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Increased LAMP2A levels correlate with a shorter disease-free survival of HER2 negative breast cancer patients and increased breast cancer cell viability

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ABSTRACT

Chaperone Mediated Autophagy (CMA) is a selective autophagy pathway deregulated in many cancers. In this study, we were aiming at understanding the importance of CMA in breast cancer. To this end, we examined the expression of the CMA markers HSP8 and LAMP2A in different breast cancer cell lines and found a wide range of LAMP2A expression levels across the cell lines analyzed. Next, we applied a specific immunohistochemical staining protocol to a tissue microarray derived from a cohort of 365 breast cancer patients. Therefore, we were able to find a correlation of high LAMP2A but not HSPA8 (HSC70) with worse disease free survival in patients with HER2 negative tumors ($p = 0.026$) which was independent prognostic parameter from pT category, pN category and grading in a multivariate model (HR = 1.889; 95% CI = 1.039–3.421; $p = 0.037$). In line, low LAMP2A levels decrease proliferation of the breast cancer cell lines T47D and MCF-7 *in* vitro. Our data suggest that LAMP2A supports a more severe breast cancer cell phenotype.

1. Introduction

Breast cancer is the most frequent cancer and the leading cause of cancer-related deaths in females worldwide [1]. In routine diagnostic settings, breast cancers are divided into different molecular subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2) [2], which has implications on systemic tumor treatment.

Early and localized breast cancers are curable and overall survival is relatively high, while metastatic disease courses are difficult to treat, which results in a low overall survival. Consequently, there is an urgent need for better treatment options and a better understanding of resistance mechanisms. One pathway, which has been related to treatment resistance [3] is autophagy. Autophagy is an essential intracellular degradation and recycling process [4]. There are three major forms, namely macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy [5]. There is a growing number of reports relating autophagy alterations to a variety of diseases [6,7] including cancer [8–10]. Interestingly, CMA is upregulated in different cancer cell lines compared to normal counter parts. In agreement, concentrations of the CMA-related protein LAMP2A are increased in different human tumors compared to normal tissue [8]. On the other hand, CMA can prevent cellular transformation, by, for example, targeting oncogenic signaling components for degradation [11].

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On a molecular level, CMA delivers soluble cytosolic proteins across the lysosomal membrane. Cytosolic proteins harboring a KFERQ pentapeptide motif are recognized by a co-chaperone complex including the heat shock protein family A (Hsp70) member 8 (HSP8; also known as heat shock cognate 70 (HSP8)) and are targeted to the lysosomal membrane. Once at the lysosomal membrane, CMA substrates interact with the cytosolic tail of the lysosome-associated membrane protein type 2A (LAMP2A) [12] triggering multimerization of LAMP2A and subsequent substrate translocation. Lysosomal HSPA8 is required to complete the translocation of CMA target proteins [13,14]. In the lysosome, substrate proteins are degraded by proteases and amino acids are recycled. LAMP2A is the rate-limiting factor for CMA activity [15]. Consequently, LAMP2A abundance is frequently used as an indirect marker of CMA activity, and interventions that change lysosomal levels of this receptor are used to modulate CMA activity [15,16].

In the present study, we aimed at investigating the expression of the CMA markers LAMP2A and HSP8 in breast cancer to gain insight to the role of CMA in this disease.

2. Materials and methods

2.1. Cell lines and culture conditions

MCF-7 breast cancer cells were obtained from the American Type Tissue Culture (ATCC, Wesel, Germany), MDA-MB-231 and T47D were a kind gift of Prof. C. Lengerke (Department of Biomedicine, University of Basel, Switzerland) and all other breast cancer cell lines (HCC-1428, HC-1500, SKBR3, MDA-MB-361, MDA-MB-453, MDA-MB-157, MDA-MB-436, Cal-51, Cal-85-1, HCC-70) were a kind gift of Prof, E. Garattini (Mario Negri Institute for Pharmacological Research, Milano, Italy). Detailed cell culture conditions can be found in **Supplementary methods**.

2.2. Western blotting

The procedure is described in detail elsewhere [17]. The following antibodies and dilutions were used: Anti-LC3B (Novus Biologicals, NB600-1384) 1:1000 in 5% Milk or anti-LAMP2A (Abcam, ab18528) 1:1000 in 5% BSA.

