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A Role for MEK-Interacting Protein 1 in Hormone Responsiveness of ER Positive Breast Cancer Cells

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14. ABSTRACT
MEK Partner 1 (MP1 or MAPKSP1) is a scaffold protein that functions in multiple signaling pathways that play important roles in breast cancer, and is expressed in both ER-positive and negative breast cancer cell lines. We investigated MP1 function in these cells using RNA interference to silence the MP1 gene. MP1 gene silencing resulted in detachment and apoptosis of ER-positive breast cancer cell lines, but not ER-negative breast cancer or non-tumorigenic mammary epithelial cell lines. The apoptosis observed in ER-positive cells was correlated with decreased AKT activity, and expression of a constitutively active form of AKT1 partially rescued the cell death phenotype. Together, these results suggest that MP1 is required for pro-survival signaling from the PI3K/AKT pathway in ER-positive breast cancer cells, and that it may provide a novel target for the treatment of ER-positive breast tumors.

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ER-positive breast cancer, MEK Partner 1, AKT, apoptosis.

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1) Abstract from 2011 Annual Meeting of the American Association for Cancer Research.
2) Abstract from the 2011 Era of Hope Meeting.
3) Manuscript submitted for publication.

Introduction

Our original proposal was based on preliminary data suggesting that estrogen receptor alpha (ER) is present in a complex with the small scaffold protein Mek Partner-1 (MP1) in human breast cancer cell lines, and that overexpression of MP1 via transient transfection increases ER's transcriptional activity. Based on this, we proposed that MP1 would be required for proliferation of ER-positive breast cancer cells, and that understanding the ER/MP1 complex would give insights into the mechanism by which it was acting. MP1 is a widely expressed scaffold protein that interacts with several intracellular kinases that are known to impact ER function, including MEK, ERK and PAK1. It was first identified as a protein that binds to ERK and MEK, and that potentiates MAPK signaling [1]. It also binds to active PAK1 at the plasma membrane, and integrates PAK1 and Rho signaling [2]. Knockdown of MP1 inhibits spreading of fibroblasts on fibronectin [2], and also results in decreased migration of human prostate cancer epithelial cells on fibronectin [3]. Surprisingly, neither ERK nor PAK1 phosphorylation was dramatically altered when MP1 expression was inhibited in prostate cancer epithelial cells. However, the decreased migration observed upon MP1 knockdown was correlated with decreased paxillin expression and with changes in the number and turnover of focal adhesions at the migratory edge. Thus, one function of MP1 is related to cell attachment, spreading and migration.

In this research project, we investigated the function of MP1 in human breast cancer cell lines. The original aims of our proposal were **1: To test the hypothesis that MP1 is required for ER function and proliferation in human breast cancer cells, and 2: To characterize the subcellular localization and protein composition of ER/MP1 complexes.** In our previous annual reports, we described our unsuccessful efforts to reproduce the initial co-immunoprecipitation experiments demonstrating the existence of a stable complex containing both ER and MP1. Due to this, we have been unable to carry out the experiments described in Aim 2, and have concentrated our efforts on Aim 1. Many of the findings from this aim have been described in our previous annual reports, and are shown in the manuscript that is included here as Appendix 3. They can be briefly summarized as follows. 1) Inhibition of MP1 expression by RNA interference results in cell detachment and apoptosis of ER-positive breast cancer cell lines, but not ER-negative breast cancer cell lines or non-tumorigenic mammary epithelial cell lines. 2) MP1 gene silencing in ER-positive cell lines is associated with an approximate 2-3 fold decrease in ER expression and transcriptional activity, and with an approximate 2 fold decrease in the active, phosphorylated form of AKT. In agreement with the findings in prostate cancer cells, MP1 silencing does not result in decreased ERK activity in ER-positive breast cancer cells. 3) Overexpression of MP1 in ER-positive breast cancer cell lines results in increased cell migration and invasion.

For the past year, we have concentrated our efforts on determining if decreased ER and/or AKT activity is responsible for the apoptosis observed upon MP1 gene silencing. Our progress in these experiments is reported below and in the accompanying manuscript (Appendix 3).

Body: Progress on Each Task in Approved Statement of Work.

Task 1: Test hypothesis that MP1 expression is required for ER's transcriptional activity and proliferation of human breast cancer cells.

As we reported previously, both total ER and phosphorylated AKT levels decrease within 48 h of MP1 knockdown in MCF-7 cells (Appendix 3, Figures 5B and 5D). To determine if decreased ER levels are responsible for the apoptosis observed, the effects of silencing the ER gene alone or in combination with the MP1 gene were examined. Silencing of ER did not result in apoptosis of MCF-7 cells, indicating that decreased ER expression alone is not the cause of apoptosis in MP1 siRNA treated cells (Appendix 3, Figure 5C). In addition silencing ER did not prevent apoptosis induced by MP1 silencing (Appendix 3, Figure 5C), suggesting that ER expression is not required for the apoptotic response.

