



## Original Article

## GABARAPL1 is essential in extracellular vesicle cargo loading and metastasis development

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## A B S T R A C T

**Background and purpose:** Hypoxia is a common feature of tumours, associated with poor prognosis due to increased resistance to radio- and chemotherapy and enhanced metastasis development. Previously we demonstrated that GABARAPL1 is required for the secretion of extracellular vesicles (EV) with pro-angiogenic properties during hypoxia. Here, we explored the role of GABARAPL1<sup>+</sup> EV in the metastatic cascade.

**Materials and methods:** GABARAPL1 deficient or control MDA-MB-231 cells were injected in murine mammary fat pads. Lungs were dissected and analysed for human cytokeratin 18. EV from control and GABARAPL1 deficient cells exposed to normoxia (21% O<sub>2</sub>) or hypoxia (O<sub>2</sub> < 0.02%) were isolated and analysed by immunoblot, nanoparticle tracking analysis, high resolution flow cytometry, mass spectrometry and next-generation sequencing. Cellular migration and invasion were analysed using scratch assays and transwell-invasion assays, respectively.

**Results:** The number of pulmonary metastases derived from GABARAPL1 deficient tumours decreased by 84%. GABARAPL1 deficient cells migrate slower but display a comparable invasive capacity. Both normoxic and hypoxic EV contain proteins and miRNAs associated with metastasis development and, in line, increase cancer cell invasiveness. Although GABARAPL1 deficiency alters EV content, it does not alter the EV-induced increase in cancer cell invasiveness.

**Conclusion:** GABARAPL1 is essential for metastasis development. This is unrelated to changes in migration and invasion and suggests that GABARAPL1 or GABARAPL1<sup>+</sup> EV are essential in other processes related to the metastatic cascade.

## Introduction

Most cancer related deaths are attributed to the development of metastases, which therefore pose a major clinical challenge in cancer treatment [1–3]. The development of metastases is a complex cascade which requires the migration of cancer cells from the primary tumour, invasion into the surrounding tissues, intravasation, immune evasion, extravasation and colonization [4]. Hypoxia is a common feature of solid tumours caused by their continuous growth, altered energy metabolism and defective vasculature [5,6]. It is associated with increased incidence of metastasis development and therapy resistance which persists even after reoxygenation [5–7]. As such, post-hypoxic cells drive tumour recurrence after treatment [8]. This aggressive phenotype is driven by several hypoxia-induced cellular protection mechanisms such as the unfolded protein response (UPR), autophagy

and hypoxia inducible factor (HIF) signalling [5,6,9]. HIF dependent signalling contributes to the development of metastases through the induction of epithelial-mesenchymal transition (EMT), migration, invasion and development of pre-metastatic niches (PMN) [10–14]. To create these growth-favouring PMN, cancer cells manipulate immune cells, endothelial cells and stromal cells at distant locations [15–20]. This is mediated via the secretion of a variety of growth factors, chemokines, enzymes and extracellular vesicles (EV) [20].

EV are nano-sized membrane vesicles which are secreted by most cell types. They are highly enriched in tetraspanins (CD9, CD63, CD81), which are therefore widely used as markers for EV identification [21]. In addition, they contain a complex molecular cargo of proteins, lipids and nucleic acids which are transferred to surrounding and distant cells for intercellular communication [22]. Depending on the biogenesis pathway, EV can be subdivided in exosomes, produced by inward

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budding of the endosomal membrane and subsequently released by fusion of the multivesicular endosome with the plasma membrane, and microvesicles (MV), produced by direct budding of the plasma membrane [22]. Although the biogenesis of both types occurs at different subcellular locations, they share various intracellular mechanisms and sorting machineries [23]. The overlap in size and cellular machineries complicates the distinction between EV subtypes after secretion [23,24]. In addition to their physiological role in intercellular communication, EV contribute to most processes of the metastatic cascade [25–27]. Firstly, EV aid in the development of metastases by increasing cancer cell migration and invasiveness [28–30]. Secondly, they increase adherence of tumour cells to endothelial cells, suggesting a role for cancer derived EV in extravasation [17]. Thirdly, integrin composition on the membrane of EV guide organotropic metastases by targeting their uptake to specific cells and/or organs [31]. During hypoxia the molecular cargo of EV is altered, resulting in the possible transfer of hypoxia tolerance as well as suppression of anti-tumour immune reactions [32,33].

Recently we observed that expression of one of the LC3/GABARAP protein family members, Gamma-aminobutyric acid receptor-associated protein-like 1 (GABARAPL1), is increased during hypoxia and is essential for the secretion of pro-angiogenic EV during hypoxia [34]. The LC3/GABARAP protein family consist of seven orthologues which share a high sequence similarity [35]. During autophagy, LC3/GABARAP family proteins are involved in the formation of autophagosomes, directing autophagosomal cargo for degradation and fusion of autophagosomes with lysosomes [35]. In addition to their role in autophagy, the LC3/GABARAP proteins exhibit diverse functions that extend beyond autophagy, including intracellular trafficking, oncogenic/tumour suppressive functions, phagocytosis and cell motility [35,36]. As such, GABARAPL1 is required for membrane expression of EGFR during hypoxia and nuclear translocation of the androgen receptor [36,37]. Recently, GABARAPL1 was observed to increase cancer cell sensitivity to ferroptosis, a form of programmed cell death induced by, amongst others, radiotherapy [38,39]. Furthermore GABARAPL1 inhibits EMT, which plays a major role during metastasis development [40]. Nevertheless, the role of GABARAPL1 and/or GABARAPL1<sup>+</sup> EV in the development of metastases is unknown [41–43]. In the current study we therefore explored the role of GABARAPL1 in cellular migration, invasion and metastasis development.

## Material & methods

### Cell culture

MDA-MB-231 (breast adenocarcinoma) cells were cultured in DMEM (Sigma-Aldrich, D6429) supplemented with 10% fetal bovine serum (FBS) (Serana, S-FBS-SA-015). Serum derived EV were depleted by ultracentrifugation of 30% FBS at 100.000 g for 16 h and 0,22 µm filtration (Corning, 431097). Before EV isolation, cells were cultured in DMEM supplemented with 5% EV-depleted FBS for 24 h exposed to normoxia (21% O<sub>2</sub>) or hypoxia (O<sub>2</sub> < 0,02%). Inducible GABARAPL1 knockdown was performed as described previously [34].

