THE ROLES OF TRANSLOCATOR PROTEIN (TSPO) AND ADENINE NUCLEOTIDE TRANSLOCASE 2 (ANT2) IN BREAST CANCER DEVELOPMENT AND PROGRESSION

By

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ABSTRACT

THE ROLES OF TRANSLOCATOR PROTEIN (TSPO) AND ADENINE NUCLEOTIDE TRANSLOCASE 2 (ANT2) IN BREAST CANCER DEVELOPMENT AND PROGRESSION

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Mitochondria play important roles in breast cancer development and progression. Targeting mitochondria, particularly targeting mitochondrial permeability transition pore (PTP), is suggested to be an effective strategy for cancer therapy. The mitochondrial membrane proteins, translocator protein (TSPO) and adenine nucleotide translocase 2 (ANT2), are both considered to be components of PTP complex and are overexpressed in cancers compared to normal tissues. This thesis research aims to investigate the functional roles of TSPO and ANT2 in breast cancer progression and assess their potential being therapeutic targets for breast cancer treatment.

We first investigated the roles of TSPO in breast cancer progression and the potential of using TSPO ligands in treating breast cancer. Compared to mammary epithelial cells that do not express high levels of TSO, mammary epithelial cells that overexpress TSPO form larger acini structures with increased proliferation and decreased luminal apoptosis during mammary epithelial morphogenesis in 3D Matrigel culture. TSPO overexpressing acini also contain a partially filled lumen, which resembles early stage breast lesions initiating breast cancer. Our data further show that overexpression of TSPO promotes breast cancer cell migration and that silencing of TSPO reduces cell migration. Combination of TSPO ligands (PK 11195 or Ro5-4864)

and lonidamine, a clinical phase II drug targeting mitochondria, has led to significant apoptosis of ER-negative breast cancer cells, when either drug alone has very minor effect on cell viability. Our data thus suggest that targeting TSPO, particularly in combination with other mitochondria-targeting agents, could be a viable treatment strategy for ER-negative breast cancer.

We next analyzed the role of ANT2 in energy metabolism and its influence on the development of cancer cell malignancy. ANT is responsible for ATP/ADP exchange between mitochondria and cytosol. Among four human ANT isoforms, ANT2 is the only isoform overexpressed in breast cancers compared to normal breast tissue. Our data demonstrate that ANT2 promotes maximum ATP levels, cell viability and migration through oxidative phosphorylation (OXPHOS) in breast cancer cells. ANT2 depletion does not impact on anaerobic glycolysis, as the lactate levels remain unchanged upon ANT2 depletion. Oligomycin, an OXPHOS inhibitor, upregulates anaerobic glycolysis pathway and reverses the defect of ATP in ANT2 depleted breast cancer cells. However, oligomycin fails to rescue the impaired cell migration and viability due to ANT2 depletion, suggesting a specific role of OXPHOS in promoting cancer cell malignancy. Depletion of ANT2 in combination with 2-deoxyglucose (a glycolysis inhibitor) treatment leads to great reduction of breast cancer cell viability, which suggest that targeting OXPHOS, particularly ANT2, in combination with inhibition of glycolysis could be a valid strategy for breast cancer treatment.

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LIST OF ABBREVIATIONS

2D	2 dimensional
2DG	2-deoxy-D-glucose
3D	3 dimensional
ANT	adenine nucleotide translocae
ADH	atypical ductal hyperplasia
α-KG	α-ketoglutarate
ATP	adenosine triphosphate
Bcl2	B-cell lymphoma 2
Bim EL	bim-extra long
ВА	bongkrekic acid
BRCA1	breast cancer 1
BSA	bovine serum albumin
CCCP	uncoupler carbony cyanide m-chlorophenyl hydrazone
CYPD	cyclosporine-A-sensitive cyclophilin D
CAT	carboxy atractyloside
CLL	chronic lymphocytic leukemia
CRAC	cholesterol recognition amino acid consensus
CsA	cyclosporin A
DBI	diazepam-binding inhibitor
DAPI	4'-6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DCIS	ductal carcinoma in situ
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ETC	electron transport chain
FBS	fetal bovine serum
FDG	fluoro-2-deoxy-D-glucose
GRBOX	glycolysis-regulated box
HER2	Human epidermal growth factor receptor 2
НКІІ	hexokinase II
HIF	hypoxia-inducible factor
HPV E7	Human papillomaviruses E7
IDC	invasive ductal carcinoma
LON	lonidamine
MMP2	matrix metalloproteinase 2
MOMP	Mitochondrial outer membrane permeabilization
mtPTP	mitochondrial permeability transition pore
mtCK	mitochondrial creatine kinase
mTOR	mammary target of rapamycin
ND5	NADH dehydrogenase subunit 5 gene
OXPHOS	oxidative phosphorylation

OAA	oxaloacetate
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCA	tricarboxylic acid
TSPO	tranlocator protein
TNF-a	tumor necrosis factor alpha
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffer saline
PBR	peripheral-type benzodiazepine receptor
PET	positron emission tomography
PTP	permeability transition pore
PMA	phorbol-12-myristate 13 acetate
РКС	protein kinase C
PET	positron emission tomography
PR	progesterone receptor
PI3K	phosphatidylinositide 3-kinases
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
Sp1/Sp3	specificity protein 1/specificity protein 3
siRNA	small interfering ribonucleic acid
TNBC	triple negative breast cancer

ТСА	tricarboxylic acid cycle
TRAIL	TNF-related apoptosis-inducing ligand
UV	ultraviolet
VDAC	voltage dependent activated channel

CHAPTER I

LITERATURE REVIEW

1. Breast cancer progression and therapy

1.1 Breast cancer progression

Breast cancer is the most commonly diagnosed cancer in women. Based on American Cancer Society's statistics, about 1 in 8 women have a risk for breast cancer development during their lifetime [1]. About 232,340 new cases of invasive breast cancer and about 64,640 new cases of carcinoma in situ will be diagnosed in 2013 in US [1, 2].

Breast cancer originates from lobules or ducts of the breast. Breast lobules are glands for milk production and ducts connect lobules to the nipple [3, 4]. Most breast cancers arise in the ducts, representing 40-75% of patients with breast cancers [5]. Normal ducts are well-polarized acini with a hollow lumen. These acinar structures are bilayered including the inner luminal epithelial cell layer and the outer myoepithelial cell layer [6, 7]. Abnormal morphology of ducts, such as lesions at the luminal epithelial cells layer or filling of lumen, are important signs of breast cancer initiation [6].

Breast cancer stages describe the progression of cancer [8]. Stage 0 are noninvasive cancers, such as atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS). In DCIS, ductal epithelial cells are usually hyper-proliferative and the proliferative cells can deposited into the lumen [8]. Stage I, II and III describe different extents of invasive breast cancer cells that break through to surrounding breast tissue or nearby lymph nodes. In metastatic, stage IV breast cancers, cancer cells metastasize to distant organs, such as bones and lungs [8]. Patients at stage 0 usually have 93% 5year survival rate, whereas, patients with stage IV metastatic breast cancer have only 15% 5-year survival rate and higher recurrence rates [8, 9]. To understand the transition

from pre-invasive to invasive stages of breast cancer, gene expression profiles have been analyzed and compared among distinct stages. Similar gene expression profiles have been found among different tumor stages, but some genes are quantitatively correlated with the transition between tumor stages [5, 10].

In addition to tumor stages, tumor grades are used to describe the degree of malignancy of cancer cells during breast cancer progression, based on nuclear pleomorphism, glanduar/tubule formation and proliferative index [11]. Significant gene expression and chromosome changes are identified among tumor grades [5, 10, 12]. For example, chromosomal loss of 16q, gains of 1q are often present in low grade tumors, whereas, " high grade-like" tumors tend to show gain of 11q13, loss of 13q [12].

1.2 Breast cancer subtypes and treatment

Breast cancer is a heterogeneous disease with diverse morphological features, genetic, and epigenetic variations. Gene profiling conveys important messages to help construct therapeutic strategy for patient treatment. Generally, breast cancer is classified into four subtypes based in part upon the expression of the steroid hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), and the oncogenic receptor tyrosine kinase HER2, along with proliferative index (as measured by Ki-67 immunostaining) and various cytokeratin markers. The luminal A subtype breast cancers are typically ER positive and /or PR positive and Ki-67 <14%; luminal B subtype are typically ER positive and/ or PR-positive and Ki-67 > 14%; HER2 subtype are ER-negative, PR-negative, HER2-overexpressing; and triple-negative subtype are ER-negative, PR-negative, HER2-non-overexpressing, cytokeratin 5/6 positive [13-16].

Luminal A comprises approximately 40% of breast cancers, whereas luminal B accounts for 20% [13, 17, 18]. Luminal B cancers have a worse prognosis than luminal A subtype. Patients diagnosed with luminal A or B breast cancer are usually treated with anti-estrogens or other endocrine therapies [19]. For instance, tamoxifen, or fulvestrant, ER antagonists, are frequently used in premenopausal patients with ER positive breast cancer [20]. In postmenopausal women, aromatase inhibitors are used to block production of nonovarian-derived estrogens.

The HER2 overexpression subtype has been found in 15-20% of the breast cancer patients [18], and typically results from amplification of the HER2 gene locus. Patients with the HER2 subtype of breast cancer are often treated with either Herceptin (trastuzumab) or lapatinib, which targets the extracellular domain or inhibits the tyrosine kinase domain of HER2 receptor, respectively [21].

Triple-negative breast cancers (TNBC) represent 15%-20% of breast cancer cases, and are more malignant, metastatic and lack targeted therapies [22, 23]. For some TNBC that show defects in DNA repair, DNA alkylating agents and inhibitors of poly (ADP ribose) polymerase 1 (PARP1) are suggested as potential treatments. For some TNBC with amplification of EGFR, EGFR inhibitors (anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitor) are also potential anti-cancer agents [24-27].

The development of drug resistance is the main challenge in the treatment of breast cancers. Half of patients with ER-positive tumors do not respond to the treatment of tamoxifen [28]. Only 26% of patients with HER2 subtype tumors respond to Herceptin as a single therapy [29]. Several mechanisms are involved in the acquisition of

resistance to endocrine therapy or HER2-targeting therapy, such as deregulation of components of ER or HER2 pathways, upregulation of signaling pathways downstream of ER or HER2 [30], or activation of alternative pathways [31, 32]. TNBCs are especially difficult to treat, as different subsets of TNBCs tend to have distinct characteristics. For instance, some TNBCs contain mutation of BRCA1, some have loss of phosphatase and tensin homolog (PTEN), some overexpress EGFR [24]. Therefore, a single therapy is usually not effective in treating TNBCs.

Combination therapy including inhibitors that block the dysregulated pathways in resistant cancers has been proven to be an effective therapeutic strategy. For example, in a Phase II study, treatment with tamoxifen plus the mTOR inhibitor, everolimus, increased overall survival compared with tamoxifen alone in postmenopausal women with endocrine-resistant breast cancer [33]. In Herceptin-resistant breast cancer, the SRC tyrosine kinase pathway is one of the downstream pathways that are commonly activated. Treatment with a SRC inhibitor plus Herceptin has been shown to be effective in overcoming Herceptin resistance in *vitro* and in *vivo* in preclinical models [34]. For TNBC tumors with a BRCA mutation, PARP inhibitors are suggested to be a promising treatment strategy. In a study for TNBCs without BRCA mutation, treatment with the PI3K inhibitor BKM120 decreases the expression of BRCA and consequently sensitizes those TNBCs to the treatment with a PARP inhibitor, olaparib [35].

1.3 Experimental models to study breast cancer

Different experimental models are used to investigate breast cancer progression and evaluate potential treatments. These include human breast cancer cell lines,

xenograft tumors and genetically engineered mice. Cell lines may used to model breast cancer in 2D and 3D cultures, as well as xenografts [36].

Breast cancer cell lines have been mostly used in the investigation of breast cancer progression, and provide us with detailed information about how certain genes or signaling pathways may impact on cancer cell proliferation, apoptosis, migration [37]. Though the differences between cell lines and primary tumors should be noticed, different cell lines with unique molecular profiling are still useful in the research of potential cancer therapies [36, 37]. For example, MDA-MB-231 or BT549 are triplenegative human breast cancer cell lines, which are often used to evaluate potential anticancer agents for TNBC.

Most studies using cell lines are conducted on 2D plastic dishes. Plastic culture creates an environment that is markedly different from the breast microenvironment [36]. To overcome such limitations, 3D culture, which includes extracellular matrices and thus partially mimics the extracellular environment of the breast, has distinct advantages over 2D culture. For example, a Matrigel culture system has been established to model mammary epithelial morphogenesis in *vitro* [38-40]. In this 3D culture system, nontumorigenic mammary epithelial cells, such as MCF10A, can form polarized, growth-arrested acini with hollow lumens, which resemble the mammary glands [38, 41]. Abnormal formation of acini structures in 3D culture by induction of certain oncogenes could reveal some aspects of breast cancer development, such as uncontrolled proliferation and inhibition of apoptosis [41].

Studying tumor progression and development in xenografts and genetically engineered mouse models is argued to be more relevant in understanding the biology of

breast cancer, since mouse models convey the complicated stromal environment for cancer development. But deciphering the difference between mouse models and the human patients is still a hurdle to overcome. Since no single model is able to recapitulate the complexity of breast cancer, modeling breast cancer will still rely on multi-systems approach.

2. Mitochondria and cancer

2.1 Function of mitochondria in health and cancer

Mitochondria play important roles in many cell functions, including cellular biosynthesis of cellular materials, production of energy through oxidative phosphorylation (OXPHOS), generation of reactive oxygen species (ROS), maintenance of Ca²⁺ homeostasis, and regulation of apoptosis by activation of mitochondrial permeability transition pore (mtPTP) [42].

Mitochondria are essential for cancer cells. Elimination of mtDNA from multiple cancer cell lines results in decreased cellular growth rate, reduced colony formation in soft agar and inhibited tumor formation in nude mice [43-45]. Various mtDNA mutations have been found in many types of cancers, including breast cancer [42]. These mutations not only contribute to cancer cell adaption to the changing bioenergetic microenvironment (such as hypoxia), but also involve in the increase supply of energy and nutrition for cancer cell proliferation [46]. As mitochondria are critical for cell metabolism and cell apoptosis, mitochondria are strongly associated with hallmarks of cancer cells, including unrestricted proliferative signaling, insensitivity to growth

suppressors, resistance to apoptosis, capability of replicative immortality, induction of angiogenesis, and tissue invasion and metastasis [47-49].

Mitochondria are the major organelle in which many intermediate metabolites for cell proliferation and growth are produced (Figure 1.1) [50]. The tricarboxylic acid (TCA) cycle, as a hub for biosynthesis during proliferation, takes place in mitochondria. The TCA cycle produces many intermediates for macromolecular biosynthesis of lipids, proteins or nucleic acids [51]. For instance, citrate, generated from the TCA cycle, can be used for synthesis of fatty acids and cholesterol for the composition of lipid membranes of the dividing cells [52, 53]. Mitochondria are also important for fatty acid catabolism, as the fatty acid beta-oxidation occurs at the mitochondria. Beta-oxidation is the process that breaks down the fatty acid into acetyl-CoA, which could feed into the TCA cycle to generate energy when glucose is low in the cells [54]. Mitochondria also participate in glutaminolysis. Glutamine is converted to glutamate that enters the mitochondria. Glutamate can be converted to α -ketoglutarate (α -KG) by glutamate dehydrogenase in the mitochondria. α -KG enters the TCA cycle and produces oxaloacetate (OAA), which could be used to generate new amino acids during cell proliferation [55].

