A ROLE FOR MP1 SCAFFOLD PROTEIN IN BREAST CANCER CELLS

By

Mihaela Marina

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Physiology

2011

ABSTRACT

A ROLE FOR MP1 SCAFFOLD PROTEIN IN BREAST CANCER CELLS

By

Mihaela Marina

Breast cancer cells are characterized by their uncontrolled proliferation, high motility and ability to escape cell death. These pathophysiological processes are driven by aberrant expression and activity of various intracellular pathways, including ER, PI3K/AKT/mTOR, MAPK and integrins. Activated by estrogen, growth factors, nutrients, and other extra- and intracellular stimuli, these signaling pathways can cooperate or compensate for each other in order to promote tumor growth, metastasis and drug resistance. In normal cells, signaling can be tightly regulated by scaffold proteins that can turn on or off pathways, according to the cellular context. One such scaffold protein is MP1, which was initially discovered within the MAPK pathway. Subsequent studies revealed its association with additional pathways and its implication in regulating spreading, migration, proliferation, and differentiation. Since the pathways MP1 interacts with regulate breast tumorigenesis, we hypothesized and MP1 can directly participate in regulating the proliferation and survival of breast cancer cells.

A panel of human mammary epithelial cell lines, consisting of ER-positive and ER-negative tumorigenic and non-tumorigenic cells was used. MCF-7 ER-positive and MDA-MB-231 ER-negative cells represented the main models for our studies. We

confirmed that MP1 was expressed in all the mammary cell lines investigated, with collectively higher levels in ER-positive breast cancer cells compared to ER-negative ones. We then used siRNA duplexes to investigate the role of MP1 in breast cancer cells. First, we observed that MP1 silencing specifically induced cell death of ER-positive cells, whereas ER-negative cell lines examined were largely unaffected. Next, we established that the observed cell death effect was apoptosis, as indicated by PARP cleavage and downregulation of Bcl-2 pro-survival protein. These data suggested a novel pro-survival role of MP1 protein that is specific to ER-positive breast cancer cells.

After establishing the biological relevance of MP1 silencing, we undertook a mechanistic approach and investigated the effects of MP1 siRNA on several signaling pathways. Partial depletion of MP1 was correlated with significant decrease in AKT activity and ER levels and activity. In contrast, MAPK activity was not affected, whereas reduced β1 integrin expression did not reach statistical significance. In addition, the pro-apoptotic effects triggered by MP1 silencing were not dependent on ER signaling, which is also not required for survival of MCF-7 cells. Finally, we re-confirmed that in contrast to MDA-MB-231 cells, MCF-7 cells require PI3K for survival and active AKT was able to partly rescue the cell death phenotype induced by MP1 silencing.

In summary, the studies presented here indicate a novel role for MP1 scaffold protein in ER-positive breast cancer cells. MP1 silencing resulted in cell death of ER-positive breast cancer cells only, therefore, expression of MP1 appears to be a requirement for promoting survival of this subset of breast cancer cells. They further suggest that the mechanism of cell death that occurred is apoptosis and this was mediated via PI3K/AKT signaling with possible cooperation from ER and β1 integrin.

ACKNOWLEDGEMENTS

Graduate school, especially the PhD research, is often regarded as a tedious and solitary journey and my experience was no exception to this belief. While most of the time I was alone trying to unveil the secrets of breast cancer cells, there were many people I could share my excitement and frustrations with.

I would like to thank Dr. Susan Conrad for her support and patience and for modeling my scientific thinking. The lessons I learnt from her will be very valuable during my research career.

My guidance committee was extremely helpful therefore, I express my gratitude to Drs. Kathy Gallo, Richard Miksicek, Hua Xiao and Vilma Yuzbasiyan-Gürkan.

Another supportive group was the Breast Cancer Signaling Network Consortium that informally guided me especially at the beginning of my project.

Throughout my studies, I have always counted on the support provided by the Department of Physiology, especially Dr. Arthur Weber and Ms. Kim Crain.

My journey would not have been complete without the help of my student fellows and friends here, at Michigan State University.

Last but not least, I am grateful for the unconditional love and support of my family and friends from Romania.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH	atypical ductal hyperplasia
AI	aromatase inhbitor
ALH	atypical lobular hyperplasia
AMP	adenosine monophosphate
AP-1	activating protein-1
Apaf-1	apoptotic protease activating factor 1
Bcl-2	B cell lymphoma 2
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
Ccd42	cell division control protein 42
CDK	cyclin-dependent kinase
CSS	charcoal stripped serum
DAPI	4',6-diamidino-2-phenylindole
DCIS	ductal carcinoma in situ
DISC	death-inducing signaling complex
E2	estrogen, 17-β estradiol
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor alpha
ERE	estrogen response element

ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
FAK	focal adhesion kinase
Fas	fatty acid synthase
FBS	fetal bovine serum
FLIP	FLICE inhibitory protein
Gab2	GRB2-associated-binding protein 2
GIP1	GBF interacting protein 1
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
IAP	inhibitor of apoptosis
ICI	ICI 182,780
IGF	insulin-like growth factor
IFG-IR	insulin-like growth factor 1 receptor
ILK	integrin-linked kinase
JNK	Jun N-terminal kinase
LCIS	lobular carcinoma in situ
MAPK	mitogen-activated protein kinase
MAPKSP1	mitogen-activated protein kinase scaffold protein 1
MEK	MAPK/ERK kinase
MMTV	mouse mammary tumor virus

MNAR	modulator of non-genomic action of estrogen receptor
MP1	MEK binding partner 1
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAK1	p-21-activated kinase-1
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffer saline
PDK1	phopshoinositide dependent kinase
PI3K	phopshoinositide 3-kinase
PI3KCA	phopshoinositide 3-kinase constitutively active
PIP3	phosphatidylinositol-3,4,5-trisphosphate
POSH	plenty of SH3 domains
PR	progesterone receptor
PTEN	phosphatase and tensing homolog
Raptor	regulatory associated protein of mTOR
Rho	Ras homologous
Rictor	rapamycin-insensitive companion of mTOR
RTK	receptor tyrosine kinase
SERM	selective estrogen receptor modulator
siRNA	small interfering ribonucleic acid
TNBC	triple negative breast cancer
TNF	tumor necrosis factor

TNF-R1	tumor necrosis factor receptor 1
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor

CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

I. Breast cancer incidence, risk factors, and the importance of targeted therapies

Over the past decade, both the incidence and mortality rate of breast cancer have decreased, reflecting improvements in screening, diagnosis and treatment methods. In spite of this encouraging progress, breast cancer remains the most common form of cancer detected globally and the second leading cause of cancer-related deaths in women. In 2011, over 39,000 women are expected to lose their lives to this disease in the United States alone (1, 2).

It is estimated than one in eight women will develop breast cancer at some point in her lifetime. The chance that a woman will be diagnosed with breast cancer can be estimated based on reproductive factors including age at onset of menarche, pregnancy status, use of oral contraceptives and hormone replacement therapies. In addition, age, family history, *BRCA1* and *BRCA2* mutations and lifestyle factors such as physical activity, obesity, and alcohol consumption affect breast cancer risk. Fatality from breast cancer is mainly due to metastasis and the risk of metastasis and relapse is correlated with a series of parameters including tumor size and grade, lymph node involvement, hormone receptor status, HER2 overexpression, and gene expression signatures (3). A decrease in incidence and mortality rates might be made possible by a more in depth understanding of the aforementioned clinical and biological determinants. Biomedical research continues to have a tremendous impact on the management of this disease through analysis and characterization of genetic, molecular and cellular risk factors.

In addition to providing reliable data about the probability of breast cancer occurrence and mortality, basic and translational research is the driving force behind improving treatment methods. Effective treatments include single use or combinations of radiation, surgery, chemotherapy and endocrine therapy. However, there are subsets of patients who do not benefit from current therapies, and breast tumors often undergo recurrence due to chemotherapy failure and acquired resistance to all therapies. The following section reviews the major subtypes of breast cancer in terms of general mechanisms of progression, incidence, prognosis, current treatment and resistance and future directions of biomedical research.

II. Breast cancer subtypes and therapies

The uncontrolled proliferation of mammary epithelial cells leads to the formation of pre-malignant lesions, called atypical ductal or lobular hyperplasia (ADH or ALH), which carry a high risk of developing breast cancer. This condition precedes the non-invasive stages called ductal or lobular carcinoma in situ (DCIS or LCIS). Since tumors progressing from DCIS have invasive potential (4), their treatment usually requires surgery, whereas patients with LCIS are subjected to careful screening or endocrine therapy (5).

From the molecular standpoint, there are four main subtypes of breast tumors that are treated with current therapies: ER-positive, ER-negative, HER2-positive and triple negative (ER-/PR-/HER2-) breast tumors. Another classification scheme for breast tumors separates them into five subtypes: Luminal A (ER+ and/or PR+, HER2-) with 42-59% incidence; Luminal B (ER+ and/or PR+, HER2+) with 6-12% incidence; HER2+

(ER-/HER2+) with 7-12% incidence; Basal (ER-/PR-/HER2-) with 15-20% incidence; and unclassified (ER-/PR-/HER2-/CK5/6-/HER-) breast cancers with 6-10% incidence (6-13). Generally, ER-negative tumors fall into the HER2-overexpressing and triple negative breast cancers groups. Although poorly differentiated, these tumors display distinct features, including high grade, pushing margins and high p53 expression (14). Unfortunately, ER-negative tumors are more aggressive and less amenable to therapy than ER-positive tumors, and often have a poor clinical outcome.

A. Estrogen receptor-positive breast tumors

Estrogen receptor (ERα, for simplicity, ER) is a main regulator of mammary gland development and a potent stimulator of breast cancer proliferation. Expressed in over 70% of breast tumors (defined by >1% positive staining), ER is a therapeutic and prognostic marker, since its status is a good predictor of response to endocrine therapies and is correlated with low risk of metastasis. The estrogen hormone, its natural ligand, is synthesized by ovaries in premenopausal women and by peripheral tissue in postmenopausal women. Upon binding to ER, estrogen is a powerful growth stimulant that controls the early stages of breast tumor proliferation (15). Aiming to inhibit the proliferative effects of estrogens, endocrine therapy blocks further growth of breast tumors and is usually well tolerated by both pre- and postmenopausal women. In general, anti-estrogen therapy is adjuvant to combinations of surgery, radiation and chemotherapy, but can be also administered pre-surgery.

Current endocrine therapies involve the administration of selective estrogen receptor modulators (SERMS) such as tamoxifen and raloxifene, which block binding of estrogen

to ER, aromatase inhibitors (AI) such as anastrozole, letrozole or exemestane, which inhibit synthesis of estradiol, and pure antiestrogens such as fulvestrant, which induce degradation of ER (16-19).

After initially responding to ER inhibition, 30% of breast tumors can become tamoxifen insensitive, and many progress to a non-responsive state to all endocrine therapies (16, 20, 21). Therefore, innate and acquired endocrine resistance remains the greatest challenge in the management of ER-positive breast tumors. Evidence from cell-based studies, pre-clinical animal models, genome-wide arrays and proteomic analyses of breast tumors led to several proposed mechanisms for endocrine resistance. This phenomenon can be due to deregulation of the ER signaling pathway, including the receptor itself (22-26) or the switch of tamoxifen action from antagonist to agonist (27), impaired cell cycle regulation (28-30), impaired survival signals (31), or activation of compensatory cytosolic pathways that will promote tumor proliferation and survival in the presence of antiestrogens (32).

B. HER2-overexpressing breast tumors

The human epidermal growth factor (HER) family of receptors is another example of molecules that regulate cell growth in normal cells but have a potent oncogenic role in breast cancer (33, 34). Approximately 20% of breast cancer patients have tumors that overexpress HER2, an event that was historically associated with an aggressive phenotype and poor survival rate (35). HER2 signals via homo- or heterodimerization with the other HER receptors, with the strongest oncogenic unit being HER2:HER3 (36).

Current therapies targeting HER2-overexpressing breast tumors include the monoclonal antibody trastuzumab, which blocks receptor downstream signaling and the small molecule tyrosine kinase inhibitor, lapatinib, which inhibits the kinase activity of HER2. Both compounds were shown to block cell proliferation and promote cytotoxicity and were used as monotherapy or in combination with chemotherapeutic agents. Unfortunately, HER2-overexpressing breast cancers can acquire resistance to anti-HER2 therapies and this may be attributed to the expression of truncated forms of the receptor or to misregulated signaling downstream of it, which can circumvent the inhibition of proliferative and survival signals.

As evidence of their genetic and molecular heterogeneity, a number of HER2-overexpressing breast tumors are also ER-positive, thus several trials have addressed the combination of these targeted therapies with promising results in terms of progression free survival (37-40).

C. Triple negative breast tumors

Around 10-15% of breast tumors do not express estrogen receptor, progesterone receptor, or HER2 and are classified as triple negative (TNBC) (41-43). Being non-responsive to both endocrine and anti-HER2 therapies, these tumors have a more aggressive phenotype and a poor prognosis. Patients with TNBC have the highest risk of developing metastasis (44, 45) and a high risk of recurrence.

Treatment of TNBC is based on standard cytotoxic polychemotherapy (46). Cytotoxic agents target mitotic events, including microtubule assembly, blocked by the use of taxanes, and the synthesis, replication and transcription or DNA, which can be inhibited

by platinum, anthracycline antibiotics or fluorouracil. Since these compounds unselectively target all proliferating cells, some common side effects include immunosuppression, alopecia, and gastrointestinal bleeding and vomiting. In spite of the systemic off-target effects, chemotherapy has been effective in the management of TNBC as neoadjuvant, adjuvant and anti-metastatic strategy (47).

TNBCs are very heterogeneous and several biomarkers with therapeutic and prognostic relevance have been identified. For example, patients harboring *BRCA1* and *BRCA2* germ-line mutations responded positively to poly(ADP-ribose) polymerase (PARP) inhibitors. In this setting, inhibition of PARP (DNA-repairing enzyme) induced cell death, by blocking the homologous recombination DNA repair step, which is critical to the cells in the absence of BRCA1 (48).

In summary, the genetic and molecular heterogeneity of breast tumors confer on epithelial cancer cells the ability to become poorly or non-responsive, to current therapies. An important goal of biomedical research is to unravel the mechanisms that cause resistance to various therapies, and to develop new drugs that will be effective for specific breast cancer subtypes. In this era of novel targeted therapies, the identification and validation of tumor biomarkers has also become an essential component of breast cancer research. Single or panels of biomarkers can potentially indicate the onset and progression of breast tumors, and/or predict the response to therapeutic agents. Tracking changes in appropriate markers in biopsies or circulating tumor cells in response to drug administration could provide correlations between the biomarker status and the efficacy of a particular drug. Ideally, this might indicate patients who are

less likely to benefit from newly designed compounds, and these patients could be excluded from long-term clinical trials with these drugs.

III. Signaling pathways in breast cancer and the importance of scaffold proteins

At the cellular level, breast cancer is not a single disease but rather a collection of conditions that vary in their biology and response to treatment. As with other tumor cells, mammary cancer cells are characterized by high uptake of nutrients from their media, abnormal growth, uncontrolled proliferation, impaired DNA replication and repair, evasion of cell death, and the acquisition of new abilities that allow them to invade the surrounding tissues, survive in the bloodstream, migrate and colonize distant organs (49). This section reviews some of the signaling pathways that are responsible for aberrant cellular processes that contribute to malignant transformation, and emphasize the biological and clinical relevance of signal transduction form these pathways and their complex interactions.

A. Estrogen receptor

As previously stated, ER promotes proliferation of breast cancer cells. ER is a type I ligand-activated nuclear receptor that functions as a transcription factor. Ligand-activated ER dimerizes, translocates into the nucleus where it recruits coregulators and binds promoter regions of DNA that contain estrogen response elements (ERE). Direct binding to an ERE is referred to as the classical genomic pathway (50). In addition, ER

can regulate gene transcription indirectly, via protein-protein interactions with other transcription factors such as c-Fos/c-Jun, which bind activating protein 1-(AP-1) containing promoters (non-classical genomic pathway) (51). By turning on genes encoding proteins implicated in tumor proliferation, such as cyclin D1 (52), IGF-IR (53) and c-Myc (54, 55), ER promotes autocrine regulation of cell proliferation (56).

Emerging data point out the bidirectional cross-talk between ER and other signaling molecules. First, ER can be activated by phosphorylation on a number of conserved residues, and this activation does not necessarily require the presence of estrogen (57). The ligand-independent receptor activation occurs in response to signals from growth factor receptors, such as HER2, epidermal growth factor receptor (EGFR), and insulin-like growth factor receptor (IGF-IR) (58, 59). Downstream kinases, including MAPK, AKT, JNK, PAK1 and ILK phosphorylate and activate ER on specific sites (60-67) and phosphorylation of ER promotes ligand independent growth of breast cancer cells and has been linked to endocrine resistance (61, 68-70). The clinical relevance of ER phosphorylation at Ser167 includes the predictive of response to endocrine therapy and a good prognosis for overall survival in breast cancer patients (71-73).

In addition to its genomic effects, ER has non-genomic functions. By modulating the activity of several cellular kinases, ER signaling controls a subset of genes that are usually regulated by growth factor signaling (62, 63, 74, 75). These non-genomic effects are due to a small subpopulation of extranuclear ER that may be homologue to the nuclear ER or an atypical G-coupled protein receptor (76). ERs localize to the plasma membrane, activate cyclic AMP second messenger or receptor tyrosine kinases HER2,

IGFR and EGFR (77-80). Additionally, ER has functional interactions with adaptor protein Shc and with Src and PI3K kinases (59, 81-85).

In addition to its well-established proliferative role, several studies indicate that ER may be implicated in breast cancer cell survival. ER promotes survival of breast cancer cells via cross-talk with the PI3K/AKT pathway (61), or by regulating the activity of NF-κB (86, 87), Bcl-2 (88, 89), and inhibitor of apoptosis (IAP) family members (90).

In summary, ER is a crucial regulator of breast cancer cell proliferation and survival. Its activation and signal transduction are part of intricate bidirectional interactions with other signaling pathways with known roles in malignant transformation and drug resistance.

B. PI3K/AKT/mTOR

The phopshoinositide 3-kinase (PI3K) pathway is an important signaling node in breast cancer biology. Activated by signals received from various sources, such as IGF-IR, integrins or ER (91), PI3K lipid kinases phosphorylate the 3'-hydroxyl group of phosphoinositides like phosphatidylinositol-3,4,5-trisphosphate (PIP3). This second messenger recruits phopshoinositide dependent kinase (PDK1) and the serine/threonine kinase AKT to the plasma membrane (92, 93). In turn, PDK1 phosphorylates AKT on Th308, and this particular phosphorylation is essential for kinase activity (94). Complete activation of AKT is achieved by phosphorylation of Ser473 by a different kinase. The lipid phosphatase and tensin homolog PTEN is the physiological inhibitor of PI3K, since it dephosphorylates PIP3. Therefore, the levels of PIP3 are important for maintaining normal cellular functions.

Increased PI3K signaling is associated with breast tumorigenesis and can be attributed to a number of genetic and molecular alterations including high levels of constitutive active AKT1, mutations and amplifications of *PI3KCA* and mutations of *PTEN* tumor suppressor in 30% of breast cancers (95-97). AKT is the primary target of PI3K signaling. This kinase plays a pro-survival role in breast cancer cells, where it relays signals from upstream molecules such as integrins, growth factor receptors, PI3K, and mTORC1, to downstream molecules such as FLIP, Bcl-2, Bad, and NF-κB (98-101). Data from murine models of breast cancer indicate that AKT1 cooperates with other oncogenes to promote mammary tumorigenesis (102).

Both cell-based and animal models of breast tumors report the cross-talk between AKT and other oncogenic pathways, including Raf/MAPK, ER, and integrin-linked kinase (ILK). Phosphorylation of Raf by AKT inhibited the Raf-MEK-ERK pathway and induced proliferation of breast cancer cells (103). This controversial effect is supported by previous findings showing that prolonged activation of the Raf/MAPK pathway results in growth inhibition (104). Several interactions between AKT and ER signaling have been demonstrated in breast cancer cells. For example, AKT phosphorylates ER and promotes survival of breast cancer cells (61), estrogen activates AKT (105, 106) and in turn, the AKT kinase activity modulates the effects of estrogen on ER activity (107, 108). Finally, in mouse mammary glands, high levels of AKT phosphorylation are correlated with overexpression of ILK, hyperplasia, and subsequent tumor formation (109).

In addition to promoting breast tumor growth and survival, activation of PI3K/AKT signaling pathway has also been linked to resistance to antiestrogens (110-114) and trastuzumab (115-117). Thus, compounds targeting the PI3K/AKT signaling pathway

represent a potential therapeutic tool for ER-positive and HER-2 overexpressing breast tumors with primary or acquired resistance to current therapies (118).

Another downstream target of PI3K is the mammalian target of rapamycin (mTOR), which is activated in response to nutrients and growth factors in proliferating cells and is an essential regulator of nutrient uptake, protein synthesis, growth, cell cycle, and survival/apoptosis (119). Two mTOR complexes have been identified, and they differ in their protein composition and response to rapamycin, which inhibits mTORC1 but not mTORC2. Both complexes include the mTOR serine/threonine kinase and one of the associated proteins raptor and rictor, which are found in mTORC1 and mTORC2, respectively. Importantly, mTORC1 is activated by AKT (120), whereas mTORC2 signals upstream of and can phosphorylate AKT at Ser473 (121, 122). Due to its demonstrated functions in mammalian cells, it is not surprising that hyperactive mTOR signaling drives breast tumor growth by stimulating translation of mRNA and protein synthesis, cycle progression and survival of breast cancer cells (123).

In conclusion, the PI3K/AKT/mTOR pathway integrates signals from growth factor receptors, integrins and ER to regulate growth, proliferation and survival of breast cancer cells, and is also implicated in drug resistance. Therefore, pre-clinical and early clinical studies have been carried out using inhibitors of PI3K, mTOR, dual PI3K/mTOR inhibitors, and compounds that selectively target the AKT kinase (118, 123-126). Ongoing trials include blockade of PI3K/AKT combined with aromatase inhibitors or trastuzumab.

C. MAPK

The MAPK signal transduction requires sequential activation of three kinases (127, 128) and the Raf-MEK-ERK module is the most studied MAPK cascade in the context of breast cancer biology (129). The extracellular regulated kinase (ERK) receives amplified signals from its upstream partners and phosphorylates its cytosolic and nuclear targets to mediated cell proliferation (130, 131).

The role of mitogen activated protein kinases (MAPKs) in breast tumorigenesis significantly overlaps with that of growth factor receptor tyrosine kinases, since the MAPK linear cascade is activated downstream of HER2, EFGR and IGF-IR. In addition to growth factors, membrane-localized ER, G-coupled protein receptors and several ligands, including insulin, prolactin, estrogen, and progesterone can initiate responses through MAPK signaling to regulate proliferation, growth, differentiation, migration, and survival of breast cancer cells (130). Moreover, activation of MAPK was observed in human tumors and was correlated with decreased disease free survival (132).

In breast cancer cells, estrogen rapidly activates ERK, which translocates to the nucleus (133). Additional studies confirmed that proliferation of ER-positive breast cancer cells is partly due to activation of ERK by estrogen (134-137). Estrogen-induced activation of MAPK involves IGF-IR and EFGR to promote growth and survival of breast cancer cells (75, 138). Conversely, MAPK stimulates ER signaling, via phosphorylation of ER (139). A notable aspect of MAPK function is the compensatory activation after estrogen deprivation (140, 141). Both cell culture and *in vitro* models have demonstrated that ER-positive cells adapt to long term deprivation of estradiol by activating the MPAL pathway,

which promotes cell proliferation via ER transactivation and also independently from ER. Moreover, high levels of tyrosine kinase activity are correlated with estrogen independence and anti-estrogen resistance, resulting in increased ERK activity (142, 143).