### EV isolation

Differential centrifugation of conditioned medium (CM) was performed as described previously [34]. EV were purified using size exclusion chromatography (SEC) or sucrose density gradient centrifugation (DGC). For SEC, CM was concentrated by filtration (Millipore, UFC910096) to a final volume of 1 ml, loaded onto a 16 ml Sepharose® CL-2B column (Cytiva, GE17-0140-01) and eluted in PBS in 1 ml fractions. EV-containing fractions 7 and 8 were pooled, aliquoted and stored at –80 °C. The procedure for DGC was performed as described before [34]. In short, EV were pelleted at 100.000g for 1 h, resuspended in PBS and loaded on the bottom of a linear sucrose gradient (2.0 M–0.4 M).

Gradients were centrifuged for 16 h at 200.000g and separated in 12 fractions (bottom-up).

### Protein quantification

SEC purified EV were lysed in RIPA for 2 h. EV from sucrose gradient fractions were pelleted at 100.000 g for 1 h and resuspended in PBS containing 2% SDS and 1% triton. Protein concentrations were determined using the Micro BCA™ Protein Assay Kit (ThermoFisher scientific, 23235), according to the manufacturer's guidelines.

### Nanoparticle tracking analysis

NTA was performed using the ZetaView® Nanoparticle Tracking Analyzer (Particle Metrix GmbH, v8.05.11 SP4). Settings were kept constant for each acquisition and analysis: sensitivity 80, shutter 100, frame rate 30, minimal brightness 30, minimal area 10, maximal area 1000 and trace length 15.

### High resolution flow cytometry

Flow cytometric analysis of individual EVs was performed on a BD Influx flow cytometer tailored for EV analysis (Becton Dickinson, Brussels, Belgium) as described previously [34,44].

### Immunoblotting

EV were pelleted by ultracentrifugation at 100.000 g for 60 min. Cells and EV were lysed in RIPA supplemented with (non-)reducing Laemmli buffer. Protein lysates were denatured for 5 min at 96 °C, separated by SDS-PAGE and transferred to PVDF membranes. After blocking in 5% casein in PBS-T (0,2% Tween-20) for 90 min, proteins were probed for CD63 (0,5 µg/ml, BD-Pharmingen™, 556019), CD81 (0,5 µg/ml, BD-Pharmingen™, 555675), GABARAPL1 (0,5 µg/ml, Protein tech group, 110101-AP), GM130 (0,25 µg/ml, BD Biosciences, 610823), β-actin (1/10.000, Clone 4, MP-Biomedicals, 0869100-CF) overnight at 4 °C. HRP-labelled secondary antibodies (Anti-mouse, 7076S, 0,184 µg/ml; Anti-rabbit, 7074S, 0,06 µg/ml, Cell Signaling) were incubated for 90 min and bands were visualized using ECL Detection Reagent (Cytiva, RPN2232). Band intensity was quantified using ImageJ software.

### Transmission electron microscopy

A thin aqueous film of the SEC isolated EV sample was formed on a Lacey Carbon Film (Electron Microscopy Sciences) by applying 2.5 µl of sample and blotting away excess liquid. The grid was held by tweezers in the environmental chamber (20 °C and more than 95% relative humidity) of the Vitrobot mark IV [45]. After blotting, the grid with the thin aqueous film was rapidly vitrified by plunging into ethane cooled to its melting point (–180 °C) by liquid nitrogen. The vitrified specimen was transferred to a cryo-transmission electron microscope (Arctica 200KV) and pictures were taken with a Falcon III camera (adapted from [34]).

### RT-qPCR

RNA was isolated using the NucleoSpin kit (Machery-Nagel, 740955.50) and converted into cDNA using the iScript™ cDNA Synthesis Kit (Biorad, 1708891). Gene expression (Supplemental Table 1) was analysed using the SensiMix™ SYBR® Low-ROX Kit (meridian bioscience, QT625-05) in the CFX connect Real-Time System (Biorad) and normalised to RPL13A expression.

### Mass spectrometry

EV proteins were concentrated by acetone precipitation and

**Table 1**  
10 of top 20 most abundant proteins in MDA-MB-231 EV are associated with metastasis.

Protein	Metastasis association	Ref.
Thrombospondin-1 (TSP-1)	-High tumour TSP-1 is associated with metastasis development -TSP-1 deficiency reduces metastasis development -TSP-1 stimulates cancer cell migration and invasion	[79,80]
Galectin-3-binding protein (G3-BP)	-Inhibition of G3-BP (knockdown and antibodies) reduces metastasis development	[81]
Pentraxin-related protein PTX3	-High PTX3 expression in tumour associated with metastasis development -PTX3 knock-down/overexpression reduces/increases metastasis development -PTX3 promotes cancer cell migration and invasion -PTX3 promotes epithelial-mesenchymal transition -PTX3 stimulates adhesion of cancer cells to endothelial cells	[82–84]
Fibulin-1	-High Fibulin-1 expression in tumour associated with metastasis development	[85]
Vitronectin	-High Vitronectin expression in tumour associated with metastasis development	[86]
Alpha-fetoprotein (AFP)	-High AFP expression in tumour associated with metastasis development -AFP increases cancer cell migration and invasion	[87–89]
Versican (VCAN)	-High VCAN expression in tumour associated with metastasis development -VCAN increases cancer cell migration and invasion	[90–92]
Agrin	-Knockdown/overexpression of Agrin decreases/increases metastasis <i>in vivo</i> -Knock-down of Agrin reduces cancer cell migration and invasion -Agrin promotes epithelial-mesenchymal transition	[93,94]
Complement C3	-Cleaved complement C3 increases cancer cell migration, invasion and metastasis via upregulation of Nrf2 -Involved in pre-metastatic niche development	[95,96]
Kinesin-like protein KIF20B	-High KIF20B expression in tumour associated with metastasis development -Knock-down of KIF20B reduces cancer cell migration and invasion	[97]

resuspended in 30 µl 50 mM ammonium bicarbonate (ABC; Sigma-Aldrich) containing 5 M urea. Samples were processed and analysed as described previously [46].