Mitochondria are also the powerhouse where most cellular ATP is generated (Figure 1.1) [49]. A continuous supply of ATP is required for various physiological and biochemical activities in the cells. Glycolysis and oxidative phosphorylation (OXPHOS) are two major pathways for ATP synthesis. Glycolysis occurs in the cytosol and produces 2 ATP from each mole of glucose [50, 56]. Anaerobic glycolysis results in



Figure 1.1 Schematic of metabolic pathways. ATP is primarily produced through OXPHOS (oxidative phosphorylation) and glycolysis. During glycolysis, glucose is converted to pyruvate and generates about 2-4 ATP from each glucose molecule. Pyruvate can be converted to lactate when oxygen levels are low, or be transported into mitochondria if enough oxygen is present. In the mitochondria, pyruvate enters the citric acid (TCA) cycle. In addition, glutamine can be converted to glutamate and enters TCA cycle. In the TCA cycle, electrons are released from intermediate reaction products

Figure 1.1 (cont'd)

and transferred to nicotinamide adenine dinucleotide (NAD+) to yield reduced NADH. NADH can then feed the electrons into the mitochondrial electron transport chain. In the electron transport chain, electrons are released from the NADH and transferred to a series of molecules. Finally, the electrons (together with protons [H+]) are transferred to oxygen to generate water and about 36 ATP (per glucose molecule) is produced by ATP synthase. The carbon flux through glycolysis and TCA cycles provides abundant biomass intermediates for biosynthetic pathways such as synthesis of nucleotide, lipid and amino acid that are used for making new cells. accumulation of lactate as OXPHOS fails [50]. OXPHOS takes place in the mitochondria and produces up to 36 ATP from each mole of glucose [57, 58]. Thus OXPHOS is much more efficient in generating ATP. OXPHOS occurs through the electron transport chain (ETC). As the electrons travel through complexes I, III and V, protons are pumped out across the mitochondrial membrane to create an electrochemical gradient, which is then used by ATP synthase (complex V) to make ATP [59]. The inner membrane protein, adenine nucleotide translocase (ANT), exchanges ADP and ATP between the mitochondria and cytosol [60].

The Warburg effect suggests that cancer cells tend to rely on glycolysis instead of OXPHOS [61, 62]. However, this idea is still controversial. Many studies have revealed that not all tumors exhibit low OXPHOS activity; instead, different types of cancer cells show different ratio of OXPHOS and glycolysis [42]. Accumulation of FDG (2-18 fluoro-2-deoxy-D-glucose, a glucose analog tracer) in tumor tissues during PET scans could be explained, at least in part, by the glycolytic activity in the surrounding glycolytic stromal cells rather than only in the tumor cells themselves [63].

To communicate with other subcellular compartments, mitochondria utilize reactive oxygen species (ROS) or Ca^{2+} as signaling molecules [42, 64, 65].

Most of the ROS is produced in the respiratory chain at the mitochondrial membrane. The excess electrons from complexes I and III are transferred to O_2 to generate superoxide anions O_2^{-} . O_2^{-} is further converted to H_2O_2 by the mitochondrial matrix Mn^{2+} superoxide dismutase or by Cu^{2+}/Zn superoxide dismutase. H_2O_2 can be further converted to the most reactive ROS, hydroxyl radicals (.OH) [42]. Such ROS could be reduced by cellular redox sysytem, such as glutathione. Increased levels of

ROS are associated with tumorigenicity [66, 67]. For example, cell lines carrying a frame-shift of the NADH dehydrogenase (respiratory complex I) subunit 5 gene (ND5) have increased ROS levels and show enhanced tumor growth when implanted in nude mice [67].

Besides ROS, Ca^2 is another molecule that could serve as mitochondrial signaling molecule and influence cell functions. Overload of Ca^{2+} in mitochondria can induce mitochondrial membrane permeability transition and thus decrease the mitochondrial membrane potential and lead to cell apoptosis [68]. Conversely, a decrease in the mitochondrial membrane potential inhibits Ca^{2+} import into mitochondria, resulting in increased cytosolic Ca^{2+} . Cytosolic Ca^{2+} activates PI3K-AKT pathways to regulate gene expression for maintaining Ca^{2+} homeostasis, glucose metabolism, apoptosis and tumorigenesis [69]. For example, when non-tumorigenic mouse myoblast C2C12 cells are treated with either ethidium bromide or with mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), cellular mitochondria. The accumulation of cytosolic Ca^{2+} subsequently induces cell invasion measured by Matrigel invasion assays [70].

The study of Ca^{2+} effect on mitochondria led to discovery of the permeability transition pore (PTP) [71]. In response to high levels of Ca^{2+} within the matrix or the decreased concentration of adenine nucleotide, the PTP will be opened leading to the release of proapototic factors into the cytosol. Desensitization to the inducer of PTP opening in cancer cells thus could confer resistance of cancer cells to certain chemotherapeutic agents [42, 68].

2.2 Mitochondrial permeability transition pore and cancer

Mitochondrial permeability transition pore (PTP) is a multi-protein complex located at the mitochondria membrane. The composition of mitochondrial PTP is still under debate. It is thought to involve translocator protein (TSPO), voltage dependent activated channel (VDAC), adenine nucleotide translocase (ANT) and cyclosporin-Asensitive cyclophilin D (CYPD). Hexokinase II and mitochondrial creatine kinase (mtCK) are also reported to be associated with the mtPTP complex (Figure 1.2) [72-74]. Recent data also suggest that ATP synthase is a key component of the PTP complex [75].

In normal cell physiology, the mitochondrial PTP is responsible for transporting metabolites, particularly mediating ATP/ADP flux between the mitochondria and cytosol [74]. In some circumstance, the PTP controls mitochondria homeostasis via permeability transition, which is an abrupt increase of inner membrane permeability to protons, water and solutes with molecular masses of < 1500 Da [74]. These events occur along with opening of PTP, which results in mitochondrial depolarization and mitochondrial matrix swelling [74, 76].

The opening/ closure of the PTP is controlled by many factors [77]. The closure of PTP can be induced by cyclosporin A, Mg²⁺, bongkrekic acid, high ADP/ATP or acidic conditions (pH<7), while the opening of PTP can be induced by a decreased mitochondrial potential, adenine nucleotide depletion, increased mitochondrial matrix Ca²⁺, and ROS [78, 79]. When the permeability transition is activated, PTP will form an unspecific pore, allowing massive entry of water resulting in matrix swelling, remodeling of cristae and local ruptures of the outer membrane [78]. Opening of the mtPTP also



Figure 1.2 A proposed model for the permeability transition pore complex.

Mitochondrial permeability transition pore (mtPTP) complex is proposed to be composed of voltage dependent activated channel (VDAC) at the outer membrane, the tranlocator protein (TSPO) which interacts with VDAC at the outer membrane, adenine nucleotide translocase (ANT) at the inner membrane, and peptidyl–prolyl isomerase cyclophilin D (CYPD) which interacts with ANT. There is evidence that hexokinase II (HK II) and mitochondrial creatine kinase (mtCK) are also associated with this complex. During the opening of PTP, pro- and anti-apoptotic BCL-2 family members including Bcl-2 and Bax are involved and play regulatory roles. releases cytochrome c from the inter-membrane space into the cytosol to initiate apoptosis. During this process, pro- and anti-apoptotic BCL-2 family members such as Bcl-2 or Bax could block or promote the opening of PTP, respectively (Figure 1.2) [80].

Many inducers of PTP opening are elevated in cancer cells. For example, tumor cells usually confront hypoxia, in which case they have limited access to oxygen in primary tumor mass or during metastasis. Hypoxia can result in PTP opening, probably through upregulating pro-apoptotic factors by the hypoxia-inducible factor (HIF) transcription factor [81-83]. In another scenario, cancer cells need to evade detached-induced cell death or "anoikis" to survive when they detach from their original site during metastasis. Such detachment from neighboring cells or matrix could lead to activation of PTP and subsequence cell death [84]. In addition, increased levels of ROS are usually detected in cancer cells [84]. ROS are potent inducers of PTP, which may also lead to overload of cytosolic Ca²⁺ [85, 86]. Both ROS and Ca²⁺ can induce permeability transition and subsequence apoptosis. Therefore, the increased levels of PTP inducers require the ability of cancer cells to inhibit PTP opening, so that they can survive. How cancer cells become tolerant of increased PTP inducers is unclear.

Differential expression of many components of PTP in cancer cells may provide an explanation for how some cancer cells become resistant to the increased PTP inducers. For example, TSPO is overexpressed in breast, ovarian, hepatic and colon carciniomas compared to their normal tissues [87, 88]. TSPO is suggested to regulate apoptosis through its interaction with VDAC [87, 88]. Hexokinase II is upregulated in tumors, which could contribute to the increased glycolysis in cancer cells [72-74]. Increased expression of ANT2, not ANT1, is observed in various cancers [89]. Since

overexpression of ANT1 but not ANT2 induces apoptosis in HeLa cells, upregulation of ANT2 in cancer cells may confer cancer cell resistance to PTP induced apoptosis, meanwhile meeting the increased needs for ATP transport in cancer cells [90]. The interaction among the components of PTP complex is also important in the control of apoptosis. For example, the association of Bax with ANT is detected in the induction of mitochondria-involved apoptosis. Some pharmacological inhibitors, such as cyclosporin A (CsA, an inhibitor of PTP), furosemide (which inhibits mitochondrial translocation of Bax) or z-VDAD (a pan-caspase inhibitor), are reported to impair the association of Bax with ANT during apoptosis induction by etoposide (a DNA damaging agent), resulting in a decrease of apoptosis [91].

2.3 Mitochondria as a target for cancer therapy

Since mitochondria have critical impact on cancer cells, including energy production and apoptosis, mitochondria have emerged as a potential cancer therapeutic target. Several strategies have been proposed, including inhibition of tumor-specific alteration of mitochondrial metabolism, targeting mitochondrial membrane permeabilization, inducing cellular ROS, and regulation of the pro-apoptotic or antiapoptotic mitochondrial proteins [92]. Cancer cells display altered metabolism compared to normal cells. For example, solid tumors are often in a hypoxic microenvironment and show elevation of glycolysis. Thus, 2-deoxy-D-glucose (2DG), an inhibitor of glycolysis, is suggested to be useful in patients with advanced solid tumors and is currently in phase I clinical trials [93]. Some tumor cells show increased activity of mitochondrial oxidative phosphorylation (OXHPOS). Metformin, an antioxidant that inhibits

mitochondrial complex I, is currently used to treat type 2 diabetes, and is proposed to be used as a new cancer therapeutic agent for inhibiting TCA cycle and mitochondrial oxidative phosphorylation [58].

Induction of mitochondrial membrane permeabilization could be another strategy for cancer therapy. Many compounds exist that act on different components of the PTP complex to induce mitochondrial permeability transition (MPT) and subsequent apoptosis. For example, lonidamine, a putative ANT ligand, can permeabilize ANTcontaining (but not ANT-free) proteoliposomes. Combination therapy using lonidamine with a TSPO ligand, diazepam, inhibited glioblastoma growth in a Phase II clinical study [94, 95]. Agents that increase cytosolic Ca²⁺ concentration or stimulate ROS generation can also be used to trigger MPT. In addition, elimination of endogenous inhibitors of PTP opening, such as glucose, ATP, creatine phosphate and glutathione, can lead to indirect permeabilization of membrane [96].

Several compounds that induce overproduction of ROS or inhibition of antioxidant systems are under investigation for their potential in cancer treatment. For example, motexafin gadolinium has been shown to elevate ROS and inhibit antioxidants in cancer cells [97]. In a Phase III study, motexafin gadolinium plus brain radiotherapy extended the time for neurological progression in the patients with lung cancer that had metastasized to the brain [98].

In addition, imbalance of pro-apoptotic and anti-apoptotic Bcl-2 family proteins may induce cancer cell apoptosis via regulation of mitochondrial membrane permeabilization [96]. For example, the anti-apoptotic proteins, BCL-2 and BCL-XL, can interact with BH3-only pro-apoptotic proteins to inhibit apoptosis. Thus, BH3 mimetics,

such as ABT737, can compete with BH3-only protein for the binding of BCL-2 or BCL-XL to induce cell death [99].

3. Translocator protein (TSPO)

3.1 Introduction to TSPO and its ligands

Translocator Protein (TSPO), also known as peripheral-type benzodiazepine receptor (PBR), was first identified as a second binding site for the benzodiazepine, diazepam, which is a widely prescribed psychoactive drug [100, 101]. TSPO is highly expressed in secretory and glandular tissue, especially in steroidogenic cells [100, 102]. In cancer and neurological diseases, TSPO levels are upregulated [103].

Several putative transcription factor binding sites are present in the promoter of the murine TSPO. These include v-erythroblastosis virus E26 oncogene homolog, AP1, specificity protein 1/specificity protein 3 (Sp1/Sp3), GATA,SOX, AP2, Ik2,and SRY [102, 104]. The transcript levels of TSPO can be modulated by multiple stimuli, including IL-1, TNF-a, serotonin, norepinephrine, dopamine, peroxisome proliferators, and ginkgolide B [105-107]. In non-steroidogenic cells, such as NIH-3T3 fibroblasts, phorbol-12-myristate 13 acetate (PMA) induces TSPO expression through activation of PKCɛ. In steroidogenic cells, such as MA-10 Leydig, the high levels of TSPO are associated with the high constitutive expression of PKCɛ [108]. PKCɛ controls the expression of TSPO through Raf-MEK1/2-ERK1/2 signaling pathway, resulting the binding of c-Jun and STAT3 transcription factors to the TSPO promoter [108].

TSPO plays multiple roles in cells, including regulation of steroid biosynthesis, protein import, porphyrin transport and heme biosynthesis, immuno-modulation, as well as cellular respiration [100]. Targeted deletion of TSPO in mice results in early embryonic lethality, suggesting that TSPO is an indispensable gene [109].

Endogenous ligands of TSPO include cholesterol, porphyrins and the protein known as diazepam-binding inhibitor (DBI) [100, 110]. Cholesterol binds to TSPO with high affinity at the cytosolic carboxyl terminus, where a cholesterol recognition amino acid consensus (CRAC) domain is identified [111, 112]. The other ligands bind to a region at the amino terminus. The proteins in the PTP complex such as VDAC can influence the binding affinity of the ligands. Many synthetic ligands can also bind to TSPO with high affinity. These include the benzodiazepine diazepam, the isoquinoline carboxamide PK 11195, the benzodiazepine Ro5-4864, and indole derivatives FGIN-1-27 [100, 110]. TSPO ligands are commonly used to study the expression and function of TSPO.

3.2 TSPO in neuropsychiatric disorders

The expression levels of TSPO are low in the normal central or peripheral system, but are dramatically upregulated in response to damage in the peripheral nervous system, injured brain regions, neurodegenerative diseases and brain tumors [110]. Therefore, TSPO is currently under investigation as a biomarker of brain inflammation and various neuropathologies. Potent TSPO ligands for detecting brain damage, brain tumors or neurodegenerative disease are under development [110]. For example, [11C](R)-PK11195 is used for positron emission tomography (PET) studies in neuroinflammation [113]. Moreover, TSPO has been suggested as a target for neurotherapy and TSPO ligands have shown to have beneficial effects on neuronal viability or neurite

outgrowth [110]. For example, the TSPO ligand SSR180575 promotes functional neuron recovery in rats with injured facial nerves [114] and is currently under Phase II study in patients with diabetic peripheral neuropathy (ClinicalTrials.gov identifier: NCT00502515).

3.3 TSPO in cancer

Elevated levels of TSPO have been found in many types of tumors, including breast cancer, prostate cancer and colorectal cancer [87, 88]. In breast cancer, increased levels of TSPO parallel the invasive and metastatic ability of tumors when normal breast tissue, fibroadenomas, primary and metastatic adenocarcinomas are compared [88]. Levels of TSPO are also correlated with the aggressiveness of breast cancer cells. Higher levels of TSPO mRNA are detected in the more aggressive MDA-MB-231 cells, with lower TSPO levels in the less aggressive MCF7 cells [87, 115]. More aggressive cancer cells tend to have higher proliferation rates and metastatic potential. Overexpression of TSPO in MCF7 cells increases proliferation, whereas silencing of TSPO in MDA-MB-231 cells decreases proliferation [116]. In addition, overexpression of TSPO in C6 rat malignant glioma cells promotes cell migration [117]. The contribution of TSPO to breast cancer cell migration is unknown.

3.4 The application of TSPO ligands in cancer

TSPO ligands are often used to investigate the expression and function of TSPO in various cancer types. For example, a TSPO-targeted near-infrared (NIR) probe (NIR-conPK 11195) has been used to image human breast adenocarcinoma cells in xenograft models [118]. In addition, in clinical PET imaging studies of glioma, [¹⁸F]

PBR06, a high-affinity aryloxyanilide-based TSPO imaging ligand, is a candidate probe for quantitative assessment of TSPO expression [119]. Thus, TSPO ligands could be useful in cancer imaging and diagnosis.