In summary, the MAPK signaling pathway is a crucial regulator of breast tumorigenesis, particularly via complex interactions with ER, in response to estrogen and growth factor-driven stimulation. In clinical practice, MEK blockers are relatively new targets and ongoing trials are underway to investigate the effects of specific MEK inhibitors in solid tumors.

D. PAK

p21-activated kinase (PAK) is another serine/threonine kinase with well-established roles in the biology of breast cancer. In normal cells, PAK regulates actin reorganization and cell motility via binding to the small GTP-ases Rac and Cdc42, or through its scaffolding function between PDK1 and AKT (144, 145). Subsequent studies performed in fibroblasts reported a cross-talk between PAK1 and MAPK, as PAK1 activates a population of MEK1 and ERK in focal complexes during adhesion to fibronectin in the presence of MP1 scaffold protein (146, 147).

PAK1 has increased expression and activity in breast tumors (148-150) and in many breast cancer cells (151), where it promotes cell migration (152, 153). PAK1 can be activated by known mitogens such as heregulin (154). In addition, high expression of PAK1 promotes malignant transformation by inducing chromosome instability in tumorigenic epithelial cells (152) and aberrant survival and transformation of non-

tumorigenic mammary epithelial cells (65, 69, 148, 155, 156). MMTV-PAK1 transgenic mice developed malignant tumors that also expressed active (phosphorylated) MEK1 (157). Several reports have noted the interaction of PAK1 with ER signaling, since ER is a direct phosphorylation target of PAK1, and the kinase was linked to tamoxifen resistance (65, 69).

In conclusion, data emerging from human tumor samples together with functional studies provided the rationale for designing small molecule inhibitors of PAK1. Future pre-clinical investigations will evaluate the therapeutic potential of PAK1 inhibition in the context of breast cancer (158) .

E. Integrins

Proliferation and survival of breast cancer cells is highly dependent of their attachment to the extracellular matrix and this attachment is mediated by integrins. Mammalian cells have 24 distinct integrin receptors, which are dimers formed by combinations of α and β subunits. Their ligands are the extracellular matrix proteins collagen, laminin, fibronectin, and vitronectin (159, 160). While helping to maintain cell adhesion to the matrix and three-dimensional tissue structure, integrins connect extra- to intracellular molecules to regulate proliferation, survival, gene transcription and migration (161). Ligand-bound integrins function at focal adhesions, where they interact with paxillin, vinculin, talin, or alpha-actinin, intracellular proteins that regulate actin polymerization and formation of motile structures. Focal adhesions are also active signaling nodes, via activation of focal adhesion kinase (FAK), ILK, s-Src, Rho and Cdc42 as well as MAPK and PI3K (162).

Several integrin dimers are highly expressed in breast cancer cells and have been shown to contribute to malignant transformation. For example, α6β4 integrin activates PI3K through increasing HER2 activation (163), and the interaction of this integrin with another growth factor receptor was required for PI3K-mediated migration (164). In a 3D model of mammary acini, α6β4 integrin activates PAK1 to promote resistance to apoptosis (165) while β4 subunit was required to promote HER2-mediated tumorigenesis *in vivo* (166). Another laminin receptor, αvβ3 integrin, has higher expression in tumorigenic compared with nontumorigenic mammary epithelial cells (167), and its expression is correlated with the metastatic potential of breast cancer cells (168). Furthermore, inhibition of αvβ3 integrin blocks VEGF-induced angiogenesis and decreases cell proliferation (169). Finally, several studies have noted that expression and activity of another dimer, the αvβ1 integrin, is correlated with adriamycin resistance (170) and induces migration of breast cancer cells expressing Shc (171).

Individual integrin subunits have been characterized in terms of expression, activity and their roles in breast cancer cells. Thus, α2 integrin inhibits metastasis in cell culture and in mouse models and is positively correlated with ER status in breast tumors (172). Significant research investigated the role of β1 integrin in breast tumorigenesis. This subunit displays aberrant expression in 30-50% of breast tumors (173) and is associated with low disease free survival (174). β1 integrin has been linked to chemotherapy resistance (175, 176). In 3D culture, β1 integrin inhibitory antibodies induce apoptosis of several breast cancer cell lines and sensitize cells to radiotherapy in xenograft models (177, 178). Moreover, β1 integrin is required for tumor formation and growth in a mouse model of human breast cancer (179).

Integrins can indirectly regulate the biology of breast cancer cells through signals emerging from their downstream targets such as ILK and FAK kinases. FAK is a cytosolic kinase activated upon integrin ligation and is responsible for controlling motility, proliferation and survival (180-182). *In vitro* studies have shown that FAK is activated by PI3K and can in turn activate Ras/MAPK pathway (146, 183, 184). Analysis of metastatic breast tumors and DCIS revealed high levels of FAK, suggesting its role in tumor initiation and metastasis (185, 186). Subsequent studies reported that its expression in breast tumor tissue was correlated with invasiveness (186, 187). Blockade of FAK expression inhibited lung metastasis *in vivo* and the kinase also cooperated with ERK to induce angiogenesis in breast cancer cells (188, 189).

ILK is a serine/threonine kinase that binds to β1 and β3 integrin at focal adhesions (190) and requires PI3K and GSK3 to phosphorylates AKT on Ser473 (191, 192). While normal cells have low levels of ILK activity, this kinase is highly expressed in breast tumors, and its overexpression was correlated with p-AKT at Ser473 and elevated levels of IGF1 (193). Functional assays in breast cancer cells indicate that ILK suppresses anoikis, promotes cell survival through the PI3K/AKT/mTOR pathway (194-196), and phosphorylates ER to facilitate estrogen-mediated migration (66). In addition, interaction of ILK with rictor protein (of mTORC2) stimulates phosphorylation of AKT1 and promotes survival (197). Finally, overexpression of ILK causes malignant transformation *in vivo* (109).

In summary, a great body of evidence demonstrates the role of integrin signaling in breast cancer formation and progression and offers new possibilities of therapeutic interventions (198).

F. Scaffold proteins

Cells integrate information from a large number of extracellular and intracellular signals in order to elicit specific responses. As part of this integration, the stoichiometry, stability, and compartmentalization of signaling complexes are regulated by scaffold proteins. Located most often at branch points within signaling networks, scaffolds mediate cross-talk between key molecules and can potentiate or inhibit the strength of signals. Models of pathway connections through scaffold proteins vary from simple to complex. For example, a scaffold protein can function to facilitate interactions in a linear manner or it can mediate pathway branching and amplification of output to multiple downstream partners. Finally, scaffolds can be part of more elaborate interaction networks involving feedback regulation, thus directly activating or blocking signaling pathways (199). In turn, scaffold proteins can be regulated based on a cell's needs and the presence or absence of scaffold proteins therefore contributes to the diversity of possible cellular responses.

Functional studies indicate a role for several scaffold proteins in breast cancer. For instance, modulator of non-genomic action of estrogen receptor (MNAR) mediates the interactions of ER with PI3K and Src, and is required for the rapid effects of estrogen in breast cancer cells (200). POSH is another scaffold protein the proapoptotic function of which is suppressed by AKT in breast cancer cells (201). The Gab2 adaptor/scaffold protein is required to promote mammary tumor metastasis in *neu*-expressing mice (202). A recent study investigated the GIP1 scaffold protein, which was previously linked to IGF-IR and other receptors (203). GIP1 gene silencing inhibited proliferation and induced apoptosis of breast cancer cells (204).

In conclusion, scaffold proteins are essential for complex signaling networks that impact various cellular functions. Changes in the levels of scaffold protein genes or their mislocalization can potentially alter signal transduction and lead to faulty cell decisions that may ultimately contribute to breast tumorigenesis. The following section addresses some of the currently known facts about the scaffold protein MP1 and its potential relevance in the context of breast cancer.

G. MP1 scaffold protein

MP1 (MEK partner 1) is a 14 KDa (124 amino acid) scaffold protein that is present in several species with various degrees of homology to humans: mouse (124 aa, 97% homology), rat (124 aa, 96%), frog (*Xenopus laevis*; 123 aa, 87%), fruit fly (*Drosophila*; 124 aa, 45%), and worm (*Caenohrabditis elegans*; 145 aa, 20%) (205). The 16.2 kb MP1 gene is found on chromosome 4 and has seven exons and six introns. Alternative splicing results in three transcripts, with variant 1 being the longest transcript that encodes the 124 amino acid protein. According to NCBI RefSeq, transcript 3 encodes a shorter isoform (117 aa) but neither the CCDS (Consensus CoDing Sequence) database nor the UniProt database provide public data for this second isoform. MP1 was originally identified as a scaffold protein that specifically binds MEK1 and ERK1, and its ability to regulate MAPK signaling (206) and its localization to late endosomes are the most well characterized functions of this protein.

The scaffolding role of MP1 has been demonstrated in several *in vitro* studies that report its association with large signaling complexes located to different intracellular compartments (207). For example, in late endosomes, MP1 interacts with adaptor

proteins p14, p18 and KRas to induce ERK activation (208-211). p14 is required to localize MP1 to late endosomes, and although they have different primary sequences the two small proteins have similar structures and form a high affinity heterodimer (205, 212). Biochemical and crystallographic analyses found that both proteins consist of a five-stranded β-sheet flanked by three α-helices (205, 213). No specific protein interaction domains were identified. However, the structures of MP1, p14 and their heterodimer indicate the presence of several surface-exposed residues, suggesting the possibility of interaction with cytoplasmic proteins (205, 212, 213).

A more recent study reports the presence of MP1 in the mTOR pathway (214). In this study, the MP1/p14/p18 trimeric, coined Ragulator, interacts with and localizes Rag GTP-ases to lysosomes. Moreover, MP1 knockdown in HEK392T cells prevents the lysosomal recruitment of mTORC1 in the presence of amino acids and inhibits the amino acid-induced stimulation of dTORC1 in *Drosophila*, suggesting that MP1 is required for the recruitment of mTORC1 to lysosomes and is essential for the amino acid-dependent activation of the complex. MP1 is also required for PAK1 to activate a population of MEK1 in focal complexes during adhesion of rat fibroblasts to fibronectin (215) and this interaction is sufficient to activate MEK1 in the absence of Raf, but to a lesser extent than observed with active Raf or EGF (147). Coimmunoprecipitations carried out in CCL39 mice fibroblasts shown an interaction between MP1 and RACK1, a scaffold protein that is required for the presence of active ERK to focal adhesions (216). In a family (humans) with an immunodeficiency syndrome, a mutation in 3' UTR for exon 4 lead to reduced expression of p14 gene and the affected individuals had stunted growth (217).

Several cell-based and *in vivo* studies have addressed the functional role of MP1. Silencing of MP1 delayed spreading of fibroblasts and decreased migration of prostate cancer cells on fibronectin (215, 218). Furthermore, deletion of *p14* gene in mice causes defects in embryo development and embryonic lethality before gastrulation (10.5 days). Epidermis of the $p14^{-/-}$ mice displayed fewer cell layers and had a lower mitotic index which prompted the authors of this study to conclude that p14/MP1 complex regulates cellular proliferation during development (209). Finally, transgenic lines of *Drosophila,* with either silenced or over-expressing dMP1, develop ectopic veins during wing development, suggesting that MP1 ortholog is required to regulate the vein cell differentiation (219).

Taken together, these findings combined with published literature in breast cancer cell biology provide evidence for a potential role of MP1 scaffold protein in regulating survival, proliferation and motility of breast cancer cells.

IV. Apoptosis

Apoptosis is a normal cellular process in the development of breast tissue as it takes place during the formation of ducts and lumens, at the end of menstrual cycle and during involution post lactation. In breast tumors, apoptosis is the final effect of many drugs, in particular chemotherapeutic agents that are known to induce cytotoxicity. Morphologically, apoptotic cells shrink, display membrane blebbing, chromatin condensation and eventually disintegrate into apoptotic bodies which are subjected to phagocytosis (220).

At a cellular level, apoptosis is defined as the programmed cell death that involves two distinct processes, in terms of their localization and activation. The intrinsic pathway of apoptosis is activated mainly in response to DNA damage and centers on the release of cytochrome *c* from mitochondria. The decision of cell to undergo apoptosis is dependent on the functional balance between the pro-apoptotic and pro-survival members of the Bcl-2 protein family, which control the mitochondrial channels and release of cytochrome *c* (221). Once released, the cytochrome *c* binds Apaf-1 scaffold protein and facilitates the assembly of apoptosome and activation of initiator caspases 2, 8, 9 and 10. At the same time, released mitochondrial protein Smac binds to and inactivates the inhibitor of apoptosis proteins (IAP). As a result, effector caspases are activated by proteolytic cleavage and can further cleave their downstream targets, including intermediate filament proteins, nuclear lamin, and the DNA-repair enzyme, PARP.

The extrinsic pathway of apoptosis is triggered upon activation of a death receptor at the plasma membrane. TRAIL and FAS receptors bind their cognate ligands and undergo conformational changes to recruit Fas-associated death domain (FADD) protein and form the death-inducing signaling complex (DISC). FADDs recruit and mediate cleavage of caspase 8, thus activating the caspase cascade. In addition, by cleaving the pro-apoptotic Bid member of the Bcl-2 family, caspase 8 integrates the intrinsic and extrinsic apoptotic pathways. A distinct apoptotic extrinsic mechanism is represented by TNF signaling. Secreted by tumor cells or by their neighboring cells, this cytokine binds and activates TNF-R1. In turn, the receptor associates with TRADD and signals to activate caspases via interaction with FADD or to regulate gene transcription though NF-κB signaling.

Additional proteins have been shown to regulate apoptosis. Cellular FLICE inhibitory protein (c-Flip) prevents cleavage and activation of caspase 8 (222) and several cell-based studies revealed its requirement for cell survival. Activated AKT can repress apoptosis by inactivating caspase 9 and Bad, FLIP or by upregulating Bcl-2 (99, 101, 222). The Myc oncoprotein and NF-κB seem to have both pro-apoptotic and anti-apoptotic roles. A notable regulator of apoptosis is the p53 tumor suppressor, which targets many components of the apoptotic machinery, triggers apoptosis under DNA damage events, thus contributing to the tissue homeostasis. In turn, in breast cancer cells, AKT was shown to mediate degradation of p53 protein (223).

Breast cancer cells have developed strategies to evade apoptosis in order to overcome two main obstacles: to survive in the hostile environment found in the bloodstream during migration and to resist the cytotoxic effects of therapeutic agents or gamma irradiation. One way of achieving this requirement is by altering the levels of molecules directly implicated in apoptosis. For example, the Bcl-2 pro-survival protein is overexpressed in breast tumors, and this is correlated with poor prognosis (224). In addition, mutation of *TP53* and loss of p53 functional protein impedes the ability of breast cancer cells to induce pro-apoptotic genes (225). An alternative mechanism of escape occurs through constitutive activation of growth factor signaling pathways and their downstream MAPK and PI3K/AKT cascades, and this strategy accounts for drug resistance in many types of breast tumors. Although challenging, understanding the mechanisms regulating apoptotic events in subtypes of breast cancer may provide valuable clues about potential novel molecules with prognostic and therapeutic

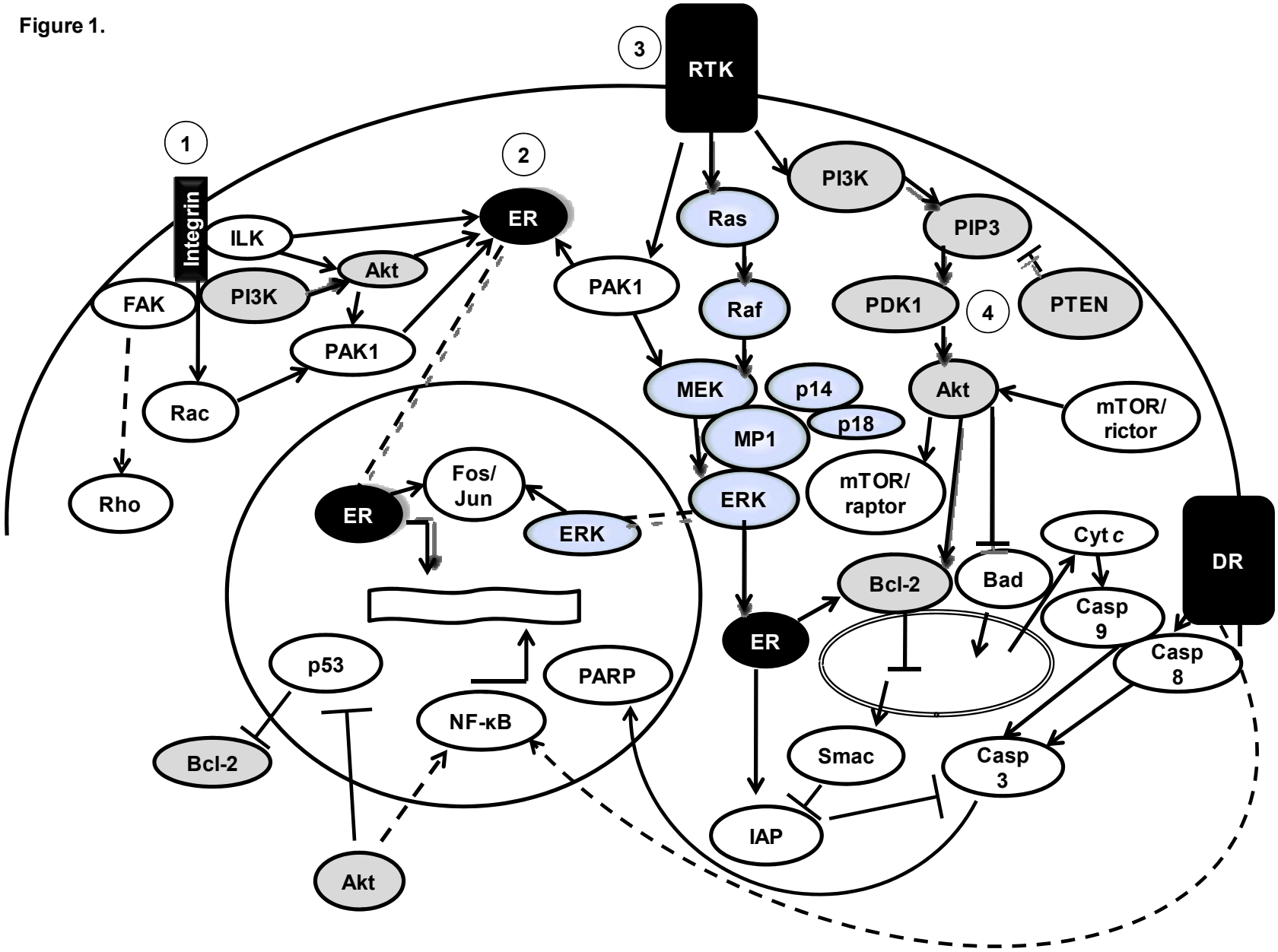
relevance. This dissertation research has focused on one such protein, MP1, which we identify as an important pro-survival molecule in ER-positive breast cancer cells.

Figure 1. Signaling pathways in breast cancer.

1. Loss of integrin expression triggers defects in cell adhesion and increased tumor cell invasiveness and metastasis.
2. Activation of ER signaling by ligands or phosphorylation leads to synthesis of cell cycle proteins and promotes cell proliferation.
3. Activation of receptor tyrosine kinases by growth factors stimulates proliferation and growth of tumor cells and also renders ER ligand-independent.
4. Activation of the PI3K/AKT pathway and of Bcl-2 pro-survival protein leads to escape from apoptosis and promotes tumor cell survival.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 1.


3
RTK
1
2
Integrin
ILK
Akt
FAK
PI3K
PAK1
Rac
Rho
ER
PAK1
Ras
Raf
MEK
p14
p18
MP1
ERK
PI3K
PIP3
PDK1
4
PTEN
Akt
mTOR/
rictor
mTOR/
raptor
ER
Fos/
Jun
ERK
p53
PARP
NF-κB
Bcl-2
Akt
ER
Bcl-2
Bad
Cyt c
DR
Casp
9
Casp
8
Casp
3
Smac
IAP

REFERENCES

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69-90.

2. Kohler BA, Ward E, McCarthy BJ, Schymura MJ, Ries LA, Eheman C, et al. Annual report to the nation on the status of cancer, 1975-2007, featuring tumors of the brain and other nervous system. J Natl Cancer Inst. 2011;103:714-36.

3. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415:530-6.

4. Dupont WD, Page DL. Risk factors for breast cancer in women with proliferative breast disease. N Engl J Med. 1985;312:146-51.

5. Espina V, Liotta LA. What is the malignant nature of human ductal carcinoma in situ? Nat Rev Cancer. 2011;11:68-75.

6. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006;295:2492-502.

7. Hannemann J, Kristel P, van Tinteren H, Bontenbal M, van Hoesel QG, Smit WM, et al. Molecular subtypes of breast cancer and amplification of topoisomerase II alpha: predictive role in dose intensive adjuvant chemotherapy. Br J Cancer. 2006;95:1334-41.

8. Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB, et al. Concordance among gene-expression-based predictors for breast cancer. N Engl J Med. 2006;355:560-9.

9. Potemski P, Kusinska R, Watala C, Pluciennik E, Bednarek AK, Kordek R. Prognostic relevance of basal cytokeratin expression in operable breast cancer. Oncology. 2005;69:478-85.

10. Yang XR, Sherman ME, Rimm DL, Lissowska J, Brinton LA, Peplonska B, et al. Differences in risk factors for breast cancer molecular subtypes in a population-based study. Cancer Epidemiol Biomarkers Prev. 2007;16:439-43.

11. Millikan RC, Newman B, Tse CK, Moorman PG, Conway K, Dressler LG, et al. Epidemiology of basal-like breast cancer. Breast Cancer Res Treat. 2008;109:123-39.

12. Stead LA, Lash TL, Sobieraj JE, Chi DD, Westrup JL, Charlot M, et al. Triple-negative breast cancers are increased in black women regardless of age or body mass index. Breast Cancer Res. 2009;11:R18.

13. Lund MJ, Butler EN, Hair BY, Ward KC, Andrews JH, Oprea-Ilies G, et al. Age/race differences in HER2 testing and in incidence rates for breast cancer triple subtypes: a population-based study and first report. Cancer. 2010;116:2549-59.

14. Putti TC, El-Rehim DM, Rakha EA, Paish CE, Lee AH, Pinder SE, et al. Estrogen receptor-negative breast carcinomas: a review of morphology and immunophenotypical analysis. Mod Pathol. 2005;18:26-35.

15. Russo IH, Russo J. Role of hormones in mammary cancer initiation and progression. J Mammary Gland Biol Neoplasia. 1998;3:49-61.

16. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;365:1687-717.

17. Cuzick J, Sestak I, Baum M, Buzdar A, Howell A, Dowsett M, et al. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. Lancet Oncol. 2010;11:1135-41.

18. Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet. 2005;365:60-2.

19. Vergote I, Robertson JF, Kleeberg U, Burton G, Osborne CK, Mauriac L. Postmenopausal women who progress on fulvestrant ('Faslodex') remain sensitive to further endocrine therapy. Breast Cancer Res Treat. 2003;79:207-11.

20. Chia S, Gradishar W. Fulvestrant: expanding the endocrine treatment options for patients with hormone receptor-positive advanced breast cancer. Breast. 2008;17 Suppl 3:S16-21.