Inhibition of MP1 expression resulted in cell death in MCF-7 cells, and this was correlated with a decrease in the phosphorylated (active) form of AKT1 (Appendix 3, Figure 5D). In contrast, MDA-MB-231 cells showed no increase in cell death in response to MP1 knockdown (Appendix 3, Figures 3 and 4). If decreased AKT activity was responsible for the cell death observed after MP1 knockdown in MCF-7 cells, 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 Appendix 3 Figure 6A, a concentration of 20 μM was sufficient to partially inhibit PI3K activity in both cell lines. MCF-7 cell viability declined upon LY294002 treatment (Appendix 3, Figure 6B), and this was the result of apoptosis as indicated by increased PARP cleavage (Appendix 3, Figure 6C). In contrast, MDA-MB-231 cell viability was unaffected by LY294002 treatment. These data indicate that MCF-7 cells are more dependent on pro-survival signaling from PI3K/AKT1 than MDA-MB-231 cells, and are in agreement with previous reports showing a differential requirement for PI3K signaling in these two cell lines (4, 5).

Since MP1 silencing resulted in decreased AKT activity in MCF-7 cells, and since these cells are highly dependent on pro-survival signals from the PI3K/AKT pathway, we hypothesized that MCF-7 cells containing a constitutively active form of AKT1 would not undergo apoptosis in response to MP1 silencing. To test this hypothesis, we generated pools of stably transfected MCF-7 cells expressing constitutively active AKT1 (MCF-7/Myr-Flag-AKT1). Phosphorylated-AKT1 (p-AKT) was highly expressed in a pool of MCF-7/Myr-Flag-AKT1 cells compared to a pool of cells containing the control pBabe-puro vector (Appendix 3, Figure 7A). These two pools of cells were transfected with MP1 siRNA or control siRNA, and the effects on cell survival were examined. As shown in Appendix 3 Figure 7B, 64% of vector 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 in response to MP1 silencing was decreased in Myr-Flag-AKT1 expressing cells relative to the control cell line (Appendix 3, Figure 7C). These experiments were repeated with clonal transfectants containing control vector or Myr-Flag-AKT1 with similar results (data not shown). Together, these findings indicate that expression of active AKT1 partially overcomes the requirement for MP1 expression for survival

of MCF-7 cells, and suggests that the apoptosis observed upon MP1 silencing is due, at least in part, to a loss of pro-survival signaling from the AKT pathway.

Tasks 2-4: As described above and in previous reports, we have been unable to reproduce our initial co-IP experiments. Since tasks 2-4 all depending on isolating ER/MP1 complexes, we could not carry out the experiments described in these tasks.

Key Research Accomplishments (entire grant period):

- Demonstrated that MP1 expression is required for survival of ER-positive breast cancer cell lines, but not ER-negative breast cancer cell lines or non-tumorigenic mammary epithelial cell lines.
- Demonstrated that silencing the MP1 gene results in decreased ER expression and activity in MCF-7 cells. However, a loss of ER function does not seem to be the cause of cell death, since no significant increase in apoptosis is observed when the ER gene itself is silenced.
- Demonstrated that silencing the MP1 gene leads to decreased AKT phosphorylation, and that expression of constitutively active AKT1 partially rescues cells from apoptosis induced by MP1 silencing. This suggests that the apoptosis observed upon MP1 gene silencing is due, at least in part, to a loss of pro-survival signaling through AKT.
- Demonstrated that overexpression of MP1 increases cell migration and invasion of ER-positive MCF-7 cells.

Reportable Outcomes (2010-2011):

1) Abstract from 2011 Annual Meeting of the American Association for Cancer Research (Appendix 1)

2) Abstract from 2011 Era of Hope Meeting (Appendix 2)

3) Marina, M. and S.E. Conrad. The Small Scaffold Protein MP1 is required for the survival of estrogen receptor positive breast cancer cells. Manuscript submitted for publication. (Appendix 3)

4) Degrees Obtained: PhD in Physiology awarded to Mihaela Marina, 2011.

5) Cell lines developed: Derivatives of MCF-7 cells expressing a constitutively active AKT1.

6) Grants submitted based on this research.

IDEA Expansion Award Application Number BC104072: "The scaffold protein MAPKSP11 is required for survival of ER positive breast cancer cells". 07/01/2011-06/30/2013. Not funded.

IDEA Expansion Award Application Number BC112716: "MEK Partner 1 as a novel therapeutic target for estrogen receptor positive breast cancer." 09/30/2012 – 09/29/2014. Pending.

Conclusions:

Our experiments have revealed a novel requirement for MP1 expression for the survival of ER-positive breast cancer cells. The pro-survival functions of MP1 seem to be mediated, at least in part, via AKT. MP1 has previously been shown to interact with a number of signaling molecules including ERK, MEK and PAK1, and to play a role in cell attachment and migration. However, the facts that it is required for optimal AKT activity and for cell survival are both novel findings, and suggest that its role in cell survival may be specific to a limited number of cell types, including ER-positive breast cancer cells.

The requirement for MP1 expression in ER-positive breast cancer cells suggests that this small scaffold protein may provide a novel target for the treatment of ER-positive breast tumors. The current targeted therapies for ER-positive tumors (antiestrogens and aromatase inhibitors) have been very effective, but the occurrence of both de novo and acquired resistance is an important clinical problem that limits their efficacy. Our results indicate that cells with acquired antiestrogen resistance (LCC9) retain dependence on MP1, suggesting that agents targeting this scaffold protein could be used to treat endocrine resistant tumors. In addition, the availability of agents targeting two different molecules (ER and MP1) in ER-positive breast cancer cells would offer the opportunity to use them in combination with each other. Since the probability of a single cell acquiring simultaneous resistance to two agents would likely be much lower than to a single agent alone, combination therapy could decrease the frequency of treatment failure and relapse. Since ER-positive tumors are the most common form of breast cancer, and since endocrine resistance is a significant cause of disease relapse and death, this work has the potential to impact a large number of breast cancer patients.

