Inflammation and cancer: the role of extracellular enolase-1

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ABC	Adenosine-triphosphate binding casette
APS	Ammonium persulfate
BABTA	1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′ tetraacetic acid
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchonininc acid
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAF	Cancer associated fibroblast
CAI	Carboxyamidotriazole
CCL	Chemokine (C-C) motif ligand
CPA	Cyclopiazonic acid
CXCL	Chemokine C-X-C motif ligand
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
ECM	Extracellular matrix
EDTA	Ethylendinitrilo-*N,N,N′,N′* tetra acetate
EGTA	Ethylene glycol-bis (2-amino-ethyleter)-*N,N,N′,N′*- tetraacetic Acid
EMT	Epithelial-mesenchymal transition
ENO-1	Enolase-1
ESCRT	Endosomal sorting complex
FITC	Fluorescein-5-isothiocyanate
FCS	Fetal calf serum
G418	Geneticin
GCSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GROα	Growth regulated alpha protein
H2B	Histone 2B
HMEC	Human mammry epithelial cells
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IgG	Immunoglobulin G

IL	Interleukin
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTCC	L-type calcium channel
MHC	Major histocompatibility complex
MMP	Matrix- metalloproteinase
MVB	Multivesicular bodies
MyD 88	Myeloid differentiation primary response gene 88
NSCLC	Non-small cell lung cancer
ORAI1	Ca^{2+} release-activated calcium modulator
P26S	26S proteasome subunit
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptor
PBGD	Porphobilinogen deaminase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal carcinoma
PDGF	Platelet derived growth factor
PLA	Plasmin
PLG	Plasminogen
PLG-R	Plasminogen receptor
PMSF	Phenylmethylsulphonyl fluoride
PSA	Prostate specific antigen
qPCR	Real time PCR
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SERCA	Endoplasmic reticulum Ca^{2+} -ATPase
siRNA	Small interfering RNA
SOCE	Store operated calcium entry
STIM1	Stromal interaction molecule 1
TBS	Tris buffered saline buffer
TBS-T	Tris buffered saline buffer + 0.1 % Tween 20

TCA	Trichloroacetic acid
TEM	Transmission electron microscope
TEMED	*N,N,N′,N′*-tetramethyl-ethane-1,2-diamine
TEMs	Tetraspanins enriched microdomains
TGF-β	Transforming growth factor beta
TLR-4	Toll like receptor-4
TNF-α	Tumor necrosis factor alfa
tPA	Tissue type plasminogen activator
uPA	Urokinase type plasminogen activator
uPAR	Urokinase type plasminogen activator receptor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

V. SUMMARY

Extracellular matrix degradation is one of the crucial steps in cancer cell invasion and spreading. A number of proteases, including plasmin, mediate disruption of stromal barriers and basement membrane and thus facilitate tumor cell movement. Formation of plasmin is a result of the plasminogen (PLG) activation cascade, which involves PLG activators and receptors. Enolase-1 (ENO-1) is one of the plasminogen receptors (PLG-R). It belongs to the so called "moonlighting protein group", which exhibits various functions at distinct cellular and extracellular sites of the cell. This primary glycolytic enzyme was found to be overexpressed in more than 20 types of human cancer and accounts for enhanced cancer progression and poor clinical outcome. Although numerous studies provide evidence for pro-tumorigenic properties of cytoplasmic ENO-1, the contribution of cell surface bound ENO-1 to cancer progression has not yet been described.

Here, we demonstrate increased expression of ENO-1 in different types of human cancer, in particular, in breast ductal carcinoma. Cell fractionation of the breast cancer cells (MDA-MB-231) revealed elevated ENO-1 cell surface levels, which correlated with enhanced migratory and invasive properties of these cells. Overexpression of wild-type ENO-1 increased invasion of MDA-MB-231 cells. This effect was not observed when ENO-1 mutant bearing the mutation in a PLG binding site was overexpressed. Exposure of MDA-MB-231 cells to LPS further potentiated ENO-1 cell surface expression and simultaneously increased release of ENO-1 to the extracellular space in the form of exosomes. These effects were independent of *de novo* protein synthesis and did not require the classical endoplasmic reticulum/Golgi pathway. LPS-triggered ENO-1 exteriorization was diminished upon pretreatment of MDA-MB-231 cells with the Ca^{2+} chelator BAPTA or an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase pump, cyclopiazonic acid. In line with this observation, STIM1 and ORAI1 were found to regulate LPS-induced ENO-1 cell surface expression and release. Accordingly, pharmacological blockage or knockdown of STIM1 or ORAI1 reduced ENO-1-dependent migration of breast cancer cells.

Collectively, these data reveal the functional consequence of extracellulary localized ENO-1 in cancer cell behaviour and the mechanism which drives ENO-1 exteriorization. Thus, targeting cell surface bound ENO-1 may offer a novel therapeutic strategy in patients suffering from cancer.

VI. ZUSAMMENFASSUNG

Der Abbau der extrazellulären Matrix ist einer der entscheidenden Schritte bei der Krebszellinvasion und –ausbreitung. Mehrere Proteasen einschließlich Plasmin vermitteln die Auflösung der stromalen Barrieren und der Basalmembran und erleichtern so die Bewegung der Tumorzellen. Die Plasminbildung ist das Ergebnis der Plasminogen (PLG)-Aktivierungskaskade, die PLG-Aktivatoren und Rezeptoren umfasst. Enolase-1 (ENO-1) ist einer dieser Plasminogenrezeptoren (PLG-R). Sie gehört zur Gruppe der sogenannten „moonlighting"-Proteine, die mehrere Funktionen in unterschiedlichen zellulären und extrazellulären Bereichen der Zelle aufweisen. Dieses primär glykolytische Enzym, das in mehr als 20 humanen Krebsarten überexprimiert ist, ist verantwortlich für ein schnelleres Fortschreiten der Krebserkrankungen und für eine schlechte klinische Prognose. Obwohl zahlreiche Studien die tumorerzeugenden Eigenschaften von zytoplasmatischer ENO-1 beweisen, wurde der Einfluss der oberflächengebundenen ENO-1 noch nicht beschrieben.

In dieser Arbeit zeigen wir nun die erhöhte Expression von ENO-1 in verschiedenen humanen Tumortypen, insbesondere im duktalen Brustkarzinom. Die Zellfraktionierung der Brustkrebszellen MDA-MB-231, ergab ein erhöhtes Niveau der ENO-1 an der Zelloberfläche, welches mit den verbesserten Invasions- und Migrationseigenschaften dieser Zellen korreliert. Die Überexpression der Wildtyp- ENO-1 erhöht die Einwanderung der MDA-MB-231. Dieser Effekt konnte nicht beobachtet werden, wenn eine ENO-1-Mutante überexprimiert wurde, die eine Mutation in der PLG-Bindungsstelle aufwies. Wurden die MDA-MB-231 mit LPS behandelt, so wurde die Expression der ENO-1 an der Zelloberfläche weiter verstärkt, was gleichzeitig zu einer erhöhten Freisetzung der ENO-1 in den extrazellulären Raum in Form von Exosomen führte. Diese Effekte waren unabhängig von der *de novo* Proteinsynthese und benötigten nicht den klassischen Pfad über das endoplasmatische Retikulum und den Golgi-Apparat. Die LPS-gesteuerte Exteriorisation wurde durch eine Vorbehandlung der MDA-MB-231 mit BAPTA einem Ca^{2+}-Chelator oder Cyclopiazonsäure dem Inhibitor der Ca^{2+}-ATPase-Pumpe des endoplasmatischen Retikulums verringert. Dies entsprach der Beobachtung, dass STIM1 und ORAI1 die LPS-induzierte Expression von ENO-1 an der Zelloberfläche und die Freisetzung der ENO-1 regulieren. Dementsprechend reduzierte eine pharmakologische Blockierung oder ein Knockdown von STIM1 oder ORAI1 die faktorabhängige Migration der Brustkrebszellen.

Zusammengefasst zeigen diese Daten den Mechanismus auf, der die Exteriorization von ENO-1 steuert und die funktionellen Auswirkungen dieser extrazellulär lokalisierten ENO-1 auf das Verhalten der Krebszellen. Folglich stellt die zelloberflächengebundene ENO-1 das Ziel einer neuen therapeutischen Strategie bei der Behandlung von Krebspatienten dar.

1. INTRODUCTION

1.1. Cancer development and progression

Transformation of a normal cell into a cancer cell is a multistep process, composed of the accumulated number of genetic mutations of the normal cell as well as physiologic changes within the cancer cell and the host immune system [1, 2]. Three major steps can be distinguished in tumorigenesis: (i) initiation; which encompasses damage to, and division of affected cells such their growth is changed irreversibly (ii) progression; natural selection of cells bearing mutations with multiple rounds of replication mediating transition into autonomous, cancerous growth (iii) metastasis; spread of malignant cells [3]. In order to acquire ability to form cancer colonies in soft agar or in the immunocompromised mice, mutations in more than two oncogenes have to take place [4]. The combined activation of oncogenes and inactivation of tumor supressor genes drives the successful progression of cancer. However, gain of multiple mutations in oncogenes does not quarantee a full malignant state. Natural selection of transformed cells with multiple cycles of replication is needed to reach the full metastatic potential [3].

After succesful gain of mutations, tumor cell invasion has to take place. However, to physically invade into blood vessels, proteolytic degradation is required. Proteases are produced by cancer cells and they can promote cancer cell invasion and intravasation in several ways. Proteases may cleave cell adhesion molecules, leading to the disruption of cell contacts. Impairment of cell contacts enables the release of either individual or groups of cells [5, 6]. Next, degradation or turnover of proteins in the extracellular matrix (ECM) facilitate invasion of cells into surrounding tissue and vasculature. All these steps require complex interactions between cancer and host cells and in particular with ECM. ECM, as a part of the tumor microenviroment, plays an important role in cell adhesion, proliferation and motility. Degradation of ECM within the tumor stroma by diverse spectrum of proteases results in disruption of stromal barriers and basement membrane and thus facilitates tumor cell movement (Figure 1) [5-7]. A positive correlation between the aggressivness of tumor and secretion of various proteases from tumor cells was reported in several studies [8, 9]. In addition, some tumor cells may induce expression of proteolytic enzymes in neighbouring nonmalignant cells, hijacking their activity to invade tissue [10]. In all steps of tumorigenesis, starting from initiation through progression and metastasis, five classes of proteases have been reported to be involved: serine, cysteine, aspartic, threonine and metalloproteases [8].

One of the best studied serine proteases involved in cancer cell progression is plasmin (PLA). PLA is a final product of the plasminogen (PLG) activation system.

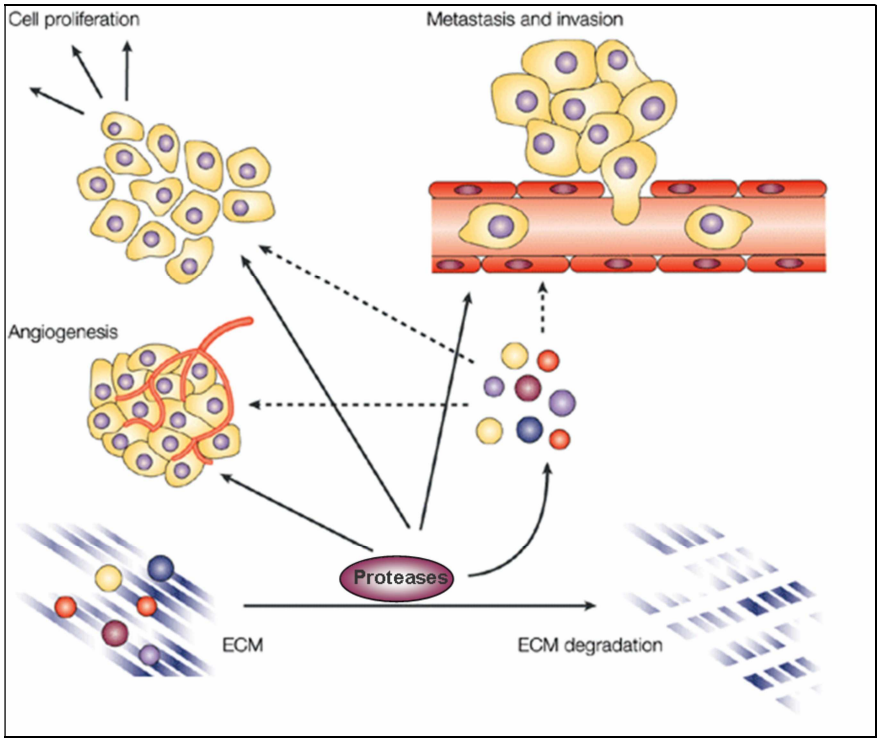

Figure 1. The role of proteases in cancer progression. (Rao JS, 2003, modified)

1.1.1. Plasminogen/plasmin system

1.1.1.1 Plasminogen/plasmin system components

Plasminogen/plasmin (PLG/PLA) system plays a crucial role in a number of biological as well as pathological events. Besides its function in fibrinolysis [11], PLG/PLA system plays an important role in processes such as tissue remodeling [12], ovulation [13], embryogenesis [14], angiogenesis [15] and tumor invasion [16].

PLG is a precursor of PLA, predominantly found in the human circulation [17] and in association with ECM [18]. PLA is a final product of the PLG activation system, which involves a precursor, cellular receptors and activators of PLG (Figure 2) [19]. The precursor of PLA is secreted as a single chain glycoprotein by the liver and circulates in blood in an activation-resistant form [20]. Binding of PLG to the cellular receptors alters the conformation of PLG and thus enables its activation. Bound PLG is subsequently cleaved by urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA) to PLA [21].

Active PLA remains associated with the cell surface, where it is protected from inhibitors [17]. Although PLG binding to the cell surface is necessary for PLA production, the presence of PLG activators is critical for successful PLG activation. Thus, only high amounts of uPA are able to convert PLG to PLA [22]. uPA is synthesized and secreted as a zymogen (pro-uPA) and its activation is accelerated upon binding to its cellular receptor (uPAR) [23]. Taken together, the efficient activation of PLG requires an active receptor bound uPA, PLG in activation-susceptible conformation and uPA-PLG binding.

Binding of PLG to the cells is mediated by several distinct receptors. Since PLG interacts with its receptors through kringle domains, which express high affinity for lysine residues [24, 25], the interaction between PLG and cellular receptors can be blocked by the lysine and lysine analogs [26]. Physiologic regulation of PLG cascade includes proteases and PLG activation inhibitors. The major inhibitor of this cascade is α2-antiplasmin. This inhibitor blocks the activity of free unbound PLA, however binding of PLA to the cellular receptor provides protection against α2-antiplasmin [17]. The activities of PLG activators are mainly regulated by two serine protease inhibitors, plasminogen activator inhibitor (PAI) type 1 and type 2. PAI-1 is a major inhibitor of tPA, whereas PAI-2 exhibits inhibitory activity mainly toward uPA and is less effective against tPA [27].

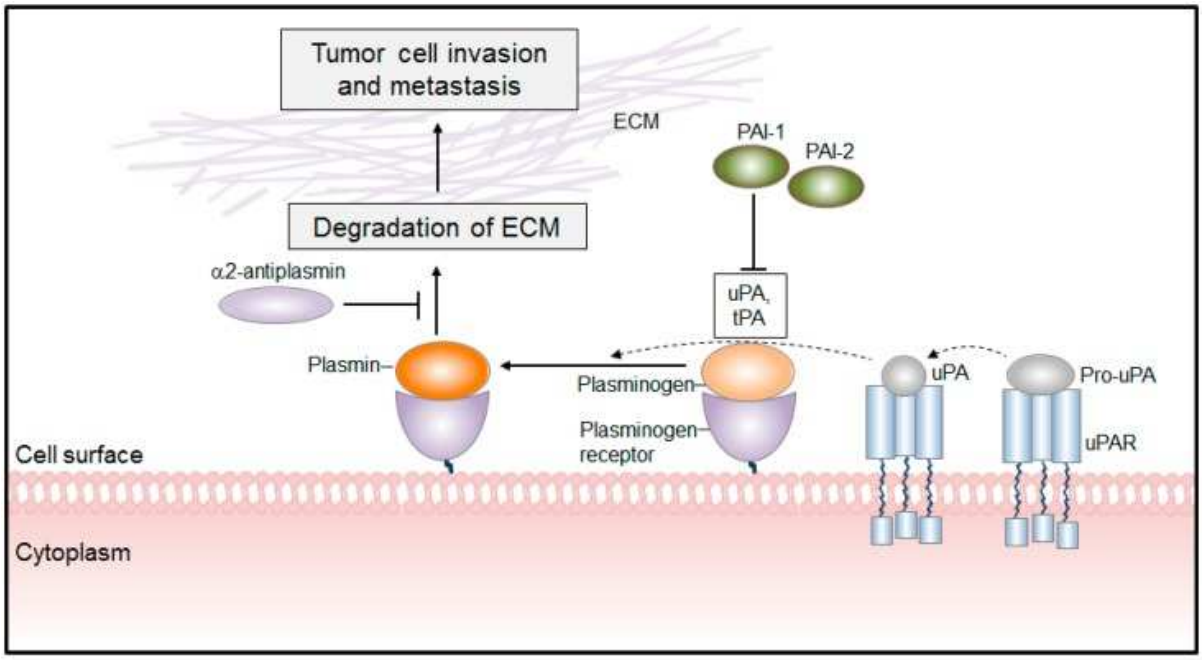

Figure 2. PLG/PLA system components in tumor cell invasion. (Didiasova *et al*., 2014)

PLA displays a broad spectrum activity. On one hand, PLA degrades ECM proteins, activates matrix-metalloproteinases (MMP) type 1, 3, 9 and processes growth factors such as transforming growth factor (TGF)-β , basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [28]. On the other hand, upon binding to the receptors, PLG/PLA activates intracellular signaling pathways and thus affects cellular processes. PLA induces neutrophil aggregation, hepatocyte proliferation [29, 30], monocyte chemotaxis [31], migration of endotheliocytes [32] as well as expression of proinflammatory [33] and growth-factor like genes [34]. There are only few studies elucidating mechanism by which PLA regulates cellular processes. Here, protease activated receptor (PAR)-1 and -4, annexin A2 and integrins such as αMβ2 and αvβ3 were found to play a pivotal role [34-36]. αMβ2 integrin was shown, for example, to regulate PLG-stimulated neutrophil survival [37] and αvβ3 integrin was reported to stimulate endotheliocytes migration [32]. Altogether, PLA together with its precursor PLG control broad spectrum of cellular activities either trough a direct processing of extracellular proteins or by activation of intracellular signaling pathways.

1.1.1.2 Plasminogen/plasmin receptors

Plasminogen receptors (PLG-R) are a heterogenous group of cell surface proteins, which binds PLG as well as PLA. They are distributed on both prokaryotic and eukaryotic cells. Various eukaryotic cells including monocytes [38], monocytoid cells [39], macrophages [40], endothelial cells [41, 42], fibroblasts [43], platelets [44] and carcinoma cells [45, 46] express PLG-R. The PLG binding capacity of a single cell is relatively high, namely around 105 binding sites [47]. Multiple PLG-R and their collective expression account for a total PLG binding capacity of a cell. The heterogenity of PLG-R and their different cell surface expression may explain, how the regulation of diverse biological processes including fibrinolysis, inflammation, wound healing and angiogenesis takes place at the same time. PLG-R can be grouped into four classes. First class includes proteins possesing preexisting C-terminal lysine residue, such as α-enolase (ENO-1) on monocytes [26, 48], neurons [49], carcinoma cells [50], lymphoid cells [51], myoblasts [52] and pathogenic bacteria [53], cytokeratin 8 on carcinoma cells [46], p11 on endothelial cells [54] and glyceraldehyde-3-phosphate dehydrogenase on bacteria [55]. Second class of PLG-R requires cleavage in order to expose a lysine residue and includes annexin A2 on endothelial cells [56] and actin on

endothelial and carcinoma cells [57, 58]. Third class includes proteins synthetized without a C-terminal lysine residue. αIIbβ3 integrin on platelets [44], activated αMβ2 integrin on PMA-stimulated neutrophils [59], amphoterin on cancer cells [60] and GP330 in kidney cells [61] belong to this class. The fourth group of the receptors binds PLG, but does not promote its activation. Tissue factor and gangliosides are members of this group [62, 63].

The majority of PLG-R belong to the so called "moonlighting proteins", which exhibit multiple functions at distinct cellular and extracellular sites [64, 65]. Indeed, the majority of proteins that bind PLG, are well characterized cytosolic or nuclear proteins with established functions in metabolism, DNA packaging or cytoskeleton organization. These proteins do not contain a signaling sequence that would direct them to the cell surface, and they do not posses hydrophobic region to be simply inserted into the membrane, with exception of annexin A2 [66]. Thus, "non-classical" protein release independent of the endoplasmic reticulum (ER) - Golgi pathway has been proposed to explain their cell surface localization [67, 68].

1.1.2. Plasminogen/plasmin system in tumorigenesis

Breakdown of ECM is one of the most important requirements for tumor development and progression. PLA, a PLG activation product, degrades ECM and facilitates tissue invasion and thus, contributes to metastasis [5, 6]. The PLG/PLA system promotes tumor spreading not only by PLA-mediated ECM breakdown but it also controls tumor angiogenesis, a process which is essential for nutrition and oxygen supply [69]. First steps in angiogenesis encompass vessel wall diassembly, basement membrane degradation and cell migration. All mentioned processes are regulated by extracellular protelysis and the PLG/PLA system [15]. In addition, PLA may directly activate VEGF [70], which is a key mediator of angiogenesis [71]. Furthermore, the PLG/PLA system affects cell adhesion, proliferation, migration [72, 73] and apoptosis [74], cellular events that are dysregulated upon tumorigenesis. Excessive production of PLA in tumor microenviroment results from the local imbalance between PLG activators and PLA [75].

A prerequisite for PLA formation is the binding of its precursor – PLG to the cell surface. Binding of PLG to the cells is mediated by a diverse spectrum of "moonlighting proteins". The importance of PLG-R for metastasis formation was highlighted in the study, describing paclitaxel-resistant variants of the invasive human cancer cell line in a superinvasive metastasis model *in vitro* [76]. Proteomic approaches revealed a number of significantly upregulated cell surface proteins including PLG-R such as ENO-1, annexin A2 and actin in

the superinvasive cells as compared to the non-invasive ones. This suggests the involvement of the afforementioned PLG-R in the regulation of invasive properties of cancer cells. One of these PLG-R, namely ENO-1, was found to be overexpressed in more than 20 types of human cancer [77].

