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Lipid Rafts as Master Regulators of Breast Cancer Cell Function

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1. Introduction

Cancer is a leading cause of death in developed countries, and is on the rise in developing countries due in part to a lack of prophylactic screening and non-universal access to medical care (Jemal et al., 2011). Breast cancer is initiated when breast epithelial cells escape growth arrest and form a proliferating tumour mass. Numerous cellular mechanisms are dysregulated in breast tumour cells, including modified cell fate, altered protein signalling and trafficking, and enhanced cell migratory potential. Although these events are complex and subject to regulation by multiple elements, recent evidence has suggested that specialised cell membrane domains termed lipid rafts are actively involved in each of these processes (Cary & Cooper, 2000; Nabi & Le, 2003; Simons & Toomre, 2000). This chapter will therefore focus on the contribution of lipid rafts to breast cancer initiation and progression under these headings.

Lipid rafts are sub-domains of the cell membrane enriched in cholesterol and glycosphingolipids (Le Moyec et al., 1992; Nohara et al., 1998). These microdomains cluster together proteins involved in the regulation of crucial cellular processes; many of which are altered in cancer cells (Pike, 2003; de Laurentiis et al., 2007). Furthermore, lipid rafts are readily modified by diet and nutrition (Schley et al., 2007; Yaqoob, 2009), and studies have shown that fatty acid supplementation sensitises human mammary tumour cells to the cytotoxic effects of anti-cancer agents *in vitro* and *in vivo* (Germain et al., 1998; Menendez et al., 2005; Colas et al., 2006). This chapter will focus on the potential regulatory functions of lipid rafts as a novel approach towards understanding mechanisms of cancer initiation, progression and cell migration, a key event preceding metastatic progression. Finally it will discuss the potential of lipid rafts as novel therapeutic targets in breast cancer.

2. What are lipid rafts?

The discovery of glycosphingolipid clustering in the Golgi apparatus and at the apical surface of polarised epithelial cells led to the hypothesis of non-random membrane compartmentalisation (Simons & Ikonen, 1997; van Meer et al., 2008). These “compartments” were termed lipid rafts.

The structure and function of lipid raft domains depend on their lipid and protein compositions. An example of this is the sub-population of lipid rafts first identified in endothelial cells as flask-shaped membrane invaginations termed caveolae, or “little caves” (Yamada, 1955), which subsequent characterisation revealed to be enriched in proteins from the caveolin family (Rothberg, K G et al., 1992). Caveolins localise in the cytoplasmic leaflet of the cell membrane, and, together with high concentrations of cholesterol, are responsible for the characteristic curvature of caveolar membranes. Lipid rafts and caveolae have different structural protein markers and different proteins associated with them (Table 1), but their lipid composition and the mechanisms of protein targeting to them are very similar.

	Lipids	Protein Markers	Receptor Proteins	Signalling Proteins	References
Non-caveolar lipid rafts	Cholesterol	Flotillin-1, -2	Fas		
	Glycosphingolipid		EGFR	Ras	
	Sphingomyelin		HER2	Src	
	Ganglioside GM1		IGF-1R	Erk2	
	Ganglioside GM3		CD44	Shc	
			ER		(Nohara et al., 1998;
Caveolae	Cholesterol	Caveolin-1, -2 and -3	Fas		de Laurentiis et al., 2007; Patra, 2008)
	Glycosphingolipid		EGFR	Ras	
	Sphingomyelin		HER2	Src	
	Ganglioside GM1		IGF-1R	eNOS	
			CD44	PI3 kinase	
			ER	Phospho-lipase C	
			uPAR		
			MMP-1, -2, -9		

Table 1. Lipid and protein contents of caveolae and non-caveolar lipid rafts.

2.1 Lipid composition of membrane rafts

The functional properties of raft sub-populations differ according to subtle variations in the types of lipid and quantities of cholesterol they contain. This has permitted differential detergent extraction of various types of lipid rafts. The cholesterol concentration in detergent-resistant membranes (rafts) is 3-5 times higher than that in total membranes (Brown & Rose, 1992; Pike & Casey, 2002), sphingomyelin represents 10-15% of total lipid content, while glycosphingolipids such as cerebroside and gangliosides account for a further 10-20% (Brown & Rose, 1992; Prinetti et al., 2000). In contrast, glycerophospholipids (including membrane phospholipids) comprise less than 30% of raft lipids despite accounting for approximately 60% of total membrane lipids (Brown & Rose, 1992; Pike & Casey, 2002). Raft-enriched lipids localise preferentially on the outer leaflet of the cell membrane, unlike glycerophospholipids (Pike, 2003). These observations suggest that lipid rafts are bilayer structures and that a variable composition of the cell membrane leaflets may play a role in recruitment of various proteins into lipid rafts. Accordingly, proteins can be targeted to rafts in many dynamically-regulated ways, including attachment of glycosylphosphatidylinositol (GPI) anchors or via lipid modifications such as prenylation and palmitoylation.

2.2 Protein targeting to lipid rafts

Perhaps the best-characterised system of protein association with lipid rafts is via modification with GPI anchors. The GPI anchor is a conserved oligosaccharide core covalently linked to a lipid moiety embedded in the outer leaflet of the cell membrane through acyl or alkyl chains (Levental et al., 2010). These anchors are added to soluble polypeptides in the lumen of the endoplasmic reticulum (ER), promoting membrane raft affiliation (Brown & Rose, 1992). Partitioning of GPI-anchored proteins into lipid rafts may allow sorting to the apical surface of polarised epithelial cells (Fiedler et al., 1993).

Another way in which proteins are targeted to lipid rafts is through addition of prenyl groups. Two types of prenyl groups, C₁₅ farnesyl and C₂₀ geranylgeranyl, are added on to C-terminal cysteine-rich domains of cytoplasmic proteins by prenyl transferases (Casey & Seabra, 1996). Although prenylated proteins are reportedly enriched in lipid rafts (Prior & Hancock, 2001; Parmryd et al., 2003), it is thought that the prenyl groups interact with raft-affiliated proteins rather than being directly incorporated into rafts (Magee & Seabra, 2003).

