Plasma membrane repair provides a new strategy for targeting metastatic cancer cells

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Stress induced plasma membrane injuries imposed by mechanical activity and the extracellular environment is a constant threat to cells that they need to cope with to stay alive. This is especially prominent for invasive cancer cells with their increased motility and capacity to navigate through the harsh tumor stroma, which further increases the risk of plasma membrane injury. The impact of these stresses on cancer cell membrane and mechanism by which tumor cells cope with this is poorly understood. Plasma membrane repair (PMR) is triggered by Ca\(^{2+}\) influx through the injury site. Depending on the cell type and extent of damage, cells use different components including yolk granules, lysosomes, mitochondria and cytoskeleton to help repair the damaged plasma membrane. Here, the involvement of annexins and S100 proteins in PMR will be discussed in light of our recent discovery showing that metastatic cancer cells require S100A11 protein and its binding partner annexin A2 for PMR. This Ca\(^{2+}\)-triggered protein complex facilitate resealing by modulating polymerization of actin cytoskeleton at the plasma membrane to enable wound closure and excision of damaged membrane. These findings demonstrate that cancer cells depend on efficient PMR, which reveal a new approach for targeting metastatic cancer cells.

**Keywords:** Plasma Membrane Repair; Cancer; Annexins; S100 proteins; S100A11; Annexin A2; Metastasis; Breast Cancer; Actin

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**Introduction**

The phospholipid bilayer that constitutes the plasma membrane surrounds and physically separates the interior structures of the cell from the extracellular environment. It is selectively permeable to ions and organic molecules and yet maintains an essential osmotic barrier to the outside. Hence, plasma membrane injury poses critically threat to both single cell- and multicellular organisms and survival requires that they rapidly repair or reseal lesions. Prevention of damage can be achieved by dynamic adaptations at a single cell or tissue level to limit the level of imposed stress, e.g., by actively modulating the epithelial layer to relieve mechanical forces. Still, many cells experience plasma membrane ruptures on a recurring basis that they need to cope with to maintain cell and tissue integrity. Disease results from defect in the repair system and is associated with cancer [1], diabetes [2], Chediak-Higashi [3] syndrome and muscular dystrophies [4].

Studies of liposomes *in vitro*, which can re-seal holes of various sizes spontaneously by re-organizing phospholipids according to their most favorable thermodynamic state led to the assumption that plasma membranes can re-seal without assistance [5]. This hypothesis was incompatible with studies in live cells where the sub-cortical cytoskeleton poses tension.
to the plasma membrane which prevents passive resealing. Accordingly, research during the last two decades have revealed that plasma membrane repair (PMR) is a complex and active process that require membrane replacements, fusion events and cytoskeletal reorganization [6]. Here I will briefly discuss the mechanisms involved in PMR, with a focus on the involvement of annexins, actin and S100 proteins. Additionally, the involvement of this process in cancer metastasis will be discussed and the potential for harnessing this new aspect of tumor metastasis for developing new therapeutic approaches to target metastatic cancers.

The membrane repair machinery

The PMR response is triggered by uncontrolled Ca\(^{2+}\) influx at the injury site, which triggers a versatile system that dependent on the extent and type of damage can employ different strategies including membrane replacement and membrane patching [7]. Fusion of intracellular vesicles around the wound perimeter to form a patch was first revealed in the repair of the sea urchin egg membrane following mechanical puncture. The patch model explains how homotypic vesicles are recruited to the wound upon Ca\(^{2+}\) entry to initiate homotypic and exocytic membrane fusion events with the plasma membrane to seal a rupture [8]. Lysosomes were originally identified as the patch source although different cell types may apply other available intracellular membrane resources to seal the wound including enlargosomes [9] and mitochondria [10].

PMR by membrane replacement occur in cells wounded by small pore forming proteins. Here, the injured membrane area is physically removed by either exocytic or/and endocytic membrane replacement. Injuries formed by the bacterial toxin streptolysin O (SLO) employ endocytosis to remove SLO-containing pores from the plasma membrane [11]. In contrast, lesions from pore-forming proteins of the membrane attack complex (MAC) produced by the immune system can be removed by shedding the injured membrane into microvesicles in, e.g. human neutrophils and platelets [12].

Cells can also sequester damaged membrane by sealing off the injured part into protrusions termed blebs. Here, blebs act as a snare that eventually is sealed off to isolate the damaged membrane from the cell body - a process that is mediated by the contraction of cortical actomyosin [13, 14].

