MEK5/ERK5 Signaling Suppresses Estrogen Receptor Expression and Promotes Hormone-Independent Tumorigenesis

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Abstract

Endocrine resistance and metastatic progression are primary causes of treatment failure in breast cancer. While mitogen activated protein kinases (MAPKs) are known to promote ligand-independent cell growth, the role of the MEK5-ERK5 pathway in the progression of clinical breast carcinoma remains poorly understood. Here, we demonstrated increased ERK5 activation in 30 of 39 (76.9%) clinical tumor samples, as well as across breast cancer cell systems. Overexpression of MEK5 in MCF-7 cells promoted both hormone-dependent and hormone-independent tumorigenesis in vitro and in vivo and conferred endocrine therapy resistance to previously sensitive breast cancer cells. Expression of MEK5 suppressed estrogen receptor (ER)α activity and ER-mediated gene transcription. Global gene expression changes associated with upregulation of MEK5 included increased activation of ER-α independent growth signaling pathways and promotion of epithelial-to-mesenchymal transition (EMT) markers. Taken together, our findings show that the MEK5-ERK5 pathway mediates progression to an ER(−), mesenchymal and endocrine therapy-resistant phenotype. Given the need for new clinical therapeutic targets, our results demonstrate the therapeutic potential of targeting the MEK5-ERK5 pathway in breast cancer.

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Introduction

Despite recent advances in endocrine therapy and the development of new agents, resistance remains a major obstacle in the treatment of breast cancer. The progression of cancer cells to a resistant phenotype is generally characterized by the acquisition of cellular or molecular changes that alter the response to therapeutic agents. Both acquired and de novo resistance occurs through enhanced cellular signaling cascades that circumvent estrogen receptor (ER)-dependent proliferation [1]. Resistance is primarily characterized by progression of ER-α(+) cancers to an ER-α(−) phenotype or the acquisition of secondary signaling networks that bypass the requirement for ER-α activity [2]. The loss of ER-α function and expression results in resistance to both primary and secondary endocrine therapeutics, creating a significant deficit of available treatment options. The development of hormone-independence and transition to a mesenchymal phenotype are hallmarks in the progression to endocrine resistance and metastasis [3,4]. A better understanding of the mechanisms involved in the progression to endocrine resistance is critical for developing new targeted breast cancer therapies.

There is mounting evidence in the literature concerning the role of mitogen activated protein kinases (MAPKs) in cancer development and response to therapeutics. Several reports have demonstrated MAPKs regulate cancer cell survival, anti-apoptotic signaling, angiogenesis, proliferation, and hormone-independence [5,6,7,8,9,10,11]. However, the majority of these studies have focused on the ERK1/2, JNK and p38 families. The MEK5-ERK5 pathway remains the least studied of the MAPK family members. A number of studies have demonstrated overexpression or activation of the MEK5-ERK5 pathway in glioblastoma, leukemia, lymphoma, medulloblastoma, and prostate cancer [12,13,14]. While some reports have suggested a role for the MEK5-ERK5 pathway in breast cancer oncogenesis this pathway’s role in breast cancer cells has not been fully explored. MEK5 has been demonstrated to be overexpressed in 50% of breast tumors and a correlation has been found between tumor overexpression of MEK5 and increased activation of STAT3, which is associated...
with proliferation and metastasis [15,18]. Additionally, ERK5 has been demonstrated to be overexpressed in 20% of patients and increased expression of ERK5 in breast tumor samples correlated with earlier relapse [16]. These data support recent findings from our laboratory reporting MEK5 overexpression in ER-α (-) breast cancer cells promotes breast cancer therapeutic resistance [17].

Both MEK5 and ERK5 are structurally and functionally distinct from other MAPKs [19,20,21]. MEK5 has a novel docking site on the N-terminus containing a different consensus motif than other MEKs [22]. Furthermore, ERK5 contains a larger C-terminus than other MAPKs, which regulates activation, auto-phosphorylation, nuclear transport and subcellular localization of the kinase [23,24]. It has been speculated that the larger C-terminus of ERK5 may allow for specific targeting by inhibitors without affecting other kinases in the pathway. ERK5 also contains a transcriptional activation domain, suggesting that the enzyme may exert direct kinase activity or induction of gene expression, unlike other ERK kinases [25]. Once in the nucleus, ERK5 can activate several transcription factors including Sap1, c-FOS, c-MYC, and MEF2 [26]. We, along with others, have also demonstrated a role for ERK5 in activation of NF-kB and AP1 mediated gene transcription [27,28]. However, to date the mechanisms of MEK-ERK5 signaling and its effects on global gene transcription are not wholly understood.

The purpose of this study is to elucidate the role of MEK-ERK5 signaling in the progression of breast cancer. Recent studies have demonstrated a correlation between ER-α expression, ERK1/2 signaling, and hormone independence [8,9,10,11]. Yet, the role of MEK5 signaling in the regulation of ER-α expression and progression to hormone independence is unclear. Defining the mechanisms of MEK5-ERK5 signaling in the regulation of ER-α expression and EMT will significantly impact our understanding of tumor progression and clinical drug resistance. A better understanding of MEK5-ERK5 and subsequent downstream signaling may lead to new therapeutic targets in the treatment of endocrine resistant breast cancer.

