Received: 28 November 2022 | Revised: 7 March 2023 | Accepted: 12 March 2023

DOI: 10.1111/boc.202200110

Biology of the Cell

RESEARCH ARTICLE

Annexin-A5 and annexin-A6 silencing prevents metastasis of breast cancer cells in zebrafish

Céline Gounou[1] | **Flora Bouvet**[1] | **Benjamin Liet**[2] | **Valérie Prouzet-Mauléon**[2,3] | **Léna d'Agata**[1] | **Etienne Harté**[4] | **Françoise Argoul**[4] | **Géraldine Siegfried**[2] | **Richard Iggo**[2] | **Abdel-Majid Khatib**[2,5,6] | **Anthony Bouter**[1]

[1]Univ. Bordeaux, CNRS, Bordeaux INP, CBMN, UMR 5248, Pessac, France

[2]Univ. Bordeaux, INSERM, BRIC, U 1312, Bordeaux, France

[3]CRISPRedit, TBMcore, UAR CNRS 3427, Inserm US 005, Univ, Bordeaux, France

[4]Univ. Bordeaux, CNRS, LOMA, UMR 5798, Talence, France

[5]XenoFish, B2 Ouest, Allée Geoffroy St Hilaire CS50023, Pessac, France

[6]Bergonié Institute, Bordeaux, France

Correspondence
Anthony Bouter, Bât. B14, Allée Geoffroy Saint Hilaire, 33600 Pessac, France.
Email: anthony.bouter@u-bordeaux.fr

Funding information
la Ligue Contre Le Cancer de Gironde, Grant/Award Numbers: 191796, 238171; Ligue Contre Le Cancer du Lot-et-Garonne, Grant/Award Number: 238294

Abstract

Background Information: During tumor invasion and metastasis processes, cancer cells are exposed to major compressive and shearing forces, due to their migration through extracellular matrix, dense cell areas, and complex fluids, which may lead to numerous plasma membrane damages. Cancer cells may survive to these mechanical stresses thanks to an efficient membrane repair machinery. Consequently, this machinery may constitute a relevant target to inhibit cancer cell dissemination.

Results: We show here that annexin-A5 (ANXA5) and ANXA6 participate in membrane repair of MDA-MB-231 cells, a highly invasive triple-negative breast cancer cell line. These crucial components of the membrane repair machinery are substantially expressed in breast cancer cells in correlation with their invasive properties. In addition, high expression of ANXA5 and ANXA6 predict poor prognosis in high-grade lung, gastric, and breast cancers. In zebrafish, the genetic inhibition of ANXA5 and ANXA6 leads to drastic reduction of tumor cell dissemination.

Conclusion: We conclude that the inhibition of ANXA5 and ANXA6 prevents membrane repair in cancer cells, which are thus unable to survive to membrane damage during metastasis.

Significance: This result opens a new therapeutic strategy based on targeting membrane repair machinery to inhibit tumor invasion and metastasis.

KEYWORDS
annexins, annexin-A5, annexin-A6, cancer, invasion, membrane repair, metastasis

INTRODUCTION

Cells continuously exposed to mechanical stress frequently exhibit membrane disruptions (P. L. McNeil et al., 2003). These physiological events occur in muscle, epithelial, and endothelial cells, which respectively undergo physical constraints due to muscle contraction/stretching, and fluid or hemodynamic shear stress. These cells are biologically adapted to cope with such events thanks to an effective membrane repair machinery, which restores membrane integrity at the minute scale (P. L. McNeil et al., 2003). Defect of membrane resealing leads to cell death and may be responsible for tissue necrosis and the development of degenerative diseases (Bansal et al., 2003). As membrane repair

Abbreviations: ANX, annexin; ANXA5, annexin-A5; ANXA6, annexin-A6; BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phénylindole; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; D-PBS, Dulbecco's phosphate-buffered saline; DPF, day post-fertilization; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HPI, hour post-injection; HR, hazard ratio; KM, Kaplan-Meier; MOI, multiplicity of infection; PVDF, polyinylidene fluoride; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis.

strongly relies on proteins binding to membrane in a Ca^{2+}-dependent manner, such as dysferlin, AHNAK, or annexins (Bouter et al., 2011; Boye et al., 2017; Lennon et al., 2003), one of the main triggers of the process is the influx of Ca^{2+} from extracellular (mM) to intracellular (μM) milieu.

The mammalian annexin family consists of 12 cytosolic proteins, numbered from ANXA1 to A13 (the number 12 has not been assigned) (Gerke & Moss, 2002). These proteins share the ability of binding to membrane-exposed negatively-charged phospholipids in a Ca^{2+}-dependent manner. They participate in various stages of membrane repair. ANXA1 and ANXA2, which have been shown to induce the Ca^{2+}-dependent aggregation of liposomes (Lambert et al., 1997), may be responsible for intracellular vesicle aggregation and the formation of the lipid patch that leads to membrane resealing (Davenport et al., 2016; Lennon et al., 2003; A. K. McNeil et al., 2006). ANXA5 self-assembles around the rupture site to strengthen the membrane and prevent the tear from spreading, hence promoting membrane resealing (Bouter et al., 2011; Carmeille et al., 2016, 2015). Even though the accurate function of ANXA4 and ANXA6 remains unclear, they are crucial in cell membrane remodeling during the resealing process (Boye et al., 2017; Croissant et al., 2020).

Invasive cancer cells may spread from the primary tumor to other organs through the blood or lymphatic system in a multi-step process known as metastasis. Invasion of distant organs by cancer cells requires the remodeling of actin cytoskeleton, which enhances membrane dynamics but reduces plasma membrane stiffness (Wirtz et al., 2011). Furthermore, significant shear stress would be experienced by invasive cancer cells while they migrate through the extracellular matrix and invade distant organs, especially during intra- and extravasation processes for bloodstream entrance and exit, respectively (Azevedo et al., 2015; Gensbittel et al., 2021; Wirtz et al., 2011).

We hypothesize that invasive cancer cells must cope with severe mechanical forces leading to plasma membrane ruptures during metastasis. These cells would possess an exacerbated membrane repair machinery suited for multiple membrane injuries, whose inhibition may lead to cell death. The validation of such a hypothesis would pave the way for the development of new therapeutic strategies to limit tumor invasion and prevent cancer metastasis. Data from the literature serve as the first basis of this working hypothesis. Although few studies have linked membrane repair to cancer, several of them have indeed found fortuitously a positive correlation between key actors of membrane repair and tumor invasion (Jaiswal et al., 2014; Lauritzen et al., 2015; Leung et al., 2013; Prieto-Fernández et al., 2022). In addition, the hypothesis is supported by our experimental data, which provided an *in cellulo* proof of principle. We have shown that MDA-MB-231 cell migration on fibrillar collagen causes plasma membrane damages, leading to cell death in the absence of ANXA5 and ANXA6 due to a defective membrane repair machinery (Bouvet et al., 2020).

Here, we investigated the effect of the silencing of ANXA5 and ANXA6 on the dissemination of cancer cells in vivo. Experiments were performed on modified MDA-MB-231 cells constitutively expressing the fluorescent tdTomato protein for live imaging in zebrafish. We show that cells, when rendered deficient for ANXA5 and ANXA6, exhibit deficiency of membrane repair. Whether we observed that simultaneous silencing of ANXA5 and ANXA6 has no effect on proliferation in vitro, we reveal a reduced ability to metastasize for these ANX-deficient cells in vivo. In addition, KM plotter database analysis shows that high expression of ANXA5 or ANXA6 predicts poor prognosis for patients suffering from gastric, lung, and breast cancer. Altogether, these results suggest that annexins may constitute relevant targets to prevent tumor invasion and metastasis.

MATERIALS AND METHODS

Survival analysis

The Kaplan–Meier plotter database (https://kmplot.com/analysis/) was used to correlate overall survival rate to the level of ANXA5, ANXA6, myoferlin, S100A11, or AHNAK mRNA expression. To determine the prognostic value, patients were split by auto-selecting the best cutoff, the period of follow-up was fixed at 120 months, the "only JetSet best probe set" option was selected, and biased arrays was excluded. Hazard ratios with a 95% confidence interval and log-rank *p*-value were calculated. The log-rank test calculates the mean chi-square for each event time from each group. Two groups are considered to be significantly different when $p < 0.05$.