21. Clarke R, Shajahan AN, Riggins RB, Cho Y, Crawford A, Xuan J, et al. Gene network signaling in hormone responsiveness modifies apoptosis and autophagy in breast cancer cells. J Steroid Biochem Mol Biol. 2009;114:8-20.

22. Kuske B, Naughton C, Moore K, Macleod KG, Miller WR, Clarke R, et al. Endocrine therapy resistance can be associated with high estrogen receptor alpha (ERalpha) expression and reduced ERalpha phosphorylation in breast cancer models. Endocr Relat Cancer. 2006;13:1121-33.

23. Naughton C, MacLeod K, Kuske B, Clarke R, Cameron DA, Langdon SP. Progressive loss of estrogen receptor alpha cofactor recruitment in endocrine resistance. Mol Endocrinol. 2007;21:2615-26.

24. Han F, Miksicek R, Clarke R, Conrad SE. Expression of an estrogen receptor variant lacking exon 3 in derivatives of MCF-7 cells with acquired estrogen independence or tamoxifen resistance. J Mol Endocrinol. 2004;32:935-45.

25. Clarke R, Liu MC, Bouker KB, Gu Z, Lee RY, Zhu Y, et al. Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. Oncogene. 2003;22:7316-39.

26. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst. 2003;95:353-61.

27. Barkhem T. Molecular mechanisms of estrogen and antiestrogen action. Department of Medical Nutrition, Karolinska Institutet, Stockholm, Sweden. 2002.

28. Prall OW, Sarcevic B, Musgrove EA, Watts CK, Sutherland RL. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J Biol Chem. 1997;272:10882-94.

29. Varma H, Conrad SE. Reversal of an antiestrogen-mediated cell cycle arrest of MCF-7 cells by viral tumor antigens requires the retinoblastoma protein-binding domain. Oncogene. 2000;19:4746-53.

30. Mukherjee S, Conrad SE. c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells. J Biol Chem. 2005;280:17617-25.

31. Riggins RB, Bouton AH, Liu MC, Clarke R. Antiestrogens, aromatase inhibitors, and apoptosis in breast cancer. Vitam Horm. 2005;71:201-37.

32. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med. 2011;62:233-47.

33. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer. 2005;5:341-54.

34. Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. Nat Rev Cancer. 2009;9:463-75.

35. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235:177-82.

36. Baselga J. Treatment of HER2-overexpressing breast cancer. Ann Oncol. 2010;21 Suppl 7:vii36-40.

37. Gluck S, Arteaga CL, Osborne CK. Optimizing chemotherapy-free survival for the ER/HER2 positive metastatic breast cancer patients. Clin Cancer Res. 2011.

38. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353:1659-72.

39. Jackisch C. HER-2-positive metastatic breast cancer: optimizing trastuzumab-based therapy. Oncologist. 2006;11 Suppl 1:34-41.

40. Orman JS, Perry CM. Trastuzumab : in HER2 and hormone receptor co-positive metastatic breast cancer. Drugs. 2007;67:2781-9.

41. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13:4429-34.

42. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, et al. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res. 2007;13:2329-34.

43. Irvin WJ, Jr., Carey LA. What is triple-negative breast cancer? Eur J Cancer. 2008;44:2799-805.

44. Lin NU, Claus E, Sohl J, Razzak AR, Arnaout A, Winer EP. Sites of distant recurrence and clinical outcomes in patients with metastatic triple-negative breast cancer: high incidence of central nervous system metastases. Cancer. 2008;113:2638-45.

45. Kassam F, Enright K, Dent R, Dranitsaris G, Myers J, Flynn C, et al. Survival outcomes for patients with metastatic triple-negative breast cancer: implications for clinical practice and trial design. Clin Breast Cancer. 2009;9:29-33.

46. Brenton JD, Carey LA, Ahmed AA, Caldas C. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? J Clin Oncol. 2005;23:7350-60.

47. Isakoff SJ. Triple-negative breast cancer: role of specific chemotherapy agents. Cancer J. 2010;16:53-61.

48. Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet. 2010;376:235-44.

49. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.

50. Osborne CK, Schiff R, Fuqua SA, Shou J. Estrogen receptor: current understanding of its activation and modulation. Clin Cancer Res. 2001;7:4338s-42s; discussion 411s-412s.

51. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, et al. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol. 2000;74:311-7.

52. Doisneau-Sixou SF, Sergio CM, Carroll JS, Hui R, Musgrove EA, Sutherland RL. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr Relat Cancer. 2003;10:179-86.

53. Lee AV, Cui X, Oesterreich S. Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. Clin Cancer Res. 2001;7:4429s-35s; discussion 11s-12s.

54. Musgrove EA, Sutherland RL. Cell cycle control by steroid hormones. Semin Cancer Biol. 1994;5:381-9.

55. Sutherland RL, Prall OW, Watts CK, Musgrove EA. Estrogen and progestin regulation of cell cycle progression. J Mammary Gland Biol Neoplasia. 1998;3:63-72.

56. Tan H, Zhong Y, Pan Z. Autocrine regulation of cell proliferation by estrogen receptor-alpha in estrogen receptor-alpha-positive breast cancer cell lines. BMC Cancer. 2009;9:31.

57. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem. 2001;276:36869-72.

58. Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, et al. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene. 1995;10:2435-46.

59. Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK, Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. Clin Cancer Res. 2004;10:331S-6S.

60. Ali S, Metzger D, Bornert JM, Chambon P. Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. EMBO J. 1993;12:1153-60.

61. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem. 2001;276:9817-24.

62. Driggers PH, Segars JH. Estrogen action and cytoplasmic signaling pathways. Part II: the role of growth factors and phosphorylation in estrogen signaling. Trends Endocrinol Metab. 2002;13:422-7.

63. Segars JH, Driggers PH. Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes. Trends Endocrinol Metab. 2002;13:349-54.

64. Lee H, Bai W. Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. Mol Cell Biol. 2002;22:5835-45.

65. Wang RA, Mazumdar A, Vadlamudi RK, Kumar R. P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium. EMBO J. 2002;21:5437-47.

66. Acconcia F, Manavathi B, Mascarenhas J, Talukder AH, Mills G, Kumar R. An inherent role of integrin-linked kinase-estrogen receptor alpha interaction in cell migration. Cancer Res. 2006;66:11030-8.

67. Lannigan DA. Estrogen receptor phosphorylation. Steroids. 2003;68:1-9.

68. Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, McInerney EM. William L. McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. Breast Cancer Res Treat. 1997;44:23-38.

69. Holm C, Rayala S, Jirstrom K, Stal O, Kumar R, Landberg G. Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients. J Natl Cancer Inst. 2006;98:671-80.

70. Kok M, Zwart W, Holm C, Fles R, Hauptmann M, Van't Veer LJ, et al. PKA-induced phosphorylation of ERalpha at serine 305 and high PAK1 levels is associated with sensitivity to tamoxifen in ER-positive breast cancer. Breast Cancer Res Treat. 2011;125:1-12.

71. Yamashita H, Nishio M, Kobayashi S, Ando Y, Sugiura H, Zhang Z, et al. Phosphorylation of estrogen receptor alpha serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. Breast Cancer Res. 2005;7:R753-64.

72. Jiang J, Sarwar N, Peston D, Kulinskaya E, Shousha S, Coombes RC, et al. Phosphorylation of estrogen receptor-alpha at Ser167 is indicative of longer disease-free and overall survival in breast cancer patients. Clin Cancer Res. 2007;13:5769-76.

73. Yamashita H, Nishio M, Toyama T, Sugiura H, Kondo N, Kobayashi S, et al. Low phosphorylation of estrogen receptor alpha (ERalpha) serine 118 and high phosphorylation of ERalpha serine 167 improve survival in ER-positive breast cancer. Endocr Relat Cancer. 2008;15:755-63.

74. Pietras RJ, Marquez-Garban DC. Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res. 2007;13:4672-6.

75. Santen RJ, Fan P, Zhang Z, Bao Y, Song RX, Yue W. Estrogen signals via an extra-nuclear pathway involving IGF-1R and EGFR in tamoxifen-sensitive and -resistant breast cancer cells. Steroids. 2009;74:586-94.

76. Levin ER. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol. 2003;17:309-17.

77. Filardo EJ. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol. 2002;80:231-8.

78. Aronica SM, Kraus WL, Katzenellenbogen BS. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci U S A. 1994;91:8517-21.

79. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev. 2008;29:217-33.

80. Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, Grohe C. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem. 2000;275:18447-53.

81. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J. 1996;15:1292-300.

82. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature. 2000;407:538-41.

83. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, et al. Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. Mol Endocrinol. 2002;16:116-27.

84. Zhang Z, Kumar R, Santen RJ, Song RX. The role of adapter protein Shc in estrogen non-genomic action. Steroids. 2004;69:523-9.

85. Edwards DP, Boonyaratanakornkit V. Rapid extranuclear signaling by the estrogen receptor (ER): MNAR couples ER and Src to the MAP kinase signaling pathway. Mol Interv. 2003;3:12-5.

86. Riggins RB, Zwart A, Nehra R, Clarke R. The nuclear factor kappa B inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells. Mol Cancer Ther. 2005;4:33-41.

87. Frasor J, Weaver A, Pradhan M, Dai Y, Miller LD, Lin CY, et al. Positive cross-talk between estrogen receptor and NF-kappaB in breast cancer. Cancer Res. 2009;69:8918-25.

88. Teixeira C, Reed JC, Pratt MA. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res. 1995;55:3902-7.

89. Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, et al. Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. J Biol Chem. 1999;274:32099-107.

90. Stanculescu A, Bembinster LA, Borgen K, Bergamaschi A, Wiley E, Frasor J. Estrogen Promotes Breast Cancer Cell Survival in an Inhibitor of Apoptosis (IAP)-Dependent Manner. Horm Cancer. 2010;1:127-35.

91. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov. 2005;4:988-1004.

92. Scheid MP, Woodgett JR. PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol. 2001;2:760-8.

93. Scheid MP, Huber M, Damen JE, Hughes M, Kang V, Neilsen P, et al. Phosphatidylinositol (3,4,5)P3 is essential but not sufficient for protein kinase B (PKB) activation; phosphatidylinositol (3,4)P2 is required for PKB phosphorylation at Ser-473: studies using cells from SH2-containing inositol-5-phosphatase knockout mice. J Biol Chem. 2002;277:9027-35.

94. Dibble CC, Manning BD. A molecular link between AKT regulation and chemotherapeutic response. Cancer Cell. 2009;16:178-80.

95. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, et al. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res. 2005;65:2554-9.

96. DeGraffenried LA, Fulcher L, Friedrichs WE, Grunwald V, Ray RB, Hidalgo M. Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/AKT pathway. Ann Oncol. 2004;15:1510-6.

97. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. Oncogene. 2008;27:5497-510.

98. Panka DJ, Mano T, Suhara T, Walsh K, Mier JW. Phosphatidylinositol 3-kinase/AKT activity regulates c-FLIP expression in tumor cells. J Biol Chem. 2001;276:6893-6.

99. Bratton MR, Duong BN, Elliott S, Weldon CB, Beckman BS, McLachlan JA, et al. Regulation of ERalpha-mediated transcription of Bcl-2 by PI3K-AKT crosstalk: implications for breast cancer cell survival. Int J Oncol. 2010;37:541-50.

100. Carracedo A, Pandolfi PP. The PTEN-PI3K pathway: of feedbacks and cross-talks. Oncogene. 2008;27:5527-41.

101. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. AKT phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91:231-41.

102. Dillon RL, White DE, Muller WJ. The phosphatidyl inositol 3-kinase signaling network: implications for human breast cancer. Oncogene. 2007;26:1338-45.

103. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by AKT (protein kinase B). Science. 1999;286:1741-4.

104. Alblas J, Slager-Davidov R, Steenbergh PH, Sussenbach JS, van der Burg B. The role of MAP kinase in TPA-mediated cell cycle arrest of human breast cancer cells. Oncogene. 1998;16:131-9.

105. Zhang S, Li X, Burghardt R, Smith R, 3rd, Safe SH. Role of estrogen receptor (ER) alpha in insulin-like growth factor (IGF)-I-induced responses in MCF-7 breast cancer cells. J Mol Endocrinol. 2005;35:433-47.

106. Stoica GE, Franke TF, Wellstein A, Czubayko F, List HJ, Reiter R, et al. Estradiol rapidly activates AKT via the ErbB2 signaling pathway. Mol Endocrinol. 2003;17:818-30.

107. Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, Iann MC, et al. Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/AKT pathway. Oncogene. 2003;22:7998-8011.

108. Stoica GE, Franke TF, Wellstein A, Morgan E, Czubayko F, List HJ, et al. Heregulin-beta1 regulates the estrogen receptor-alpha gene expression and activity via the ErbB2/PI 3-K/AKT pathway. Oncogene. 2003;22:2073-87.

109. White DE, Cardiff RD, Dedhar S, Muller WJ. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene. 2001;20:7064-72.

110. Frogne T, Jepsen JS, Larsen SS, Fog CK, Brockdorff BL, Lykkesfeldt AE. Antiestrogen-resistant human breast cancer cells require activated protein kinase B/AKT for growth. Endocr Relat Cancer. 2005;12:599-614.

111. Shin I, Arteaga CL. Expression of active AKT protects against tamoxifen-induced apoptosis in MCF-7 Cells. IUBMB Life. 2006;58:664-9.

112. Ghayad SE, Vendrell JA, Ben Larbi S, Dumontet C, Bieche I, Cohen PA. Endocrine resistance associated with activated ErbB system in breast cancer cells is reversed by inhibiting MAPK or PI3K/AKT signaling pathways. Int J Cancer. 2010;126:545-62.

113. Ellis MJ, Lin L, Crowder R, Tao Y, Hoog J, Snider J, et al. Phosphatidyl-inositol-3-kinase alpha catalytic subunit mutation and response to neoadjuvant endocrine therapy for estrogen receptor positive breast cancer. Breast Cancer Res Treat. 2010;119:379-90.

114. Ma CX, Crowder RJ, Ellis MJ. Importance of PI3-kinase pathway in response/resistance to aromatase inhibitors. Steroids. 2011;76:750-2.

115. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell. 2004;6:117-27.

116. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12:395-402.

117. O'Brien NA, Browne BC, Chow L, Wang Y, Ginther C, Arboleda J, et al. Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. Mol Cancer Ther. 2010;9:1489-502.

118. Baselga J. Targeting the phosphoinositide-3 (PI3) kinase pathway in breast cancer. Oncologist. 2011;16 Suppl 1:12-9.

119. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell. 2010;40:310-22.

120. Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J. 1999;344 Pt 2:427-31.

121. Chen XG, Liu F, Song XF, Wang ZH, Dong ZQ, Hu ZQ, et al. Rapamycin regulates AKT and ERK phosphorylation through mTORC1 and mTORC2 signaling pathways. Mol Carcinog. 2010;49:603-10.

122. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129:1261-74.

123. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011;12:21-35.

124. Garcia-Echeverria C, Sellers WR. Drug discovery approaches targeting the PI3K/AKT pathway in cancer. Oncogene. 2008;27:5511-26.

125. Wong KK, Engelman JA, Cantley LC. Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev. 2010;20:87-90.

126. Wander SA, Hennessy BT, Slingerland JM. Next-generation mTOR inhibitors in clinical oncology: how pathway complexity informs therapeutic strategy. J Clin Invest. 2011;121:1231-41.

127. Winston LA, Hunter T. JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. J Biol Chem. 1995;270:30837-40.

128. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol. 1997;9:180-6.

129. O'Neill E, Kolch W. Conferring specificity on the ubiquitous Raf/MEK signalling pathway. Br J Cancer. 2004;90:283-8.

130. Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, et al. The role of mitogen-activated protein (MAP) kinase in breast cancer. J Steroid Biochem Mol Biol. 2002;80:239-56.

131. Whyte J, Bergin O, Bianchi A, McNally S, Martin F. Key signalling nodes in mammary gland development and cancer. Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development. Breast Cancer Res. 2009;11:209.

132. Mueller H, Flury N, Eppenberger-Castori S, Kueng W, David F, Eppenberger U. Potential prognostic value of mitogen-activated protein kinase activity for disease-free survival of primary breast cancer patients. Int J Cancer. 2000;89:384-8.

133. Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP. Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci U S A. 1999;96:4686-91.

134. Lobenhofer EK, Huper G, Iglehart JD, Marks JR. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ. 2000;11:99-110.

135. Clarke R, Brunner N. Acquired estrogen independence and antiestrogen resistance in breast cancer: estrogen receptor driven phenotypes? Trends Endocrinol Metab. 1996;7:291-301.

136. Castoria G, Barone MV, Di Domenico M, Bilancio A, Ametrano D, Migliaccio A, et al. Non-transcriptional action of oestradiol and progestin triggers DNA synthesis. EMBO J. 1999;18:2500-10.

137. Zhang Z, Maier B, Santen RJ, Song RX. Membrane association of estrogen receptor alpha mediates estrogen effect on MAPK activation. Biochem Biophys Res Commun. 2002;294:926-33.

138. Song RX, Chen Y, Zhang Z, Bao Y, Yue W, Wang JP, et al. Estrogen utilization of IGF-1-R and EGF-R to signal in breast cancer cells. J Steroid Biochem Mol Biol. 2010;118:219-30.

139. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst. 2004;96:926-35.

140. Jeng MH, Yue W, Eischeid A, Wang JP, Santen RJ. Role of MAP kinase in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. Breast Cancer Res Treat. 2000;62:167-75.

141. Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Kmar R, et al. Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology. 2000;141:396-405.

142. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. Cancer Res. 2008;68:826-33.

143. Osborne CR, Duncan A, Sedlacek S, Paul D, Holmes F, Vukelja S, et al. The addition of hormone therapy to tamoxifen does not prevent hot flashes in women at high risk for developing breast cancer. Breast Cancer Res Treat. 2009;116:521-7.

144. Higuchi M, Onishi K, Kikuchi C, Gotoh Y. Scaffolding function of PAK in the PDK1-AKT pathway. Nat Cell Biol. 2008;10:1356-64.

145. Bokoch GM. Biology of the p21-activated kinases. Annu Rev Biochem. 2003;72:743-81.

146. Slack-Davis JK, Eblen ST, Zecevic M, Boerner SA, Tarcsafalvi A, Diaz HB, et al. PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. J Cell Biol. 2003;162:281-91.

147. Park ER, Eblen ST, Catling AD. MEK1 activation by PAK: a novel mechanism. Cell Signal. 2007;19:1488-96.

148. Balasenthil S, Sahin AA, Barnes CJ, Wang RA, Pestell RG, Vadlamudi RK, et al. p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells. J Biol Chem. 2004;279:1422-8.

149. Ong CC, Jubb AM, Zhou W, Haverty PM, Harris AL, Belvin M, et al. p21-activated kinase 1: PAK'ed with potential. Oncotarget. 2011;2:491-6.

150. Dummler B, Ohshiro K, Kumar R, Field J. Pak protein kinases and their role in cancer. Cancer Metastasis Rev. 2009;28:51-63.

151. Mira JP, Benard V, Groffen J, Sanders LC, Knaus UG. Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. Proc Natl Acad Sci U S A. 2000;97:185-9.

152. Vadlamudi RK, Adam L, Wang RA, Mandal M, Nguyen D, Sahin A, et al. Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. J Biol Chem. 2000;275:36238-44.

153. Adam L, Vadlamudi R, Mandal M, Chernoff J, Kumar R. Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. J Biol Chem. 2000;275:12041-50.

154. Adam L, Vadlamudi R, Kondapaka SB, Chernoff J, Mendelsohn J, Kumar R. Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. J Biol Chem. 1998;273:28238-46.

155. Menard RE, Jovanovski AP, Mattingly RR. Active p21-activated kinase 1 rescues MCF10A breast epithelial cells from undergoing anoikis. Neoplasia. 2005;7:638-45.

156. Li Q, Mullins SR, Sloane BF, Mattingly RR. p21-Activated kinase 1 coordinates aberrant cell survival and pericellular proteolysis in a three-dimensional culture model for premalignant progression of human breast cancer. Neoplasia. 2008;10:314-29.

157. Wang RA, Zhang H, Balasenthil S, Medina D, Kumar R. PAK1 hyperactivation is sufficient for mammary gland tumor formation. Oncogene. 2006;25:2931-6.

158. Yi C, Maksimoska J, Marmorstein R, Kissil JL. Development of small-molecule inhibitors of the group I p21-activated kinases, emerging therapeutic targets in cancer. Biochem Pharmacol. 2010;80:683-9.

159. DeSimone DW, Stepp MA, Patel RS, Hynes RO. The integrin family of cell surface receptors. Biochem Soc Trans. 1987;15:789-91.

160. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. Science. 1987;238:491-7.

161. Aplin AE, Juliano RL. Integrin and cytoskeletal regulation of growth factor signaling to the MAP kinase pathway. J Cell Sci. 1999;112 (Pt 5):695-706.

162. Giancotti FG. Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr Opin Cell Biol. 1997;9:691-700.

163. Folgiero V, Bachelder RE, Bon G, Sacchi A, Falcioni R, Mercurio AM. The alpha6beta4 integrin can regulate ErbB-3 expression: implications for alpha6beta4 signaling and function. Cancer Res. 2007;67:1645-52.

164. Gambaletta D, Marchetti A, Benedetti L, Mercurio AM, Sacchi A, Falcioni R. Cooperative signaling between alpha(6)beta(4) integrin and ErbB-2 receptor is required to promote phosphatidylinositol 3-kinase-dependent invasion. J Biol Chem. 2000;275:10604-10.

165. Friedland JC, Lakins JN, Kazanietz MG, Chernoff J, Boettiger D, Weaver VM. alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini. J Cell Sci. 2007;120:3700-12.

166. Pylayeva Y, Guo W, Giancotti FG. Analysis of integrin signaling in genetically engineered mouse models of mammary tumor progression. Methods Enzymol. 2007;426:439-61.

167. Liapis H, Flath A, Kitazawa S. Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. Diagn Mol Pathol. 1996;5:127-35.

168. Wong NC, Mueller BM, Barbas CF, Ruminski P, Quaranta V, Lin EC, et al. Alphav integrins mediate adhesion and migration of breast carcinoma cell lines. Clin Exp Metastasis. 1998;16:50-61.

169. Glinskii AB, Ma J, Ma S, Grant D, Lim CU, Sell S, et al. Identification of intergenic trans-regulatory RNAs containing a disease-linked SNP sequence and targeting cell cycle progression/differentiation pathways in multiple common human disorders. Cell Cycle. 2009;8:3925-42.

170. Nista A, Leonetti C, Bernardini G, Mattioni M, Santoni A. Functional role of alpha4beta1 and alpha5beta1 integrin fibronectin receptors expressed on adriamycin-resistant MCF-7 human mammary carcinoma cells. Int J Cancer. 1997;72:133-41.

171. Mauro L, Sisci D, Bartucci M, Salerno M, Kim J, Tam T, et al. SHC-alpha5beta1 integrin interactions regulate breast cancer cell adhesion and motility. Exp Cell Res. 1999;252:439-48.

172. Ramirez NE, Zhang Z, Madamanchi A, Boyd KL, O'Rear LD, Nashabi A, et al. The alphabeta integrin is a metastasis suppressor in mouse models and human cancer. J Clin Invest. 2011;121:226-37.

173. Zutter MM, Krigman HR, Santoro SA. Altered integrin expression in adenocarcinoma of the breast. Analysis by in situ hybridization. Am J Pathol. 1993;142:1439-48.