1.1.3 Role of ENO-1 in tumorigenesis

ENO is a key glycolytic enzyme that catalyzes conversion of 2-phosphoglycerate into phosphoenolpyruvate in the cytoplasm. Besides its role in glycolysis, ENO may be transported from the cytoplasm to the cell surface where it acts as a PLG-R on various cell types [78]. In verterbrates, this enzyme possess three distinct subunits and can form homo- or heterodimers [79]. Whereas the αα isoenzyme of ENO, also referred to as ENO-1, is ubiquitously expressed, the ββ isoenzyme is found predominantly in muscles and the γγ isoenzyme is characteristic for nervous tissue.

Growing body of evidence suggests that ENO-1 does not only exert its house-keeping function, but indeed plays a role in numerous pathophysiological processes. Under pathological conditions, ENO-1 is translocated to the cell surface, where it acts as a PLG-R and thus controls pericellular proteolysis [26, 48]. Cell surface expression of ENO-1 has been reported on several cell types including monocytes, T and B cells, neuronal cells as well as cancer cells [80]. Cell surface associated proteolysis is frequently observed during physiological and pathological events. Binding of PLG to the cell surface, leads to PLA production that activates collagenases, degrades fibrin and several other matrix proteins [81]. ENO-1-dependent pericellular proteolytic activity allows many pathogens [82] but also immune [83] and cancer cells [84] to invade tissue, consequently leading to infection, inflammation or metastasis formation. Furthermore, high titer of anti-ENO-1 antibodies in the plasma has been associated with different systemic and invasive autoimmune diseases including viral hepatitis, retinopathy, systemic lupus erythematosus as well as rheumatoid arthritis [85-87].

ENO-1 was found to be overexpressed in more than 20 types of human cancer [77] and several mechanisms seem to account for the indicated changes in ENO-1 production. Firstly, ENO-1 is located in the chromosomal region 1p36 [88], which is frequently rearranged or deleted in human malignancy. Secondly, hypoxia drives transcription of ENO-1 gene through hypoxia-inducible factor 1 binding element [89]. Thirdly, the expression of ENO-1 is elevated in c-Myc overexpressing cells, suggesting the critical role of c-Myc in the regulation of ENO-

1 expression in cancer cells [90]. Finally, increased levels of ENO-1 in cancer cells may be explained by the Warburg effect, which describes the increase in anaerobic glycolysis under hypoxic conditions, a common feature of most solid tumors [91].

High ENO-1 mRNA expression correlates with cancer progression and poor clinical outcome of the affected patients. Ectopic overexpression of ENO-1 promotes cell proliferation, migration, invasion, and colony formation thereby contributing to metastasis formation [80, 92]. ENO-1 was found to be significantly overexpressed in effusion-derived tumor cells and tumor specimens of lung cancer [93, 94]. Moreover, levels of the cell surface bound ENO-1 were higher in the late and end stage of non-small cell lung cancer (NSCLC). ENO-1 cell surface expression negatively correlated with survival and disease reccurence in NSCLC patients [93]. Furthermore, subset of NSCLC patients with advanced stages of NSCLC demonstrated significantly higher titers of autoantibodies directed against ENO-1 [95].

Increased expression of ENO-1 [77, 96] and elevated titers of anti-ENO-1 antibodies in the sera were also found in patients suffering from breast cancer [97]. Similarly to lung cancer, those patients whose tumors displayed high ENO-1 levels had poor prognosis with greater tumor size, poor nodal status, and a shorter disease-free interval [98]. Additionaly, an *in vitro* study demonstrated that, transformation of a less metastatic breast cancer cell line MCF-7 into a more invasive phenotype was accompanied by increased ENO-1 protein expression [96]. Consequently, depletion of ENO-1 expression by small interfering RNA (siRNA) significantly decreased proliferation and increased sensitivity to anti-cancer drugs in tamoxifen-resistant breast cancer cells [96]. The possible correlation between ENO-1 expression and invasiveness of breast cancer cells was futher stressed by the observation demonstrating that cell surface expression of ENO-1 is significantly elevated in a superinvasive cell line as compared to a less invasive cell line [99].

Upregulated ENO-1 level was also observed in head and neck cancers [100, 101]. Again, increased levels of ENO-1 positively correlated with poor prognosis and development of recurrence. An *in vitro* study revealed, that ectopic overexpression of ENO-1 in oral cancer cells promotes their proliferation, migration and invasion in a chemokine (C-C motif) ligand (CCL)20-dependent manner. This, together with the fact that ENO-1 expression positively correlated with CCL20 content in oral cancer cells, led to the conclusion, that CCL20 is a downstream target of ENO-1 that plays a role in ENO-1-mediated cell transformation [100].

Elevated expression of ENO-1 together with its cell surface localization were also observed in pancreatic ductal adenocarcinoma (PDAC) [102-104], in different kinds of neoplasms of central nervous system [105] as well as in ovarian, uterus and cervic cancer [77, 106]. Collectively, elevated expression of ENO-1 together with its cell surface localization were shown to be a good prognostic marker in human cancers [98].

1.2. Tumor microenviroment

Formation of a clinically relevant tumor requires support from a surrounding stroma, also reffered to as tumor microenviroment. Two major steps limiting metastasis formation are: access to the vasculature at the site of primary tumor [107] and tumor formation at the secondary site. Thus, a permissive tumor microenviroment must be present in order to promote vascularization at the primary site and proliferation at the secondary site of tumor [108]. In other words, a metastatic cell requires an appopriate enviroment in order to create tumor at the secondary site [109]. Tumor microenviroment contributes to tumor growth mainly by blood supply [110], however its composition may affect tumor progression. The role of microenviroment in tumor progression may be positive as well as negative. On one hand, tumor cells may reside for decades in a dormant state since the microenviroment at the secondary site supresses their growth. Furthermore, such microenviroment may even stimulate phenotypic reversion of fully metastatic cells into non-malignant cells [111]. On the other hand, pathologic changes in the tissue microenviroment can drive tumor progression [112-114]. This idea has been developed by Coussens and Werb, who demonstrated the predominant role of inflammation in tumor progression. In fact, many types of cancer may even arrise from chronic inflammation, chronic irritation or infection [115]. Cancer cells may also regulate the microenviroment, for example, by producing factors that create permissive surrounding, so called prometastatic niche, where the metastasis can be seeded [116]. Thus, due to cell-cell contact, material exchange or vesicle mediated cell to cell communication, an active cross-talk between cancer cells and stroma is achieved [117, 118]. In this context, exosomes represent one of major players in cell to cell comunication in cancer cells [119-121].

1.2.1. Inflammation and cancer

Inflammation plays a critical role in cancer progression. Many cancers arise from sites of infection, chronic irritation or inflammation [115]. Up to 15 % of malignancies worldwide can be attributed to infectious agents [122]. An ongoing infection within the host tissue induces inflammation and recruitment of inflammatory cells. Recruited leukocytes and phagocytic cells release reactive oxygen (ROS) and nitrogen species, which in turn induce DNA damage [123]. Persistent tissue injury and regeneration in the presence of ROS triggers proliferation of epithelial cells accompanied by genetic alterations [124]. However, ROS are not the only factors released by inflammatory cells that induce or promote tumor progression. Cytokines and chemokines secreted from inflammatory cells may support tumor growth as well. The most prominent group of cytokines that promotes tumor progression are proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-17.

TNF-α is produced by immune cells and can promote tumor survival through induction of expression of antiapoptotic genes [125]. It may also play a role in the transformation of normal cells into cancerous cells by stimulating the production of molecules, for example ROS, that can directly cause genetic damage or mutations [126]. Genetic predisposition leading to TNF-α synthesis was found to be associated with increased risk of the development of bladder, gastric, breast cancer as well as poor prognosis [127]. In addition, TNF-α was shown to be important in later stages of cancer, where it promotes angiogenesis and metastasis formation [128].

IL-6 is a key growth promoting and antiapoptotic molecule [129]. This cytokine contributes to tumor progression mainly by increasing proliferation of cancer cells [130]. In addition, not only the cytokine itself, but also its soluable receptor contributes to cancer progression. Soluable IL-6 receptor promotes T cell survival and enhances production of IL-6 by T cells [131]. The production of IL-6 is also regulated by IL-17. IL-17 induces expression of other proinflammatory cytokines including TNF-α, IL-6 and IL-1β thereby amplifying inflammatory responses [132, 133]. Furthermore, IL-17 enhances tumorigenic growth and angiogenesis [134, 135].

Expression of chemokines and their receptors were also reported to be dysregulated in many types of cancer [136]. Chemokines released from inflammatory cells may regulate cancer growth in many ways. For instance, growth regulated alpha protein (GROα) also

known as C-X-C motif ligand 1 (CXCL1), GROβ/CXCL2, GROγ/CXCL3 and IL-8/CXCL8 have been shown to dramatically induce cancer cell proliferation [137]. Blockage of GROα or CXCR2 receptor attenuates proliferation of cancer cells [138], whereas their overexpression enhances cancer cell colony forming activity and invasive potential [139, 140]. Noteworthy, cancer cells produce chemokines as well. They do so, to recruit inflammatory cells and to promote tumor growth. Chemokines (especially CXCL) released by cancer or inflammatory cells are pro-angiogenic and induce endothelial cell chemotaxis [141, 142]. The role of CXCL chemokines was extensively studied in the breast cancer metastasis model, where interaction between CXCR4 and its ligand CXCL12 triggered metastasis formation [143]. Collectively, pro-tumorigenic activities of inflammatory cells include release of chemokines and growth factors, as well as stimulation of angiogenesis, DNA damage and ECM remodelling to facilitate cancer cell invasion [115].

Despite the large body of evidence demonstrating the important role of inflammation in cancer progression, only little is known about the initial trigger of inflammatory processes. In general, infectious agents are though to be the main cause of inflammation in the host enviroment. Supporting this concept, about 20 % of cancer cases are directly linked to infectious agent [144].

1.2.1.1. Lipopolysaccharide in cancer

Endotoxin is a cell wall component of gram-negative bacteria composed of protein, lipids and lipopolysaccharide (LPS), which is released when bacteria are lysed [145]. However, mainly LPS is responsible for most of the biological properties of bacterial endotoxins [146, 147]. Bacterial LPS is able to potentiate inflammatory responses in host environment. Macrophages and monocytes stimulated by LPS release several pro-inflammatory cytokines, including TNF-α, IL-1 and IL-6 [148, 149]. Upon internalization, LPS binds to CD14 protein [145]. LPS-CD14 complex activates toll like receptor-4 (TLR-4) and thus initiates downstream signaling pathways [145]. However, the immune cells are not the first ones, that confront the LPS presence. Epithelial cells function as a barrier restricting pathogen entry and actively participate in numerous defence reactions. Upon contact with LPS, epithelial cells produce pro-inflammatory cytokines, chemokines and antimicrobial peptides [150]. Released mediators attract the immune cells to the site of infection, thereby potentiating inflammatory responses [145]. Several studies demonstrated that inflammation initiated by LPS is involved in cancer progression [145].

Recent studies have addressed the role of LPS in accelarated metastatic burden after surgery. Bacterial constituents, for example LPS are frequently shed into the enviroment [151]. Already low concentrations of LPS (0.2 ng/m^3) in the atmosphere cause harmful effects [152]. LPS contaminations are common problem in the clinical settings. During laparotomy or air laparoscopy, LPS may contaminate peritoneal cavity and enter the circulation. Few studies demonstrated a positive correlation between LPS contamination during surgery and increased metastasis formation. Not only LPS from atmosphere, but also gut bacteria, which frequently translocate from the gut during operation, represent another post-surgical source of LPS. Consequently, mice which underwent air laparoscopy had elevated serum levels of LPS and increased metastatic burden [153, 154]. Moreover, a direct LPS injection into mice suffering from breast cancer significantly elevated levels of serum VEGF, implicating that LPS may increase metastatic potential through stimulation of angiogenesis [153].

Couple of mechanisms may account for positive effect of LPS on cancer cell progression. LPS presence, sensed by both inflammatory and cancer cells, creates an inflammatory enviroment, which favours tumor growth. Firstly, LPS attracts inflammatory cells and thus aggravates inflammatory reactions [145]. Pro-inflammatory cytokines and ROS produced by immune and epithelial cells have pro-tumorigenic properties and may affect different processes such as migration, invasion and angiogenis. Depletion of neutrophils in LPS-treated mice significantly reduces adhesion of circulating cancer cells [155]. Similarly, LPS-activated monocytes increase adhesion of cancer cells to endothelial cells by acting as so called “bridging cells”. Here, binding of cancer intercellular adhesion molecule (ICAM)-1 to monocyte β2 integrin and binding of endothelial ICAM-1 or vascular cell adhesion molecule (VCAM)-1 to monocyte β1 or β2 integrin, play a pivotal role [156].

Secondly, LPS sensed by cancer cells may initiate signalling processes leading to enhanced tumor growth. Cancer cells are mostly arising from epithelial cells, which are though to be the first sensors of bacterial infection. Thus, cancer cells may also sense LPS and promote inflammatory responses. LPS stimulation of cancer cells positively correlates with NF-κB activity [157]. NF-κB is involved in the regulation of pro-inflammatory cytokine gene expression, cellular adhesion, apoptosis and oncogenesis [158]. Enhanced activation of NF-κB in cancer cells is associated with overproduction of VEGF and IL-8, two mediators stimulating tumor growth [159]. In addition, LPS-mediated activation of NF-κB promotes adhesion of tumor cells in a β_1 integrin-dependent manner [160]. Finally, LPS activates oncogene, metadherin, which in turn initiates NF-κB signaling, thereby inducing IL-8 and

MMP-9 expression. Noteworthy, IL-8 and MMP-9 are important for cancer cell invasion [161].

The link between LPS and cancer progression is well established. However, in order to induce all above mentioned processes, LPS must be sensed by the cells. This happens through the transmembrane receptor TLR-4 [162]. Binding of LPS to its receptor leads to activation of the adaptor proteins: myeloid differentiation primary response gene (MyD) 88 or TIR-domain-containing adapter-inducing interferon-β (TRIF) [162]. Downstream targets of TLR-4 activation include NF-κB, mitogen activated protein kinases p38 and ERK1, which can induce production of pro-inflammatory cytokines [163]. Couple of studies revealed that there is a constitutive expression of TLR-4 in cancer cells [164-168]. LPS-mediated TLR-4 signaling can promote tumor cell adhesion and metastasis in colorectal cancer cells by increased β1 integrin expression [160]. In addition, activation of TLR-4 promotes expression of VEGF and TGF-β [168], two molecules, which influence tumor progression, neovascularization and immunosupression [169]. Binding of LPS to TLR-4 is also important for activation of uPA-uPAR system, which in turn increases tumor cell adhesion and invasion [170]. Thus, TLR-4 may serve as a good target for anti-tumor therapies [160].

1.2.2. Exosomes and cancer

Exosomes are small membranous vesicles with the size ranging from 30-150 nm in diameter [171]. They are produced by various cell types under both physiological and pathological conditions, and in particular by tumor and hematopoetic cells [172]. The biogenesis of exosomes (Figure 3) [173] is controlled by the endosomal sorting complex (ESCRT). Recycling of many membrane receptors leads to the formation of plasma membrane coated with a clathrin protein [174]. These invaginations evolve into early endosomes and then mature into late endosomes, which are also termed multivesicular bodies (MVB). Proteins trapped inside MVB may be (i) recycled back to the cell membrane (ii) sequestered in intraluminal vesicles within MVB [175, 176] (iii) degradated through fusion with lysosomes or (iv) released in the form of exosomes during fusion of MVB with the plasma membrane. Exosome secretion is regulated by Ca^{2+} current. Increase in intracellular levels of Ca^{2+} triggers exosome production as well as their fusion with the plasma membrane [177]. Only little is known about signals, which direct proteins into MVB and then control their release in the form of exosomes. Mono-ubiquitinylation is one of them and ESCRT have been shown to control the sorting of ubiquitinated proteins into intraluminal vesicles [178].

However, not all the proteins present in exosomes are ubiquitinylated and thus, a passive mechanism enriching MVB has been considered. It has been shown that tetraspanin family members could participate in sorting of proteins into the exosomes as well. Tetraspanins belong to the membrane proteins, that may cluster among each other or with other membrane and cytosolic proteins, thus creating tetraspanin-enriched microdomains (TEMs). For instance, CD9 and CD82 tetraspanins promote β-catenin secretion in exosomes. Similarly, loading of metalloprotease CD10 into exosomes, depends on its interaction with tetraspanin CD9. CD63 tetraspanin is responsible for the package of epstein-barr virus into exosomes.

Exosomal proteins are located either at the surface of exosomal membrane or in the lumen. The cargo of exosomes largely depends on their cellular origin and contains cytosolic and membrane proteins [172]. In addition, exosomes also contain microRNAs, mRNAs and DNA fragments, which can be shuttled from a secreting cell to a recipient cell [179]. Some proteins are ubiquitosly expressed in all the types of exosomes regardless of their origin and can be thus used as markers of exosomes. The most commonly used markers of exosomes are: major histocompatibility complex (MHC) class I molecules [180], heat shock proteins (Hsp) such as Hsp70 and Hsp90 [181] and tetraspanins including CD9, CD63, CD81 and CD82 [182]. Mitochondrial, ER or nuclear proteins are not detected in exosomes [182]. Growing body of evidence suggests that exosomes play a pivotal role in cell-to-cell communication by transporting proteins and nucleic acids from one to another cell [120]. Although the specific sorting of proteins into exosomes is poorly understood, the protein composition of exosomes depends on enviromental conditions and thus determines the outcome of the communication.

Uptake of exosomes by recipient cells occurs usually randomly and depends on the type of transmembrane proteins located on the recipient cell [183]. The binding of exosomes to the recipient cells is mediated mostly by adhesion molecules such as integrins or ICAM-1. Three possible ways of exosome-mediated cell-to-cell communication have been described thus far [173] (Figure 3) (i) juxtacrine signaling through receptor-ligand binding [180] (ii) direct fusion of an exosome with a membrane of the recipient cells and release of its cargo into cytoplasm [184] (iii) internalization of intact exosomes [183]. The internalization of intact exosomes may occur through three possible pathways: (i) phagocytosis (ii) pinocytosis or (iii) clathrin/dynamin/caveolae-dependent endycytosis.

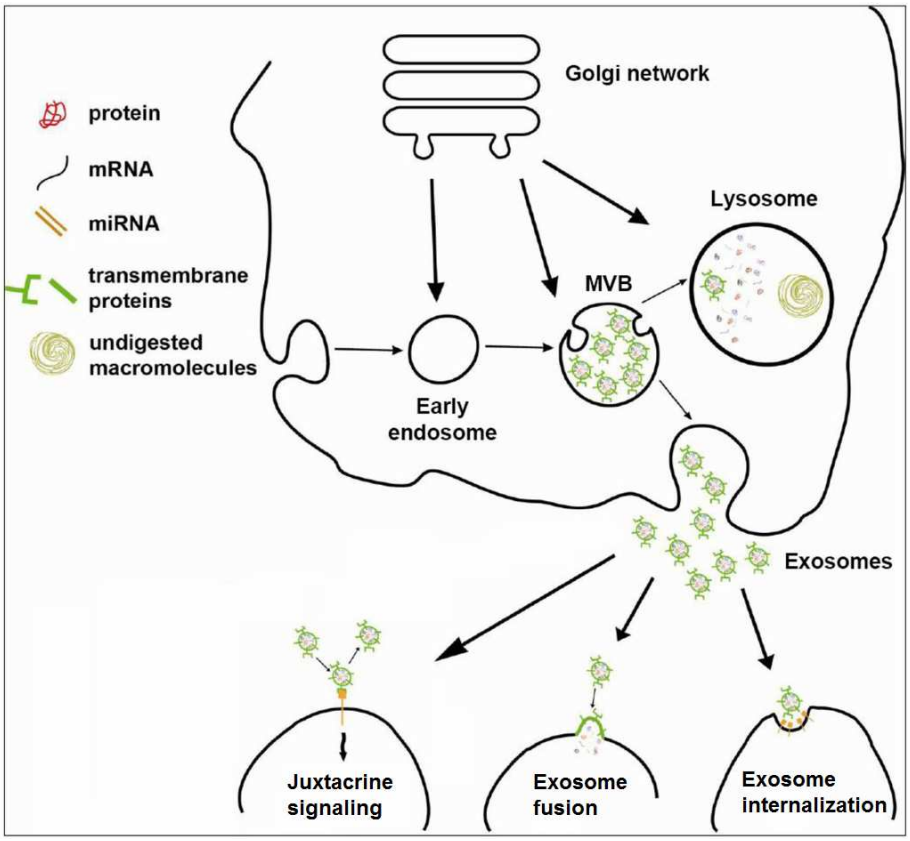

Figure 3. Formation and uptake of exosomes in target cell. (Urbanelli L *et al*., 2013, modified)

Exosomes display a wide variety of biological functions. Beside their immunomodulatory properties, they also play a role in the development, protein shedding and tumorigenesis [185-188]. However, tumor-derived exosomes display a bimodal role. On one hand, exosomes produced by cancer cells manipulate tumor microenviroment, favoring processes such as adhesion, migration and angiogenesis thereby promoting tumor progression. On other hand, exosomes released by cancer cells stimulate immune cells leading to tumor restriction.

Anti-tumorigenic properties of exosomes are associated with their ability to interact with immune cells. Protein cargo of tumor-derived exosomes usually reflects the content of parental cells, and thus is rich in tumor specific antigens [189, 190]. Therefore, exosomes enrinched in tumor antigens may prime dendritic cells (DCs), which in turn can induce CD8+ T cell dependent anti-tumor responses [189, 191]. Furthermore, exosomes may directly trigger apoptosis of tumor cells by their ability to increase expression of the pro-apoptotic *bax*

gene and decrease expression of the anti-apoptotic *bcl-2* gene [192]. Despite these facts, exosome involvement in tumor regression in cancer patients is rather marginal [172].

Pro-tumorigenic properties of exosomes are most frequently observed in the mouse models of cancer and different mechanisms seem to account for these effects. Regulation of immunological responses is one of them. On one hand, exosomes participate in the recruitment of immune cells in the tumor microenviroment and thus enhance release of pro-inflammatory cytokines [193]. On other hand, exosomes are able to suppress activity of cytotoxic T cells. Moreover, exosomes derived from several types of tumors may inhibit proliferation of lymphocytes or natural killer cells [194, 195]. In addition some types of exosomes may express on their surface Fasl or TRAIL, ligands, which may induce apoptosis of cytotoxic T cells [195].

