One possible explanation might be that an increased secretion of ECM proteins leads to ER stress, activation of the UPR, and transcriptional upregulation of ER folding factors. However, this scenario is not readily reconciled with our data, which indicate that only the PERK branch of the UPR, but not IRE1 or ATF6, was activated in mammospheres (see Fig. 3). Previous studies demonstrated that ATF6 and/or IRE1, but not PERK alone play a critical role in the induction of major ER chaperones during the UPR [53–55]. Thus, the lack of IRE1 or ATF6 activation (see Fig. 3) argues against a robust UPR in mammospheres and cannot account for the observed increased mRNA and protein levels shown in Fig. 1d and Fig. 2. Another explanation for the elevated mRNA and protein levels of selected ER folding factors in mammospheres might be a gradual selection of cells with high expression levels of those genes during anchorage-independent growth. Such cells would have a selective advantage in responding to dramatically increased demands for efficient folding of ECM proteins and an increased likelihood of survival.

EMT is a key regulator of breast tumor progression, as it promotes anoikis-resistance, expansion of cancer stem cell subpopulations, metastatic potential, drug resistance, and disease recurrence [56–59,14]. The EMT gene signature of human mammary epithelial cells (HMLE) partially overlaps with the ECM signature [60], and the hallmark EMT gene set at MolSigDB (<http://www.broadinstitute.org/gsea/msigdb/>) contains many genes of secreted proteins. Not surprisingly, a recent study demonstrated that HMLE cells induced to undergo EMT synthesize and secrete large quantities of ECM proteins [61]. Consistent with their increased secretory output and a higher ER load, HMLE-EMT cells were more sensitive to perturbations of the ER function. For example, downregulation of BiP expression, a major ER chaperone of the HSP70 family, in HMLE-EMT, but not the parental cells, decreased cell survival and caused a significant reduction of cell growth [61].

Our results suggest that the functions of PDIs, and possibly other components of the ER quality control system, in detached cells extend beyond their role in supporting the secretory phenotype of EMT. Firstly, the two breast cancer lines used in our study, MCF10DCIS.com and SUM159PT, are of the basal/claudin-low subtype [62,63] and they already express phenotypic and molecular features associated with EMT. Yet, these cells further upregulate expression (or select for elevated expression) of ER folding factors upon anchorage-independent growth. Secondly, a knock-down of PDI, ERp44, or ERp57 does not affect the

proliferation of attached cells in 2D, but it impairs the anchorage-independent growth of mammospheres. Thus, PDI, ERp44, ERp57, and possibly other PDI family members, help resolve the demand for enhanced ER folding capacity during anchorage-independent growth of cancer cells.