Cell culture

Cell culture media and reagents were from ThermoFisher Scientific (Waltham, MA, USA) except when otherwise stated. MDA-MB-231 human breast tumor cells expressing tdTomato protein were established by lentiviral transduction as previously described (Iggo, 2022) using the pDRM18 LTN plasmid (Addgene number 174721). Cells were cultured in Dulbecco modified Eagle's minimal essential medium (DMEM) containing 4 mM Glutamax and supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 μg/mL). Cells were maintained at 37°C in a 5% CO_2 humidified incubator.

Generation of ANXA5 and ANXA6 deficient MDA-MB-231 cells

For each gene, three sgRNAs were designed with CRISPOR algorithm (crispor.tefor.net [Haeussler et al., 2016]): ANXA5-sgRNA#1 (AGGGTACTACCAGCGGATGT), ANXA5-sgRNA#2 (GCCTGGAAGATGACGTGGTG), ANXA5-sgRNA#3 (CACACTTCGTGTTCCAAAGA), ANXA6-sgRNA#1 (GCTCTGTACACTGCCATGAA), ANXA6-sgRNA#2 (TAATCACCTCACGGAGCAAC) and ANXA6-sgRNA#3 (CGTAGAGGGACTTGTAGCTC). The following scrambled sgRNA was used as control: ATATTTCGGCAGTTGCAGCA. Oligonucleotides (Eurogentec) containing the gene-targeting gRNA with 5'overhang BsmBI digestion sites were annealed and cloned into the lentiCRISPRv2 plasmid (a gift from Feng Zang; Addgene plasmid #52961) (Sanjana et al., 2014). ANXA5-sgRNA#1, ANXA5-sgRNA#2, ANXA6-sgRNA#1, and ANXA6-sgRNA#2 sequences were used in this study. Lentiviral production was done for each individual sgRNA and they were used separately or in combination to infect cells thereafter selected with puromycin treatment. Lentiviral-based particles were produced by Bordeaux University Lentiviral Vectorology Platform (US005, Bordeaux, France) by transient transfection of 293T cells. 2.10^5 MDA-MB-231 cells were cultured in a 30 mm Petri Dish for 24 h and co-transduction was carried out by adding single sgRNA or a mixture of ANXA5- and ANXA6-targeting sgRNAs lentiviral particles to the cells at multiplicity of infection (MOI) of 20 in 2 mL Opti-MEM for 24 h. Transduced cells were cultured for 24 h in growth medium and then selected in selection medium composed by 2 μg/mL puromycin in DMEM for 48 h. Cells were passaged and subsequently cultured in 25 cm^2 cell culture flask in selection medium. At each passage, a fraction of cells was used for preparing protein extracts for western-blot analysis of the expression of ANXA5 and ANXA6.

Western-blot

2.10^6 cells were trypsinized, pelleted, and re-suspended in 300 μL of D-PBS supplemented with 1 mM EGTA. Protein extracts were obtained by sonicating ice-cold cell suspension with a Branson digital sonifier (amplitude 20%, duration 2 min, interval 5 s, and pulse 5 s). Two successive centrifugations at 13,000 g for 1 min allowed to remove cell debris. 10 μg protein extracts were separated on a 10% SDS-PAGE. Semi-dry electrophoretic transfer (Bio-Rad, Hercules, CA, USA) onto PVDF membrane was performed for 1 h at 100 V. The cellular content in ANXA4 (35 kDa), ANXA5 (35 kDa), ANXA6 (68 kDa), and actin (42 kDa) or GAPDH (37 kDa) was detected with mouse anti-ANXA4 monoclonal antibody (SAB4200121, Sigma, Saint-Louis, MO, USA), mouse anti-ANXA5 monoclonal antibody (AN5, Sigma), mouse anti-ANXA6 monoclonal antibody (sc-271859, Santa cruz Biotechnology), rabbit anti-actin polyclonal antibody (A2066, Sigma), and rabbit anti-GAPDH polyclonal antibody (G9545, Sigma), respectively. All primary antibodies were diluted 1:1,000 in saturation solution composed by Tris buffer saline (10 mM Tris, 150 mM NaCl, pH 8.0) supplemented with 0.1% Tween20 and 5% non-fat dry milk. Revelation was performed using secondary antibodies conjugated to horse-radish peroxidase (GE-Healthcare) diluted 1:2,000 in saturation solution and Opti-4CN colorimetric kit (Bio-Rad). ImageJ software was used to measure the relative intensity of protein bands.

Proliferation analysis

In 6-well clear plates (Corning, NY, USA), 1.6×10^5 cells/well were seeded in duplicate in growth medium without red phenol to avoid disturbance of the phase measurement. Quantitative phase images were acquired with a lens-free microscope (Cytonote, Iprasense, Montpellier, France) in an incubator at 37°C, 5% CO_2. The acquisition period was 25 min. Phase images were analyzed using Trackmates2 on Fiji and MATLAB. Three independent experiments were performed for each cell line.

Membrane repair assay

Membrane repair assay was performed as previously described (Bouvet et al., 2020). MDA-MB-231 cells were irradiated at 820 nm with a tunable pulsed depletion laser Mai Tai HP (Spectra-Physics, Irvine, USA) of an upright two-photon confocal scanning microscope (TCS SP5, Leica) equipped with an HCX PL APO CS 63.0 × 1.40 oil-objective lens. Irradiation consisted of 1 scan (1.6 s) of a 1 μm × 1 μm area with a power of 110 (±5) mW. 512 × 512 images were acquired at 1.6 s intervals with pinhole set to 1 Airy unit. FM1-43 was excited by the 488-nm laser line (intensity set at 30% of maximal power) and fluorescence emission was measured between 520 nm and 650 nm. For quantitative analysis, the fluorescence intensity was integrated over the whole cell surface and corrected for the fluorescence value recorded before irradiation, using ImageJ software.

Traffic of ANXA5 and ANXA6 in laser-injured cells

For the subcellular trafficking analysis of ANXA5-GFP or ANXA6-GFP, MDA-MB-231 cells cultured in 35-mm glass bottom dishes (MatTek, Ashland, USA) were

transfected by the pA5-GFP or pA6-GFP plasmid (Croissant et al., 2022). Membrane damage was performed by laser ablation as described above, without FM1-43. At least three independent experiments were performed and each experiment included the analysis of at least five damaged cells.

MDA-MB-231 dissemination in zebrafish

Experiments were performed using the Casper zebrafish stain (ZIRC, Oregon, USA). Adult zebrafish were produced in accordance with the French Directive (Ministère de l'Agriculture et de l'alimentation) under permit number A33-063-935. All the procedures were conducted in compliance with the European Communities Council Directive (2010/63/EU). Adult fish aged 6 months to 2 years were crossed to produce embryos. Embryos were cultured in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) at 28°C. Dechorionated 2-day post fertilization (dpf) zebrafish embryos were anaesthetized with 0.003% tricaine (Sigma, St. Louis, MO, USA) and positioned in 3% methylcellulose on a dich coated with 1% agarose. Cells were treated with versene solution and resuspended in PBS containing 1% phenol red at the density of 4×10^7 cells per milliliter. The cell suspension was loaded into borosilicate glass capillary needles (Femtotip II, Eppendorf) and injections were performed using a pump (Femtojet 4i; Eppendorf) and micromanipulator (Phymep). Around 500 cells/embryos were injected above the duct of cuvier in perivitelline space of the embryo. After checking the implantation with mammalian cells, zebrafish embryos were maintained at 35.5°C. Tumor imaging was done at 3-, 28-, and 52-h post injection (hpi).

Fluorescence microscopy

Cell imaging was performed using the conventional fluorescence microscope IX81 (Olympus) equipped with the UPLFLN20X/0.50/WD2.1 mm and the UPLFLN60XO/0.65/0.65-1.25/D0.12 objectives. DAPI was observed using the U-MWU2 cube containing a band-pass excitation filter (330–385 nm), a dichroic mirror (50 % transmission from 400 nm), and a long-pass emission filter (threshold 420 nm). GFP was observed using the U-MINIBA2 cube containing a band-pass excitation filter (470–490 nm), a dichroic mirror (threshold 505 nm), and a band-pass emission filter (510–550 nm). tdTomato was observed using the cube U-MWG2 containing a band-pass excitation filter (510–550 nm), a dichroic mirror (threshold 570 nm), and a long-pass emission filter (threshold 590 nm).