Sequencing

After SEC, EV RNA was isolated using the miRNeasy Micro Kit (Qiagen, 217084). The cDNA library was generated using the TruSeq Small RNA library prep kit (Illumina) according to the manufacturer’s protocol. Input of total RNA for library preparation was between 3 and 16 ng. Small RNAs were enriched by size selection on the PippinHT (Sage Science). Samples were pooled in equimolar ratio and sequenced using NextSeq 500/550 High-Output v2.5 Kit (75cycles) on Illumina NextSeq 500. Analyses of sequencing datasets were performed as previously described [47]. The small RNA reads were aligned to the mirBase database (v22) and summed per transcript. MicroRNAs were considered being expressed when at least five reads aligned to a microRNA and were present in all samples of the respective group.

Migration

Cells were seeded at a density of 62,500 cells/cm<sup>2</sup>, allowed to attach overnight and scratched using the IncuCyte Wound maker (Essen

Bioscience). Migration of cancer cells was assessed after GABARAPL1 knockdown or 18 h of EV stimulation (derived from 1x10<sup>6</sup> cells). Cell migration was monitored using the Incucyte FLR (Essen Bioscience).

Invasion

Invasion of cancer cells was assessed after GABARAPL1 knockdown or after 24 h of EV stimulation (2.5 µg/ml). Inserts (Sarstedt, 833932800) were coated with Matrigel® (0.9 mg/ml, 2 h, 37 °C, Corning, 354234), after which cells were seeded (10,000 cells/insert) in 0% FBS (with 10% FBS in the bottom well as chemoattractant) and allowed to invade for 17–24 h. Cells were fixed with 4% para-formaldehyde and stained with Hoechst. The number of invaded cells was quantified using ImageJ software.

Tumour models

All animal experiments were conducted in accordance with national guidelines and approved by the animal ethics committee of Maastricht University. Inducible GABARAPL1 knockdown or control MDA-MB-231 cells were injected in the mammary fat pad of female NMR1<sup>nu/nu</sup> mice (1.0x10<sup>6</sup> cells in 50 µl Matrigel®). To induce knockdown, doxycycline was administered via drinking water (2 g/L, 5% sucrose, *ad libitum*). Animals were injected intra-peritoneally with pimonidazole (60 mg/kg) 1 h prior to, and intra-venously with Hoechst (3 mg/kg) 1 min prior to, killing. When tumours reached 1000 mm<sup>3</sup>, animals were killed, tumours and lungs were dissected and paraffin embedded. Metastases were visualized by human cytokeratin 18 immunohistochemistry on three levels (200 µm depth difference). Tumour necrosis, vessel density, hypoxia and perfusion were assessed via haematoxylin-eosin, 9F1, pimonidazole and Hoechst staining, respectively.

Statistical analysis

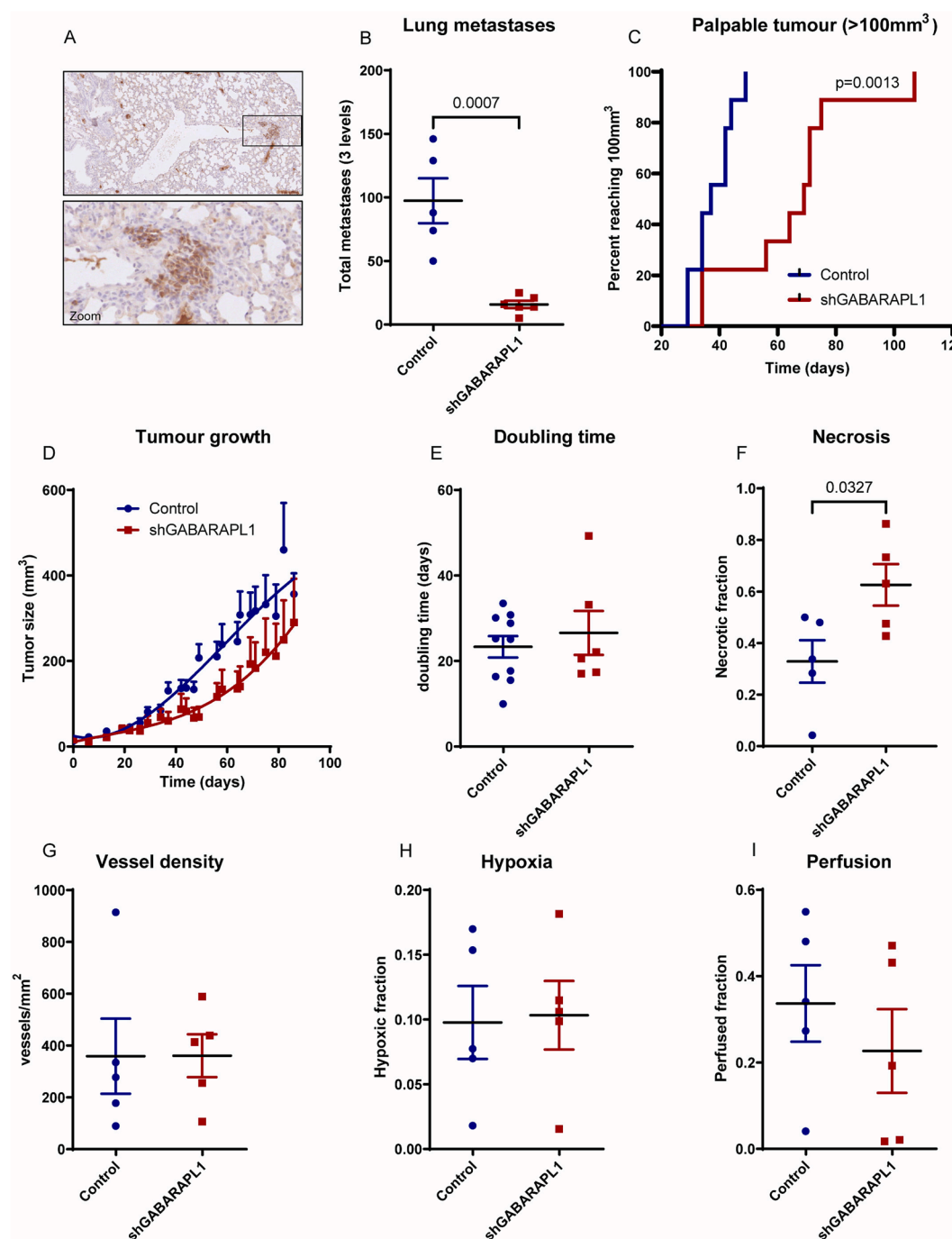
Statistical analysis were performed using GraphPad Prism (v9.5.0). Differences were considered significant when p < 0.05. Data is represented as mean ± SEM.