Another lipid post-translational modification, palmitoylation, is dynamically regulated by enzymes (Kang et al., 2008; Wan et al., 2007), and controls raft targeting of certain proteins in physiological and pathophysiological settings. Palmitoylation involves the addition of palmitic acid moieties to integral and peripheral membrane proteins through esterification (Bhatnagar & Gordon, 1997). Membrane-associated palmitoyl acyltransferases (PATs) are the most studied palmitoylation enzymes (Planey & Zacharias, 2009), while the palmitoyl thioesterase family removes palmitate groups (Camp & Hofmann, 1995). Close proximity of protein cysteine residues to the membrane is thought to facilitate palmitoylation by PATs, whereas membrane-distal residues are more likely to be prenylated or N-myristoylated (Bijlmakers & Marsh, 2003). PAT activity has been linked to lipid rafts (Dunphy et al., 2001), with palmitoylated proteins found either at the cell membrane or on intracellular membranes (Lobo et al., 2002; Roth et al., 2002). Palmitoylated proteins are naturally more lipophilic, thus their affinity for lipid rafts is increased. Many proteins are targeted to lipid rafts through palmitoylation, including flotillins, Src family kinases, endothelial nitric oxide synthase and various transmembrane receptors (Ghosh et al., 1998; Gong et al., 2003).

2.2.1 Flotillin-1 and -2 mark non-caveolar lipid rafts

Non-caveolar lipid rafts are associated with expression of the reggie family of proteins, flotillin-1 and flotillin-2 (Figure 1a). Palmitoylation of flotillin-1 is essential for localisation to lipid rafts (Morrow et al., 2002), while two hydrophobic stretches also contribute to its raft affiliation (Liu et al., 2005). On the other hand, flotillin-2 is raft-targeted via both myristoylation and palmitoylation. Flotillins were originally thought to be localised in caveolae (Bickel et al., 1997), however later studies excluded this (Neumann-Giesen et al., 2004; Stuermer et al., 2004). Much remains to be determined about the functionality of flotillins in lipid rafts, in particular, the significance of their highly conserved N-terminal domain (Babuke & Tikkanen, 2007; Tavernarakis et al., 1999).

2.2.2 Caveolins – selective markers for caveolar lipid rafts

Along with glycosphingolipids (Tran et al., 1987) and increased cholesterol (Rothberg, K. G. et al., 1990), caveolae are enriched in the family of 21-24 kDa integral membrane proteins known as caveolins. There are three known caveolins: caveolin-1 (Rothberg, J. M. & Artavanis-Tsakonas, 1992), caveolin-2 (Okamoto et al., 1998) and caveolin-3 (Tang et al., 1996).

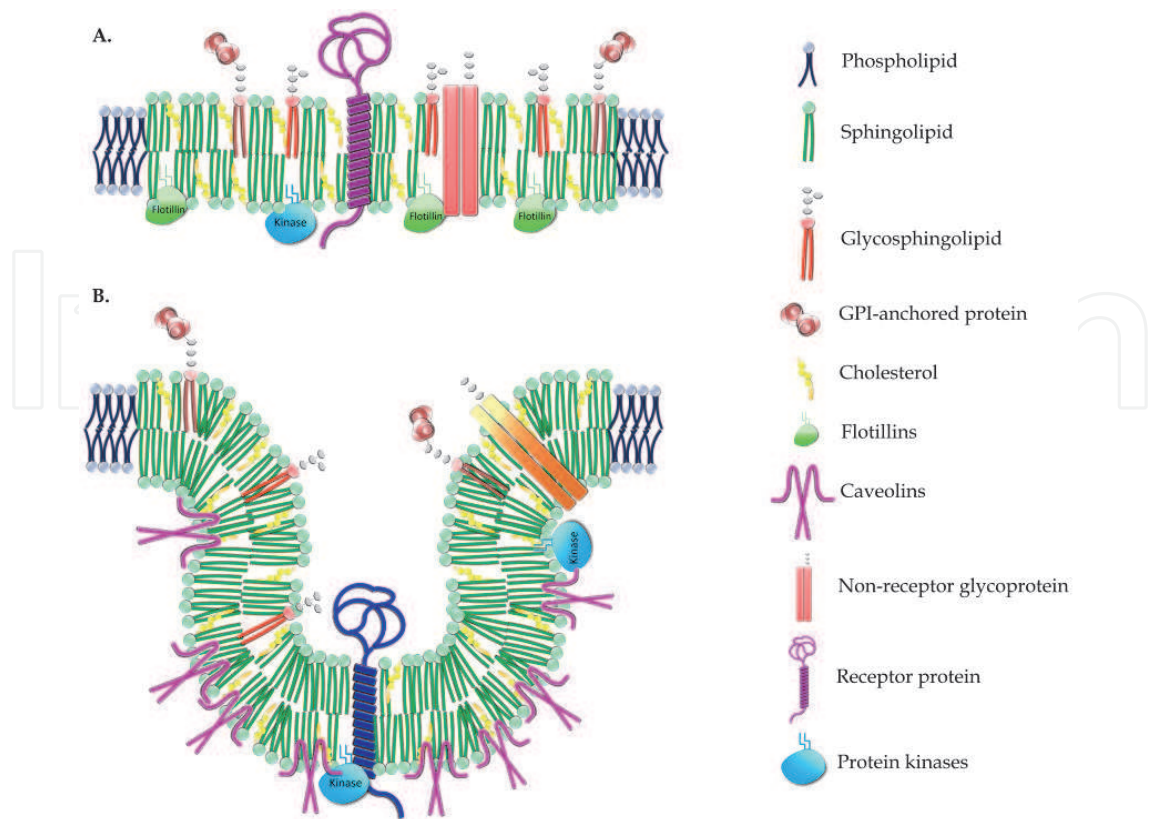


Fig. 1. Lipid and protein components of (A.) non-caveolar lipid rafts and (B.) caveolae.

Caveolins form “omega” structures in the membrane via cytoplasmic localization of both their N- and C-termini (Figure 1b). Aside from palmitoylation, the ability of caveolins to bind sphingolipids (Fra et al., 1995) and cholesterol (Murata et al., 1995) can also explain their high affinity for caveolar lipid rafts. Caveolins -1 and -2 are abundantly expressed in most cell types, including adipocytes, fibroblasts, endothelial and epithelial cells (Fan et al., 1983; Galbiati et al., 2001). Caveolin-3 expression however is restricted to muscle (Rubin et al., 2007). The N-terminal region of caveolin-1 contains a scaffolding domain which aids interactions with various signalling molecules, illustrating the potential importance of caveolin-1 for the signalling functions of caveolar lipid rafts (Everson & Smart, 2006).

2.3 Physiological functions of lipid rafts

Lipid rafts and caveolae are often viewed as organisation centres or signalling platforms, and Table 1 outlines examples of raft-associated proteins which will be discussed in the text. In this chapter, we will concentrate on dynamic raft regulation of a variety of physiological processes, including membrane trafficking, cell polarisation and signal transduction.