Materials and Methods

Ethics Statement

All procedures involving animals were conducted in compliance with State and Federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by the Tulane University Animal Care and Use Committee. The facilities and laboratory animal program of Tulane University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Tulane institutional review board approved the use of animals and human tissues in this study. The Tulane institutional review board approved the use of human tissue samples and written informed consent for the original human work that produced the tissue samples.

Patient Samples Staining and Analysis

Breast slides (T-BO-1) were obtained from the tissue array research program (TARP) (National Cancer Institute and National Human Genome Research Institute). Thirty-nine infiltrating breast carcinomas were represented in the array. Immuno-staining for phospho-ERK5 was performed in house and the results were examined using an immunohistochemical (IHC) histologic score (H-score) incorporating intensity and distribution of staining. The H-score is described by: HS 1/4 (p+t)/100, where p denotes the percentage of stained cells and t denotes the intensity of the staining [29]. The H-score scale was 0–3. Staining scale: 0, none; 1, weak; 2, moderate; and 3, strong. Scoring was performed blinded by trained pathologists [30]. A tumor was scored as 0 if there were no appreciable staining in tumor cells compared with stromal elements, as 1 if there were barely detectable staining in cytoplasm and/or nucleus compared with stromal elements, as 2 if there were readily appreciable brown staining distinctly marking tumor cell cytoplasm and/or nucleus, and as 3 if there was dark brown staining in tumor cells completely obscuring cytoplasm and/or nucleus.

Immunohistochemistry of Xenograft Tissue

Immunohistochem was performed as previously described [31,32]. Briefly, slides from tumors were deparaffinized and rehydrated. For antigen retrieval sections were heated for 25 minutes at 95°C in the presence of Rodent decloaker. Samples were then allowed to cool for 20 minutes at room temperature. Slides were then blocked in rodent block for 30 minutes and then with primary PgR and ER-α antibodies for one hour. Mouse-on-mouse HRP-polymer secondary antibody was added to the sections for 15 minutes at room temperature. Subsequently, slides were incubated in DAB for one minute and then counterstained for 30 seconds. A Leica DM IRB Inverted Research microscope and SPOT RT color camera were used to view slides; original magnification at 400×. For staining quantification numbers of positively stained cells were represented as percentage total number of cells per field of view.

Cells and Reagents

MCF-7N cell variant [subclone of MCF-7 human adenocarcinoma line from American Type Culture Collection (ATCC)] was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN) in 1996 [33]. The MCF-7-MEK5 cells (MCF-7 cells stably overexpressing MEK5) were generated as previously described [34]. The breast cancer cell lines MDA-MB-231, MDA-MB-361 (ER-α (-)), ZR75, T47D, and SKBR3 were acquired from ATCC in 2004. Liquid nitrogen stocks were made upon receipt and maintained until the start of each study. Estrogen response element–luciferase and/or qPCR for ER and progesterone receptor (PgR) were used to confirm cell lines sustained estrogen responsiveness. Morphology and doubling times were also recorded regularly to ensure maintenance of phenotype. Cells were used for no more than 6 months after being thawed. Cells were cultured as previously described [35]. ICI 182,780 was purchased from Tocris Bioscience (Ellisville, MO). Dimethylsulfoxide (DMSO) and 17β-estradiol (E2, estrogen) were purchased from Fisher Scientific (Waltham, MA). 4-Hydroxytamoxifen (tamoxifen, OHT) was purchased from Sigma-Aldrich (St. Louis, MO). Dosing for these reagents was E2 (1 nM), tamoxifen (100 nM) and ICI 182,780 (100 nM) unless otherwise indicated.

Western Blot Analysis

Western blot analyses were conducted as published [35]. Cells were maintained in 10% FBS DMEM for 24 hours prior to harvesting for protein extraction. Membranes were probed with primary antibodies according to manufacturer’s protocol. Antibodies: ER-β, AP1, and β-actin were purchased from Cell Signaling (Danvers, MA) (dilution 1:1000) and total ER-α, ERK5, and GAPDH were purchased from Santa Cruz Biotechnology (Dallas, Texas) (dilution 1:250). IR-tagged secondary antibodies were purchased from LiCor Biosciences (Lincoln, Nebraska). Blots were analyzed by the Odyssey Infrared Imaging System (LiCor Biosciences). Experiments were conducted in triplicate with representative blots shown.
Animal Studies