174. Yao ES, Zhang H, Chen YY, Lee B, Chew K, Moore D, et al. Increased beta1 integrin is associated with decreased survival in invasive breast cancer. Cancer Res. 2007;67:659-64.

175. Aoudjit F, Vuori K. Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. Oncogene. 2001;20:4995-5004.

176. Morozevich GE, Kozlova NI, Preobrazhenskaya ME, Ushakova NA, Eltsov IA, Shtil AA, et al. The role of beta1 integrin subfamily in anchorage-dependent apoptosis of breast carcinoma cells differing in multidrug resistance. Biochemistry (Mosc). 2006;71:489-95.

177. Park CC, Zhang HJ, Yao ES, Park CJ, Bissell MJ. Beta1 integrin inhibition dramatically enhances radiotherapy efficacy in human breast cancer xenografts. Cancer Res. 2008;68:4398-405.

178. Park CC, Zhang H, Pallavicini M, Gray JW, Baehner F, Park CJ, et al. Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. Cancer Res. 2006;66:1526-35.

179. White DE, Kurpios NA, Zuo D, Hassell JA, Blaess S, Mueller U, et al. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell. 2004;6:159-70.

180. Parsons JT. Focal adhesion kinase: the first ten years. J Cell Sci. 2003;116:1409-16.

181. Hanks SK, Ryzhova L, Shin NY, Brabek J. Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. Front Biosci. 2003;8:d982-96.

182. Schlaepfer DD, Mitra SK. Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev. 2004;14:92-101.

183. Chen HC, Appeddu PA, Isoda H, Guan JL. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J Biol Chem. 1996;271:26329-34.

184. Shen TL, Guan JL. Differential regulation of cell migration and cell cycle progression by FAK complexes with Src, PI3K, Grb7 and Grb2 in focal contacts. FEBS Lett. 2001;499:176-81.

185. Owens LV, Xu L, Craven RJ, Dent GA, Weiner TM, Kornberg L, et al. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. Cancer Res. 1995;55:2752-5.

186. Lightfoot HM, Jr., Lark A, Livasy CA, Moore DT, Cowan D, Dressler L, et al. Upregulation of focal adhesion kinase (FAK) expression in ductal carcinoma in situ (DCIS) is an early event in breast tumorigenesis. Breast Cancer Res Treat. 2004;88:109-16.

187. Oktay MH, Oktay K, Hamele-Bena D, Buyuk A, Koss LG. Focal adhesion kinase as a marker of malignant phenotype in breast and cervical carcinomas. Hum Pathol. 2003;34:240-5.

188. Mitra SK, Lim ST, Chi A, Schlaepfer DD. Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. Oncogene. 2006;25:4429-40.

189. Mitra SK, Mikolon D, Molina JE, Hsia DA, Hanson DA, Chi A, et al. Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. Oncogene. 2006;25:5969-84.

190. Li F, Zhang Y, Wu C. Integrin-linked kinase is localized to cell-matrix focal adhesions but not cell-cell adhesion sites and the focal adhesion localization of integrin-linked kinase is regulated by the PINCH-binding ANK repeats. J Cell Sci. 1999;112 (Pt 24):4589-99.

191. Dedhar S, Williams B, Hannigan G. Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. Trends Cell Biol. 1999;9:319-23.

192. Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, et al. Regulation of protein kinase B/AKT-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. J Biol Chem. 2001;276:27462-9.

193. Hannigan G, Troussard AA, Dedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. Nat Rev Cancer. 2005;5:51-63.

194. Troussard AA, Costello P, Yoganathan TN, Kumagai S, Roskelley CD, Dedhar S. The integrin linked kinase (ILK) induces an invasive phenotype via AP-1 transcription factor-dependent upregulation of matrix metalloproteinase 9 (MMP-9). Oncogene. 2000;19:5444-52.

195. Troussard AA, McDonald PC, Wederell ED, Mawji NM, Filipenko NR, Gelmon KA, et al. Preferential dependence of breast cancer cells versus normal cells on integrin-linked kinase for protein kinase B/AKT activation and cell survival. Cancer Res. 2006;66:393-403.

196. Persad S, Attwell S, Gray V, Delcommenne M, Troussard A, Sanghera J, et al. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/AKT and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. Proc Natl Acad Sci U S A. 2000;97:3207-12.

197. McDonald PC, Oloumi A, Mills J, Dobreva I, Maidan M, Gray V, et al. Rictor and integrin-linked kinase interact and regulate AKT phosphorylation and cancer cell survival. Cancer Res. 2008;68:1618-24.

198. Cordes N, Park CC. beta1 integrin as a molecular therapeutic target. Int J Radiat Biol. 2007;83:753-60.

199. Good MC, Zalatan JG, Lim WA. Scaffold proteins: hubs for controlling the flow of cellular information. Science. 2011;332:680-6.

200. Cheskis BJ, Greger J, Cooch N, McNally C, McLarney S, Lam HS, et al. MNAR plays an important role in ERa activation of Src/MAPK and PI3K/AKT signaling pathways. Steroids. 2008;73:901-5.

201. Lyons TR, Thorburn J, Ryan PW, Thorburn A, Anderson SM, Kassenbrock CK. Regulation of the Pro-apoptotic scaffolding protein POSH by AKT. J Biol Chem. 2007;282:21987-97.

202. Ke Y, Wu D, Princen F, Nguyen T, Pang Y, Lesperance J, et al. Role of Gab2 in mammary tumorigenesis and metastasis. Oncogene. 2007;26:4951-60.

203. Ligensa T, Krauss S, Demuth D, Schumacher R, Camonis J, Jaques G, et al. A PDZ domain protein interacts with the C-terminal tail of the insulin-like growth factor-1 receptor but not with the insulin receptor. J Biol Chem. 2001;276:33419-27.

204. Chittenden TW, Pak J, Rubio R, Cheng H, Holton K, Prendergast N, et al. Therapeutic implications of GIPC1 silencing in cancer. PLoS One. 2010;5:e15581.

205. Lunin VV, Munger C, Wagner J, Ye Z, Cygler M, Sacher M. The structure of the MAPK scaffold, MP1, bound to its partner, p14. A complex with a critical role in endosomal map kinase signaling. J Biol Chem. 2004;279:23422-30.

206. Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science. 1998;281:1668-71.

207. Sharma C, Vomastek T, Tarcsafalvi A, Catling AD, Schaeffer HJ, Eblen ST, et al. MEK partner 1 (MP1): regulation of oligomerization in MAP kinase signaling. J Cell Biochem. 2005;94:708-19.

208. Teis D, Wunderlich W, Huber LA. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev Cell. 2002;3:803-14.

209. Teis D, Taub N, Kurzbauer R, Hilber D, de Araujo ME, Erlacher M, et al. p14-MP1-MEK1 signaling regulates endosomal traffic and cellular proliferation during tissue homeostasis. J Cell Biol. 2006;175:861-8.

210. Lu A, Tebar F, Alvarez-Moya B, Lopez-Alcala C, Calvo M, Enrich C, et al. A clathrin-dependent pathway leads to KRas signaling on late endosomes en route to lysosomes. J Cell Biol. 2009;184:863-79.

211. Nada S, Hondo A, Kasai A, Koike M, Saito K, Uchiyama Y, et al. The novel lipid raft adaptor p18 controls endosome dynamics by anchoring the MEK-ERK pathway to late endosomes. EMBO J. 2009;28:477-89.

212. Kurzbauer R, Teis D, de Araujo ME, Maurer-Stroh S, Eisenhaber F, Bourenkov GP, et al. Crystal structure of the p14/MP1 scaffolding complex: how a twin couple attaches mitogen-activated protein kinase signaling to late endosomes. Proc Natl Acad Sci U S A. 2004;101:10984-9.

213. Cui Q, Sulea T, Schrag JD, Munger C, Hung MN, Naim M, et al. Molecular dynamics-solvated interaction energy studies of protein-protein interactions: the MP1-p14 scaffolding complex. J Mol Biol. 2008;379:787-802.

214. Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010;141:290-303.

215. Pullikuth A, McKinnon E, Schaeffer HJ, Catling AD. The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals. Mol Cell Biol. 2005;25:5119-33.

216. Vomastek T, Iwanicki MP, Schaeffer HJ, Tarcsafalvi A, Parsons JT, Weber MJ. RACK1 targets the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway to link integrin engagement with focal adhesion disassembly and cell motility. Mol Cell Biol. 2007;27:8296-305.

217. Bohn G, Allroth A, Brandes G, Thiel J, Glocker E, Schaffer AA, et al. A novel human primary immunodeficiency syndrome caused by deficiency of the endosomal adaptor protein p14. Nat Med. 2007;13:38-45.

218. Park ER, Pullikuth AK, Bailey EM, Mercante DE, Catling AD. Differential requirement for MEK Partner 1 in DU145 prostate cancer cell migration. Cell Commun Signal. 2009;7:26.

219. Mouchel-Vielh E, Bloyer S, Salvaing J, Randsholt NB, Peronnet F. Involvement of the MP1 scaffold protein in ERK signaling regulation during Drosophila wing development. Genes Cells. 2008;13:1099-111.

220. Gewirtz DA, Holt SE, Grant S. Apoptosis, senescence, and cancer. 2nd ed. Totowa, N.J.: Humana Press; 2007.

221. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer. 2002;2:277-88.

222. Juo P, Kuo CJ, Yuan J, Blenis J. Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. Curr Biol. 1998;8:1001-8.

223. Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, et al. AKT enhances Mdm2-mediated ubiquitination and degradation of p53. J Biol Chem. 2002;277:21843-50.

224. Chang J, Clark GM, Allred DC, Mohsin S, Chamness G, Elledge RM. Survival of patients with metastatic breast carcinoma: importance of prognostic markers of the primary tumor. Cancer. 2003;97:545-53.

225. Weinberg RA. The biology of cancer. New York: Garland Science; 2007.

CHAPTER 2

The Role of MP1 Scaffold Protein in Survival of Breast Cancer Cells

ABSTRACT

MEK Partner 1 (MP1 or MAPKSP1) is a scaffold protein that has been reported to function in multiple signaling pathways, including the ERK, PAK and mTORC pathways. Several of these pathways influence the biology of breast cancer, but the functional significance of MP1 in breast cancer cells has not been investigated. In this report, we demonstrate a specific requirement for MP1 expression in estrogen receptor (ER) positive breast cancer cells. MP1 is widely expressed in both ER-positive and negative breast cancer cell lines. However, inhibition of its expression using siRNA duplexes resulted in detachment and apoptosis of ER-positive, but not ER-negative, breast cancer cells. Inhibition of MP1 expression in ER-positive cells resulted in reduced AKT1 activity, and expression of a constitutively active form of AKT1 partially rescued the cell death phenotype observed when the MP1 gene was silenced. Together, these results suggest that MP1 is required for pro-survival signaling from the PI3K/AKT pathway specifically in ER-positive breast cancer cells.

INTRODUCTION

The small protein MEK Partner 1 (MP1, also known as Map Kinase Scaffold Protein 1 and LAMTOR3) was originally identified as a scaffold protein that potentiates MAPK signaling by binding to MEK1 and ERK1 (1). MP1 interacts with another small protein p14, and together these two proteins are localized to endomembrane compartments as part of larger signaling complexes. For example, an MP1-p14-MEK1 complex is localized to late endosomes, and this localization is required for EGF-induced ERK1/2 signaling (2-4). A second MP1-p14-p18 Ragulator complex is required for the

recruitment of mTORC1 to the lysosomal surface, and is essential for its amino acid-dependent signaling (5). In addition to these trimeric complexes, MP1 has been reported to bind PAK1 at the plasma membrane, and the MP1-PAK1 interaction is required for MEK phosphorylation by PAK1 in the absence of Raf (6, 7). Thus, the scaffold protein MP1 can regulate the function of several intracellular kinases in different subcellular locations.

Both *in vitro* and *in vivo* approaches have been taken to investigate the biological functions of MP1. Transient inhibition of MP1 expression using RNA interference in fibroblasts resulted in decreased Rho activity and delayed cell spreading on fibronectin (7). Similar knockdown experiments in DU145 prostate cancer cells resulted in decreased migration on fibronectin (6). This effect on migration was independent of the ability of MP1 to activate ERK and PAK1, since the levels of phosphorylated ERK and PAK1 were unchanged upon MP1 knockdown. However, inhibition of MP1 expression was associated with both decreased expression of paxillin and decreased number and turnover of focal adhesions at the migratory edge. Together, these data indicate that one function of MP1 in cell culture is related to cell adhesion and migration. Studies performed in conditional p14 knockout mice and in *Drosophila* have addressed the *in vivo* functions of MP1. The endosomal p14-MP1-MEK1 complex is required for cell proliferation in the epidermis during mouse embryogenesis (3). In *Drosophila*, the MP1/ERK complex regulates cell differentiation during development of the wing, since both down-regulation and overexpression of *dMAPKSP1* lead to an ectopic wing vein phenotype (8). In summary, MP1 is a widely expressed protein that interacts with multiple protein kinases and may impact various cellular processes including

proliferation, spreading, migration, and differentiation. Since many of these processes play important roles in cancer biology, we used a loss of function approach to evaluate the role of MP1 in regulating the proliferation and/or survival of breast cancer cells.

Analysis of publicly available gene expression datasets indicates that MP1 RNA is expressed in both normal mammary epithelial cells and in breast cancer cells. In this report, MP1 protein expression was investigated in a panel of human mammary epithelial cell lines. The data indicate that MP1 is expressed in both estrogen receptor alpha (ER)-positive and ER-negative breast cancer cell lines, as well as in non-transformed cells. However, the effects of inhibiting MP1 expression by transient transfection with siRNA duplexes differed between the cell lines. MP1 knockdown induced apoptosis of the ER-positive breast cancer cells, but not ER-negative breast cancer or non-tumorigenic cell lines. The apoptosis observed in ER-positive cells was associated with cell detachment, and with decreased ER expression and AKT activation. The phenotype could be partially reversed by overexpressing a constitutively active form of AKT1, suggesting that MP1 plays a novel role in promoting survival of ER-positive breast cancer cells via the AKT pathway.

MATERIALS AND METHODS

Cell lines and culture conditions

MCF-7 and LCC9 cells were obtained from the Lombardi Cancer Center. T47D, ZR-75-1, MDA-MB-231, BT-549, and Sk-Br-3 cells were purchased from the American Type Culture Collection. Cells were maintained in Improved Modified Eagle's Medium (IMEM)

containing phenol red (GIBCO-Invitrogen-Applied Biosystems), supplemented with 5% fetal bovine serum (HyClone), and 100 Units/ml Penicillin/100 µg/ml Streptomycin (Invitrogen) and incubated at 37 ºC with 5% CO_2.

siRNA transfections

All siRNA transfection reagents were purchased from Dharmacon-Thermo Scientific. Two independent MP1 siRNA duplexes (ON-TARGETplus), ER siRNA duplexes (ON-TARGETplus siRNA Human ESR1) and a non-targeting siRNA (ON-TARGETplus siCONTROL) were used. Cells were plated in six-well plates at 10^5-3 x 10^5 cells per well in FBS containing medium. After 24 h, cells were transfected with 30-150 nM of either control or MP1 siRNA using DharmaFECT 1 transfection reagent. After 48 h, cells were harvested by scraping on ice cold PBS and prepared for protein extraction.

Determination of cell death

Cell death was assessed at 48 h post transfection using Trypan blue exclusion assays. Briefly, floating cells were collected, centrifuged, and resuspended in PBS, while attached cells were trypsinized, centrifuged, and resuspended in PBS. For each cell suspension, 18 µl were incubated with 2 µl trypan blue for 15 min and both total number and the number of dead cells were counted with a hemacytometer. The remaining harvested cells were processed for protein determination and immunoblotting.

Immunoblotting

Cell pellets were lysed in CelLytic M lysis buffer (Sigma), supplemented with cocktail tablets of protease (Roche - Complete Mini EDTA-free) and phosphatase inhibitors

(Roche – PhosSTOP). Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Total protein (10-20 µg) was subjected to 4-20% Tris-HCl SDS-PAGE (Bio-Rad), transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore), blocked with Odyssey Blocking Buffer and then incubated with the appropriate primary antibodies. Alexa Fluor 680 anti-goat, anti-rabbit, and anti-rabbit (Invitrogen) and IRDye 800CW anti-mouse and anti-rabbit (LI-COR) secondary antibodies were used for two-color detection of proteins. Membranes were scanned and analyzed using the LI-COR Odyssey system.

Luciferase assay

MCF-7 cells were cultured in six-well plates at 3 x 10^5 cells per well. The following day they were co-transfected with 0.5 µg of ERE2-tk109-luc and 0.06 µg of pβgal-Basic, using Superfect transfection reagent (Qiagen). After 3 h, the medium was changed to transfection mixes of either control or MP1 siRNA and cells were incubated overnight. The transfection medium was then replaced with phenol red-free IMEM supplemented with 5% CSS for 24 h, then cells were stimulated with 10 nM 17β-estradiol (Sigma) for 8 h. Cells were lysed and assayed for luciferase (Promega) and β-galactosidase (Clonetech) activity as suggested by each manufacturer.

RNA isolation and quantity assessment

Total RNA was isolated using the Trizol/Chloroform method. Briefly, cells were lysed and homogenized with Trizol, followed by a phase separation using chloroform. Next, the samples were precipitated with isopropanol, washed and redissolved in RNA

storage solution (Ambion). After a new precipitation step using sodium citrate and ethanol, RNA pellets were subjected to DNase digestion (Ambion). Total RNA was quantified at 260 nm using a Nanodrop ND-1000 spectrophotometer. The purity of RNA was verified by a 260/280 ratio of 2.0 ± 0.25 and a 260/230 ratio of 1.8 ± 0.15.

Reverse transcription, semi-quantitative PCR and DNA gel electrophoresis

A hundred ng (100 ng) total RNA were reverse-transcribed using Superscript One Step kit according to the manufacturer's protocol (Invitrogen). To perform a semi-quantitative PCR analysis, 25 µl of cDNA-containing reaction solutions were subjected to various PCR conditions using a Bio-Rad Thermal cycler. The PCR products were loaded on 1.5% agarose gel containing 100 ng/ml ethidium bromide (Sigma) and bands were visualized using a thermal gel imaging system (FUJIFILM).

Primers and PCR conditions

The following human primers were used for PCR reactions:

- MP1 forward 5'-AACGGATCCATGGCGGATGACCTAAA-3'; reverse 5'-GCCGAATTCCAGAAACTTCCACAACTTG-3'.
- ER forward 5'-CATTATGGAGTCTGGTCCTGTGA-3'; reverse 5'-GTTTCAACATTCTCCCTCCTCTT-3'.
- Actin forward 5'-CTGGGACGACATGGAGAAAA-3'; reverse 5'-AAGGAAGGCTGGAAGAGTGC -3'.

The following PCR conditions were used:

- MP1 and actin primers: 56 ºC annealing temperature (30 seconds) and 30 cycles
- ER primers: 54 ºC annealing temperature (30 seconds) and 36 cycles

The PCR products were 391 bp for MP1, 214 bp for ER, and 563 bp for actin.

Antibodies and reagents

The following primary antibodies were used for Western blotting: MP1 (A-19, Santa Cruz), actin (AC-40, SIGMA), estrogen receptor alpha (AB-17, Lab Vision-Thermo Scientific, or F-10, Santa Cruz), PARP (Cell Signaling), p-AKT (T308, Cell Signaling), AKT1 (BDI111, Santa Cruz), ERK (C-16, Santa Cruz), p-ERK (Cell Signaling), Flag M2 (Sigma), β1 integrin (N-20, Santa Cruz) or Bcl-2 (BD Biosciences).

Pan caspase inhibitor z-VAD-FMK was obtained from BD Biosciences, Trizol from Invitrogen and PI3K inhibitor LY294002 was purchased from Sigma.

Retroviral infection of MCF-7 cells

pBabe-puro (Addgene plasmid 1764) or pBabe-puro-Myr-Flag-AKT1 (Addgene plasmid 15294, (9)) were transfected into 293GPG packaging cells and retroviral stocks were prepared as previously described (10). These virus stocks were used to infect MCF-7 cells (1 ml per 10 cm dish), in the presence of polybrene (8 μg/ml), and stable colonies were selected with 0.5 μg/ml puromycin. Both single colonies and pools of 50-100 colonies were selected and propagated. Stable cell lines/pools were routinely maintained in medium supplemented with 0.25 μg/ml puromycin and plated in puromycin-free conditions for siRNA transfections.

Statistical Analysis

Data are expressed as the mean ± S.D. Most experiments were performed three times. Paired evaluations were made for experimental and control conditions within each experiment and for comparing groups of cell lines an unpaired two-tailed evaluation was done. Significance was determined by Student's *t* test. Significance level was set at $p < 0.05$.

RESULTS

MP1 expression profiling in human mammary epithelial cells

Expression of MP1 protein was assessed by immunoblotting in the following human mammary epithelial cell lines: MCF10A and 184B5 (nontumorigenic), MCF-7, LCC9, T47D, and ZR-75-1 (tumorigenic, ER-positive), and MDA-MB-231, BT-547, Hs579T, and Sk-Br-3 (tumorigenic, ER-negative) (Figure 2). MP1 was present in all cell lines, although the level was variable. Actin expression also varied between cell lines, but was consistent between experiments. A comparison between ER-positive (mean=3.8±0.4) and ER-negative (mean=2±1.1) breast cancer cell lines indicated a small but significantly ($p=0.03$) higher ratio of MP1/Actin protein levels in the ER-positive group. Since the number of samples investigated here is small, we also queried publicly available databases for MP1 mRNA expression that were downloaded from GEO including: GSE2034, GSE3494, GSE6532, GSE4922, GSE11121, GSE7390, GSE2603 and GSE14020. Data was normalized using RMA in Affymetrix Expression console and batch effects were removed. In agreement with our protein results, MP1 was widely expressed, but showed a small but statistically significant elevation in both ER and PR

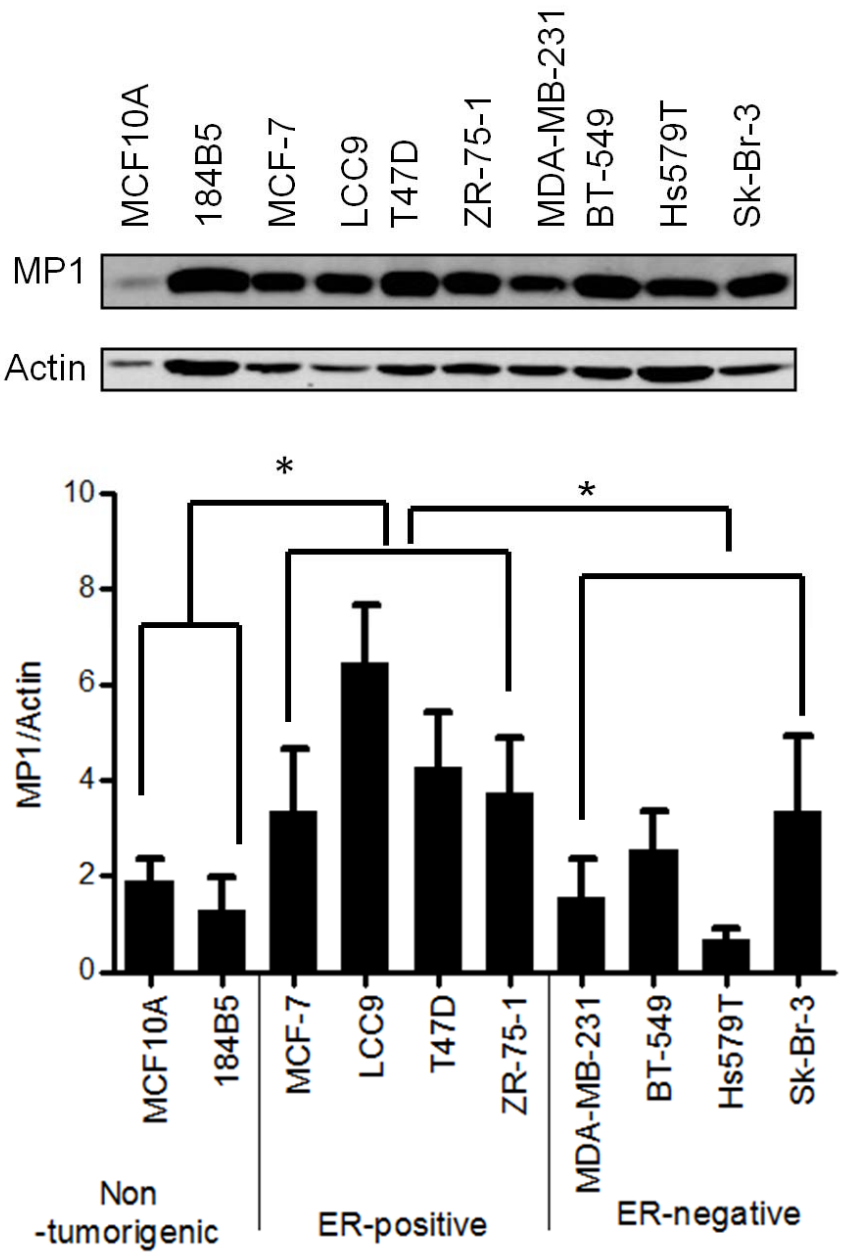


Figure 2. MP1 expression in breast cancer cell lines. Human mammary epithelial cell lines were grown in exponential culture and whole-cell lysates were prepared. Top panel: Immunoblot from a representative experiment. Lower panel: Quantitation of MP1/Actin ratios in three independent experiments (mean ± SD, *p<0.05).

positive breast cancer samples ($p<0.0001$ by t-test for both). One clinical study identified MP1 as a gene associated with poor prognosis in sporadic lymph-node negative breast cancer patients (11), suggesting a putative role in the context of breast tumors. However, our analysis did not reveal a correlation between high MP1 expression and disease outcome (time to distant metastasis or disease free survival).