2.3.1 Membrane trafficking and cell polarity

Membrane trafficking allows exchange of cellular components between cell sites and cellular organelles. In polarised epithelial cells the trafficking machinery is highly polarised, targeting plasma membrane proteins to separate apical and basolateral domains (Mellman & Nelson, 2008). Following lipid and protein synthesis in the ER, vesicular transfer mediates transport to subsequent or final destinations (Rodriguez-Boulau et al., 2005). The formation and distribution of vesicles requires organised stabilisation of the membrane to allow

deformation and fusion with the target compartment. The most studied mechanism involves coating of the vesicle with clathrin oligomers (Gorelick & Shugrue, 2001), which is thought to be vital in basolateral protein sorting (Deborde et al., 2008; Folsch et al., 2009). Apical trafficking is less understood, but lipid rafts have been proposed to play a decisive role (Schuck & Simons, 2004). Conformational changes in the membrane are proposed to occur via oligomerisation and fusion of many small lipid raft domains (Lipowsky, 1993). Attachment of GPI anchors may also contribute to the polarisation of membrane trafficking (Paladino et al., 2008).

2.3.2 Cell signalling

Lipid raft-mediated trafficking of lipids and proteins facilitates dynamic regulation of cellular signalling cascades. Several frameworks have been suggested to link rafts to signal transduction. The simplest interpretation views lipid rafts as platforms where signalling molecules are co-localised, aiding their structural interactions and influencing downstream signalling (Lingwood et al., 2009). The nature of a signal may be modified by the type of lipid raft the target molecule is localised in and also the primary location of the raft, which in turn enhances the specificity of the signal. Rafts can also control cellular signalling by altering the function of their affiliated proteins. Accumulating evidence suggests that raft-associated proteins behave differently whether localized inside or outside of rafts. Modified signal transduction following lipid raft/caveolar disruption has been reported in the case of several signalling cascades involving Erk (Furuchi & Anderson, 1998), EGFR (Ringerike et al., 2002; Schley et al., 2007), insulin receptor (Parpal et al., 2001), and PDGF receptor (McGuire et al., 1993).

Finally, some lipid rafts are actively involved in endocytosis (reviewed in Lajoie & Nabi, 2010), which promotes internalisation of receptors and signalling molecules. This process may be facilitated by clustering of caveolin or receptor proteins (Paladino et al., 2004). Internalisation of ligands or receptors modifies downstream signal transduction, and is associated with the termination of extracellular ligand-driven signalling events via transient receptor desensitisation.

The ability of lipid rafts to traffic proteins, control cell polarity and alter cell signalling underlies their emerging roles as crucial regulators of cellular processes including cell fate, growth, adhesion and migration. Since all are dysregulated in cancer, it is reasonable to suggest that lipid rafts may modify tumorigenic processes. This will next be addressed.

3. Lipid raft regulation of key processes in breast cancer cells

Alterations in cell fate, growth, adhesion and migration play central roles in the initiation and progression of breast cancer. We next outline how lipid rafts may regulate such processes during the initial stages of cancer development, during tumour growth and during the possible progression to a migratory and metastatic phenotype.

3.1 Apoptosis and regulation of cell fate

Defects in apoptosis allow tumour cells to escape growth-inhibitory signals and to progress through the cell cycle. Two major apoptotic pathways have been described, extrinsic (mediated by activation of death receptors) and intrinsic (mediated by mitochondria). Both may require lipid rafts for successful signal transduction (Li et al., 1998).

Death receptors are located at the cell membrane, and once activated, trigger apoptotic signal transduction. Perhaps the best-characterised death receptor is Fas (CD95 or APO-1), which has been implicated in the apoptotic events that drive physiological remodelling of the mammary gland after breast feeding (Song et al., 2000). Down-regulation of Fas has also been associated with poor prognosis in breast cancer patients (Reimer et al., 2000), and inhibition of Fas activity has been linked to drug resistance (Landowski et al., 1997). Activation of Fas results in receptor aggregation, and recruitment of procaspase-8 to form the death-inducing signalling complex DISC (Peter & Krammer, 2003) (Figure 3a). Recent studies have shown that Fas is translocated into lipid rafts, where apoptotic receptor aggregation takes place (Gajate et al., 2004; Gajate & Mollinedo, 2005). This is the mode of action of a pro-apoptotic drug, edelfosine; with cholesterol depletion being shown to abolish apoptosis (Gajate & Mollinedo, 2001; Gajate & Mollinedo, 2007; Gajate et al., 2009).

Whether rafts could be used as targets to re-trigger Fas-dependent apoptosis in tumour cells is an intriguing concept. A recent study demonstrated that nitric oxide (NO) can reverse apoptotic resistance via increasing Fas S-nitrosylation (Leon et al., 2011). In breast cancer cells that overexpressed wild-type Fas, NO incubation resulted in enhanced recruitment of the receptor into lipid rafts, which in turn sensitized cancer cells to the death-inducing Fas ligand. In fact, DISC formation is impaired in cells expressing nitrosylation (Leon et al., 2011) and palmitoylation (Chakrabandhu et al., 2007) mutants of Fas.

Acquired resistance of breast cancer cells to Fas-induced apoptosis may alternatively result from activation of survival pathways, such as the PI3 kinase pathway. Its engagement leads to activation of the serine-threonine kinase Akt, which negatively regulates apoptosis by inactivating pro-apoptotic proteins such as Bad and caspase-9 (Datta et al., 1999). Lipid raft localisation of Akt has been implicated in facilitating its activation (Hill et al., 2002; Elhyany et al., 2004). Raft disruption has been reported to reduce the sensitivity of normal-like MCF-10a cells to apoptosis, suggesting that tumour cells rely on cholesterol for growth and malignant signalling (Li et al., 2006). Interestingly the cholesterol analogue ginsenoside Rh2 has been shown to reduce lipid raft abundance and increase internalisation, decreasing Akt-dependent survival signalling (Park et al., 2010). It has also been demonstrated that cholesterol depletion induces anoikis-like apoptosis via down-regulation of focal adhesion kinase and hypoxia inducible factor-1 (Lee et al., 2009; Park et al., 2009).