The cortical cytoskeleton associated with the plasma membrane poses membrane tension, which prevents spontaneous resealing upon injury [15]. Thus, spatial and temporal remodeling of cortical cytoskeleton around the wound site is essential for efficient PMR. Ca\(^{2+}\) influx at the injury site triggers a decrease in membrane tension through depolymerization of cortical actin as observed in Xenopus oocytes and Drosophila Embryos [16]. This is followed by assembly of a contractile actomyosin “purse string” that assembles around wound borders and closes the wound in a purse-string manner. Xenopus oocytes wounds generated by needle puncture assumes a circular structure and constricts circumferentially, coincident with the recruitment of filamentous actin (F-actin) and myosin-II to the wound borders [17]. Disruption of the actin cytoskeleton by either Cytochalasin D or Latrunculin B prevents actomyosin ring assembly and impairs wound closure [17]. PMR in Drosophila embryos also involves formation of actomyosin complex and a plasma membrane plug that is rapidly recruited from the surrounding edges of the membrane. Further, intracellular vesicles are drafted to the wound perimeter to form a membrane patch within the actin string to seal the wound [6, 16].

Repair of membrane lesions also requires a coordinated interaction between annexin-containing multiprotein complexes and the inner phospholipid surface to seal the rupture [18]. The members of the annexin protein family function as intracellular Ca\(^{2+}\) sensors. They interact with multiple proteins including S100 proteins and distinct anionic phospholipids to promote membrane segregation, vesicle trafficking, vesicle fusion as well as membrane and cytoskeletal organization in a Ca\(^{2+}\)-dependent manner [19, 20].

Annexin and S100 proteins in membrane repair

Members of the annexin protein family have been identified in major eukaryotic phyla including plants and animals but are absent from yeast and prokaryotes [21].

Plant annexins originated approximately a billion years ago and have developed into a remarkable abundant and varied family [21]. Recent findings suggest that plant annexins are up-regulated in connection with abiotic stress responses to cope with drought and oxidative stress conditions [22, 23]. To this end, accumulating evidence suggest that annexins are instrumental in dealing with membrane stress.

There are 12 different annexin proteins in humans (ANXA1-ANXA11 and ANXA13) with orthologues in most vertebrates [19]. They contain an unique COOH-terminal core domain that consist of four preserved structural repeats, each composed of five α-helices of about 70-75 residues in length [24]. The repeats form a convex surface structure on which type-2 Ca\(^{2+}\) binding domains are located. Upon Ca\(^{2+}\) binding annexins bind the negatively charged phospholipids of the membrane to form a ternary complex bridging annexins and
membrane together via Ca\(^{2+}\)\(^{25}\). The NH2-terminal region is variable in length and sequence between family members and enables the protein to interact with distinct cytoplasmic partners such as S100 proteins \(^{26}\).

S100 proteins are small (10-14 kDa), EF-hand-type Ca\(^{2+}\)-binding proteins that upon Ca\(^{2+}\) activation exert both intracellular and extracellular functions. S100 genes are exclusively found in vertebrates and are clustered on chromosome 1q21 in humans (S100A1-S100A16) \(^{27}\). The majority of the protein family members form symmetric noncovalent homodimers, a feature that is exclusive to the S100 proteins within the EF-hand family of calcium binding proteins \(^{28}\). These proteins undergo a profound calcium-induced structural conformation exposing a hydrophobic domain that can interact with the NH2-terminal region of some annexins such as ANXA1 and ANXA2 \(^{26}\). This interaction facilitates close apposition of adjacent phospholipid membranes and promotes membrane fusion events \(^{19}\). Several pair of S100-annexin complexes have been identified including the complex of S100A10 and annexin A2 (S100A10-ANXA2) that bind to cytoskeletal components and has been associated with intracellular vesicle fusion \(^{29}\). S100A10 and ANXA2 are known to exist as a heterotetrameric complex where an S100A10 dimer resides in the center of the complex, interconnecting two annexin A2 molecules \(^{26}\). Similarly, annexin A1 and S100A11 (originally named S100C or calgizzarin) has also been found to interact in a temporal Ca\(^{2+}\)-dependent manner \(^{30}\). Several other pairs of annexin and S100 proteins have been discovered and it seems plausible that some S100 proteins are able to bind several annexins to exert its biological role.