Additional insight into the role of ER folding factors in breast cancer progression, as well as validation of our *in vitro* results, comes from our analysis of the gene expression profiling of CTCs present in the bloodstream of an index breast cancer patient at different time points during the disease progression (see Fig. 1f and ref. [6]). In this patient with metastatic breast cancer, disease progression correlated with upregulation of a number of ER folding factors. While the original report highlighted the upregulation of many EMT genes at the time point of disease progression, the gene set enrichment analysis (GSEA) identified the ECM module as the most significantly enriched among the 170 genes upregulated at that time point [6]. The disease progression was accompanied not only by an increased number of CTCs, by also by the appearance of multicellular CTC clusters [6]. The presence of CTC clusters might have been facilitated, in part, by increased production of ECM proteins, increased proliferation of single CTCs, or both. Whereas it is generally believed that CTCs are quiescent and reside at the G0 phase of the cell cycle [64–66], CTCs may actually represent a more heterogeneous population with regard to the degree of proliferation [67]. Intriguingly, the mRNA levels of Ki-67 and cyclin D3, an important regulator of the G1/S transition during the cell cycle, also increase in CTCs at the time of disease progression in the patient dataset shown in Fig. 1f, together with ECM genes and ER folding factors [6]. We propose, therefore, that the disease progression in this patient might have marked an exit of CTCs from quiescence and initiation of their proliferation. However, more studies involving larger patient populations, with serial isolations of CTCs, are needed to further validate the apparent correlation between increased expression of ECM and ER quality control genes, disease progression, and enhanced CTC proliferation. Furthermore, to directly test a role of ER quality control genes in metastasis, the mammosphere-based model of anoikis resistance utilized in the current study should be extended into *in vivo* approaches, such as experimental metastasis assay employing mouse tail vein injection of breast cancer cells with downregulated expression of ER quality control genes. Given the critical role of CTCs in breast cancer metastasis, better understanding of the mechanisms controlling anoikis resistance of cultured cancer cells and CTC survival *in vivo* should remain a high priority in the field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant R01CA172222 to AZ and Innovative Research Award from Terry C. Johnson Center for Basic Cancer Research at KSU to MZ. This is contribution 15-424-J from Kansas Agricultural Experiment Station.