Next mechanism supporting the role of exosomes in cancer progression is related to their ability to control cancer associated fibroblasts (CAF), the most prominent cell type in tumor microenviroment. CAFs are spindle shaped mesenchymal cells that share characteristics with smooth muscle cells and fibroblasts. They may originate from a variety of different progenitor cells, including locally residing fibroblasts, epithelial and endothelial cells via epithelial-to-mesenchymal transition (EMT) or bone marrow-derived mesenchymal cells [196]. This process is regulated by TGF-β, which is transported from cancer cells into neighbouring cells in the form of exosomes [196, 197]. Increased number of CAFs in tumor microenviroment contributes to active remodeling of stroma supporting tumor growth and vascularization [198]. Furthermore, tumor-derived exosomes may regulate stroma remodeling by themselves. Namely, exosomes are rich in MMPs, which may degrade ECM and thus increase tumor motility [199, 200].

Neovascularization is one of the prerequisite for tumor expansion. Formation of new vessels is a result of hypoxic conditions and inflammatory responses in tumor enviroment [201]. Couple of studies demonstrated that exosomes are rich in pro-angiogenic factors such as VEGF, FGF, TGF-β, platelet derived growth factor (PDGF) and IL-8 [202]. In addition, exosomes contain multiple angiogenic microRNAs [203]. Grange et al. extended these observations by reporting that exosomes may activate endothelial cells to organize capillary-like structures on matrigel [204]. In addition, exosomes stimulated organizition of endothelium seems to operate through their ability to induce expression of pro-angiogenic factors including IL-1α, FGF, granulocyte colony stimulating factor (GCS-F), TNF-α, Leptin, TGF-α and VEGF [205].

Together, increasing evidence suggests that exosomes may exert anti- as well as pro-tumorigenic effects. Although the mechanism of exosomal uptake and action in the recipient cells is poorly understood, the protein composition of these vesicles is studied in detail. Numerous studies demonstrated the presence of ENO-1 in exosomes as well as on the cell surface of cancer cells. Despite this fact, the mechanism that drives exteriorization of ENO-1 and the contribution of the cell surface and exosomal ENO-1 to cancer progression are largely unknown.

2. AIM OF THE STUDY

It is well established, that ENO-1 plays an important role during cancer progression. Its direct involvement in cancer cell migration and proliferation was demonstrated in several studies. However, only little is known about the contribution of cell surface associated ENO-1 to the metastatic potential of cancer cells. Cell surface bound ENO-1 participates in PLA formation and thus increases pericellular proteolytic activity of cancer cells. To accomplish this function, ENO-1 must be transported to the cell surface. ENO-1 is primarily a cytoplasmic protein, which lacks a signal sequence, and thus cannot be translocated to the cell surface through the classical ER-Golgi pathway leaving the mechanism of ENO-1 exteriorization largely unknown.

In this context, the aim of the study was:

1. to characterize the cell surface expression of ENO-1 in different human cancer tissues as well as in breast cancer cell lines
2. to investigate the role of cell surface ENO-1 in migration and invasion of cancer cells
3. to decipher the molecular mechanism that drives exteriorization of ENO-1
4. to elucidate whether inhibition of ENO-1 transport to the cell surface has an effect on cancer cell migration and invasion

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Equipment

Name	Company
Bacteria culture incubator	Heraeus, Germany
Cell culture incubator	Heraeus, Germany
Desk Digital Slide Scanner Miramax	Zeiss, Germany
Electrophoresis chambers	Biometra, Germany
Falcon tubes	Greiner-Bio-One, Germany
Film casette	Kodak, New York
Filter tips: 10; 100; 1000 µl	Eppendorf, Germany
Fluorescence and light microscope	Leica, Germany
Gel Blotting paper	Amersham Biosciences, UK
Inverted epifluorescence microscope	Zeiss, Germany
Multifuge centrifuge	Heraeus, Germany
PCR-thermocycler	Biometra, Germany
Petri dishes	Greiner-Bio-One, Germany
Pipetboy	Eppendorf, Germany
Pipets	Eppendorf, Germany
Power suply	Biometra, Germany
Real-time PCR machine	Applied Biosystems, Germany
Tissue culture chamber slides	Greiner Bio-One, Germany
Tissue culture dishes	Greiner Bio-One, Germany
Transmission electron microscope	Zeiss, Germany
Ultra Microplate Reader EL 808	Biotek-instruments, Germany
Ultracentrifuge Optima LE-80K	Beckman, Germany
Water bath for cell culture	Medingen, Germany
Western Blot chambers	Biometra, Germany
Vortex machine	VWR, Germany

3.1.2. Reagents

Name	Company
Ammonium persulfate	Sigma-Aldrich, Germany
1-butanol (n-butyl alcohol)	Sigma-Aldrich, Germany
2-mercapto-ethanol	Sigma-Aldrich, Germany
2-propanol	Fluka, Germany
Acetic acid	Sigma-Aldrich, Germany
Acetone	Roth, Germany
Acrylamide solution, Rotiphorese gel 30	Sigma-Aldrich, Germany
Agarose	Fluka, Germany
Albumin, bovine serum	Sigma-Aldrich, Germany
Ammonium acetate	Sigma-Aldrich, Germany
Brillant Blue G	Sigma-Aldrich, Germany
Calcium chloride	Sigma-Aldrich, Germany
DMEM	Gibco, Germany
Dimethyl sulfoxide	Roth, Germany
DNA ladder (100 bp, 1 kb)	Fermentas, Germany
Ethanol absolut	Roth, Germany
Ethidium bromide	Sigma-Aldrich, Germany
Ethylene glycol bis(2-aminoethyl ether) tetraacetic acid (EGTA)	Sigma-Aldrich, Germany
ECL plus Western blotting detection kit	Amersham Biosciences, UK
Fetal calf serum	Hyclone, UK
Formaldehyde	Sigma-Aldrich, Germany
Glucose	Sigma-Aldrich, Germany
Glutamaxx	Invitrogen, Germany
Glycerol	Roth, Germany
Glycine	Roth, Germany
Hepes	Roth, Germany
High fidelity DNA polymerase	Fermentas, Germany
Lipid transfection reagent	Biorad- Laboratories, Germany

Magnesium chloride	Sigma-Aldrich, Germany
Methanol	Roth, Germany
Milk powder	Roth, Germany
MuLV reverse transcriptase	Applied Biosystems, California
Penicillin/Streptomycin	Invitrogen, Germany
PCR nucleotide mix	Fermentas, Germany
Potassium Chloride	Roth, Germany
Potassium phosphate monobasic	Sigma-Aldrich, Germany
Potassium phosphate dibasic	Sigma-Aldrich, Germany
Random hexamers	Applied Biosystems, Germany
Rnase Inhibitor	Applied Biosystems, Germany
RPMI	Gibco, Germany
Sodium chloride	Sigma-Aldrich, Germany
Sodium deoxycholate	Sigma-Aldrich, Germany
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Germany
Sodium fluoride	Sigma-Aldrich, Germany
Sodium phosphate dibasic	Sigma-Aldrich, Germany
Sodium vanadate	Sigma-Aldrich, Germany
TEMED	Roth, Germany
Trichloroacetic acid (TCA)	Sigma-Aldrich, Germany
Tris	Roth, Germany
Triton X-100	Sigma-Aldrich, Germany
Trypsin/EDTA	PAA Laboratories, Austria
Tween 20	Sigma-Aldrich, Germany

3.2. Methods

3.2.1. Cell culture

Human MDA-MB-435 highly metastatic breast carcinoma, human MCF-7 breast adenocarcinoma (both from ATCC, Manassas, VA), and human MDA-MB-231 metastatic breast carcinoma (LGC Standards GmbH, Wesel, Germany) cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10 % heat-inactivated fetal calf serum (FCS) (Hyclone, Cramlington, UK), 2 mM Glutamax and 1 % Penicilin/Streptomycin (both from Invitrogen Life Technologies). Human mammary epithelial cells (HMEC) (Invitrogen Life Technologies) were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) (Invitrogen Life Technologies) supplemented with FBS and 1 % Penicilin/Streptomycin. Cell cultures were maintained at 37 °C in a humidified incubator with 5 % CO_2.

3.2.2. Immunohistochemistry

Formalin-fixed tissues were obtained from patients with ductal breast carcinoma (n=6), squamos cell lung carcinoma (n=5), colon adenocarcinoma (n=11), bronchoalveolar carcinoma (n=5) and lung adenocarcinoma (n=12) who underwent surgical resection. The investigations have been conducted according to the Declaration of Helsinki principles and were approved by the local institutional review board and ethics committee. Five μm tissue sections were deparaffinized in xylene and rehydrated through graded ethanol washes. Antigen retrieval was performed by the treatment of tissue sections with Fast Enzyme (Zymed Laboratories Inc., San Francisco, CA) for 10 min at room temperature. Immunohistochemistry was performed using a ZytoChem-Plus AP Polymer-Kit according to the manufacturer´s instruction (Zymed Laboratories Inc). A rabbit anti-ENO-1 antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA; catalog number: sc-15343) was applied overnight at 4 °C. Negative control was performed by replacing the primary antibody with a species matched isotype control (1:200) (Sigma-Aldrich, Hamburg, Germany; catalog number: I8140). Slides were scanned with a Mirax Desk Digital Slide Scanner (Zeiss, Goettingen, Germany) and analyzed using a Mirax Viewer (Zeiss).

3.2.3. Western Blotting

Hundred µg of biotinylated proteins or 20 µl of exosomal fraction were separated on a 10 % SDS PAGE under reducing conditions, followed by electrotransfer to a PVDF membrane (GE Healthcare, Munich, Germany). After blocking the membrane with 5 % non-fat milk (Sigma-Aldrich) in TBS-T (5 mM Tris-Cl, 150 mM NaCl, 0.1 % Tween 20, pH 7.5), the membrane was probed with one of the following antibodies: rabbit anti-ENO-1 (1:5000) (Santa Cruz Biotechnology; catalog number: sc-15343), mouse anti-green fluorescence protein (1:1000) (GFP; Santa Cruz Biotechnology; catalog number: sc-9996), mouse anti-26S proteasome subunit (1:1000) (P26S; Abcam, Berlin, Germany, catalog number: ab58115), mouse anti-β1-integrin (1:1000) (BD Biosciences; catalog number: 610467), mouse anti-CD63 (1:500) (Millipore, Schwalbach, Germany; catalog number: CBL553), mouse anti-heat shock protein 70 (1:500) (Hsp70; generous gift from Dr. M. Korfei, Department of Internal Medicine, University of Giessen Lung Centre, Giessen, Germany). Afterwards, the membrane was incubated with peroxidase labeled secondary antibody (1:5000) [all from Dako, Gostrup, Denmark; catalog number: P044701-2 (mouse) and P021702-02 (rabbit)]. Final detection of proteins was performed using an ECL Plus Kit (Amersham Biosciences, Freiburg, Germany). To determine the amounts of protein loaded on the gel, blots were stripped and reprobed using a mouse anti-β-actin antibody (1:10000). (Sigma-Aldrich; catalog number: A1978).

3.2.4. Cell surface biotinylation assay

MDA-MB-231, MCF-7 and MDA-MB-435 cells were treated for 2, 4 and 6 h with 10 µg/ml LPS serotype O111:B4 (Calbiochem, Darmstadt, Germany) or 50 ng/ml TNF-α (R&D, Wiesbaden, Germany). In other experiments MDA-MB-231 cells were pretreated for 1 h with brefeldin A (BD Biosciences, Heidelberg, Germany), glyburide, methylamine, ouabain, ionophore A23187, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′ tetraacetic acid (BAPTA), cyclopiazonic acid (CPA), or YM58483 (all from Sigma-Aldrich) and then stimulated with 10 µg/ml LPS for 2 h. Afterwards, the cells were labeled with 1 mg/ml EZ-link NHS-SS-biotin (Thermo Scientific, Schwerte, Germany) for 1 h at 4 °C, rinsed 3× with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) containing 100 mM glycine and solubilized in cell-lysis buffer [50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 0.1 % Triton X-100, pH 7.4 containing protease inhibitor cocktail (Roche Diagnostics, Penzberg, Germany)]. Protein

concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Hundred μg of proteins were incubated overnight at 4 °C with end-over-end shaking with the Neutravidin Agarose Resin Beads (Thermo Scientific). Finally, beads were washed and resuspended in 25 μl of 2× Laemmli sample buffer (2 % SDS, 20 % glycerol, 120 mM TRIS, 0.02 % bromphenol blue, 4 % β-mercaptoethanol). The samples were analyzed by Western blotting as described above.

3.2.5. Transient transfection

ENO-1 wild type (WT) was recloned from pcDNA3.1 plasmid [83] into the pEGFP-C1 expression vector (Clontech Laboratories, Inc., Mountain View, CA) using *EcoR I* and *BamH I* restriction enzymes (Fermentas GmbH, St. Leon-Rot, Germany). ENO-1 WT sequence was used as a template to generate ENO-1K434R and ENO-1K434G using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The correct sequence and orientation of the inserts were confirmed by sequencing. The MDA-MB-231 cells were seeded onto 6-well tissue culture plates in RPMI 1640 medium to obtain 60-70 % confluence. After 16 h, medium was exchanged and the cells were incubated overnight in RPMI 1640 medium containing 0.1 % FBS. Cells were transfected using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions.

3.2.6. Generation of stable cell lines

MDA-MB-231 cells were transfected with pEGFP-C1 (GFP-EV) and pEGFP-C1-ENO-1WT (GFP-ENO-1) vectors using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. Positive clones were selected using 800 μg/ml of Geneticin disulphate (G418) (Roth, Karlsruhe, Germany). After selection, stable transfectans were kept in medium supplemented with 400 μg/ml of G418. Positive clones were tested for GFP and ENO-1 expression by Western blotting.

3.2.7. Proliferation assay

Proliferation of MDA-MB-231 cells and stable transfectants was determined by a DNA synthesis assay based on the uptake of [^{3}H] thymidine (PerkinElmer, Waltham, MA). Cells were cultured in 48-well plates, growth-arrested in serum-free RPMI medium and left

unstimulated or stimulated with 10 μg/ml LPS for 8 h. Subsequently, cells were pulsed with 0.2 μCi/ml [^{3}H] thymidine for 16 h. Afterwards, cells were solubilized in 0.5 M NaOH, and [^{3}H] thymidine incorporation was determined by liquid scintillation spectrometry.

3.2.8. Trichloroacetic acid precipitation of proteins

Proteins present in conditioned cell culture media were precipitated with trichloroacetic acid (TCA; Sigma-Aldrich). Briefly, MDA-MB-231 cells were stimulated with 10 μg/ml LPS for 2, 4 or 6 h. After indicated time points, supernatants were collected, mixed with TCA (final concentration 10 %), vortexed and incubated for 10 min at 4 ºC. The precipitated proteins were collected by centrifugation at 20,000 g for 45 min at 4 ºC. The pellets were washed twice with 70 % ice-cold ethanol, air dried, and resuspended in 5× Laemmli sample buffer.

3.2.9. Exosome isolation

Exosomes were isolated either from unstimulated GFP-EV and GFP-ENO-1 cells or stimulated MDA-MB-231, MCF-7 and MDA-MB-435 cells. Briefly, MDA-MB-231, MCF-7 and MDA-MB-435 cells were treated for 24 h with 1 μg/ml LPS or 50 ng/ml TNF-α. In other experiments MDA-MB-231 cells were preincubated with 1 μM A23187, 20 μM BAPTA, or 5 μM YM58483 for 1 h and then stimulated with 1 μg/ml LPS for 24 h. Exosomes were isolated from 10 ml of conditioned cell culture media which were first centrifuged at 800 g for 10 min at room temperature to sediment cells, and then centrifuged at 10,000 g for 10 min at 4 ºC (Optima LE-80K Ultracentrifuge, Beckman, Ramsey, MN) to remove the cellular debris. Exosomes were pelleted by centrifugation at 100,000 g for 3 h at 4 ºC. Finally, the exosome pellet was washed once with PBS and resuspended in 100 μl of PBS. Twenty μl exosomal fraction was mixed with 5× Laemmli sample buffer and analyzed by Western blotting. The viability of the treated cells was assessed in each experiment using a Cytotoxicity Detection Kit (Roche Diagnostics).

3.2.10. Exosome uptake

Exosomes were purified from cell culture supernatants of GFP-EV and GFP-ENO-1 stably transfected cells according to the above mentioned protocol. The purified exosomes were resuspended in 100 μl of PBS. MDA-MB-231 cells were cultured in complete RPMI medium on the microscope coverslips in the 6-well plates. Cells were serum-starved overnight and

then incubated with purified exosomes for 30 min at 37 ºC. Subsequently, cells were washed 3× with cold PBS, fixed with 4 % paraformaldehyde for 10 min at 4 ºC, incubated with rhodamin-conjugated phalloidin (Invitrogen Life Technologies) for 10 min at room temperature, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Nuclei were visualized by DAPI staining. Images were captured by a Leica DMR microscope (Leica, Heidelberg, Germany). Post processing and image analyses were done with a MetaMorph™ (Leica Microsystems, Wetzlar, Germany).

3.2.11. Electron microscopy

Exosomes were fixed with 2 % paraformaldehyde and deposited onto the butvar-carbon coated grids. The vesicle-coated grids were washed twice with PBS, twice with PBS containing 50 mM glycine, and finally with PBS containing 0.5 % BSA (PBS/0.5 % BSA), stained with 2 % uranyl acetate, and then viewed with a transmission electron microscope (TEM; Zeiss EM900; Zeiss, Jena, Germany). For the immuno-gold labeling exosome samples were absorbed onto a carbon coated butvar film on 300 mesh nickel grids, washed in PBS buffer containing 10 mM glycine for 5 min and then washed in PBS buffer. Samples were placed onto 25 µl drops of 0.4 % skim milk in water for 5 min, blotted dry on filter paper and then placed on 25 µl drops of a rabbit anti-ENO-1 antibody (1:25) (Santa Cruz Biotechnology; catalog number: sc-15343) and incubated for 1 h at 30 °C. After washing with PBS samples were incubated with 0.4 % skim milk for 5 min, blotted dry and then placed onto 25 µl drops of a mixture of protein A/G gold and goat-anti rabbit gold nanoparticles (1:75 dilution) and incubated for 30 min at room temperature. Samples were then washed with PBS and TE-buffer (20 mM TRIS, 2 mM EDTA, pH 7.0) before air-drying. Samples were examined in a TEM 910 Zeiss at an acceleration voltage of 80 kV.

3.2.12. Live cell Ca^{2+} imaging

The cells were cultured on 25 mm glass cover slips which were loaded with 2 µM fura-2/AM in dark for 45 min followed by a washing step in Ringer solution (5.8 mM KCl, 141 mM NaCl, 0.5 mM KH_2PO_4, 0.4 mM NaH_2PO_4, 11.1 mM glucose, 10 mM Hepes, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4). After 15 min, the single glass cover slip was mounted on the stage of a Zeiss 200M inverted epifluorescence microscope coupled to a PolyChrome V monochromator (Till Photonics, Munich, Germany) light source in a sealed temperature-controlled RC-21B imaging chamber (Warner Instruments, Hamden, CT) and perfused with

prewarmed solution (32 °C). Fluorescence images were obtained with alternate excitation at 340 and 380 nm. The emitted light was collected at 510 nm by an air-cooled Andor Ixon camera (Andor Technology, Belfast, Ireland). Measurements were made every 3 s. Background fluorescence was recorded from each cover slip and subtracted before calculation. The acquired images were stored and processed offline with TillVision software (Till Photonics). Calcium concentration $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [206]. Maximal and minimal ratio values were determined at the end of each experiment by first treating the cells with 10 μM ionomycin (maximal ratio) and then chelating all free Ca^{2+} with 20 mM EGTA (minimal ratio). Cells that did not respond to ionomycin were discarded. After 3 min of baseline measurement, the cells were stimulated with 10 μg/ml LPS for 10 min in the absence or presence of extracellular Ca^{2+}. In the next set of experiments, after baseline measurement, cells were pretreated with 20 μM BAPTA or 50 μM CPA followed by 10 min of 10 μg/ml LPS treatment. For data analysis, the basal level of Ca^{2+} was determined as an average value of the first 50 seconds of the curve. Then, after subtracting the baseline, the LPS-induced Ca^{2+} peak height was calculated and is presented as $\Delta[Ca^{2+}]i$. All chemicals were dissolved and diluted to the desired concentrations in Ringer solution. All the solutions were freshly prepared on the day of the experiment and stored at 4 °C until they were used.

3.2.13. Calculating the intracellular calcium concentration

The $Ratio_{340/380}$ and F_{380} (fluorescence at 380 nm excitation) curves were exported offline either as *.txt or *.xls files. The intracellular calcium concentration was determined using the following equation *(2)*:

$$[Ca]_i(nM) = K_d \times \beta \times \frac{Ratio_{340/380} - R_{\min}}{R_{\max} - Ratio_{340/380}}$$

K_d=224 nM, is the dissociation constant of Fura-2

$\beta = \frac{F_{380}\max}{F_{380}\min}$ from the 380 nm excitation curve

R_{min} is the minimal ratio, determined by 20 mM EGTA

R_{max} is the maximal ratio, determined by 10 μM ionomycin

$Ratio_{340/380}$ is the ratio of the fluorescence at 340 nm and 380 nm excitation.

3.2.14. Antisense oligonucleotide

Commercially available siRNA sequence directed against human stromal interaction molecule (STIM) 1 (Thermo Scientific), human Ca^{2+} release-activated calcium modulator (ORAI) 1 (Life Technologies) and a universal negative-control siRNA (Thermo Scientific) were employed. Cells were starved overnight and then treated with 100 nM siRNA using the siLentFectTM Lipid transfection reagent (Biorad Laboratories, Munich, Germany) according to the manufacturer's instructions. After 72 h cells were splitted and seeded onto 6 cm cell culture dishes, serum-starved overnight and then treated one more time with 100 nM siRNA for 48 h. The efficacy of STIM1 and ORAI1 knockdown was assessed by real time PCR.

3.2.15. RNA isolation and reverse transcriptase (RT) reaction

Isolation of RNA from formalin-fixed, paraffin-embedded tumor tissue and adjacent non-tumorous tissue was performed as previously described [207]. Isolation of RNA from MDA-MB-231 cells was performed using a peqGOLD Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instruction. One µg of RNA obtained from parafin-embedded tissue or MDA-MB-231 cells was used in a reaction containing: 2 µl 10× RT buffer, 0.8 µl dNTP Mix (100 mM), 2 µl 10× RT Random Primers (25 µM), 1 µl MultiScribe Reverse transcriptase (200 U/µl), 1 µl Rnase Inhibitor (20 U/µl), 3.2 µl nuclease-free water. The reaction was incubated at 25 ºC for 10 min followed by 37 ºC for 2 h and then 85 ºC for 5 min (Tgradient Thermocycler, Biometra, Goettingen, Germany).