Inhibition of MP1 expression induces cell death and detachment of ER-positive breast cancer cells

To study the effect of inhibiting MP1 expression in breast cancer cells, short interfering RNA (siRNA) duplexes were used. Initial experiments were carried out in ER-positive MCF-7 cells. By 48 h post-transfection, cells treated with two independent MP1 siRNAs displayed a dramatic phenotype involving cell rounding and detachment (Figure 3A). As shown in Figure 3B, MP1 protein levels were reduced more than 50% by 48 h with these two MP1 siRNAs relative to control siRNA.

To determine if this response to MP1 knockdown was a general feature of ER-positive breast cancer cells, two additional ER-positive cell lines were examined: LCC9 and T47D. The LCC9 cell line is an estrogen independent and antiestrogen resistant derivative of MCF-7 cells (12), and T47D is an independently derived ER-positive cell line. The siRNA sequence that consistently yielded better knockdown (MP1 siRNA #1) was chosen for these experiments and MP1 was silenced in all of the cell lines investigated (Figure 4A). As shown in Figure 4B, LCC9 and T47D cells exhibited a similar phenotype to MCF-7. To quantitate the effect of MP1 knockdown, attached and

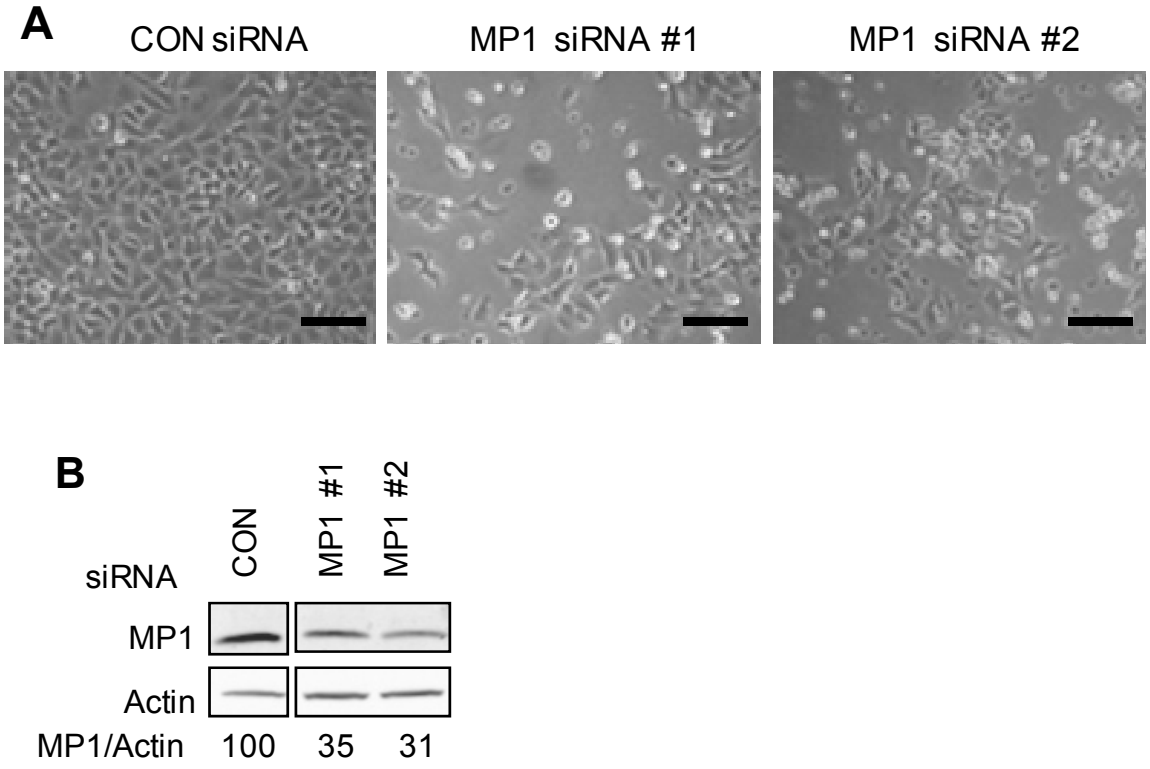


Figure 3. MP1 silencing using two independent siRNA sequences. MCF-7 cells were transfected with 40 nM control or MP1 siRNAs. At 48 h cells were photographed, then harvested for counting and extract preparation. A) Photographs of MCF-7 cells transfected with two different MP1 siRNA and control siRNA sequences. B) Immunoblot of transfected samples. Numbers represent the relative MP1/Actin ratios.

detached cells were collected at 48 h following siRNA transfection, stained with trypan blue, and counted (Figure 5). As determined by trypan blue staining, MCF-7 cells were the most sensitive to MP1 knockdown, with more than 70% of cells detached by 48 h, and the majority of these were dead. In contrast, only 10% of cells were detached in the control siRNA transfections. Although LCC9 and T47D cells were less sensitive than MCF-7, both showed a significant increase in dead/floating cells upon MP1 knockdown, with the average percentage of dead cells being 70% for MCF-7, 42% for LCC9 and 49% for T47D.

Inhibition of MP1 expression does not induce death of ER-negative breast cancer cells or non-tumorigenic cells

Since MP1 is expressed in ER-negative breast cancer cells and in non-tumorigenic mammary epithelial cells (Figure 2), the effects of MP1 knockdown in representatives of these cell types were also examined. Three ER-negative breast cancer cell lines (MDA-MB-231, BT-549, and Sk-Br-3) and one non-tumorigenic mammary epithelial cell line (184B5) were transfected with either control or MP1 siRNA and examined at 48 h. Although MP1 levels were decreased to the same or greater extent as that obtained in the ER-positive lines (Figure 6), no obvious changes in cell morphology were seen, and cell counting/trypan blue exclusion indicated that there was no significant increase in cell detachment or death in MP1 siRNA transfected cells compared with control samples (Figure 7). Thus, the requirement for MP1 expression for cell attachment and survival may be specific to ER-positive breast cancer cells.

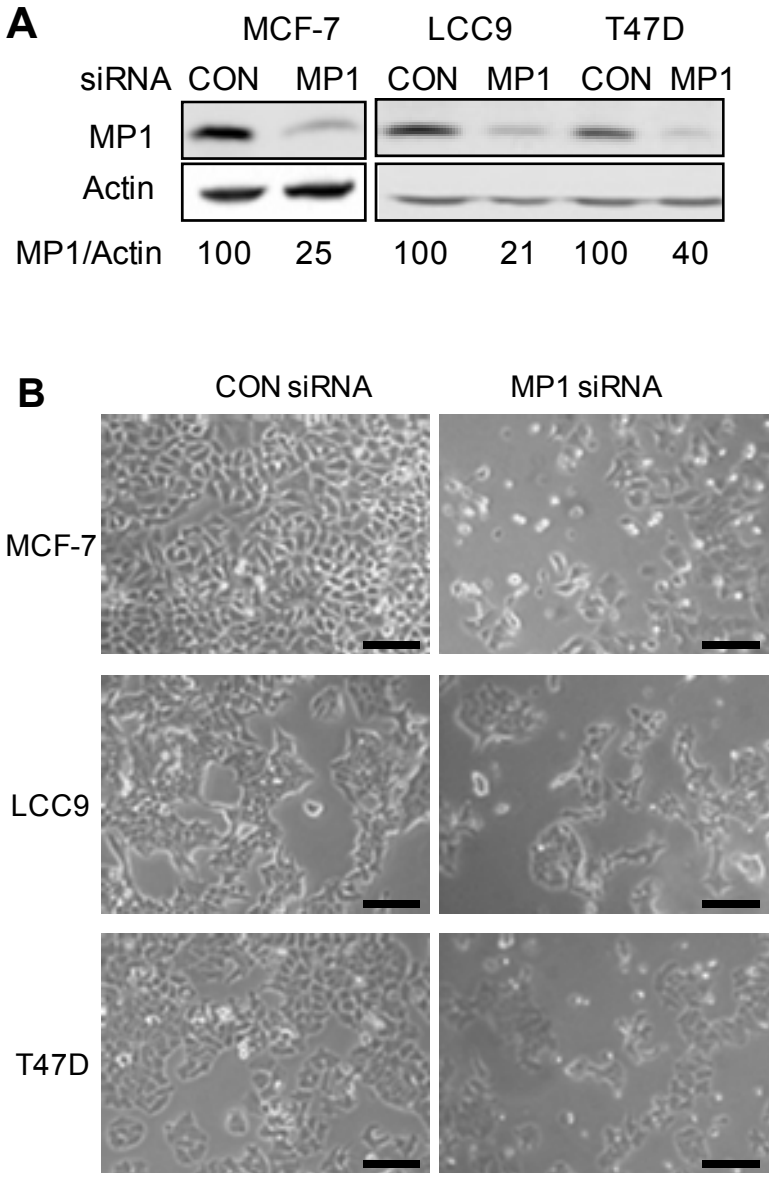


Figure 4. MP1 expression is required for attachment and survival of ER-positive breast cancer cells. A) Immunoblots of MCF-7, LCC9 and T47D cells transfected with 40 nM control or MP1 siRNA. B) Representative photographs of transfected cells. Scale bar = 100 μm.

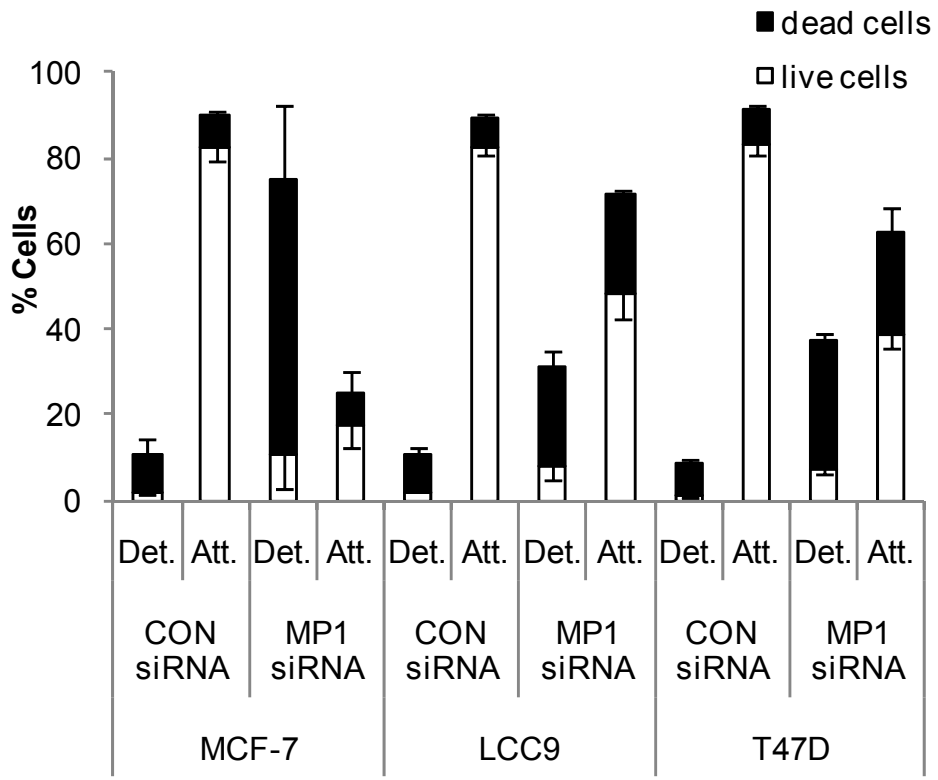
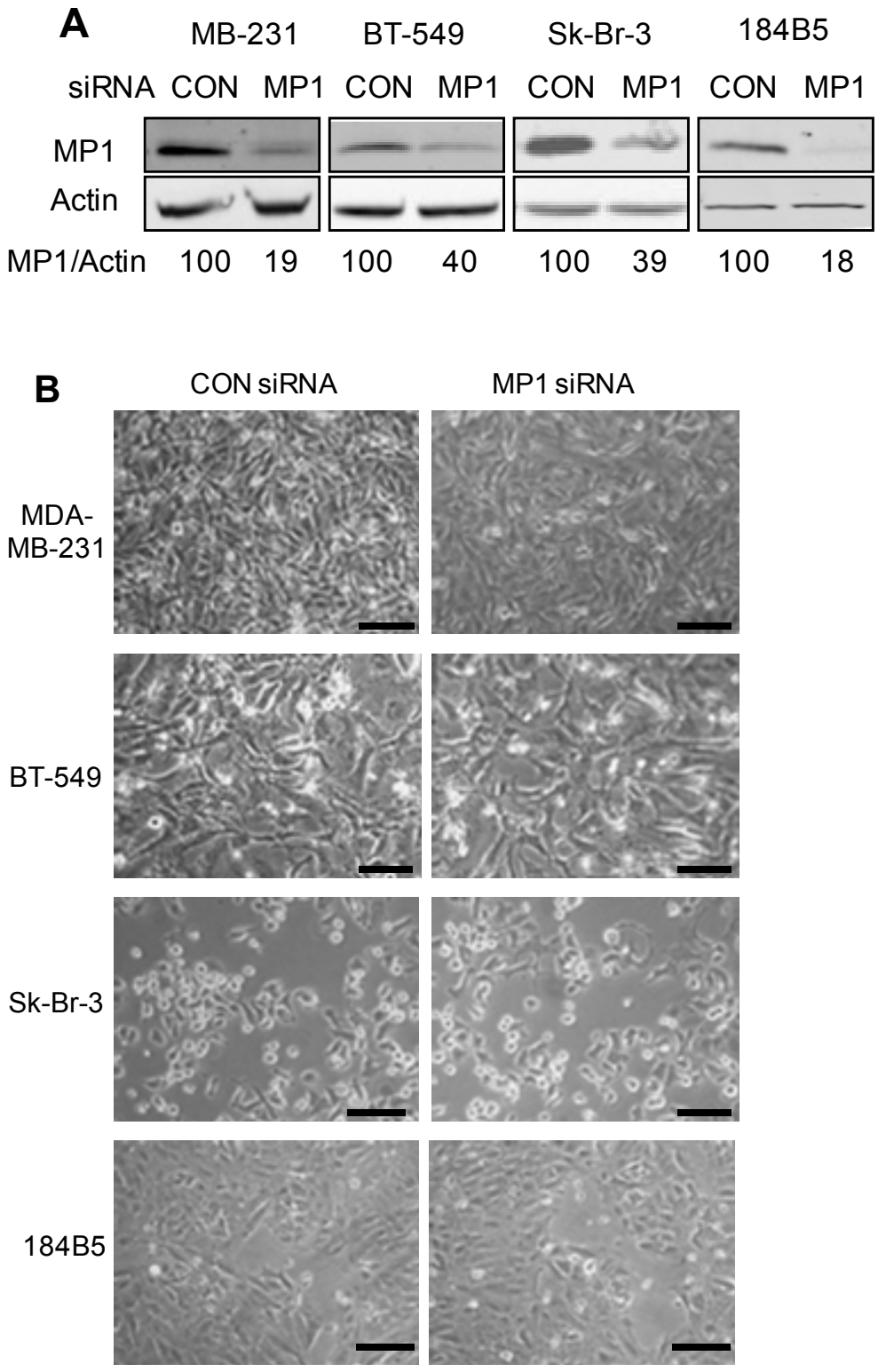


Figure 5. Quantitation of detachment and death induced by silencing of MP1 in ER-positive mammary epithelial cells. Attached and floating cells were collected and analyzed. The percentage of dead cells (black bars) and live cells (white bars) in each population was determined by trypan blue exclusion assays. Error bars represent the mean ± SD of three independent experiments.

Figure 6. MP1 expression is not required for attachment or survival of ER-negative mammary epithelial cells. MDA-MB-231, BT-549, Sk-Br-3, and 184B5 cells were transfected with 40 nM control or MP1 siRNAs for all cell lines except 184B5, where 150 nM siRNAs were used. At 48 h cells were photographed, then harvested for counting and extract preparation. A) Immunoblots of transfected samples. Numbers represent the MP1/Actin ratios expressed as percentage of control samples. B) Representative photographs of transfected cells. Scale bar = 100 μm.

Figure 6.


A
MB-231
BT-549
Sk-Br-3
184B5
siRNA CON MP1 CON MP1 CON MP1 CON MP1
MP1
Actin
MP1/Actin 100 19 100 40 100 39 100 18
B
CON siRNA
MP1 siRNA
MDA-MB-231
BT-549
Sk-Br-3
184B5

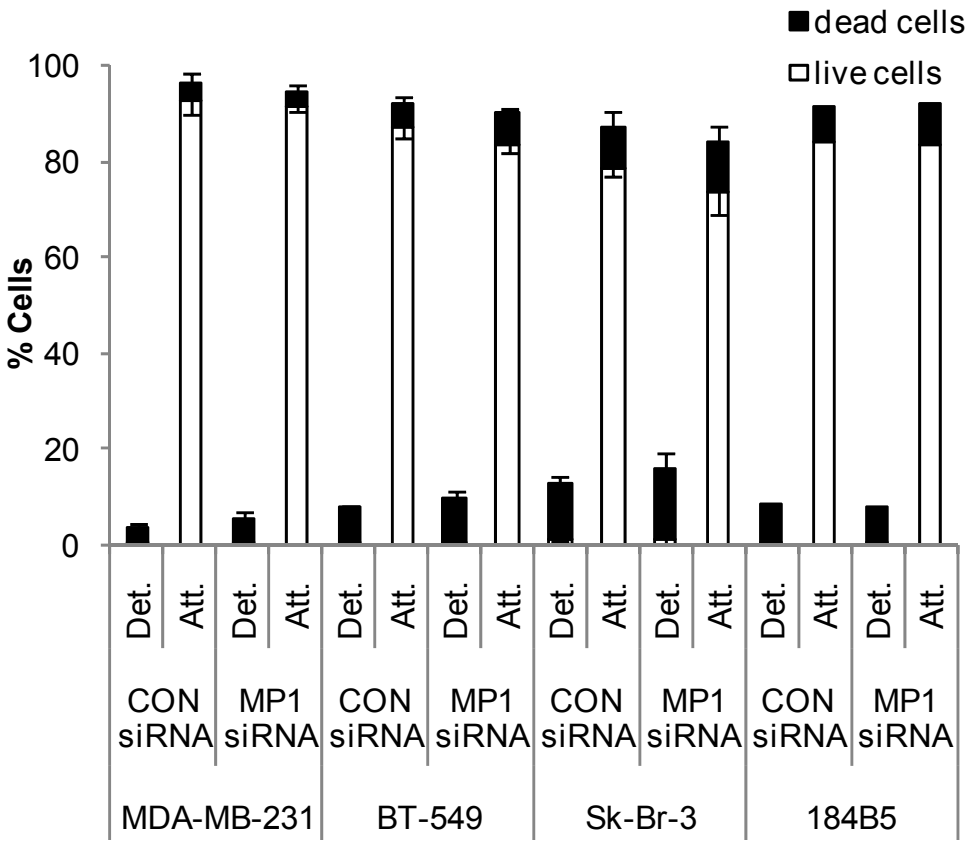


Figure 7. Quantitation of detachment and death induced by silencing of MP1 in ER-negative mammary epithelial cells. Attached and floating cells were collected and analyzed. The percentage of dead cells (black bars) and live cells (white bars) in each population was determined by trypan blue exclusion assays. Error bars represent the mean ± SD of three independent experiments for all samples except 184B5 cells. For this cell line the numbers shown represent the average of two independent experiments.

Inhibition of MP1 expression results in apoptosis of MCF-7 cells

To examine the mechanism of the cell death observed in MCF-7 cells upon MP1 silencing, expression of the anti-apoptotic protein Bcl-2 was examined. As shown in Figure 8A, Bcl-2 levels decreased approximately two fold in MP1 siRNA treated cells. In addition, cleavage of poly (ADP-ribose) polymerase *(*PARP), which is a marker of apoptosis, occurred in MCF-7 cells but not in MDA-MB-231 cells (Figure 8B). To further confirm that death was via apoptosis, cells were treated with the pan-caspase inhibitor z-VAD-FMK concurrently with siRNA transfection. As shown in Figures 8C and 8D, this treatment prevented PARP cleavage and cell rounding/detachment in MCF-7 cells.

MP1 knockdown reduces ER protein and mRNA levels and transcriptional activity

Because the most robust effect of MP1 silencing after prolonged treatment with siRNA is apoptosis and since ER was shown to mediate survival of breast cancer cells, the effect of MP1 knockdown on ER expression and activity was investigated. MP1 silencing reduces the protein and mRNA levels of ER (Figure 9A and B) at 48 and 30 hours, respectively. In addition, relative luciferase activity in the presence of estrogen was significantly decreased following siRNA treatment (Figure 9C). These data suggest that MP1 is required to maintain ER levels and facilitate ER-driven transcription on ERE-containing promoters.