Results

Although GABARAPL1 is involved in several processes related to metastasis development [41–43], the exact and overall effect is unknown. We first determined the impact of GABARAPL1 on metastasis development *in vivo*. Strikingly, the number of pulmonary metastases resulting from GABARAPL1 deficient tumours decreased by 84% (Fig. 1A,B). In line with previous results [34], GABARAPL1 deficiency reduces tumour growth in early development but not when the tumour is fully established. This is illustrated by delayed palpability, but no effect on doubling time (Fig. 1C-E). As observed previously, GABARAPL1 deficiency is associated with increased tumour necrosis (Fig. 1F). Other micro-environmental parameters, such as vessel density, hypoxia and vessel perfusion remain unaltered (Fig. 1G-I). In conclusion, we show that GABARAPL1 fulfils an essential role in the development of metastases *in vivo*.

Metastasis development is the result of a sequential series of events including migration, invasion, intravasation, extravasation and colonization. To evaluate if GABARAPL1 is involved in cellular migration and invasion, scratch- and transwell invasion-assays were performed. The migratory capacity of GABARAPL1 deficient cells was decreased (Fig. 2A). No differences in cellular invasion were observed (Fig. 2B, Supplemental Fig. 2), suggesting that migration is not rate limiting in the invasive process.

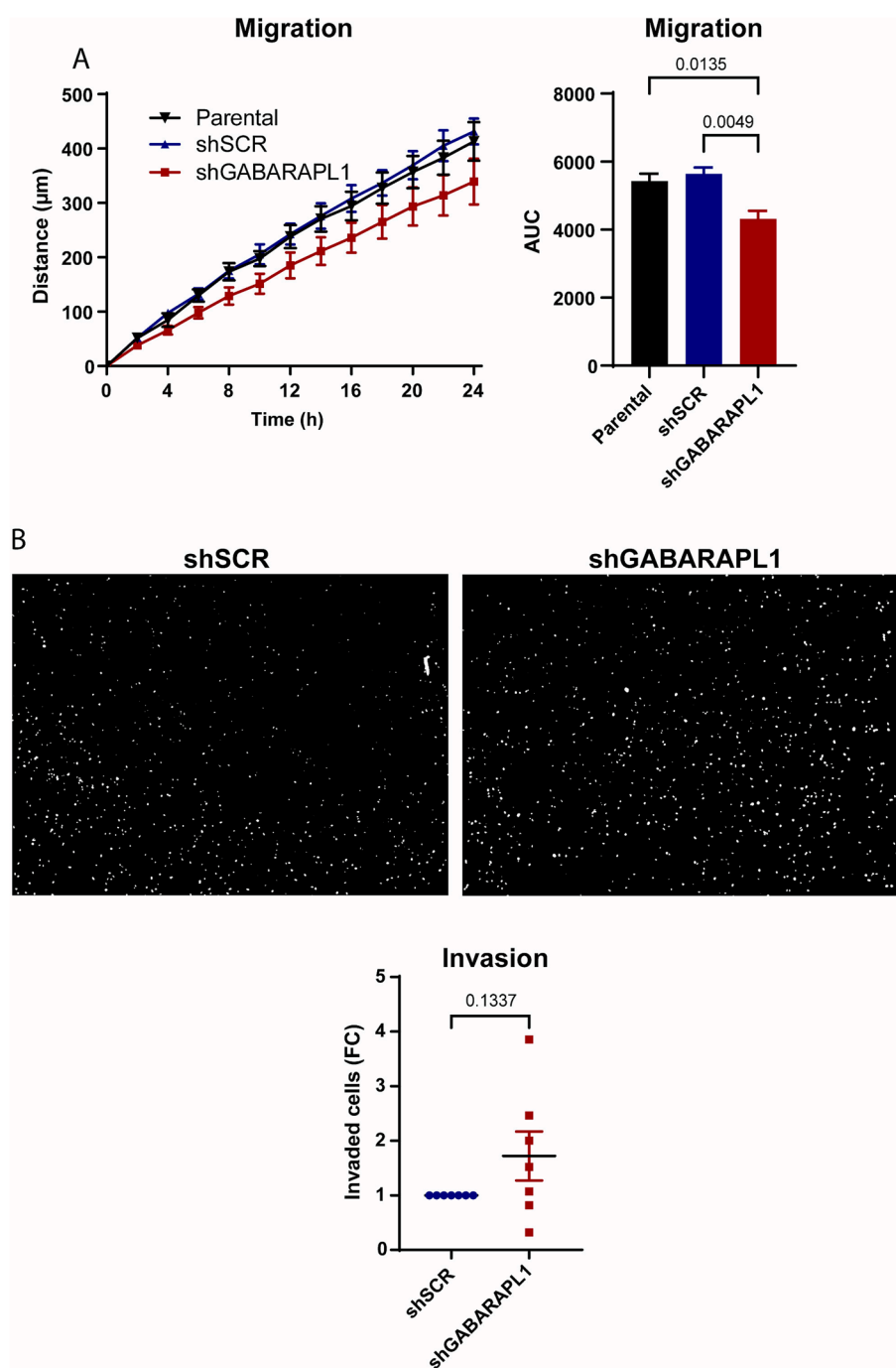
Although a reduction in migration was observed in GABARAPL1 deficient cells, invasion is not affected and therefore does not explain the large difference in metastases formation *in vivo*. Previously we showed that GABARAPL1 controls biogenesis of EV, in particular those released



**Fig. 1.** GABARAPL1 contributes to metastasis development *in vivo*. A) Lung metastases in mice with control and shGABARAPL1 xenografts visualised by human cytokeratin 18 immunohistochemistry. B) Quantification of lung metastases in mice with control (n = 5) and shGABARAPL1 (n = 6) xenografts, unpaired *t* test. C) Kaplan-Meier plot of the time for control (n = 9) and shGABARAPL1 (n = 9) tumours to reach 100 mm<sup>3</sup> after implantation. D) Growth curve of control (n = 12) and shGABARAPL1 (n = 11) xenografts. E) Doubling time of control (n = 10) and shGABARAPL1 (n = 6) xenografts. F) Tumour necrosis of MDA-MB-231 xenografts was morphologically determined by H&E staining, n = 5 per group, unpaired *t* test. G) Vessel density was determined by 9F1 immunohistochemistry, n = 5 per group. H) The hypoxic fraction of xenografts was determined using pimonidazole staining, n = 5 per group. I) Perfusion of control and shGABARAPL1 xenografts was determined by Hoechst, n = 5 per group.