Bibliography (entire grant period):

Publications:

Marina, M. and S.E. Conrad. The Small Scaffold Protein MP1 is Required for the Survival of Estrogen Receptor Positive Breast Cancer Cells. Manuscript submitted for publication. (Appendix 3)

Meeting Abstracts:

Conrad, S.E., Marina, M. and J.E. Lee. **2008**. A Role for Mek-Interacting Protein 1 in Estrogen Receptor Positive Breast Cancer Cells. 2008. DOD-Era of Hope Meeting, Baltimore, MD.

Marina, M. and S.E. Conrad. **2009**. “Inhibition of MP1 induces apoptosis of ER-positive but not of ER-negative breast cancer cells”. Midwest Regional Breast Cancer Symposium, Iowa City, IA.

Marina, M. and S.E. Conrad. **2010**. MEK Partner 1 is required for survival of ER-positive Breast Cancer Cells. Great Lakes Nuclear Receptor Conference, Ann Arbor, MI.

Wang, L. and S.E. Conrad. **2010**. The function of CDCA4 in proliferation of breast cancer cells. Great Lakes Nuclear Receptor Conference, Ann Arbor, MI.

Marina, M. and S.E. Conrad**. 2011.** Inhibition of MP1 expression induces apoptosis of ER positive breast cancer cells. Annual Meeting of the American Association of Cancer Research, Orlando, FL.

Marina, M., L. Wang and S.E. Conrad. **2011**. Mp1 is required for survival of ER-positive breast cancer cells. DOD-Era of Hope Meeting, Orlando, FL.

List of Personnel:

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Limin Wang (Research Associate)

Jung-Eun Lee (Visiting Scholar)

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Appendix/Supporting Data:

4) Abstract from 2011 Annual Meeting of the American Association for Cancer Research.
5) Abstract from the 2011 Era of Hope Meeting.
6) Manuscript submitted for publication.

INHIBITION OF MP1 EXPRESSION INDUCES APOPTOSIS OF ER-POSITIVE BREAST CANCER CELLS

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MEK Partner 1 (MP1 or MAPKSP1) is a widely expressed scaffold protein that functions in several signaling pathways (MAPK and PAK1) that are known to impact ER function and breast cancer biology. We therefore hypothesized that MP1 might play an important role in ER positive breast cancer cells. To test this hypothesis, we used transient transfection with siRNA to investigate the function of MP1 in a panel of human mammary epithelial cell lines that includes non-tumorigenic, ER-positive and ER-negative breast cancer cell lines. After 48 hours, ER-positive cell lines transfected with MP1siRNA (but not control siRNA) rounded up and detached from the plate, and trypan blue staining indicated the majority of MP1siRNA treated cells were dead. No significant increase in cell detachment or death was observed in ER-negative cells treated with MP1 siRNA relative to control siRNA, although immunoblotting confirmed that MP1 protein levels were successfully reduced in all cell lines. The phenotype observed when MP1 expression was inhibited in ER-positive cells was further characterized using the MCF-7 cell model. Inhibition of MP1 expression in this cell line resulted in cleavage of PARP-1, and both this cleavage and the cell rounding/detachment phenotype could be rescued with the pan-caspase inhibitor z-VAD-FMK. We therefore conclude that inhibition of MP1 expression induces apoptosis in MCF-7 cells. Additional experiments were carried out to identify pro-survival pathways that are regulated by MP1 in MCF-7 cells. Inhibition of MP1 expression resulted in decreased expression of ER, integrin beta 1, and phosphor-AKT1. Direct inhibition of ER expression with ER siRNA did not result in cell death, making this an unlikely mechanism of cell death. However, MCF-7 cells were shown to be very dependent on PI3K signaling, and the cell death phenotype was partially rescued by expression of constitutively active AKT1. These results suggest that the inhibition of MP1 expression results in a loss of pro-survival signaling from integrin beta-1 and/or PI3K-AKT pathways, and that MP1 expression is required specifically for survival of ER-positive, but not ER-negative, breast cancer cells.