MP1 siRNA induced apoptosis does not require ER

Given the results on ER levels and activity upon silencing of MP1, a double knockdown approach was undertaken in order to test whether ER is required for MP1 siRNA

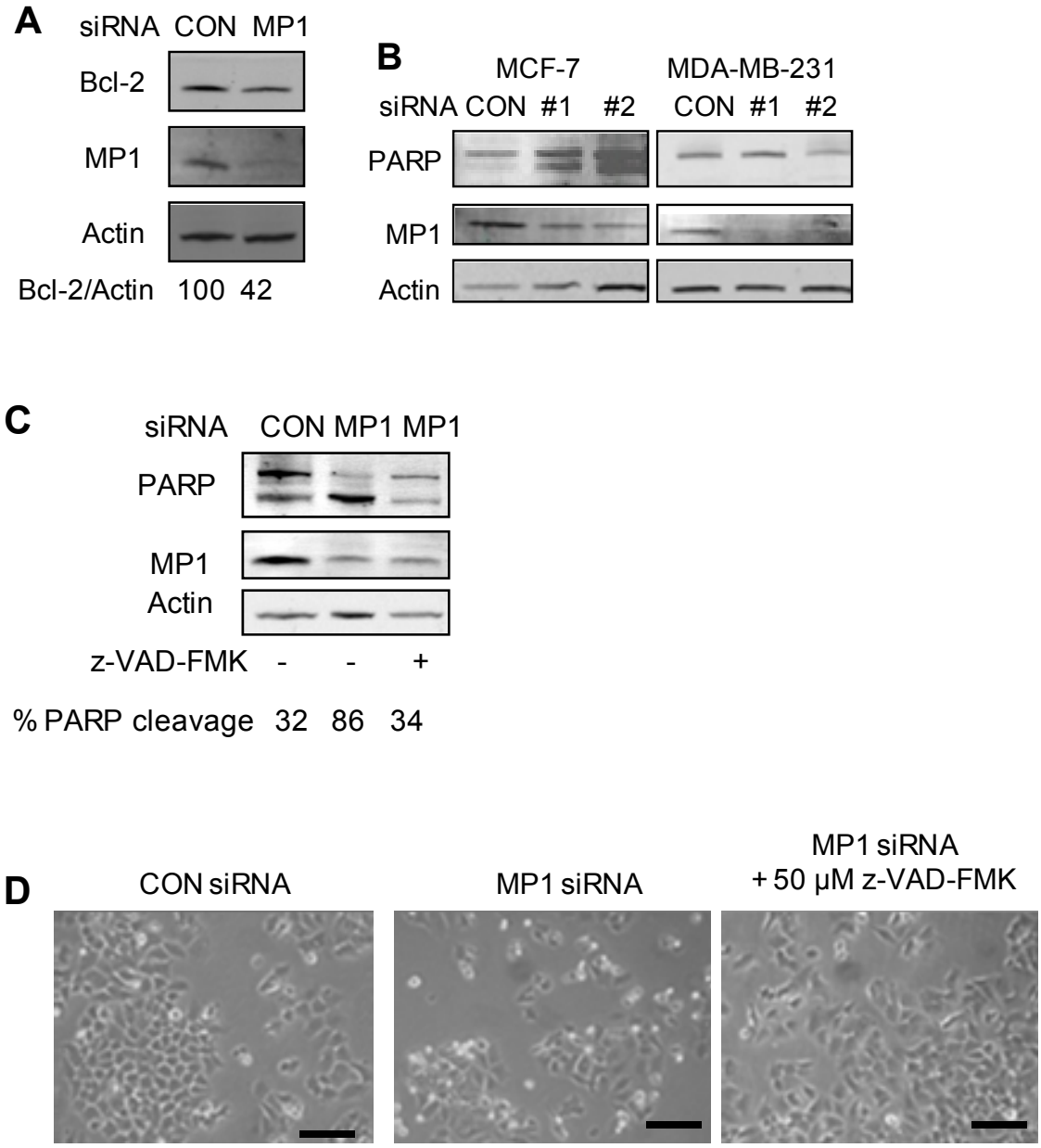


Figure 8. MP1 knockdown induces apoptosis of MCF-7 but not of MDA-MB-231 cells. MCF-7 and/or MDA-MB-231 cells were transfected for 48 h with 30 nM control or MP1 siRNA, and cell extracts were prepared. A) Immunoblot of Bcl-2 protein in MCF-7 cells. Numbers represent the average MP1/Actin ratios expressed as percentage of control samples (n=3). B) Immunoblot of PARP in MCF-7 and MDA-MB-231 cells. C) Immunoblot and quantification of PARP cleavage in MCF-7 cells transfected with MP1 siRNA in the absence or presence of 50 µM z-VAD-FMK. D) Representative photographs of the samples analyzed in panel (C). Scale bar = 100 µm.

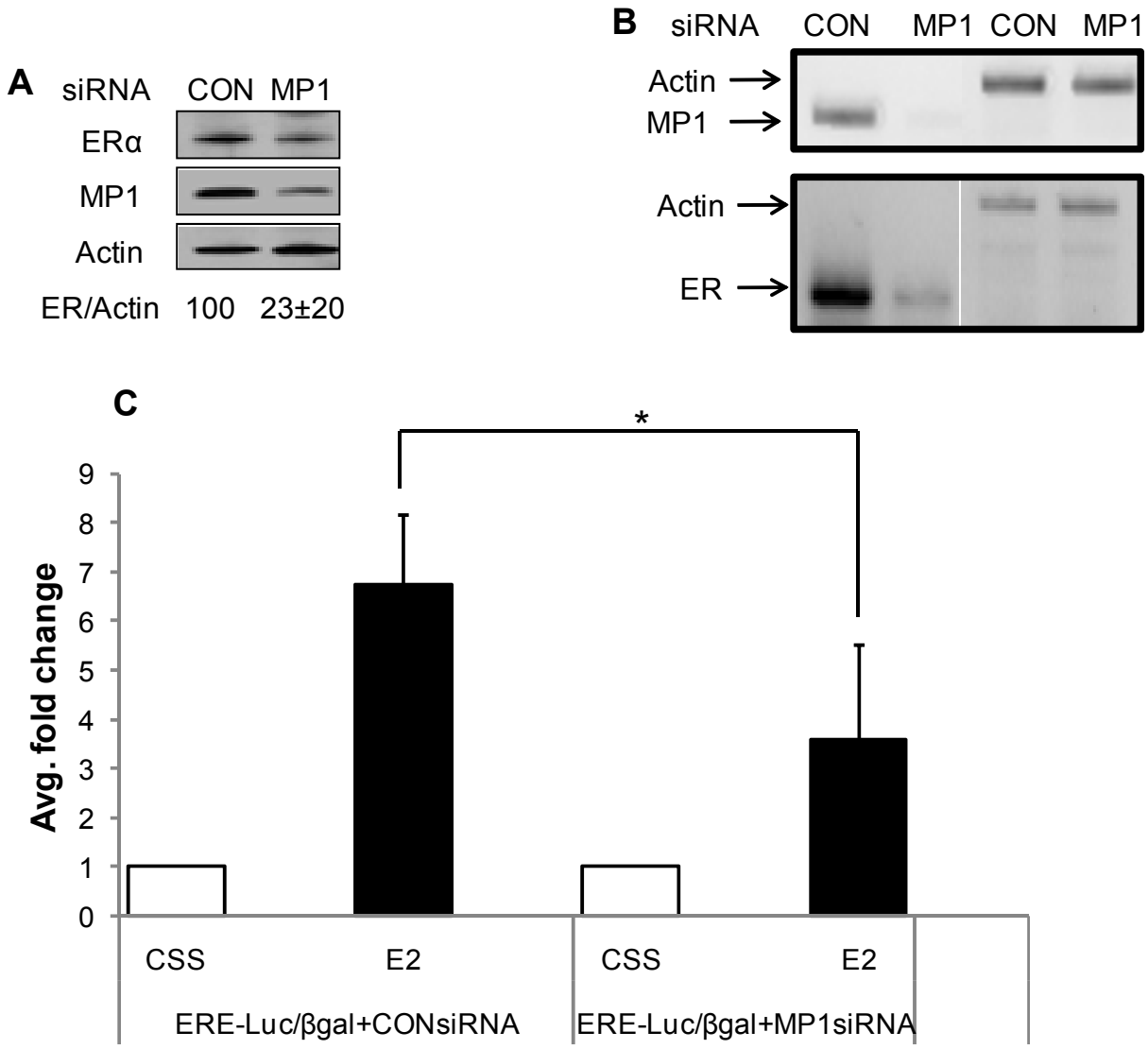


Figure 9. MP1 silencing decreases ER mRNA levels and transcriptional activity. A) Immunoblots of ER, MP1 and actin in MCF-7 cells transfected with MP1 siRNA for 48 hours. B) Semi-quantitative PCR indicating mRNA levels of MP1, ER and actin in MCF-7 cells transfected with siRNA for 30 h. C) Quantification of luciferase/β galactosidase activity in MCF-7 cells cotransfected with plasmids expressing ERE2-tk109-luc and pβgal-Basic then treated with siRNA and stimulated with estrogen. Bars represent normalized luciferase activity in estrogen-stimulated cells relative to CSS (n=4 ± SD, *p<0.5).

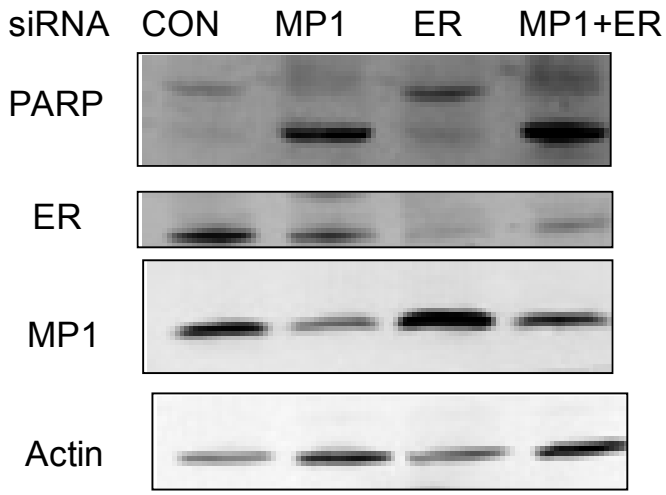


Figure 10. ER silencing does not rescue the apoptosis induced by MP1 siRNA in MCF-7 cells. Cells were transfected with control, MP1, ER or combination of MP1 and ER siRNAs for 48 h then harvested and probed for PARP cleavage, ER, MP1, and actin by immunoblotting.

induced apoptosis of MCF-7 cells. If this process was dependent on ER, then the siRNA inhibition of ER expression concurrently with MP1 silencing, would prevent it. As illustrated by the immunoblots in figure 10, both MP1 and ER proteins were decreased with siRNA. Inhibition of MP1 expression induced PARP cleavage, which is indicative of apoptosis. Silencing of ER did not result in PARP cleavage and apoptosis, suggesting that MCF-7 cells do not depend on ER signals alone for survival. Interestingly, double silencing of MP1 and ER did not rescue cells from the MP1 siRNA-induced PARP cleavage and apoptosis, indicating that the ER is not required for apoptosis induced by MP1 silencing.

MP1 knockdown reduces β1 integrin expression and decreases AKT activity but does not impact ERK expression or activity in MCF-7 cells

To identify pathways affected by MP1 knockdown, expression of total and phosphorylated ERK and AKT1 and β1 integrin were examined (Figure 11). AKT1 is a pro-survival protein with a well-established role in the biology of cancer. ERK signaling is typically associated with proliferation, but may also be involved in regulating cell survival. The level of phospho-ERK was unaffected by MP1 knockdown (Figure 11A), suggesting that a loss of ERK signaling is not responsible for the observed detachment and cell death . In contrast, both phospho-AKT1 and β1 integrin levels decreased within 48 h of MP1 knockdown (Figure 11B and C).

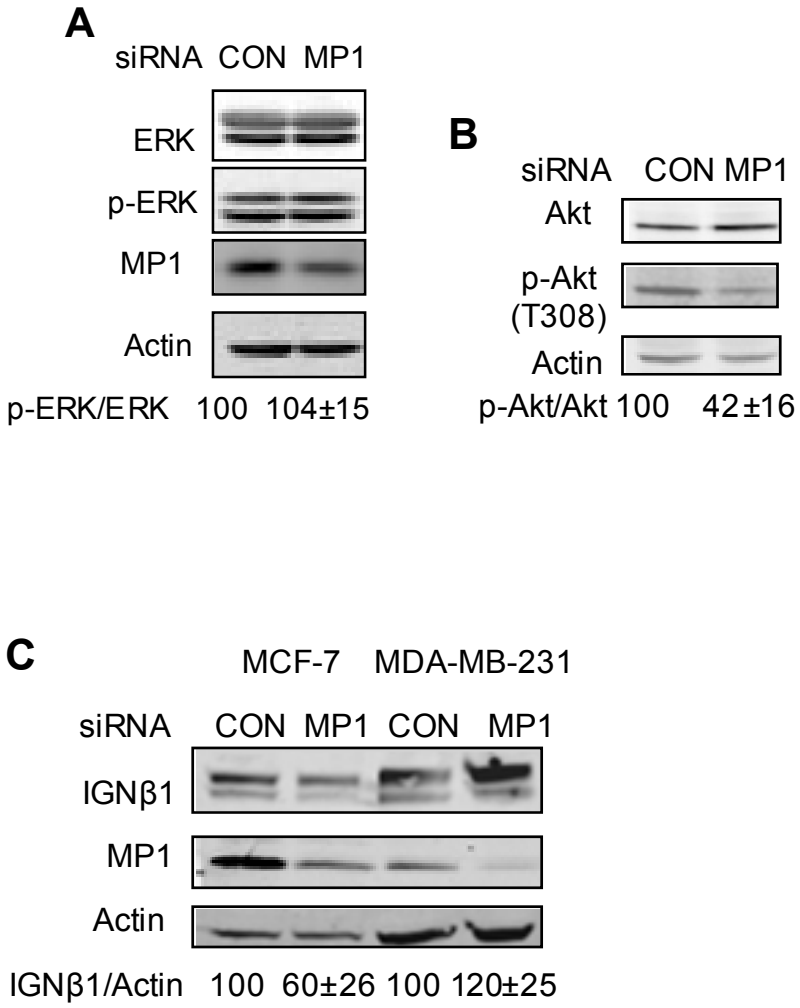


Figure 11. Effect of MP1 knockdown on cellular signaling pathways. MCF-7 and/or MDA-MB-231 cells were transfected with 30 nM MP1 siRNA for 48 h. A) Immunoblot of total and phospho-ERK in MCF-7 cells. The average p-ERK/total ERK ratios are expressed as percentage of control samples (n=3). B) Immunoblot of total and phospho-AKT in MCF-7 cells (n=4). C) Immunoblot of β1 integrin in MCF-7 and MDA-MB-231cells (n=3, p=0.1).

Differential requirement for PI3K/AKT pathway for survival of MCF-7 and MDA-MB-231 cells

Inhibition of MP1 expression resulted in cell death in MCF-7 cells, and this was correlated with decreased phosphorylated (active) AKT1. In contrast, MDA-MB-231 cells showed no such response to MP1 knockdown. If decreased AKT activity is responsible for the cell death observed after MP1 knockdown, the lack of death in MDA-MB-231 cells could be due to the fact that AKT activity is not dependent on MP1 in MDA-MB-231 cells, or that survival of these cells is not dependent upon active AKT. To test the latter possibility, MCF-7 and MDA-MB-231 cells were treated with various concentrations of the PI3K inhibitor LY294002, and the effects on AKT1 phosphorylation and cell viability were examined. As shown in Figure 12A, a concentration of 20 μM was sufficient to partially inhibit PI3K activity in both cell lines, as indicated by decreased p-AKT1 levels. MCF-7 cell viability declined upon LY294002 treatment, and this was the result of apoptosis as indicated by increased PARP cleavage (Figure 12B and C). In contrast, MDA-MB-231 cells were unaffected by LY294002 treatment. These data indicate that MCF-7 cells are more dependent on PI3K/AKT1 pro-survival signaling than MDA-MB-231 cells, and are in agreement with previous reports showing a differential requirement for PI3K signaling in these two cell lines (13, 14) .

Constitutively active AKT1 partially rescues MP1 siRNA induced apoptosis of MCF-7 cells

MP1 knockdown was correlated with decreased activation of AKT1 in MCF-7 cells, which are highly dependent on pro-survival signals from the PI3K/AKT pathway. To

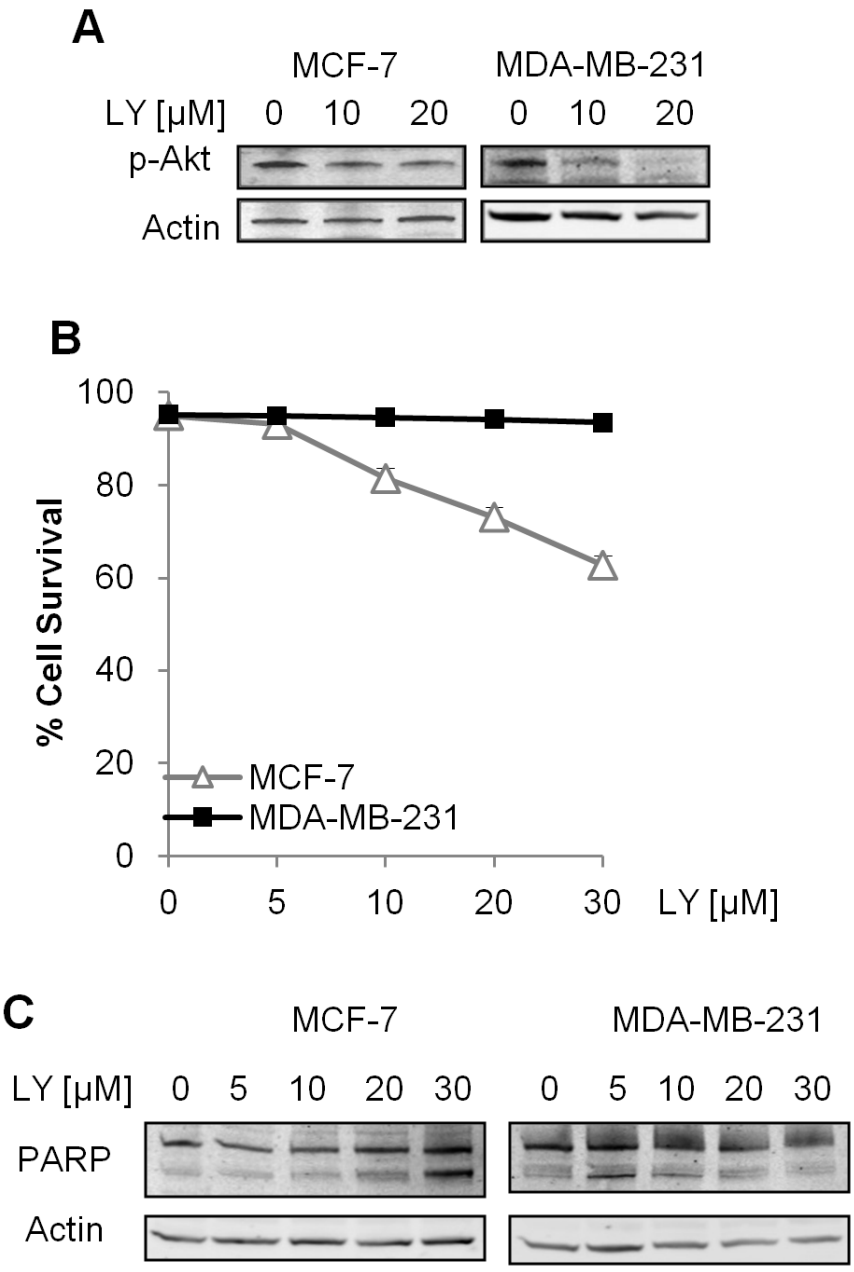


Figure 12. The PI3K/AKT pathway is required for survival of MCF-7 but not MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were treated with various concentrations of LY294002 for 48 h. A) Immunoblot of p-AKT in MCF-7 and MDA-MB-231 cells treated LY294002. B) Effects of LY294002 treatment on viability as determined by trypan blue exclusion assays. C) Immunoblot of PARP cleavage in MCF-7 and MDA-MB-231 cells treated with LY294002.

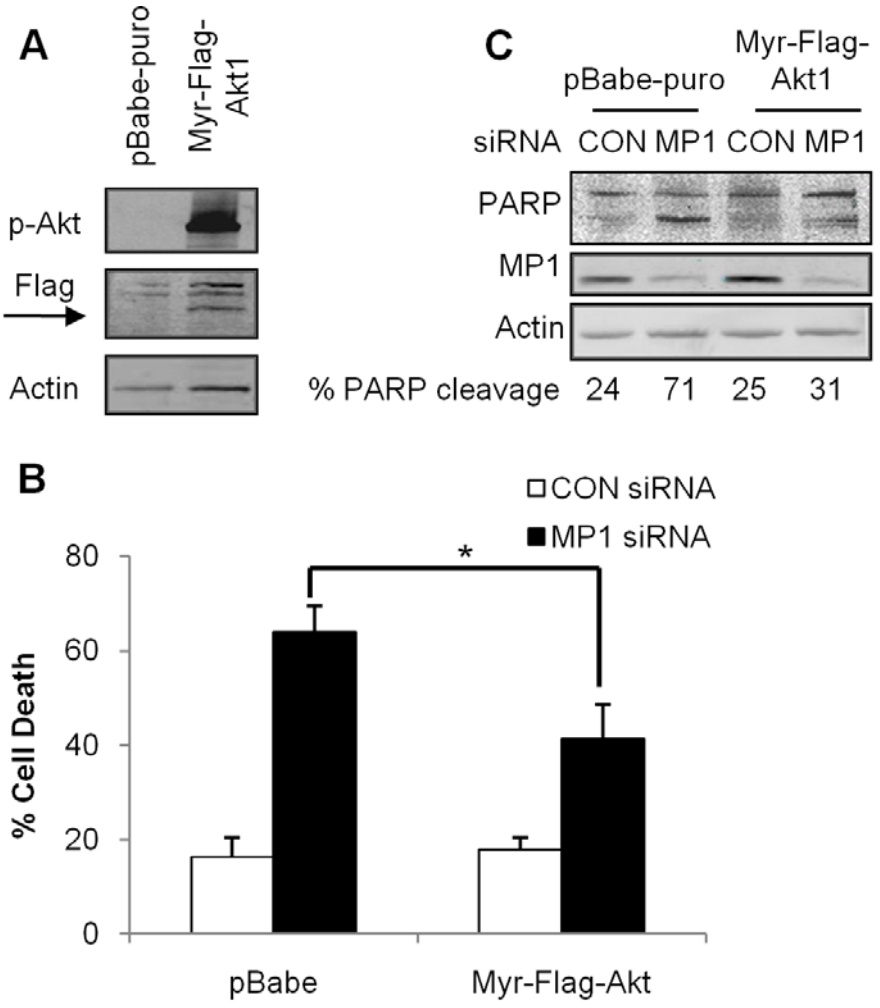


Figure 13. Constitutively active AKT1 partially rescues MCF-7 cells from the apoptosis induced by MP1 siRNA. A) Immunoblot of p-AKT and Flag in stable pools of MCF-7 cells infected with control (pBabe-puro) or Myr-Flag-AKT1 expression vector as described in Materials and Methods. B) The stable pools of cells described in (A) were transfected with 30 nM control siRNA or MP1 siRNA for 48 h, and cell viability was determined by trypan blue exclusion assay. Bars represent the percentage of trypan blue-positive cells. Error bars represent the mean ± SD for three independent experiments, *p<0.05. C) Immunoblot of PARP and MP1 in a representative experiment described in (B).

examine whether active AKT1 is sufficient to maintain cell viability in the absence of MP1 we generated MCF-7 cells expressing constitutively active AKT1 (MCF-7/pBabe-puro/Myr-Flag-AKT1). p-AKT1 was highly expressed in a pool of MCF-7/pBabe-puro/Myr-Flag-AKT1 cells compared to a pool of cells containing the control pBabe-puro vector (Figure 13A). These pools of cells were transfected with MP1 siRNA or control siRNA, and the effects on cell survival were examined. As shown in Figure 13B, 64% of pBabe-puro containing cells were dead in the MP1 siRNA treated sample, but this decreased to 41% in cells expressing constitutively active AKT1. In addition, the extent of PARP cleavage was decreased in Myr-Flag-AKT1 expressing cells (Figure 13C).