In addition to the potential use in tumor detection, TSPO ligands are also being investigated as possible cancer treatment agents. Multiple studies show that TSPO ligands impact cell proliferation and apoptosis. For example, PK 11195 is reported to cause cell cycle arrest and inhibit proliferation in human hepatocellular carcinoma cells [120]. PK 11195 induces apoptosis in some cancer cells including breast cancer MCF7 cells [121], C6 glioma cells [117], chronic lymphocytic leukemia (CLL) cells [122] and human prostate cancer cells [123]. The apoptosis induced by PK 11195 has been attributed to mitochondrial membrane depolarization along with cytochrome c release [122]. Moreover, TSPO ligands such as PK 11195 and Ro5-4864 can also sensitize cancer cells to the apoptosis induced by various chemotherapeutic agents [120]. For instance, PK 11195 sensitizes human hepatocellular carcinoma cells to induction of apoptosis in combination with chemotherapeutic agents, including paclitaxel, docetaxel, and doxorubicin [120]. Ro5-4864 enhances apoptosis induced by chemotherapeutic agents in Jurkat cells [124]. Taken together, TSPO ligands could potentially serve as anti-cancer agents [96, 122, 125]

The mechanism of action of PK 11195 and Ro5-4864 is not yet well understood. Whether these ligands act via TSPO is still under debate. Knockdown of the TSPO gene did not influence death of nutrient-depleted HeLa cells induced by PK 11195 [125]. Low-affinity binding sites for PK 11195 and Ro5-4864 have been identified in TSPO

knockdown cells, which may indicate additional potential acting sites of PK 11195 and Ro5-4864 [125].

4. Adenine nucleotide translocase (ANT)

4.1 Introduction of ANT

Adenine nucleotide translocator (ANT) resides in the mitochondrial inner membrane with six transmembrane helices [60]. ANT controls ADP/ATP flux between mitochondria and cytosol. In most cases, ANT is responsible for simultaneously transporting ATP out of mitochondria and importing ADP from the cytosol. When yeast cells have failure in OXPHOS, ATP is imported into matrix and the mitochondrial membrane potential is maintained [126, 127]. The reversed nucleotide transport is important for cell viability when mitochondrial is damaged. Based on theses findings, it has been suggested that ANT can exchange ADP/ATP in a reverse direction [126].

During ATP/ADP transport, ANT undergoes conformational changes toward the cytoplasm (c-conformation) or toward the mitochondrial matrix (m-conformation) [128, 129]. ANT ligands have been used to study ANT structure and function. For example, bongkrekic acid (BA) and carboxyatractyloside (CAT) are ANT inhibitors, which bind to ANT at the m- and c-conformation respectively, and block the conformation change required for ATP transport [130, 131]. It is still controversial whether ANT functions as a monomer or a dimer. Several studies using native gel electrophoresis, ultracentrifugation and cross-links have suggested a dimeric structure of ANT [129, 132, 133]. Some other reports however have suggested that ANT can function as a

monomer [134]. Furthermore, the crystal structure of bovine ANT revealed that ANT is monomeric [135].

Four ANT isoforms are present in the human genome. ANT1, 2, and 3 share 80% amino acid similarity in their sequences, while ANT4 shares 66%-68% identity with the others[136]. ANT1, ANT2, and ANT4 are expressed in a tissue-specific manner, whereas ANT3 is ubiquitously expressed [137]. ANT1, which is highly expressed in differentiated tissue such as skeletal muscle, heart and brain, is the best-studied ANT isoform [89]. ANT2 is specifically expressed in undifferentiated tissues or highly proliferating cells, such as such as liver, kidney, as well as in tumors [89]. ANT4 can be detected in brain, liver and testis [138, 139]. Whether ANT isoforms can compensate for each other remains obscure. The expression shift between ANT1 and ANT2 is only found in dilated cardiomyopathy, where the expression of ANT1 increased while ANT2 expression decreased [140]. Knockout of ANT1 in mice leads to mitochondrial myopathy, while ANT2 knockout mice results in embryonic lethality [141].

ANT has two major identified cellular roles, transporting ADP/ATP and potentially participating in the permeability transition during apoptosis. The function of ANT is possibly regulated by the interaction between ANT and other mitochondrial membrane proteins [142]. For example, a study using ANT-containing proteoliposomes shows that the anti-apoptotic protein Bcl-2 enhances the ADP/ATP exchange, whereas, pro-apoptotic protein Bax inhibits such translocase activity of ANT [143].

4.2 ANT and energy metabolism
ANT supplies energy for cellular functions by exporting ATP from the mitochondria. ANT therefore is important in cellular energy metabolism. Both ANT1 and ANT2 are found in the portion of the innner membrane of the mitochondria that is associated with the outer membrane, whereas only the ANT2 isoform localizes to the cristae where the oxidative respiration occurs, suggesting that ANT2 may be more coupled to energy production [144]. The physical interaction between ANT2 with ATP synthase, named the "ATP synthasome", revealed the importance of ANT2 in the ATP generation from OXPHOS [145]. In contrast, some studies have suggested a role of ANT2 in glycolysis. ANT2 shares 53% sequence identity with AAC3 gene, one of the three isoforms of the yeast ADP/ATP carriers [146]. AAC3 is essential for yeast to grow on a fermentable substrate and is regulated by heme through the ROX1 factor which is related to oxygen repression [147]. When transformed with ANT2, the yeast cells that were depleted of all AAC genes were able to restore growth under anaerobic (oxygen free) conditions [148]. These data suggested that ANT2 could be important in glycolysis under hypoxic conditions. In addition, comparison of proximal promoter between yeast AAC3 gene and human ANT2 led to the recognition of the glycolysis-regulated box (GRBOX) which is a ROX1-like binding motif involved in aerobic glycolysis [148]. Despite these findings in yeast, no direct evidence exists for the for ANT2 regulation of glycolysis in human cancer cells.

4.3 ANT and apoptosis

A number of studies have suggested that ANT is involved in the opening of PTP and the subsequent apoptosis. Overexpression of ANT1 in cervical carcinoma HeLa

cells results in disruption of membrane potential, ATP depletion and subsequent cell apoptosis [90]. Overexpression of ANT3 also leads to apoptosis in HeLa cells apoptosis [149]. In contrast, overexpression of ANT2 does not result in cell apoptosis [90]. However, depletion of ANT2 by transient transfection of ANT2 siRNA synergizes the apoptogenic effect by lonidamine on cell death in HeLa cells [150]. Moreover, stable knockdown of ANT2 by shRNA transfection disrupted mitochondrial membrane potential and induced apoptosis [151]. Thus it is hypothesized that ANT2 may serve as a therapeutic target for breast cancer.

4.4 ANT2 in cancer

The levels of ANT2 are upregulated in cancer tissues compared with normal tissues, as well as in cancer cell lines, such as HeLa, MCF7 cells, MDA-MB-231 cells, compared with non-tumorigenic cell lines, such as fibroblasts or the epithelial cell line MCF10A [150, 151]. Silencing of ANT2 modestly increases reactive oxygen species, as well as reduces ATP levels without affecting glycolysis in HeLa cells. Moreover, the effect by ANT2 silencing is not compensated by other endogenous ANT isoforms [150]. In the aggressive breast cancer cell line, MDA-MB-231, silencing of ANT2 by vector-based gene silencing reduces cellular ATP levels, induces cell cycle arrest, and decreases cell proliferation [151]. In HER2-amplified SKBR3 cells, silencing of ANT2 using vector-based RNA interference approach inhibited cell migration and invasion. This phenotype is proposed to be due to inhibition of PI3K/AKT and subsequent decreased expression of matrix metalloproteinase 2 (MMP2) and MMP9 upon ANT2

silencing [152]. Suppression of migration and invasion by ANT2 silencing is also observed in MDA-MB-231 and MCF7 cells (data not shown) [152].

Several studies have provided evidence about targeting ANT2 as a potential cancer treatment strategy. Silencing ANT2, but not ANT1, overcomes the protection from Bcl-2 overexpression, and sensitizes cancer cells to death induced by lonidamine [150]. Silencing of ANT2 in breast cancer cells confers sensitivity to the apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL) [153]. Moreover, depletion of ANT2 also inhibits tumor growth in xenograft mouse models [151].

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CHAPTER II

THE 18-kDA TRANSLOCATOR PROTEIN (TSPO) DISTRUPTS MAMMARY EPITHELIAL MORPHOGENESIS AND PROMOTES BREAST CANCER CELL MIGRATION

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1. Abstract

Mitochondria play important roles in cancer progression and have emerged as viable targets for cancer therapy. Increasing levels of the outer mitochondrial membrane protein, 18-kDa translocator protein (TSPO), are associated with advancing breast cancer stage. In particular, higher TSPO levels are found in estrogen receptor (ER)negative breast tumors, compared with ER-positive tumors. In this study, we sought to define the roles of TSPO in the acquisition of breast cancer malignancy. Using a threedimensional Matrigel culture system, we determined the impact of elevated TSPO levels on mammary epithelial morphogenesis. Our studies demonstrate that stable overexpression of TSPO in mammary epithelial MCF10A acini drives proliferation and provides partial resistance to luminal apoptosis, resulting in enlarged acinar structures with partially filled lumen that resemble early stage breast lesions leading to breast cancer. In breast cancer cell lines, TSPO silencing or TSPO overexpression significantly altered the migratory activity. In addition, we found that combination treatment with the TSPO ligands (PK 11195 or Ro5-4864), and lonidamine, a clinical phase II drug targeting mitochondria, decreased viability of ER-negative breast cancer cell lines. Taken together, these data demonstrate that increase in TSPO levels at different stages of breast cancer progression results in the acquisition of distinct properties associated with malignancy. Furthermore, targeting TSPO, particularly in combination with other mitochondria-targeting agents, may prove useful for the treatment of ER-negative breast cancer.

2. Introduction

Breast cancer is the second most frequently diagnosed cancer and one of the leading causes of cancer death among U.S. women [1]. Estrogen receptor (ER)negative breast cancers are typically more aggressive than ER-positive tumors [2,3]. In the absence of HER2 overexpression, there are no currently available targeted therapies to treat ER-negative breast cancer. Chemotherapeutic agents can be useful in treating patients with ER-negative breast tumors but resistance and toxicity limit efficacy [1,2,4]. Mitochondria play central roles in regulating bioenergetics, metabolism and cell death. Dysregulation of mitochondria in cancer contributes to the acquisition of multiple malignant phenotypes, including aberrant proliferation, impaired apoptosis and enhanced invasion and metastasis [5,6,7]. Therefore, targeting mitochondria has emerged as a potential strategy for breast cancer therapy [5,7].

Translocator protein (TSPO), first known as the peripheral-type benzodiazepine receptor, is a five-trans-membrane domain protein that resides primarily in the outer mitochondrial membrane [8,9]. As a component of the mitochondrial permeability transition pore (PTP) complex, TSPO is believed to be involved in the opening of the PTP, a critical step in initiating apoptosis [10,11,12]. In addition, TSPO participates in multiple cellular activities, including cholesterol transport, steroidogenesis, cell proliferation, and cellular respiration [8]. Elevated TSPO levels are found in multiple types of cancer. Increased TSPO levels are found in both prostate and colorectal tumors compared with their surrounding non-tumoral tissues [13,14,15]. Progressive elevation of TSPO levels is associated with the degree of invasiveness of breast cancer

[13,15,16]. For instance, higher levels of TSPO are found in ductal carcinoma *in situ* (DCIS) compared with normal breast tissue; and invasive breast tumors have higher TSPO than do DCIS. In particular, higher TSPO is found in ER-negative than in ER-positive breast tumors and cell lines [13,16,17]. Overexpression of TSPO increases proliferation of ER-positive, luminal MCF7 cells, whereas silencing of TSPO leads to a decrease of proliferation of ER-negative, claudin-low MDA-MB-231 cells [18].

Synthetic TSPO ligands have been reported to inhibit proliferation and induce apoptosis in multiple cancer cell lines, including MCF7 cells [19]. Both the isoquinoline PK 11195 and the benzodiazepine Ro5-4864 facilitate apoptosis induced by certain chemotherapeutic agents [20,21,22]. For instance, PK 11195 sensitizes human hepatocellular carcinoma cells to apoptosis induction by paclitaxel, docetaxel, and doxorubicin [21].

The functional impact of increased TSPO levels on mammary morphogenesis and early stage breast cancer has not been investigated. The morphogenesis of mammary epithelial cells in 3D Matrigel culture shares many features with mammary gland development *in vivo* and hence has been used to investigate the impact of oncogenes on breast cancer development [23,24]. In 3D Matrigel, a single immortalized, non-transformed mammary epithelial MCF10A cell undergoes a well-defined morphogenic program to form a growth-arrested, well-polarized, hollow acinus that resembles the acinar structure of mammary lobules [23,24,25]. During MCF10A 3D morphogenesis, proliferation continues for about 15 days and diminishes thereafter. Apoptosis of luminal cells typically initiates between day 6 and day 8, and luminal clearance is complete by about day 20 yielding hollow acinar structures. Cessation of

proliferation as well as apoptosis of luminal cells is required for lumen formation [26]. Overexpression of certain oncogenes in MCF10A, including ErbB2/HER2, leads to increased proliferation and deficient luminal apoptosis in 3D Matrigel, resulting in enlarged structures with filled lumens, resembling phenotypes found in early stages of breast cancer, such as atypical hyperplasia and DCIS [26,27,28].

To better understand the potential roles of TSPO in breast cancer development and progression, the morphogenesis of MCF10A cells stably overexpressing TSPO was evaluated in 3D Matrigel culture. MCF10A-TSPO cells develop larger acini with enhanced proliferation and impaired luminal apoptosis when compared to control MCF10A acini. We also demonstrate that increased TSPO levels promote breast cancer cell migration, suggesting that TSPO may contribute to the development of invasive breast cancer. Finally, combining TSPO ligands (PK 11195 or Ro5-4864) with the mitochondrial targeting agent, lonidamine, potentiated apoptosis in ER-negative breast cancer cell lines. These studies, taken together, provide evidence that elevation of TSPO levels is sufficient to promote multiple malignant phenotypes, including increased proliferation, resistance to apoptosis, and enhanced migration. Furthermore, TSPO ligands, in combination with other agents that target the mitochondria, might be an effective approach for treating advanced breast cancer.

3. Experimental procedures

3.1 Cell lines, Antibodies, Chemical compounds, and siRNAs

The human mammary epithelial cell line, MCF10A, a gift from Dr. Joan Brugge (Harvard Medical School, Boston, MA, USA), was maintained as previously described

[23]. Breast cancer cell lines (MCF7, MDA-MB-231 and BT549) were obtained from ATCC (Manassas, VA, USA). MDA-MB-231, MCF7 and BT549 cells were cultured in DMEM (Gibco BRL, Paisley, PA, USA) supplemented with 10% FBS and antibiotics (Penicilin/Streptomycin, 50 µg/ml). Antibody against TSPO was obtained from Novus Biological (Littleton, CO, USA). Anti-Flag M2, HA and actin monoclonal antibodies were from Sigma-Aldrich (St Louis, MO, USA). Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 680-conjugated anti-goat IgG were from Li-COR Biosciences (Lincoln, NE, USA). Horseradish peroxidase-conjugated anti-rabbit IgG was from Bio-Rad (Hercules, CA, USA). The active caspase-3 and PARP antibodies were from Cell Signaling Technologies, Inc. (Danvers, MA, USA), and the Ki-67 antibody was from Abcam (Cambridge, MA, USA). PK 11195, Ro5-4864 and Ionidamine were also from Sigma. TSPO siRNAs (siTSPO #1: 5'-GAGAAGGCUGUGGUUCCCC-3' and siTSPO #2: 5'-CACUCAACUACUGCGUAUG-3') were synthesized based upon previously published sequences [29] by Dharmacon (Lafayette, CO, USA).