The first direct demonstration and insight into the role of annexins in PMR response in human cells came from studying annexin A1 in HeLa cells. Disruption of the plasma membrane by laser injury triggered recruitment of ANXA1 to the wound perimeter to seal the injured site in a Ca\(^{2+}\)-dependent manner. The healing capability of ANXA1 could be directly inhibited by a specific antibody, an inhibitory peptide or by expressing a dominant-negative ANXA1 mutant deficient in Ca\(^{2+}\) binding \(^{31}\). Furthermore, ANXA1 and ANXA2 have been associated with the repair of sarcolemmal membrane in muscular dystrophy type-2 and Miyoshi myopathy caused by mutations in the dystrophin gene \(^{32}\).

To this end, the discovery and characterization of the first annexin originally named “synexin” (annexin A7) in 1978 by Creutz et al.,\(^{33}\) revealed its ability to aggregate vesicles. Thus, the protein family was named “annexin” according to their ability to “annex” cellular membranes together in a Ca\(^{2+}\)-dependent manner.

Annexin A5 promotes PMR by binding and assembles into two-dimensional arrays to the sites of membrane injury upon Ca\(^{2+}\) activation. ANXA5 was found to bind and form 2D arrays exclusively around the edges of the wound perimeter. Further, perivascular mouse cells deficient of ANXA5 exhibited a severe membrane repair defect that was rescued by the addition of exogenous recombinant ANXA5 protein \(^{34}\). The 2D ANXA5 array formed at the torn membrane is proposed to prevent wound expansion by restricting membrane tension and keeping the membrane edges together to promote resealing.

The shedding of microvesicles to remove lesions induced by pore forming streptolysin O was shown to involve annexin A6 (ANXA6) which is activated and recruited to the site of injury at lower Ca\(^{2+}\) concentration as compared to ANXA1. Upon binding around the SLO pore at the injured plasma membrane ANXA6 is sealed off with damaged portions, which are subsequently released in the form of ANXA6-containing microvesicles \(^{35}\).

**ANXA2 and S100A11 in the PMR response of cancer cells**

We have recently discovered that ANXA2 and S100A11 play a significant role in the PMR response in invasive breast cancer cells \(^{11}\). Both proteins are over expressed in various tumors and S100A11 is associated with tumor metastasis as well as poor prognosis in pancreatic, lung and colon cancers \(^{36-41}\). We hypothesized that cancer cells are exposed to increased plasma membrane stress due to enhanced membrane dynamics and oxidative stress. Furthermore, invasion through compact extracellular matrix may expose metastatic cells to stretch-induced injuries that they need to cope with to survive and spread. Malignant transformation also alter membrane stiffness by increasing the proportion of saturated phospholipids in the plasma membrane, which combined with enhanced membrane dynamics can lead to membrane lesions \(^{42}\). We studied mobile and invasive MCF7 breast cancer cells ectopically expressing a truncated ErbB2 oncogene (p95ErbB2) \(^{43}\), which mimics the constitutively active cleaved form of ErbB2 oncoprotein commonly found in aggressive breast cancers \(^{44}\). Here we found that these cells require enhanced PMR to cope with recurrent lesions and up-regulate ANXA2 and 100A11 proteins to improve their membrane repair capacity. As a result cells are highly efficient at maintaining plasma membrane integrity following various stresses and injuries and loss of S100A11-ANXA2 complex reduces viability and compromises their invasive ability.
Upon laser injury, ANXA1, a binding partner of S100A11, was recruited directly to the injured cell membrane in a Ca\textsuperscript{2+}-dependent manner, to initiate repair at the site of injury. However, the recruitment of ANXA1 was independent of S100A11 binding, as it persisted in cells lacking S100A11. Instead, ANXA2 colocalized with S100A11 at the injury site and their presence at the repair site was mutually dependent. This is in line with the reported fivefold tighter Ca\textsuperscript{2+}-dependent in vitro interaction of S100A11 with ANXA2 as compared with ANXA1. Our data demonstrate that the key function of S100A11-ANXA2 complex in PMR is to aid in remodeling of actin cytoskeleton at the site of injury to facilitate repair and excision of the damaged cell membrane. This function of S100A11-ANXA2 complex is in agreement with work showing individual interactions of S100A11 and ANXA2 proteins with the actin cytoskeleton. The important role of actin dynamics in this process is further supported by inhibition of PMR by drugs that block actin polymerization or depolymerization. We present evidence that this is due to the role of membrane-associated cortical actin cytoskeleton in maintaining cell membrane stability. While cortical F-actin is necessary to support the cell membrane, it also creates tension that would inhibit passive rescaling after injury. Thus, depolymerizing cortical actin after injury would not only keep the tension from further damaging the injured cell membrane, but will also facilitate membrane fusion to enhance the repair of the wounded membrane. In resting cell, ANXA1, ANXA2 and S100A11 are predominantly cytosolic and there is a layer of cortical actin under the cell membrane. Following cell membrane injury, the cortical F-actin consistently decreases around the injury site concomitant with the recruitment of ANXA1 at the injured plasma membrane. We propose that Ca\textsuperscript{2+}-triggered local loss of F-actin by actin severing proteins reduces membrane tension and brings together the wounded edges of the cell membrane. This is followed by Ca\textsuperscript{2+}-dependent accumulation of S100A11-ANXA2 complex near the injury site. As S100A11 and ANXA2 bind F-actin and decrease the depolymerization rate of preformed actin filaments, the S100A11-ANXA2 complex restricts F-actin depolymerization, preserving and allowing new buildup of F-actin around the injury site. ANXA2 is also capable of Ca\textsuperscript{2+} dependent binding to phospholipids, which enables aggregation of endosomes and other vesicles. At these membranes, the S100A11-ANXA2 complex helps nucleate polymerization of cortical F-actin by enabling buildup of cortical F-actin. The buildup of the cortical F-actin together with presence of vesicular endomembrane and the wounded edges of the plasma membrane, all participate in the fusion of the wounded cell membrane at the repair site marked by the S100A11-ANXA2 complex and the excision of the damaged part of the cell membrane by ANXA1. The buildup of cortical actin is analogous to F-actin drawstring formation during healing of injured Xenopus oocyte cell membrane. Thus, the F-actin buildup allows pulling the wounded membrane edges together during excision to facilitate repair. Recently, it was shown that Ca\textsuperscript{2+}-regulated cyclic assembly and disassembly of F-actin at the cell membrane regulates vesicle fusion. Thus, the vesicle aggregation and F-actin assembly mediated by the S100A11-ANXA2 complex may help with PMR by facilitating vesicle fusion and cortical actin buildup. These findings demonstrate that invasive cancer cells are dependent on efficient PMR system to cope with elevated rate of cell injury and that they rely on the S100A11-ANXA2 complex to enable plasma membrane repair.