References

1. Joosse SA, Gorges TM, Pantel K. Biology, detection, and clinical implications of circulating tumor cells. *EMBO Mol Med*. 2015; 7:1–11. DOI: 10.15252/emmm.201303698 [PubMed: 25398926]
2. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to isolation and characterization. *J Cell Biol*. 2011; 192:373–382. DOI: 10.1083/jcb.201010021 [PubMed: 21300848]
3. McInnes LM, Jacobson N, Redfern A, Dowling A, Thompson EW, Saunders CM. Clinical implications of circulating tumor cells of breast cancer patients: role of epithelial-mesenchymal plasticity. *Front Oncol*. 2015; 5:42.doi: 10.3389/fonc.2015.00042 [PubMed: 25767772]
4. Yap TA, Lorente D, Omlin A, Olmos D, de Bono JS. Circulating tumor cells: a multifunctional biomarker. *Clin Cancer Res*. 2014; 20:2553–2568. DOI: 10.1158/1078-0432.CCR-13-2664 [PubMed: 24831278]
5. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. *Cancer Discov*. 2014; 4:650–661. DOI: 10.1158/2159-8290.CD-13-1014 [PubMed: 24801577]
6. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, Concannon KF, Donaldson MC, Sequist LV, Brachtel E, Sgroi D, Baselga J, Ramaswamy S, Toner M, Haber DA, Maheswaran S. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*. 2013; 339:580–584. DOI: 10.1126/science.1228522 [PubMed: 23372014]
7. 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*. 2014; 158:1110–1122. DOI: 10.1016/j.cell.2014.07.013 [PubMed: 25171411]
8. Buchheit CL, Weigel KJ, Schafer ZT. Cancer cell survival during detachment from the ECM: multiple barriers to tumour progression. *Nature Rev Cancer*. 2014; 14:632–641. DOI: 10.1038/nrc3789 [PubMed: 25098270]
9. Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol*. 2003; 5:733–740. DOI: 10.1038/ncb1026 [PubMed: 12844146]
10. Buchheit CL, Rayavarapu RR, Schafer ZT. The regulation of cancer cell death and metabolism by extracellular matrix attachment. *Semin Cell Dev Biol*. 2012; 23:402–411. DOI: 10.1016/j.semcdb.2012.04.007 [PubMed: 22579674]
11. Nagaprashantha LD, Vatsyayan R, Lelsani PC, Awasthi S, Singhal SS. The sensors and regulators of cell-matrix surveillance in anoikis resistance of tumors. *Int J Cancer*. 2011; 128:743–752. DOI: 10.1002/ijc.25725 [PubMed: 20949625]
12. Taddei ML, Giannoni E, Fiaschi T, Chiarugi P. Anoikis: an emerging hallmark in health and diseases. *J Pathol*. 2012; 226:380–393. DOI: 10.1002/path.3000 [PubMed: 21953325]
13. Horbinski C, Mojesky C, Kyprianou N. Live free or die: tales of homeless (cells) in cancer. *Am J Pathol*. 2010; 177:1044–1052. DOI: 10.2353/ajpath.2010.091270 [PubMed: 20639456]
14. Frisch SM, Schaller M, Cieply B. Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis. *J Cell Sci*. 2013; 126:21–29. DOI: 10.1242/jcs.120907 [PubMed: 23516327]
15. Thiery JP, Lim CT. Tumor dissemination: an EMT affair. *Cancer Cell*. 2013; 23:272–273. DOI: 10.1016/j.ccr.2013.03.004 [PubMed: 23518345]
16. Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, Gao S, Puigserver P, Brugge JS. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature*. 2009; 461:109–113. DOI: 10.1038/nature08268 [PubMed: 19693011]
17. Grassian AR, Metallo CM, Coloff JL, Stephanopoulos G, Brugge JS. Erk regulation of pyruvate dehydrogenase flux through PDK4 modulates cell proliferation. *Genes Dev*. 2011; 25:1716–1733. DOI: 10.1101/gad.16771811 [PubMed: 21852536]