during hypoxia [34]. As EV and hypoxia contribute to many hallmarks of cancer, we next investigated whether (hypoxic) MDA-MB-231 derived EV play a role in the development of metastases [26,31]. Cancer cell derived EV were isolated using SEC and tested for EV marker expression (Fig. 3A, Supplemental Fig. 3A). Immunoblot analysis indicated enrichment of EV-markers CD81 and CD63 in EV preparations. The absence of the golgi associated protein, GM130, confirms the absence of cellular contaminants. Particle size distribution was determined by

nanoparticle tracking analysis. No differences in size distribution were observed (Fig. 3B-C, Supplemental Fig. 3B-C). Cryo-TEM imaging confirms the isolation of lipid-bilayer vesicles (Fig. 3D). Protein mass spectrometry analysis of EV revealed that 9 out of the 20 most abundant proteins in EV derived from both normoxic and hypoxic cells are associated with metastasis development (Table 1). In addition, miRNA sequencing demonstrated that 7 out of 20 most abundant miRNAs in these EV are involved in metastatic processes. These miRNAs have been



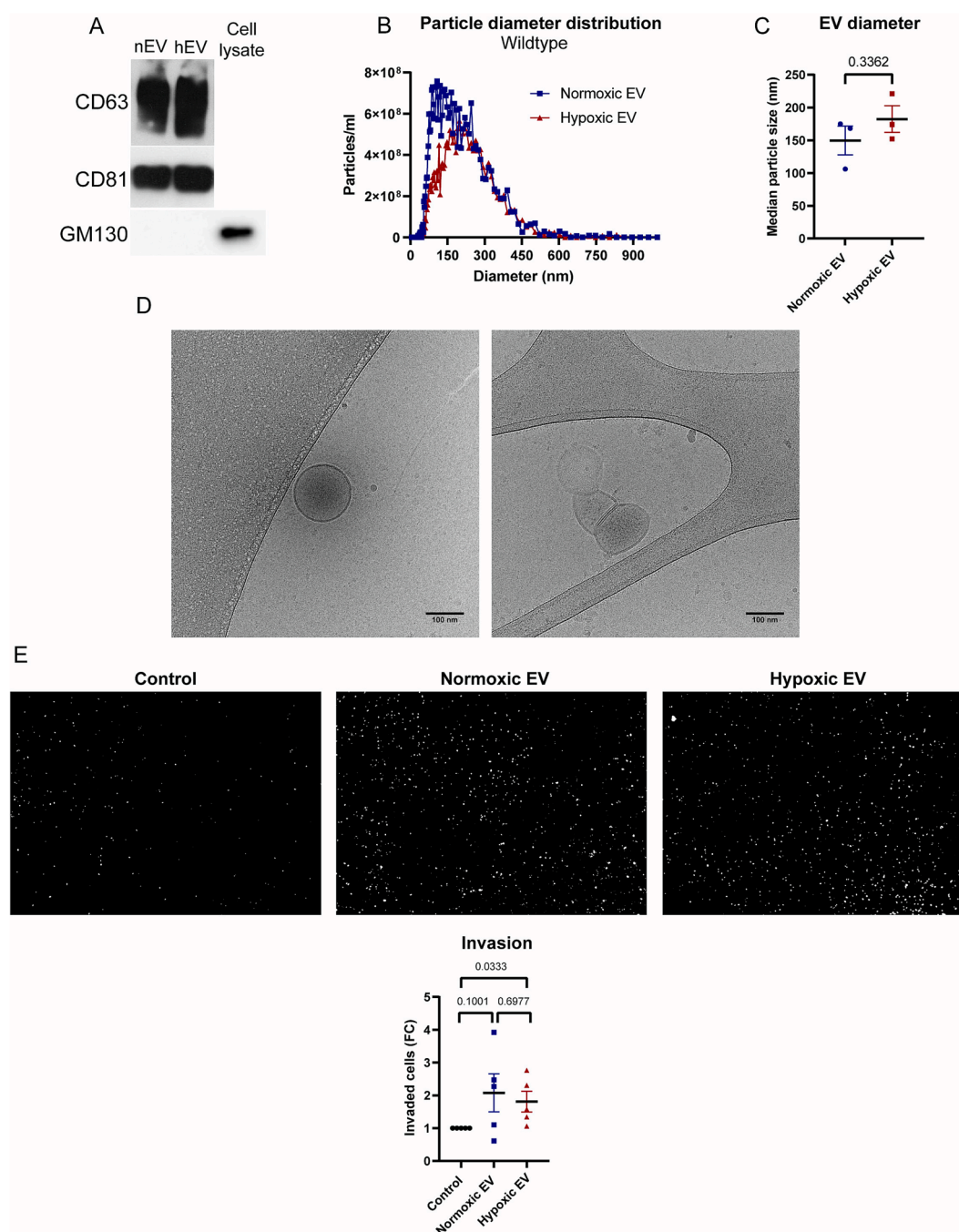
**Fig. 2.** GABARAPL1 increases cancer cell migration but does not affect invasion. A) Migration rate of MDA-MB-231 parental, control (shSCR) and GABARAPL1 deficient cells,  $n = 3$ , one-way ANOVA with Tukey's multiple comparisons test. B) Representative binary images of transwell inserts after invasion by control or GABARAPL1 deficient cells. For each individual experiment, fold-change (FC) was calculated compared to control (set to 1),  $n = 7$ , unpaired  $t$  test.

found to influence various cellular events, including cell migration, invasion, and epithelial-mesenchymal transition (EMT) (Table 2). We therefore examined EV mediated effects on migration and invasion. MDA-MB-231 migration was not altered in response to EV stimulation (Supplemental Fig. 4 A-B). In contrast, cancer cell invasion was significantly increased upon stimulation with cancer cell derived EV from hypoxic cells (Fig. 3E, Supplemental Figure 5). Collectively, these data suggest the involvement of EV in the development of metastases.