Another apoptotic death receptor is TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 and 2, referred to as DR4 and DR5 respectively (Yang et al., 2010). Studies have demonstrated that translocation of these receptors into lipid rafts after TRAIL engagement is involved in apoptotic signal transduction (Merino et al., 2006; Dumitru et al., 2007; Song et al., 2007). In metastatic MDA-MB-231 cells, for instance, DR4 palmitoylation (Rossin et al., 2009) is crucial not only for its localisation into lipid rafts but also for receptor aggregation, both of which are essential for TRAIL-induced cell death (Merino et al., 2006). Therefore while much remains to be understood about the role of rafts in regulating apoptosis in breast cancer cells, it may offer a novel therapeutic target (see Section 4).

3.2 Growth and metabolism

In conjunction with altered apoptosis, abnormal signalling by growth factor receptors can facilitate breast tumour proliferation and growth. Lipid rafts modulate the signalling functions of several growth factor receptors, including the ErbB (HER) family of receptors.

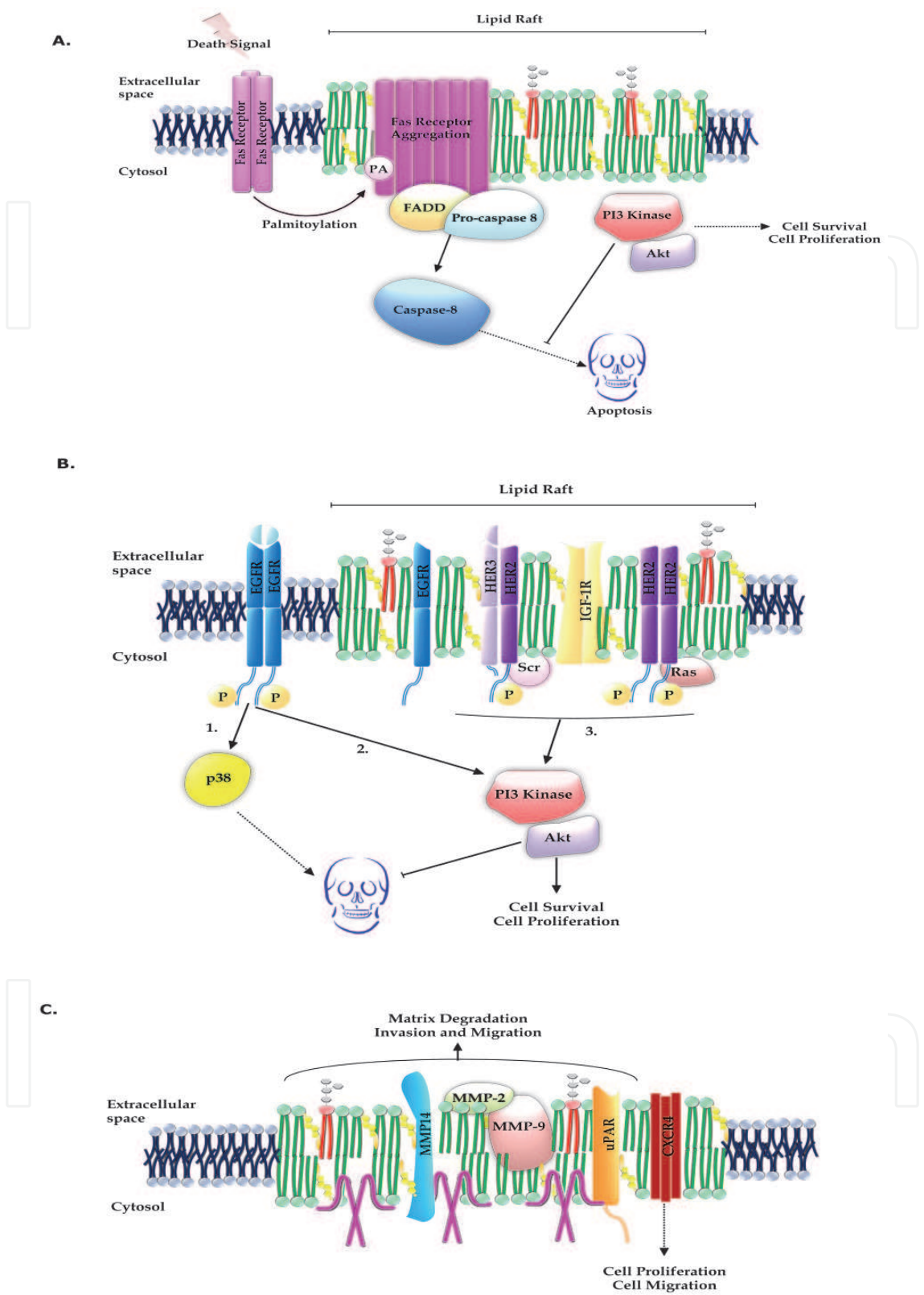


Fig. 2. Lipid raft regulation of breast cancer cell processes. (A.) Apoptotic signals are transduced via lipid rafts. (B.) EGFR signalling may induce apoptosis (1.) or proliferation (2.) outside of lipid rafts. Predominant oncogenic signalling is transduced through rafts (3.). (C.) Rafts cluster many degradation enzymes and proteins crucial for cell migration.

ErbB receptors are often mutated, amplified and/or overexpressed in breast cancer (Troyer & Lee, 2001). In particular, epidermal growth factor receptor, EGFR, and HER2 homo- and heterodimerisations have been described to promote oncogenic proliferation (Barros et al., 2010). Both of these receptors are associated with lipid rafts, and raft modifications can alter their signal transduction (Chen & Resh, 2002; Freeman et al., 2007).

EGFR functionality in particular is largely dependent upon its affiliation with rafts. Upon ligand binding, EGFR translocates out of caveolin-positive raft domains to stimulate downstream signalling (Mineo et al., 1999) and caveolin-1 raft domains negatively regulate EGFR activation (Lajoie et al., 2007). Accordingly, raft disruption via cholesterol depletion reportedly results in EGFR activation (Pike & Casey, 2002; Westover et al., 2003). Although EGFR phosphorylation is associated with oncogenic proliferation, sustained activation of EGFR outside of lipid rafts in fact correlates with increased p38 activation, which is pro-apoptotic. Potential modulations of EGFR activation status by dietary lipids will be discussed in Section 4.

Some studies have examined the activation of downstream targets of EGFR signalling, such as the small GTPase Ras (Rogers et al., 2010), after exposure to fatty acids. Ras associates with lipid rafts via palmitoylation (Calder & Yaqoob, 2007), and is involved in cell survival, growth and proliferation (Downward, 2006). Furthermore, enhanced anti-proliferative effects were seen upon co-treatment with an EGFR inhibitor and DHA (Rogers et al., 2010).