18. Grassian AR, Coloff JL, Brugge JS. Extracellular matrix regulation of metabolism and implications for tumorigenesis. *Cold Spring Harbor Symp Quantit Biol.* 2011; 76:313–324. DOI: 10.1101/sqb.2011.76.010967
19. Whelan KA, Schwab LP, Karakashev SV, Franchetti L, Johannes GJ, Seagroves TN, Reginato MJ. The oncogene HER2/neu (ERBB2) requires the hypoxia-inducible factor HIF-1 for mammary tumor growth and anoikis resistance. *J Biol Chem.* 2013; 288:15865–15877. DOI: 10.1074/jbc.M112.426999 [PubMed: 23585570]
20. Douma S, Van Laar T, Zevenhoven J, Meuwissen R, Van Garderen E, Peeper DS. Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. *Nature.* 2004; 430:1034–1039. DOI: 10.1038/nature02765 [PubMed: 15329723]
21. Grassian AR, Schafer ZT, Brugge JS. ErbB2 stabilizes epidermal growth factor receptor (EGFR) expression via Erk and Sprouty2 in extracellular matrix-detached cells. *J Biol Chem.* 2011; 286:79–90. DOI: 10.1074/jbc.M110.169821 [PubMed: 20956544]
22. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol.* 2001; 3:785–792. DOI: 10.1038/ncb0901-785 [PubMed: 11533657]
23. Toruner M, Fernandez-Zapico M, Sha JJ, Pham L, Urrutia R, Egan LJ. Antianoikis effect of nuclear factor-kappaB through up-regulated expression of osteoprotegerin, BCL-2, and IAP-1. *J Biol Chem.* 2006; 281:8686–8696. DOI: 10.1074/jbc.M512178200 [PubMed: 16407217]
24. Park SH, Riley P, Frisch SM. Regulation of anoikis by deleted in breast cancer-1 (DBC1) through NF-kappaB. *Apoptosis.* 2013; 18:949–962. DOI: 10.1007/s10495-013-0847-1 [PubMed: 23588592]
25. Vigneron AM, Ludwig RL, Vousden KH. Cytoplasmic ASPP1 inhibits apoptosis through the control of YAP. *Genes Dev.* 2010; 24:2430–2439. DOI: 10.1101/gad.1954310 [PubMed: 21041411]
26. Zhao B, Li L, Wang L, Wang CY, Yu J, Guan KL. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes Dev.* 2012; 26:54–68. DOI: 10.1101/gad.173435.111 [PubMed: 22215811]
27. Kenific CM, Debnath J. Cellular and metabolic functions for autophagy in cancer cells. *Trends Cell Biol.* 2015; 25:37–45. DOI: 10.1016/j.tcb.2014.09.001 [PubMed: 25278333]
28. Avivar-Valderas A, Salas E, Bobrovnikova-Marjon E, Diehl JA, Nagi C, Debnath J, Aguirre-Ghiso JA. PERK integrates autophagy and oxidative stress responses to promote survival during extracellular matrix detachment. *Mol Cell Biol.* 2011; 31:3616–3629. DOI: 10.1128/MCB.05164-11 [PubMed: 21709020]
29. Avivar-Valderas A, Bobrovnikova-Marjon E, Alan Diehl J, Bardeesy N, Debnath J, Aguirre-Ghiso JA. Regulation of autophagy during ECM detachment is linked to a selective inhibition of mTORC1 by PERK. *Oncogene.* 2013; 32:4932–4940. DOI: 10.1038/onc.2012.512 [PubMed: 23160380]
30. Debnath J. Detachment-induced autophagy during anoikis and lumen formation in epithelial acini. *Autophagy.* 2008; 4:351–353. [PubMed: 18196957]
31. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol.* 2012; 196:395–406. DOI: 10.1083/jcb.201102147 [PubMed: 22351925]
32. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. *EMBO Rep.* 2014; 15:1243–1253. DOI: 10.15252/embr.201439246 [PubMed: 25381661]
33. Rutkevich LA, Williams DB. Participation of lectin chaperones and thiol oxidoreductases in protein folding within the endoplasmic reticulum. *Curr Opin Cell Biol.* 2011; 23:157–166. DOI: 10.1016/j.ceb.2010.10.011 [PubMed: 21094034]
34. Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem.* 2005; 74:739–789. DOI: 10.1146/annurev.biochem.73.011303.074134 [PubMed: 15952902]
35. Wang M, Kaufman RJ. The impact of the endoplasmic reticulum protein-folding environment on cancer development. *Nature Rev Cancer.* 2014; 14:581–597. DOI: 10.1038/nrc3800 [PubMed: 25145482]