To characterize if EV secretion by breast cancer cells is dependent on GABARAPL1, we isolated EV from control and GABARAPL1 deficient cells by sucrose density gradient centrifugation. EV were quantified by high-resolution flow cytometry [34,44]. Contrary to findings in other

cell types, no differences were observed in the total EV counts of secreted EV (Fig. 4A). Immunoblot analysis demonstrated increased expression of EV-markers CD63 and CD81 in EV secreted by GABARAPL1 deficient cells (Fig. 4B). Total EV protein content was not significantly altered (Fig. 4C). miRNA profiling revealed that EV derived from GABARAPL1 deficient cells contain a different miRNA profile compared to control cells (shSCR) (Fig. 4D). Due to GABARAPL1's essential role in biogenesis and cargo recruitment to hypoxia-regulated EV subsets [34], differences in EV miRNA profiles from GABARAPL1 deficient cells (Fig. 4D, right) were expected. Surprisingly, miRNA profiles in EV derived from normoxic cells were also altered (Fig. 4D, left). These alterations appear to be specific for the oxygenation status. From the 67





**Fig. 3. Extracellular vesicles increase MDA-MB-231 cell invasiveness.** A) Immunoblot for the presence of EV markers CD63 and CD81, and absence of Golgi marker GM130, in EV isolates from normoxic (N) or hypoxic (H) MDA-MB-231 cells. B) Particle size distribution as measured by nanoparticle tracking analysis (NTA). C) Median particle size as measured by NTA,  $n = 3$ , unpaired  $t$  test. D) Cryo-TEM imaging of EV. E) EV increase MDA-MB-231 invasiveness in transwell invasion assays, representative binary images of transwell inserts. For each individual experiment, FC was calculated compared to control (set to 1),  $n = 5$ , unpaired  $t$  test.

miRNA that were altered, 60 are only represented in either normoxia or hypoxia. Of the miRNA observed in both groups, only miR-126-5p was decreased in GABARAPL1 deficient EV independent of oxygenation status. miR-146a-5p, miR-151a-5p and miR-1307-5p were enriched in EV derived from GABARAPL1 deficient cells independent of oxygenation status. Hypoxia is an important contributor to metastasis development. Of the 33 differentially recruited miRNA to GABARAPL1 deficient EV during hypoxia, 19 are described to affect metastatic processes (Table 3). Interestingly miR-148b-3p, miR-24-3p, miR-183-5p, miR-130b-3p, miR-345-5p, miR-27a-3p, miR-20a-5p, miR-425-5p and miR-101-3p (reduced expression in hypoxic control EV compared to

normoxic EV) were enriched in EV from hypoxic GABARAPL1 deficient cells. Conversely, miR-451a and miR-150-5p (increased expression in hypoxic control EV) are recruited less to EV from hypoxic GABARAPL1 deficient cells.

In addition, hypoxic GABARAPL1 deficient cells increase the secretion of miR-148b-3p, miR-24-3p, miR-130b-3p, miR-345-5p, miR-29a-3p, miR-146a-5p, miR-138-5p and miR-101-3p via EV. Overall, these miRNA have been previously described to reduce the development of metastases, reduce cancer cell migration and invasion and inhibit EMT [48–61].

Conversely, GABARAPL1 deficient EV contain lower levels of miR-

**Table 2**  
7 of top 20 most abundant miRNA in MDA-MB-231 EV are associated with metastasis.

miRNA	Metastasis association	Ref.
hsa-miR-92a-3p	-High expression is associated with lymph node metastasis	[98–100]
hsa-miR-21-5p	-Increases cancer cell migration and invasion -Promotes cancer cell invasiveness -Inhibition reduces cancer cell migration and invasion -Overexpression induces metastasis development <i>in vivo</i> -High exosomal abundance is associated with metastasis development	[101–103]
hsa-miR-181a-3p	-High exosomal abundance is associated with metastasis development	[104,105]
hsa-miR-191-5p	-High expression is associated with lymph node metastasis	[106]
hsa-miR-222-3p	-Increases cancer cell migration and invasion -High exosomal abundance is associated with lymph node metastasis	[107]
hsa-miR-423-5p	-Increases cancer cell migration and invasion	[108,109]
hsa-miR-1246	-High serum abundance associated with lymph node metastasis -Inhibition of miR-1246 reduces cancer cell migration, invasion and metastasis development	[110,111]

411-5p a miRNA previously related to increased development of metastases, cancer cell migration, invasion and EMT [62]. In short, these data suggest that GABARAPL1 is important for EV cargo sorting and suggest a role for GABARAPL1<sup>+</sup> EV in metastasis development. To evaluate whether the changes in GABARAPL1 deficient EV contribute to the observed reduction metastases *in vivo*, their effects on migration and invasion were determined. Unexpectedly, GABARAPL1 deficient EV did not alter cancer cell migration or invasion (Fig. 4E, Supplemental Fig. 3,6). Given the essential role of GABARAPL1 in metastasis formation *in vivo*, our data suggests that GABARAPL1 regulates other mechanisms related to the metastatic cascade.

**Discussion**

With studies describing both pro- and anti-metastatic effects, the role of GABARAPL1 in the development of metastasis remains unclear [40–43]. Here, we demonstrate that GABARAPL1 is essential in the metastatic cascade as illustrated by 84% reduction in metastases derived from GABARAPL1 deficient tumours. In agreement with previous studies in colorectal and glioblastoma xenografts [34], MDA-MB-231 GABARAPL1 deficient tumours show increased necrosis, probably due to a decreased stress tolerance. This could in part explain the decrease in metastases due to a reduction in viable and potentially migratory cells, however, the increase in necrosis is not proportional to the decrease in pulmonary metastases. Interestingly, GABARAPL1 deficiency significantly reduces migration, yet no effects were observed on invasiveness. This is surprising as invasion depends on degradation of extracellular matrix (ECM) combined with migration, suggesting that ECM degradation is rate limiting. Furthermore, these results suggest that GABARAPL1 is essential in other processes of the metastatic cascade, rather than cellular migration and invasion.