MP1 IS REQUIRED FOR SURVIVAL OF ER-POSITIVE BREAST CANCER CELLS

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Approximately 70% of breast tumors express estrogen receptor (ER), and the majority of these require estrogen for proliferation and/or survival. Many functions of the ER rely on cross-talk with cellular signaling molecules, including ERK, PAK1, and AKT1. These intracellular kinases can all phosphorylate ER, and the resulting phosphorylation events may alter ER activity and/or ligand dependence. In addition, ER can affect the activity of intracellular kinases via protein-protein interaction, indicating that the cross talk between ER and intracellular kinases is bidirectional. Understanding the molecular basis and functional consequences of this cross talk is important in order to understand ER's role in breast cancer. MEK Partner 1 (MP1, also known as Map Kinase Scaffold Protein 1 or MAPKSP1) is a widely expressed scaffold protein that interacts with several protein kinases that impact ER function and breast cancer biology, including ERK1 and PAK1. We investigated the role of MP1 in breast cancer cells using both RNA interference and overexpression approaches. To study the effects of inhibiting MP1 expression, a panel of human mammary epithelial cell lines that included non-tumorigenic cells, ER-positive breast cancer cells and ER-negative breast cancer cells were transiently transfected with MP1 siRNA or control siRNA. Within 48 hours, up to 80% of ER-positive breast cancer cells transfected with MP1siRNA rounded up and detached from the plate, and trypan blue staining indicated that the majority of detached cells were dead. No significant increase in cell detachment or cell death was observed in ER-negative breast cancer cells or non-tumorigenic cells as a result of inhibiting MP1 expression. The mechanism of cell death in ER-positive breast cancer cells was characterized using the MCF-7 cell line. Increased PARP-1 protein cleavage was detected in MP1 siRNA- treated MCF-7 cells. Both PARP cleavage and cell detachment/death were prevented by pre-treatment with the pan-caspase inhibitor z-VAD-FMK, indicating an apoptotic mechanism of death. Inhibition of MP1 expression in MCF-7 cells also resulted in decreased levels of ER and p-AKT, suggesting a loss of pro-survival signaling from the ER and/or PI3K-AKT pathways. The effect of overexpressing MP1 in MCF-7 cells was investigated using transient and stable transfection of Flag-MP1 constructs. Overexpression of MP1 led to an increase in both cell migration and invasion in Boyden chamber assays. In summary, our experiments suggest that MP1 expression is required for survival of ER-positive, but not ER-negative breast cancer cells, and may also play a role in migration and invasion of ER-positive cells. Previous studies in fibroblasts and prostate cancer cells identified roles for MP1 in focal adhesion turnover, and in cell attachment, spreading and migration. A role in cell survival has not been previously reported, and may be specific to ER-positive breast cancer cells. Our current experiments are directed towards identifying the signaling pathways affected by MP1 in ER-positive breast cancer cells, and evaluating the potential of these pathways as targets to inhibit cell survival and/or migration.

The Small Scaffold Protein MP1 is Required for the Survival of Estrogen Receptor Positive Breast Cancer Cells.

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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 MP1's functional significance in breast cancer cells has not been investigated. In this report, we demonstrate a 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 also 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 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 (8). This effect on migration was independent of MP1's ability to activate ERK and PAK1, since the levels of phosphorylated ERK and PAK1 were unchanged upon MP1 knockdown. However, MP1 gene silencing in prostate cancer cells 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 spreading 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 (2). In *Drosophila*, the MP1/ERK complex regulates cell differentiation during development of the wing, since both down-regulation and overexpression of *dMAPKSP1* led to an ectopic wing vein phenotype (9). 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. Many of these processes play important roles in cancer biology, and we therefore investigated the role of MP1 in breast cancer cells.

Analysis of publicly available gene expression datasets indicates that MP1 mRNA 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-tumorigenic 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 cell lines examined, 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 activity. The cell death 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), a non-targeting siRNA (ON-TARGETplus siCONTROL) and an ER siRNA (ON-TARGETplus) were used. Cells were plated in six-well plates at 10^5 to 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. For MP1 siRNA and ER siRNA cotransfection cells were treated with a 30 nM mix of two duplexes. Cells were harvested after 48 h, then lysed in CelLytic M lysis buffer (Sigma), supplemented with cocktail tablets of protease (Roche - Complete Mini EDTA-free) and phosphatase inhibitors (Roche – PhosSTOP).

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. 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 and anti-rabbit (Invitrogen) and IRDye 800CW anti-mouse (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), 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), or Bcl-2 (BD Biosciences). Pan caspase inhibitor z-VAD-FMK was obtained from BD Biosciences 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, (10)) were transfected into 293GPG packaging cells and retroviral stocks were prepared as previously described (11). 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.

Gene expression analysis. To examine gene expression across human breast cancer, datasets 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. MP1 was examined in the resulting dataset within the various clinical parameters associated with the datasets including ER and PR status.

Statistical analysis. Data are expressed as the mean ± S.D. Experiments were performed three times unless otherwise indicated. Paired evaluations were made for experimental and control conditions within each set of experiments. 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 1). 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 the three categories of cell lines indicated significantly higher levels of MP1 protein in the ER-positive breast cancer cells than in ER-negative breast cancer or non-tumorigenic cell lines. Since the number of samples investigated here is small, we also queried publicly available databases for MP1 mRNA expression. In agreement with our protein results, MP1 was widely expressed, but showed a statistically significant elevation in both ER and PR positive breast cancer samples ($p<0.0001$ by t-test for both). One clinical study

identified MP1 as a gene associated with a poor prognosis signature in sporadic lymph-node negative breast cancer patients (12), suggesting a putative role in the context of breast tumors. However, our analysis did not reveal a correlation between high MP1 expression and either 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 2A), and by 72 h virtually all cells had detached from the plates (not shown). As shown in Figure 2B, 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 (13), and T47D is an independently derived ER-positive cell line. MP1 siRNA #1 was used in these experiments. As shown in Figure 2C, LCC9 and T47D cells exhibited a similar phenotype to MCF-7. To quantitate the effect of MP1 knockdown, attached and detached cells were collected at 48 h following siRNA transfection, stained with trypan blue, and counted. As shown in Figure 2E, MCF-7 cells were the most sensitive to MP1 knockdown. More than 70% of cells had detached by 48 h, and the majority of these were dead as determined by trypan blue staining. 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 (Figure 2D).