36. Gutierrez T, Simmen T. Endoplasmic reticulum chaperones and oxidoreductases: critical regulators of tumor cell survival and immunorecognition. *Front Oncol.* 2014; 4:291.doi: 10.3389/fonc.2014.00291 [PubMed: 25386408]
37. Luo B, Lee AS. The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene.* 2013; 32:805–818. DOI: 10.1038/onc.2012.130 [PubMed: 22508478]
38. Clarke R, Cook KL, Hu R, Facey CO, Tavassoly I, Schwartz JL, Baumann WT, Tyson JJ, Xuan J, Wang Y, Warri A, Shajahan AN. Endoplasmic reticulum stress, the unfolded protein response, autophagy, and the integrated regulation of breast cancer cell fate. *Cancer Res.* 2012; 72:1321–1331. DOI: 10.1158/0008-5472.CAN-11-3213 [PubMed: 22422988]
39. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, Rimm DL, Wong H, Rodriguez A, Herschkowitz JI, Fan C, Zhang X, He X, Pavlick A, Gutierrez MC, Renshaw L, Larionov AA, Faratian D, Hilsenbeck SG, Perou CM, Lewis MT, Rosen JM, Chang JC. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci USA.* 2009; 106:13820–13825. 0905718106 [pii]. DOI: 10.1073/pnas.0905718106 [PubMed: 19666588]
40. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.* 2003; 17:1253–1270. [pii]. DOI: 10.1101/gad.106180317/10/1253 [PubMed: 12756227]
41. Flanagan L, Van Weelden K, Ammerman C, Ethier SP, Welsh J. SUM-159PT cells: a novel estrogen independent human breast cancer model system. *Breast Cancer Res Treat.* 1999; 58:193–204. [PubMed: 10718481]
42. Barnabas N, Cohen D. Phenotypic and molecular characterization of MCF10DCIS and SUM breast cancer cell lines. *Int J Breast Cancer.* 2013; 2013:872743.doi: 10.1155/2013/872743 [PubMed: 23401782]
43. Miller FR, Santner SJ, Tait L, Dawson PJ. MCF10DCIS.com xenograft model of human comedo ductal carcinoma *in situ*. *J Natl Cancer Inst.* 2000; 92:1185–1186.
44. Li H, Duhachek-Muggy S, Dubnicka S, Zolkiewska A. Metalloproteinase-disintegrin ADAM12 is associated with a breast tumor-initiating cell phenotype. *Breast Cancer Res Treat.* 2013; 139:691–703. DOI: 10.1007/s10549-013-2602-2 [PubMed: 23771733]
45. Hynes RO, Naba A. Overview of the matrisome--an inventory of extracellular matrix constituents and functions. *Cold Spring Harb Perspect Biol.* 2012; 4:a004903.doi: 10.1101/cshperspect.a004903 [PubMed: 21937732]
46. Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, Sheth N, Bradshaw J, Burchard J, Kulkarni A, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, Hannon GJ. Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet.* 2005; 37:1281–1288. DOI: 10.1038/ng1650 [PubMed: 16200065]
47. Shaw FL, Harrison H, Spence K, Ablett MP, Simoes BM, Farnie G, Clarke RB. A detailed mammosphere assay protocol for the quantification of breast stem cell activity. *J Mammary Gland Biol Neoplasia.* 2012; 17:111–117. DOI: 10.1007/s10911-012-9255-3 [PubMed: 22665270]
48. Feige MJ, Hendershot LM. Disulfide bonds in ER protein folding and homeostasis. *Curr Opin Cell Biol.* 2011; 23:167–175. DOI: 10.1016/j.ceb.2010.10.012 [PubMed: 21144725]
49. Solda T, Garbi N, Hammerling GJ, Molinari M. Consequences of ERp57 deletion on oxidative folding of obligate and facultative clients of the calnexin cycle. *J Biol Chem.* 2006; 281:6219–6226. DOI: 10.1074/jbc.M513595200 [PubMed: 16407314]
50. Rutkevich LA, Cohen-Doyle MF, Brockmeier U, Williams DB. Functional relationship between protein disulfide isomerase family members during the oxidative folding of human secretory proteins. *Mol Biol Cell.* 2010; 21:3093–3105. DOI: 10.1091/mbc.E10-04-0356 [PubMed: 20660153]
51. Anelli T, Sannino S, Sitia R. Proteostasis and “redoxstasis” in the secretory pathway: Tales of tails from ERp44 and immunoglobulins. *Free Radic Biol Med.* 2015; 83:323–330. DOI: 10.1016/j.freeradbiomed.2015.02.020 [PubMed: 25744412]