RESULTS

High expression of ANXA5 or ANXA6 in tumor cells predicts poor patient prognosis

ANXA5 and ANXA6 have been associated with either tumor-promoting or tumor-suppressing activity (Grewal et al., 2017, 2021; Korolkova et al., 2020; Peng et al., 2014; Qi et al., 2015). This apparent discrepancy may be result from the different stage and grade in which the cancer cells have been analyzed. We hypothesize indeed that cancer cells within a growing primary tumor do not require a large amount of proteins involved in motility, invasiveness, or membrane repair processes. In contrast, a metastatic nodule is likely composed of cells that are mobile and invasive, potentially enriched in membrane repair proteins.

To investigate the involvement of ANXA5 and ANXA6 in promoting cancer invasion and metastasis, we first examined whether their expression was correlated with the prognostic value in various advanced cancers. Advanced cancer is characterized by high grade and/or stage, which correlates with the velocity of the disease development and the extent of affected tissues, respectively. High-grade and -stage cancers are often very aggressive, with strong tumor invasion and the presence of metastatic cells. The impact of ANXA5 and ANXA6 mRNA expression on overall survival of four human cancer types, ie breast, gastric, lung, and ovarian cancer, was analyzed according to the Kaplan–Meier Plotter database (Lánczky & Győrffy, 2021). We observed that high expression of ANXA5 (Figure 1a) and ANXA6 (Figure 1b) positively correlated with a lower survival in breast, gastric, and lung advanced cancer patients.

Given the multifunctionality of both annexins, at this stage, no correlation between low survival rate and membrane repair could be made. To support the hypothesis that a correlation may exist between a reinforced membrane-repair machinery and poor patient prognosis, we analyzed the impact of the expression of other membrane-repair genes on survival curves. To date, myoferlin, S100A11, and AHNAK have been reported to belong to the membrane repair machinery of cancer cells (Davis et al., 2014; Jaiswal et al., 2014; Leung et al., 2013; Li et al., 2012), together with ANXA5 and ANXA6 (Bouvet et al., 2020). We analyzed therefore the impact of the expression of these genes on survival curve and showed that they exhibited a similar trend than ANXA5 and ANXA6 (Figure S1), suggesting that high expression of membrane-repair genes affects survival of advanced cancer patients and is a predictor of poor prognosis.

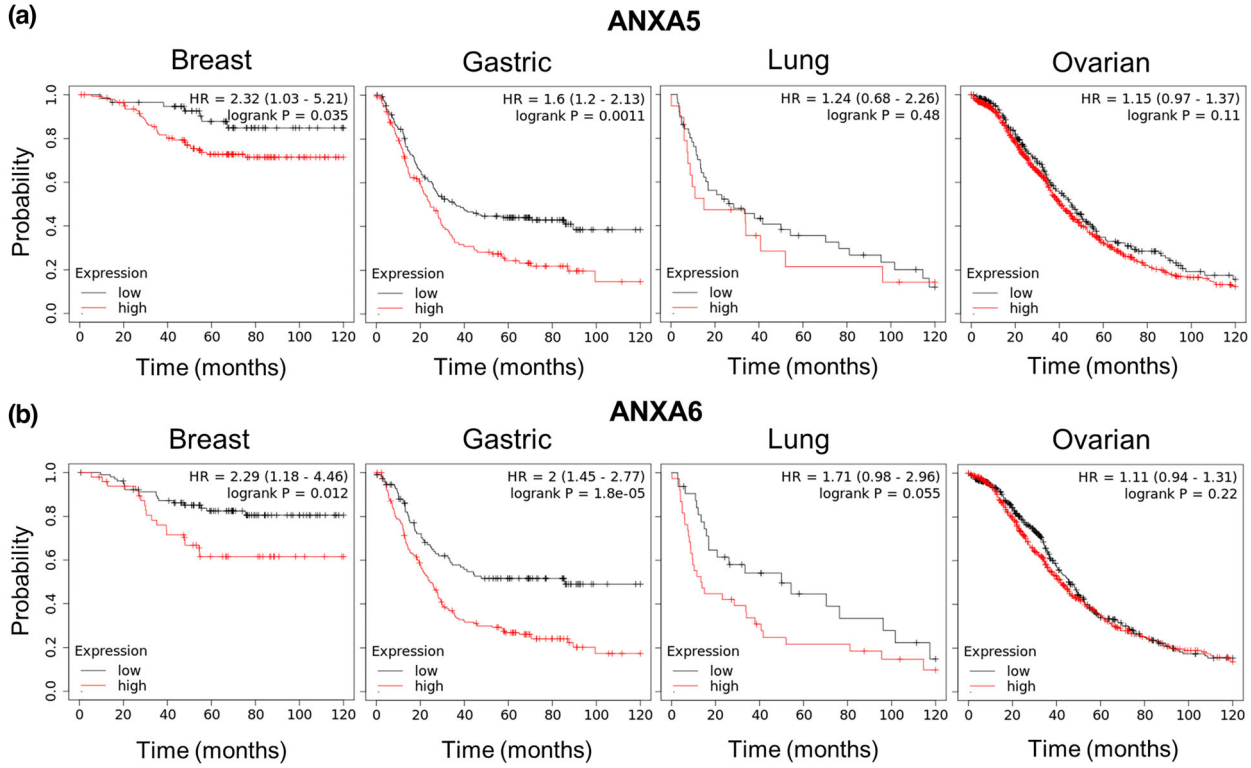

FIGURE 1 High ANXA5 or ANXA6 expression in advanced cancer patients is a poor prognosis factor. (a,b) Survival curves from Kaplan–Meier plot profiles for breast (n = 201), gastric (n = 305), lung (n = 70), or ovarian (n = 1220) high-grade and -stage cancer patients, stratified by high (red line) and low expression (black line) of ANXA5 (a) or ANXA6 (b) mRNA (Affymetrix identifier, 200782_at and 200982_s_at, respectively). In each analysis, hazard ratio (HR) and log-rank probability were calculated. A HR significantly different from 1 means that survival was better in one of both groups. For example, if a HR is 1.5, the survival probability in group 1 is 1.5 higher than in group 2. To note, cancer grade (typically numbered from 1 to 3) correlates with the velocity of the disease development while stage (from 1 to 4) characterizes the extent of affected tissues. Advanced cancers were triple-negative subtype, stage 3, stage 3 + grade 4, and stage 3, for breast, lung, ovarian, and gastric cancer, respectively. HR, hazard ratio.

ANXA5 or ANXA6 expression correlates with invasiveness properties of breast cancer cells

Our study focused on MDA-MB-231 cells, which are highly invasive cells established from triple-negative breast cancer. We have previously reported that ANXA1, ANXA2, ANXA5, and ANXA6, which are four annexins reported to belong to the sarcolemma repair machinery (Croissant et al, 2021), are similarly expressed in MDA-MB-231 cells and placental BeWo cells. The placenta should be the richest organ in annexins, from which most of them have been initially isolated and identified (Buhl et al., 1991; Funakoshi et al., 1987; Römisch & Heimburger, 1990). To complete this study, we compared the expression of ANXA4, ANXA5, and ANXA6 between MDA-MB-231 and MCF7 cells. Like MDA-MB-231, MCF7 cells were established from breast cancer but exhibit lower invasiveness properties (Thompson et al., 1992, 1988). We observed that ANXA5 and ANXA6 were expressed at higher levels in MDA-MB-231 cells compared to MCF7 (Figure 2). Our results corroborate previous findings that showed the presence of higher ANXA6 levels in MDA-MB-231 or BT549 compared to MCF7 or MCF10 cell lines (Sakwe et al., 2011). Nevertheless, they contradict a previous study of Vila de Muga et al., in which it was reported that ANXA6 expression is lower in ER-negative compared ER-positive breast cancer cell lines (Vilá de Muga et al., 2009). Interestingly, we recently reported that ANXA1 and ANXA2 were also expressed at higher level in MDA-MB-231 compared to MCF7 cells (Gounou et al., 2022), suggesting that stronger invasive properties were associated with higher expression of membrane repair proteins. Nevertheless, we could not rule out the possibility that another cell process, such as cell growth, proliferation, motility, or lipid/glucose homeostasis, may be dysregulated and explain modified invasiveness (Grewal et al., 2021).