**PMR as target for therapeutic intervention**

Targeting regulators of the PMR response may prove advantageous to control tumor metastasis for future cancer therapy. Our recent findings suggest that the S100A11-ANXA2 complex, injury-triggered actin remodeling and other steps in the PMR response are novel and viable approaches for targeting cancer cells. Given that annexins and S100 proteins are often differential regulated in neoplasia and overexpressed in various cancers make them attractive candidates to compromise PMR. Annexin A11, A8, A6, A4, A3, A2, A1 was shown up-regulated in different cancers types and four of these family members (annexin A1, A2, A5 and A6) has so far been reported involved in PMR as described above. Further, ANXA2 overexpression is directly correlated with aggressive clinical stage in colorectal, pancreatic and brain tumors and linked to metastatic progression. Changes in annexin A3 and A4 expression has also been associated with chemo resistance in ovarian cancer cells. The relative high sequence homology of the annexins suggests the likelihood of functional redundancy between the family members. In line with this, changes in the expression of one annexin can profoundly affect the expression levels of another suggesting a strict functional fail-safe mechanism. Thus, efficient pharmacologically strategies to target annexins may require that several family members are inhibited simultaneously, e.g. by targeting the conserved annexin core domain. Alternatively, annexin function can be compromised by restricting the interaction with its S100 protein binding partners, e.g. by blocking the interaction between ANXA2 and S100A11. Aside from annexins, S100 proteins offer another potential target. These proteins are implicated in multiple stages of cancer and are commonly up-regulated and associated with tumor progression in various cancers. However, with S100A11 as an exception...
[1] their direct regulatory role in PMR has not yet been characterized. Some S100 family members may work as Ca\(^{2+}\) triggered switches that upon injury can bind and regulate the function of several annexin family members at the plasma membrane to facilitate wound closure.

In summary, it is the hope that the findings reviewed here will attract more researchers to study plasma membrane repair in cancer, which should disseminate a strong translational interest for future cancer therapy.

**Conflicting interests**

The authors have declared that no competing interests exist.

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