3.2 Stable cell populations

Stable pools of FLAG tagged TSPO-expressing and control MCF10A cells were generated after retroviral transduction with pLXSN-TSPO-FLAG or an empty pLXSN vector. The TSPO-FLAG fragment was subcloned from pLH-Z₁₂I-PL²-TSPO-FLAG vector to the retroviral vector pLXSN to construct pLXSN-TSPO-FLAG vector. pLH-Z₁₂I-PL²-TSPO-FLAG was constructed by PCR amplification of the TSPO coding sequence from pReceiver-TSPO-HA-HIS (a kind gift from Drs. Lookingland and Goudreau, Michigan State University, MI, USA) and subcloning into pLH-Z₁₂I-PL² empty vector. The

coding sequence for the FLAG epitope tag was incorporated into PCR primers to generate the expression vector for TSPO with a C-terminal FLAG epitope tag. The construct was fully verified by sequencing. To generate retrovirus, pLXSN-TSPO-FLAG was transfected into the 293GPG packaging cell line (a gift from R. Mulligan, Harvard Medical School, Children's Hospital, Boston, MA, USA) [30]. The retrovirus with integrated TSPO was used to infect MCF10A to generate MCF10A-TSPO cells. The stable pools of MCF10A-TSPO cells were selected in 300 µg/ml G418 and maintained in 50 µg/ml G418. The control MCF10A cell line pool expressing the empty vector pLXSN (MCF10A-pLXSN) was generated in an analogous fashion.

3.3 Transfection and cell lysis

Transfection of siRNAs (20 nM) or a universal control siRNA was performed using INTERFERin (Polyplus-transfection, New York, NY, USA) according to the manufacturer's instructions. Transfection of the TSPO expression vector or a control vector was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells cultured on 2D plastic were lysed in Triton X-100 lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM Na₃VO₄, 2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Protein concentrations of cellular lysates were measured by Bradford protein assays (Biorad, Pierce, Rockford, IL, USA).

3.4 Gel electrophoresis and immunoblotting analysis

Proteins were subjected to SDS–polyacrylamide gel electrophoresis and transferred from gel to Immobilon-FL PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% milk or Odyssey blocking buffer (Li-COR Biosciences, Lincoln, NE, USA) and incubated with appropriate antibodies, followed by incubation with a horseradish peroxidase-conjugated or an IRDye-conjugated secondary antibody, and developed by chemiluminescence method or visualized by fluorescence using the Li-COR Odyssey infrared scanner (Li-COR Biosciences, Lincoln, NE, USA), respectively.

3.5 Immunofluorescence

For 2D cultures, cells were seeded on coverslips for 24 h, incubated with MitoTracker Red (Sigma-Aldrich, St Louis, MO, USA) for 30 min, and fixed with 3.7% formaldehyde. After fixation, cells were permeabilized with 0.5% v/v Triton X-100 for 5 min and blocked in 4% w/v bovine serum albumin (BSA) in PBS for 30 min at room temperature. Coverslips were then incubated overnight at 4 °C with anti-FLAG antibody or anti-TSPO antibody (1:1000 dilution) in PBS containing 2% BSA. Coverslips were washed three times for 5 min each with PBS, followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG (1:200 dilution) or Alexa Fluor 680-conjugated anti-goat IgG (1:500 dilution) for 60 min at room temperature. After washing for three times with PBS, the cells were stained with 4'-6-Diamidino-2-phenylindole (DAPI, 0.5 µg/ml) for 15 min and mounted. Images were taken using an Olympus FV1000 confocal laserscanning microscope.

3.6 3D morphogenesis assay

A single cell suspension of 3000 cells was seeded per well on solidified Matrigel (BD Biosciences, San Jose, CA, USA) in overlay medium [23,30] (DMEM/F12 supplemented with 2% horse serum; 1.5 ng/ml EGF (Peprotech, Rocky Hill, NJ, USA); 10 µg/ml insulin; 100 µg/ml hydrocortisone; 1 ng/ml cholera toxin; 50 U/ml streptomycin/penicillin and 3% Matrigel). Cultures were replenished with fresh medium every four days [23,30]. Phase contrast images were acquired with QCapturePro. All immunofluorescence procedures were performed as previously described [23] for antibodies against Ki-67 and cleaved caspase-3. Nuclei were stained with 5 µg/ml DAPI and cells were mounted with anti-fade reagent Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Fluorescence microscopy was performed on an Olympus Fluoview laser-scanning microscope. Acinar structures were analyzed with ImageJ to determine size, by digitally tracing the circumference of acini and expressing the cross sectional area as pixels. Statistical analysis was performed with Student's *t*-test.

3.7 Transwell migration

Chemotactic transwell migration was performed using Boyden transwell chambers (8 µm pore size; Corning Costar, Cambridge, MA, USA). Cells were deprived of serum overnight, trypsinized and introduced into the upper chamber (5 X10⁴ for MDA-MB-231 and BT549; 10⁵ for MCF7). The chemoattractant in the lower chamber was medium supplemented with 5% FBS (for MDA-MB-231 and MCF7 cells), or 1% FBS (for BT549 cells). After a certain time of migration (24 h for MCF7, 6 h for MDA-MB-231, 4 h for BT549), cells were fixed and stained with crystal violet. Migrated cells were

quantified by counting five randomly chosen fields. The experiments were performed in duplicate wells and each experiment was performed at least three times as indicated.

3.8 Trypan blue assay

MDA-MB-231 or BT549 cells (1X10⁵) were seeded in 12-well plates and after 16 h were treated with PK 11195 or Ro5-4864 and/or lonidamine or DMSO (vehicle) at the indicated concentrations. After 24 h, 0.4% solution of trypan blue in buffered isotonic salt solution was used to stain dead cells. The number of blue-staining cells and the number of total cells were quantified using a hemacytometer. Percentage of cell death was expressed as blue-staining cells/total cells × 100%.

3.9 Statistical analysis

For 3D morphogenesis assays, box plots were generated to represent the quantified data using either OriginLab software or web-based programs from http://www.physics.csbsju.edu/stats/t-test.html. For migration assays and trypan blue assays, bar graphs with mean±standard deviation were generated to represent the data. To compare the difference among experimental groups, student's *t*-tests were conducted and p<0.05 was considered as statistically significant.

4. Results

4.1 Stable expression of TSPO in human mammary epithelial MCF10A cells.

To investigate the role of TSPO in mammary morphogenesis, a stable pool of TSPO-overexpressing mammary epithelial cells, MCF10A-TSPO, was generated after

retroviral transduction with a pLXSN construct encoding recombinant TSPO with a Cterminal FLAG epitope tag. The control MCF10A cell line pool expressing the empty vector pLXSN (MCF10A-pLXSN) was generated at the same time. The levels of ectopically expressed and endogenous TSPO in the stable MCF10A populations were evaluated by immunoblotting using an anti-FLAG (Figure 2.1A, top panel) or anti-TSPO antibody (Figure 2.1A, bottom panel). Ectopically expressed TSPO did not affect the endogenous level of TSPO. Based upon densitometry, total TSPO levels were 2.5 fold higher in the MCF10A-TSPO population compared to the control MCF10A-pLXSN population. The subcellular localization of endogenous and ectopically expressed TSPO in the population of MCF10A-TSPO cells showed similar distribution as judged by immunofluorescence staining with anti-FLAG (Figure 2.1B, top panel) or anti-TSPO (Figure 2.1B, *bottom* panel) antibody. FLAG-tagged TSPO was stably expressed in about 90% of the cells in the stable MCF10A population (data not shown). Confocal imaging revealed that both the endogenous and the expressed TSPO co-localized largely with MitoTracker Red, a mitochondrial marker, indicating that both endogenous and exogenous TSPO localize to the mitochondria.

4.2 Elevation of TSPO levels increases acinar size and promotes proliferation during mammary epithelial morphogenesis.

To evaluate the potential role(s) of TSPO in breast cancer development, the morphogenesis of MCF10A-TSPO and control MCF10A-pLXSN cells in 3D Matrigel culture was monitored over time. Images of cultures at day 15 and day 20 were acquired and used to evaluate the size of acini (Figure 2.2A). Both control and TSPO-



Figure 2.1 MCF10A cells stably overexpressing TSPO. Stable vector control (pLXSN) and TSPO-FLAG overexpressing cells (TSPO) were generated as described under *Experimental procedures*. A. Expression of TSPO was detected by immunoblotting using antibodies against FLAG tag (*top* panel) or TSPO (*bottom* panel), respectively, with actin as a loading control. B. Immunostaining of ectopically expressed and endogenous TSPO using antibodies against FLAG tag (*top* panel) or TSPO (*bottom* panel) or TSPO (*bottom* panel). MitoTracker Red was used to stain mitochondria and cells were imaged by confocal microscopy. Scale bar: 10 μm.



Figure 2.2 Overexpression of TSPO increases acini size. Control MCF10A-pLXSN and MCF10A-TSPO cells were seeded in Matrigel as described under *Experimental procedures*. A. Images were acquired on day 15 and day 20 of culture. Scale bar: 50 µm. B. Maximal cross-sectional area in pixels of individual acini was determined using

Figure 2.2 (cont'd)

ImageJ software, and plotted as a box plot. *Black line*, median value; *box*, interquartile range; *solid square*, mean; *open circles*, outliers. Data are from a representative set of three independent experiments in which ~100 acini per condition were measured. P-value determined by Student's *t*-test. *** p<0.001 indicates a significant difference between TSPO and pLXSN control. C. The cross-sectional area of stable TSPO-overexpressing acini relative to the control acini (=100%). Each column represents results from 3 independent experiments in which a total of at least 300 acini per condition were measured. Error bar: SD. P-value was determined by Student's *t*-test. *

overexpressing acini reached their maximal size by day 15 (Figure 2.2B). The elevation of TSPO expression resulted in a 30% increase on average in acinar cross-sectional area compared to the control vector-expressing acini (Figure 2.2 C). Quantitative analysis of maximal cross sections obtained by confocal imaging showed that the outer layer of MCF10A-TSPO acini had both a greater number of cells and a higher cell density than the control pLXSN acini (Figure 2.3). At earlier times (day 5 and day 8) TSPO-expressing structures were also modestly larger than pLXSN-control structures (Figure 2.4).

To investigate whether TSPO-induced proliferation contributes to the enlarged acinar phenotype, the number of proliferating cells within individual acini was quantified from immunofluorescence images acquired after staining with the proliferation marker Ki-67 at day 15, when cells of MCF10A acini are known to cease proliferation [26]. While no Ki-67 staining was observed in the majority of the control structures, most MCF10A-TSPO structures were Ki-67 positive (Figure 2.5A), with at least one Ki-67 positive cell in 84% of MCF10A-TSPO acini, compared to 41% of control structures. More than 5 Ki-67 positive cells were observed in 43% of MCF10A-TSPO acini, compared to 5% of control MCF10A structures (Figure 2.5B). In agreement with our findings at day 15, more Ki-67 positive cells were observed in MCF10A-TSPO acini at day 10 of mammary morphogenesis compared with control pLXSN structures (Figure 2.6). These data demonstrate that elevated expression of TSPO promotes proliferation during mammary epithelial morphogenesis, and that the enlarged acini observed upon TSPO overexpression is due, at least in part, to enhanced proliferation.



Figure 2.3 Stable TSPO overexpression increases the number and density of cells within the outer layer of acini. MCF10A-pLXSN and MCF10A-TSPO cells were seeded in Matrigel as described under *Experimental procedures*. Confocal images were acquired on day 15. The number of outer layer cells within, and the circumference of the maximal acinar cross section were quantified from confocal images using ImageJ software. The results are expressed relative to number of cells in the outer layer in the circumference of MCF10A-pLXSN acini (=100%) (*left* panel). Cell density within the outer layer was calculated as cell number of outer layer/circumference in arbitrary units. The results are expressed relative to outer layer cell density of MCF10A-pLXSN acini (=100%) (*right* panel). Results are based on 300 acinar structures for each condition, combined from three independent experiments. P-value was determined by Student's *t*-test. * p<0.05 indicates significant differences between TSPO-expressing acini and pLXSN control acini.



Figure 2.4 Overexpression of TSPO increases acini size at early stages of mammary morphogenesis. MCF10A-pLXSN (control) and MCF10A-TSPO cells were seeded in Matrigel as described under *Experimental procedures*. Confocal images were acquired on the indicated days. Cross-sectional area in pixels of individual acini from day 5 and day 8 was determined using ImageJ software, and plotted as a box plot (*left* panel). *Black* line, median value; *box*, interquartile range; *solid square*, mean; *open circles*, outliers. Data are combined from ~70 acini per condition. P-value was determined by Student's *t*-test. *** p<0.001 indicates a significant difference between TSPO-expressing and control (pLXSN) acini. Representative confocal images of control (pLXSN) and TSPO-expressing acini at day 8 are shown (*right* panel).


Figure 2.5 Overexpression of TSPO enhances proliferation during mammary epithelial morphogenesis. Control MCF10A-pLXSN cells and MCF10A-TSPO cells

Figure 2.5 (cont'd)

were seeded and cultured in Matrigel as described *Experimental procedures*. A. On day 15, the cultures were fixed and stained with 4',6'-diamidino-2-phenylindole (DAPI, *blue*) and anti-Ki-67 (*green*). Representative fluorescent images of control vector (pLXSN) and TSPO expressing acini are shown. Scale bar: 50 µm. Expanded image of a single acinus from pLXSN control or TSPO are shown. Scale bar: 10 µm. B. Cultures were scored for the number of acini containing 0, 1 to 5, or more than 5 Ki-67-positive cells based on at least 250 acini from each condition, combined from three independent experiments. Error bar: SD. P-value was determined by Student's *t*-test. * p<0.05 indicates a significant difference between TSPO and pLXSN control.



Figure 2.6 Overexpression of TSPO increases proliferation at early stage of mammary morphogenesis. MCF10A-pLXSN (control) and MCF10A-TSPO cells were seeded in Matrigel as described under *Experimental procedures*. At day 10, cultures of acini were fixed and stained with DAPI (*blue*) and anti-Ki-67 (*green*). Confocal images were acquired and representative images of control vector (pLXSN) and TSPO-expressing acini are shown (*left* panel). Scale bar: 20 µm. The number of Ki67-positive cells were quantified from at least 70 acini from each condition, and plotted as a box plot (*right* panel). *Black* line, median value; *box*, interquartile range; *open circles*, outliers. P-value was determined by Student's *t*-test. *** p<0.001 indicates a significant difference between TSPO and pLXSN control.

4.3 Increased expression of TSPO results in partially filled lumen during morphogenesis.

To investigate the impact of increased TSPO levels on lumen formation during morphogenesis, nuclei of control and TSPO-overexpressing acini were stained with DAPI at day 20, and serial confocal cross sections were examined for the presence of DAPI-stained cells within the lumen. While MCF10A-control acini were hollow at day 20 as expected, the TSPO-overexpressing acini were partially filled (Figure 2.7A). Both enhanced proliferation and inhibition of apoptosis can result in the accumulation of cells within the lumen [26]. We speculated that overexpression of TSPO may prevent apoptosis of luminal cells, resulting in incomplete clearance of cells from the lumen, a process that has been shown to involve caspase-3 [26]. To assess whether increased expression of TSPO inhibits luminal apoptosis, the presence of cleaved, activated caspase-3 in luminal cells was detected by immunofluorescence. Activated caspase-3 was detected in the lumen of both control and TSPO-expressing acini during morphogenesis at day 10, day 15 (data not shown) and day 20 (Figure 2.7B), suggesting TSPO overexpression does not completely suppress apoptosis. At the late stage of morphogenesis (day 20), only rarely were cells detected in the lumen of control acini, whereas large numbers of both viable (activated caspase-3 negative) and apoptotic (activated caspase-3 positive) cells were present in the lumen of the TSPOoverexpressing acini (Figure 2.7B). Quantification showed that TSPO-overexpressing acini retained on average 10-fold more viable cells within their lumen compared to control acini (Figure 2.7). Of the luminal cells in control acini nearly 90% were active caspase-3 positive, whereas in TSPO-overexpressing acini only 40% of the luminal



Figure 2.7 Overexpression of TSPO leads to partial filling of lumen during morphogenesis. Control MCF10A-pLXSN and MCF10A-TSPO cells were seeded and cultured in Matrigel. On day 20, cultures were fixed and stained with DAPI and anticleaved caspase-3 as described in *Experimental procedures*. A. Schematic diagram (*top* panel) of serial confocal cross sections of an acinus structure showing the relative position of the sections with respect to z-axis. Serial confocal cross-sections images of

Figure 2.7 (cont'd)

MCF10A-pLXSN and MCF10A-TSPO acini stained with DAPI (*bottom* panel). Scale bar: 20 μ m. B. Representative images of cleaved caspase-3 staining (*green*) were acquired using confocal microscopy. Scale bar: 25 μ m. C. The number of viable cells (active caspase-3 negative) per lumen was quantified, and plotted as a box plot. *Black* line, median value; *box*, interquartile range; *solid square*, mean; *open circles*, outliers. Data are from a representative set of three independent experiments in which ~90 acini per condition were measured. D. The percent of active caspase-3 positive cells in the lumen was quantified based on at least 250 acini combined from three independent experiments. Error bar: SD. P-value was determined by Student's *t*-test. ** p<0.01, *** p<0.001 indicate significant differences between TSPO and pLXSN control.

cells expressed active caspase-3 (Figure 2.7D). These data, taken together, suggest that increasing TSPO levels partially suppresses luminal apoptosis.