Membrane repair in MDA-MB-231 cells requires ANXA5 and ANXA6

MDA-MB-231 cells were modified to constitutively express the tdTomato fluorescent protein, enabling in vivo follow-up of cell dissemination in zebrafish by fluorescence microscopy. These cells were rendered

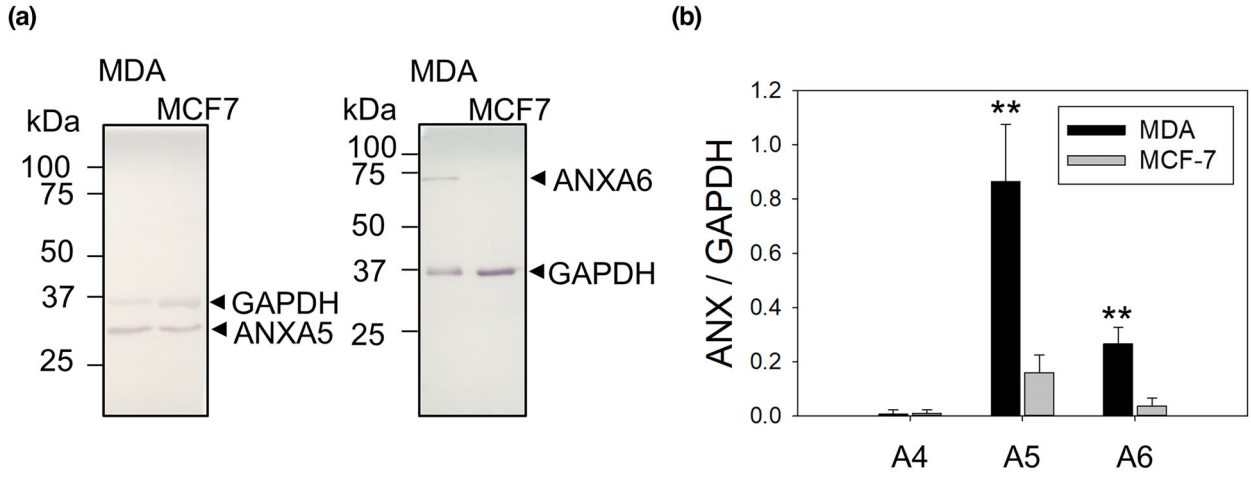

FIGURE 2 Invasiveness properties in breast cancer cells are correlated with high expression of ANXA5 and ANXA6. 10 μg of protein extracts from MDA-MB-231 and MCF7 cells were analyzed by western-blotting. (a) Representative image of western-blot analysis showing the revelation of ANXA5 and ANXA6 in MDA-MB-231 and MCF7 cells, compared to GAPDH (loading control). The whole membranes are presented in Figure S2. (b) The histogram presents mean values ($\pm$ SEM) of the ratio ANX/GAPDH from five independent experiments, analyzed by the gel analysis plugging of ImageJ. Student t-test for independent samples. $^{**}p < 0.01$.

deficient for ANXA5 and/or ANXA6 using the CRISPR/Cas9 strategy, leading to cell lines hereafter named sgANXA5, or sg ANXA6, or sgANXA5/A6 MDA-MB-231, in which ANXA5 and ANXA6 expression was substantially reduced (Figures 3a and S3). The study of the double knock-out cell line was particularly interesting because we have shown previously that the migration on fibrillar collagen triggered numerous unrepaired plasma membrane damages when cells were deficient in both ANXA5 and ANXA6, while the single silencing led to a more moderate phenotype (Bouvet et al., 2020).

Fluorescence microscopy imaging of the tdTomato protein revealed no significative difference in the morphology of control or sgANXA5/A6 MDA-MB-231 cells (Figures 3b and S4). In addition, we observed that silencing of ANXA5 and ANXA6 has no effect on cell growth in vitro (Figure 3c). This result was surprising, since both annexins have been associated with growth-promoting and -inhibiting features (Grewal et al., 2021; Prieto-Fernández et al., 2022). Actually, we showed that sgANXA6 MDA-MB-231 cells proliferate more rapidly than control cells, while sgANXA5 MDA-MB-231 cells exhibited a slower growth than both other cell types (Figure S5). This result suggests that the absence of both ANXA5 and ANXA6 cancels their respective inhibiting and promoting effect on proliferation.

We have previously shown that ANXA5 and ANXA6 were required for membrane resealing in MDA-MB-231 cells (Bouvet et al., 2020). In order to confirm the participation of ANXA5 and ANXA6 in membrane repair of this modified tdTomato-positive MDA-MB-231 cell line, a standard membrane repair assay was performed using laser ablation in the presence of Ca^{2+} and FM1-43 (Bouvet et al., 2020; Carmeille et al., 2017). After laser injury, the entry of FM1−43 molecules into the cytosol, and the subsequent increase of the intracellular fluorescence intensity, is stopped in case of membrane resealing, whereas a continuous entry of FM1-43 into the cytosol, and therefore a strong increase in the fluorescence intensity, is observed in the absence of membrane resealing. We observed that most control MDA-MB-231 cells are able to reseal a laser-induced membrane damage in about 60 s (Figure 4a,c). In contrast, laser-damaged sgANXA5, sgANXA6, or sgANXA5/A6 MDA-MB-231 cells exhibited systematically a strong and large increase of the intracellular fluorescence intensity (Figures 4b,c and S6), which indicated that they were unable to reseal cell membrane damage. To complete this set of experiments, we then analyzed the traffic of ANXA5 and ANXA6 in membrane-damaged MDA-MB-231 cells, since most key membrane repair proteins have been reported of being rapidly recruited to the disruption site (Croissant et al., 2021; Croissant et al., 2022). In MDA-MB-231 cells transfected with either ANXA5-GFP or ANXA6-GFP vector, cell membrane was damaged by laser ablation and the subcellular trafficking of each annexin was analyzed. We observed that ANXA5-GFP and ANXA6-GFP were systematically recruited to the disruption in a few seconds after membrane damage (Figure 4d,e). We conclude that ANXA5 and ANXA6 participate in membrane repair in MDA-MB-231 cells.

Inhibition of ANXA5 and ANXA6 prevents metastasis in zebrafish

To investigate the role of ANXA5 and ANXA6 on tumoral progression, we compared the ability of MDA-MB-231 cells, either wild-type or deficient for ANXA5 and ANXA6, to disseminate and proliferate when injected into the yolk sac of zebrafish larvae and we