3.2.16. Real time PCR

Real-time PCR (qPCR) was performed to amplify transcripts of human *ENO-1*, human *STIM1*, human *ORAI1,* human subunit of L-type calcium channnel (*LTCC $Ca_v1.2$*), human porphobilinogen deaminase (*PBGD*) and human beta-actin (*β-ACT)* (Table 1). *PBGD* or *β-ACT* were used as reference genes. Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curve analysis and gel electrophoresis were performed to confirm the exclusive amplification of the expected PCR product. All changes in the target gene mRNA levels are presented as delta Ct (ΔCt) which was calculated by subtracting the Ct value of the target gene from the Ct value of the reference gene. The higher values of ΔCt correspond to higher relative expression of the gene of interest.

Table 1. List of primers used for qRT-PCR

Gene	Accession number	Nucleotide sequences (5'→3')	[1]T_m (°C)	Amplicon size [2](nt)
ENO-1	NM_001428.3	[3]F: GAA TAA AGA AGG CCT GGA GC [4]R: TAG ACA CCA CTG GGT AGT CC	60	217
STIM1	NM_001277961.1	F: AGT GAG AAG GCG ACA GGA R: ATG TTA CGG ACT GCC TCG	60	130
ORAI1	NM_032790.3	F: ACC TCG GCT CTG CTC TCC R: CAG GCA CTG AAG GCG ATG	60	86
$Ca_v1.2$	NM_001167625.1	F: TGG TCC ATG GTC AAT GAG R: CGC ATT GGC ATT CAT GTT	60	107
PBGD	NM_000190.3	F: CCC ACG CGA ATC ACT CTC AT R: TGT CTG GTA ACG GCA ATG CG	60	69
β-ACT	NM_001101.3	F: ATT GCC GAC AGG ATG CAG GAA R: GCT GAT CCA CAT CTG CTG GAA	60	149

[1]T_m, melting temperature; [2]nt, nucleotide; [3]F, forward; [4]R, reverse

3.2.17. Lactate dehydrogenase (LDH) release or cytotoxicity assay

MDA-MB-231 cells were stimulated with 10 µg/ml LPS for 2, 4 or 6 h. After indicated time points, LDH release was assessed using a Cytotoxicity Detection Kit (Roche Diagnostics) according to the manufacturer's instruction. MDA-MB-231 cells treated with 1 % Triton X-100 for 5 min were used as a positive control.

3.2.18. Wound healing assay

MDA-MB-231 cells or cells stably transfected with GFP-EV and GFP-ENO-1 were seeded onto 6-well tissue culture plates and serum-starved overnight. MDA-MB-231 cells were stimulated either with 10 µg/ml LPS alone or in combination with a peptide blocking binding of PLG to ENO-1 (KFAGRNFRNPLAK; kindly provided by Dr. S. Bergmann, Institute of

Microbiology, Technical University Braunschweig, Braunschweig, Germany) or a scramble peptide (KFAGRNFRNPLA; Thermo Scientific). Cells were washed with PBS and wounds were incised by scratching the cell monolayers using a pipette tip. Images of the scratch were captured with a microscope immediately after incision (0 h) and 8 h after scratching to assess the rate of gap closure.

3.2.19. Transwell inasion assay

Invasion assay was performed either with unstimulated GFP-EV and GFP-ENO-1 cells or stimulated MDA-MB-231 cells. Briefly, MDA-MB-231 cells were starved overnight and stimulated either with 1 µg/ml LPS or with exosomes isolated from GFP-EV and GFP-ENO-1 cells. Cells (5×10^4) were added into the upper chamber containing the fibronectin-coated polycarbonate membrane (8 µM pore size, BD Biosciences). Five hundred µl RPMI medium containing 2 % FBS was added into the lower chamber of the transwell. Cells were then cultured for 16 h at 37 °C. Afterwards, cells on the upper surface of the polycarbonate membrane of the transwell were removed with a cotton swab and the cells that migrated onto the underside of the membrane were fixed with aceton/methanol (1:1) solution, washed with PBS and stained with 0.5 % crystal violet for 30 min. Cells that migrated to the lower surface of the filter were counted.

3.2.20. Statistics

The statistical analysis was performed using a GraphPad Prism version 5.02 for Windows (GraphPad Software, La Jolla, CA). Data are presented as mean values ± S.E.M. unless otherwise stated. Differences between two groups were tested using a Student's *t*-test. Comparison of multiple groups was performed by analysis of variance (ANOVA) followed by Tukey's post hoc test. All tests were performed with an undirected hypothesis. p value less than 0.05 was considered as statistically significant.

4. RESULTS

4.1. Expression of ENO-1 is elevated on the cell surface of cancer cells and contributes to cancer cell invasion

To investigate the role of cell surface bound ENO-1 in cancer cell invasion, we first determined the expression level of this protein in different tumor types. Immunohistochemical staining of ductal breast carcinoma, squamos cell lung carcinoma, colon adenocarcinoma, bronchoalveolar carcinoma and lung adenocarcinoma revealed high ENO-1 protein expression in cancer cells (Fig. 4.1A, indicated by arrowheads). qPCR confirmed high ENO-1 mRNA expression in tumor tissue as compared to adjacent non-tumorous tissue (Fig. 4.1B).

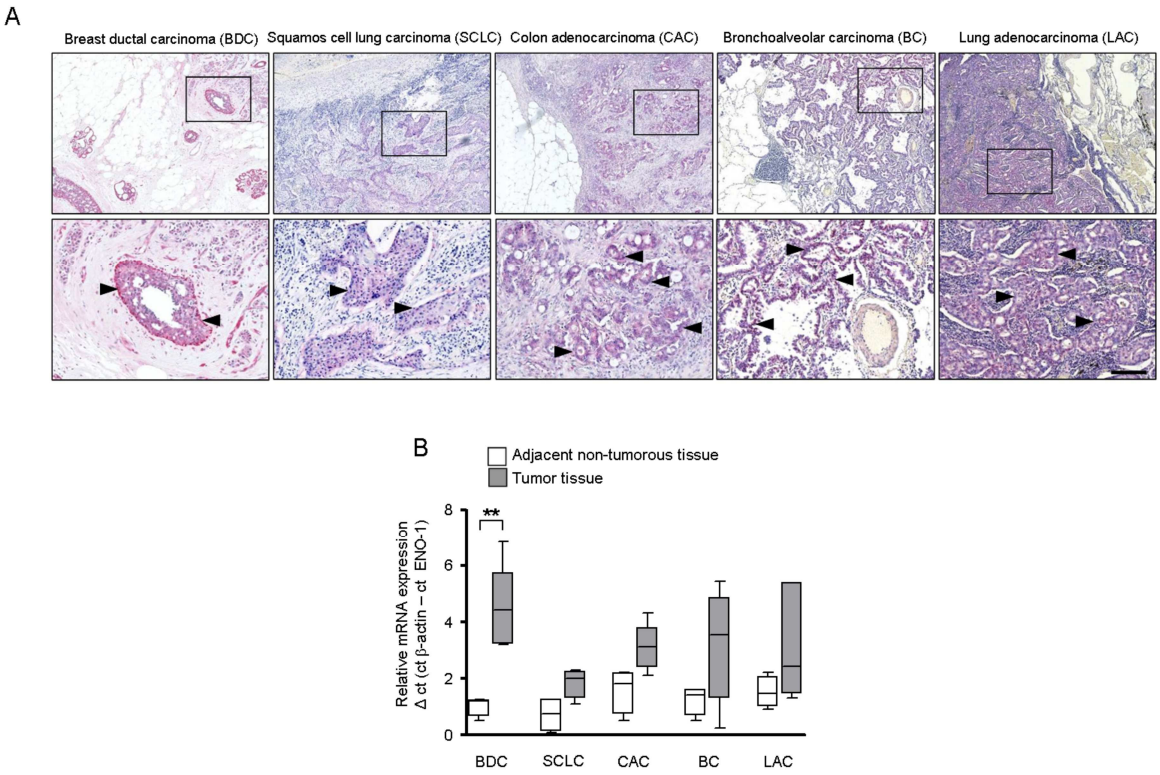

Figure 4.1. Expression of ENO-1 in different human cancer tissue and breast cancer cell line.
(A) Representative breast ductal carcinoma (BDC; n=6), squamos cell lung carcinoma (SCLC; n=5), colon adenocarcinoma (CAC; n=11), bronchoalveolar carcinoma (BC; n=5), and lung adenocarcinoma (LAC; n=12) tissue sections stained for ENO-1. ENO-1 positive staining is indicated by arrowheads. Bar size 100 µM. (B) qPCR analysis of ENO-1 mRNA expression in tumor and adjacent non-tumorous tissue. qPCR data are expressed as ΔCt using β-actin as a reference gene. n=5 per group.

As the highest ENO-1 expression was detected in ductal carcinoma of the breast, in the further studies we focused on the role of ENO-1 in breast cancer cells. In order to demonstrate subcellular localization of ENO-1 in cancer cells, various experimental approaches were conducted. Cell fractionation revealed markedly increased levels of cell surface associated ENO-1 in a highly metastatic breast cancer cell line MDA-MB-231 as compared to primary human mammary epithelial cells (HMEC) (Fig. 4.2A). Flow cytometry analysis confirmed high cell surface abundance of ENO-1 on MDA-MB-231 cells (Figure 4.2B). To verify ENO-1 distribution in cancer cells, MDA-MB-231 cells were transfected with a vector carrying either GFP alone (GFP-EV) or GFP tagged ENO-1 (GFP-ENO-1) and the localization of ENO-1 was examined by fluorescence microscopy. While GFP was exclusively expressed in the cytoplasm (Fig. 4.2C, bottom panel), GFP-ENO-1 was present in the cytoplasm as well as on the cell surface (Fig. 4.2C, upper panel, indicated by arrows).

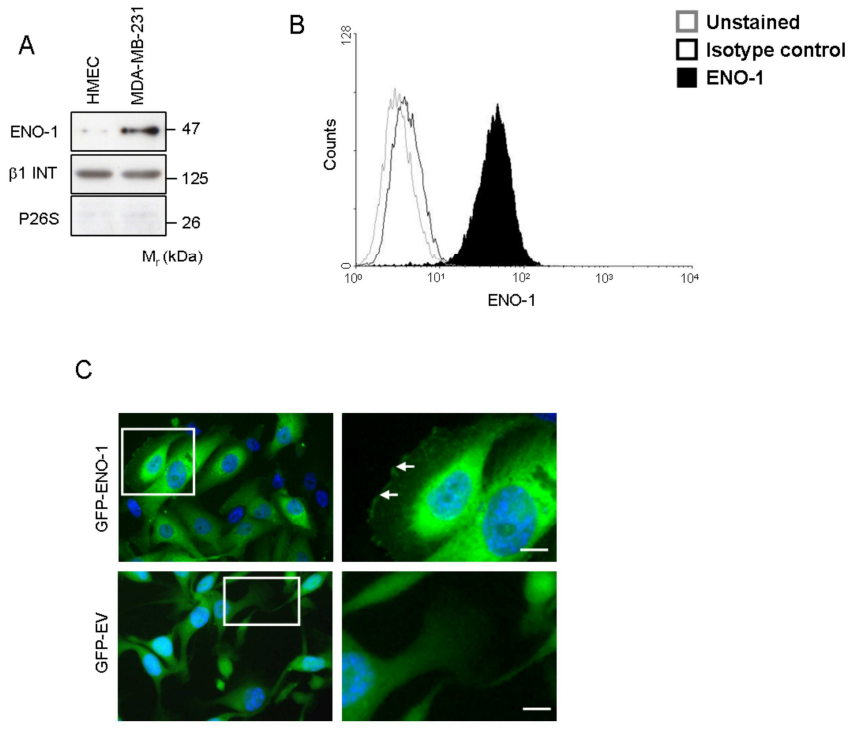

Figure 4.2. Localization of ENO-1 in a highly invasive MDA-MB-231 breast cancer cell line.
(A) Cell surface expression of ENO-1 in primary human mammary epithelial cells (HMEC) and in MDA-MB-231 cells. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1-integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are shown. (B) Cell surface expression of ENO-1 in MDA-MB-231 cells as assessed by flow cytometry. n=3. (C) Distribution of ENO-1 in MDA-MB-231 cells stably transfected with GFP-EV or GFP-ENO-1. Arrows indicate the cell surface-associated GFP tagged ENO-1. Bar size 5 μm.

In order to explore the functional consequence of increased ENO-1 cell surface expression in cancer cells, wound healing and transwell invasion assays with MDA-MB-231 cells that overexpress GFP-ENO-1 were performed. Transfection of MDA-MB-231 cells with GFP-ENO-1 enhanced their migratory (Fig. 4.3A, B) and invasive (Fig. 4.3C, D) properties. These effects were dependent on the impact of ENO-1 on cancer cell motility as overexpression of GFP-ENO-1 did not increase the number of cells (Fig. 4.3E).

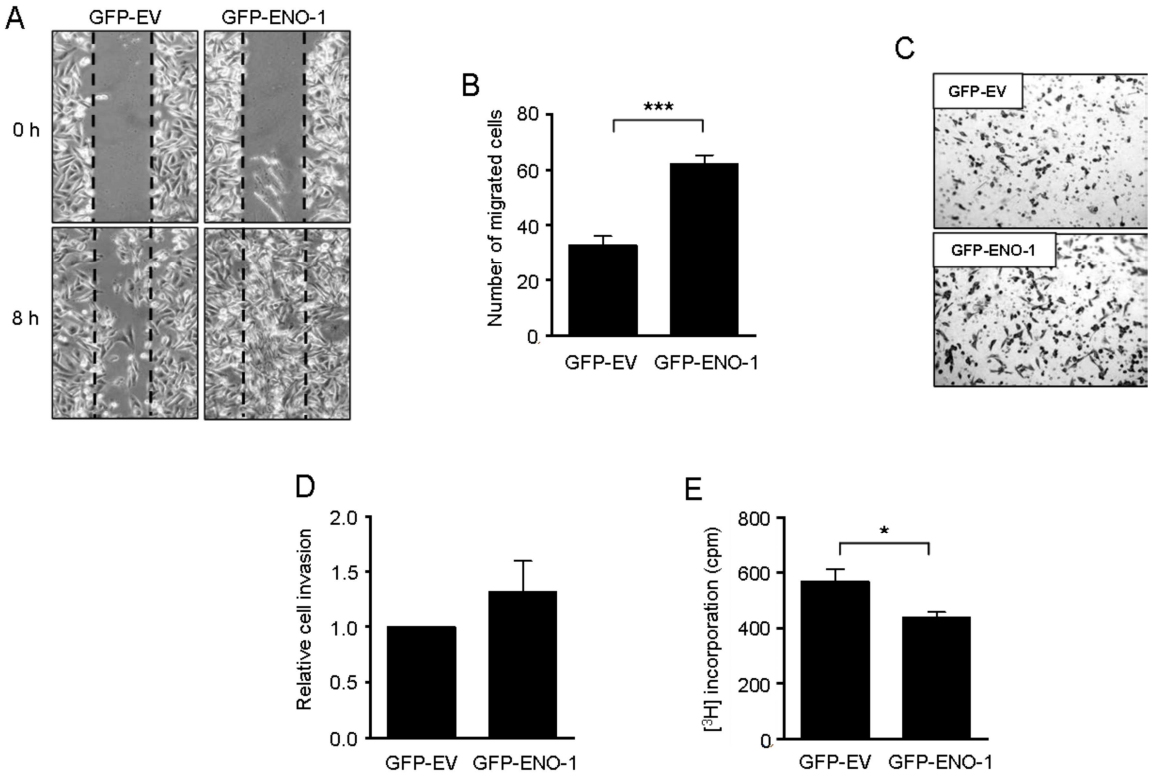

Figure 4.3. Overexpression of ENO-1 correlates with migratory and invasive properties of MDA-MB-231 breast cancer cells.
(A) Confluent monolayers of stable transfectants expressing either GFP (GFP-EV) or GFP tagged ENO-1 (GFP-ENO-1) were scratched and incubated for 8 h at 37 °C in serum-free RPMI medium. Representative pictures from the wound-healing assay at time 0 h and 8 h are shown. (B) The rate of wound closure was assessed by counting the cells that migrated into the same-sized square fields. Data represent mean values ± S.E.M. n=3; ***, $p<0.001$. (C) Stable transfectants expressing either GFP (GFP-EV) or GFP tagged ENO-1 (GFP-ENO-1) were seeded onto a fibronectin-coated membrane and allowed to invade for 16 h. Representative images of the cells that invaded the underside of the membrane are shown. (D) Cells that invaded the underside of the membrane were counted. Data represent mean values ± S.E.M. n=3. (E) Proliferation of GFP-EV and GFP-ENO-1 overexpressing cells as assessed by [^{3}H] thymidine incorporation. Data represent mean values ± S.E.M. n=3; *, $p<0.05$.

4.2. C-terminal lysine residue in PLG binding site of ENO-1 controls invasion of MDA-MB-231 cells

Pericellular proteolytic activity is critical for cancer cell migration and invasion. Binding of PLG to the cell surface associated ENO-1 facilitates its conversion to PLA and enables invasion of cancer cells [50]. C-terminal lysine residue of ENO-1 was identified to be responsible for the binding to PLG [26]. To explore, whether cell surface ENO-1 regulates cancer cell motility through a PLG-dependent mechanism, C-terminal lysine residue in PLG-binding region of ENO-1 was mutated to glycine or arginine (Fig. 4.4A). Overexpression of ENO-1 wild type (GFP-ENO-1) and ENO-1 mutants (GFP-ENO-1K434G; GFP-ENO1-K434R) in MDA-MB-231 cells was confirmed by Western blotting (Fig. 4.4B).

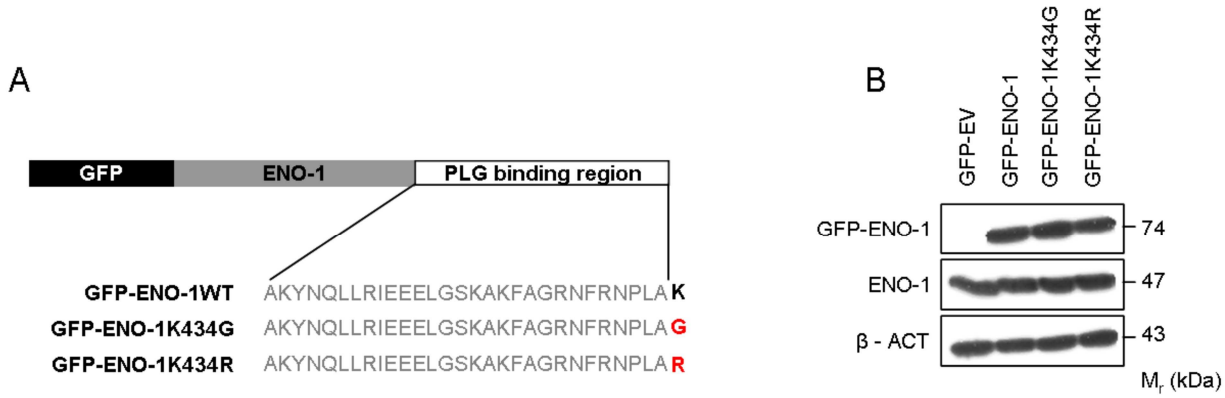

Figure 4.4. Overexpression of ENO-1 mutants.
(A) Schematic representation of ENO-1 mutants. C-terminal lysine (K) residue was replaced by glycine (G) or arginine (R). (B) Overexpression of ENO-1 mutants in MDA-MB-231 cells as assessed by Western blotting. β-actin (β-ACT) served as a loading control. Representative blots are demonstrated. n=3.

In order to explore the functional consequence of ENO-1 mutation, MDA-MB-231 cells were transfected with GFP-ENO-1, GFP-ENO-1K434G or GFP-ENO-1K434R and a transwell invasion assay was performed. Cells transfected with GFP-ENO-1 exhibited increased invasion as compared to GFP-EV transfected cells. Substitution of ENO-1 C-terminal lysine residue by glycine or arginine slightly diminished invasion of cancer cells as compared to the cells transfected with wild type ENO-1 (Fig. 4.5A, B). To exclude, that the observed effect is not a result of altered cell proliferation, cell growth of MDA-MB-231 cells overexpressing wild type and mutated forms of ENO-1 was controled. However, no change in cell proliferation was observed (Fig. 4.5C).