Another member of the ErbB family, HER2, is the favoured dimerisation partner of other ErbB proteins for receptor activation (Tzahar et al., 1996; Park, B. W. et al., 2000). Oncogenic HER2 dimerisation in breast cancer cells takes place in lipid rafts (Nagy et al., 2002), and forced exclusion of HER2 from rafts (via crosslinking of the raft-associated ganglioside GM1) has been shown to decrease HER2 dimerisation and tyrosine phosphorylation (Nagy et al., 2002). Another possible avenue of HER2 signalling regulation by lipid rafts relates to protein trafficking. HER2 is rapidly recycled back to the cell membrane if endocytosed (Worthylake et al., 1999), which maintains its overexpression at the cell membrane of breast cancer cells. Modulation of lipid metabolism may control HER2 overexpression by increasing its endocytosis and preventing redistribution back to the cell membrane (Paris et al., 2010). For instance, phospholipase C (PLC) has been shown to co-localise with HER2 in lipid raft domains of HER2-overexpressing breast cancer cell lines, and PLC antagonism enhances HER2 internalisation and delays its recycling to the cell membrane, reducing breast cancer cell proliferation (Paris et al., 2010). This implies that lipid rafts play a key role in transduction of this oncogenic signal.

Another protein known to localise in lipid rafts with HER2, in addition to EGFR, is the estrogen receptor (ER) (Marquez et al., 2006). Estrogen signalling is linked to lipid rafts, where ER co-localises with ErbB receptors to modulate growth events (Marquez et al., 2006). Both these receptors may be activated by membrane-bound ER (Razandi et al., 2003), resulting in MAP kinase-dependent ER phosphorylation (Pietras, 2003). As these receptors are reportedly lipid raft-affiliated, interference of this union with lipid rafts may prove to be useful in targeting endocrine resistance in breast cancer.

Insulin-like growth factor-1 receptor (IGF-1R) is another receptor tyrosine kinase whose activation leads to proliferation and differentiation via MAP kinase and PI3 kinase/Akt pathways (Adams et al., 2000). IGF-1R activity has been linked to lipid raft affiliation, particularly caveolae. Stable expression of caveolin-1 in MCF7 breast cancer cells, while decreasing cell attachment (Fiucci et al., 2002), results in enhanced matrix-independent

cell survival via upregulation of IGF-1R and subsequent activation of p53 and p21 (Ravid et al., 2005). Caveolin-1 further drives IGF-1R-induced recruitment of β_1 -integrin into lipid rafts (Salani et al., 2009), which could in turn regulate the influence of β_1 -integrin on cell fate (Li et al., 2005). In fact segregation of IGF-1R in and out of rafts has been shown to dynamically regulate overall signalling potency of the protein (Remacle-Bonnet et al., 2005), which is emerging as a promising pharmaceutical target in breast cancer (Weroha & Haluska, 2008).

Sigma receptors are a novel family of receptors whose physiological and pathophysiological roles are only beginning to emerge. They inhibit proliferation, induce apoptosis and can decrease cell adhesion in mammary carcinoma cell lines (reviewed in Aydar et al., 2004). Sigma receptors were proposed to have the ability to remodel lipid rafts by modulating raft cholesterol levels via cholesterol-binding motifs (Gebreselassie & Bowen, 2004; Takebayashi et al., 2004). Accordingly, raft cholesterol levels are reduced following sigma-1 gene knockdown (Palmer et al., 2007). Sigma-1 receptors also form a complex with β_1 -integrin in MDA-MB-231 cells, and their translocation outside rafts decreases breast cancer cell growth and adhesion (Palmer et al., 2007). Because cancer cells have elevated levels of lipid rafts and cholesterol (Li et al., 2006), and because many growth signalling molecules depend on rafts to exert their functions, dietary fat modifications could affect signal transduction in breast cancer cells (as will be further addressed in Section 4).

3.3 Cell migration and metastasis

Cancer progression does not depend solely on increased growth and reduced apoptosis, but also on the ability of tumours to seek out new niches to support their continued survival. Accordingly, cells often activate pathways that reduce adhesion and promote cell migration, increasing the likelihood of the metastatic spread of breast cancer.

Kinases play a significant role in regulating cell adhesion and migration. The Src family of kinases (SFK) integrates signal transduction from many receptor tyrosine kinases, including EGFR, IGF-1R and HER2 (Belsches-Jablonski et al., 2001; Parsons & Parsons, 2004) to multiple downstream targets including PI3-kinase, Ras and focal adhesion kinase (Parsons & Parsons, 2004). SFK activation has been linked to lipid rafts in breast cancer cells (Hitosugi et al., 2007), fuelling speculation that selective targeting of raft-affiliated SFK may offer a more potent therapy than conventional SFK inhibitors such as dasatinib. Src has also been shown to phosphorylate the raft marker flotillin-2 in an EGF-dependent manner, resulting in Src translocation into endosomes and the enhancement of cell spreading (Neumann-Giesen et al., 2007). Conversely, the finding that flotillin-2 knockdown reduces cell spreading further highlights the potential regulatory influence of lipid rafts on cell adhesion and actin dynamics (Neumann-Giesen et al., 2007).

Lipid rafts and caveolin-1 have also been shown to be crucial for the formation of invadopodia, membrane protrusions that penetrate the surrounding matrix through a combination of matrix remodelling and physical force (Buccione et al., 2009). Invadopodia cluster together proteins involved in actin cytoskeleton organisation, signalling, cell-ECM adhesion and membrane remodelling (Gimona et al., 2008). Lipid rafts have been reported to be concentrated at the leading edge of invadopodia in a panel of breast cancer cell lines, and disruption of lipid rafts may suppress invadopodia formation (Yamaguchi et al., 2009). Invasive potential has also been linked with the raft-affiliated proteins caveolin-1 and membrane type 1 matrix metalloproteinase (MMP14) in both breast (Annabi et al., 2001) and

prostate (Wang et al., 2009) cancer cells. In fact caveolin-1 and MMP14 have been shown to co-associate (Labrecque et al., 2004) and to be co-trafficked in invasive breast cancer cell lines (Yamaguchi et al., 2009). Accordingly, a reduction in matrix degradation activity of MMP14 has been reported in MDA-MB-231 cells following disruption of lipid rafts by cholesterol depletion or after knockdown of caveolin-1 in MMP14-overexpressing MDA-MB-231 cells (Yamaguchi et al., 2009). Together these results highlight that lipid rafts and caveolin-1 are important for invadopodia function in breast cancer cells.