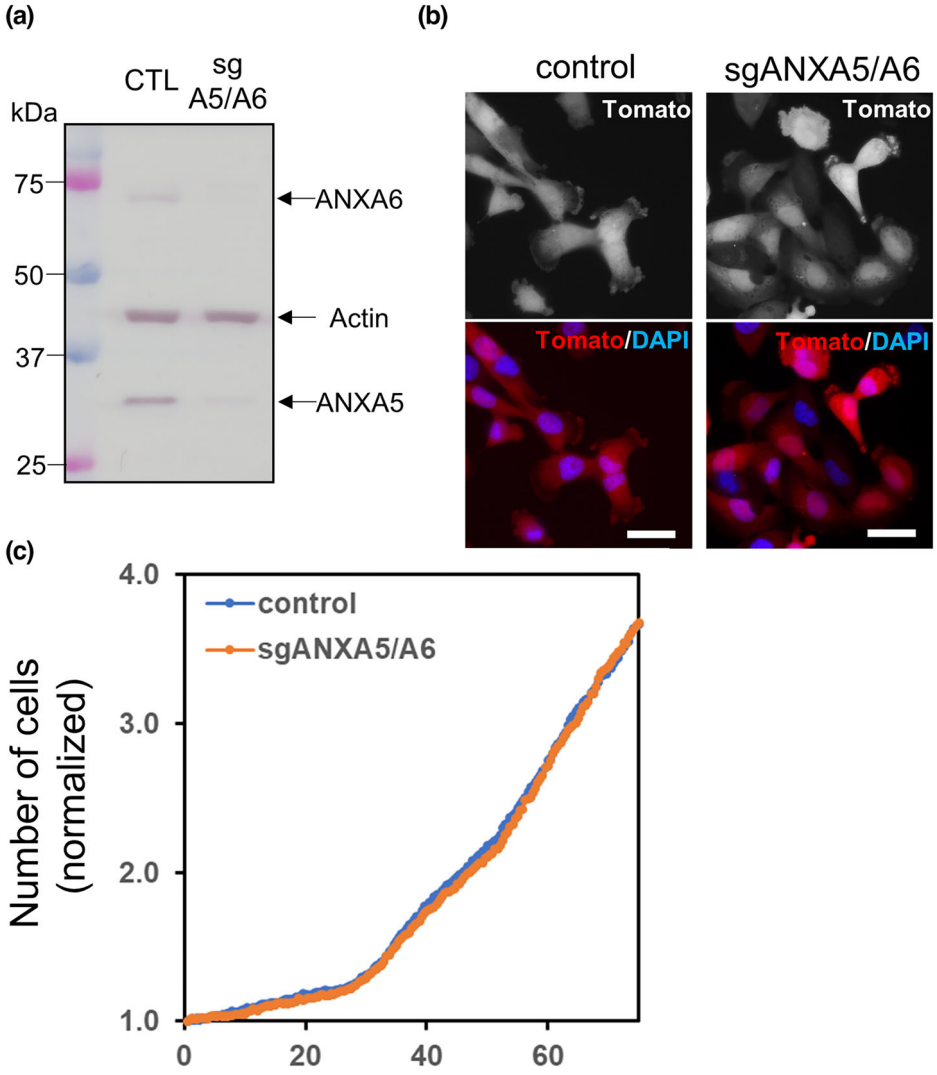

FIGURE 3 sgANXA5/A6 MDA-MB-231 cells exhibit morphology and growth properties similar to control cells. (a) ANXA5/A6 deficient MDA-MB-231 cells were established by the CRIPSR-Cas9 strategy. The cellular content of ANXA5 and ANXA6 in sgANXA5/A6 MDA-MB-231 cells was quantified by western-blotting. The expression of ANXA5 and ANXA6 is decreased of more than 95% and 85% in sgANXA5/A6 MDA-MB-231 cells, respectively. (b) Control and sgANXA5/A6 MDA-MB-231 cells, which expressed constitutively the tdTomato fluorescent protein (red), were imaged by fluorescence microscopy. Scale bars = 40 μm. (c) Proliferation of control or sgANXA5/A6 MDA-MB-231 cells was monitored with a lensfree microscope enabling long-term observation of a large cell population (Allier et al., 2017). The averaged number of cells (+/− SEM) normalized to the number of seeded cells from three independent experiments is presented.

quantified metastases formed in tail and/or head of fish. We focused on the double knock-out sgANXA5/A6 MDA-MB-231 cell line, which exhibited proliferation properties similar to control cells and a higher sensitivity to membrane damages than single knock-out when cells are submitted to migration on fibrillar collagen (Bouvet et al., 2020), which may correspond to constraints encountered by cancer cells in vivo. At 24 h post-injection (hpi), we observed that 30% of fishes injected with control cells showed caudal and/or cranial metastases with 4 metastases on average (Figure 5a,c), compared to 4% for sgANXA5/A6-cells-injected fishes containing a single metastasis (Figure 5b,c). Finally, we observed that the mortality rate reached 80% for fishes injected with control cells and only 25% for those injected with sgANXA5/A6 cells at 48 hpi (Figure 5c). Together, these results indicate that ANXA5 and ANXA6 deficiency renders MDA-MB-231 cells less likely to undergo metastasis in zebrafish. We, therefore, conclude that ANXA5 and ANXA6 are required for engraftment and metastasis in zebrafish.

DISCUSSION

We hypothesized that invasive cancer cells are submitted to membrane damages during tumor invasion and metastasis, and require an efficient membrane repair machinery to cope with mechanical constraint. As a result, the membrane repair machinery could be

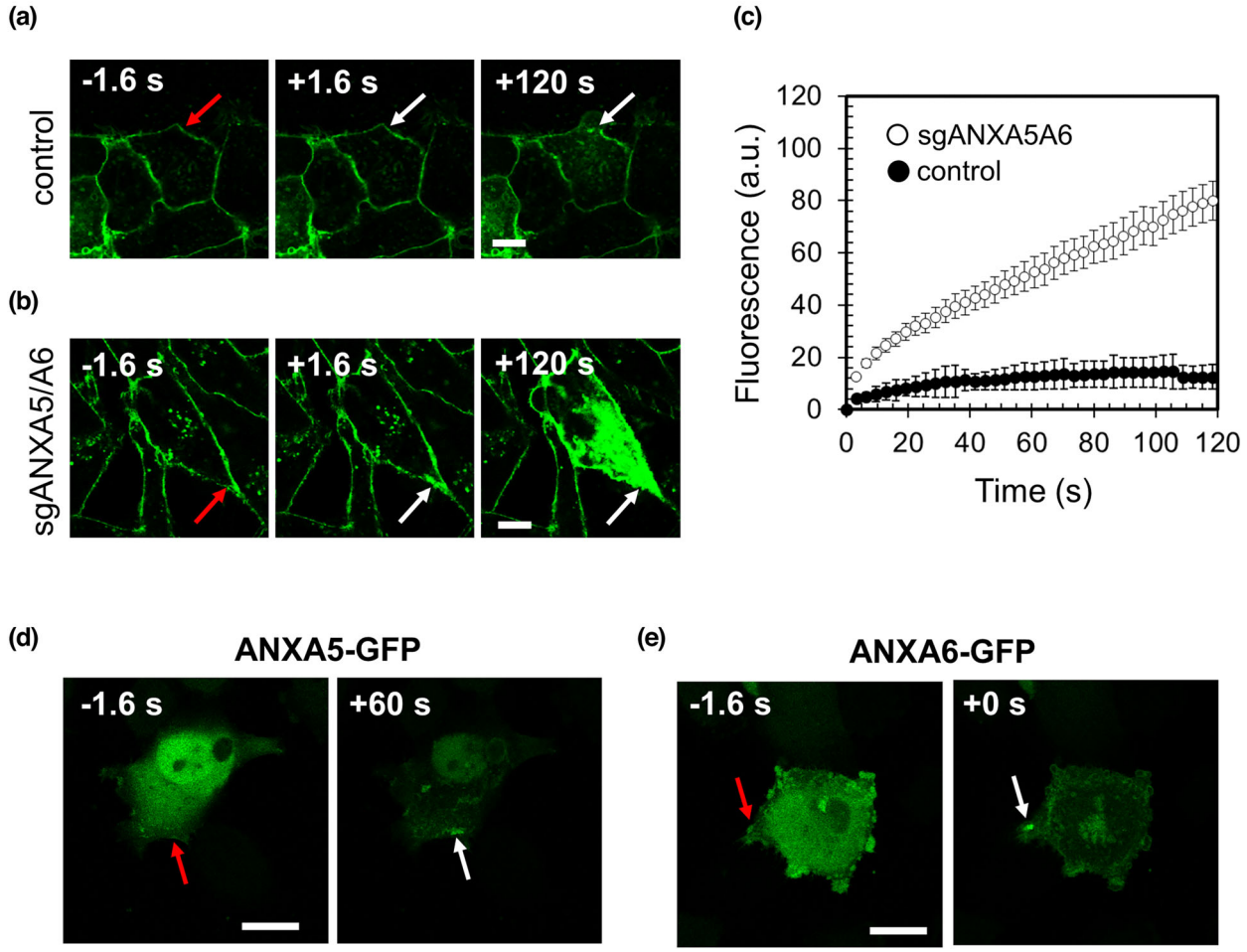

FIGURE 4 ANXA5 and ANXA6 are involved in membrane repair of MDA-MB-231 cells. (a,b) Sequences of representative images showing the response of a control (a) or sgANXA5/A6 (b) MDA-MB-231 cell to a membrane damage performed by 110-mW infrared laser irradiation, in the presence of FM1-43 (green). In all figures, the area of membrane irradiation is marked with a red arrow before irradiation and a white arrow after irradiation. Scale bars: 10 μm. (c) Kinetic data represent the FM1−43 fluorescence intensity integrated over whole cell sections, averaged for about 30 cells (+/− SEM). For a majority of control MDA-MB-231 cells, the fluorescence intensity reached a plateau after about 60 s (black filled circles). For sgANXA5/A6 MDA-MB-231 cells, a continuous and large increase of the fluorescence intensity was observed (empty circles), indicating the absence of membrane resealing. (d,e) Recruitment of ANXA5-GFP (d) and ANXA6-GFP (e) to the site of membrane injury. Red arrow, area before irradiation; white arrow, area after irradiation. After laser injury, MDA-MB-231 cells exhibited an accumulation of ANXA5 and ANXA6 at the disruption site. Scale bars: 20 μm.

considered as a relevant target to annihilate tumor progression. We confirm this hypothesis in zebrafish, in which the genetic inhibition of ANXA5 and ANXA6 drastically reduced the metastasis process of breast cancer cells.