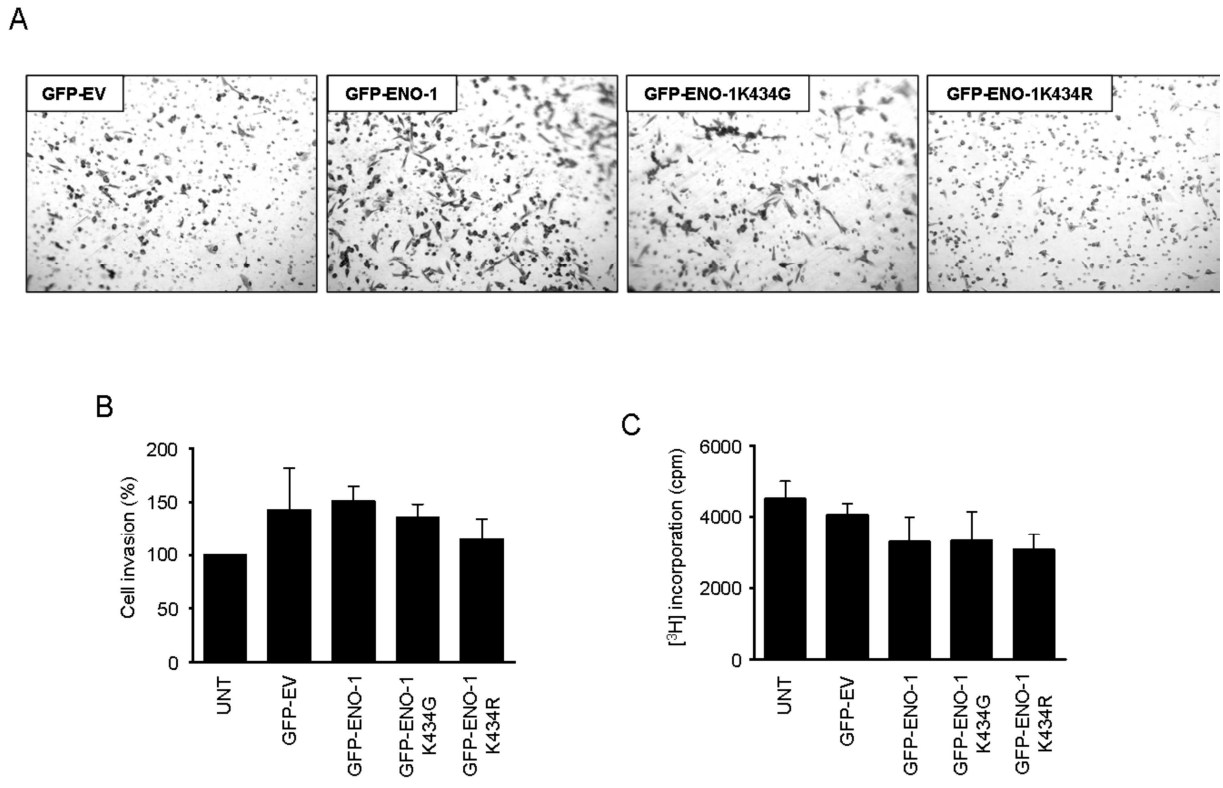

Figure 4.5. Substitution of C-terminal lysine residue by glycine or arginine in ENO-1 PLG binding site impairs cancer cell invasion.
(A) Untransfected MDA-MB-231 cells (UNT) or cells overexpressing GFP (GFP-EV), GFP tagged ENO-1 (GFP-ENO-1) or GFP tagged ENO-1 mutants (GFP-ENO-1K434G and GFP-ENO-1K434R) were seeded onto a fibronectin-coated membrane and allowed to invade for 16 h. Representative images of the cells that invaded the underside of the membrane are shown. (B) Cells that invaded the underside of the membrane were counted. Data represent mean values ± S.E.M. n=3. (C) Proliferation of untransfected MDA-MB-231 cells (UNT) or GFP-EV, GFP-ENO-1, GFP-ENO-1K434G and GFP-ENO-1K434R overexpressing cells as assessed by [^{3}H] thymidine incorporation. Data represent mean values ± S.E.M. n=3.

4.3. LPS induces translocation of ENO-1 to the cell surface and to the extracellular space

Inflammation has long been thought to contribute to tumor progression, for example, by influencing the invasive potential of cancer cells (23). In line with this notion, treatment of MDA-MB-231 cells with LPS markedly augmented their migratory and invasive properties (Fig. 4.6A-D). The increased migration of MDA-MB-231 cells observed in the presence of LPS was abolished by a peptide directed against C-terminal part of ENO-1 involved in the

binding of PLG (Fig. 4.6A, B). Stimulation of cells with LPS did not affect their growth (Fig. 4.6E).

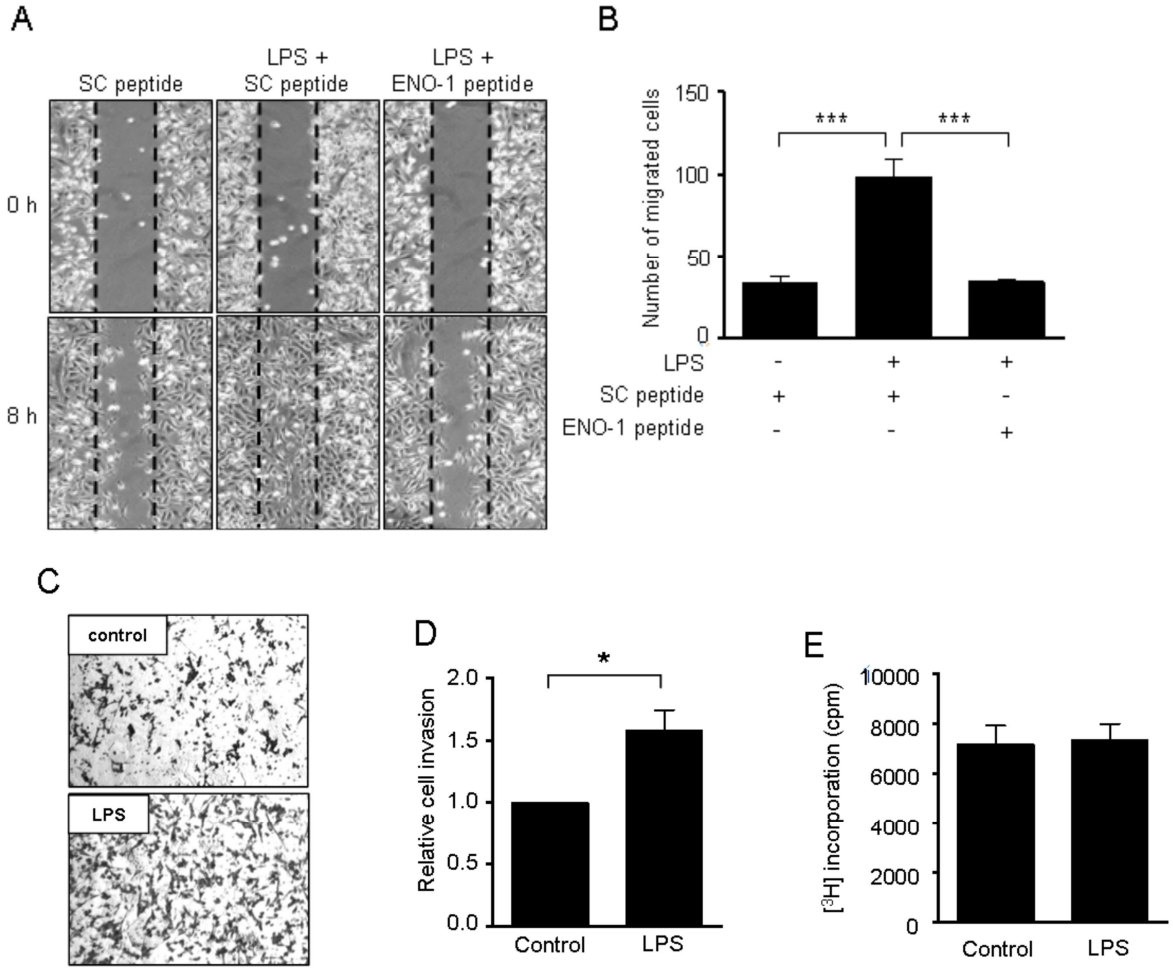

Figure 4.6. ENO-1 mediates LPS-triggered migration and invasion of cancer cells.
(A) Impact of ENO-1 peptide on LPS-driven MDA-MB-231 cell migration. Representative pictures from the wound healing assay at time 0 h and 8 h are shown. (B) The rate of wound closure was assessed by counting the cells that migrated into the same-sized square fields. Data represent mean values ± S.E.M. n=4; ***, $p<0.001$. SC, scramble. (C) LPS stimulated MDA-MB-231 cells were seeded onto a fibronectin-coated membrane and allowed to invade for 16 h. Representative images of cells that invade the underside of the membrane are demonstrated. (D) Cells that invaded the underside of the membrane were counted. Data represent mean values ± S.E.M. n=3; *, $p<0.05$. (E) Proliferation of MDA-MB-231 cells stimulated with LPS for 8 h as assessed by [^{3}H] thymidine incorporation. Data represent mean values ± S.E.M. n=3.

LPS-mediated increase in cell migration was accompanied by elevated cell surface expression of ENO-1. Alterations in cell membrane bound ENO-1 levels were visible 2 h after stimulation and remained unchanged thereafter (Fig. 4.7A). Moreover, LPS-induced

mobilisation of ENO-1 on the cell surface was TLR-4-dependent as preincubation of cells with an anti-TLR-4 blocking antibody inhibited ENO-1 translocation (Fig. 4.7B).

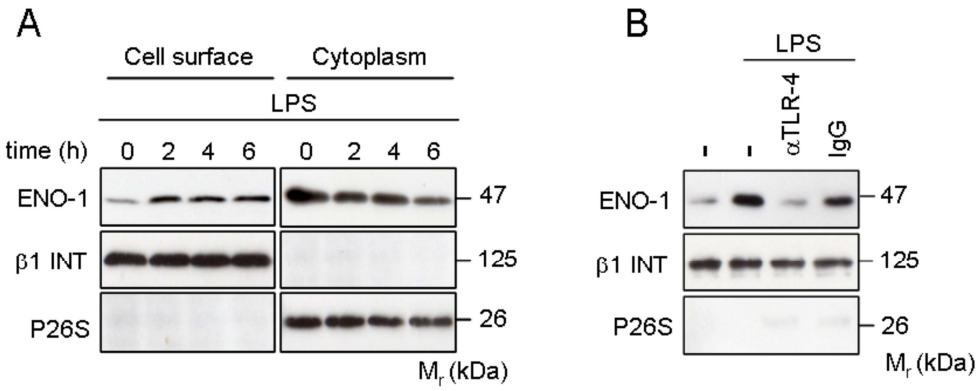

Figure 4.7. LPS upregulates cell surface expression of ENO-1.
(A) Cell surface expression of ENO-1 in MDA-MB-231 cells exposed to LPS for the indicated time points. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1 integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are shown. (B) Cell surface expression of ENO-1 in MDA-MB-231 cells stimulated with LPS in the absence or presence of an anti-TLR-4 antibody. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1 integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are demonstrated. TLR, toll-like receptor; IgG, isotype control.

Augmented levels of cell surface bound ENO-1 occcured in the absence of detectable changes in total cellular expression of ENO-1 mRNA and protein (Fig. 4.8A, B), suggesting that alterations in cell surface ENO-1 abundance are independent of the new protein synthesis.

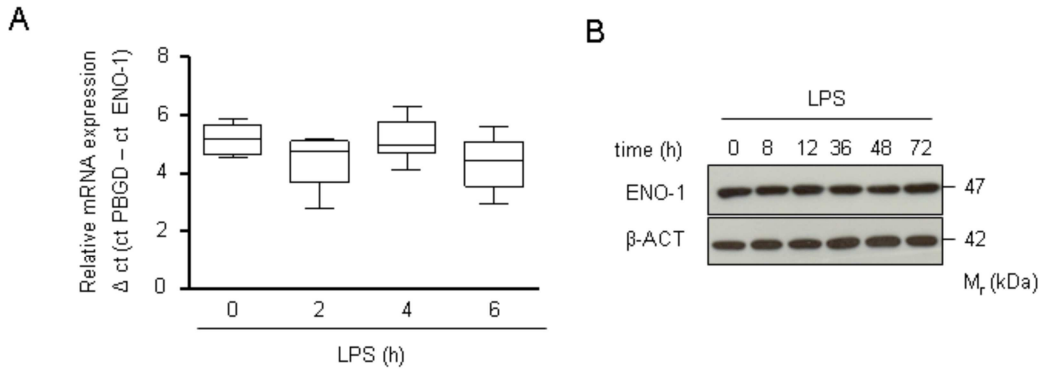

Figure 4.8. LPS does not influence ENO-1 mRNA and protein expression.
(A, B) Time course of ENO-1 mRNA and protein expression after treatment of MDA-MB-231 cells with LPS for indicated time points as assessed by qPCR (A) and Western blotting (B) qPCR data are expressed as ΔCt using PBGD as a reference gene. n=3. β-actin served as loading control for Western blotting. n=3. Representative Western blots are shown.

Interestingly, there was not only an increased ENO-1 translocation to the cell surface, but also an increased release into the extracellular space upon LPS stimulation (Fig. 4.9A). Elevated extracellular levels of ENO-1 were positively correlated with the markers of exosomes, CD63 and Hsp70 implying that ENO-1 is secreted from LPS-treated MDA-MB-231 cells in the form of vesicles (Fig. 4.9A). To exclude that the release of ENO-1 is a result of cell damage, a LDH assay was performed. This experimental procedure revealed no impact of LPS on cell viability (Fig. 4.9B).

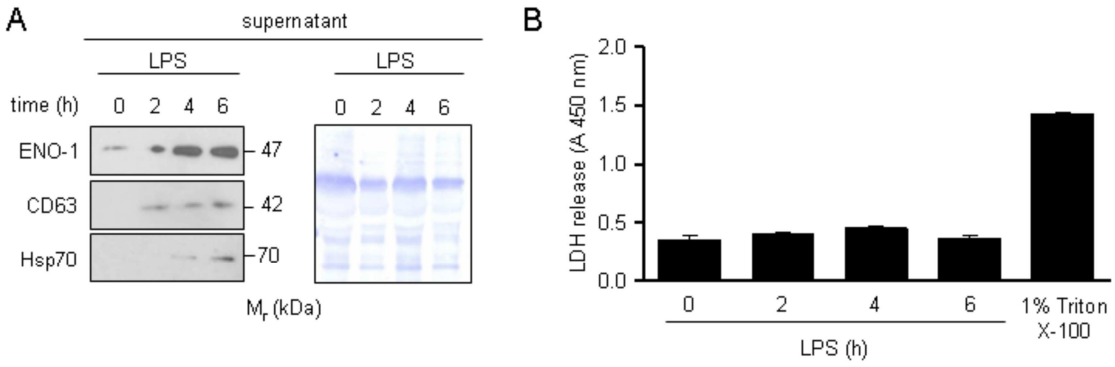

Figure 4.9. LPS induces release of ENO-1 into the extracellular space.
(A) Levels of ENO-1 in conditioned culture media collected after stimulation of MDA-MB-231 cells with LPS for indicated time points. Hsp70 and CD63 served as exosome markers. Coomassie brilliant blue (CBB) staining served as a loading control. n=3. Representative Western blots are shown. (B) Release of LDH to cell culture media collected from the cells exposed to LPS for indicated time points. Triton X-100 was used as a positive control. Data represent mean values ± S.E.M. n=3. LDH, lactate dehydrogenase.

To examine whether the level of cell surface ENO-1 associates with the metastatic potential of cancer cells, ENO-1 cell surface expression was explored in a less metastatic breast cancer cell line, MCF-7, and a highly metastatic breast cancer cell line, MDA-MB-435. In contrast to MDA-MB-435 cells, which exhibited high cell surface bound ENO-1 levels under basal conditions, cell membrane associated ENO-1 was not detectable on MCF-7 cells (Fig. 4.10). Following LPS exposure, levels of cell surface bound ENO-1 increased on MCF-7 cells but remained unchanged on MDA-MB-435 cells (Fig. 4.10). This intimates that the level of ENO-1 on the cell surface of MDA-MB-435 cells is already saturated under basal condition. Altogether, these results imply that i) LPS-triggered ENO-1 exteriorization aggravates the malignant behavior of tumor cells and that ii) there is a positive correlation between the level of cell surface ENO-1 and the metastatic potential of cancer cells.

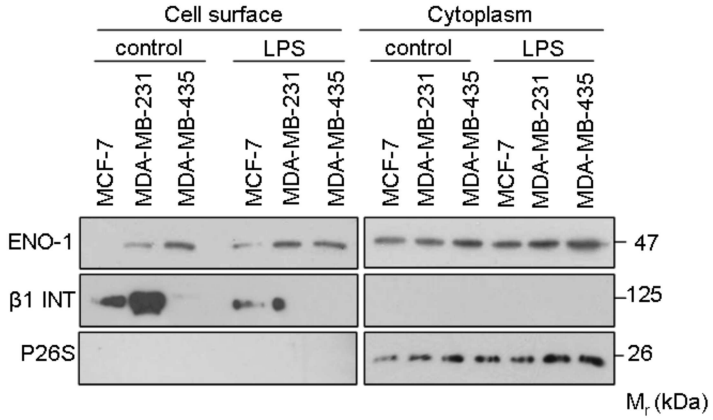

Figure 4.10. Cell surface-bound ENO-1 associates with the metastatic potential of breast cancer cells.
Cell surface expression of ENO-1 in MCF-7, MDA-MB-231 and MDA-MB-435 cells exposed to LPS for 2 h. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1-integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are shown.

To study whether ENO-1 exteriorization is stimulus dependent, MDA-MB-231 cells were treated with TNF-α and cell surface ENO-1 mobilisation as well as exosome release were analysed. In contrast to LPS, TNF-α did not promote ENO-1 translocation to the cell membrane (Fig. 4.11A). However, it potentiated exosome production and thus the release of ENO-1 into the extracellular space (Fig. 4.11B).

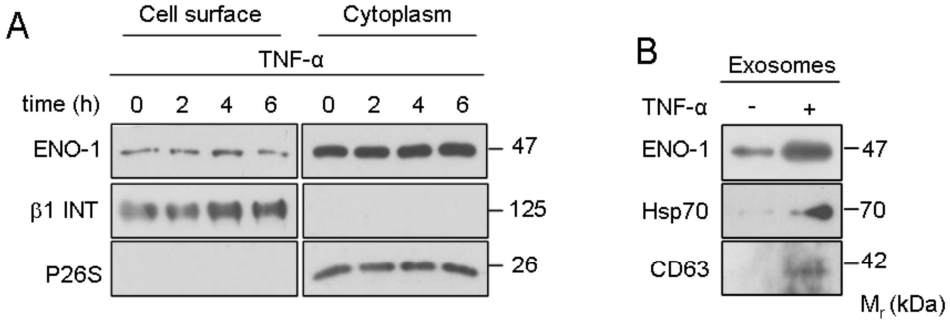

Figure 4.11. TNF-α induces release of ENO-1 into conditioned medium without affecting its cell surface abundance.
(A) Cell surface expression of ENO-1 in MDA-MB-231 cells exposed to 50 ng/ml TNF-α for the indicated time points. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1-integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are shown. (B) Abundance of ENO-1 in exosomes isolated from MDA-MB-231 cells exposed to 50 ng/ml TNF-α as assessed by Western blotting. Hsp70 and CD63 served as exosome markers. n=3. Representative Western blots are demonstrated.

4.4. ENO-1 released from MDA-MB-231 cells in the form of exosomes enhances tumor cell migration in a paracrine manner

Accumulating evidence suggests that cancer cells secrete abundant levels of exosomes, small (30-150 nm in diameter) membranous vesicles [171]. Thus, we examined whether ENO-1 detected in the cell culture media of MDA-MB-231 cells is associated with exosomes. Exosomes were isolated by serial ultracentrifugation steps from cell culture supernatants of MDA-MB-231 cells overexpressing GFP-EV or GFP-ENO-1 (Fig. 4.12.).

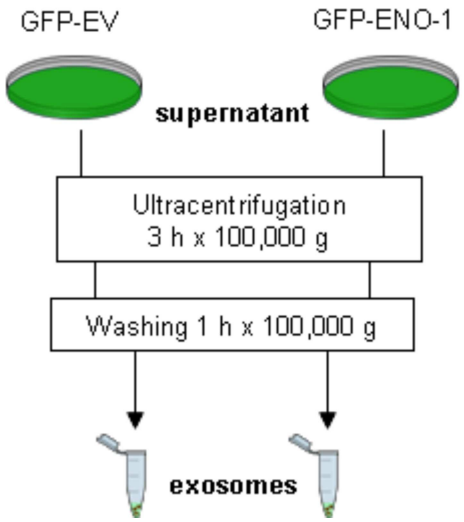

Figure 4.12. Exosome isolation.
Schematic representation of exosome purification from the cells overexpressing GFP-EV or GFP-ENO-1.

Electron microscopy confirmed the presence of double membrane vesicles with a size range between 100 and 150 nm in the recovered high speed pellets (Fig. 4.13A, upper panel). ENO-1 detected by an anti-ENO-1 antibody coupled to gold particles was observed on the surface of exosomes (Fig. 4.13A, lower panel). Purified exosomes were further analyzed by Western blotting. Despite similar expression levels of GFP and GFP tagged ENO-1 in MDA-MB-231 total cell lysates, only GFP-ENO-1 was found in exosomes (Fig. 4.13B). Isolated exosomes were also positive for CD63 and Hsp70 (Fig. 4.13B). In order to assess whether LPS-triggered increase in the amount of extracellular ENO-1 is a result of augmented exosome secretion, exosomes were isolated from LPS-treated MDA-MB-231 cells and subjected to Western blotting with an anti-ENO-1 antibody. Stimulation of cells with LPS

resulted in increased levels of ENO-1, which was accompanied by a concomitant increase in the levels of Hsp70 and CD63 (Fig. 4.13C). As LPS did not potentiate cell proliferation (Fig. 4.6E), changes in the cell numbers could not account for the observed effect.

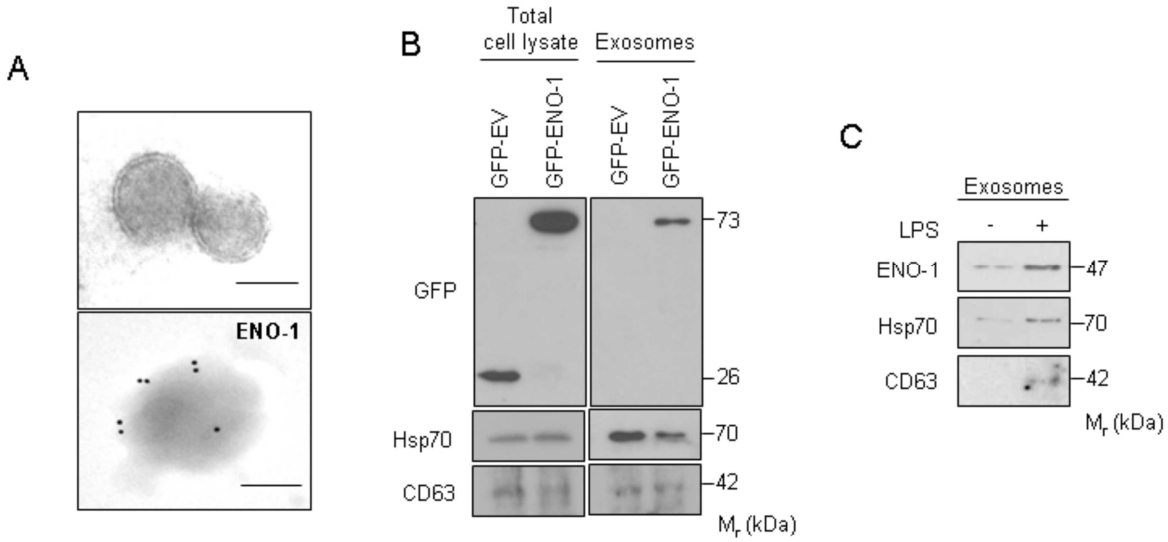

Figure 4.13. ENO-1 is released from MDA-MB-231 cells in the form of exosomes.
(A) Transmission electron micrographs of exosomes isolated from MBA-MB-231 cells. Exosomes negatively stained with 2 % uranyl acetate (upper panel). Exosomal ENO-1 visualized by a gold particle-coupled anti-ENO-1 antibody (lower panel). Bar size 100 nm. (B) Levels of ENO-1 in total protein extracts (left panel) and exosomes (right panel) isolated from GFP-EV and GFP-ENO-1 overexpressing MDA-MB-231 cells as assessed by Western blotting. Hsp70 and CD63 served as exosome markers. n=3. Representative Western blots are shown. (C) Abundance of ENO-1 in exosomes isolated from MDA-MB-231 cells exposed to LPS as assessed by Western blotting. Hsp70 and CD63 served as exosome markers. n=3. Representative Western blots are demonstrated.