4.4 TSPO promotes breast cancer cell migration.

In addition to enhanced proliferation and resistance to apoptosis, breast cancer cells must acquire migratory ability for invasion and metastasis [6]. To examine the contribution of TSPO to breast cancer cell migration, we modulated the levels of TSPO by transient overexpression or silencing and performed transwell migration assays. Migrated cells were stained with crystal violet and quantified as described in Experimental procedures. TSPO was overexpressed in a poorly migratory ER-positive breast cancer cell line, MCF7, by transient transfection and migration was assessed in a transwell assay. Ectopic expression of HA-tagged TSPO was confirmed by immunoblotting (Figure 2.8A). Overexpression of TSPO increased migration of MCF7 cells by 1.5 fold (Figure 2.8B). In complementary experiments, we examined whether silencing of TSPO could suppress migration of highly invasive ER-negative breast cancer cells. Two different siRNAs, siTSPO #1 and siTSPO #2, were used to silence the TSPO gene in MDA-MB-231 cells. Transient transfection of each siRNA decreased TSPO expression by 70%, as determined by quantitative immunoblotting (Figure 2.9A). Using a commercially available TSPO antibody, we observed reduction of the immunoreactive 18 kDa band upon TSPO silencing, but did not observe changes in other immunoreactive bands (Figure 2.11). Transwell migration of the TSPO-depleted



Figure 2.8 Overexpression of TSPO promotes the migration of MCF7 cells. A. Control or TSPO expression vectors were transiently expressed in MCF7 cells. HAtagged TSPO was detected by immunoblotting using an antibody against the HA tag, with actin as a loading control. B. The control and HA-TSPO-expressing MCF7 cells were allowed to migrate toward 10% FBS for 24 h in a transwell assay as described under *Experimental procedures*. Images of crystal violet-stained migratory cells were taken from five random chosen fields (*upper* panel). The quantification results are expressed relative to the migration of control cells (=100%). Column (*bottom* panel): Mean of three experiments. Error bar: SD. P value was determined by Student's *t*-test. * p<0.05 indicates a significant difference between control and TSPO-overexpressing MCF7 cells.



А

В



Figure 2.9 Silencing of TSPO impairs migration of MDA-MB-231 breast cancer cells. A. Control siRNA and siRNA sequences against TSPO (siTSPO #1 or siTSPO #2) were used to transfect MDA-MB-231 cells. The extent of silencing was determined by immunoblotting with antibodies against TSPO, with actin used as a loading control. B. Control and TSPO-depleted cells were then subjected to transwell assays as described

Figure 2.9 (cont'd)

under *Experimental procedures*. Images of crystal violet-stained migratory cells were taken from five random chosen fields (*upper* panel). The quantification results are expressed relative to the migration of control cells (=100%). Column (*bottom* panel): Mean of three experiments. Error bar: SD. P-value was determined by Student's *t*-test. * p<0.05 indicates significant differences between control and TSPO-depleted cells.

MDA-MB-231 cells decreased by about 35% compared with control siRNA-transfected MDA-MB-231 cells (Figure 2.9B). Similar results were obtained upon silencing of TSPO in another ER-negative breast cancer cell line, BT549, which resulted in 40% decrease in migration in a transwell assay (Figure 2.10).

4.5 Combination of TSPO ligands with lonidamine potentiates apoptosis in breast cancer cells.

Our data demonstrate important roles of the mitochondrial protein TSPO in driving phenotypes associated with breast cancer malignancy, suggesting it could be a potential therapeutic target. The isoquinoline carboxamide TSPO ligand, PK 11195 sensitizes hepatocellular carcinoma cells to cytotoxic chemotherapy agents [5,21]. Ro5-4864, a benzodiazepine TSPO ligand, enhances apoptosis induced by chemotherapeutic agents in Jurkat cells [22]. Here, we tested the effect of combining PK 11195 or Ro5-4864 with lonidamine, another drug that targets the mitochondria, on viability using two ER-negative human breast cancer cell lines, MDA-MB-231 and BT549. Cells were treated with the indicated concentrations of PK 11195 and Ionidamine for 24 h. The percentage of cell death was determined using trypan blue exclusion assays. As shown in Figure 2.12 A and B, Ionidamine alone (grey bars) or PK 11195 alone, over a range of concentrations, failed to induce cell death, whereas combining both PK 11195 and lonidamine induced cell death dramatically. For instance, in MDA-MB-231 cells, either 50 µM PK 11195 or 400 µM lonidamine alone did not induce cell death compared to vehicle control, whereas a combination of 50 µM PK



Figure 2.10 Silencing of TSPO impairs migration of BT549 breast cancer cells. A. Control siRNA and siRNA sequence against TSPO (siTSPO #2) were used to transfect BT549 cells. The extent of silencing was determined by immunoblotting with antibodies against TSPO, with actin used as a loading control. B. Control and TSPO-depleted cells were then subjected to transwell assays as described under *Experimental procedures*. Images of crystal violet-stained migratory cells were taken from five random chosen fields (*upper* panel). The quantification results are expressed relative to the migration of control cells (=100%). Column (*bottom* panel): Mean of three experiments. Error bar: SD. P-value was determined by Student's *t*-test. * p<0.05 and ** p<0.01 indicate significant differences between control and TSPO-depleted cells.



Figure 2.11 Full immunoblot of cellular lysates from MDA-MB-231 cells after TSPO silencing. The full TSPO immunoblot from Figure 2.5 is shown. The 18 kDa TSPO band, as well as molecular weight markers, are indicated. The actin blot from Figure 2.9 is shown as a loading control. See Figure 2.9 and *Experimental procedures* for details. Control siRNA and siRNA against TSPO (siTSPO #1 or siTSPO #2) were used to transfect MDA-MB-231 cells as described under *Experimental procedures*. After 24 h, cellular lysates were harvested and subjected to immunoblotting assays using an anti-TSPO antibody, with actin as a loading control.



Figure 2.12 Combination of TSPO ligands and lonidamine potentiates apoptosis in breast cancer cells. BT549 (A) or MDA-MB-231 (B) cells were treated with vehicle (DMSO) or different concentrations of PK 11195 combined with different concentrations of lonidamine (LON) as indicated for 24 h. C. MDA-MB-231 cells were treated with DMSO or Ro5-4864 combined with different concentrations of LON, as indicated, for 24 h. Cell death was determined using trypan blue exclusion assays as described in *Experimental procedures*. Column: Mean of three independent experiments. Error bar: SD. D. MDA-MB-231 cells were treated with DMSO, PK 11195 or Ro5-4864 alone or in combination with LON at the indicated concentrations for 24 h and cellular lysates were generated. Immunoblotting of cellular lysates was performed using antibodies against PARP. Actin was used as loading control. PK: PK 11195; Ro: Ro5-4864.



MDA-MB-231

11195 and 400 µM lonidamine resulted in 40% cell death. In both MDA-MB-231 and BT549 cells, combining 100 µM PK 11195, with lonidamine resulted in 55% cell death at 200 µM and 90% cell death at 400 µM Ionidamine. Like PK 11195, treatment with 100 µM Ro5-4864 alone had no effect on viability of MDA-MB-231 cells, whereas the combination of 100 µM Ro5-4864 and 400 µM lonidamine resulted in 60% cell death (Figure 2.12C). To determine whether the cytoxicity induced by the combination of TSPO ligands with lonidamine is due to apoptosis, cleavage of poly (ADP-ribose) polymerase (PARP) was monitored (Figure 2.12D). During apoptosis, the 116 kDa full length PARP is cleaved by caspase-3 to generate an 89 kDa fragment which is a wellvalidated apoptotic marker [31]. Upon treatment with a TSPO ligand, PK 11195 or Ro5-4864, or lonidamine alone, full length intact PARP (116 kDa) was observed. However, combined treatment with 100 µM PK 11195 and 200 µM lonidamine, or 100 µM Ro5-4864 and 400 µM lonidamine, resulted in PARP cleavage, as evidenced by the presence of the 89 kDa PARP fragment. These data demonstrate that combining a TSPO ligand and lonidamine efficiently induces apoptosis of ER-negative breast cancer cells. To determine whether mammary acini overexpressing TSPO would show similar sensitivity to TSPO ligands and lonidamine, the acini were treated for 48 h with PK 11195 alone or with PK 11195 and Ionidamine. As shown in Figure 2.13, treatment with PK 11195 alone did not increase apoptosis as judged by active caspase-3 staining, but the combination of PK 11195 and lonidamine dramatically increased apoptosis of cells both in the lumen and in the outer layer.



Figure 2.13 Combination of PK 11195 and Ionidamine increases apoptosis during mammary morphogenesis. MCF10A-TSPO cells were seeded in Matrigel as described under *Experimental procedures*. At day 13, cultures were treated with vehicle (DMSO), or 100 μ M PK 11195 or a combination of 100 μ M PK 11195 and 200 μ M Ionidamine. After 48 h treatment, the cultures were fixed and stained with DAPI (*blue*) and anti-active caspase-3 (*green*). Representative fluorescence images are shown. Scale bar: 20 μ m.

5. Discussion

Understanding the roles of the mitochondrial proteins in breast cancer, particularly those with differential expression in the diseased state, is critical for developing treatment strategies [5,7]. Increased levels of TSPO correlate with increasing invasiveness of breast cancer tissues and breast cancer cells [13,15,16]. We hypothesized that increasing TSPO levels at each stage of breast cancer may confer distinct malignant properties to mammary epithelial and breast cancer cells that promote breast cancer progression. Thus, we used non-tumorigenic, immortalized mammary epithelial MCF10A cells, non-invasive ER-positive MCF7 breast cancer cells and invasive ER-negative MDA-MB-231 and BT549 cells as our cell line models for different stages of breast cancer.

Hyperproliferation and filling of the luminal space are key features of atypical hyperplasia and DCIS [24,25]. Oncogene-induced breast cancer phenotypes have been modeled using 3D Matrigel culture of MCF10A cells [24]. During morphogenesis of MCF10A acini, some of the proliferative outer layer cells are deposited into the lumen [26]. To form and maintain a hollow lumen, luminal cells must undergo apoptosis, presumably due to the lack of matrix-derived survival signals [23,24]. Inappropriate proliferation of the outer layer cells along with decreased apoptosis of the luminal cells can lead to formation of aberrant acini with filled lumen [26]. Overexpression of cyclin D1 or HPV-E7 in MCF10A cells leads to formation of larger acini but the lumens remain hollow, consistent with the established role of these oncogenes in proliferation [26]. In these model systems, luminal filling is only observed upon coexpression with anti-

apoptotic Bcl family members, supporting the notion that increased proliferation and resistance to apoptosis are both required for luminal filling. In contrast, elevation of TSPO levels is sufficient to produce larger acini with partially filled lumens, and in these respects more closely resembles the phenotypes induced by activated Akt [27], mixed-lineage kinase 3 [32], ErbB2/HER2 [26,28] or B-Raf^{V600E} [33].

Our data are consistent with the idea that elevation of TSPO promotes both proliferation of outer layer cells and survival of cells within the lumen. At day 15 expression of TSPO dramatically increases proliferation compared to control acini (Figure 2.3). By day 20, however, only rarely are proliferative cells observed in TSPOexpressing acini (data not shown), yet large numbers of viable cells remain in the lumen (Figure 2.4). We speculate that the increased levels of TSPO in these viable luminal cells afford at least partial protection from apoptosis. Although the role of TSPO in the regulation of apoptosis is not completely defined, a body of evidence supports an antiapoptotic function of TSPO. TSPO physically associates with the mitochondrial permeability transition pore (PTP) complex and thus increased TSPO levels may increase resistance to mitochondrial membrane permeability and subsequent cell death [12]. In chronic lymphocytic leukemia (CLL) cells, PK 11195 has been shown to induce apoptosis by mitochondrial membrane depolarization along with cytochrome c release [34].

Increased migratory ability is critical in the transition from localized to invasive breast cancer. Our data demonstrate that increasing expression of TSPO promotes migration of poorly migratory MCF7 cells, whereas, silencing of TSPO decreased migration of invasive MDA-MB-231 cells. These data provide evidence for the idea that

increased TSPO levels contribute to acquisition of an invasive phenotype in breast cancer cells. These findings are consistent with studies in rat glioma cells [35], suggesting that TSPO might regulate migration and invasion in a wide range of tumor types. In 3D Matrigel mammary, acini derived from TSPO-overexpressing nontumorigenic MCF10A mammary epithelial cells do not show an invasive phenotype, suggesting that, at least in this context, TSPO alone is not sufficient to induce invasion. While the underlying mechanism whereby TSPO influences cell migration is unknown, it is conceivable that TSPO affects cellular energy supply necessary for migration by perturbing the function of adenine nucleotide translocase (ANT), which is also found in the PTP complex. ANT is responsible for ATP and ADP exchange between mitochondria and cytosol and plays an essential role in cellular energy metabolism [36,37].

Lonidamine is a mitochondria-targeting agent that disrupts aerobic glycolysis [5], likely through inhibition of the mitochondrial bound hexokinase 2 [38,39], although ANT has also been suggested as a target [40,41]. Our data show that combined treatment of TSPO ligands, PK 11195 or Ro5-4864, with lonidamine greatly reduces viability of MDA-MB-231 cells, compared with either single agent, consistent with findings in myeloid cells [41]. Our findings that PK 11195 and Ro5-4864, which represent different classes of TSPO ligands, give similar results when combined with lonidamine imply that TSPO is a potential drug target and suggest that dual targeting of mitochondria could be a useful therapeutic approach for treating ER-negative breast cancer. While no toxicity of PK 11195 has been observed in small human trials [42], a phase II trial of lonidamine in metastatic breast cancer patients was halted due to toxicity [43]. Our data suggest

that lower doses of lonidamine might be therapeutically efficacious when combined with TSPO ligands.

Clinical observations show incremental increases in TSPO levels from normal breast tissues through advancing stages of breast cancer. Using a 3D mammary epithelial morphogenesis model, our studies suggest a novel role for TSPO in luminal cell survival, a key feature of DCIS. Increased TSPO levels promote proliferation in the 3D mammary acini, consistent with findings in 2D culture of breast cancer cell lines [16,19]. Our studies further show that altering TSPO levels impacts breast cancer cell migration, a necessary property for invasion and metastasis. Our results suggest that PK 11195, Ro5-4864 or other TSPO ligands, might be useful in developing combination therapies for breast cancer treatment.