We have previously reported that ANXA5 and ANXA6, which belong to a protein family crucial for membrane repair at least in skeletal muscle cells (Croissant et al., 2021), are significantly expressed in cancer cells (Bouvet et al., 2020). We show here in addition that the expression level of both annexins is positively correlated with invasiveness properties of breast cancer cells. Since ANXA5 and ANXA6 may participate in many physiological processes, including cell proliferation (Ding et al., 2017; Grewal et al., 2017; Qi et al., 2015; Sun et al., 2018), difficulty of breast cancer cells to disseminate in vivo could have resulted from a process different from membrane repair. Nevertheless, we show that MDA-MB-231 cells deficient for ANXA5 and ANXA6 exhibit proliferation properties similar to control cells. In contrast, we observe that ANXA5 and ANXA6 are required for membrane repair in these breast cancer cells. In skeletal muscle cells, ANXA5 and ANXA6 participate in strengthening (Bouter et al., 2011; Carmeille et al., 2017) and remodeling (Croissant et al., 2020) the damaged cell membrane, respectively. To date, the mechanism of membrane repair remains poorly documented beside muscle cells and there is little evidence that ANXA5 and ANXA6 work similarly in cancer cells.

We have shown that ANXA5 and ANXA6 are highly expressed in poor prognosis tumors. We speculate that higher expression of key membrane repair proteins corresponds to an adaptation of cancer cells to cope with mechanical injuries during invasion and

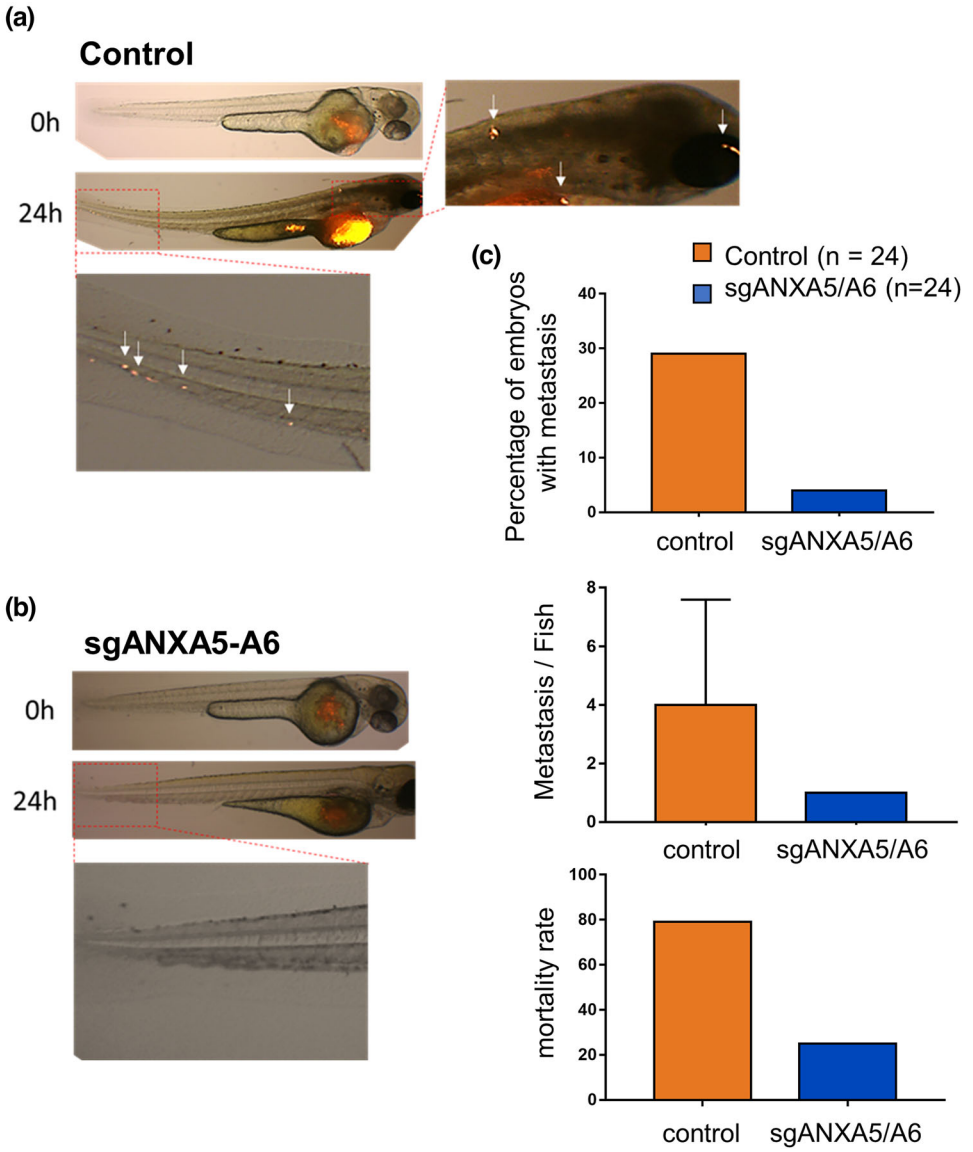

FIGURE 5 Silencing of ANXA5 and ANXA6 prevents metastasis process of MDA-MB-231 cells in zebrafish. (a,b) Control (a, $n = 24$) or sgANXA5/A6 MDA-MB-231 (b, $n = 24$) cells were injected in the perivitelline space of Casper zebrafish embryos. Tumor imaging was done by fluorescence microscopy at 24- and 48-hpi through the tdTomato fluorescent protein constitutively expressed in MDA-MB-231 cells and merged with bright-field images. Insets display magnified images of a portion of the tail and head with metastases (white arrows). (c) The percentage of embryos, either injected by control or sgANXA5/A6 MDA-MB-231 cells, which presented caudal or head metastases was quantified at 24 hpi. Mortality rate was calculated at 48 hpi.

metastasis. We propose that inhibition of membrane repair will disproportionately affect invasive and metastatic cancer cells, since normal cells are not exposed to these stresses.

To date, no potential therapy targeting ANXA5 or ANXA6 has been reported (Prieto-Fernández et al., 2022). Inhibiting membrane repair of cancer cells with therapeutic antibodies would be a good option since it has been shown that a monoclonal anti-ANXA6 antibody significantly decreases the invasiveness of pancreatic, lung, and breast cancer cells in vitro (O'Sullivan et al., 2017). In addition, we and other have shown that anti-ANXA2 antibodies may affect membrane repair in cancer cells and prevent tumor growth and metastasis (Gounou et al., 2022; Lokman et al., 2013). Mechanistically, we hypothesize that antibodies enter the cell at sites of membrane damage and interfere with the membrane repair machinery. Intriguingly, antibodies should only affect cells with membrane damage, potentially further increasing their therapeutic index. Effect of anti-ANXA5 and/or anti-ANXA6 antibodies should be tested in order to investigate whether they may inhibit tumor progression in vivo. Should antibodies fail to inhibit membrane repair, pharmacological drugs able to weaken this process, such as phenotiazines (Heitmann et al., 2021), could be tested.

AUTHOR CONTRIBUTIONS

Céline Gounou, Flora Bouvet, and Anthony Bouter performed the majority of experiments with assistance from Léna d'Agata (Western-blot analysis), Benjamin Liet, Géraldine Siegfried, and Abdel-Majid Khatib (zebrafish experiments), Etienne Harté and Françoise Argoul (proliferation assays), Valérie Prouzet-Mauléon (establishment of sgANXA5/A6 MDA-MB-231 cells), Richard Iggo (establishment of tdTomato+ MDA-MB-231 cells). Anthony Bouter coordinated the entire project and designed the experiments. Anthony Bouter wrote the manuscript. Léna d'Agata, Richard Iggo, Géraldine Siegfried, and Abdel-Majid Khatib contributed to the writing of the manuscript.