52. Sannino S, Anelli T, Cortini M, Masui S, Degano M, Fagioli C, Inaba K, Sitia R. Progressive quality control of secretory proteins in the early secretory compartment by ERp44. *J Cell Sci.* 2014; 127:4260–4269. DOI: 10.1242/jcs.153239 [PubMed: 25097228]
53. Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H, Harada A, Mori K. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev Cell.* 2007; 13:365–376. DOI: 10.1016/j.devcel.2007.07.018 [PubMed: 17765680]
54. Shoulders MD, Ryno LM, Genereux JC, Moresco JJ, Tu PG, Wu C, Yates JR, Su AI, Kelly JW, Wiseman RL. Stress-independent activation of XBP1s and/or ATF6 reveals three functionally diverse ER proteostasis environments. *Cell Rep.* 2013; 3:1279–1292. DOI: 10.1016/j.celrep.2013.03.024 [PubMed: 23583182]
55. Lu PD, Jousse C, Marciniak SJ, Zhang Y, Novoa I, Scheuner D, Kaufman RJ, Ron D, Harding HP. Cytoprotection by pre-emptive conditional phosphorylation of translation initiation factor 2. *EMBO J.* 2004; 23:169–179. DOI: 10.1038/sj.emboj.7600030 [PubMed: 14713949]
56. Brabletz T. To differentiate or not--routes towards metastasis. *Nature Rev Cancer.* 2012; 12:425–436. DOI: 10.1038/nrc3265 [PubMed: 22576165]
57. De Craene B, Berx G. Regulatory networks defining EMT during cancer initiation and progression. *Nature Rev Cancer.* 2013; 13:97–110. DOI: 10.1038/nrc3447 [PubMed: 23344542]
58. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nature Rev Cancer.* 2009; 9:265–273. DOI: 10.1038/nrc2620 [PubMed: 19262571]
59. Creighton CJ, Chang JC, Rosen JM. Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. *J Mamm Gland Biology Neoplasia.* 2010; 15:253–260. DOI: 10.1007/s10911-010-9173-1
60. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, Hartwell K, Onder TT, Gupta PB, Evans KW, Hollier BG, Ram PT, Lander ES, Rosen JM, Weinberg RA, Mani SA. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci USA.* 2010; 107:15449–15454. DOI: 10.1073/pnas.1004900107 [PubMed: 20713713]
61. Feng YX, Sokol ES, Del Vecchio CA, Sanduja S, Claessen JH, Proia TA, Jin DX, Reinhardt F, Ploegh HL, Wang Q, Gupta PB. Epithelial-to-mesenchymal transition activates PERK-eIF2alpha and sensitizes cells to endoplasmic reticulum stress. *Cancer Discov.* 2014; 4:702–715. DOI: 10.1158/2159-8290.CD-13-0945 [PubMed: 24705811]
62. Behbod F, Kittrell FS, LaMarca H, Edwards D, Kerbawy S, Heestand JC, Young E, Mukhopadhyay P, Yeh HW, Allred DC, Hu M, Polyak K, Rosen JM, Medina D. An intraductal human-in-mouse transplantation model mimics the subtypes of ductal carcinoma in situ. *Breast Cancer Res.* 2009; 11:R66. bcr2358 [pii]. doi: 10.1186/bcr2358 [PubMed: 19735549]
63. Prat A, Karginova O, Parker JS, Fan C, He X, Bixby L, Harrell JC, Roman E, Adamo B, Troester M, Perou CM. Characterization of cell lines derived from breast cancers and normal mammary tissues for the study of the intrinsic molecular subtypes. *Breast Cancer Res Treat.* 2013; 142:237–255. DOI: 10.1007/s10549-013-2743-3 [PubMed: 24162158]
64. Giancotti FG. Mechanisms governing metastatic dormancy and reactivation. *Cell.* 2013; 155:750–764. DOI: 10.1016/j.cell.2013.10.029 [PubMed: 24209616]
65. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: an awakening field. *Nature Rev Cancer.* 2014; 14:611–622. DOI: 10.1038/nrc3793 [PubMed: 25118602]
66. Muller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, Janicke F, Pantel K. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. *Clin Cancer Res.* 2005; 11:3678–3685. DOI: 10.1158/1078-0432.CCR-04-2469 [PubMed: 15897564]
67. Aurilio G, Sciandivasci A, Munzone E, Sandri MT, Zorzino L, Cassatella MC, Verri E, Rocca MC, Nole F. Prognostic value of circulating tumor cells in primary and metastatic breast cancer. *Expert Rev Anticancer Ther.* 2012; 12:203–214. DOI: 10.1586/era.11.208 [PubMed: 22316368]