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CHAPTER III

ADENINE NUCLEOTIDE TRANSLOCASE 2 (ANT2) PROMOTES MAXIMUM ATP PRODUCTION, CELL MIGRATION AND VIABILITY THROUGH OXPHOS IN BREAST CANCER CELLS

1. Abstract

Mitochondrial function is critical for survival, energy supply, and proliferation in breast cancer cells. During tumor progression, metabolic pathways are frequently altered. Cancer cells increase glycolysis and glutaminolysis for biomass synthesis and ATP production, which are both critical for acquisition of malignant phenotypes, such as increased proliferation and migration. Adenine nucleotide translocase (ANT) is responsible for ATP/ADP exchange between the mitochondria and cytosol. Among the four ANT isoforms, ANT2 is the only isoform that is overexpressed in breast cancer. This study aims to understand the special role of ANT2 in cancer cell energy metabolism and its potential influence on the development of malignant phenotypes in breast cancer cells. Silencing of ANT2 limits the maximum levels of ATP in MDA-MB-231 and MCF7 breast cancer cells in culture media containing both glucose and glutamine, whereas overexpression of ANT2 increases ATP levels in MCF7 cells. Along with the ATP change, depletion of ANT2 decreases migration of MDA-MB-231 cells, whereas overexpression of ANT2 enhances migration of MCF7 cells. To analyze the energy pathway(s) that ANT2 may regulate, lactate levels were measured to assess anaerobic glycolytic activity. Depletion of ANT2 from MDA-MB-231 cells does not affect lactate production. Nevertheless, oligomycin (an OXPHOS inhibitor) increases lactate levels in both control and ANT2-depleted MDA-MB-231 cells. The increased glycolytic activity resulted in increased ATP in ANT2-depleted cells upon oligomycin treatment. However, as oligomycin alone inhibits cancer cell migration and viability, the defect of migration and viability caused by ANT2 depletion could not be rescued by oligomycin,

which suggests that functional OXPHOS is important in cancer cell migration and viability. In addition, silencing of ANT2 in combination with 2-deoxy-glucose (2DG, a glycolysis inhibitor) results in greater inhibition of breast cancer cell viability, compared to either treatment alone. Overall, our study provides evidence that elevation of ANT2 levels in breast cancer cells allows for maximal OXPHOS dependent ATP production and enhances cancer cell migration and viability. These data suggest that ANT2 could serve as a viable target for breast cancer treatment, particularly in combination with glycolytic inhibitors.

2. Introduction

Mitochondrial function is essential for cancer cell survival and proliferation [1, 2]. For example, tumor cells with depleted mitochondrial DNA have reduced tumor-forming capability in mouse xenograft studies [3-5]. Oligomycin, an ATP synthase inhibitor, impairs cancer cell proliferation [6] and migration [7]. As cancer cells acquire malignant features, such as hyper-proliferation or increased migratory capability, they require additional biomass synthesis and increased energy [8, 9].

The tricarboxylic acid (TCA) cycle, which occurs in the mitochondria, prepares many building blocks for the synthesis of macromolecules such as lipids, amino acids and nucleotides [8]. Glucose and glutamine, which are two key nutrients for cancer cell growth and proliferation, can be converted to pyruvate and glutamate, respectively, and enter the TCA cycle [10]. Highly proliferating cancer cells often show increased uptake of glucose and glutamine [8, 10, 11].

Maximal cellular ATP production occurs through oxidative phosphorylation (OXPHOS) in the inner mitochondrial membrane [12]. Compared to OXPHOS, glycolysis is a less efficient pathway for ATP production. During glycolysis, the conversion of a single glucose molecule results in the production of 2 ATP molecules [9, 13]. Pyruvate can either enter the mitochondrial OXPHOS pathway to generate 36 ATP per molecule, or be reduced to lactate under anaerobic conditions [12, 14]. The Warburg effect suggests that cancer cells tend to perform glycolysis instead of OXPHOS even in the presence of oxygen [15, 16]. However, this concept has been challenged, as multiple studies have revealed that OXPHOS, compared with glycolysis, is the major contributor to ATP production in most cancer cell lines and tumors [17-19]. For example, 79% of total ATP generated in cervical carcinoma HeLa cells is through the OXPHOS pathway and 91% for breast carcinoma MCF7 cells [20]. Moreover, based on about 2,000 human breast cancer samples, cancer cells show more OXPHOS activity than the adjacent stromal tissues, evidenced by the increased activity of OXPHOS complex IV and upregulation of a set of mitochondria-specific genes [21]. Nevertheless, the profile of metabolic activity varies among cancer cell lines. Highly aggressive MDA-MB-231 breast cancer cells generate more of the glycolytic product, lactate, than do MCF7 cells [22, 23] reflecting their higher glycolytic activity. In addition, increased OXPHOS activity confers resistance to clinically used B-RAF inhibitors in melanomas [24, 25] suggesting that inhibition of OXPHOS could render cancer cells more vulnerable to some treatments. Toxicity precludes the clinical use of currently available OXPHOS inhibitors, and thus identifying and investigating selective targets in the OXPHOS pathways for cancer treatment is needed.

Adenine nucleotide translocase (ANT) is important in cellular energy metabolism since it is responsible for ATP/ADP exchange between the mitochondria and cytosol [26]. There are four human ANT isoforms, ANT1, ANT2, ANT3 and ANT4. ANT1 is the major isoform found in differentiated tissues such as heart and muscle; whereas ANT2 is overexpressed in tissues with high proliferative potential, such as liver, kidney, and in tumors [27, 28]. ANT3 is ubiquitously expressed in various tissues [27, 29], whereas ANT4 is predominantly found in brain, liver and testes [29]. Although ANT1 and ANT2 share about 80% amino acid sequence similarity [30], overexpression of ANT1, but not ANT2, promotes apoptosis in HeLa cells [31].

ANT2 is the only isoform that is overexpressed in tumor cells such as MCF7 and MDA-MB-231 breast cancer cells, compared to the nontumorigenic breast epithelial cells [32]. Depletion of ANT2 has been shown to reduce cancer cell malignant phenotypes. For example, silencing of ANT2 inhibits the proliferation of MDA-MB-231 cells [32] and decreases the migration and invasion in HER2/neu-overexpressing SK-BR3 cells [33]. Moreover, depletion of ANT2 inhibits tumor growth in a xenograft model [32]. ANT2 is therefore suggested to be a potential target for cancer therapy. Silencing of ANT2 in cancer cells confers sensitivity to cell death induced by lonidamine, a mitochondria-targeting agent [34], or TNF-related apoptosis-inducing ligand (TRAIL) [35].

Our study demonstrates important roles of ANT2 in generating ATP through OXPHOS, and promoting cell viability and migration in breast cancer. Depletion of ANT2 leads to a decrease of cellular ATP levels and cell migration of aggressive, invasive MDA-MB-231 cells, whereas, overexpression of ANT2 results in elevation of

ATP and enhanced migration of poorly migratory MCF7 cells. Depletion of ANT2 does not change lactate production, suggesting a dominant role for ANT2 in OXPHOSderived ATP, rather than in glycolysis-derived ATP. Oligomycin, an OXPHOS inhibitor, increases lactate production and restores ATP levels of ANT2 depleted cells; but it fails to rescue the impaired migration and cell viability in ANT2 depleted MDA-MB-231 cells, suggesting a critical role of OXPHOS in cancer cell malignancy. Furthermore, simultaneously inhibition of glycolysis with 2DG and OXPHOS with ANT2 depletion results in a profound decrease of cell viability. Taken together, these studies suggest that ANT2 could serve as a potential mitochondrial OXPHOS target for breast cancer treatment,

3. Experimental procedures

3.1 Cell Lines, Cell Culture, Transfection

Breast cancer cell lines, MCF7 and MDA-MB-231, were from ATCC (Manassas, VA, USA). MCF7 and MDA-MB-231 cells were cultured in DMEM (Gibco BRL, Paisley, PA, USA) with 10% FBS. Oligonucleotides of siRNA against ANT2 (nucleotides 127-147: 5'-GCAGAUCACUGCAGAUAAGdTdT-3' and 5'-

CUUAUCUGCAGUGAUCUGCdTdT-3') were synthesized from Invitrogen (Carlsbad, CA, USA) [34]. Transfection of siRNA (ANT2-specific siRNA (20 nM) and a universal control siRNA) was performed using InterferIN (Polyplus-transfection, New York, NY, USA) according to the manufacturer's instructions. Transfection of HA-ANT2 vector and the control vector was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. 3.2 RNA extraction and semi-quantitative RT-PCR

RNA was extracted from the cells using Trizol reagent. RT-PCR was performed using SuperScript[™] III One-Step RT-PCR system (Invitrogen, Carlsbad, CA, USA). The primers for ANT2 are 5'-GCTGATGTGGGTAAAGCTGGAGCTGAAAGGGA-3' and 5'-ACAAAAGCACCACCCATGCCTCT-3'. The primers for ANT1 are 5'-GCTGATGTGGGCAGGCGCGCCCAGCGTGA-3' and 5'-

ACAAAAGCACCGCCCATGCCTCT-3'. Primers for ANT3 PCR were as follows: 5'-GGGAAAGTCAGGCACAGAGCG-3' and 5'-CGTACAGGACCAGCACGAAGG-3'. The primers for GAPDH are 5'-GGTCGGAGTCAACGGATTTGGTCG-3', 5'-CCTCCGACGCCTGCTTCACCAC-3' [34]. For semi-quantitative PCR, different cycle numbers were tested and conditions were optimized to detect unsaturated signals for quantification; 26 cycles and 30 cycles were used for amplification of ANT2 and GAPDH respectively according to Stancy Liou (Gallo Lab, MSU) unpublished data.

3.3 Cell lysis and Immunoblotting

Cells were lysed in Triton X-100 lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM Na₃VO₄, 2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Protein concentrations of cellular lysates were measured by Bradford protein assays (Biorad, Rockford, IL, USA). Cell lysates were clarified by centrifugation at 14,000 rpm for 15 min prior to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred from gel to Immobilon-FL PVDF membranes (Millipore,

Billerica, MA, USA). The membranes were then blocked with odyssey blocking buffer (Li-COR Biosciences, Lincoln, NE, USA) and incubated with appropriate antibodies, followed by incubation with an IRDye-conjugated secondary antibody. The blots were developed by fluorescence using the Li-COR Odyssey infrared scanner (Li-COR Biosciences, Lincoln, NE, USA).

3.4 Immunofluorescence

Cells were seeded on coverslips for 24 h, incubated with MitoTracker Red (Sigma-Aldrich, St Louis, MO, USA) for 30 min, and fixed with 3.7% formaldehyde. After fixation, cells were permeabilized with 0.5% v/v Triton X-100 for 5 min and blocked in 4% w/v bovine serum albumin (BSA) in PBS for 30 min at room temperature. Coverslips were then incubated overnight at 4 °C with anti-HA antibody (1:1000 dilution) in PBS containing 2% BSA. Coverslips were washed three times for 5 min each with PBS, followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG (1:200 dilution) for 60 min at room temperature. After washing for three times with PBS, the cells were stained with 4'-6-Diamidino-2-phenylindole (DAPI, 0.5 µg/ml) for 15 min and mounted. Images were taken using a fluorescent microscope.

3.5 ATP assay

ATP was extracted from cells using boiling water as previously described [36] and measured using an ATP Enliten Kit (Promega, Madison, WI, USA) according to the manual. Briefly, cells were cultured on 12-well plates in the indicated medium for 48 h. At the time for ATP extraction, media was removed and 200 µl of boiling water was

added into each well. After boiling for 10 min, cells were chilled on ice briefly before subjecting to centrifugation at 12,000 g for 5 min at 4°C. A bioluminescence assay was used to measure ATP content of the supernatant. The ATP levels were determined by conversion of luminescence signals based upon an ATP standard curve performed at the same time. The total amount of ATP was finally normalized to the cell number.

3.6 Lactate assay

ANT2 was first silenced using siRNA transfection in MDA-MB-231 cells. After 24 h, cells were split and transferred to a 24-well plate $(1X10^5 \text{ cells/well})$ in the indicated media. After another 24 h, media were replaced as indicated (400 µl media/well) and cells were incubated for 6 h. Media were then collected and lactate was measured. For lactate measurement, the reaction mix includes 300 mM hydrazine buffer, 2.5 mM ß-NAD, lactate dehydrogenase. The reaction time is 30 min. The end point was measured using a spectrophotometer at 340 nm.

3.7 Transwell migration

Chemotactic transwell migration was performed using Boyden transwell chambers (8 μ m pore size; Corning Costar, Cambridge, MA, USA). Cells were serum starved overnight, trypsinized and placed in the upper chamber (5 × 10⁴ for MDA-MB-231 and 10⁵ for MCF7). Medium supplemented with 5% FBS was used as the chemoattractant in the bottom chamber. After 24 h for MCF7 or 6 h for MDA-MB-231, migrated cells were fixed, stained with crystal violet and quantified by counting five randomly chosen fields. The experiments were performed in duplicate wells and each
experiment was performed at least three times. For the oligomycin treatment, MDA-MB-231 cells were treated with 5 µg/ml oligomycin for 20 min prior to and during the transwell assays.

3.8 Cell viability assay

Control or ANT2 depleted MDA-MB-231 and MCF7 cells were seeded into 12well plates with 16,000 cells/well for MDA-MB-231 cells and 25000 cells/well for MCF7 cells. After 16 h, cells were treated with indicated concentration of oligomycin and 2DG in media containing 25 mM glucose and 4 mM glutamine for 72 h in MDA-MB-231 or 48 h for MCF7 cells. Total cell number under different culture conditions was quantified using a hemocytometer, and 0.4% solution of trypan blue in buffered isotonic salt solution was used to distinguish live and dead cells.

3.9 Wound healing assay

Control and ANT2 depleted MDA-MB-231 cells were grown to confluence. The monolayer of cells was scratched using a 200 μ l pipette tip and growth medium was replaced with fresh medium supplemented with Mitomycin C (1 μ g/ml). The closure of wound was monitored over time by microscopy. Percentage wound closure was expressed as [1-(width of the wound at 24 h/width of the wound at t=0)] x100%.

3.10 Statistical analysis

Bar graphs with mean±standard deviation were generated to represent the data in migration assays and cell viability assays. Student's *t*-tests were conducted to

compare the difference between experimental groups and p<0.05 was considered as statistically significant. Bonferroni method or Benjamini-Hochberg method was used to control the increased type 1 errors when multiple comparisons were performed. The p-value of individual test was compared to the adjusted p-value from Bonferroni method or Benjamini-Hochberg method to determine the statistical significance.

4. Results

4.1 ANT2 enhances ATP production in breast cancer cells in medium containing both glucose and glutamine.

As an ATP/ADP transporter between cytosol and mitochondria, ANT2 is reported to regulate cellular ATP levels [32, 34, 37]. To investigate the function of ANT2, ANT2 levels were manipulated by siRNA transfection. Levels of ANT1, ANT2 and ANT3 mRNA were detected using semi-quantitative RT-PCR. Upon ANT2 silencing, ANT2 mRNA levels were diminished while ANT1 and ANT3 mRNA levels remained constant. In addition, upon ANT1 silencing, levels of ANT1 decreased while ANT2 and ANT3 remained constant (Figure 3.1). These data suggest that all knockdowns are isoformspecific.

To investigate how ANT2 may impact ATP production from different nutrient sources, ANT2 was first silenced in MDA-MB-231 cells by siRNA transfection (Figure 3.2A *left* panel). A portion of control and ANT2 depleted cells were used for RNA extraction to confirm the silencing effect, and the remaining control and ANT2-depleted cells were used for ATP measurement. Based upon semi-quantitative RT-PCR, siRNA



Figure 3.1 Silencing of ANT2 or ANT1 does not affect the mRNA levels of other ANT isoforms. (A) MDA-MB-231 cells were transfected with control or ANT2 siRNA. Total RNA was extracted and semi-quantitative RT-PCR was performed to detect ANT1, ANT2 and ANT3 mRNA levels. (B) MDA-MB-231 cells were transfected with control or ANT1 siRNA. Total RNA was extracted and semi-quantitative RT-PCR was performed to detect ANT1 ANT2 ANT1, ANT2 and ANT3 mRNA levels.