ACKNOWLEDGMENTS

The authors acknowledge la Ligue Contre Le Cancer de Gironde (grant 191796 and 238171 to A.B.) and Ligue Contre Le Cancer du Lot-et-Garonne (grant 238294 to A.B.) for their financial support. Christel Poujol and Sébastien Marais are acknowledged for the help in membrane repair assays that were done in the Bordeaux Imaging Center a service unit of the CNRS-INSERM and Bordeaux University, member of the national infrastructure France BioImaging supported by the French National Research Agency (ANR-10-INBS-04). The authors thank the staff of Vect'UB and CRISPR-edit technology platforms (INSERM US 005 – CNRS UAR 3427- TBM-Core, University of Bordeaux, France) for technical assistance. The authors thank Christine Varon for her help in KMplotter analysis.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information.

INSTITUTIONAL REVIEW BOARD STATEMENT

Zebrafish were produced in accordance with the French directive (Ministère de l'Agriculture et de l'alimentation) under permit number A33-063-935. All the procedures were conducted in compliance with the European Communities Council Directive (2010/63/EU).

ORCID

Françoise Argoul https://orcid.org/0000-0001-9157-3565
Anthony Bouter https://orcid.org/0000-0002-0068-2780

REFERENCES

Allier, C., Morel, S., Vincent, R., Ghenim, L., Navarro, F., Menneteau, M., Bordy, T., Hervé, L., Cioni, O., Gidrol, X., Usson, Y., & Dinten, J.M. (2017) Imaging of dense cell cultures by multiwavelength lens-free video microscopy. *Cytometry Part A*, 91(5), 433–442. https://doi.org/10.1002/CYTO.A.23079

Azevedo, A.S., Follain, G., Patthabhiraman, S., Harlepp, S. & Goetz, J.G. (2015) Metastasis of circulating tumor cells: favorable soil or suitable biomechanics, or both? *Cell Adhesion & Migration*, 9(5), 345–356. https://doi.org/10.1080/19336918.2015.1059563

Bansal, D., Miyake, K., Vogel, S.S., Groh, S., Chen, C.C., Williamson, R., McNeil, P.L., & Campbell, K.P. (2003) Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nature*, 423(6936), 168–172. https://doi.org/10.1038/nature01573

Bouter, A., Gounou, C., Bérat, R., Tan, S., Gallois, B., Granier, T., d'Estaintot, B.L., Pöschl, E., Brachvogel, B., & Brisson, A.R. (2011) Annexin-A5 assembled into two-dimensional arrays promotes cell membrane repair. *Nature Communications*, 2(1), 270. https://doi.org/10.1038/ncomms1270

Bouvet, F., Ros, M., Bonedeau, E., Croissant, C., Frelin, L., Saltel, F., Moreau, V., & Bouter, A. (2020) Defective membrane repair machinery impairs survival of invasive cancer cells. *Scientific Reports*, 10(1), 21821. https://doi.org/10.1038/s41598-020-77902-5

Boye, T.L., Maeda, K., Pezeshkian, W., Sønder, S.L., Haeger, S.C., Gerke, V., Simonsen, A.C., & Nylandsted, J. (2017) Annexin A4 and A6 induce membrane curvature and constriction during cell membrane repair. *Nature Communications*, 8(1), 1623. https://doi.org/10.1038/s41467-017-01743-6

Buhl, W.J., García, M.T., Zipfel, M., Schiebler, W. & Gehring, U. (1991) A series of annexins from human placenta and their characterization by use of an endogenous phospholipase A2. *European Journal of Cell Biology*, 56(2), 381–390.

Carmeille, R., Bouvet, F., Tan, S., Croissant, C., Gounou, C., Mamchaoui, K., Mouly, V., Brisson, A.R., & Bouter, A. (2016) Membrane repair of human skeletal muscle cells requires Annexin-A5. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1863(9), 2267–2279. https://doi.org/10.1016/j.bbamcr.2016.06.003

Carmeille, R., Croissant, C., Bouvet, F. & Bouter, A. (2017) Membrane repair assay for human skeletal muscle cells. *Methods in Molecular Biology*, 1668, 195–207. https://doi.org/10.1007/978-1-4939-7283-8_14

Carmeille, R., Degrelle, S. A., Plawinski, L., Bouvet, F., Gounou, C., Evain-Brion, D., Brisson, A.R., & Bouter, A. (2015) Annexin-A5 promotes membrane resealing in human trophoblasts. *Biochimica et Biophysica Acta*, 1853(9), 2033–2044. https://doi.org/10.1016/j.bbamcr.2014.12.038

Croissant, C., Carmeille, R., Brévart, C. & Bouter, A. (2021) Annexins and membrane repair dysfunctions in muscular dystrophies. *International Journal of Molecular Sciences*, 22(10), 5276. https://doi.org/10.3390/ijms22105276

Croissant, C., Gounou, C., Bouvet, F., Tan, S. & Bouter, A. (2020) Annexin-A6 in membrane repair of human skeletal muscle cell: a role in the cap subdomain. *Cells*, 9(7), 1742. https://doi.org/10.3390/cells9071742

Croissant, C., Gounou, C., Bouvet, F., Tan, S. & Bouter, A. (2022) Trafficking of annexins during membrane repair in human skeletal muscle cells. *Membranes*, 12(2), 153. https://doi.org/10.3390/MEMBRANES12020153

Davenport, N.R., Sonnemann, K.J., Eliceiri, K.W. & Bement, W.M. (2016) Membrane dynamics during cellular wound repair. *Molecular Biology of the Cell*, 27(14), 2272–2285. https://doi.org/10.1091/MBC.E16-04-0223/MC-E16-04-0223-S23.MOV

Davis, T.A., Loos, B. & Engelbrecht, A.M. (2014) AHNAK: the giant jack of all trades. *Cellular Signalling*, 26(12), 2683–2693. https://doi.org/10.1016/J.CELLSIG.2014.08.017

Ding, X.M.M., Li, J.X.X., Wang, K., Wu, Z.S.S., Yao, A.H.H., Jiao, C.Y.Y., Qian, J.J., Bai, D.S. & Li, X.C. (2017) Effects of silencing annexin

A5 on proliferation and invasion of human cholangiocarcinoma cell line. *European Review for Medical and Pharmacological Sciences*, 21(7), 1477–1488.

Funakoshi, T., Heimark, R.L., Hendrickson, L.E., McMullen, B.A. & Fujikawa, K. (1987) Human placental anticoagulant protein: isolation and characterization. *Biochemistry*, 26(17), 5572–5578.

Gensbittel, V., Kräter, M., Harlepp, S., Busnelli, I., Guck, J. & Goetz, J.G. (2021) Mechanical adaptability of tumor cells in metastasis. *Developmental Cell*, 56(2), 164–179. https://doi.org/10.1016/J.DEVCEL.2020.10.011

Gerke, V. & Moss, S.E. (2002) Annexins: from structure to function. *Physiological Reviews*, 82(2), 331–371. https://doi.org/10.1152/PHYSREV.00030.2001

Gounou, C., Léna D'agata, F.B., Derieppe, M.A., Rouyer, L., Bouton, L., Mélane, M., Chapeau, D., Harté, E., Martineau, J., Prouzet-Mauleon, V., Tan, S., Souleyreau, W., Saltel, F., Argoul, F., Siegfried, G., Khatib, A., Brisson, A., Iggo, R., & Bouter, A. (2022) Inhibition of the membrane repair protein annexin-A2 prevents tumour invasion and metastasis. *Research Square*. https://doi.org/10.21203/rs.3.rs-2252278/v1

Grewal, T., Hoque, M., Conway, J.R.W., Reverter, M., Wahba, M., Beevi, S.S., Timpson, P., Enrich, C., & Rentero, C. (2017) Annexin A6-A multifunctional scaffold in cell motility. *Cell Adhesion & Migration*, 11(3), 288–304. https://doi.org/10.1080/19336918.2016.1268318

Grewal, T., Rentero, C., Enrich, C., Wahba, M., Raabe, C.A. & Rescher, U. (2021) Annexin animal models-from fundamental principles to translational research. *International Journal of Molecular Sciences*, 22(7), 3439. https://doi.org/10.3390/IJMS22073439

Haeussler, M., Schönig, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J.B., Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J., Joly, J.S., & Concordet, J.P. (2016) Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology*, 17(1), 148. https://doi.org/10.1186/S13059-016-1012-2