2.3. Immunofluorescence staining and confocal microscopy

Cells were seeded on 18 × 18mm glass slides inserted to 6-well plates at a density of 0.3 x 10^6 cells/well. 24h post-seeding cells were stained for LAMP1 (Thermofisher (eBioscience), 14-1079-80) and LAMP2A (Abcam, ab18528) according to the protocol described elsewhere [18]. Pictures were taken on a confocal microscope (Olympus FluoView1000 with a 63x objective).

2.4. Lentiviral vectors

pLKO.1-puro lentiviral vector expressing shRNA targeting *HSP8* (sh*HSC70*_1: NM_006597.3–2040s21c1) were purchased from Sigma-Aldrich. Sequences of shRNAs to target LAMP2A (shRNA#1: CTGCAACCTGATTGATTA; shRNA#2: GGCAGGAGTACTTATTCTAGT; shRNA#3: GACTGCAGTGCAGATGACG) were derived from Massey et al., 2006 and Han et al., 2017 [10,19] and cloned with an U6 promoter into an EF1α-IRES-Hygro lentiviral vector backbone. The pSIN-PA-mCherry-KFERQ-NE [20] construct was as a gift from Shu Leong Ho (Addgene plasmid # 102365). Lentivirus production and transduction were done as described [21,22].

2.5. PA-mCherry-KFREQ degradation assay

T47D and MCF-7 stably expressing the PA-mCherry-KFERQ construct were seeded at a density of 2x10^5 cells/well of a 6-well plate. The day after, cells were exposed to UV light for 2.5 min and media was changed. In each experiment, we included inactivated cells as a negative control. 0, 6, 24 and 30 h post-activation, cells were detached using Accutase, washed with PBS containing 1% FBS and fixed with 4% PFA for 10 min. After fixation, cells were