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 1), 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) (14) were transfected with either control or MP1 siRNA and examined at 48 h. Although MP1 levels were decreased to the same or greater extent than that obtained in the ER-positive lines, 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 3). Thus, the requirement for MP1 expression for cell attachment and survival may be specific to ER-positive breast cancer cells.

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

To determine if the cell death observed in MCF-7 cells upon MP1 silencing was due to apoptosis, expression of the anti-apoptotic protein Bcl-2 was examined. As shown in Figure 4A, 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 4B). 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 4C and 4D, this treatment prevented both PARP cleavage and cell rounding/detachment in MCF-7 cells.

MP1 knockdown reduces ER protein 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 ER and of total and phosphorylated ERK and AKT1 were examined (Figure 5). AKT1 is a pro-survival protein with a well-established role in the biology of cancer. Both ER and ERK are typically associated with proliferation, but may also be involved in regulating cell survival. The level of phospho-ERK was unaffected by MP1 knockdown (Figure 5A), suggesting that a loss of ERK signaling is not responsible for the cell detachment and death observed. In contrast, both total ER and phosphor-AKT levels decreased within 48 h of MP1 knockdown (Figure 5B and 5D). To determine if decreased ER levels were responsible for the apoptosis observed, the effects of silencing the ER gene alone or in combination with the MP1 gene were examined. Silencing of ER did not result in apoptosis of MCF-7 cells, indicating that decreased ER expression alone is not the cause of apoptosis in MP1 siRNA treated cells (Figure 5C). In addition silencing ER did not prevent apoptosis induced by MP1 silencing (Figure 5C), suggesting that ER expression itself is not required for the apoptotic response.

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 (Figure 5D). In contrast, MDA-MB-

231 cells showed no increase in cell death in response to MP1 knockdown. If decreased AKT activity is responsible for the cell death observed after MP1 knockdown in MCF-7 cells, 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 6A, 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 (Figure 6B), and this was the result of apoptosis as indicated by increased PARP cleavage (Figure 6C). In contrast, MDA-MB-231 cell viability was 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 (15, 16) .

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 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/Myr-Flag-AKT1). Phosphorylated-AKT1 (p-AKT) was highly expressed in a pool of MCF-7/Myr-Flag-AKT1 cells compared to a pool of cells containing the control pBabe-puro vector (Figure 7A). These two pools of cells were transfected with MP1 siRNA or control siRNA, and the effects on cell survival were

examined. As shown in Figure 7B, 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 in response to MP1 silencing was decreased in Myr-Flag-AKT1 expressing cells relative to the control cell line (Figure 7C). These experiments were repeated with clonal transfectants containing control vector or Myr-Flag-AKT1 with similar results (Supplementary Figure 1). Together, these findings indicate that expression of active AKT1 partially overcomes the requirement for MP1 expression for survival of MCF-7 cells.

DISCUSSION

The results presented here reveal a novel role for the small scaffold protein MP1 in ER-positive breast cancer cells. Although MP1 is expressed in both ER-positive and ER-negative breast cancer cells, its depletion using RNAi-mediated gene silencing led to detachment and death of several ER-positive cell lines, including one (LCC9) with acquired estrogen independence and antiestrogen resistance. In contrast, MP1 gene silencing had no detectable effect in three ER-negative breast cancer cell lines 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 or death was not reported in either case (7, 8). Therefore, MP1 expression seems to be required for survival in a subset of cell types, including ER-positive breast cancer cells. The mechanism of cell death that occurred as a result of inhibiting MP1 expression in MCF-7 cells was shown to be apoptosis, 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 activity was somewhat surprising, since MP1 was originally identified as a scaffold protein that increases ERK signaling (1), but is consistent with results obtained in prostate cancer cells (8). In contrast, inhibition of MP1 expression resulted in a greater than two fold decrease in AKT phosphorylation. 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 function in breast cancer cells, where it relays signals from upstream molecules including integrins, growth factor receptors, PI3K and mTORC1 to downstream molecules such as Bcl-2 and NF-κB (17-22). The fact that it 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.

Depletion of MP1 in MCF-7 cells also resulted in decreased levels of ERα protein and mRNA (data not shown). 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 (23). The fact that LCC9 cells, which are estrogen independent and antiestrogen resistant, die in response to MP1 gene silencing also supports a model in which loss of ER expression is not the sole cause of cell death. Several studies indicate that ER may be implicated in breast cancer cell survival via cross-talk with the PI3K/AKT pathway (24), or by regulating the activity of NF-κB (25, 26), Bcl-2 (27, 28), or IAP family members (29). We

therefore cannot rule out the possibility that decreased ER expression may contribute in some way to the apoptosis observed in MCF-7 cells.

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. 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 (15, 16).

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 motility. Since the phenotype involves cell rounding and detachment, inhibition of MP1expression may disrupt cell adhesion signals, which could then trigger cell death. Preliminary PCR array experiments indicated that inhibiting MP1 expression leads to decreased expression of molecules involved in cell adhesion in MCF-7 cells, including several integrins (data not shown). Immunoblotting analysis indicated a small but reproducible decrease in beta 1 integrin protein levels upon MP1 silencing (Supplementary Figure 2). Since integrins can initiate pro-survival signaling (30), future experiments will investigate if a loss of integrin expression plays a role in the decreased AKT activation and/or apoptosis observed as a result of MP1 knockdown.

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 both endocrine sensitive and endocrine resistant ER-positive breast tumors.