Heitmann, A.S.B., Zanjani, A.A.H., Klenow, M.B., Mularski, A., Sønder, S.L., Lund, F.W., Boye, T.L., Dias, C., Bendix, P.M., Simonsen, A.C., Khandelia, H., & Nylandsted, J. (2021) Phenothiazines alter plasma membrane properties and sensitize cancer cells to injury by inhibiting annexin-mediated repair. *The Journal of Biological Chemistry*, 297(2), 101012. https://doi.org/10.1016/J.JBC.2021.101012

Iggo, R. (2022) Lentiviral transduction of mammary epithelial cells. *Methods in Molecular Biology (Clifton, N.J.)*, 2471, 159–183. https://doi.org/10.1007/978-1-0716-2193-6_8

Jaiswal, J.K., Lauritzen, S.P., Scheffer, L., Sakaguchi, M., Bunkenborg, J., Simon, S.M., Kallunki, T., Jäättelä, M. & Nylandsted, J. (2014) S100A11 is required for efficient plasma membrane repair and survival of invasive cancer cells. *Nature Communications*, 5, 3795. https://doi.org/10.1038/ncomms4795

Korolkova, O.Y., Widatalla, S.E., Williams, S.D., Whalen, D.S., Beasley, H.K., Ochieng, J., Grewal, T. & Sakwe, A.M. (2020) Diverse roles of annexin A6 in triple-negative breast cancer diagnosis, prognosis and EGFR-targeted therapies. *Cells*, 9(8), 1855. https://doi.org/10.3390/CELLS9081855

Lambert, O., Gerke, V., Bader, M.F., Porte, F. & Brisson, A. (1997) Structural analysis of junctions formed between lipid membranes and several annexins by cryo-electron microscopy. *Journal of Molecular Biology*, 272(1), 42–55. https://doi.org/10.1006/jmbi.1997.1183

Lánczky, A. & Győrffy, B. (2021) Web-based survival analysis tool tailored for medical research (KMplot): development and implementation. *J Med Internet Res*, 23(7), e27633. https://doi.org/10.2196/27633

Lauritzen, S.P., Boye, T.L. & Nylandsted, J. (2015) Annexins are instrumental for efficient plasma membrane repair in cancer cells. *Seminars in Cell & Developmental Biology*, 45, 32–38. https://doi.org/10.1016/j.semcdb.2015.10.028

Lennon, N.J., Kho, A., Bacskai, B.J., Perlmutter, S.L., Hyman, B.T. & Brown, R.H. (2003) Dysferlin interacts with annexins A1 and A2 and mediates sarcolemmal wound-healing. *The Journal of Biological Chemistry*, 278(50), 50466–50473. https://doi.org/10.1074/jbc.M307247200

Leung, C., Yu, C., Lin, M.I., Tognon, C. & Bernatchez, P. (2013) Expression of myoferlin in human and murine carcinoma tumors: role in membrane repair, cell proliferation, and tumorigenesis. *The American Journal of Pathology*, 182(5), 1900–1909. https://doi.org/10.1016/j.ajpath.2013.01.041

Li, R., Ackerman IV, W E., Mihai, C., Volakis, L.I., Ghadiali, S. & Kniss, D.A. (2012) Myoferlin depletion in breast cancer cells promotes mesenchymal to epithelial shape change and stalls invasion. *PloS One*, 7(6). https://doi.org/10.1371/JOURNAL.PONE.0039766

Lokman, N.A., Elder, A.S.F., Ween, M.P., Pyragius, C.E., Hoffmann, P., Oehler, M.K. & Ricciardelli, C. (2013) Annexin A2 is regulated by ovarian cancer-peritoneal cell interactions and promotes metastasis. *Oncotarget*, 4(8), 1199–1211. https://doi.org/10.18632/ONCOTARGET.1122

McNeil, A.K., Rescher, U., Gerke, V. & McNeil, P.L. (2006) Requirement for annexin A1 in plasma membrane repair. *The Journal of Biological Chemistry*, 281(46), 35202–35207. https://doi.org/10.1074/jbc.M606406200

McNeil, P.L. & Steinhardt, R.A.R.A. (2003) Plasma membrane disruption: repair, prevention, adaptation. *Annual Review of Cell and Developmental Biology*, 19, 697–731. https://doi.org/10.1146/annurev.cellbio.19.111301.140101

O'Sullivan, D., Dowling, P., Joyce, H., Mcauley, E., Mccann, A., Henry, M., McGovern, B., Barham, P., Kelleher, F.C., Murphy, J., Kennedy, S., Swan, N., Moriarty, M., Clynes, M., & Larkin, A. (2017) A novel inhibitory anti-invasive MAb isolated using phenotypic screening highlights AnxA6 as a functionally relevant target protein in pancreatic cancer. *British Journal of Cancer*, 117(9), 1326–1335. https://doi.org/10.1038/bjc.2017.306

Peng, B., Guo, C., Guan, H., Liu, S. & Sun, M.Z. (2014) Annexin A5 as a potential marker in tumors. *Clinica Chimica Acta*, 427, 42–48. https://doi.org/10.1016/j.cca.2013.09.048

Prieto-Fernández, L., Menéndez, S.T., Otero-Rosales, M., Montoro-Jiménez, I., Hermida-Prado, F., García-Pedrero, J.M. & Álvarez-Teijeiro, S. (2022) Pathobiological functions and clinical implications of annexin dysregulation in human cancers. *Frontiers in Cell and Developmental Biology*, 0, 1905. https://doi.org/10.3389/FCELL.2022.1009908

Qi, H., Liu, S., Guo, C., Wang, J., Greenaway, F.T. & Sun, M.Z. (2015) Role of annexin A6 in cancer. *Oncology Letters*, 10(4), 1947–1952. https://doi.org/10.3892/OL.2015.3498

Römisch, J. & Heimburger, N. (1990) Purification and characterization of six annexins from human placenta. *Biological Chemistry Hoppe-Seyler*, 371(5), 383–388.

Sakwe, A.M., Koumangoye, R., Guillory, B. & Ochieng, J. (2011) Annexin A6 contributes to the invasiveness of breast carcinoma cells by influencing the organization and localization of functional focal adhesions. *Experimental Cell Research*, 317(6), 823–837. https://doi.org/10.1016/j.yexcr.2010.12.008

Sanjana, N.E., Shalem, O. & Zhang, F. (2014) Improved vectors and genome-wide libraries for CRISPR screening. *Nature Methods*, 11(8), 783–784. https://doi.org/10.1038/NMETH.3047

Sun, X., Liu, S., Wang, J., Wei, B., Guo, C., Chen, C. & Sun, M.Z. (2018) Annexin A5 regulates hepatocarcinoma malignancy via CRKI/II-DOCK180-RAC1 integrin and MEK-ERK pathways. *Cell Death & Disease*, 9(6). https://doi.org/10.1038/S41419-018-0685-8

Thompson, E.W., Paik, S., Brünner, N., Sommers, C.L., Zugmaier, G., Clarke, R., Shima, T.B., Torri, J., Donahue, S., Lippman, M.E., Martin, G.R., & Dickson, R.B. (1992) Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *Jour-*

nal of Cellular Physiology, 150(3), 534–544. https://doi.org/10.1002/jcp.1041500314

Thompson, E.W., Reich, R., Shima, T.B., Albini, A., Graf, J., Martin, G.R., Dickson, R.B., & Lippman, M.E. (1988) Differential regulation of growth and invasiveness of MCF-7 breast cancer cells by antiestrogens. *Cancer Research*, 48(23), 6764–6768.

Vilá de Muga, S., Timpson, P., Cubells, L., Evans, R., Hayes, T.E., Rentero, C., Hegemann, A., Reverter, M., Leschner, J., Pol, A., Tebar, F., Daly, R.J., Enrich, C., & Grewal, T. (2009) Annexin A6 inhibits Ras signalling in breast cancer cells. *Oncogene*, 28(3), 363–377. https://doi.org/10.1038/onc.2008.386

Wirtz, D., Konstantopoulos, K. & Searson, P.C. (2011) The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nature Reviews Cancer*, 11(7), 512–522. https://doi.org/10.1038/nrc3080

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Gounou, C. et al. (2023) Annexin-A5 and annexin-A6 silencing prevents metastasis of breast cancer cells in zebrafish. *Biology of the Cell*, 115, 2200110. https://doi.org/10.1111/boc.202200110