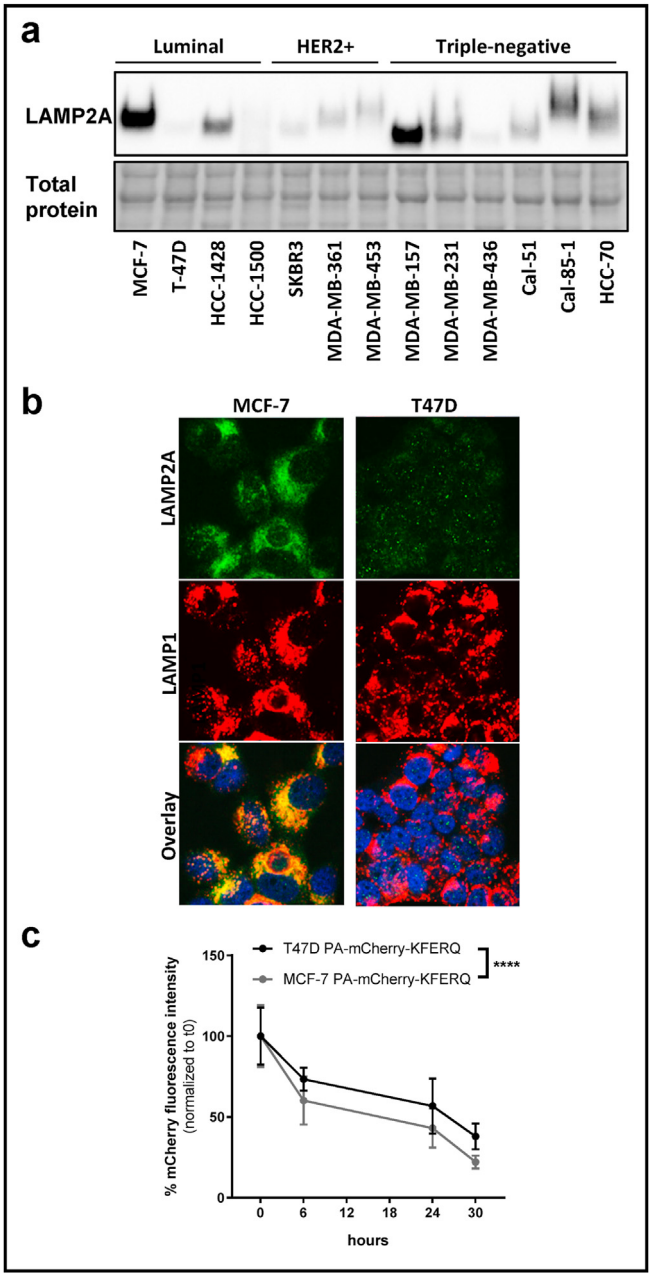

Fig. 1. LAMP2A expression levels differ across a panel of breast cancer cell lines. **(a)** Proteins from 13 different breast cancer cell lines were harvested and Western blot for LAMP2A was performed. n = 2. Luminal, Human epidermal growth factor receptor 2 (HER2), and triple negative breast cancer subtypes are indicated. Luminalll, and . **(b)** Immunofluorescent staining of LAMP2A (green) and LAMP1 (red) in MCF-7 and T47D. **(c)** MCF-7 and T47D stably expressing the PA-mCherry-KFERQ construct were exposed to UV to switch on mCherry fluorescence and degradation was monitored by FACS analysis at indicated time points. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