Together, these findings indicate that expression of active AKT1 partially overcomes the requirement for MP1 expression for survival of MCF-7 cells. They also suggest that AKT1 signals, at least in part, downstream of MP1.

DISCUSSION

The results presented here reveal a novel role for the small scaffold protein MP1 in breast cancer cells. Although MP1 is expressed in both ER-positive and ER-negative breast cancer cells, its depletion using RNAi-mediated suppression led to detachment and death of several ER-positive cell lines, but not three ER-negative breast cancer or a non-tumorigenic mammary epithelial cell line. Although this is a limited sample, MP1 has also been depleted in rat fibroblasts and human prostate cancer cells, and cell detachment/death was not reported in either case (6, 7). Therefore, MP1 expression seems to be required for survival in a subset of cell types, including ER-positive breast cancer cells. The observed cell death that occurred as a result of inhibiting MP1

expression in MCF-7 cells was shown to be due to an intrinsic apoptotic mechanism, as demonstrated by decreased Bcl-2 expression, increased PARP cleavage, and rescue of the death phenotype by treatment with the pan-caspase inhibitor z-VAD-FMK.

Several interesting questions are raised by these results. One is what pro-survival pathways are affected by loss of MP1 expression in MCF-7 cells. Depletion of MP1 did not result in decreased ERK activation, indicating that its pro-survival functions are not mediated by the ERK pathway. The lack of an effect on ERK activation was somewhat surprising, since MP1 was originally identified as a scaffold protein that increased ERK signaling, but is consistent with results obtained in prostate cancer cells (6). In addition, since the expression of MP1 protein was evaluated in whole cell extracts it is possible that impaired ERK activity was localized to endosomes only, as reported by previous studies. Immunoblotting data demonstrated that inhibition of MP1 expression resulted in a greater than two fold decrease in AKT phosphorylation, suggesting that AKT1 signals, at least in part, downstream of MP1. The extent of AKT inhibition may be an underestimate, since by 48 h a majority of cells were dead, and the remaining live cells might represent ones with the lowest extent of MP1 knockdown. AKT plays a known pro-survival role in breast cancer cells, where it functions to relay signals from integrins or growth factor receptors, PI3K, or mTORC1, to downstream molecules such as Bcl-2 and NF-κB (15-20). The fact that AKT may also play a role in MP1 mediated survival is supported by the fact that expression of a constitutively active AKT1 partially rescued the cell death phenotype observed upon MP1 knockdown. There are two possible explanations for the lack of a full rescue: first, it is possible that not all of the MCF-7 cells in the pool selected here express the constitutively active AKT1 and second, the

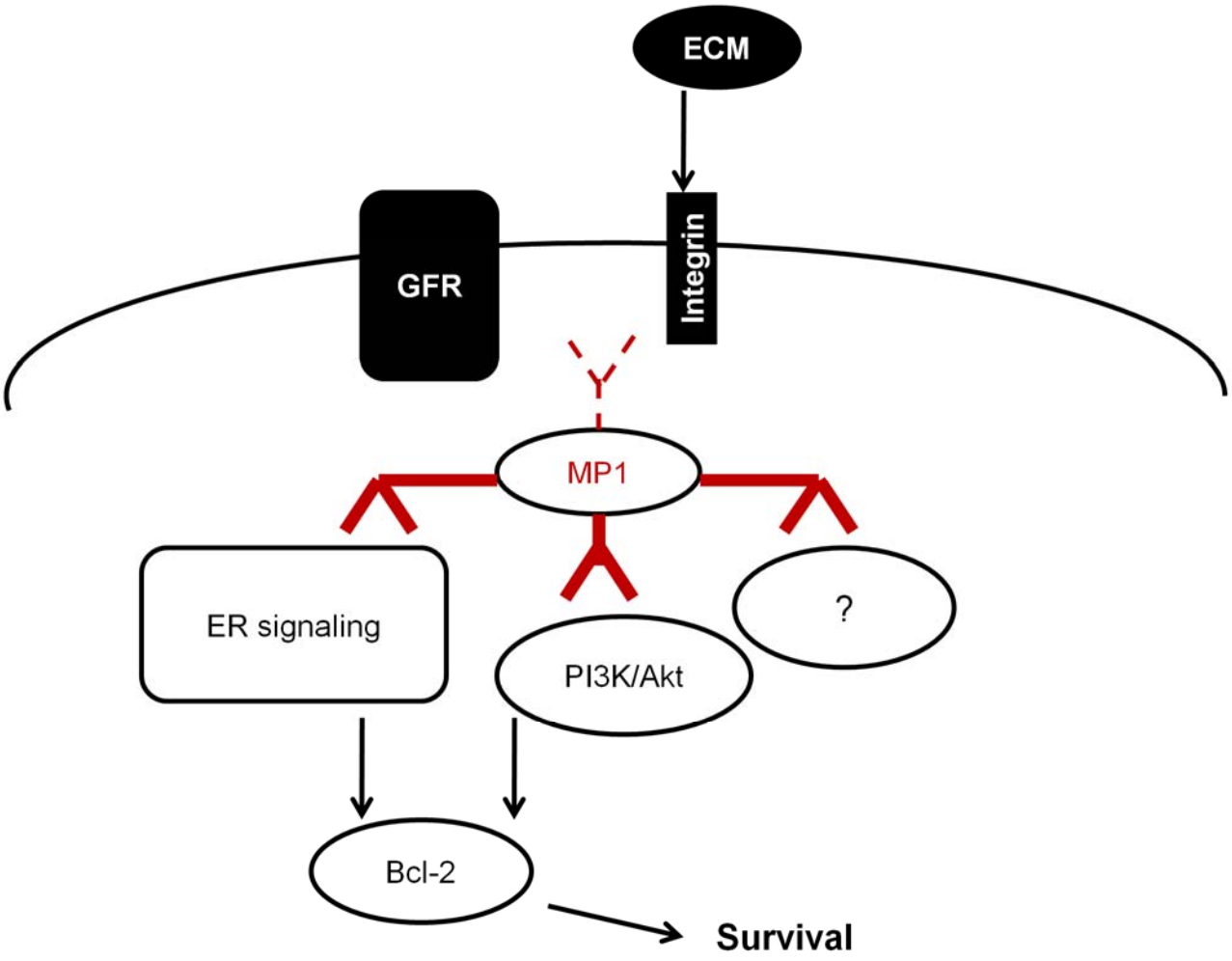


Figure 14. Proposed model of MP1 siRNA-induced apoptosis of ER-positive breast cancer cells. Although not directly proven, inhibition of MP1 expression may decrease the expression of IGNβ1 and possibly other molecules (GFR, RTK) at the plasma membrane. This in turn decreases signaling from the PI3K/AKT and/or other downstream pro-survival pathways that ER-positive breast cancer cells in particular are highly dependent on, leading to Bcl-2 downregulation and apoptosis.

PI3K/AKT may not be the only pro-survival pathway in MCF-7 cells and silencing MP1 may trigger the activity of parallel AKT-independent apoptotic signals.

Depletion of MP1 in MCF-7 cells also resulted in decreased levels of ERα protein, mRNA and reduced the transcriptional activity of the receptor, suggesting that MP1 may promote ER expression and activity. However, the apoptosis observed is unlikely to be due solely to a loss of ER signaling, since we and others have found that inhibition of ER expression using siRNA does not result in MCF-7 cell death (21). Furthermore, concurrent silencing of MP1 and ER did not rescue the cell death observed, indicating that the apoptosis induced by MP1 silencing does not depend on ER. Several studies indicate that ER may be implicated in breast cancer cell survival via cross-talk with the PI3K/AKT pathway (22), or by regulating the activity of NF-κB (23, 24), Bcl-2 (25, 26), or IAP family members (27). We therefore cannot rule out the possibility that decreased ER expression may contribute to the apoptosis observed.

A second question raised by these results is the molecular basis for the differential requirement for MP1 for survival of ER-positive vs. ER-negative breast cancer cells. One possibility is that activation of pro-survival proteins such as AKT is not dependent on MP1 in ER-negative cells, and a second is that the ER-negative cells are less dependent on these pro-survival signaling pathways. Unfortunately we were not able to test the impact of MP1 silencing on AKT activity in MDA-MB-231 cells. The fact that LY294002 caused a concentration-dependent apoptotic response in MCF-7 cells, but did not affect MDA-MB-231 cells supports the latter hypothesis. This is in agreement with previous reports describing a differential sensitivity to this compound between the

two breast cancer cell lines (13, 14). Our findings suggest that MP1 promotes survival in part via PI3K/AKT1 signaling in MCF-7 cells.

A final question is whether the cell death that we have observed is related to the previously identified roles of MP1 in cell spreading and cell motility. Since the apoptotic phenotype involves cell rounding and detachment, inhibition of MP1expression may disrupt cell adhesion signals, which could then trigger cell death.

MP1 silencing in MCF-7 cells was correlated with decreased levels of β1 integrin protein expression. This receptor is of a particular interest, since 30-50% of breast tumors display aberrant expression of β1 integrin and αvβ1 heterodimer is the primary receptor for fibronectin. Data from literature address the interactions between β1 integrin and molecules that regulate cell survival. However, the direct effect of β1 integrin on breast cancer cell viability, is far from being elucidated and may be ligand- and cell-type dependent, and involve only a subpopulation of the receptor. Although not statistically significant, the decrease in β1 integrin suggests that inhibition of MP1 expression in MCF-7 cells may decrease the availability of pro-survival β1 integrin at the plasma membrane, which in turn, may trigger the activation of downstream apoptotic signals. This data partly fit a more recent study in which β1 integrin inhibitory antibodies induce apoptosis of several breast cancer cells in a 3D culture system, regardless of ER status (28).

In summary, this is the first report investigating the role of the small scaffold protein MP1 in mammary epithelial cells. We have identified a novel functional interaction between MP1 and AKT1, and demonstrated that a loss of MP1 expression results in

apoptosis in ER-positive cells that are highly dependent upon the AKT pathway for survival. Future studies will further examine the molecular mechanism(s) by which MP1 promotes survival of ER-positive breast cancer cells, and evaluate its potential as a therapeutic target for ER-positive breast tumors.

REFERENCES

REFERENCES

1. Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science. 1998;281:1668-71.

2. Pullikuth AK, Catling AD. Scaffold mediated regulation of MAPK signaling and cytoskeletal dynamics: a perspective. Cell Signal. 2007;19:1621-32.

3. Teis D, Taub N, Kurzbauer R, Hilber D, de Araujo ME, Erlacher M, et al. p14-MP1-MEK1 signaling regulates endosomal traffic and cellular proliferation during tissue homeostasis. J Cell Biol. 2006;175:861-8.

4. Teis D, Wunderlich W, Huber LA. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev Cell. 2002;3:803-14.

5. Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010;141:290-303.

6. Park ER, Pullikuth AK, Bailey EM, Mercante DE, Catling AD. Differential requirement for MEK Partner 1 in DU145 prostate cancer cell migration. Cell Commun Signal. 2009;7:26.

7. Pullikuth A, McKinnon E, Schaeffer HJ, Catling AD. The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals. Mol Cell Biol. 2005;25:5119-33.

8. Mouchel-Vielh E, Bloyer S, Salvaing J, Randsholt NB, Peronnet F. Involvement of the MP1 scaffold protein in ERK signaling regulation during Drosophila wing development. Genes Cells. 2008;13:1099-111.

9. Boehm JS, Zhao JJ, Yao J, Kim SY, Firestein R, Dunn IF, et al. Integrative genomic approaches identify IKBKE as a breast cancer oncogene. Cell. 2007;129:1065-79.

10. Ory DS, Neugeboren BA, Mulligan RC. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A. 1996;93:11400-6.

11. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415:530-6.

12. Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, et al. MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to

the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res. 1997;57:3486-93.

13. Bartucci M, Morelli C, Mauro L, Ando S, Surmacz E. Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. Cancer Res. 2001;61:6747-54.

14. Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible AKT activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol Cancer Ther. 2002;1:707-17.

15. Bratton MR, Duong BN, Elliott S, Weldon CB, Beckman BS, McLachlan JA, et al. Regulation of ERalpha-mediated transcription of Bcl-2 by PI3K-AKT crosstalk: implications for breast cancer cell survival. Int J Oncol. 2010;37:541-50.

16. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest. 2008;118:3065-74.

17. Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. AKT phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol. 2001;21:893-901.

18. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129:1261-74.

19. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol Cell. 2006;21:749-60.

20. Nam JM, Onodera Y, Bissell MJ, Park CC. Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. Cancer Res. 2010;70:5238-48.

21. Wang X, Belguise K, Kersual N, Kirsch KH, Mineva ND, Galtier F, et al. Oestrogen signalling inhibits invasive phenotype by repressing RelB and its target BCL2. Nat Cell Biol. 2007;9:470-8.

22. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem. 2001;276:9817-24.

23. Frasor J, Weaver A, Pradhan M, Dai Y, Miller LD, Lin CY, et al. Positive cross-talk between estrogen receptor and NF-kappaB in breast cancer. Cancer Res. 2009;69:8918-25.

24. Riggins RB, Zwart A, Nehra R, Clarke R. The nuclear factor kappa B inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells. Mol Cancer Ther. 2005;4:33-41.

25. Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, et al. Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. J Biol Chem. 1999;274:32099-107.

26. Teixeira C, Reed JC, Pratt MA. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res. 1995;55:3902-7.

27. Stanculescu A, Bembinster LA, Borgen K, Bergamaschi A, Wiley E, Frasor J. Estrogen Promotes Breast Cancer Cell Survival in an Inhibitor of Apoptosis (IAP)-Dependent Manner. Horm Cancer. 2010;1:127-35.

28. Park CC, Zhang H, Pallavicini M, Gray JW, Baehner F, Park CJ, et al. Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. Cancer Res. 2006;66:1526-35.

APPENDIX

The Role of MP1 Scaffold Protein in Estrogen-regulated Functions of Breast Cancer Cells

APPENDIX

ABSTRACT

Proliferation and migration of breast cancer cells are regulated by various cytosolic kinases, including the members of the MAPK pathway and PAK1. In turn, as shown by studies carried out in fibroblasts, the activity of these molecules requires the participation of MP1 scaffold protein. Moreover, in ER-positive breast tumors, intracellular kinases potentiate the function of ER, even in the absence of ligands, and trigger various cellular responses. Because MP1 interacts with signaling molecules that are known to promote breast tumorigenesis, the initial aim was to investigate the role that MP1 plays in regulating the proliferation and migration of breast cancer cells. The effects of inhibiting MP1 expression by siRNA-mediated silencing were investigated. First, we observed that inhibition of cell cycle progression seemed to have a protective effect against apoptosis. Under these experimental conditions, we observed that neither short-term proliferation nor migration of MCF-7 cells requires MP1. Taken together, these studies indicate that although it is required for ER activity, MP1 scaffold protein is not essential for estrogen-mediated proliferation and migration after release from cell cycle arrest.

INTRODUCTION

Uncontrolled proliferation is one of the acquired attributes of cancer cells and this tumoral process is promoted by mitogens such as estrogens and growth factors. Their mechanism of action involves increased transcription of molecular components of the cell-cycle machinery. For over 70% of breast tumors, ER is a major regulator of cell proliferation. Activated by estrogen, ER stimulates the expression of genes encoding the cyclins that are involved in the transition from G_1 to S phase of the cell cycle Therefore, antiestrogens like fulvestrant (ICI) block cell proliferation by arresting the cells in G_0/G_1 phase (1). Regardless of ER status, growth factors and their receptors as well as integrins can regulate cell cycle progression, hence stimulate proliferation, mainly through the MAPK and PI3K pathways.

Another hallmark of epithelial cancers is the acquisition of motility. In many breast cancers, including some ER-positive tumors, this feature is a prerequisite for the switching of neoplastic cells to a metastatic phenotype. Compelling data reveal numerous mechanisms accounting for cell migration. In addition to its proliferative function, ER also influences motility. In MCF-7 cells, estrogen-stimulated ERα positively regulates cell migration (2). In MDA-MB-231 cells transfected with ERα and ERβ, migration and invasion are inhibited in a ligand-independent manner (3). Notably, Raf, MEK and ERK, members of the mitogen activated protein kinase (MAPK) cascade, and the p-21 activated kinase-1 (PAK1) regulate cell motility in normal cells and are implicated in breast cancer signaling. Several studies have reported the direct involvement of MEK and ERK in the motility of tumor cells (4-7). PAK1 is downstream

of Rac and Cdc42, and its activation induces the formation of membrane protrusions (8, 9). PAK1 may also play a role in breast cancer by causing tumorigenesis and hyperplasia of the mammary epithelium (10, 11), and by regulating spreading and migration (12, 13). In addition, MAPK and PAK1 are known to trigger ligand independent effects of ER-signaling by directly activating ER (10, 14).

MEK binding partner 1 (MP1) is a small scaffold protein that promotes MEK1 activation by B-Raf (15). The overexpression of MP1 enhances ERK activation in cultured fibroblasts (15). In HeLa cells and mouse fibroblasts, the adaptor protein p14 recruits MP1 to late endosomes where the MP1/p14 heterodimer is required for ERK activation (16, 17). Recently, crosstalk between PAK1 and MEK1 was demonstrated in rat fibroblasts, where PAK1 activates a population of MEK1 and ERK in focal complexes during adhesion to fibronectin. This interaction requires MP1 (18) and is sufficient to activate MEK1 in the absence of Raf, but to a lesser extent than observed with active Raf or EGF (19). Of particular interest is the involvement of MP1 in regulating attachment of fibroblasts. Knocking down MP1 inhibited their acute spreading on fibronectin, thus indicating that MP1 allows the rapid cytoskeletal reorganization necessary for membrane protrusions and cell spreading to occur. The existence of this new interaction between MP1 and PAK1, which was required for subsequent MEK1 activation, suggested a role for MP1 in coupling MAPK signaling to Rho (18).

The pathways that MP1 interacts with control the proliferation and motility of breast cancer cells, therefore we reasoned that MP1 might have similar functions in mammary tumor epithelial cells and might directly contribute to the proliferation and motility of

breast cancer cells. To test this hypothesis, the effects of MP1 silencing on MCF-7 cell short-term proliferation and migration were examined. Our results indicate that inhibition of cell cycle appears to have a protective effect against the apoptosis induced by MP1 silencing. Consequently, when cells are released from arrest using estrogen, MP1 is not required to promote the entry in the S phase or migration. Taken together, these studies indicate that MP1 scaffold protein is not essential for estrogen-mediated proliferation and migration of breast cancer cells following arrest.

MATERIALS AND METHODS

Cell lines and culture conditions

MCF-7 cells were obtained from the Lombardi Cancer. Cells were maintained in Improved Modified Eagle's Medium (IMEM) containing phenol red (GIBCO-Invitrogen-Applied Biosystems), supplemented with 5% fetal bovine serum (HyClone), and 100 Units/ml Penicillin/100 µg/ml Streptomycin (Invitrogen) and incubated at 37 ºC with 5 % CO_2.

siRNA transfections

All siRNA transfection reagents were purchased from Dharmacon-Thermo Scientific. MP1 (ON-TARGETplus siRNA Human MAP2K1IP1) and a non-targeting siRNA (ON-TARGETplus siCONTROL) were used. Cells were plated in six-well plates at 10^5-3 x 10^5 cells per well in FBS containing medium. After 24 h, cells were transfected or co-transfected with 30 nM siRNA using DharmaFECT 1 transfection reagent. After 48 h,

cells were harvested by scraping on ice cold PBS or trypsinization and prepared for protein extraction.

Cell cycle arrest

After 5-6 h, siRNA transfection mixes were replaced with charcoal-stripped serum (CSS) medium supplemented with 10 nM ICI182,780 for 24 hours, which induced their growth-arrest in the G_0/G_1 phase of the cell cycle and abolished ER signaling.

BrdU incorporation

MCF-7 cells were plated on glass coverslips in 6-well plates, then transfected and pre-arrested as previously described. Following ICI pre-arrest for 24 hours, cells were treated with 10 nM 17-β estradiol or vehicle in CSS-containing medium for 19 hours. During the last five hours of treatment, cells were labeled with 25 μM BrdU, then fixed for 15 minutes with 3% methanol-free formaldehyde (Polysciences) and subjected to immunofluorescence. In parallel, cells were plated, treated and harvested at the same time point for protein extraction.

Immunofluorescence

Formaldehyde-fixed cells were washed with PBS, then fixed for 10 minutes with ice-cold pure methanol at -20ºC. DNA was denatured using 2N HCl for 30 min at 37ºC, then slides were neutralized by 0.1 M borax solution pH 8.5 (Sigma) for 30 min. After a brief wash with PBS, cells were blocked in 2% BSA, then incubated with anti-BrdU/Alexa Fluor conjugated antibody (Invitrogen). A DAPI nuclear counterstain was performed.

Eight random fields were visualized and the percentage of BrdU positive cells was calculated using the Metamorph program.

Immunoblotting

Cell pellets were lysed in 1% Triton XC-100/HEPES or CelLytic M lysis buffer (Sigma), supplemented with cocktail tablets of protease (Roche - Complete Mini EDTA-free) and phosphatase inhibitors (Roche – PhosSTOP). Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Total protein (10-20 µg) was subjected to 4-20% Tris-HCl SDS-PAGE (Bio-Rad), transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore), blocked with Odyssey Blocking Buffer and then incubated with the appropriate primary antibodies overnight. Alexa Fluor 680 anti-goat, anti-rabbit, and anti-rabbit (Invitrogen) and IRDye 800CW anti-mouse and anti-rabbit (LI-COR) secondary antibodies were used for two-color detection of proteins. Membranes were scanned and analyzed using the LI-COR Odyssey system.

Antibodies and reagents

The following primary antibodies were used for Western blotting: MP1 (A-19, Santa Cruz), actin (AC-40, SIGMA) and estrogen receptor alpha (AB-17, Lab Vision-Thermo Scientific, or F-10, Santa Cruz)

CDK2 inhibitor SU9516 was obtained from Calbiochem, ICI from Tocris, 17-β estradiol, DAPI and BrdU were purchased from Sigma.

Migration assay

Cell migration or invasion was analyzed using a Boyden chamber transwell assay (Corning). Cells were plated, transfected, and synchronized in G_0/G_1 phase as previously described. Pre-arrested cells were then incubated overnight in 10 nM estrogen/CSS. Untransfected cells treated with estrogen were used as a positive control for migration, while cells in steroid-depleted media (CSS) represented the negative migration control. To prepare them for migration, cells were trypsinized, quenched with soybean trypsin inhibitor, counted and resuspended in serum-starvation media. Uncoated polycarbonate transwell permeable inserts were pre-treated with BSA in order to block the attachment to the membrane. Fifty thousand cells were seeded in the upper chambers and allowed to migrate for 24 h. The chemoattractant in the lower chamber was medium supplemented with 10% FBS. The permeable membranes were then fixed, stained with crystal violet and the migrating cells were visually scored under a phase-contrast microscope equipped with a grid-containing eyepiece, at 200X. The results are the average number of cells invading through the membrane as counted per five random grid areas, in duplicate inserts

Statistical Analysis

Data are expressed as the mean ± S.D. Most experiments were performed three times. Paired evaluations were made for experimental and control conditions within each experiment. Significance was determined by Student's t test and set at $p<0.05$.