MMP14 is not the only lipid raft-affiliated proteinase implicated in breast cancer progression. Aberrant expression of MMP2 and MMP9, which localize in rafts during cancer cell migration (Patra, 2008), have been associated with high-grade breast cancer (Mira et al., 2004). Downregulation of MMP2 and MMP9 has been shown to decrease tumour cell invasion (Patra, 2008). Similarly, the serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR), which have been linked to breast cancer progression and metastasis (Patra, 2008; Sahores et al., 2008), localise to lipid rafts during cancer cell migration (Sahores et al., 2008). A recent study investigating the importance of lipid rafts in regulating uPAR and MMP9 functionality in breast cancer has demonstrated that cholesterol depletion reduces co-localisation of uPAR and MMP9 with lipid rafts and significantly decreases their total protein and mRNA levels (Raghu et al., 2010). Lipid raft disruption in breast cancer cells resulted in reduced amounts of active Src, FAK, Akt and ERK and increased uPAR co-localisation with lysosomal markers, which was reversed after cholesterol repletion (Raghu et al., 2010). This is in agreement with previous observations of differences in MMP9-driven cell migration according to its sub-cellular localisation inside or outside rafts (Mira et al., 2004).

Although controversial, another approach to understanding breast cancer metastasis comes from the observation that disseminated tumour cells have progenitor-like properties, termed “cancer stem cells” (Acconcia et al., 2004). Low expression of CD24, a ligand for P-selectin on cancer and myeloid cells (Aigner et al., 1997), has been proposed as a marker for cancer stem cells, and breast cancer patients with aggressive triple-negative disease reportedly have higher percentages of cancer stem-like cells (May et al., 2011; Reuben et al., 2011). CD24 appears to govern the localization and function of the chemokine receptor CXCR4, which regulates proliferation in primary and metastatic breast cancer (Smith et al., 2004). CXCR4 must localise in lipid rafts for effective signalling (Manes et al., 2001; Wysoczynski et al., 2005), and CD24 reportedly reduces cellular responsiveness to CXCR4 signalling in a metastatic breast cancer cell line by excluding the latter from lipid rafts (Schabath et al., 2006). Thus low CD24 levels in putative cancer stem cells would have a positive effect on CXCR4-driven proliferative signalling, via enhanced raft affiliation of CXCR4. Accordingly, high expression of CD24 has been shown to reduce tumour growth and spread in mice (Schabath et al., 2006). Therefore CXCR4 metastatic potential may be modulated by altering its affiliation with rafts independently of its expression levels.

Another proposed marker for breast cancer stem cells is CD44 (Blick et al., 2010; May et al., 2011), a multi-functional lipid raft-affiliated transmembrane glycoprotein expressed in a variety of tissues (Murai et al., 2011). CD44 is the major receptor for the extracellular matrix component hyaluronan (HA) (Herrera-Gayol & Jothy, 1999); but it can also act as a co-receptor for growth factors (Bourguignon et al., 1997; Orian-Rousseau et al., 2002) and organise the cellular actin cytoskeleton through cytoplasmic linker proteins (Ponta et al., 2003). CD44 abnormalities have been associated with aggressive histological features of

breast cancer (Joensuu et al., 1993; Diaz et al., 2005), and the association of CD44 with MMP9 in breast tumour cells promotes tumour cell migration and invasion (Bourguignon et al., 1998). It is possible that this matrix-degrading association takes place in lipid rafts, where both CD44 and MMP9 localize. Interactions between CD44 and HA also stimulate a variety of events leading to tumour progression, including Rho kinase activation, Ras signalling and others (reviewed in Bourguignon, 2008). Although the exact mechanisms of these events have yet to be clarified, one proposed method involves CD44 interaction with ankyrin within lipid rafts (Singleton & Bourguignon, 2004). Another matrix glucosaminoglycan, osteopontin, can also activate CD44 to promote cell survival and increased endothelial adhesion by recruiting Src and integrins into lipid rafts (Lee et al., 2008).

The influence of CD44 on cancer progression also extends to other growth factor signalling pathways, since it has previously been demonstrated that the growth factor receptor c-Met cannot be activated by its ligand alone, but also requires CD44 co-expression (van der Voort et al., 1999). c-Met, which has been shown to localise in lipid rafts and whose signalling is sensitive to cholesterol depletion (Coleman et al., 2009), is frequently dysregulated in metastatic breast cancer (Gastaldi et al., 2010; Elnagar et al., 2011). It can thus be hypothesised that lipid rafts are required for successful transduction of growth factor-mediated oncogenic signals through formation of functional CD44 complexes.

Taken altogether, several key molecules frequently implicated in breast cancer initiation, growth and migration are regulated by lipid rafts via sequestration, endocytosis or termination of protein interactions. Considering the ongoing need to develop drugs which selectively attack cancer cells while leaving normal cells unaltered, the imbalance in lipid raft composition between tumour and normal cells may suggest rafts as attractive and novel pharmacological targets in the battle against breast cancer.

4. Lipid rafts as novel therapeutic targets in breast cancer

This section will focus on current cancer treatments targeting lipid rafts, the potential importance of lipid raft modulation to overcome mechanisms of drug resistance, and new mechanisms associated with lipid raft physiology that could be targeted by novel drugs. Finally it will discuss the importance of the diet in influencing lipid raft physiology as a potential mechanism to prevent or reduce cancer dissemination.

4.1 Current drug treatments targeting lipid rafts

There is growing interest in the possibility of targeting lipid rafts for cancer treatments due to their role in the regulation of many steps of tumour transformation and progression, such as the apoptotic pathways initiated by FasL and TRAIL. Current treatments targeting lipid rafts are mainly focused on activating these apoptotic pathways in cancer cells. However many classes of drugs routinely used in breast cancer chemotherapy have been shown to exert some of their effects by modulating lipid rafts.

One key example is cisplatin, whose mechanism of action is still incompletely understood, but which has been described to exert some of its actions through modulation of ceramide lipid rafts. In a human colon cancer cell line, cisplatin induced clustering of the Fas receptor in membrane lipid rafts by activating the acid sphingomyelinase (ASMase), which produces ceramide and is responsible for induction of apoptosis. Nystatin, a compound which disrupts lipid rafts, completely reversed this effect (Lacour et al., 2004). It has also been

reported that a combination of cisplatin and an anti-Fas antibody in cells expressing sphingomyelinase induced marked apoptosis of cancer cells (Huang et al., 2010).