Fig. 1. Elevated expression of ECM and ER quality control genes in mammospheres. **a** Representative images of SUM159PT and MCF10DCIS.com cells grown under adherent conditions (ADH) or in suspension as mammospheres for 10 days (MMS). **b** mRNA ratios of selected ECM genes in mammospheres (MMS) *versus* adherent (ADH) SUM159PT and MCF10DCIS.com cells were evaluated by qRT-PCR; *ACTIN* was used as a normalization control. The results represent the mean values from at least 2 independent experiments, $\pm$ S.E.M. Asterisks indicate mRNA fold changes significantly greater than 1 (*, $P < 0.05$, one sample *t*-test). **c** mRNA expression levels of selected ECM genes in primary breast tumor cells isolated from 11 breast cancer patients, grown as mammospheres (M) or adherent cultures (A), based on ref. [39]. The results of microarray profiling were retrieved from Gene Expression Omnibus (GEO) using the accession number GSE7515. Downloaded data were $\log_2$ -transformed and mean-centered. **d** mRNA levels of selected ER folding factors in SUM159PT and MCF10DCIS.com cells were quantified by qRT-PCR; *ACTIN* was used as

normalization control. The average fold change of each mRNA in MMS *versus* ADH cells $\pm$ S.E.M was calculated based on 3 (SUM159PT) or 2 (MCF10DCIS.com) independent experiments. Asterisks indicate mRNA fold changes significantly greater than 1 (*, $P < 0.05$, one sample *t*-test). **e** Comparison of the mRNA expression levels of selected ER folding factors in mammospheres (M) and adherent cultures (A) of primary breast cancer cells. The data were retrieved and processed as in panel **c**. **f** Elevated expression of ECM genes coincides with upregulated expression of the ER folding machinery genes in circulating tumor cells (CTCs). mRNA expression levels of ECM genes, ER chaperones/foldases/lectins, and selected cytoplasmic chaperones in CTCs from a breast cancer patient (columns 1–5) and identically processed blood specimens from 10 healthy donors (columns 6–15), based on ref. [6]. The index breast cancer patient was sampled at five time points during treatment. At points 1, 2, and 3, there was a response (R) to a targeted therapy. Time point 4 corresponds to the therapy resistance and disease progression (P). After switching to chemotherapy, a response was observed at time point 5. The expression data were retrieved from GEO:GSE41245. Gene names are on the right, gene name aliases are provided in parentheses. The expression values for each gene were normalized between 0 and 1, with the minimal value out of the 15 column values set as 0 and the maximal value set as 1

Fig. 2.

Comparison of the protein levels of selected ER folding factors in mammospheres *versus* adherent cells. SUM159PT (**a**) or MCF10DCIS.com (**b**) cell lysates were analyzed by Western blotting using antibodies specific for the following ER folding factors: PDI, 57 kDa; ERp44, 44 kDa; ERp57, 57 kDa; ERp72, 72 kDa; Calreticulin, 48 kDa; Calnexin, 80 kDa; BiP, 78 kDa; GRP-94, 94 kDa; GRP-170, 170 kDa. Tubulin is a gel-loading control. In panel **a**, the following ER proteins were analyzed in the same gel and share the same loading control: PDI and GRP-170; ERp44 and Calnexin. In panel **b**, the following ER proteins were analyzed in the same gel and share the same loading control: ERp57 and GRP-170; ERp44, Calreticulin, Calnexin, and BiP; PDI and ERp72. Band intensities from 3 independent experiments were quantified using ImageJ. The average fold change in the ER folding factor levels in mammospheres *versus* adherent cells $\pm$ S.E.M is indicated in each panel

Fig. 3.

Evaluation of the three branches of the unfolded protein response in mammospheres (MMS) and adherent (ADH) SUM159PT and MCF10DCIS.com cells. **a** Activation of the PERK branch of the UPR was assessed based on a change in mobility of PERK in immunoblots and the extent of phosphorylation of eIF α (p-eIF2 α). **b** The status of the IRE1 branch of the UPR was evaluated by measuring the extent of splicing of *XBPI* mRNA by semi-quantitative PCR. “u XBP1” and “s XBP1” indicate the un-spliced and spliced forms of *XBPI* mRNA, respectively. **c** The status of the ATF6 branch of the UPR was probed using an ATF6 reporter. Cells were co-transfected with ATF6-firefly luciferase reporter and control *Renilla* luciferase plasmids, and the relative luciferase activity was determined using a dual luciferase assay. Tunicamycin (Tun) was used as a positive control for the ER stress-

mediated activation of IRE1 and ATF6. Mammospheres were grown for 10 days in experiments shown in panels **a** and **b**, and for 3 days in panel **c**