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FIGURE LEGENDS

Figure 1. 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, $*p<0.05$).

Figure 2. MP1 expression is required for attachment and survival of ER-positive breast cancer cells. Cells were transfected with 40 nM control or MP1 siRNAs as described in **Materials and Methods**. 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)** Immunoblots of extracts prepared from cells shown in panel **(A)**. Numbers represent the relative MP1/Actin ratios. **(C)** Photographs of MCF-7, LCC9 and T47D cells transfected with MP1 siRNA or control siRNA. Scale bar = 100 µm. **(D)** Immunoblots of extracts prepared from cells shown in panel **(C)**. **(E)** Attached and floating cells

were collected and analyzed as described in **Materials and Methods**. 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 3. MP1 expression is not required for attachment or survival of ER-negative mammary epithelial cells. Cells were transfected with 40 nM control or MP1 siRNAs for all cell lines except 184B5, where 150 nM siRNAs were used, as described in **Materials and Methods**. At 48 h cells were photographed, then harvested for counting and extract preparation. **(A)** Photographs of transfected MDA-MB-231, BT-549, Sk-Br-3, and 184B5 cells. Scale bar = 100 µm. **(B)** Immunoblots of transfected samples. Numbers represent the MP1/Actin ratios expressed as percentage of control samples. **(C)** Cell counting and trypan blue exclusion assays were carried out as described in the legend to Figure 2. 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.

Figure 4. 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 Bcl-2/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 5. Effect of MP1 knockdown on cellular signaling pathways. MCF-7 cells were transfected with 30 nM control or 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 ER in MCF-7 (n=3). **(C)** Double knockdown of MP1 and ER in MCF-7 cells; immunoblots of PARP, ER, MP1, and actin. **(D)** Immunoblot of total and phospho-AKT in MCF-7 cells. The average p-AKT/total AKT ratios are expressed as percentage of control samples (n=4).

Figure 6. 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 (n=3). **(C)** Immunoblot of PARP cleavage in MCF-7 and MDA-MB-231 cells treated with LY294002.

Figure 7. Constitutively active AKT1 partially rescues MCF-7 cells from apoptosis induced by MP1 gene silencing. **(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)**.

Figure 1

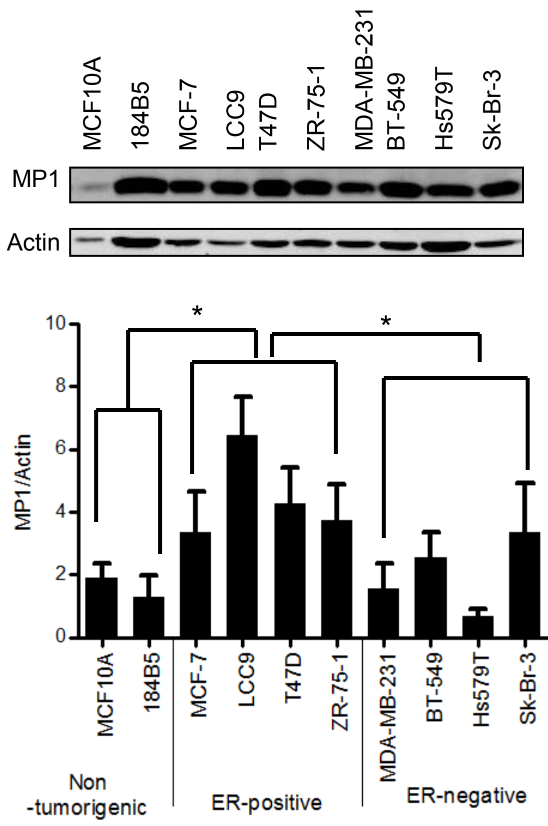
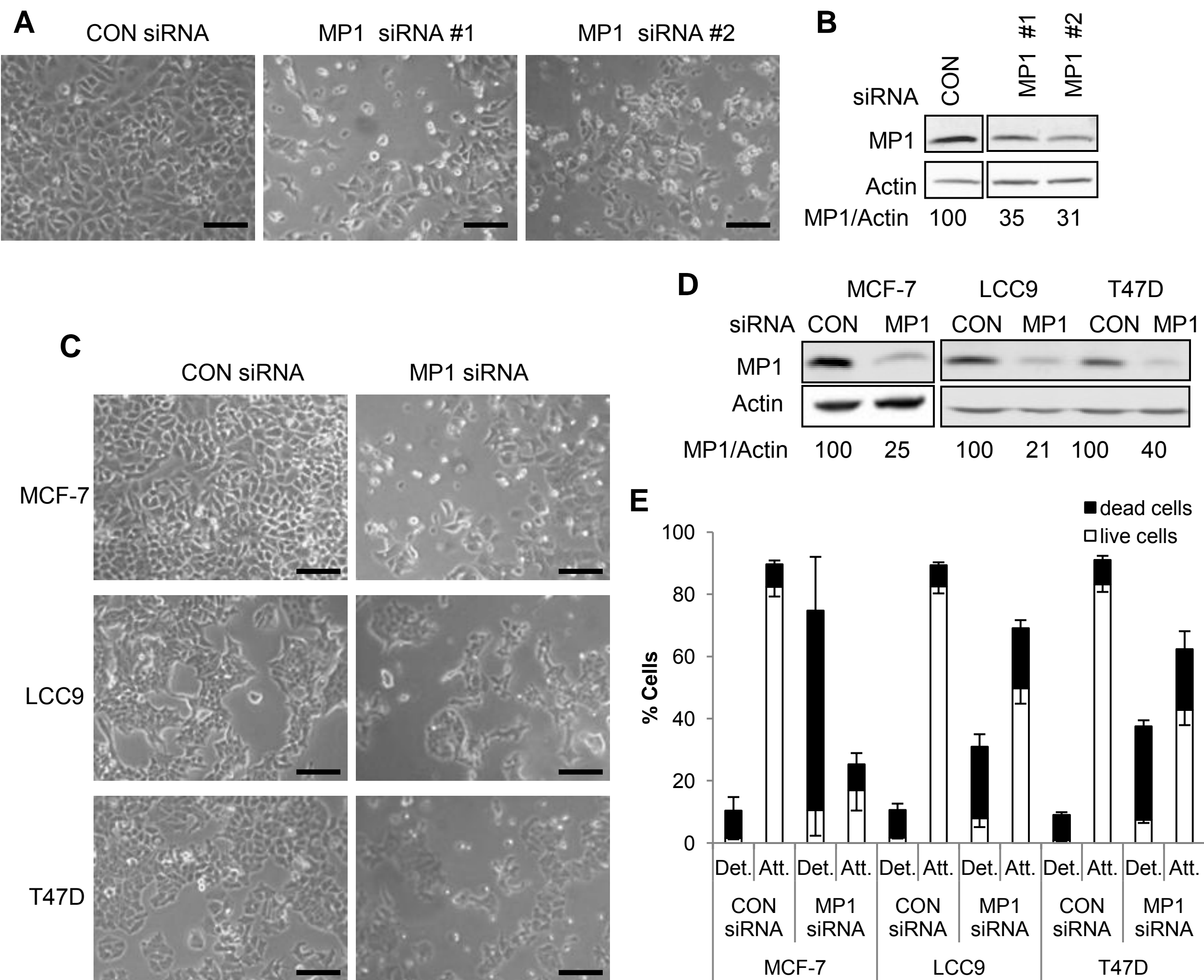