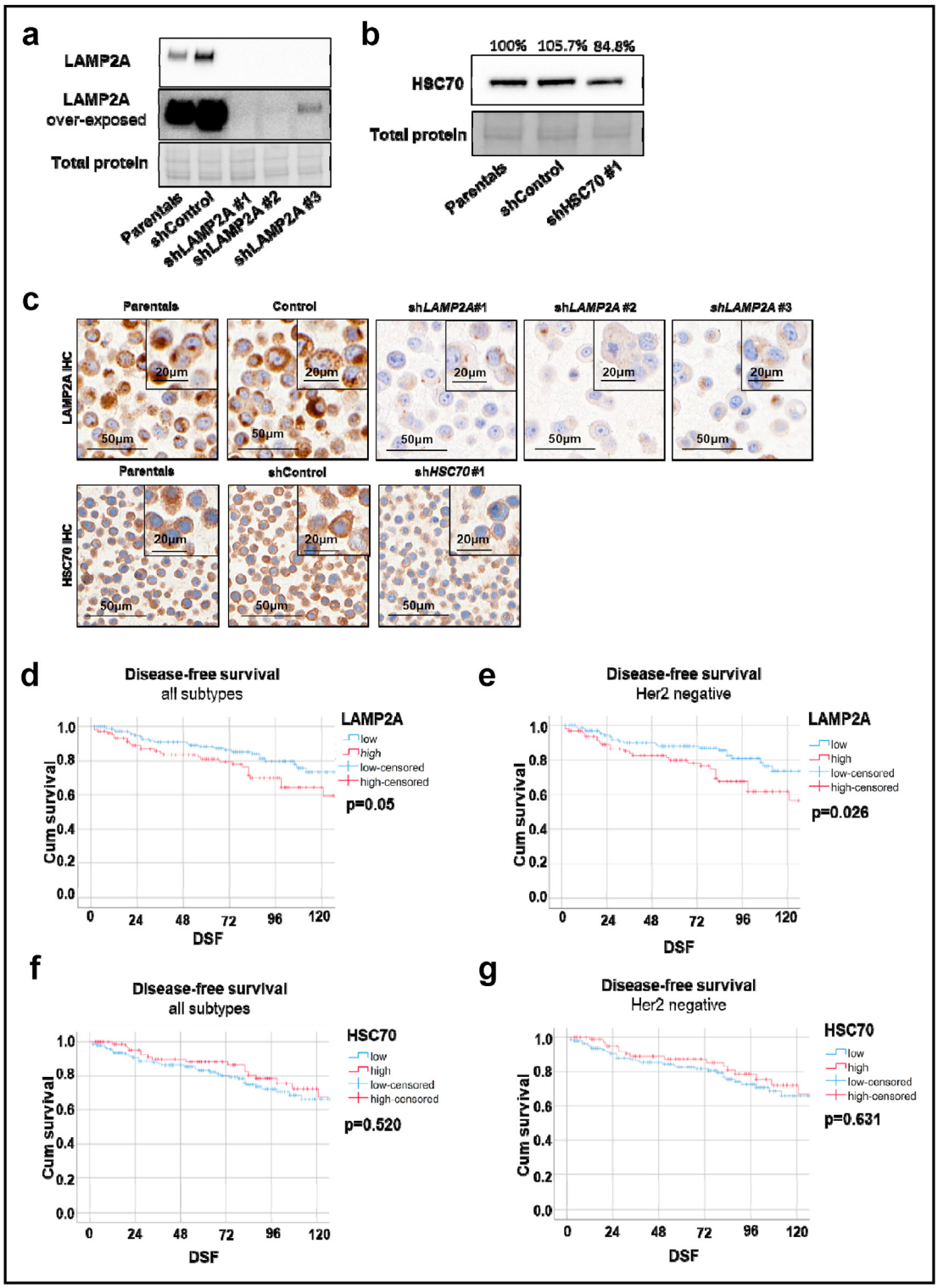

Fig. 2. LAMP2A staining correlates with a decreased disease-free survival in HER2-negative breast cancer patients. **(a–d)** MCF-7 cells were transduced with different lentiviral vectors containing either, an empty vector control (Control), a scrambled shRNA (shCtrl) or shRNAs targeting LAMP2A or HSP8, respectively. After selection cells were subjected to **(a–b)** Western blot analysis or **(c)** immunohistochemistry (IHC) for LAMP2A (upper panel) or HSP8 (lower panel). **(d–g)** Disease free survival curves for the expression of (**d-e**) LAMP2A and (**f-g**) HSP8 (HSC70) in breast cancer patients are shown. DFS; disease free survival, Cum survival; cumulative survival.

Table 1
Patient collective.

Parameter		Frequency	%
Age at diagnosis	median 67 yrs (range:31–98)		
Histological subtypes	No special type (NST)	253	71.1
	Lobular	51	14.3
	Other	52	14.6
Grading	G1	41	11.5
	G2	194	54.5
	G3	121	34.0
pT category	pT1	150	42.1
	pT2	167	46.9
	pT3	21	5.9
	pT3	18	5.1
pN category	pN0	146	41
	pN1mi	18	5.1
	pN1	102	28.6
	pN2	27	7.6
	pN3	22	6.2
	no lymph nodes		11.5
Molecular Subtype	Lum A	240	67.4
	Lum B	68	19.1
	Her2	12	3.4
	basal/oth	36	10.1
Her2 status	Negative	320	89.9
	Positive	36	10.1
Anti Hormone Therapy	Yes	229	64.3
	No	60	16.9
	no data	67	18.8
chemotherapy	Yes	123	34.6
	No	168	47.2
	no data	65	18.3
anti Her2 therapy	Yes	21	5.9
	No	270	75.8
	no data	65	18.3
	Total	356	100

washed, resuspended in PBS/1% FBS and stored at 4 °C until FACS analysis. FACS analysis was performed on a BD LSR-II flow cytometer (BD Biosciences). Living and single cells were gated based on FSC and SSC and SSC-H and SSC-W respectively. Living, single cells were analyzed for mCherry median fluorescent intensity.

2.6. CAM assay

A schematic overview of the procedure is shown in Supplementary Fig. 3 and methods.

3. Results and discussion

3.1. LAMP2A is differently expressed across a panel of breast cancer cell lines

First, we aimed at assessing LAMP2A expression in a panel of breast cancer cell lines. We found a high degree of variation in the levels of LAMP2A expression across different cell lines from different molecular subtypes (Fig. 1a). LAMP2A bands were normalized to total protein and quantified using ImageJ and the expression in MCF7 cells was set to 100%. LAMP2A expression varied from less than 10%, as seen in T47D (7.2%), HCC1500 (9.1%), SKBR3 (5.2%) and MDA-MB-436 (3.9%) to 100% and more in MCF-7 (100%) and MDA-MB-157 (108.6%). LAMP2A protein expression in MCF-7 and T47D were confirmed by immunofluorescence (Fig. 1b). In agreement with the Western Blot data, MCF-7 cells display high and T47D low levels of LAMP2A, respectively. Importantly, LAMP2A is found as a dot-like staining in the cytoplasm, as seen for another lysosomal marker, namely LAMP1 (Fig. 1b), suggesting that LAMP2A is present at lysosomes. Next, we assessed CMA activity in MCF-7 and T47D by analyzing the degradation of a photoactivatable mCherry construct containing a KFERQ motif [20]. As expected from the high levels of LAMP2A, degradation of the CMA targeted mCherry construct was significantly faster in MCF-7 than T47D cells. The effect was already observed at 6h post activation of the mCherry signal (Fig. 1c). We conclude, that CMA activity correlates with LAMP2A expression in MCF-7 and T47D breast cancer cell lines. Since the effect starts at 6h post activation, we exclude that proliferation differences play a role. Although, we cannot rule out that the construct is degraded via other pathways.