RESULTS

The initial transfection protocol resulted in cell detachment and death, preventing the performance of additional functional assays under these conditions. We therefore modified the procedure, so that transfection mixes containing control or MP1siRNA were replaced with ICI-containing medium after five-six hours. This change partially rescued the observed phenotype, causing fewer cells to detach. Under these new conditions, we have investigated the requirement for MP1 in two estrogen-mediated cellular processes in MCF-7 cells: S phase entry and migration.

MP1 siRNA induced apoptosis is rescued by cell cycle arrest

Our observations indicate that the replacement of transfection media with ICI/CSS reduced the cell death phenotype in MCF-7 cells. ICI anti-estrogen decreases the levels of ER and also induces cell cycle arrest in G_0. The same effect can be achieved by treating cells with SU9516, that specifically blocks CDK2, which is required from the transition of G_1 to S phase. This latter experiment lacks a negative control, which would be represented by MCF-7 cells transfected with MP1 siRNA for 48 hours, in the absence of SU9516. Such experiment would have resulted in PARP cleavage in the presence of MP1 siRNA but not in control samples. Both ICI and SU9516 prevented PARP cleavage upon MP1 silencing, as demonstrated in figure 15, suggesting that cell cycle arrest counteracts the apoptotic signals mediated by loss of MP1 expression.

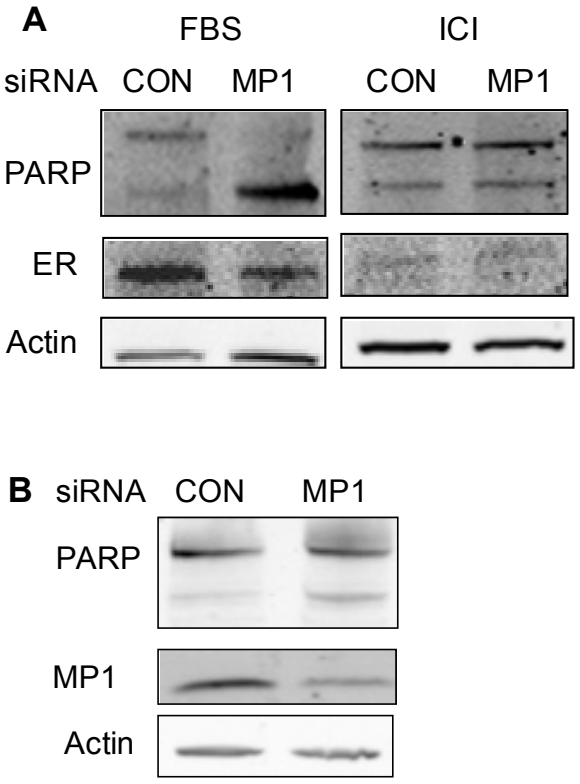


Figure 15. Cell cycle arrest rescues the apoptosis induced by MP1 siRNA in MCF-7 cells. A) Immunoblot of PARP, ER and actin in cells transfected with siRNA mixes in FBS or ICI. B) Immunoblot of PARP, MP1 and actin in cells transfected with MP1 siRNA concurrently with 5 μM SU9516, specific CDK2 inhibitor, for 48 h.

Silencing of MP1 does not affect estrogen-induced S phase entry following arrest in MCF-7 cells

Since the best described role of MP1 involves the scaffolding between MEK1 and ERK1 and because these kinases have been linked to ER signaling, we asked whether endogenous MP1 was necessary for MCF-7 cells to proliferate in the presence of estrogen. To better delineate the role of estrogen in S phase entry, cells were synchronized in the G_0 quiescent phase of their cell cycle, then released with estrogen. The results from three independent experiments revealed that transfection with MP1 siRNA did not alter estrogen-stimulated DNA synthesis (Figure 16). Under estrogen stimulation, there were 34.5% BrdU-positive cells in control siRNA-treated wells and 30.5% with MP1 siRNA transfection. In the absence of estrogen, there were only 12% BrdU-positive cells in control wells and 10.8% in MP1siRNA transfected wells. These data suggest that basal expression of MP1 scaffold protein is not required for the estrogen-dependent S phase entry of the cell cycle.

Effects of MP1 silencing on estrogen-stimulated migration after cell cycle arrest

Estrogen promotes migration of MCF-7 breast cancer cells independently from its effects on proliferation (20). As shown in figure 17, our results suggest that high levels of MP1 may not be required for migration of MCF-7 cells following release from arrest. Although chemoattractants were present in the bottom wells, the migratory potential of untransfected cells increased four-fold when treated with estrogen compared to CSS alone, whereas control and MP1 siRNA transfected cells migrated at comparable levels. A specific mitosis inhibitor, such as mitomycin D, was not used in order to clearly

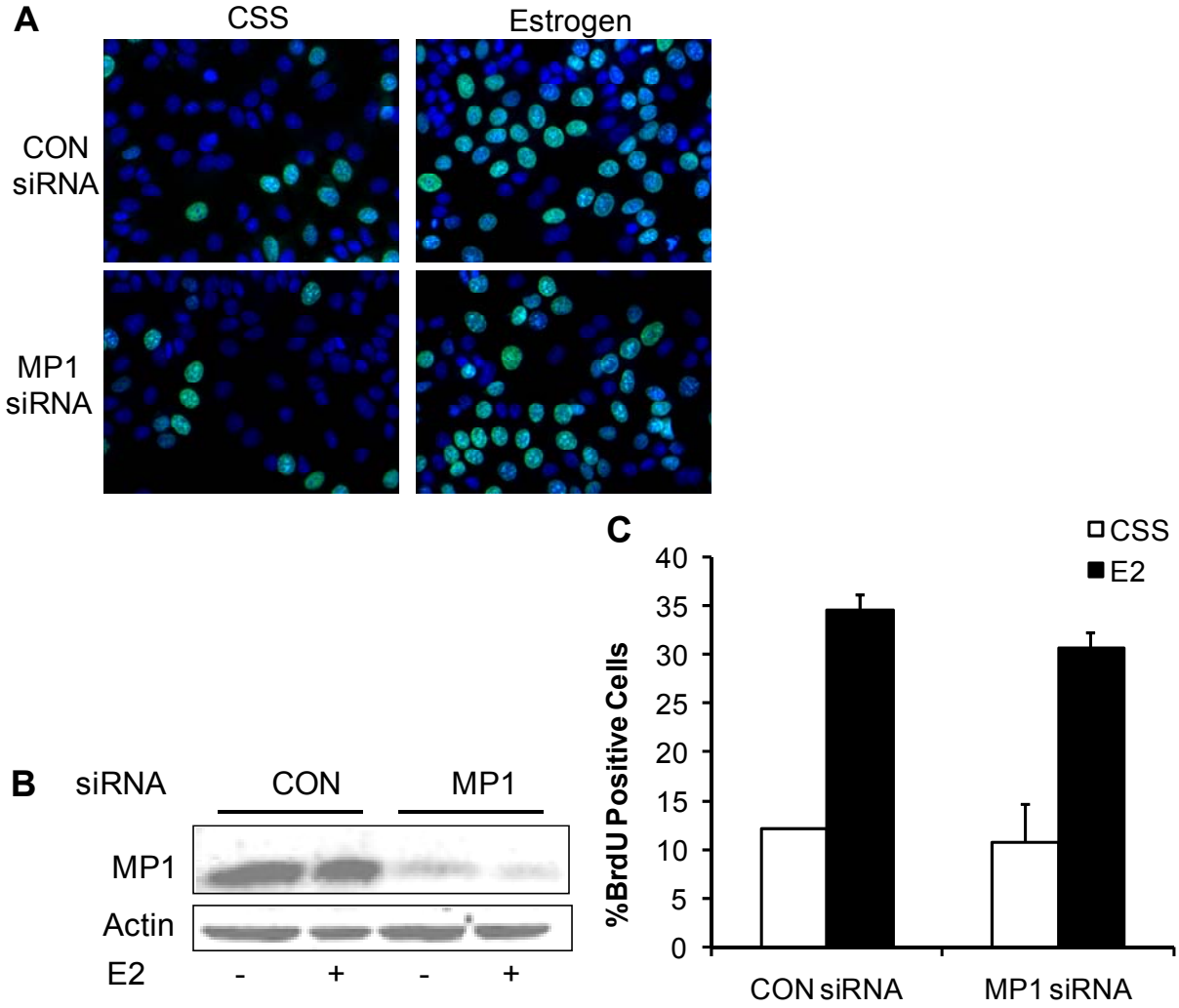
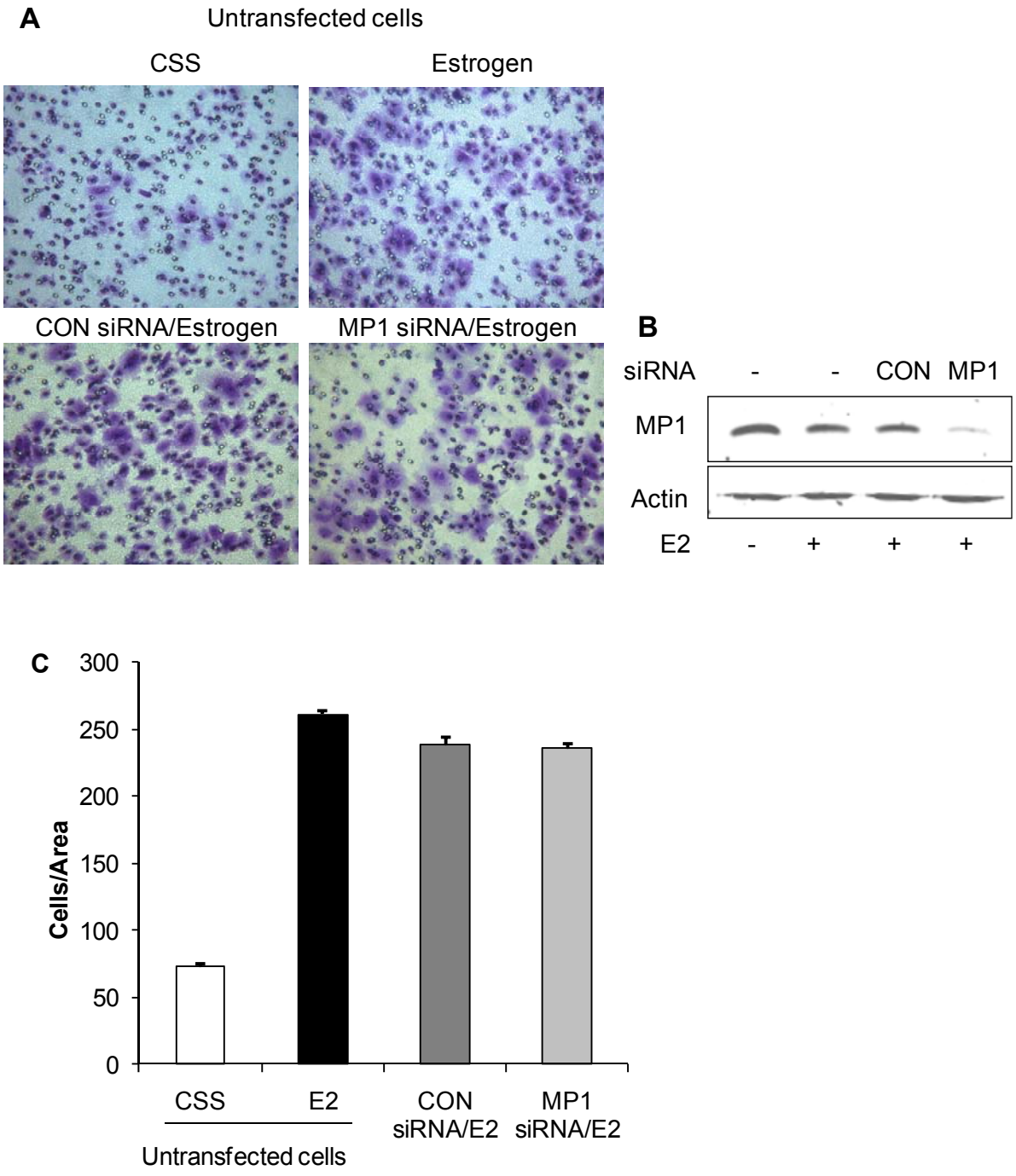


Figure 16. Effect of MP1 knockdown on DNA synthesis. MCF-7 cells were plated, pre-arrested and stimulated with estrogen. A) Representative overlaid immunofluorescent images of pre-arrested MCF-7 cells transfected with MP1 siRNA (lower panels) or control siRNA (upper panels) in the absence (left) or presence of estrogen (right). Green=anti BrdU Alexa Fluor 480; blue=DAPI. B) Immunoblots of MP1 and actin in samples plated, treated and harvested under similar conditions as the BrdU labeled cells. C) Quantification of BrdU incorporation. Bars represent percentage of BrdU-positive cells (mean ±SD, n=3).

Figure 17. Effect of MP1 knockdown on estrogen-stimulated migration of MCF-7 cells. A) Representative images of migrating MCF-7 cells at 200X magnification. Untransfected cells (upper panels) were treated with CSS alone (left) or 10 nM estrogen (right). Cells transfected with control siRNA (lower left) or MP1 siRNA (lower right) were stimulated with estrogen. B) Immunoblots of MP1 and actin of samples shown in (A). C) Quantification of cell migration. Bars represent number of migrating cells/area. Error bars are the mean standard error of replicate wells.

Figure 17.


A
Untransfected cells
CSS
Estrogen
CON siRNA/Estrogen
MP1 siRNA/Estrogen
B
siRNA - - CON MP1
MP1
Actin
E2 - + + +
C
Cells/Area
300
250
200
150
100
50
0
CSS
E2
CON siRNA/E2
MP1 siRNA/E2
Untransfected cells

distinguish between proliferation and migration. However, even if the cells had completed a new cell cycle after their 24 h-long movement through the permeable inserts, the migration would have been masked by proliferation in both MP1 siRNA and control siRNA treated samples, since the knockdown of MP1 resulted in similar numbers of BrdU-positive cells.

DISCUSSION

The additional findings reported here help delineate the functions of MP1 protein in breast cancer cells. Our data indicate that its partial inhibition using siRNA transfection for two days results in apoptosis. This effect prevented us from examining additional physiologically relevant processes, such as proliferation and migration. Previous studies have clearly established the role of estrogen in promoting proliferation and migration of MCF-7 cells. Therefore, in order to investigate these functions we undertook a well-established strategy of pre-arresting the cells in the G_0/G_1 phase of the cell cycle, then releasing them with estrogen to further stimulate their proliferation or migration. Since cell transfection requires active proliferation, the ICI-mediated cell cycle arrest was done at 5-6 hours post siRNA transfection with MP1 siRNA.

Interestingly, replacement of transfection media with ICI/CSS diminished the observed cell death effect and prevented the cleavage of PARP. Similar effects were noted when MCF-7 cells were transfected with MP1 siRNA in the presence of a specific CDK2 inhibitor. Cell cycle arrest is not always followed by apoptosis, since these are two distinct processes that share some regulatory factors (21). However, the molecular mechanisms that determine cell decision between proliferation and apoptosis are still

elusive and may be cell type and context dependent. In our experiments, is appears that inhibition of cell cycle progression has a protective effect against pro-apoptotic signals induced by MP1 silencing. Although we do not provide direct evidence to support this, it is possible that inhibition of apoptosis occurred via transcriptional regulation of pro-apoptotic molecules by highly active inhibitors of cell cycle, such as p21. Such mechanisms have been noted in different cell types (22, 23). Alternatively, exit of G_1 phase by estrogen may activate cytosolic pro-survival pathways, such as PI3K/AKT, that can override the pro-apoptotic signals initiated by MP1 silencing (24).

Under these conditions of partial MP1 silencing and reduced cell death, we carried out functional studies to establish the role of MP1 in short-term proliferation and migration of estrogen-stimulated MCF-7 cells. BrdU labeling during release from arrest revealed that MP1 siRNA transfection did not significantly decreased the percentage of proliferating cells compared to control, suggesting that sustained levels of MP1 are not required for S phase entry. MP1 was reported to regulate Rho function and its inhibition delayed spreading of fibroblasts (18) and decreased migration of prostate cancer cells (25). Generally, a suppression of tumor cell spreading is believed to be correlated with increased migration. However, there are conflicting reports on the significance of spreading relative to the metastatic potential (12, 20, 26). Our data indicate that inhibition of MP1 expression using siRNA duplexes does not influence the migration of breast cancer cells in Boyden chamber assays. Thus, MP1 may not be required for estrogen-stimulated proliferation or migration of MCF-7 cells, following cell cycle arrest.

REFERENCES

REFERENCES

1. Clarke R, Leonessa F, Welch JN, Skaar TC. Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacol Rev. 2001;53:25-71.

2. Acconcia F, Manavathi B, Mascarenhas J, Talukder AH, Mills G, Kumar R. An inherent role of integrin-linked kinase-estrogen receptor alpha interaction in cell migration. Cancer Res. 2006;66:11030-8.

3. Lazennec G, Bresson D, Lucas A, Chauveau C, Vignon F. ER beta inhibits proliferation and invasion of breast cancer cells. Endocrinology. 2001;142:4120-30.

4. Garcia R, Franklin RA, McCubrey JA. EGF induces cell motility and multi-drug resistance gene expression in breast cancer cells. Cell Cycle. 2006;5:2820-6.

5. Guvakova MA, Adams JC, Boettiger D. Functional role of alpha-actinin, PI 3-kinase and MEK1/2 in insulin-like growth factor I receptor kinase regulated motility of human breast carcinoma cells. J Cell Sci. 2002;115:4149-65.

6. Krueger JS, Keshamouni VG, Atanaskova N, Reddy KB. Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion. Oncogene. 2001;20:4209-18.

7. van Golen KL, Bao LW, Pan Q, Miller FR, Wu ZF, Merajver SD. Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer. Clin Exp Metastasis. 2002;19:301-11.

8. Edwards DC, Sanders LC, Bokoch GM, Gill GN. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol. 1999;1:253-9.

9. Parsons M, Monypenny J, Ameer-Beg SM, Millard TH, Machesky LM, Peter M, et al. Spatially distinct binding of Cdc42 to PAK1 and N-WASP in breast carcinoma cells. Mol Cell Biol. 2005;25:1680-95.

10. Wang RA, Mazumdar A, Vadlamudi RK, Kumar R. P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium. EMBO J. 2002;21:5437-47.

11. Wang RA, Zhang H, Balasenthil S, Medina D, Kumar R. PAK1 hyperactivation is sufficient for mammary gland tumor formation. Oncogene. 2006;25:2931-6.

12. Nasarre P, Constantin B, Rouhaud L, Harnois T, Raymond G, Drabkin HA, et al. Semaphorin SEMA3F and VEGF have opposing effects on cell attachment and spreading. Neoplasia. 2003;5:83-92.

13. Vadlamudi RK, Adam L, Wang RA, Mandal M, Nguyen D, Sahin A, et al. Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. J Biol Chem. 2000;275:36238-44.

14. Lannigan DA. Estrogen receptor phosphorylation. Steroids. 2003;68:1-9.

15. Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science. 1998;281:1668-71.

16. Teis D, Taub N, Kurzbauer R, Hilber D, de Araujo ME, Erlacher M, et al. p14-MP1-MEK1 signaling regulates endosomal traffic and cellular proliferation during tissue homeostasis. J Cell Biol. 2006;175:861-8.

17. Teis D, Wunderlich W, Huber LA. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev Cell. 2002;3:803-14.

18. Pullikuth A, McKinnon E, Schaeffer HJ, Catling AD. The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals. Mol Cell Biol. 2005;25:5119-33.

19. Park ER, Eblen ST, Catling AD. MEK1 activation by PAK: a novel mechanism. Cell Signal. 2007;19:1488-96.

20. Adam L, Vadlamudi R, Mandal M, Chernoff J, Kumar R. Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. J Biol Chem. 2000;275:12041-50.

21. Evan GI, Brown L, Whyte M, Harrington E. Apoptosis and the cell cycle. Curr Opin Cell Biol. 1995;7:825-34.

22. Suzuki A, Tsutomi Y, Akahane K, Araki T, Miura M. Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. Oncogene. 1998;17:931-9.

23. Steinman RA, Johnson DE. p21WAF1 prevents down-modulation of the apoptotic inhibitor protein c-IAP1 and inhibits leukemic apoptosis. Mol Med. 2000;6:736-49.

24. Sears RC, Nevins JR. Signaling networks that link cell proliferation and cell fate. J Biol Chem. 2002;277:11617-20.

25. Park ER, Pullikuth AK, Bailey EM, Mercante DE, Catling AD. Differential requirement for MEK Partner 1 in DU145 prostate cancer cell migration. Cell Commun Signal. 2009;7:26.

26. Gauthier ML, Torretto C, Ly J, Francescutti V, O'Day DH. Protein kinase Calpha negatively regulates cell spreading and motility in MDA-MB-231 human breast cancer cells downstream of epidermal growth factor receptor. Biochem Biophys Res Commun. 2003;307:839-46.

CONCLUDING REMARKS

CONCLUDING REMARKS

The main goal of this dissertation was to investigate how MP1 protein affects tumoral processes in breast cancer cells. Given its scaffolding function, and therefore its contribution to potentiating signals that regulate proliferation, spreading, migration, and differentiation, we reasoned that MP1 might play similar roles in breast cancer cells.

We propose a novel pro-survival role for MP1 that is specific to some ER-positive cell lines. Our results indicate that MP1 silencing results in detachment and death of ER-positive breast cancer cells. In contrast, in three independent ER-negative breast cancer cells, the same approach had no significant effect on survival. More importantly, non-tumorigenic mammary epithelial cells were unaffected by MP1 silencing. In MCF-7 cells, a widely used model of estrogen-stimulated, antiestrogen sensitive human breast cancer, apoptosis induced by inhibition of MP1 expression was correlated with decreased activity of AKT and decreased levels of the Bcl-2 pro-survival protein. Interestingly, we demonstrate that MCF-7 cells depend on the PI3K but not on ER signaling for survival. Moreover, concurrent silencing of MP1 and ER failed to rescue the observed cell death whereas this phenotype was partly rescued with the expression of a constitutively active AKT1. This suggests that PI3K/AKT activity is not sufficient to counteract the pro-apoptotic signals initiated by MP1 silencing, and that ER is not required for this phenomenon. However, MP1 promotes ER expression and activation in response to estrogen, therefore, the ER signaling pathway may play a contributing role in promoting cell survival.

Although our query did not reveal any significant correlations between the levels of MP1 protein expression and disease outcome, this is a biological meaningful molecule that may have a potential clinical relevance for **ER-positive breast tumors**:

- MP1 could be a potential therapeutic target, since ER-positive breast cancer cells are more susceptible to apoptosis upon MP1 depletion. In general, the physiological response of drugs targeting ER-positive tumors involves inhibition of or interference with tumor proliferation and growth. Inhibition of MP1 expression could specifically trigger apoptosis of ER-expressing tumor cells, without affecting non-tumorigenic cells. However, this strategy would be ineffective during treatment with fulvestrant (ICI), since in our cell model, this compound and the alternative cell cycle inhibitor SU9516 were able to prevent apoptosis induced by MP1 silencing.

- MP1 expression in ER-positive breast cancer cells could predict the response to specific kinase inhibitors, since these tumors would be more likely to respond to PI3K inhibition than to blockade of MAPK.