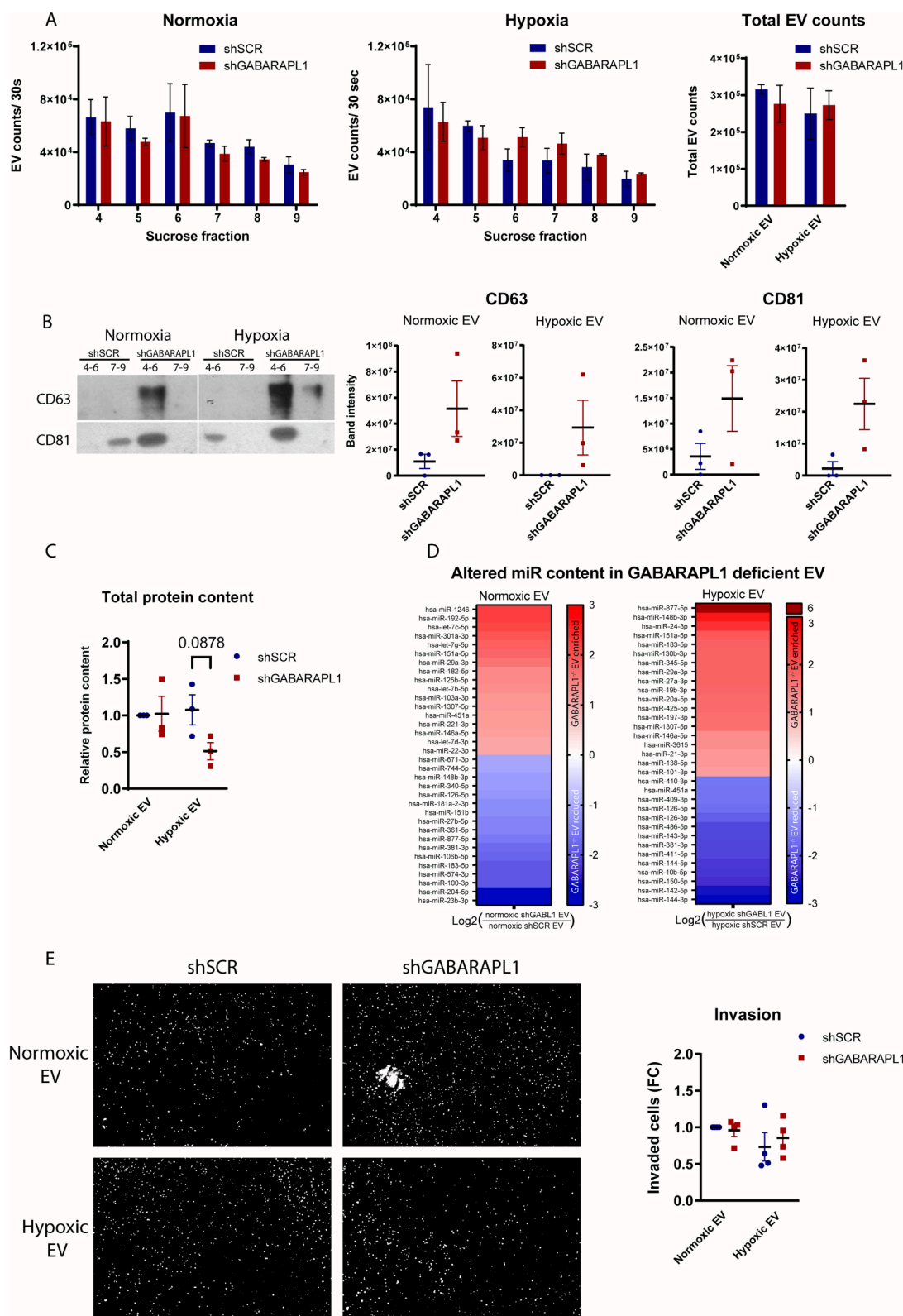
The development of metastases is a complex sequence of events. In addition to leaving the primary tumour and invading tissues, cells need to enter and survive the blood- and lymphatic- circulation after which they extravasate and colonize distant organs. As part of the pro-survival autophagy machinery, GABARAPL1 may play an essential role in the survival of circulating tumour cells [9,63]. Considering its role in receptor trafficking, GABARAPL1 may modulate protein composition on the cellular membrane, altering the cell's ability to adhere to the surrounding extracellular matrix and to intra-/extravasate [36,37]. In line, altering receptor expression on the membrane or their nuclear

translocation could alter the cell's ability to respond to surrounding stimuli [36,37]. As such, GABARAPL1 may affect different processes in the metastatic cascade.

Additionally, GABARAPL1 may contribute to metastasis development via modulation of intercellular communication. At least two members of the LC3/GABARAP protein family are involved in cargo recruitment to EV [34,64]. We previously demonstrated the role of GABARAPL1 in EV biogenesis, in particular during hypoxia [34]. Here, we confirm that GABARAPL1 is involved in EV cargo loading. We demonstrate that GABARAPL1 deficiency counteracts the loading of hypoxia responsive miRNA into EV, supporting a role for GABARAPL1 in EV cargo sorting. As such, increased loading of miR-148b-3p, miR-24-3p, miR-130b-3p, miR-345-5p and miR-101-3p (involved in suppression of cancer cell migration, invasion and metastasis formation) in GABARAPL1 deficient EV could contribute to a metastasis suppressing EV phenotype [48–55,60,61,65–67]. However, GABARAPL1 deficient cells also increase the loading of, amongst others, miR-183-5p, miR-27a-3p and miR-425-5p (known to promote cancer cell migration, invasion and metastasis formation) into EV [65–73]. In line, we demonstrate that autocrine EV-mediated stimulation of cancer cell invasion is not GABARAPL1 dependent. Nevertheless, these results do not exclude an essential role for GABARAPL1 dependent EV in other aspects of the metastatic process, including intravasation, anoikis, extravasation, matrix remodelling, PMN formation and neovascularization of the growing metastasis. Firstly, GABARAPL1 dependent EV may aid in the preparation of distant cells into a tumour-supportive environment, referred to as the pre-metastatic niche. In this line, EV fulfil an important role during the formation of metastasis [25–27]. Expression of different integrins on the EV membrane drives organotropic metastasis [31]. Furthermore, cancer cells prepare the PMN via the secretion of EV which reprogram stromal cell metabolism, immune cell populations and matrix remodelling [15,16,74]. In addition, we identified several GABARAPL1 dependent miRNAs in EV known to modulate immune cell behaviour. For example miR-381-3p and miR-23-3p skew macrophages towards the anti-inflammatory, metastasis promoting M2 phenotype [75,76]. Furthermore, miR-23-3p decreases natural killer- and T-cell effector functions [77,78]. As such, GABARAPL1 mediated loading of miRNA into EV may suppress anti-tumor immune reactions and promote the survival of circulating tumor cells, thereby promoting the development of metastases. Furthermore, EV increase the adherence of tumour cells to endothelial cells and thereby aid in cancer cell extravasation, and increase cancer cell migration and invasiveness [17,28–30]. We previously reported that GABARAPL1<sup>+</sup> EV support tumour progression via the induction of angiogenesis [34]. After colonization of distant tissues, developing metastases require angiogenesis to progress. GABARAPL1 inhibition may therefore result in decreased establishment of metastases. In conclusion, we demonstrate that GABARAPL1 is essential for the development of metastases and controls cargo loading into EV. However, more research is required to elucidate the exact role of GABARAPL1 and GABARAPL1<sup>+</sup> EV in the metastatic cascade.