To assess a functional role of exosomal ENO-1 in cancer cells, an exosome uptake assay was performed. Immunofluorescence analysis demonstrated uptake of GFP-ENO-1 loaded exosomes by parental MDA-MB-231 cells and their redistribution in the perinuclear region (Fig. 4.14A). To determine whether exosomal ENO-1 is able to influence migratory and invasive properties of recipient cells, wound healing and transwell invasion assays were performed in the presence of exosomes isolated from cells overexpressing either GFP alone or GFP tagged ENO-1. The rate of wound closure was markedly increased when GFP-ENO-1 loaded vesicles were applied (Fig. 4.14B, C). Similarly, invasion of MDA-MB-231 cells was enhanced when exosomes isolated from cells overexpressing GFP-ENO-1 were used (Fig. 4.14D, E). These results support the functional role of exosomal ENO-1 in the regulation of cancer cell motility.

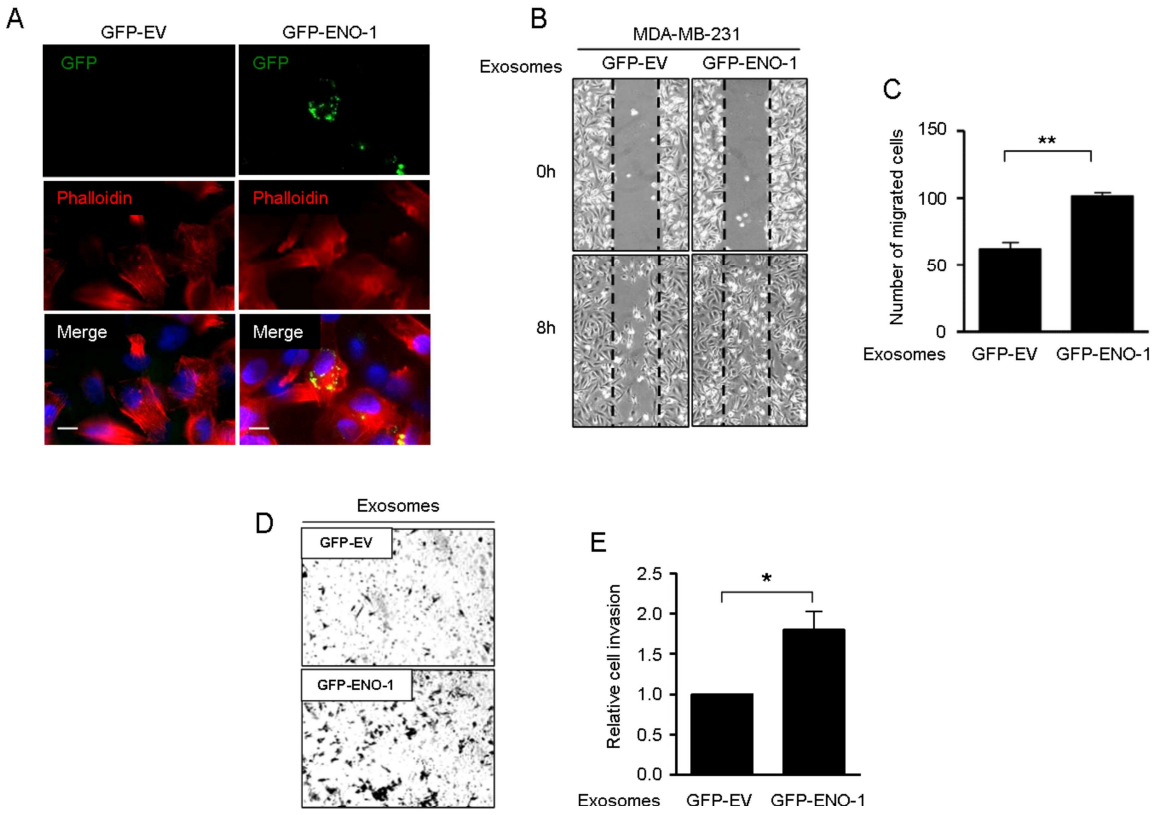

Figure 4.14. ENO-1 released in the form of exosomes enhances tumor cell migration and invasion.
(A) Representative pictures demonstrating uptake of exosomes isolated from GFP-EV and GFP-ENO-1 overexpressing cells by MDA-MB-231 cells. Bar size 5 µM. n=3. (B) Impact of exosomes isolated from GFP-EV or GFP-ENO-1 cells on MDA-MB-231 cell migration. Representative pictures from the wound-healing assay at time 0 h and 8 h are shown. (C) The rate of wound closure was assessed by counting the cells that migrated into the same-sized square fields. Data represent mean values ± S.E.M. n=3; **, $p<0.01$. (D) MDA-MB-231 cells stimulated with exosomes isolated from cells overexpressing either GFP-EV or GFP-ENO-1 were seeded onto a fibronectin-coated membrane and allowed to invade for 16 h. Representative images of cells that invaded the underside of the membrane are shown. (E) Cells that invaded the underside of the membrane were counted. Data represent mean values ± S.E.M. n=3; *, $p<0.05$.

In order to assess whether LPS potentiates release of ENO-1 into the extracellular space from other breast cancer cell lines, MCF-7 and MDA-MB-435 cells were analyzed for exosome production. ENO-1, Hsp70 and CD63 were not detected under basal conditions and upon LPS stimulation when MCF-7 cells were employed (Fig. 4.15). MDA-MB-435 displayed high extracellular levels of ENO-1, Hsp70, and CD63 already under basal condition. LPS stimulation did not further increase exosome production in these cells (Fig.

4.15). Observed differences in exosome secretion cannot arise from dissimilarities in cell growth as exosomes were always isolated from the same number of cells, irrespective of the conditions applied. Together, our results suggest that not only the level of cell surface bound ENO-1 but also the amount of ENO-1 released into the extracellular space in the form of exosomes correlate with the metastatic potential of cancer cells.

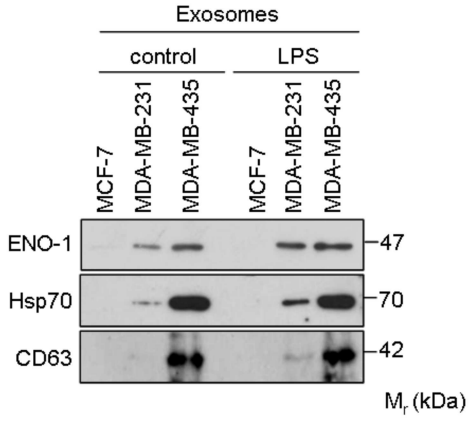

Figure 4.15. LPS-triggered release of exosomal ENO-1 from different breast cancer cell lines.
Levels of ENO-1 in exosomes isolated from MCF-7, MDA-MB-231 and MDA-MB-435 cells exposed to LPS as assessed by Western blotting. Hsp70 and CD63 served as exosome markers. n=3. Representative Western blots are demonstrated.

4.5. Translocation of ENO-1 to the cell surface of MDA-MB-231 cells occurs via a nonclassical secretory pathway

As ENO-1 lacks a N-terminal signal peptide motif, which is required for ER/Golgi targeting, a nonconventional protein secretion pathway has been suggested to explain transport of ENO-1 to the cell surface. To verify this notion, several biochemical approaches were applied. Preincubation of MDA-MB-231 cells with brefeldin A, a known blocker of ER/Golgi transport, did not inhibit translocation of ENO-1 to the cell surface upon exposure of cells to LPS, implying that a nonclassical secretion pathway is involved (Fig. 4.16A). Several mediators of nonconventional cell surface protein expression have been described, including ABC transporter, endosomal recycling, and Na^+/K^+ ATPase. However, glyburide, an ABC transporter inhibitor, methylamine, an endosomal recycling blocker, and ouabain, a Na^+/K^+ ATPase antagonist had no effect on LPS-driven transport of ENO-1 to the cell surface (Fig. 4.16B - D).

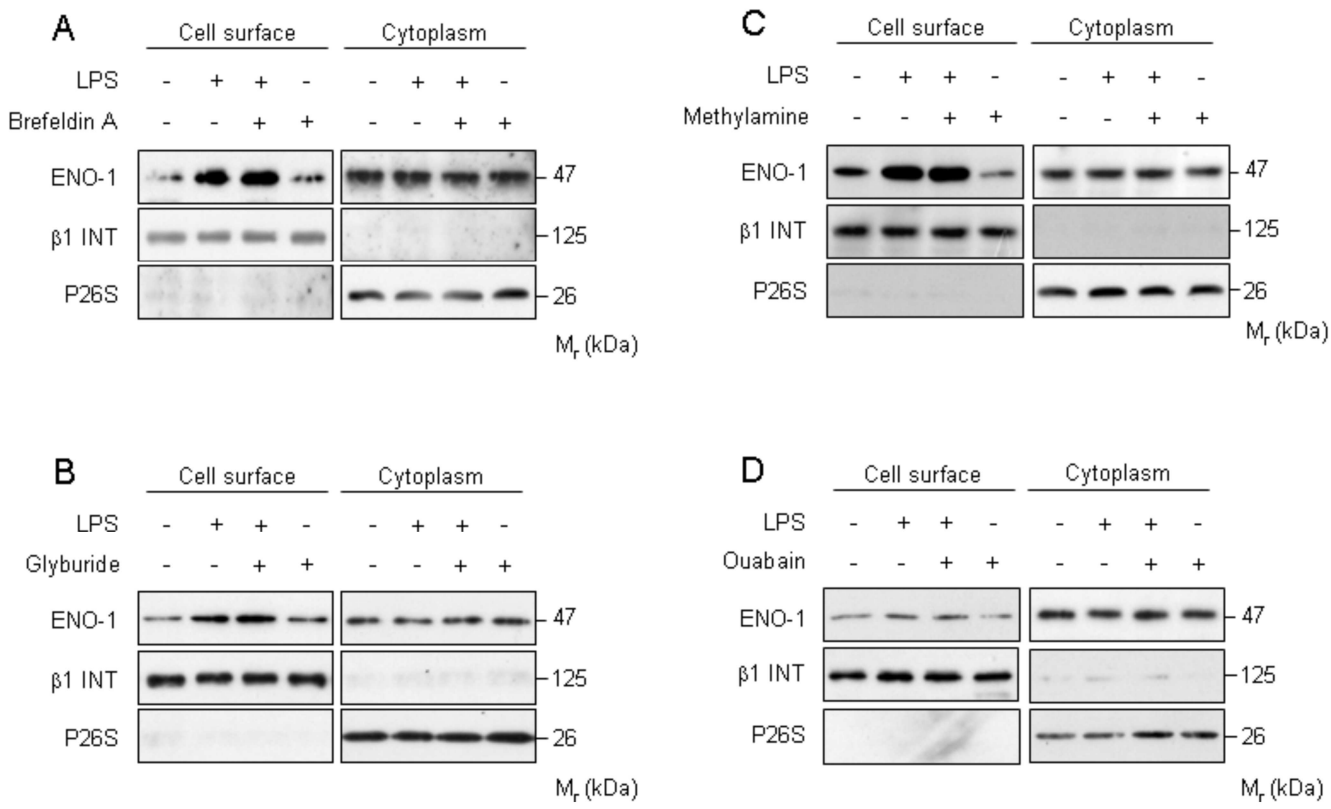

Figure 4.16. Translocation of ENO-1 to the cell surface of MDA-MB-231 cells occurs a via nonclassical secretory pathway.
(A-D) Cell surface expression of ENO-1 in MDA-MB-231 cells stimulated with LPS in the absence or presence of 10 μg/ml brefeldin A (A), 5 mM glyburide (B), 5 mM methylamine (C) or 100 nM ouabain (D). The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1-integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are demonstrated.

4.6. LPS-driven ENO-1 exteriorization is mediated by Ca^{2+}

As L-type Ca^{2+} channels (LTCC) have been implicated in exteriorization of another PLG receptor, histone 2B (H2B) [208], we next investigated the role of Ca^{2+} in the transport of ENO-1 to the cell surface. First, preincubation of MDA-MB-231 cells with the Ca^{2+} ionophore A23187 induced a time and concentration dependent translocation of ENO-1 to the cell surface (Fig 4.17A). To elucidate whether LPS and A23187 share a common pathway to regulate ENO-1 exteriorization, MDA-MB-231 cells were simultanously treated with these two reagents. Although A23187 and LPS increased transport of ENO-1 to the cell surface, this effect was not further increase when both stimuli were applied at the same time (Fig 4.17B), indicating that A23187 and LPS use a similar mechanism to regulate ENO-1 cell

surface abundance. To further explore the involvement of Ca^{2+} in LPS-triggered transport of ENO-1 to the cell surface, the levels of intracellular Ca^{2+} were depleted by pretreatment of MDA-MB-231 cells with the Ca^{2+} chelator BAPTA. As depicted in Figure 4.17C, BAPTA blocked LPS-driven ENO-1 translocation. Similar results were obtained when cyclopiazonic acid (CPA), an inhibitor of ER Ca^{2+}ATPase pump was used (Fig 4.17D).

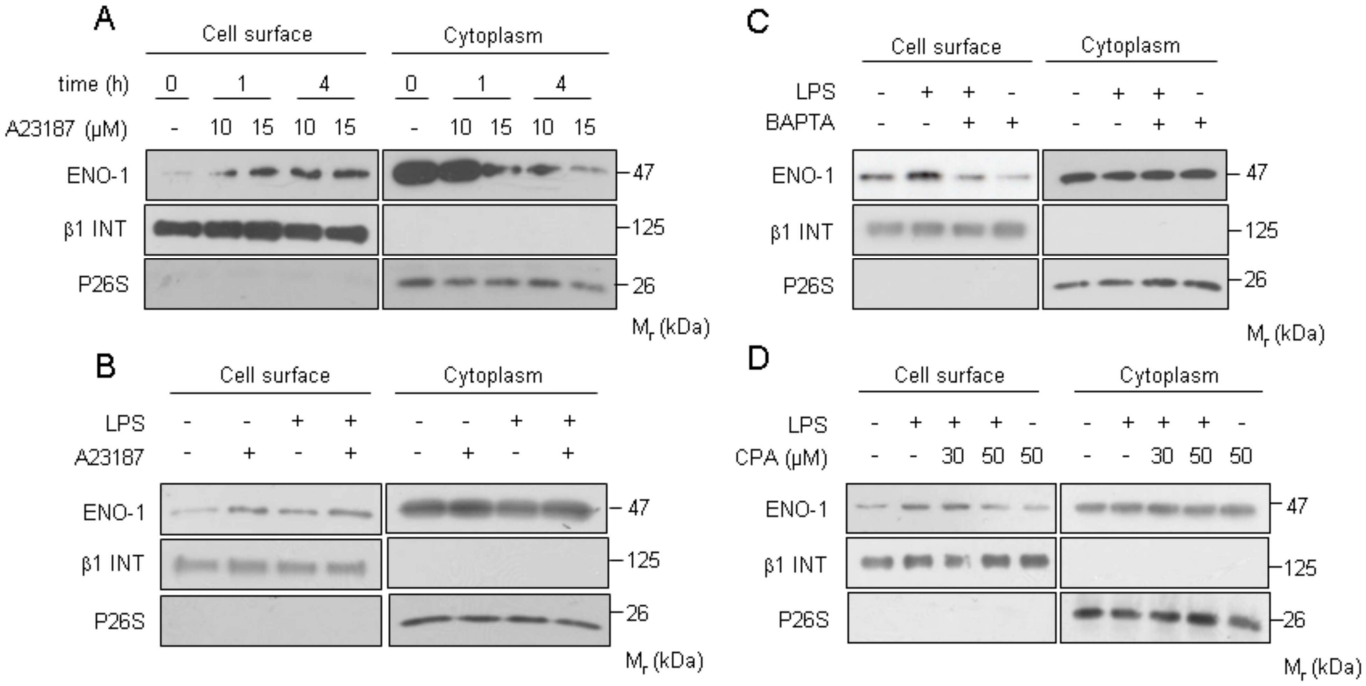

Figure 4.17. LPS-driven translocation of ENO-1 to the cell surface is dependent on intracellular Ca^{2+} levels.
(A-D) Cell surface expression of ENO-1 in MDA-MB-231 cells stimulated with LPS in the absence or presence of 10 μM A23187 (A, B), 20 μM BAPTA (C), or CPA (D). The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1-integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are demonstrated.

In order to investigate whether the release of ENO-1 into the extracellular space also depends on Ca^{2+} levels, MDA-MB-231 cells were first preincubated with the Ca^{2+} ionophore A23187. A23187 augmented exosomal levels of ENO-1, which positively correlated with elevated levels of CD63 and Hsp70 (Fig. 4.18A). Similar results were obtained when MDA-MB-231 cells were exposed to LPS. Moreover, LPS-driven release of exosomal ENO-1 was blocked by BAPTA. Notably, BAPTA alone completely abolished release of exosomes into the extracellular space (Fig. 4.18B).

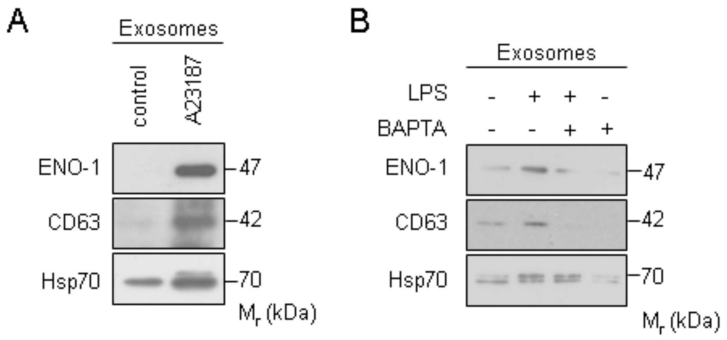

Figure 4.18. The release of exosomes is regulated by intracellular Ca^{2+} levels.
(A, B) Levels of ENO-1 in exosomes isolated from MDA-MB-231 cells stimulated with 1 μM A23187 (A) or with combination of LPS and 20 μM BAPTA (B) as assessed by Western blotting. Hsp70 and CD63 served as exosome markers. n=3. Representative Western blots are shown.

To demonstrate the direct effect of LPS on Ca^{2+} levels, cells were treated either with LPS alone or in combination with BAPTA or CPA and intracellular Ca^{2+} was measured by live cell imaging. There was no difference in the basal intracellular calcium level of the cells, regardless of the treatment or presence of extracellular Ca^{2+} (Fig. 4.19A, B). The LPS-induced intracellular Ca^{2+} increase was significantly diminished by removal of the extracellular Ca^{2+} or 10 minutes of BAPTA or CPA treatment (Fig. 4.19A, C). These data indicate that the LPS-induced ENO-1 exteriorization is Ca^{2+} -dependent.

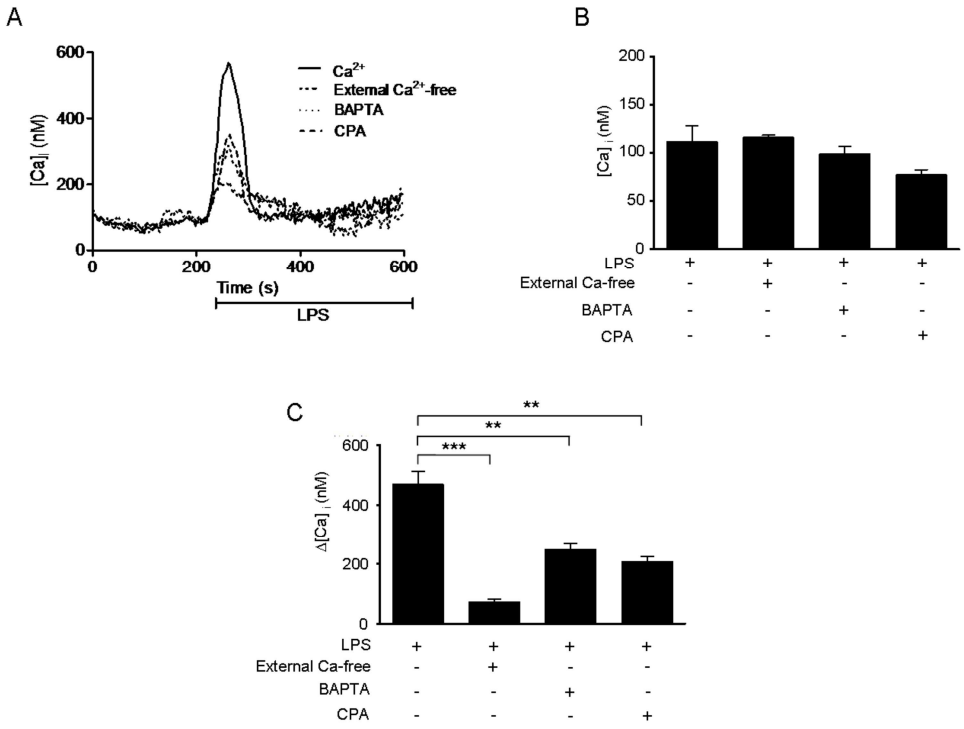

Figure 4.19. LPS-mediated increase in intracellular Ca^{2+} level.
(A) Representative traces of live cell calcium measurements upon LPS stimulus in the presence and absence of extracellular Ca^{2+}, 20 μM BAPTA and 50 μM CPA. (B, C) Quantitative data illustrating basal Ca^{2+} levels (B) and LPS-induced intracellular Ca^{2+} change (C) in MDA-MB-231 cells. Data represent mean values ± S.E.M. n=4; n cells=26-52 in each group; **, $p<0.01$; ***, $p<0.001$.