Moreover, histone deacetylase inhibitors (HDACi), powerful tumour suppressors for many different solid and hematologic cancers, have also been shown to modulate lipid raft physiology. These compounds act on tumour cells by inducing accumulation of acetylated proteins that can cause growth arrest, apoptosis and ROS-induced cell death (Marks & Xu, 2009). Since normal cells are resistant to these treatments, HDACi have been widely tested in clinical trials alone or in combination with other drugs (Marks & Xu, 2009). Van Oosten and colleagues observed a drastic increase in the expression and localization of TRAIL in lipid rafts after treatment of prostate cancer cells with a HDACi depsipeptide, inducing elevated cell apoptosis (Vanoosten et al., 2005). Similarly, in preclinical mouse breast cancer models the HDACi vorinostat, in combination with administration of monoclonal antibodies against the mouse TRAIL receptor (DR5), induced robust cell apoptosis (Frew et al., 2008).

Likewise, some derivatives of doxorubicin, an anthracycline widely used in breast cancer adjuvant chemotherapy, exercise their actions by activating lipid raft-associated pathways. Aroui and colleagues illustrated that treatment of MDA-MB-231 cells with Dox-CPP, obtained by conjugating doxorubicin to a cell-penetrating peptide, sensitised cells to TRAIL-induced apoptotic pathways by increasing TRAIL clustering and its inclusion in ceramide lipid rafts (Aroui et al., 2009). Lipid raft-mediated activation of TRAIL is also important because it reduces resistance mechanisms associated with doxorubicin resistance.

4.2 Overcoming drug resistance by targeting lipid rafts

One major drawback of current cancer treatment regimens is the development of drug resistance. Two main mechanisms have been described to be involved in this multifactorial process: 1) alterations in tumour cell physiology that cause either insensitivity to drug-induced apoptosis or induction of drug-detoxifying mechanisms; 2) expression of energy-dependent transporters that detect and eject anti-cancer drugs from cells (Wang et al., 2010).

Recent evidence points to the correlation of both mechanisms with lipid raft physiology. Mechanisms of breast cancer resistance to the anti-HER2 therapeutic antibody Herceptin/Trastuzumab may involve compensatory signalling through dimerisation of HER2 with other ErbB family members, cross-talk between HER2 and the IGF-1R pathway, coverage of the antibody binding site by MUC4 overexpression, and constitutive activation of the PI3 kinase/Akt pathways (Nahta & Esteva, 2006). Interestingly, all these events take place in lipid raft compartments and are involved in the activation of alternative oncogenic pathways to overcome the inhibitory effects of cancer treatments. Therefore, treatments altering lipid raft physiology could provide hope in preventing or reducing mechanisms of resistance to specific anti-cancer drugs.

HER2 over-expression in breast cancer cells has also been reported to stimulate fatty acid synthase (FASyn) gene expression (Menendez et al., 2004). FASyn is a major lipogenic enzyme catalyzing the synthesis of long-chain saturated fatty acids, which localize to lipid rafts in epithelial cells. Accordingly, FASyn inhibitors work by altering lipid raft physiology through deregulation of fatty acid synthesis; and have been reported to re-sensitise cells to Trastuzumab, causing growth inhibition and apoptotic cell death. Since these inhibitors also affect EGFR1 localization to lipid rafts, they may also disrupt the cross-talk between HER2 and EGFR which is involved in Trastuzumab resistance (reviewed in Menendez, 2005).

Correspondingly, since ER can localize to lipid rafts through post-translational lipid modifications termed acylations, lipid rafts have been suggested as the possible location of

interactions between ER and growth factor receptors which occur during resistance to endocrine therapy (Acconcia et al., 2004; Weinberg et al., 2005; Arpino et al., 2008). Mutations of ER acylation sites impair the ability of ER to activate transcription and cell proliferation in response to estradiol stimulation (Pietras et al., 2005). It is appealing to speculate that combinations of hormonal therapies and treatments altering ER localization to lipid rafts could prevent cross-talk between ER and EGFR pathways; and may be a future therapeutic strategy to reduce drug resistance arising in hormonal therapy. Furthermore, it has been shown that poor responsiveness of breast cancer cells to treatment with the EGFR inhibitor gefitinib may be due to increased localization of EGFR in lipid raft domains. Treatment of breast cells with lipid raft-disrupting agents (such as lovastatin) induced cellular sensitivity to gefitinib, and abrogated proliferative pathways initiated by Akt phosphorylation (Irwin et al., 2010).

However, along with single drug resistance in breast cancer therapy, the phenomenon of multidrug resistance has also been described (Ogretmen & Hannun, 2001). This occurs when resistance to one drug is accompanied by resistance to drugs whose structures and mechanisms of action may be completely different. Several studies have observed alterations of lipid raft components in multidrug-resistant cells, such as increased caveolin-1 expression, larger numbers of caveolae (Lavie et al., 1998) and elevated membrane cholesterol content (Gayet et al., 2005). Lavie and colleagues suggested that an increased number of caveolae may be more of a cause than an effect of multidrug resistance. In fact, caveolae, being capable of effluxing cholesterol, may be used by cancer cells to efflux lipophilic drugs. Since caveolar efficiency to efflux cholesterol or any cytotoxic drug is very low, they hypothesized that a higher number of caveolae is therefore necessary to compensate for drug cytotoxic effects and efflux them efficiently (Lavie et al., 1998).

Cellular lipid changes are also often accompanied by increased expression of ABC transporters which can localize to lipid rafts (Klappe et al., 2009). One member of this family, BCRP/ABCG2, which was discovered and cloned in breast cancer cells (Doyle et al., 1998), has been recently described to localize to lipid rafts. Interestingly, disruption of lipid rafts using methyl- β -cyclodextrin has been shown to cause a 40% decrease in BCRP activity (Storch et al., 2007); further highlighting the potential value of pharmacological raft targeting as a mechanism of reducing multi-drug resistance.

Overall these examples demonstrate the importance of lipid rafts in clustering oncogenic signalling molecules that are involved in breast cancer resistance to current conventional treatments. It is also evident that treatments aimed at preventing or disrupting the localisation of oncogenic signalling mediators (such as HER2, ER, IGF-1R or ABC transporters) in lipid rafts may prove therapeutically useful in combination with current treatments in order to prevent the development of resistance.