3.2. LAMP2A, but not HSP8 staining correlates with a decreased disease-free survival in breast cancer

Next, we aimed at analyzing our primary breast cancer cohort for the CMA markers LAMP2A and HSP8. First we confirmed the specificity of a previously established IHC staining protocol for the two CMA-related proteins LAMP2A and HSP8 [28] using RNAi. All three shLAMP2a constructs caused and efficient knockdown in MCF-7 cells, whereas knockdown efficiency for HSP8 (HSC70) was only around 25% (Fig. 2 a-c).

Next, we applied the staining protocol to a TMA derived from a cohort of 365 breast cancer patients. Clinicopathological details of the case collection can be found in Table 1. Expression levels for LAMP2A and HSP8 ranged from IRS 0–12, with a median of 4. There was no significant correlation between the expression levels of LAMP2A and HSP8 among each other ($r = 0.102$; $p = 0.062$ for IRS; $p = 0.355$ for dichotomized categories). There were no associations for LAMP2A levels with pathological parameters including HER2 or molecular subtype. In contrast, HER2 enriched subtypes and luminal B tumors (including HER2 positive and hormone receptor positive tumors) more frequently showed higher HSP8 levels ($p < 0.001$). For other pathological parameters (e.g. TNM category) no associations with HSP8 expression were found.

Survival data were available from 293 patients with 44 deaths, 38 metastases and 22 recurrences recorded during the follow up period. The mean overall survival time (OS) was 76 months. Mean disease-free survival time (DFS) was 70 months. For subsequent survival analysis only patients with completely resected tumors, without perioperative mortality (survival >1month) and without distant metastases at the time of surgery were analyzed ($n = 234$). We did not find a significant association between LAMP2A or HSP8 expression and overall survival of the patients ($p = 0.232$, $p = 0.667$, Supplementary Figs. 1a–c). However, for disease-free survival, high LAMP2A expression was associated with a trend to worse outcome ($p = 0.05$, Fig. 2d), which turned significant when analyzing the group of HER2 negative tumors only ($p = 0.026$, Fig. 2e). For this subgroup, LAMP2A expression was also an independent prognostic parameter in a multivariate model encompassing pT category, pN category and grading (HR = 1.889; 95% CI = 1.039–3.421; $p = 0.037$ for LAMP2A). For HSP8, no significant impact for DFS was seen, neither in the total cohort ($p = 0.52$, Fig. 2f) nor in the subgroup of HER2 positive tumors ($p = 0.631$, Fig. 2g).

The results of our *ex vivo* study point towards an oncogenic role of LAMP2A in breast cancer. This is in line with previously published papers, showing increased LAMP2A levels in most breast