Figure 3.2 ANT2 promotes ATP production in breast cancer cells in medium condition containing both glucose and glutamine. A. Control and siRNA against ANT2

Figure 3.2 (cont'd)

were transfected into MDA-MB-231 cells. The levels of ANT2 upon silencing were evaluated by semi-quantitative and GADPH is shown as a control (*left* panel). Control and ANT2 depleted cells were cultured in different culture media conditions as indicated for 24 h. Total cellular ATP was extracted and measured as described in *Experimental* procedures. For each experiment, samples with control or ANT2 depleted cells were done in triplicate. Column (right panel): combination of three independent experiments. B. Control and siRNA against ANT2 were transfected into MCF7 cells. Semi-quantitative RT-PCR was performed to detect ANT2 mRNA levels (*upper* panel). Control and ANT2 depleted cells were cultured in the DMEM media that contained glucose and glutamine. Total cellular ATP was extracted and measured (bottom panel). For each experiment, samples from control or ANT2 depleted cells were done in triplicate. Column: combination of three independent experiments. C. Control vector and vector encoded with HA-tagged ANT2 were transient expressed in MCF7 cells. The expression of HAtagged ANT2 is detected with immunoblotting using HA antibody, actin is used as the loading control (upper panel). ATP levels were measured as cells are cultured in medium containing both glucose and glutamine (bottom panel). Column: combination of three independent experiments. Error bar: SD. Student's *t*-test was used to determine p value. Bonferroni method was used to determine the statistical significance of multiple comparisons. * indicates significant difference between two treatment groups.

mediated silencing results in about 80-90% reduction of ANT2 mRNA levels. ATP levels of ANT2-depleted cells were measured under different nutrient conditions. Cells were then incubated in media containing either combined glucose and glutamine, or glucose alone, or glutamine alone. Silencing of ANT2 decreased ATP production by 30% from cells in medium containing both glucose and glutamine. Culture medium containing either glucose or glutamine alone produced about 50% or 60% ATP respectively, compared to medium containing both nutrient sources (Figure 3.2A *right* panel). Notably, cells grown on either glucose or glutamine as the sole carbon source show about 50% reduction in ATP levels; and silencing of ANT2 did not further decrease ATP levels (Figure 3.2A *right* panel). These data suggest that silencing of ANT2 limits the maximum ATP production that occurs in the medium condition containing both glucose and glutamine.

To confirm the result that decreased levels of ANT2 limit ATP levels, the impact of ANT2 on a less glycolytic breast cancer cell line, MCF7, was evaluated. ANT2 was silenced in MCF7 cells by siRNA transfection and semi-quantitative RT-PCR was performed to determine the efficiency of knockdown (Figure 3.2B *upper* panel). Control and ANT2 depleted MCF7 cells were cultured in the media containing both glucose and glutamine before being subjected to ATP assay. Silencing of ANT2 in MCF7 cells decreased total cellular ATP by 30% (Figure 3.2B *bottom* panel).

We next investigated the effect of ANT2 overexpression on cellular ATP levels in MCF7 cells. Control vector or HA-tagged ANT2 was transfected into MCF7 cells. Immunoblotting was performed to detect the overexpression of HA-tagged ANT2; actin was served as the loading control (Figure 3.2C *upper* panel). Increased expression of

ANT2 resulted in a 40% increase in ATP in MCF7 cells (Figure 3.2C *bottom* panel). Overall, these data suggest that ANT2 promotes maximum ATP production in breast cancer cells. To confirm that the recombinant ANT2 could properly localize to mitochondria, immunostaining was performed in MCF7 and HeLa cells after HA-tagged ANT2 transfection (data not shown). The co-localization of HA tagged ANT2-HA with MitoTracker Red, a mitochondria marker, revealed that HA-tagged ANT2 localized to the mitochondria. Similar localization was observed in MCF10A cells (Figure 3.3).

4.2 ANT2 promotes breast cancer cell migration.

As energy is required for all cellular functions, the ATP levels controlled by ANT2 might be expected to impact many aspects of breast cancer malignancy. It has been demonstrated previously that silencing of ANT2 decreases proliferation of MDA-MB-231 cells [38]. Here, we tested the effect of ANT2 silencing on migratory capability of MDA-MB-231 cells. ANT2 silencing was performed by siRNA transfection, and verified by the diminished mRNA levels upon ANT2 silencing using semi-quantitative RT-PCR (Figure 3.4A *upper* panel). Control and ANT2-depleted MDA-MB-231 cells were cultured in the medium containing both glucose and glutamine, and then subjected to transwell migration assays. Silencing of ANT2 resulted in 30% decrease of migration of MDA-MB-231 cells (Figure 3.4A *bottom* panel). The effect of ANT2 depletion on cell migration was also evaluated in a wound-healing assay of MDA-MB-231 cells. After 24 h, the control wound had completely closed, whereas silencing of ANT2 inhibited the wound closure by 30% (Figure 3.4B). Mitomycin C was added to prevent any potential cell proliferation contributing to the observed phenotype. As ANT2 silencing caused about



Figure 3.3 Ectopically expressed ANT2 is localized to mitochondria. Control vector

(pLXSN) and vector encoded HA tagged ANT2 were expressed in MCF10A cells.

Immunostaining was performed using antibodies against HA tag to detect the ectopically expressed ANT2, MitoTracker Red was used to stain mitochondria and cells were imaged by fluorescent microscopy. Scale bar: 10 µm.



Figure 3.4 ANT2 promotes breast cancer cell migration. A. MDA-MB-231 cells were transfected with control or ANT2 siRNA and serum deprived for 24 h. Total RNA was

Figure 3.4 (cont'd)

extracted and semi-quantitative RT-PCR was performed to detect ANT2 mRNA levels, where GADPH is shown as an internal control (upper panel). Control- and ANT2depleted-cells were then subjected to transwell migration assays as described under *Experimental procedures.* Column (*bottom* panel): Mean of three experiments. Error bar: SD. Student's *t*-test was used to determine p-value. * p<0.05 indicate significant differences between the two groups. B. Control- or ANT2-depleted-MDA-MB-231 cells were subjected to a wound-healing assay as described in *Experimental procedures*. Representative images at the indicated time points from 3 independent experiments are shown (*upper* panel). Magnification 10X. The width of remaining wounds after 24 h migration (bottom panel) was measured. Percentage wound recovery was expressed as a [1-(width of the wound at t=24 /width of the wound at t=0)] x100%. Column: mean of three experiments. Error bar: SD. * p<0.05 indicate significant differences between the two groups. C. Control vector or vector encoding HA-tagged-ANT2 was transiently expressed in MCF7 cells. Immunoblotting was used to determine the expression of HA tagged ANT2 with actin as a loading control (*left* panel). Starved cells were then subjected to transwell migration assays. Column (right panel): mean of three experiments. Error bar: SD. Student's *t*-test was performed to determine p-value. * p<0.05 indicate significant differences between the two groups.

30% reduction of ATP in MDA-MB-231 cells, the defect in cell motility could be due to the lower cellular ATP levels caused by ANT2 silencing.

To confirm the role of ANT2 in breast cancer cell migration, ANT2 was overexpressed by transfection of a construct encoding the recombinant HA-tagged ANT2 in poorly migratory MCF7 cells. Immunoblotting was performed to assess protein expression using anti-HA antibody (Figure 3.4C *left* panel). Control and ANT2 overexpressing MCF7 cells were cultured in media containing glucose and glutamine and then subjected to transwell migration assays. Mitomycin C, a cell cycle inhibitor, was added to prevent cell proliferation during the 24 h migration assay. Overexpression of ANT2 in MCF7 cells increased cell migration by approximately 1.4 fold (Figure 3.4C *right* panel). Consistent with the role of ANT2 in MDA-MB-231 cell migration, these data suggest that increasing levels of ANT2 promote breast cancer cell migration. Moreover, the elevation of ATP induced by ANT2 overexpression (Figure 3.2C) may contribute to the increased migration of ANT2 overexpressed MCF7 cells.

4.3 Oligomycin restores ATP levels in ANT2 depleted cells.

As depletion of ANT2 reduced ATP levels and migration in breast cancer cells, we next analyzed which energy pathways could be regulated by ANT2, resulting in the change of ATP levels and migration ability. OXPHOS and glycolysis are two major ATP producing pathways. To assess the effect of ANT2 silencing on glycolysis, the amount of lactate was measured. Glucose and serum in the culture media can affect the production of cellular lactate [39]. Therefore, lactate was measured under different nutrient conditions: with or without serum and with or without 25 mM glucose [9]. All



Figure 3.5 Silencing of ANT2 has no effect on cellular lactate production. A. ANT2 was first silenced by siRNA transfection and the knockdown effect was evaluated by semiquantitative RT-PCR. GADPH is shown as a control (*right* panel). The lactate production under indicated medium conditions was measured (*left* panel) as described under Figure 3.5 (cont'd)

Experimental procedures. B. MDA-MB-231 cells were treated with 5 µg/ml oligomycin in the medium containing both glucose and glutamine. After 6 h incubation, lactate in the media was measured. For each experiment, each measurement was done in triplicate. Data are a representative result from two independent experiments. Error bar: SD.

medium contained 4 mM glutamine. ANT2 was silenced in MDA-MB-231 grown in complete media (Figure 3.5A *right* panel). Control and ANT2-depleted MDA-MB-231 cells were cultured overnight under the nutrient conditions as indicated in Figure 3.5. The next day, medium was replaced with fresh corresponding medium. After 6 h, medium was collected and secreted lactate was measured. Deprivation of serum alone led to a dramatic decrease of lactate production (Figure 3.5A *left* panel). Largely decreasing levels of lactate were also observed when cells are deprived of glucose in the serum-containing media. In any of these conditions, silencing of ANT2 did not change lactate levels (Figure 3.5A *left* panel). These data indicated that ANT2 depletion had no impact on glycolysis. Together with the previous finding that ANT2 depletion led to ATP reduction upon media containing glucose and glutamine, these data suggest that ANT2 regulates cellular ATP levels through OXPHOS pathway.

To confirm that ANT2 does not influence glycolysis, oligomycin, an OXPHOS inhibitor that blocks function of ATP synthase, was used to force cell to untilize glycolysis for ATP supply [40]. MDA-MB-231 cells were treated with oligomycin for 6 h in the media containing both glucose and glutamine, and lactate secretion in the media was subsequently assessed. Oligomycin treatment resulted in increased lactate secretion both in the control or ANT2 silencing cells, which is possibly due to increased rates of glycolysis as a result of OXPHOS inhibition (Figure 3.5B). Silencing of ANT2 had no effect on lactate production in MDA-MB-231 cells treated with or without oligomycin (Figure 3.5B). Together with the finding that depletion of ANT2 reduced ATP,

these data suggest the ATP defect caused by ANT2 silencing cannot be compensated by increased activation of glycolysis pathway.

To further test whether the increased glycolysis by oligomycin treatment could reverse the defect of ATP levels caused by ANT2 depletion, ATP was measured in control and ANT2 depleted cells upon the oligomycin treatment. ANT2 is first silenced in MDA-MB-231 cells (Figure 3.6 *upper* panel), knockdown efficiency was measured by the semi-quantitative RT-PCR; and ATP was measured. ANT2 silencing consistently decreased the ATP production. Oligomycin treatment did not change the ATP levels in control cells, and even reversed the reduced ATP levels in ANT2 depleted cells back to the control levels (Figure 3.6 *bottom* panel). Combined with the result that lactate is increased upon oligomycin treatment (Figure 3.5B), the increased levels of ATP in the ANT2 depleted cells upon oligomycin treatment could be due to the enhanced glycolysis activity induced by oligomycin.

4.4 Oligomycin fails to rescue the defect of cell migration and viability induced by ANT2 depletion.

Since oligomycin treatment increases ATP levels in ANT2-depleted cells, we next investigated whether oligomycin could enhance the migration of ANT2-depleted cells. The migration of ANT2-depleted cells with or without oligomycin treatment was therefore analyzed using transwell migration assays as described above, except that cells were treated with oligomycin to block OXPHOS. MDA-MB-231 cells were treated with or without oligomycin for 20 min prior to and during transwell migration assays. The





Figure 3.6 Oligomycin increases ATP levels in ANT2 depleted cells. Control and siRNA against ANT2 were transfected into MDA-MB-231 cells. Semi-quantitative RT-PCR was performed to analyze the knockdown effect (*upper* panel). The control and ANT2 silencing cells were then treated with 5 μg/ml oligomycin. After 6 h, ATP levels were measured (*bottom* panel). Column: combination of three independent experiments. Error bar: SD. * (p<0.05) indicates significant difference between control and ANT2 depleted cells.

efficiency of ANT2 knockdown was verified by semi-quantitative RT-PCR (Figure 3.7A *left* panel). Oligomycin treatment alone caused approximately 40% reduction of cell migration. Silencing of ANT2 consistently resulted in 30% decrease of cell migration. However, combined oligomycin treatment and ANT2 depletion only caused a 40% decrease of cell migration, similar to the effect of oligomycin treatment alone (Figure 3.7A). These data, taken together with the findings that oligomycin treatment did not change ATP levels, suggest that OXPHOS, but not the levels of total ATP, is critical for migration of MDA-MB-231 cells.

We further analyzed the cell viability of ANT2-depleted cells upon oligomycin treatment. ANT2 was silenced in MDA-MB-231 cells and confirmed with semiquantitative RT-PCR (Figure 3.7B *left* panel). Control and ANT2-depleted cells were then treated with or without oligomycin. Total cell number was quantified to assess cell viability after 72 h treatment. Silencing of ANT2 decreased cell numbers of MDA-MB-231 by about 30% compared to the control (Figure 3.7B *right* panel). Oligomycin led to about 40% reduction of cell viability of control cells, and similarly 45% decrease of cell viability of ANT2 depleted cells. There was no additive effect on cell viability observed when combining ANT2 silencing with oligomycin treatment.

4.5 Combination of 2DG and ANT2 depletion significantly decreases breast cancer cell viability.

Combined inhibition of OXPHOS and glycolysis was suggested to be a promising strategy for breast cancer treatment [41, 42]. One of the glycolytic inhibitors, 2deoxyglucose (2DG), has been suggested to treat breast cancer, but was limited due to



Figure 3.7 Oligomycin fails to rescue the defect of cell migration and viability induced by ANT2 depletion. A. MDA-MB-231 cells were transfected with ANT2 siRNA and cells were serum-starved 24 h in DMEM. Total RNA was extracted using portion of the culture and ANT2 mRNA levels was analyzed by semi-quantitative RT-PCR, where GADPH is shown as an internal control (*left* panel). Oligomycin treated control and Figure 3.7 (cont'd)

ANT2 depleted cells were subjected to transwell migration. Each experiment was done in duplicate wells per condition. Column (*right* panel): Mean of three experiments. Error bar: SD. P-value was determined by Student's *t*-test. * p<0.05 indicate significant differences between the two groups. B. MDA-MB-231 cells were transfected with control and siRNA against ANT2. Semi-quantitative RT-PCR was used to determine the knockdown efficiency (*left* panel). Equal number of control and ANT2 depleted cells was treated with oligomycin. Cell number was quantified after 72 h treatment for MDA-MB-231. Relative cell number of was normalized to the DMSO treated control cells (*right* panel). Column: Mean of three experiments. Error bar: SD. Student's *t*-test was performed to determine p-value. * p<0.05 indicate significant differences between the two groups. systemic toxicity [43]. As there are currently no safe OXPHOS inhibitors, identifying specific OXPHOS targets for breast cancer cells is necessary. As we showed that ANT2 is important in OXPHOS pathway for ATP production, cell migration and viability. Here, we test whether simultaneously inhibiting ANT2 and glycolysis could lead to greater inhibition of breast cancer cell viability. We treated control or ANT2-depleted MDA-MB-231 and MCF7 cells with 2DG and analyzed cell viability. The knockdown efficiency of ANT2 in MDA-MB-231 and MCF7 cells were confirmed with semi-quantitative PCR (Figure 3.8A and B left panel). The viability of control MDA-MB-231 cells were not affected by 0.5 mM 2DG, but decreased by 65% upon 5 mM 2DG treatment. ANT2 depletion led to 35% and 75% decrease of cell viability compared to the control untreated cells (Figure 3.8A right panel). MCF7 cells appeared more sensitive to 2DG than MDA-MB-231 cells, which could be because MCF7 cells undergo more glycolysis dependent OXPHOS. The cell viability of MCF7 cells was reduced by 45% upon treatment of 2DG at 0.5 mM, and 65% when treated with 5 mM 2DG. ANT2 depletion caused further decrease of cell viability, 70% and 80% decrease of cell viability with 2DG treatment at the concentration of 0.5 mM and 5 mM, respectively (Figure 3.8B right panel). Taken together, our data show that combining ANT2 depletion with 2DG treatment could lead to greater inhibition of breast cancer cell viability, compared to either single treatment.