4.3 Novel lipid raft- mediated approaches in breast cancer treatment

Recently, glycomic studies have highlighted an emerging and critical importance of glycans in influencing lipid raft physiology. Therefore potential future cancer treatments may also indirectly target lipid rafts by targeting glycans. For example, in breast cancer cells, gangliosides (such as the ganglioside GM1 or the O-glycosylated protein MUC4) regulate the formation of EGFR growth factor-responsive heterodimer complexes in lipid rafts (Komatsu et al., 2001). Lipid rafts play a fundamental role in providing a microenvironment favouring functional interactions between glycans and HER2 / HER3, which drive tumour

progression (Komatsu et al., 2001; Nagy et al., 2002). Antibodies targeting MUC4 or treatments targeting gangliosides (i.e. ceramide glycosylation inhibitors or ganglioside-targeted vaccines such as NeuGcGM3 and Theratope) may therefore be possible treatments to interfere with ErbB-driven proliferation of breast cancer cells via modulation of lipid raft physiology (Carr et al., 2003; Ibrahim & Murray, 2003; Julien et al., 2009; Mulens et al., 2010). Raft-dependent endocytosis of paclitaxel-conjugated autocrine motility factor is elevated in metastatic breast cancer cells and has been shown to induce tumour regression and promote survival of tumour-bearing mice (Kojic et al., 2008; Kojic et al., 2007). In addition, some new generation treatments have considered the use of nanoparticles to target lipid raft-affiliated proteins involved in tumour progression and invasion. Conjugated nanoparticles present the advantage of selectively targeting cancer cells without affecting the physiology of normal cells. For example, a study in glioma cells using magnetic nanoparticles conjugated to chlorotoxin (which targets MMP2, a protein highly expressed in glioma tumour cells) demonstrated the induction of lipid-raft mediated endocytosis of MMP2 together with Cl⁻ and K⁺ channels (Veisheh et al., 2009), the latter proteins being involved in regulating cell volume during cell invasion. Using this system, a 98% reduction in invasion was observed in nanoparticle-treated cells compared to controls. In the same manner, MMP2 has also been shown to play a role in breast cancer progression and invasion and has been described as a marker for poor prognosis in ER- negative patients (Ma et al., 2009). Therefore, it is conceivable that an approach similar to the one used by Veisheh et al. might be useful as a companion drug strategy for breast cancer chemotherapy.

4.4 Lipid rafts and diet in cancer progression

Cancer cells generally possess higher levels of saturated fatty acids and cholesterol than normal cells (Li et al., 2006). Emerging evidence thus suggests the potential of diet to influence raft composition and the role of rafts in cancer pathophysiology (section 3.2).

Several studies have shown that polyunsaturated fatty acids (PUFAs) may have anti-carcinogenic properties (Sauer et al., 2007). The mechanism by which PUFAs work is still incompletely understood, but two mechanisms have been proposed. The first suggests that PUFAs affect the palmitoylation status of lipid raft proteins (Webb et al., 2000); while the second model suggests that PUFAs, having a low affinity for cholesterol due to their bulky structure, reduce raft cholesterol levels and cause displacement of raft proteins (Stulnig et al., 2001). In fact, it has been described that *in vivo* PUFA supplementation affects lipid raft composition by depleting up to 50% of cholesterol and by altering lipid raft/caveolar protein composition. In comparison to chemical disruption of lipid rafts (e.g. with methyl- β -cyclodextrin and nystatin), PUFA treatment is very selective and depletes only membrane cholesterol without affecting other cellular sources of cholesterol (Ma et al., 2004).

It has also been shown that PUFAs such as EPA and DHA inhibit protein palmitoylation of selected T cell lipid raft proteins (such as Fyn) similarly to the chemical compound 2-bromopalmitate (Webb et al., 2000). Interestingly, in MDA-MB-231 breast cancer cells, PUFAs have been shown to decrease cell proliferation and induce apoptotic cell death probably by decreasing Akt/NF κ B signal transduction (Schley et al., 2005). Furthermore, PUFA treatment has been shown to increase EPA and DHA concentrations in lipid rafts, with a corresponding decrease of sphingomyelin, cholesterol and diacylglycerol (Schley et al., 2007). In particular, PUFAs reduce sphingomyelin levels by inducing its hydrolysis to ceramide, which (as discussed) activates pro-apoptotic pathways (Schley et al., 2005).

Section 3 discussed how lipid raft disruption negatively modulates EGFR localization and signalling, and, similarly, PUFAs have been shown to exert pro-apoptotic properties by reducing EGFR localization within lipid rafts, thus inducing a sustained activation of EGFR and p38 phosphorylation in breast cancer cells (Schley et al., 2007). In the same way, (-)-epigallocatechin-3-gallate (EGCG), the active compound contained in green tea, can alter lipid raft domain composition. EGCG has been described to prevent EGF interactions with its receptor, to prevent EGFR dimerisation and to inhibit its localisation within rafts. This would promote pro-apoptotic signalling via p38. Altogether, EGFR translocation alters its activation status and may have anti-cancer effects (Patra et al., 2008). EGCG also exerts further anti-cancer activity by suppressing proliferation and enhancing apoptosis by interfering with lipid raft remodelling (Patra et al., 2008). Indeed, EGCG has been described to block the laminin-1 receptor, a raft-affiliated protein (Tachibana et al., 2004) whose activation is connected with a kinase/phosphatase cascade involved in tumour progression (Patra et al., 2008). Interestingly, EGCG also seems to play a role in modulating multidrug resistance. In fact it has been described that EGCG causes a dose-dependent increase in apoptosis in Trastuzumab- (Eddy et al., 2007), Tamoxifen- and multidrug-resistant breast cancer cells (Farabegoli et al., 2010).

Taken together, we have summarized putative anti-tumour mechanisms involving interference with lipid rafts, and highlighted the importance of rafts as targets for cancer therapy. Development of drugs directed to specific raft components could facilitate widespread use of these treatments; while dietary modification alone could influence tumorigenic behaviour through modulation of lipid raft composition. Overall we suggest that lipid rafts play a key role both in the prevention and treatment of breast cancer, and are confident that further studies in this area will prove highly fruitful in the future.

5. References

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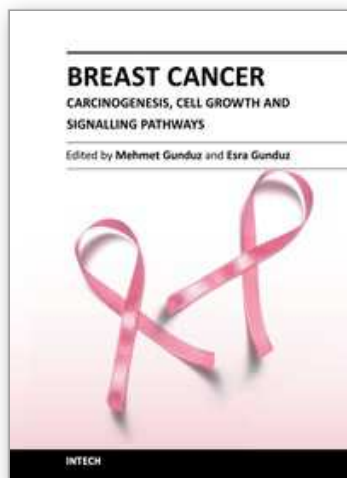
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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