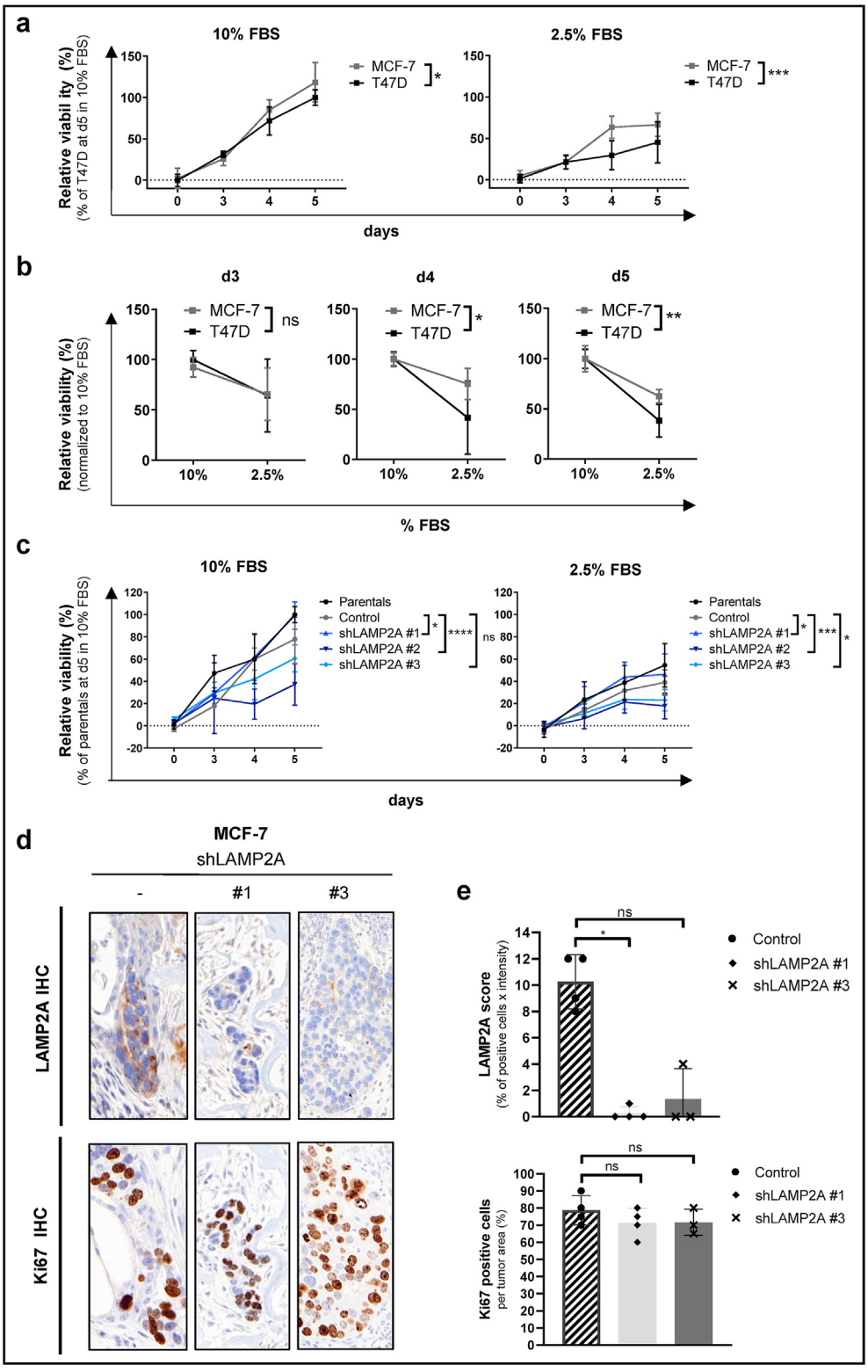

Fig. 3. LAMP2A expression is positively associated with breast cancer cell viability *in vitro*. (**a**–**b**) MCF-7 and T47D cells were seeded and cultured in presence of 10% or 2.5.% FBS. Cell viability was monitored at day 0, 3, 4 and 5 post-seeding by determining metabolic activity using Alamarblue® assay. **(a)** Growth curves of MCF-7 and T47D in normal (10% FBS, left panel) and low serum (2.5% FBS, right panel) conditions are shown. Values were normalized to T47D cells cultured in 10% FBS at day 5. Regular Two-way ANOVA was applied to compare MCF-7 and T47D cells. n = 3 **(b)** Another representation of the data shown in a. Relative cell viability in 10% and 2.5% FBS is compared at different time points of the assay (day 3, 4, 5). On each day values were normalized to 10% FBS. Statistical analysis was performed as described in a. **(c)** MCF-7 cells were transduced with a lentiviral control vector (Control) or lentiviral vectors expressing three independent shRNAs targeting LAMP2A. Cell viability was monitored over time in presence of complete (10% FBS) or low-serum (2.5% FBS) medium using an Alamarblue® assay. Parental MCF-7 cells were included in the experiment as an additional control. n = 3. Two-way ANOVA followed by Dunn's test for multiple comparisons was used to compare the groups. **(d**–**e)** MCF-7 and 2 different shLAMP2A cell lines were cultured *in ovo*, followed by staining and scoring of the tumor cells for LAMP2A (upper panel) and Ki-67 (lower panel) expression. **(d)** Representative IHC staining in MCF-7 shLAMP2A cells and the corresponding control are shown and **(e)** quantification thereof. The error bars represent SD of 3 or 4 pictures/samples from one experiment. Statistical significance was determined by Kruskal-Wallis test.

cancer compared to normal breast tissues [9,10] and the correlation of LAMP2A expression and histological grade [10]. The association of LAMP2A with worse clinical parameters was also found in other cancer types such as resected squamous cell carcinomas of the lung [23], esophageal squamous cell carcinoma [24] or non-small cell lung cancer [25].