**CRedit authorship contribution statement**

**Joel E.J. Beaumont:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. **Jinzhe Ju:** Conceptualization, Methodology, Formal analysis, Investigation. **Lydie M.O. Barbeau:** Conceptualization, Formal analysis, Investigation. **Imke Demers:** Formal analysis, Investigation. **Kim G. Savelkoul:** Project administration, Formal analysis, Investigation. **Kasper Derks:** Formal analysis. **Freek G. Bouwman:** Formal analysis, Data curation, Formal analysis. **Marca H.M. Wauben:** Data curation, Methodology, Writing – review & editing. **Marijke I. Zonneveld:** Conceptualization, Methodology, Writing – review & editing. **Tom G.H. Keulers:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. **Kasper M.A.**



**Fig. 4.** GABARAPL1 determines cargo of EV secreted by MDA-MB-231 cells. A) High-resolution flow cytometry analysis of EV secreted by MDA-MB-231 cells exposed to normoxia or hypoxia,  $n = 2$ . B) Immunoblots of EV markers CD63 and CD81 in EV isolated from control (shSCR) and shGABARAPL1 MDA-MB-231 cells exposed to normoxia or hypoxia,  $n = 3$ . C) Relative protein content of EV isolated from control (shSCR) and shGABARAPL1 MDA-MB-231 cells exposed to normoxia or hypoxia. For each individual experiment, protein content was compared to normoxic control EV (set to 1)  $n = 3$ , 2-way ANOVA with Šidák's multiple comparisons test. D) Log<sub>2</sub> transformed differentially expressed miRNAs (absolute Log<sub>2</sub>(FC) > 1) in EV isolated from shGABARAPL1 MDA-MB-231 cells (compared to control cells (shSCR)) exposed to either normoxia (left) or hypoxia (right),  $n = 3$ . E) Transwell invasion assay of MDA-MB-231 cells after stimulation with EV from control (shSCR) and shGABARAPL1 MDA-MB-231 cells exposed to normoxia or hypoxia, representative binary images of transwell inserts. For each individual experiment, fold-change (FC) was calculated compared to normoxic control EV (set to 1)  $n = 4$ .



Table 3

Differentially expressed (absolute Log<sub>2</sub>(FC) > 1) miRNA in EV from hypoxic GABARAPL1 deficient cells, compared to EV derived from hypoxic control cells (shSCR), and their role in metastasis.

miRNA	Log <sub>2</sub> <sup>(hypoxicshGAB1EV)</sup> <sub>(hypoxicshSCREV)</sub>	Metastasis association	Ref.
miR-148b-3p <sup>#</sup>	2,687974	-Reduces metastasis development	[48–50]
miR-24-3p <sup>#</sup>	2,349701	-Reduces metastasis development -Reduces cancer cell migration and invasion	[51–53]
miR-151a-5p	1,928523247	-Promotes cancer cell invasion -Enhances epithelial-mesenchymal transition	[71]
miR-183-5p <sup>#</sup>	1,815906	-Promotes cancer cell migration and invasion	[65]
miR-130b-3p <sup>#</sup>	1,799508	-Reduces cancer cell migration and invasion	[54]
miR-345-5p <sup>#</sup>	1,797919	-Reduces metastasis development -Reduces cancer cell migration and invasion	[55]
miR-29a-3p	1,796581	-Reduces cancer cell migration and invasion	[56,57]
miR-27a-3p <sup>#</sup>	1,796546	-High expression is associated with metastasis -Promotes cancer cell migration and invasion	[66,67]
miR-20a-5p <sup>#</sup>	1,729199	-Promotes epithelial-mesenchymal transition -High expression is associated with metastasis -Promotes cancer cell migration and invasion	[72,73]
miR-425-5p <sup>#</sup>	1,689548	-Promotes epithelial-mesenchymal transition -Promotes cancer cell migration and invasion	[68–70]
miR-146a-5p	1,326102	-Promotes epithelial-mesenchymal transition -Reduces cancer cell migration and invasion	[58]
miR-138-5p	1,171039	-Inhibits epithelial-mesenchymal transition -Reduces cancer cell migration and invasion	[59]
miR-101-3p <sup>#</sup>	1,109953	-Inhibits epithelial-mesenchymal transition -Reduces cancer cell migration and invasion	[60,61]
miR-451a <sup>*</sup>	−1,62602	-Inhibits epithelial-mesenchymal transition -Reduces metastasis development -Reduces cancer cell migration and invasion	[112,113]
miR-409-3p	−1,62635	-Reduces metastasis development -Reduces cancer cell migration and invasion	[114,115]
miR-126-3p	−1,86027	-Reduces metastasis development -Reduces cancer cell migration and invasion	[116,117]
miR-486-5p	−2,07169	-Reduces cancer cell invasion -Inhibits epithelial-mesenchymal transition	[118,119]
miR-143-3p	−2,0717	-Reduces metastasis development	[120]

Table 3 (continued)

miRNA	Log <sub>2</sub> <sup>(hypoxicshGAB1EV)</sup> <sub>(hypoxicshSCREV)</sub>	Metastasis association	Ref.
miR-411-5p	−2,11388	-Reduces cancer cell migration and invasion -High expression is associated with metastasis -Promotes cancer cell migration and invasion	[62]
miR-150-5p <sup>*</sup>	−2,32631	-Promotes epithelial-mesenchymal transition -Reduces metastasis development -Reduces cancer cell migration and invasion	[121,122]
miR-144-3p	−2,70755	-Reduces metastasis development -Reduces cancer cell migration and invasion -Inhibits epithelial-mesenchymal transition	[123,124]

\* Indicates increased expression in EV during hypoxia.  
# Indicates reduced expression in EV during hypoxia.

**Rouschop:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.radonc.2023.109968>.

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