Figure 2



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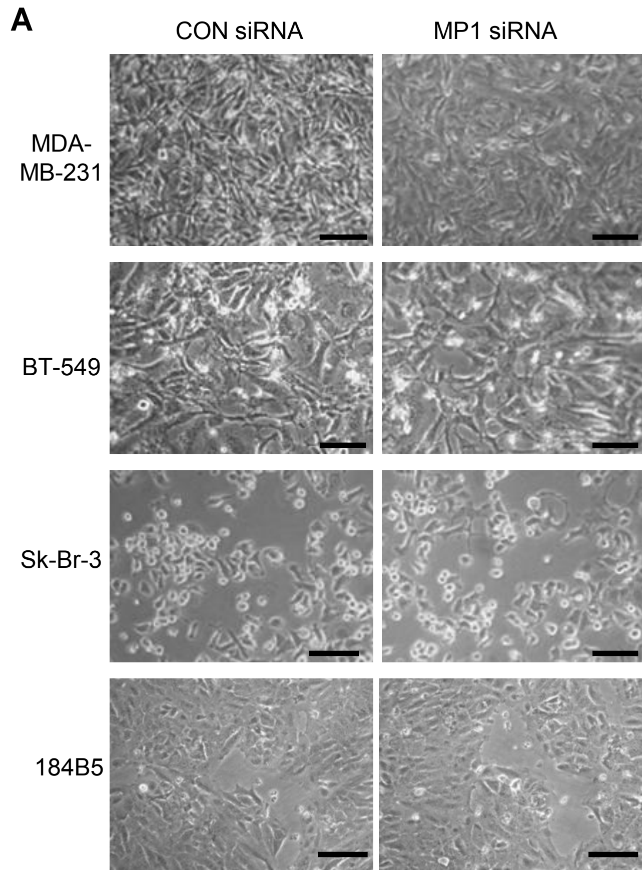