Interestingly, the levels of another CMA associated protein, HSP8, did not correlate with DFS. This might be due to the versatile, homeostatic functions of HSP8 that are not related to CMA [26]. Is the effect of LAMP2A on clinical outcome mediated via its role in CMA? Currently we can only determine CMA activity in FFPE-derived tissue by analyzing steady state analysis of proteins [27]. Until we establish markers, which are associated with an active CMA status, assessing LAMP2A and HSP8 expression, is still the best option. Importantly, LAMP2A levels at lysosomes correlate with CMA activity [28].

3.3. High LAMP2A expression confers a survival advantage to MCF-7 cells in vitro

Next, we aimed at validating our *ex vivo* findings in an *in vitro* model. Since CMA can be activated upon serum withdrawal [28], we next compared proliferation of LAMP2A-high MCF-7 and LAMP2A-low T47D cells (Fig. 1 a-b) in presence of high (10%) and low (2.5%) serum. Our data indicate that high-LAMP2A MCF-7 cells have a proliferation advantage in low serum conditions (Fig. 3 a-b). First, MCF-7 cells are proliferating significantly faster than T47D cells as monitored by an Alamarblue assay over a time course of 5 days (Fig. 3a). Importantly, T47D are more sensitive to low serum conditions as compared to MCF-7 (Fig. 3b): At day 5 of proliferation, cell viability of MCF-7 is reduced to ~62% in 2.5% FBS compared to 100% if grown in 10%, whereas viability of T47D is reduced to ~38% in presence of 2.5% FBS (Fig. 3b, right panel). The higher sensitivity of T47D cells to low serum conditions might be attributed to the amino acid recycling function of CMA [18], which is presumably more efficient in the high-LAMP2A MCF-7 cells. As a proof of principle, we knocked down *LAMP2A* in MCF-7 cells and again assessed viability of cells over-time in high and low serum media. In line with the previous data, 2 out of 3 *LAMP2A* knockdown cell lines (*shLAMP2* #2 and #3) exhibit decreased viability compared to parental and control cells no matter whether grown in 10% or 2.5% FBS (Fig. 3c). In a next experiment, we were interested to determine clonogenic growth of the different cell lines. We found no significant difference in the number of colonies between control and LAMP2A depleted MCF-7 cells, but the colonies were smaller in LAMP2A KD#2 and #3, as assessed by the total area covered of colonies (Supplementary Fig. 2). This result suggests a lower proliferation rate of these two cell lines, which is in agreement with the viability assay. Next, we assessed the tumorigenic properties of two LAMP2A-manipulated MCF-7 cells (KD#1 and #3) if grown on a chick chorioallantoic membrane (CAM) to mimic the 3D architecture (Supplementary Fig. 3). As expected, we found a reduction of LAMP2A levels in both LAMP2A knockdown cells (IRS of 0.25 and 1.333 respectively) if compared to control cells (IRS of 10.25) (Fig. 3 d-e, upper panel), supporting the Western blot data. In contrast to our 2D data, proliferation of LAMP2A depleted MCF-7 cells was only slightly decreased to control cells as determined by Ki-67 staining (Fig. 3d–e, lower panel).

There are fundamental differences between *in vitro* 2D culture and *in ovo* 3D culture, such as different cell to cell contact, interaction with extra cellular matrix components in the CAM, as well as a different nutrient supply, which may explain that we only see a slight decrease in proliferation, which was more pronounced in 2D. Triple-negative breast cancer cells, for example, differentially respond to autophagy inhibition whether they are cultured in 2D or 3D [29] and there are clear differences in gene expression profiles between 2D and 3D cultured neuroblastoma cells [30]. Furthermore, we may not be looking at the same aspects of tumor development with these two models. It is well established that autophagy can have different roles depending on the stage of tumor formation [31].

In summary, we found that high LAMP2A levels correlate with a worse outcome in HER2 negative breast cancer patients. *In vitro* experiments further demonstrate that low LAMP2A levels in the HER2 negative cell lines, T47D and MCF-7, decrease proliferation.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.06.082.

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