5. Discussion

Development of resistance to current chemotherapeutic agents is a prominent hurdle for breast cancer treatment. Thus new therapeutic targets are required.



Figure 3.8 Combination of 2DG and ANT2 depletion significantly decreases breast cancer cell viability. MDA-MB-231 (A) and MCF7 (B) cells were transfected with control and siRNA against ANT2. Semi-quantitative RT-PCR was used to determine the knockdown efficiency (*left* panel). Equal number of control and ANT2 depleted cells was treated with 2DG at the indicated concentration. Cell number was quantified after 72 h

Figure 3.8 (cont'd)

treatment for MDA-MB-231 or 48 h treatment for MCF7 cells. Relative cell number of was normalized to the DMSO treated control cells (*right* panel). Column: Mean of threeexperiments. Error bar: SD. Student's *t*-test was performed to determine p-value. Benjamini-Hochberg method was used to determine the statistical significance of multiple comparisons. * Indicates significant difference between two treatment groups.

Mitochondria are essential for cancer cells to utilize nutrients and produce energy, and changes in mitochondria in cancer cells can contribute to the resistance to apoptosis [1]. Therefore, mitochondria have emerged as a new therapeutic target [44, 45], and ANT2 is suggested to be one of the potential targets located in mitochondria [46]. Our study has extended the current literature about ANT2 in energy metabolism and its relationship with cancer progression. We have demonstrated the importance of ANT2 in the OXPHOS pathway to promote maximal ATP production, cancer cell migration and viability.

We have modulated the levels of ANT2 and examined the change of malignant phenotypes in breast cancer cells with different levels of ANT2. Since high quality, commercially available ANT2 antibodies do not exist. Semi-quantitative RT-PCR was used to determine the efficiency of ANT2 silencing after ANT2 siRNA transfection. The ANT2 siRNA has been previously validated by demonstrating efficient reduction of protein levels of V5 epitope-tagged ANT2 that correspond with decreased in ANT2 mRNA levels as determined by RT-PCR [34]. The mRNA levels of ANT1 and ANT3 were also analyzed at the same time and were not changed upon ANT2 silencing (Figure 3.1). Thus silencing of ANT2 using ANT2 siRNA specifically reduces ANT2 mRNA levels. These data suggest that expression of other ANT isoforms is not affected by the depletion of ANT2; and that the observed phenotypes using siRNA against ANT2, including decreased ATP, cell migration and viability, were specific to the depletion of ANT2.

Glucose and glutamine are both carbon sources that contribute to the synthesis of ATP [10]. When cells are grown in media containing only one carbon source, cells

generate 50% less ATP, compared to when cells are grown in media containing both glucose and glutamine. Silencing of ANT2 under conditions of a single carbon source (glucose or glutamine) does not further reduce ATP levels. It is possible that the inability of ANT2 to influence ATP levels when cells already have low ATP levels is independent of carbon source. Lack of effect of ANT2 silencing on ATP levels may be due to the presence of only a single carbon, or may be due to the lower ATP under those conditions. It would be interesting to determine whether ANT2 still controls ATP production under low ATP levels when limited concentration of glucose and glutamine is present. Nevertheless, depletion of ANT2 limits the maximal production of ATP when media contains both glucose and glutamine. These data suggest that increased levels of ANT2 could enable cancer cells to generate high levels of energy as ATP.

The process of cell migration requires an abundance of ATP [47] and mitochondrial ATP production potentially provides important local energy for breast cancer cell migration [48]. As ANT2 controls cellular ATP levels, we therefore assessed cancer cell migration upon change in ANT2 levels. The cell migration ability shows positive correlation with the ATP levels regulated by ANT2. Silencing of ANT2 decreased MDA-MB-231 cell migration along with a reduction of ATP, whereas overexpression of ANT2 increased MCF7 cell migration along with an elevation of ATP. It is thus possible that the ATP regulated by ANT2 could contribute to breast cancer migration.

To analyze the underlying energy pathways through which ANT2 regulates ATP and cell migration, we have measured lactate production in ANT2-depleted cells. The levels of lactate reflect the extent of anaerobic glycolysis, an alternative energy pathway.

Lactate production was not affected by ANT2 silencing in MDA-MB-231 cells under conditions using different carbon sources. This result is consistent with the previous finding that knockdown of ANT2 in HeLa cells reduces cellular ATP, but does not influence glycolysis [34]. These data also suggest that the decrease in ATP caused by ANT2 silencing cannot be compensated for by increased glycolysis. Since glycolytic activity remains unchanged in ANT2 depleted cells, we thus conclude that silencing of ANT2 decreases the ATP produced through OXPHOS. This is also supported by the fact that ANT2 is the only isoform of ANT family found at the mitochondrial cristae inner membrane to where F_0F_1 -ATP synthase also locates; and ANT2 physically interacts with F₀F₁-ATP synthase [49-51]. The precise mechanism by which ANT2 depletion reduces cellular ATP levels in cancer cells is not known. ANT2 is the major ATP-ADP co-transporter in cancer cells. It is conceivable that depletion of ANT2 leads to limiting mitochondrial ADP due to the decreased ATP-ADP exchange, thus increasing the ATP/ADP ratio within the inner mitochondria membrane. The increased mitochondrial ATP/ADP ratio could limit ATP synthesis from ATP synthase by reducing substrate ADP concentration, resulting in decreased ATP synthase activity and decreased ATP production. Our data provide evidence that ANT2 enhances ATP supply from OXPHOS in cancer cells.

Oligomycin is an OXPHOS inhibitor that targets at ATP synthase. Breast cancer cells when treated with oligomycin have compromised OXPHOS and switch to anaerobic glycolysis, evidenced by the increased lactate production (Figure 3.5B). Cells that have been depleted of ANT2 and treated with oligomycin have increased lactate production to a similar level as observed in the oligomycin-treated control cells. Such

increased glycolytic activity may contribute to the enhanced ATP levels in the oligomycin treated cells with ANT2 depletion (Figure 3.6). These data indicate that ANT2 and oligomycin control the same pool of ATP, which is presumably produced through OXPHOS. The mechanism through which oligomycin is able to increase anaerobic glycolysis to compensate for the blocked OXPHOS pathway whereas depletion of ANT2 cannot is unknown. However, oligomycin treatment is reported to activate AMPK pathway which is known to increase the activity of the glycolysis pathway [43]. AMPK pathway is activated in response to high AMP levels. The levels of AMP are regulated by adenylate kinase, which converts ADP into AMP and ATP [43, 52]. When ANT2 is silenced, however, the activity of mitochondrial adenylate kinase dependent ATP synthesis is not influenced according to Brenner et al. [34].

We next examined whether the oligomycin-induced increase in ATP could rescue the defect of migration and cell viability caused by ANT2 depletion. Firstly, silencing of ANT2 consistently inhibits MDA-MB-231 cell migration and viability. Based on trypan blue staining (data not shown), the decreased cell viability observed upon ANT2 silencing in breast cancer cells is due to decreased cell proliferation. Secondly, oligomycin treatment alone inhibited cancer cell migration and viability, which could potentially explain why oligomycin treatment failed to rescue the defect of migration and cell viability in ANT2-depleted cells. In addition, no additive effect was observed when combining oligomycin treatment with ANT2-depletion, supporting the idea that both oligomycin treatment and depletion of ANT2 inhibit a same pathway, OXPHOS. Moreover, these data suggest that the compensatory ATP production from glycolysis is not sufficient to promote migration and cell proliferation. Instead, functional OXPHOS or

the localized OXPHOS produced ATP may be critical for breast cancer migration and cell proliferation.

Emerging evidence shows that epithelial tumor cells such as breast cancer cells have increased OXPHOS activity compared to their adjacent stromal tissue [21, 24, 53]. This suggests that inhibition of OXPHOS could make tumor cells vulnerable and thus OXPHOS could be a potential treatment target. Moreover, cancer cells have high flexibility in switching between OXPHOS and glycolysis to adapt to a different microenvironment [47, 54]. Simultaneous inhibition of OXPHOS and glycolysis could therefore be a valid therapeutic strategy [41, 42]. However, no current OXPHOS inhibitors can be used due to their high cytotoxicity. We here proposed that ANT2 could serve as a selective target for blocking OXPHOS in breast cancer cells since ANT2 is specially overexpressed in cancer cells and silencing of ANT2 limits ATP production through OXPHOS, cancer cell migration, and viability. Our data demonstrate that combination of treatment of the glycolytic inhibitor, 2 deoxyglucose, with ANT2 depletion results in a great reduction of breast cancer cell viability. Therefore, inhibition of OXPHOS pathway, particularly through targeting ANT2, could be a viable therapy strategy for treating breast cancer.

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CHAPTER IV

CONCLUSIONS AND PERSPECTIVES

Mitochondria are essential for cancer cell functions. Mitochondria prepare many intermediates for macromolecular synthesis that are necessary for cell proliferation, provide ATP supply through OXPHOS, and control cell apoptosis through the mitochondrial permeability transition pore (PTP) complex. Mutation of mitochondrial DNA, defect in mitochondrial enzymes and reprogramming of mitochondrial bioenegetic metabolism are previously reported to be associated with cancer progression. The mitochondrial permeability transition pore (PTP) is particularly critical for mitochondrial function in cancer cells due to its involvement in energy metabolism and apoptosis. TSPO and ANT2, two proteins that are associated with the PTP complex, are upregulated in tumor tissues compared to normal tissues. Herein, we evaluated the functional roles of TSPO and ANT2 in breast cancer progression and their potential as therapeutic targets for treating breast cancer.

We first investigated the functional roles of TSPO in breast cancer development and progression. Using a 3D Matrigel culture, we assessed the impact of TSPO overexpression on mammary epithelial morphogenesis. Non-tumorigenic MCF10A epithelial cells, when grown in 3D culture, form well-polarized acini structures containing a hollow lumen. In contrast, MCF10A epithelial cells engineered to stably overexpress develop enlarged acini with a partial filled lumen. These TSPO overexpressing acini also show increased proliferation and decreased percentage of luminal apoptosis. The phenotypes of TSPO overexpressing acini overall resemble an early stage breast lesion that could potentially lead to breast cancer initiation. In breast cancer cells, our data show that TSPO promotes cell migration. Silencing of TSPO decreases migration of highly aggressive MDA-MB-231 breast cancer cells, whereas, overexpression of TSPO

enhances migration of poorly migratory MCF7 breast cancer cells. Moreover, TSPO ligands including PK 11195 and Ro5-4864 potentiate the apoptogenic effect of lonidamine in ER-negative BT549 and MDA-MB-231 breast cancer cells, whereas, TSPO ligands or lonidamine alone does not influence cell viability.

We next analyzed the roles of ANT2 in the cancer energy metabolism and its association with cancer cell malignancy. As the ATP/ADP transporter in the mitochondrial inner membrane, ANT2 is important for ATP generation in breast cancer cells. Silencing of ANT2 reduces levels of cellular ATP when breast cancer cells are cultured in medium containing both glucose and glutamine, whereas overexpression of ANT2 enhances ATP levels. The ATP controlled by ANT2 is probably produced from OXPHOS, as silencing of ANT2 does not affect anaerobic glycolysis judged by the unchanged lactate upon ANT2 depletion. Oligomycin (an OXPHOS inhibitor) enhances the ATP levels in ANT2 depleted cells through upregulation of glycolysis, but fails to rescue the impaired migration and cell viability in ANT2 depleted cells, suggesting that functional OXPHOS or OXPHOS produced ATP is important for cancer cell migration and viability. As current OXPHOS inhibitors are highly toxic, ANT2 could be a selective cancer cell target for future breast cancer therapy. Our data further show that depletion of ANT2 combined with 2-deoxyglucose (a glycolysis inhibitor) treatment results a significant decrease of breast cancer cell viability.

Overall, our study demonstrated that mitochondrial membrane proteins, TSPO and ANT2, contribute to the acquisition of cancer cell malignancy such as increased proliferation and enhanced migration, suggesting that TSPO and ANT2 could serve as potential therapeutic targets for breast cancer treatment.
While the signaling pathways through which mitochondria contribute to proliferation and migration remain obscure, roles of TSPO and ANT2 in promoting cell proliferation and migration may provide some hints. Both TSPO and ANT2 are putative components of PTP complex and physically interact with VDAC. The PTP complex is important for cellular functions due to its critical roles in energy metabolism, regulation of cellular ROS and Ca²⁺ homeostasis, as well as cell apoptosis. Both TSPO and ANT2 can promote cancer cell proliferation and migration, which could be due to their involvement in the PTP complex and their respective interaction with VDAC. The possible functional interaction between TSPO and ANT2 will be interesting to investigate. Furthermore, the combination of TSPO ligands and lonidamine, which is known to be an inhibitor of hexokinase II, as well as an ANT inhibitor, is reported to result in significant apoptosis in breast cancer cells. These studies suggest that targeting ANT2 and TSPO simultaneously could be a potential effective strategy for breast cancer treatment.

In chapter II, we have proposed TSPO ligands in combination with lonidamine could be useful in the treatment of breast cancer. We have shown that combination treatment of a TSPO ligand (PK 11195 or Ro5-4864) and lonidamine, leads to significant apoptosis in MDA-MB-231 and BT549 cells, whereas, either drugs alone does not influence cell viability. We further analyzed the effect of the combination of PK 11195 and lonidamine on MCF10A mammary epithelial morphogenesis in 3D culture, which significantly induces apoptosis within the luminal cells as well as cells at the outer layer, evidenced by the increased expression of caspase-3. Compared to the acini structures derived from MCF10A cells, breast cancer cells such as MDA-MB-231 could

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form invasive structures with poor cell-cell contact in 3D culture. Since depletion of TSPO impairs breast cancer cell migration in transwell migration, it will be interesting to investigate whether silencing of TSPO or TSPO ligands could block the invasive potential of breast cancer cells in 3D culture and whether TSPO ligands in combination with londiamine could influence the viability of breast cancer cells in 3D culture.

In chapter III, we reported the roles of ANT2 in promoting ATP production, migration and viability in breast cancer cells. Among four ANT isoforms, ANT2 is the only isoform that is overexpressed in cancer cells. ANT1 and ANT2 have shared about 80% sequence similarity. Despite of high similarity, ANT1 and ANT2 seem to have different functions. For example, ANT1 is reported to be pro-apoptotic and ANT2 is hypothesized to confer bioenergy advantage for cancer cells while protecting from cell apoptosis. The special roles of ANT2 in cancer cells are still poorly understood. For instance, whether reduction of ANT2 levels could be compensated by increasing expression of other ANT isoforms remains an open question. Our data show that ANT1 and ANT3 mRNA levels were unchanged when ANT2 was silenced in MDA-MB-231 cells, which indicated that the depletion of ANT2 did not result in increased expression of other ANT and the effect that we observed such decreased of ATP levels, cell migration and cell viability is specific induced by the depletion of ANT2. In addition, our lab has previously examined the putative promoter regions of the ANT isoforms for transcription factor binding sites present in ANT2, but not in ANT1, which revealed that c-Myc is one of the possible candidates that regulate the expression of ANT2. Activation of c-Myc expression is associated with enhanced proliferation and migration in many breast cancer cells. It will be interesting to investigate whether c-Myc is an upstream

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regulator of ANT2 and how c-Myc associated with ANT2 may contribute to cancer cell energy metabolism.

We have provided evidence to suggest an important role of ANT2 in OXPHOS in cancer cells. Our data support the hypothesis that cancer cells upregulate ANT2 to promote OXPHOS by facilitating ATP transport, which in turn possibly enhances cancer cell migration and proliferation. Warburg effect has previously suggested that cancer cells tend to use glycolysis instead of OXPHOS. It is possible that cancer cells acquire metabolic flexibility such that they are able to switch metabolism among glycolysis and OXPHOS depending on their needs and surrounding microenvironment. Expression of ANT2 has been reported to restore the growth of yeast with depletion of ANT in the absence of oxygen, suggesting ANT2 confers yeast cells the ability to carry out glycolysis. However, no direct data have supported that ANT2 promotes glycolysis in human cells. Our study instead has revealed an important role of ANT2 in promoting OXPHOS in human breast cancer cells, but we could not exclude that ANT2 may be involved in glycolysis in certain circumstance, such as hypoxia.

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