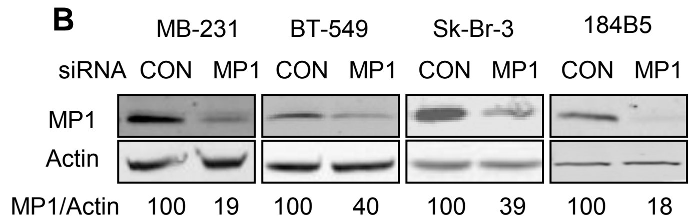

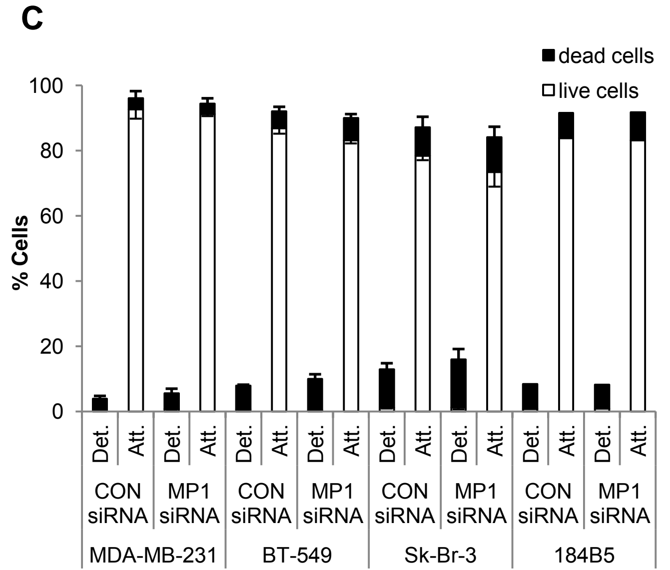

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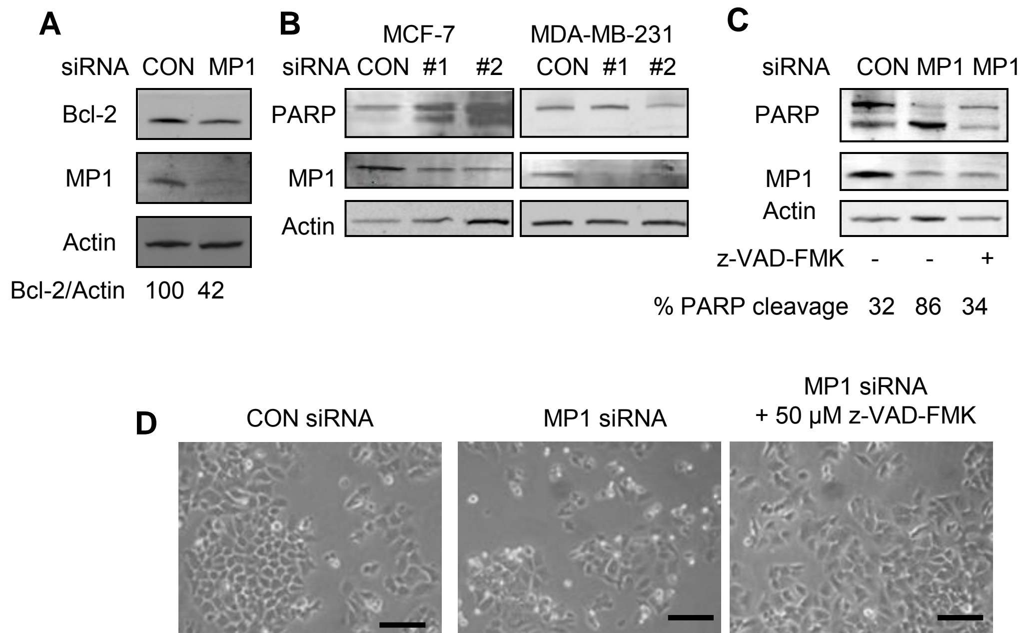


Figure 5

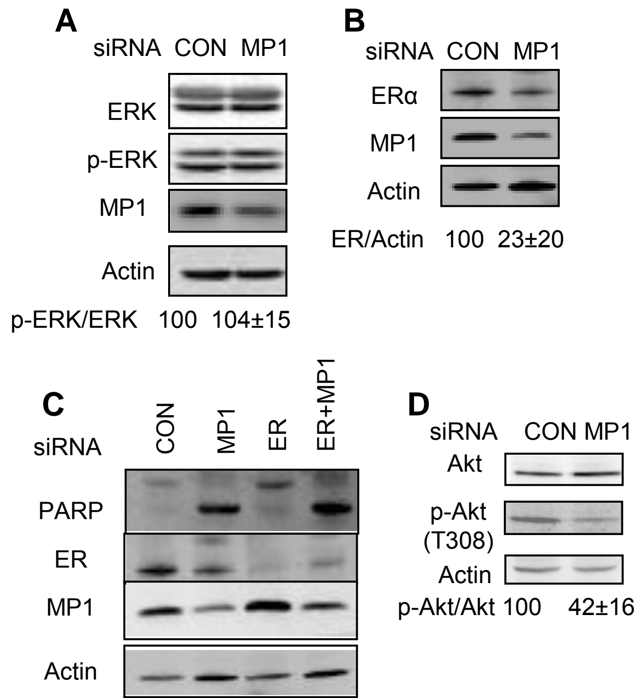
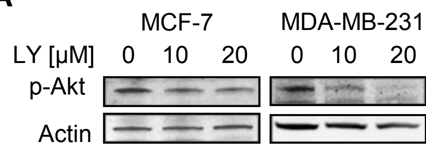
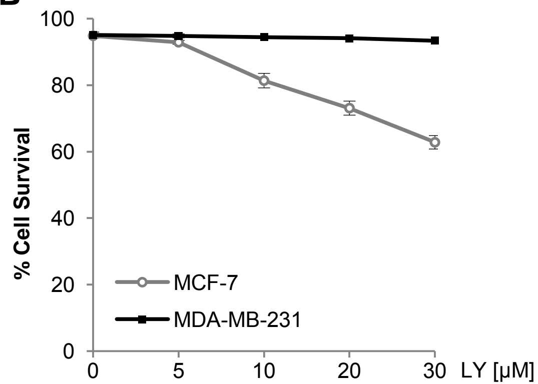


Figure 6





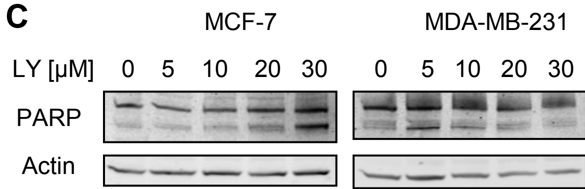


Figure 7