4.7. Blockage of STIM1/ORAI1 inhibits LPS-induced ENO-1 exteriorization

To further investigate the role of Ca^{2+} in the regulation of ENO-1 translocation, the expression STIM1 and its interacting partner - ORAI1, a member of store-operated calcium (SOC) channels, as well as the $Ca_v1.2$ subunit of LTCC was assessed. We focused on STIM1, ORAI1, and $Ca_v1.2$ LTCC since these Ca^{2+} channels have been found to control translocation of other PLG receptors to the cell surface and to regulate LPS-induced inflammatory responses [208-210]. Whereas STIM1 and ORAI1 mRNA was detected in MDA-MB-231 cells, $Ca_v1.2$ was not expressed in this cell line (Fig. 4.20A). In order to determine the potential role of STIM1 and ORAI1 in LPS-triggered transport of ENO-1 to the cell surface, a selective SOC channel inhibitor YM58483 was employed. Pretreatment of MDA-MB-231 cells with YM58483 suppressed, in a dose dependent manner, LPS-induced translocation of ENO-1 to the cell surface (Fig. 4.20B). Concomitantly, YM58483 inhibitor reduced release of exosomal ENO-1 into the extracellular space (Fig. 4.20C).

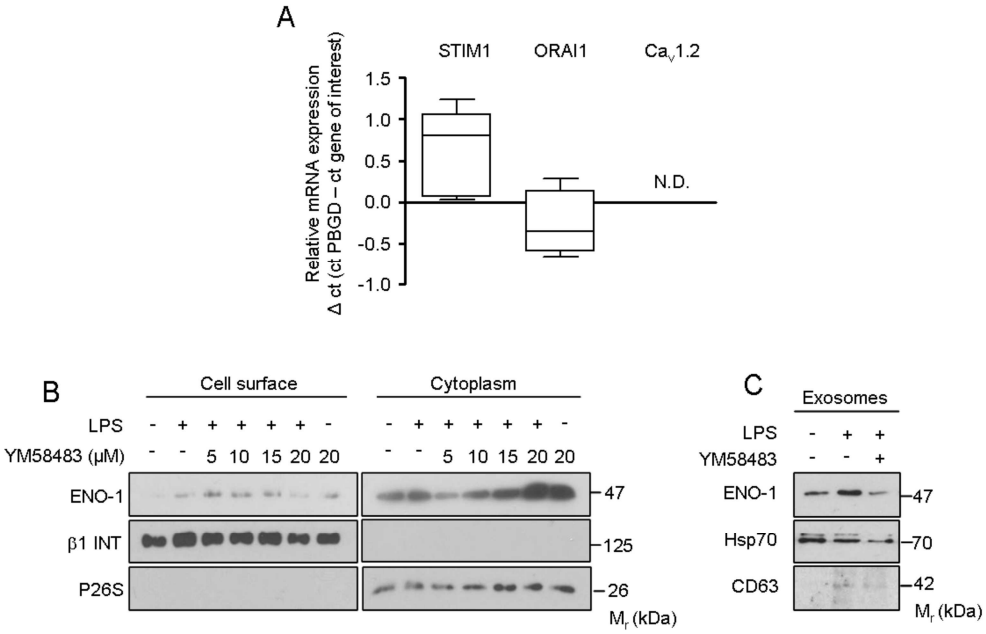

Figure 4.20. Blockage of STIM1/ORAI1 inhibits LPS-induced ENO-1 exteriorization. (A) qPCR analysis of STIM1, ORAI1 and Cav1.2 mRNA expression in MDA-MB-231 cells. Data are expressed as ΔCt using PBGD as a reference gene. n=3. N.D; not detectable. (B) Cell surface expression of ENO-1 in MDA-MB-231 cells exposed to LPS in the absence or presence of YM58483. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1 integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are shown. (C) Levels of ENO-1 in exosomes isolated from MDA-MB-231 cells exposed to LPS in the absence or presence of 5 μM YM58483 as assessed by Western blotting. Hsp70 and CD63 served as exosome markers. n=3. Representative Western blots are shown.

To exclude nontarget effects of the YM58483 inhibitor, siRNA directed against STIM1 and ORAI1 was applied. Depletion of STIM1 markedly decreased LPS-driven translocation of ENO-1 to the cell surface (Fig. 4.21A). Similar results were obtained when ORAI1 siRNA was employed (Fig. 4.21B). Efficiency of STIM1 and ORAI1 knockdown in MDA-MB-231 cells is demonstrated in figure 4.21C, D.

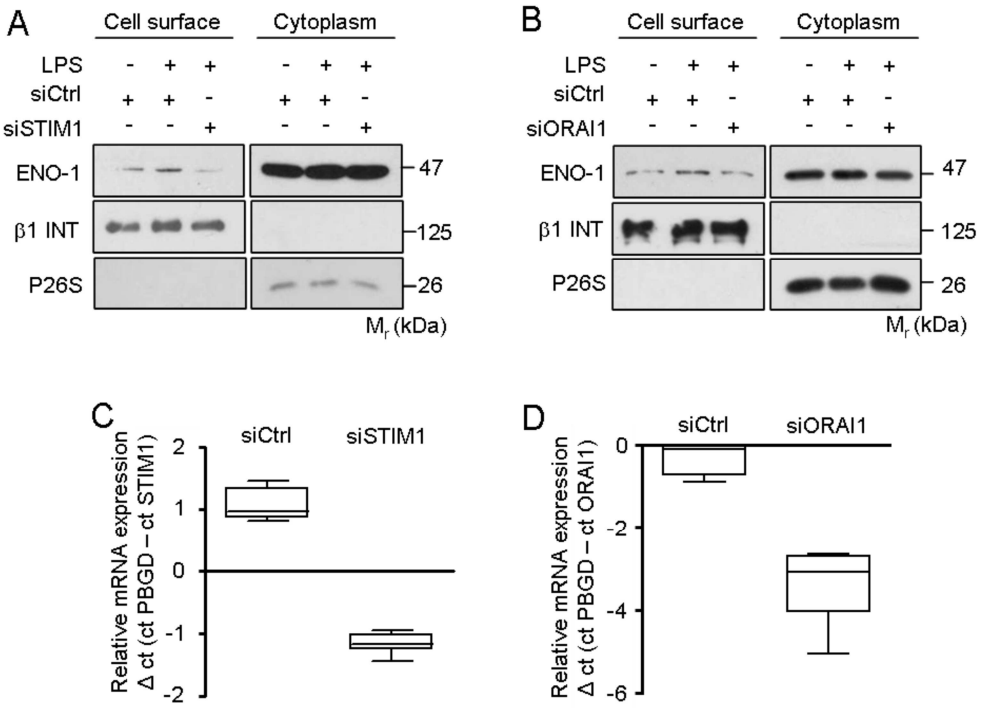

Figure 4.21. Knock down of STIM1/ORAI1 inhibits LPS-induced ENO-1 exteriorization. (A) Effect of STIM1 depletion on ENO-1 cell surface levels in MDA-MB-231 stimulated with LPS. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1 integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are demonstrated. siSTIM1, siRNA directed against STIM1; siCtrl, control siRNA. (B) Effect of ORAI1 depletion on ENO-1 cell surface levels in MDA-MB-231 stimulated with LPS. The purity of cytosolic and cell membrane fractions was assessed by probing the samples for β1 integrin (β1 INT) and P26S, respectively. n=3. Representative Western blots are demonstrated. siORAI1, siRNA directed against ORAI1. (C, D) Efficacy of STIM1 and ORAI1 knockdown in MDA-MB-231 cells as assessed by qPCR. Data are expressed as ΔCt using PBGD as a reference gene. n=3.

To prove the direct involvement of STIM1/ORAI1 in LPS-mediated Ca^{2+} influx in GFP-EV and GFP-ENO-1 cells, cells were transfected with STIM1 or ORAI1 siRNA and changes in intracellular Ca^{2+} were measured by live cell imaging. GFP-ENO-1 overexpressing cells were characterized by increased basal intracellular Ca^{2+} levels as compared to GFP-EV cells. Transfection of cells with STIM1 or ORAI1 siRNA slightly diminished basal intracellular Ca^{2+} levels in GFP-ENO-1 and GFP-EV cells (Fig. 4.22A). Upon LPS stimulation, GFP-ENO-1 cells displayed increased intracellular Ca^{2+} levels as compared to LPS-treated GFP-EV cells. Depletion of STIM1 or ORAI1 significantly reduced LPS-triggered Ca^{2+} influx as opposed to the cells treated with control siRNA in both cell types (Fig. 4.22B). These results

imply that ENO-1 overexpression in MDA-MB-231 cells triggers STIM1/ORA1-dependent Ca^{2+} entry following LPS stimulation.

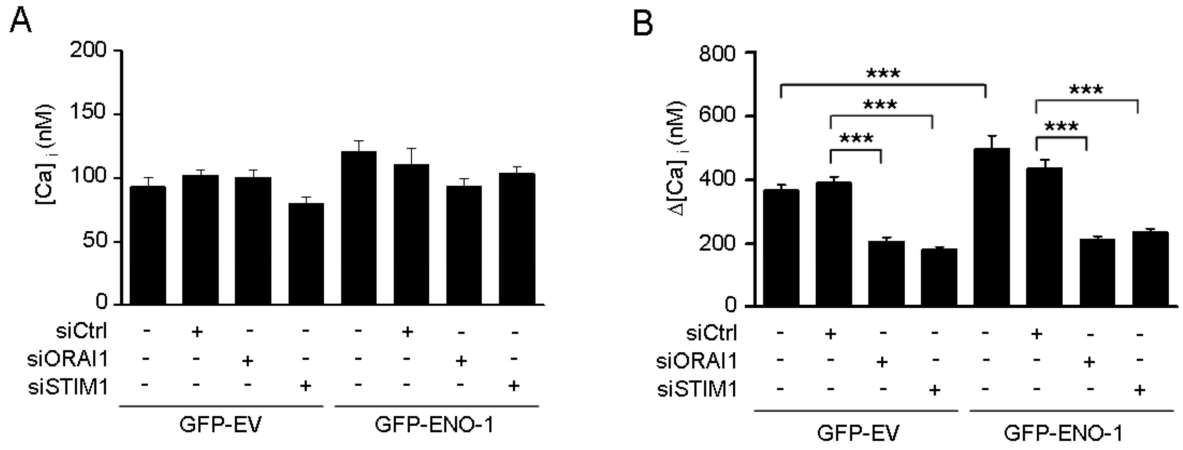

Figure 4.22. Depletion of STIM1/ORAI1 inhibits LPS-induced Ca^{2+} entry.
(A) Quantitative data illustrating basal Ca^{2+} levels in GFP-EV and GFP-ENO-1 cells treated with control siRNA or siRNA directed against ORAI1 or STIM1. Data represent mean values ± S.E.M. n=3; n cells=44-119 in each group. (B) Quantitative data illustrating LPS-induced changes in intracellular Ca^{2+} level in GFP-EV and GFP-ENO-1 cells upon STIM1 or ORAI1 depletion. Data represent mean values ± S.E.M. n=3; n cells=44-119 in each group; ***, $p<0.001$.

4.8. Blockage of STIM1/ORAI1 reduces ENO-1-dependent MDA-MB-231 cell motility

To elucidate the potential role of STIM1/ORAI1 in LPS-driven ENO-1 exteriorization and thus in the regulation of cell motility, a wound healing assay employing MDA-MB-231 cells overexpressing GFP alone or GFP-ENO-1 was performed. Overexpression of GFP-ENO-1 markedly increased the rate of wound closure as compared to the cells transfected with GFP-EV. YM58483 inhibited the effect of GFP-ENO-1 overexpression, decreasing cell migration to the level observed in YM58483-treated GFP-EV overexpressing MDA-MB-231 cells (Fig. 4.23A,B). To confirm these results, cells transfected either with GFP-EV or GFP-ENO-1 were treated with siRNA directed against STIM1 or control siRNA. A clear tendency towards reduced cell migration was observed in ENO-1 overexpressing cells treated with STIM1 siRNA as compared to the control siRNA transfected cells (Fig. 4.23C, D).

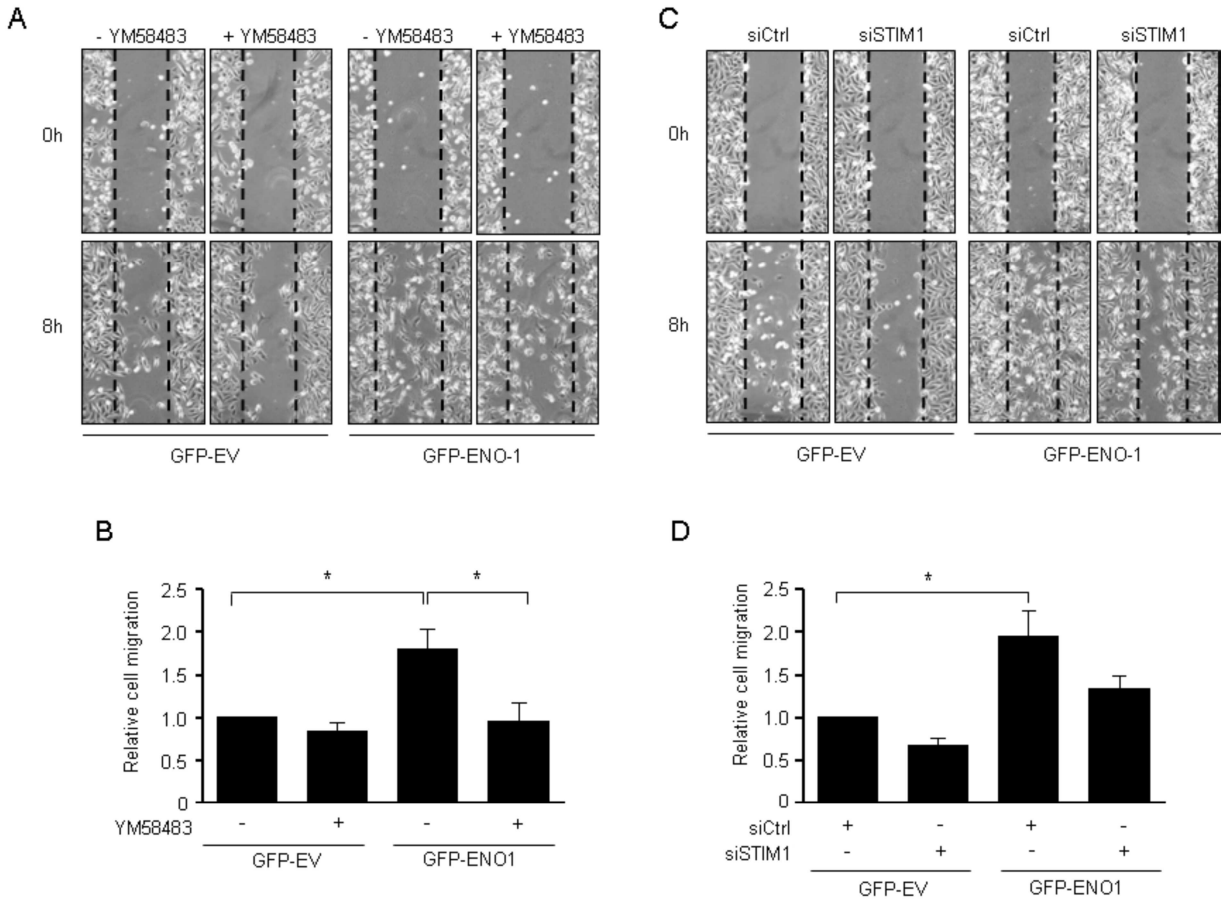

Figure 4.23. Suppression of STIM-1 reduces ENO-1-mediated migration of MDA-MB-231 cells. (A, B) Effect of 5 μM YM58483, a SOC channel inhibitor, on non-directional migration of MDA-MB-231 cells stably transfected with GFP-EV or GFP-ENO-1. Cells were scratched and incubated for 8 h at 37 °C in serum-free RPMI medium. Representative pictures from the wound-healing assay at time 0 h and 8 h are shown. The rate of wound closure was assessed by counting the cells that migrated into the same-sized square fields. Data represent mean values ± S.E.M. n=5; *, $p<0.05$ (C, D) Impact of STIM1 knockdown on nondirectional migration of MDA-MB-231 cells stably transfected with GFP-EV or GFP-ENO-1. Cells were scratched and incubated for 8 h at 37 °C in serum-free RPMI medium. Representative pictures from the wound-healing assay at time 0 h and 8 h are shown. The rate of wound closure was assessed by counting the cells that migrated into the same-sized square fields. Data represent mean values ± S.E.M. n=3; *, $p<0.05$.

Similar results were obtained when ORAI1 siRNA was employed (Fig. 4.24A, B). To further confirm that suppressed migration of MDA-MB-231 cells is a result of decreased ENO-1 cell surface expression, STIM1 depleted non-overexpressing MDA-MB-231 cells were treated with a peptide directed against C-terminal part of ENO-1 involved in the binding of PLG and a wound healing assay was performed. Treatment of cells with the ENO-1 peptide or STIM1 siRNA considerably reduced their migratory properties, however, this effect was

not potentiated when these two reagents were applied at the same time (Fig. 4.24C, D). These results support the crucial role of STIM1-mediated Ca^{2+} influx in ENO-1-dependent cell migration.

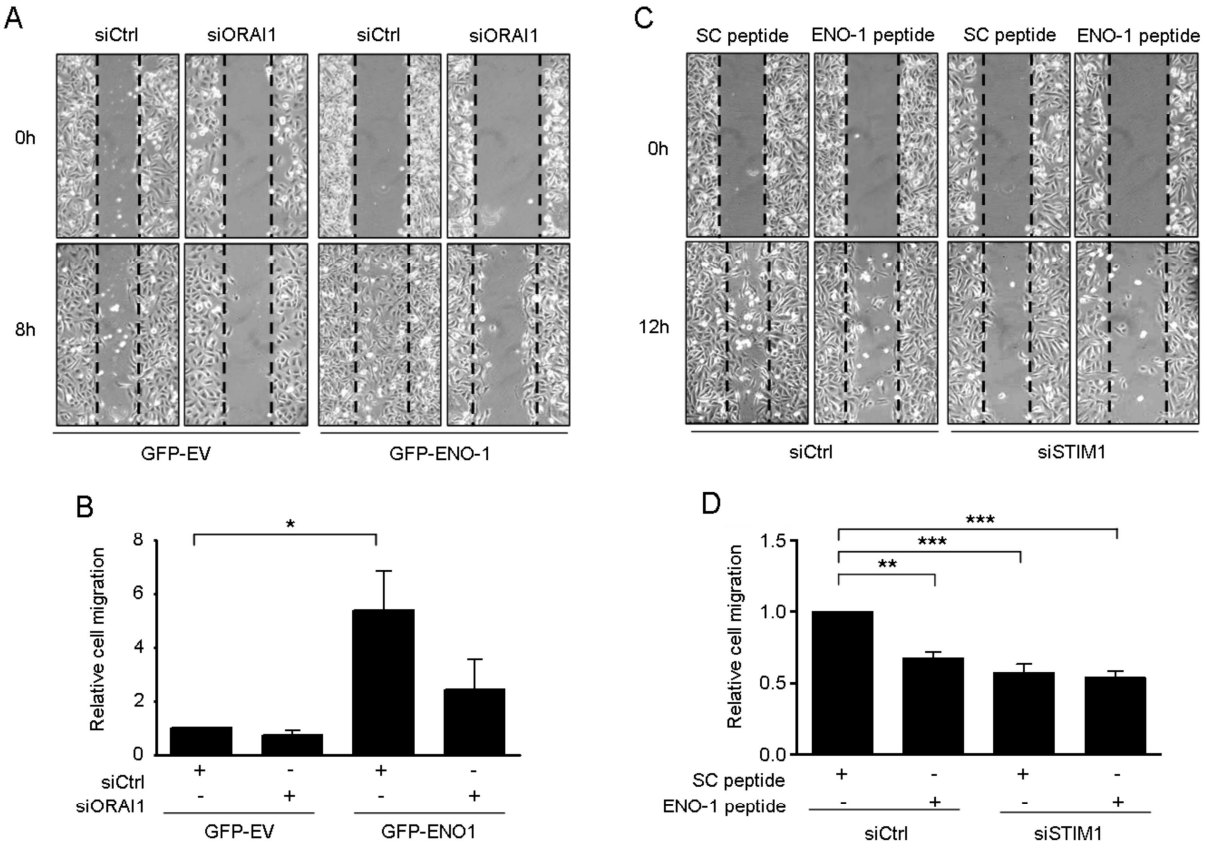

Figure 4.24. ENO-1-mediated migration of MDA-MB-231 cells depends on ORAI1 and STIM1 expression.
(A) Effect of ORAI1 knockdown on nondirectional migration of MDA-MB-231 cells stably transfected with GFP-EV or GFP-ENO-1. Cells were scratched and incubated for 8 h at 37 °C in serum-free RPMI medium. Representative pictures from the wound-healing assay at time 0 h and 8 h are shown. (B) The rate of wound closure was assessed by counting the cells that migrated into the same-sized square fields. Data represent mean values ± S.E.M. n=3; *, $p<0.05$. (C) Effect of STIM1 knockdown and/or ENO-1 peptide on nondirectional migration of MDA-MB-231 cells. Cells were scratched and incubated for 12 h at 37 °C in serum-free RPMI medium. Representative pictures from the wound-healing assay at time 0 h and 12 h are shown. SC; scramble. (D) The rate of wound closure was assessed by counting the cells that migrated into the same-sized square fields. Data represent mean values ± S.E.M. n=3; **, $p<0.01$; ***, $p<0.001$.

5. DISCUSSION

5.1. Cell surface expression of ENO-1 is elevated on breast cancer cells

Cancer cell invasion is a multistep process which requires proteolytic activity, in order to invade tissue and metastasize. Various proteases have been associated with the movement of cancer cells [5, 6], the best characterized is a serine protease PLA. PLA results from PLG activation. Accumulation of PLG on the cell surface and its subsequent conversion to PLA contributes to the modulation of pericellular proteolytic activity and thus to the regulation of migratory properties of numerous cell types including cancer cells [57, 83]. Binding of PLG to the cell surface is mediated by a number of molecules, including ENO-1, annexin A2, H2B, cytokeratin 8 or β-actin. ENO-1 is a glycolytic enzyme primary localized in the cytoplasm of prokaryotic and eukaryotic cells [78]. Besides its participation in glycolysis, ENO-1 may be translocated to the cell surface where it binds PLG and thus takes part in cell surface proteolysis. Dysregulated expression of ENO-1 has been reported in many cancer types and several mechanisms seem to account for the indicated changes. Firstly, *ENO-1* gene is located in the chromosomal region 1p36 [88], which is frequently rearranged in human cancers. Secondly, *ENO-1* gene expression may be induced by hypoxia through a hypoxia-inducible factor 1 binding element [89]. Thirdly, the expression of ENO-1 has been observed to be increased in cells overexpressing prooncogenic c-Myc [90]. In line with these observations, high levels of ENO-1 were found in many types of cancer, including head, neck, breast and lung cancer [77]. Increased levels of ENO-1 in cancer cells correlate with cancer progression and poor clinical outcome of the affected patients [98]. Ectopic overexpression of ENO-1 promotes cell proliferation, migration, invasion, and colony formation thereby contributing to metastasis formation [80, 92]. The ability of ENO-1 to regulate so many processes related to cancer cell biology results from its participation in glucose metabolism and therefore in energy production as well as its capability to modulate expression of genes involved in cell growth and inflammation [80]. Although increased abundance of ENO-1 was described on the cell surface of cancer cells, the contribution of cell surface bound ENO-1 to the increased migratory and invasive properties of tumor cells as well as the mechanism underlying ENO-1 exteriorization have not yet been reported.