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. PDI, ERp44, or ERp57 knock-down in SUM159PT cells. **a** Diagram of the doxycycline-inducible lentiviral shRNA construct (TRIPZ) targeting PDI, ERp44, or ERp57, or a construct harboring a non-targeting shRNA (shControl). 5'-LTR, 5'-long terminal repeat; TRE, tetracycline-inducible promoter; tRFP, turbo red fluorescent protein; miR30-5' and shERp57/shPDI/shERp44, micro-RNA-30 adapted shRNA targeting ERp57/PDI/ERp44; Ubc, human ubiquitin C promoter; rtTA3, reverse tetracycline-transactivator 3; IRES, internal ribosomal entry site; Puro, puromycin resistance gene; 3'-LTR, 3'-long terminal repeat. **b** PDI, ERp44, or ERp57 knock-down in adherent SUM159PT cells. Cells were stably transduced with two different shRNA constructs targeting PDI, ERp44, or ERp57, or with shControl. Cells were incubated for 4 days without or with 1 μ g/ml doxycycline (Dox), and the levels of indicated proteins in total cell lysates were evaluated by Western blotting; tubulin is a gel-loading control. **c** Monitoring doxycycline-inducible shRNA expression in mammospheres based on the RFP fluorescence. SUM159PT-shControl cells were pre-incubated for 4 days without or with doxycycline and were further grown for 10 days in mammosphere media in the absence or presence of doxycycline. Representative phase contrast and fluorescent images are shown. Analogous levels of RFP fluorescence were obtained for shPDI, shERp44, or shERp57-expressing cells (see Supplementary Fig. 1). **d**

PDI, ERp44, or ERp57 knock-down is maintained in SUM159PT cells grown as mammospheres and is not compensated for by other members of the PDI family

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5.

The effect of PDI, ERp44, or ERp57 knock-down on cell growth under adherent *versus* mammosphere conditions. **a** PDI, ERp44, or ERp57 knock-down does not have a significant effect on the growth rate of adherent cells. SUM159PT cells stably transduced with two different shRNA constructs targeting PDI, ERp44, or ERp57, or with a construct harboring a non-targeting shRNA (shControl) were seeded into 96-well plates at the density of 3000 cells/well and incubated under adherent conditions in the absence or presence of 1 μ g/ml doxycycline (Dox). Cell viability at the indicated times was measured using the CellTiter Glo assay. The results (arbitrary units, a.u.) are shown as means from 3 determinations, $\pm$ S.E.M. **b** PDI, ERp44, or ERp57 knock-down reduces the growth of cells under mammosphere conditions. Each cell type was seeded into 4 wells of a 24-well plate. After 10 days, spheres were visualized by phase contrast imaging, counted using ImageJ, and the total sphere volume was estimated as described in Methods. The results are shown as mean values obtained from 3 independent experiments. *, $P < 0.05$.

Fig. 6.

The effect of PDI, ERp44, or ERp57 knock-down on mammosphere formation and size. **a** The number of spheres in the absence or presence of doxycycline. Each cell type was seeded into 4 wells of a 24-well plate. After 10 days, spheres were visualized by phase contrast imaging and counted using ImageJ, as described in Methods. The numbers of mammospheres with diameters >50 μ m are indicated by solid color bars. The numbers of mammospheres with diameters <50 μ m and >15 μ m are shown with white bars. The results are shown as mean values obtained from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$. **b** Size distribution of RFP-positive spheres. RFP-positive spheres formed in the presence of doxycycline were visualized by fluorescence microscopy. Sphere diameters were determined using ImageJ. For each cell type, a box-and-whisker plot of the diameters of 100 randomly selected spheres formed under identical conditions is shown. ****, $P < 0.0001$