Our results indicate, that ENO-1 is highly expressed in different human cancers, including breast ductal carcinoma, squamos cell lung carcinoma, bronchoalveolar carcinoma, lung and colon adenocarcinoma. Among tested human cancers the highest expression of ENO-1 was

observed in breast ductal carcinoma. Cell fractionation and immunofluorescence analysis revealed, that ENO-1 is not only present in the cytosolic but also in the membrane fractions. Although breast cancer cells were characterized by high cell surface levels of ENO-1, augmented ENO-1 cell surface abundance was not noted on the primary mammary epithelial cells, indicating that cell surface expression of ENO-1 is associated with the pathological condition. Cell surface bound ENO-1 was also detected on immune cells (neutrophils, monocytes, T cells and B cells), neurons and pathogenic bacteria [80]. Due to its ability to concentrate PLG on the cell surface, ENO-1 contributes to acquisition of the invasive phenotype and thus allows pathogens, immune cells and cancer cells to spread [80]. Consequently, higher cell surface expression of ENO-1 in patients with NSCLC was found to correlate with aggressive behaviour of cancer cells, shorter progression-free period and overal survival of the patients [84, 211].

The significance of increased cell surface ENO-1 expression in human cancer tissue was analyzed in breast cancer cells overexpressing ENO-1. Cells transfected with GFP tagged ENO-1 (GFP-ENO-1) diplayed increased migratory and invasive properties, suggesting the role of ENO-1 in the transformation of breast cancer cells into more aggressive phenotype. This notion is futher supported by the experiments, in which the mutated forms of ENO-1 were used. Mutation of ENO-1 in PLG binding site reduced motility of MDA-MB-231 cells. The importance of PLG/PLA system in cancer cell invasion has been well documented [212]. Mice lacking PLG were shown to develop smaller primary tumors with delayed metastasis formation [213]. The PLG/PLA system may contribute to tumor progression in a direct as well as an indirect way. The direct effect is associated with the ability of PLA to degrade ECM. The indirect contribution involves degradation of ECM proteins and subsequent activation of tumor promoting factors, including MMPs, TGF-β, bFGF and VEGF. Most of these molecules are stored in the ECM in the inactive form. PLA mediated proteolytic proccessing leads to their release and activation [19]. Thus interference with PLG binding to the cell surface could represent one of the approaches in anti-cancer therapies.

5.2. LPS increases cell surface expression of ENO-1

The role of inflammation in cancer development and progression has been discussed in several studies [115, 122, 123]. Infection, chronic irritation or factors released by the inflammatory cells may cause or accelarate cancer [115]. In particular, ROS and nitrogen species, cytokines such as TNF-α, IL-6 and -17, chemokines (CXCL family) or

infectious agents (*Helicobacter pylori*, human papilomavirus) have been described as pro-oncogenic factors. LPS, which can be released from bacteria may cause inflammatory responses within host tissue. Recent studies have documented association of LPS with a marked acceleration of the metastatic phenotype. Systemic administration of LPS into animals suffering from breast cancer increased adhesion and enhanced angiogenesis in a VEGF-dependent manner. In addition, tumor cells in LPS-treated animals displayed increased invasion [155, 170], implying that LPS contributes to accelerated spreading of cancer cells.

Our study demonstrates a link between LPS treatment and increased migration and invasion of breast cancer cells. LPS-triggered increased motility of cancer cells was blocked by a peptide directed against C-terminal part of ENO-1, involved in PLG binding, suggesting a crucial role of this glycolytic enzyme in LPS-driven cancer cell migration. Cell surface bound rather than cytosolic ENO-1 seemed to be more important in this procces, as PLG binding and its subsequent activation takes place on the cell surface. Indeed, cell fractionation of breast cancer cells after LPS treatment revealed increased levels of ENO-1 on the cell surface and reduced abundance of ENO-1 in the cytosolic compartment, suggesting that LPS potentiates cell membrane expression of ENO-1 through its translocation from the cytoplasm to the cell surface. This notion is supported by the results demonstrating no change in ENO-1 mRNA and total protein expression following exposure of cancer cells to LPS. Altogether, our data imply that LPS-triggered ENO-1 translocation is independent of its *de novo* protein synthesis.

Although high cell surface expression of ENO-1 has been detected on cancer cells, our results indicate that inflammatory stimuli may further increase cell surface levels of this glycolytic enzyme. LPS-induced inflammatory responses have been shown to regulate cancer development/progression by several ways, they may (i) potentiate expression of proteins involved in the breakdown of the ECM [214], ii) increase adhesive properties of cancer cells that are essential for the metastatic colonization of a normal tissue [160], iii) induce recruitment of inflammatory cells into the tumor microenvironment which in turn can contribute to ECM turnover [215], iv) regulate angiogenesis [168], and v) modulate, as indicated by our study, cell surface associated proteolysis by triggering PLG receptor exteriorization. This notion is further supported by a study, in which a murine model of LPS-induced lung injury was used [83]. In this study, LPS application promoted recruitment of monocytes overexpressing ENO-1 to the acutely inflammed lung but not the cells that were overexpressing truncated form of ENO-1 lacking PLG binding site. These findings together

with our results, implicate that (i) LPS-triggered translocation of ENO-1 to the cell surface is cell type independent and (ii) further support the link between cell surface bound ENO-1 and invasive potential of cells. In addition, our study revealed causal relationship between exposure of breast cancer cells to LPS and their metastatic potential.

LPS-mediated increase in cell surface levels of ENO-1 could be readily blocked by an anti-TLR-4 antibody, which blocks LPS binding to its receptor. Although our study did not analyze the effect of the TLR-4 blocking antibody on cancer cell migration, numerous studies pointed out an essential role of TLR-4 in cancer progression. Expression and activation of TLR-4 has been demonstrated in breast, colon, prostate, lung and melanoma cancer cells [216]. Activation of TLR-4 in mouse colon carcinoma cells allowed tumor cells to avoid the host immune system [216]. Furthermore, supernatants from LPS-stimulated cancer cells were shown to inhibit T-cell proliferation. Moreover, administration of siRNA directed against TLR-4 or a TLR-4 blocking peptide prolonged the survival of mice bearing transplantable prostate carcinoma cells [217].

Surprisingly, LPS did not only act on the cell surface levels of ENO-1, but also increased amount of exosomal ENO-1 in cell culture conditioned media. Although, the direct involvement of LPS in exosome production has not been documented yet, some of the studies reported a link between exosomes and inflammation [218, 219]. A very recent study characterized exosomes present in BALF obtained from the patients suffering from sarcoidosis [218]. Sarcoidosis is a systemic disease with unknow etiology characterized by inflammation and damage of the lung. Patients with sarcoidosis displayed significantly higher exosome production in the lung. In addition, exosomes from these patients exhibited different protein composition and appeared to have pro-inflammatory effects towards monocytes and bronchial epithelial cells.

In our study, we also explored the potential impact of TNF-α on cell surface bound and exosomal ENO-1. TNF-α is a pro-inflammatory cytokine, which was found to be important during the early events of tumorigenesis, due to its ability to regulate activity of MMPs and processes such as cell adhesion and angiogenesis [115]. Although, stimulation with TNF-α did not change the level of cell surface bound ENO-1, it increased the abudance of exosomal ENO-1. These findings are in line with another study, which demonstrated alterations in exosome content after stimulation of endothelial cells with TNF-α. In particular, exposure of endothelial cells to TNF-α changed protein and mRNA composition of exosomes [220]. The major changes observed in exosomes released from the stimulated cells, were associated with

increased abundance of ICAM-1 and tumor necrosis factor alpha-induced protein 3 (TNFAIP3). Besides proteins, gene expression analysis of the exosomes revealed increased amount of mRNA of 18 genes, mostly involved in stress response, such as superoxide dismutase, IL-8, VCAM-1 and NF-κB pathway members. Altered exosome composition was also demonstrated in recent study, describing elevated levels of cytokines and miRNAs in macrophages stimulated with LPS [219]. Although these two studies reported changes in exosome composition rather than exosome amount upon stimulatory conditions, our findings demonstrated for the first time that both, LPS and TNF-α, may increase exosome abundance and thus ENO-1 amount in conditioned media of cancer cells.

5.3. Exosomal bound ENO-1 enhances tumor cell migration and invasion

Exosomes are small extracellular vesicles (30-150 nm) formed by endosomal budding from most of the cell types including cancer cells [171]. They contain proteins and nucleic acids of the parental cells and are actively involved in the intercellular communication. Most frequently, exosomes contain proteins involved in the membrane transport and membrane fusion, proteins present in lipid microdomains such as integrins and tetraspanins, but also cytoskeletal proteins and proteins involved in metabolic and signalling pathways. Although exosomes contain broad spectrum of proteins, their content is dynamic and can vary with regard to enviromental conditions. The molecular mechanism of protein targetting to exosomes is unknown thus far.

Strikingly, our results imply that ENO-1 is packed into the exosomes and actively released into the extracellular space. A growing body of evidence suggests that exosomes may act as regulators of cell-to-cell communication. This concept is based on the finding demonstrating that exosomes released from a given cell type may interact with other cells, leading to their stimulation [173]. Several mechanisms have been proposed to explain effects of exosomes on target cells. Firstly, exosome surface proteins may interact with receptors on target cells and thus activate signalling pathways. Secondly, exosome membrane proteins can be cleaved by cellular proteases allowing soluable ligands to bind to the respective receptors. Thirdly, exosomes may be internalized by target cells, releasing their content directly to the cytoplasm [173]. Exosomes were found to regulate a variety of processes, including cell proliferation, differentiation, migration, and invasion [173]. Tumor cells were shown to release large amounts of exosomes [221]. Exosomes derived from tumor cells may transfer oncogenes and pro-angiogenic molecules to stromal cells, and thus promote tumor vascularization.

Additionally, they can contribute to stromal remodelling and tumor cell invasion by carrying active MMPs [173]. In view of these findings, it is tempting to speculate that exosomal ENO-1 could contribute to tumor progression either by concentrating proteolytic activity on the cancer cell surface or by enlarging the cytoplasmic pool of ENO-1 thereby regulating expression of genes involved in cell growth, migration, and inflammation. Our results demonstrate, that GFP-ENO-1 loaded exosomes may alter cancer cell behaviour by increasing cancer cell migration and invasion. Yet, it is unclear whether exosomal ENO-1 increases the migratory and invasive properties of target cells by being reattached to the cell membrane or by being endocytosed and consequently translocated to the cell surface. Although stimulation of MDA-MB-231 cells with GFP-ENO-1 loaded vesicles led to their uptake and subsequent perinuclear redistribution of GFP-ENO-1, the fate of uptaken GFP-ENO-1 in recipient cells is unknown and needs future investigation.

The fact that the mechanism of ENO-1 release into the extracellular space in response to LPS is similar to the mechanism responsible for translocation of this glycolytic enzyme to the cell surface implies that these two processes may occur simultaneously or successively. Namely, cell surface localization of ENO-1 may assure its association with the intralumenal vesicles of the multivesicular endosomes and its further release into the extracellular space in the form of exosomes. Rebinding of exteriorized ENO-1 to the cell surface closes the cycle of ENO-1 extracellular transport. Similar mechanism has been proposed to explain extracellular localization of annexin A2 [222]. Nonetheless, it still remains to be elucidated which part of ENO-1 stays on the cell surface and which one is packed into exosomes and where the decision about ENO-1 sorting is made.

5.4. Transport of ENO-1 to the cell surface and to the extracellular space is regulated by intracellular levels of Ca^{2+}

ENO-1 is a cytoplasmic protein with established function in a glucose metabolism. As ENO-1 lacks a N-terminal signal peptide motif, which is required for ER/Golgi targeting [223], a nonconventional protein secretion pathway has been suggested to explain transport of ENO-1 to the cell surface. Four potentional mechanisms describing translocation of cytosolic proteins into the extracellular space have been reported thus far [224]. Two of them involve intracellular vesicles such as secretory lysosomes and exosomes. Other two mechanisms are characterized either by direct translocation of cytoplasmic proteins across the plasma membrane with the help of ABC transporters or by membrane blebbing. Our data clearly

demonstrate, that the transport of ENO-1 into the extracellular space may occur in the form of exosomes. To further support this notion, we employed pharmacological blockers of known secretory pathways and analyzed cell-surface levels of ENO-1. However, blockers of ER/Golgi transport, ABC transport, endosomal recycling and Na^+/K^+ ATPase antagonist had no effect on LPS-driven transport of ENO-1 to the cell surface. These data suggest that translocation of ENO-1 to the cell surface occurs through a nonconventional secretion pathway.

Despite the evidence of the nonclassical transport of ENO-1 to the extracellular milieu, the precise mechanism, which underlies ENO-1 exteriorization remains unknown. As exosome release is tightly regulated at the level of intracellular Ca^{2+}, we verified the importance of Ca^{2+} for ENO-1 exteriorization. LPS-mediated increase of cell surface bound and exosomal ENO-1 was reduced upon pretreatment of the cells with Ca^{2+} blockers, implying an essential role of this ion in ENO-1 transport. Intracellular Ca^{2+} modulates various cellular functions including proliferation, differentiation and apoptosis. The level of intracellular Ca^{2+} is a result of Ca^{2+} entry from the extracellular space and intracellular stores, such as ER and mitochondria [225]. Some human diseases including Alzheimer´s disease, diabetes and cancer have been associated with dysregulated levels of intracellular Ca^{2+}. Although abnormal changes in intracellular levels of Ca^{2+} may not necessary trigger the malignant phenotype, Ca^{2+} may orchestrate processes leading to tumor progression, including proliferation, migration and invasion [225]. This notion is supported by the fact that agents targeting Ca^{2+} signaling pathways are currently tested in clinical trials. Here, the best known example is carboxyamidotriazole (CAI), an inhibitor of non-voltage-operated Ca^{2+} channels and Ca^{2+} channel-mediated signaling pathways. The efficacy of CAI was tested in patiens suffering from epithelial ovarian cancer and renal cancer [226-228]. Although this agent was shown to stabilize the progression of the disease, treatment of the patients with CAI was associated with severe side effects. To narrow the adverse effects of Ca^{2+} channels blockers, new therapeutic strategies for the patients suffering from prostate cancer use the prostate specific antigen (PSA) to target an inhibitor of SERCA to the cancer cells only [229]. Our hypothesis stating an important role of intracellular Ca^{2+} in ENO-1 exteriorization is supported by the following findings: (i) depletion of intracellular stores of Ca^{2+} reduced cell surface and exosomal levels of ENO-1 (ii) inhibition of SERCA decreases cell surface bound ENO-1 (iii) increase in intracellular Ca^{2+} concentration elevated cell surface and extracellular abundance of ENO-1. In addition, our study demonstrates, that breast cancer cells transfected with ENO-

1 display increased intracellular levels of Ca^{2+} and are characterized by enhanced migratory and invasive properties. Taking this into consideration, it is tempting to speculate, that one of the mechanisms explaining the positive effect of Ca^{2+} blockers on tumor regression may lie in their ability to interfere with ENO-1 exteriorization.

Our findings are in line with previously published studies, demonstrating the importance of Ca^{2+} in exteriorization of H2B, another PLG receptor [230, 231]. Moreover, the release of annexin A2 in the form of exosomes was also shown to be regulated at the level of intracellular Ca^{2+}. All these observations underlie the important role of Ca^{2+} in the mobilization of PLG-R to the cell surface and suggest, that PLG-R may simultaneously be exteriorized. Recently published results support this idea, by showing the interaction of ENO-1 with annexin A2 [92]. As both of these proteins were reported to be present in exosomes, it is tempting to speculate that exosomal pathway could represent one of the mechanism, which is reponsible for PLG-R exteriorization. How exactly PLG-R are anchored to the cell membrane is unclear. However, annexin A2 via its ability to bind phospholipids could serve as a docking site for other PLG-R, including ENO-1. The detailed mechanism of annexin A2 and ENO-1 transport across the membrane needs future investigation.

Although we show the critical role of Ca^{2+} in ENO-1 exteriorization, mediators and molecular pathways responsible for increased Ca^{2+} entry following LPS stimulation need to be explored. Our investigations, point out an essential role of STIM1 and ORAI1 in this process. Pharmacological inhibition of SOC mediated Ca^{2+} entry and depletion of STIM1 or ORAI1 disturbed ENO-1 transport to the cell surface and into the extracellular space following LPS stimulation. STIM1 molecules in the ER and ORAI1 proteins in the plasma membrane are two main components of SOC entry, which can couple into a pore forming complex to regulate levels of intracellular Ca^{2+}. Stimulation of STIM1 can activate SOC entry, leading to sustained extracellular calcium influx. It has been documented, that SOC mediated Ca^{2+} entry accounts for a number of LPS-triggered negative effects in host enviroment. In particular, LPS can induce Ca^{2+} entry into the endothelial cells via STIM1 and thus Ca^{2+} overload, which finally may lead to inflammation and cell injury [209]. In agreement with these findings, our study demonstrates a direct involvement of STIM1 and ORAI1 in LPS-triggered Ca^{2+} entry into breast cancer cells. Furthermore, we show increased basal intracellular Ca^{2+} levels in ENO-1 overexpresing cells. This suggests, that overexpression of ENO-1 in cancer cells may alter Ca^{2+} levels. Dysregulation of intracellular Ca^{2+} homeostasis is often observed in cancer cells in particular in those cells which have high metastatic potential [225]. Taking this into

account, changes in the level of intracellular Ca^{2+} observed between GFP-EV and GFP-ENO-1 cells may be explained by the different phenotype of these cells. ENO-1 overexpression in MDA-MB-231 cells potentiated their migratory and invasive properties and thus aggravated their malignant behaviour.

Ca^{2+} entry pathways are important for the modulation of cell migration and invasion. STIM1/ORAI1 mediated SOC represents the major Ca^{2+} influx mechanism in epithelium originating cancer cells. In view of these findings, we demonstrate, that depletion of both SOC components, STIM1 and ORAI1, markedly reduced ENO-1-mediated cancer cell migration. The relationship between STIM1 depletion and ENO-1-driven migration of breast cancer cells was demonstrated by simultaneous application of STIM1 siRNA and the ENO-1 peptide. This experimental proccedure did not further decreased migration of MDA-MB-231 cells, implying that the presence of STIM1 is essential for ENO-1-mediated motility of cancer cells. Several studies demonstrated a critical role of STIM1 in cancer cell migration and metastasis formation [232, 233]. For instance, pharmacological blockage or depletion of STIM1 was reported to reduce proliferation of cancer cells and to decrease tumor metastasis in animal models. Concomitantly, overexpression of STIM1 has been observed in various types of human cancer and it was found to have diagnostic as well as prognostic value [225]. Given the fact that STIM1 and ORAI1-dependent ENO-1 exteriorization markedly contributes to the increased breast cancer cell motility, pharmacological blockers of either STIM1 or ORAI1 could represent one of the possible approaches in anti-cancer therapies. Supporting this concept, suppression of STIM1 in the animal model of human glioblastoma significantly inhibited tumor growth and metastasis formation [232].

Collectively, present study provides new insights into the mechanism responsible for translocation of ENO-1 to the cell surface of cancer cells and its release into the extracellular space. The pivotal role of STIM1/ORAI1-mediated Ca^{2+} influx in aforementioned processes may, in part, explain the beneficial effect of STIM1 inhibition in the experimental models of cancer [232, 233].

6. CONCLUSIONS

Acquisition of a metastatic phenotype by cancer cells is a complex process, which requires deattachment of cancer cells from underlying basement membrane, migration and invasion into the surrounding tissue. All these steps are carried out with the help of proteases. PLA is a serine protease, which was found to be dysregulated during cancer progression. PLA activation occurs upon binding of its precursor PLG to the cell surface. Molecules, called "moonlighting proteins", which exhibit various functions at distinct cellular and extracellular compartments, have been proposed to bind PLG on the cell membrane. The glycolytic enzyme ENO-1 is one of them. Overexpression of ENO-1 was observed in more than 20 types of human cancer. In addition, high levels of ENO-1 in cancer cells were reported to correlate with cancer progression and poor clinical outcome of the affected patients. Although, numerous investigations supported the role of ENO-1 in cancer progression, the contribution of cell surface localized ENO-1 to tumorigenesis and the mechanism of its translocation to the cell surface has not yet been addressed.

In the present study, increased levels of ENO-1 were observed in ductal breast carcinoma and on the cell surface of highly metastatic breast cancer cell line MDA-MB-231. Elevated cell surface abundance of ENO-1 correlated with augmented MDA-MB-231 cell migratory and invasive properties. Exposure of MDA-MB-231 cells to LPS potentiated translocation of ENO-1 to the cell surface and its release into the extracellular space in the form of exosomes. LPS-triggered ENO-1 exteriorization was supressed by pretreatment of MDA-MB-231 cells with the Ca^{2+} blockers - BAPTA and cyclopiazonic acid. In line with these findings, STIM1 and ORAI1-mediated Ca^{2+} entry was found to regulate LPS-induced ENO-1 exteriorization. Pharmacological blockage or knockdown of STIM1 or ORAI1 reduced ENO-1-dependent migration of MDA-MB-231 cells. Altogether, our results demonstrate an essential role of STIM1/ORAI1-mediated Ca^{2+} influx in ENO-1 exteriorization and in ENO-1-driven cancer cell migration and invasion. Thus, our study proposes the mechanism explaining, in part, the beneficial effects of drugs interfering with Ca^{2+} influx in patients suffering from cancer.

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7. REFERENCES

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8. DECLARATION

I declare that I have completed this dissertation single-handedly without the unathorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Miroslava Didiasova

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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