Xenograft tumor studies were conducted as previously described [32,35]. Immune-compromised female ovariectomized mice (29–32 days old) were obtained from Charles River Laboratories (Wilmington, MA). The animals were allowed a period of adaptation in a sterile and pathogen-free environment with food and water ad libitum. When stated, placebo or E2 pellets (0.76 mg, 60-day release; Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the lateral area of the neck in the middle point between the ear and shoulder using a precision trochar (10 gauge). MCF-7-vector, MCF-7-MEK5, MCF-7-MEK5-(empty shRNA), MCF-7-MEK5-(ERK5 shRNA) cells were harvested and viable cells mixed with Matrigel Reduced Factors (BD Biosciences, San Jose, CA). Injections (5×10^6 cells/injection) were made bilaterally into the mammary fat pad. All the procedures in animals were carried out under anesthesia using a mix of isoflurane and oxygen delivered by mask. Tumor size was measured every 2 days using a digital caliper. The volume of the tumor was calculated using the following formula: \( V = \frac{4}{3}\pi LM^2 \), where \( L \) is the larger radius and \( M \) is the smaller radius. At necropsy animals were euthanized by cervical dislocation after CO₂ exposure. Tumors were removed and either frozen in liquid nitrogen or fixed in 10% formalin for further analysis.

Microarray Data Analysis and Validation

MCF-7-vector and MCF-7-MEK5 cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) (10% DMEM) for 24 hours prior to extraction for microarray analysis. Microarray analysis was performed according to previously published protocols [36]. The hybridized Human Genome U133A 2.0 Array was scanned and analyzed using the Affymetrix Microarray Analysis Suite version 5.0. The average density of hybridization signals from four independent samples was used for data analysis and genes with signal density <300 pixels were omitted from the data analysis. P-values were calculated with two-sided t-tests with unequal variance assumptions, and a p-value of <0.001 was considered to be significant. The fold-change was described as a positive value when the expression level was increased and a negative value when the expression level was reduced. False discovery rate (FDR) was set at 0.1 in the data analysis. To confirm the gene expression data from microarray analysis, quantitative PCR was used to examine the mRNA levels of a subset of genes. The quantitative PCR results showed a high degree of correlation to the microarray data.

RNA Isolation and Quantitative Real-Time PCR

RNA Isolation and qRT-PCR was performed as previously described [36]. Briefly, RNA was isolated from cultured cells using RNeasy kit as per manufacture’s protocol (Qiagen, Germantown, MD) and evaluated spectrophotometrically by absorbance (260, 280 nm). 1 μg total RNA was reverse-transcribed (iScript kit; BioRad, Hercules, CA) as previously published [36]. Primer sequences are available in Supplementary Materials and Methods. Data analyses compare relative target expression to β-actin control and relative gene expression was analyzed using the 2^ΔΔCT method (24). Treatments and time points are specified in the figure legends. Experiments were conducted in triplicate.

Figure 1. Phosphorylated ERK5 in human breast carcinoma. (A) Thirty nine human breast carcinoma tissue samples were stained with anti-p-ERK5 (Thr218/Tyr220) and H&E, results demonstrate representative samples with histological score (H-score) 0–2. (B) Western Blot for protein expression levels of phospho- and total ERK5 and ER-α across breast cancer cell lines. (C) MCF-7 cells expressing either MEK5 (MCF7-MEK5) or empty vector were injected into the mammary fat pad of Nu/Nu mice in the presence of E2 pellets (0.76 mg, 60 day release) (n = 10/group). Points represent mean tumor volume ± SEM; * significantly different from vector p<0.05; ** significantly different from vector p<0.01. doi:10.1371/journal.pone.0069291.g001
Estrogen Response Element (ERE)-Luciferase Assay

As previously described [32], the cells were seeded in 24-well plates at a density of 50,000 cells per well in 5% charcoal/dextran treated FBS DMEM and allowed to attach overnight. After 18 h, cells were transfected with 300 ng pGL2-ERE2X-TK-luciferase plasmid, using 6 μl Effectene (Qiagen) per microgram of DNA. After 5 hours, cells were treated with vehicle or E2 and incubated at 37°C. After 18 hours, the medium was removed, and 100 μl lysis buffer was added per well and then incubated for 15 min at room temperature. Luciferase activity for the cell extracts was determined using luciferase substrate (Promega Corp., Madison, WI) in an Autoluminat Plus luminometer (Berthold Technologies, Bad Wildbad, Germany).

Cell Viability Assay

Viability assays were performed as previously described [32]. Briefly, cells were plated at a density of 7,500 cells per well in a 96-well plate in phenol-free DMEM supplemented with 5% charcoal/dextran treated FBS (5% CS-DMEM) and allowed to attach overnight. Cells were then treated with ICI 182,780 or tamoxifen for 24 hours. After treatment, 20 μl 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) reagent was incubated in each well for 4 hours. Cells were lysed with 20% sodium dodecyl sulfate (SDS) in 50% dimethylformamide. The pH and absorbance values were read on an EL×808 Microtek plate reader (Bio-Tek Instruments, Winooski, VT) at 550 nm, with a reference wave-length of 630 nm.

Clonogenic Survival Assay

Colony assays were performed as described in previously published methods [37]. Cells were serum starved for 24 hours prior to being plated in 6-well plates at a density of 1,000 cells per well in 5% CS-DMEM. 24 hours later cells were treated with indicated concentrations of vehicle, ICI 182,780, or tamoxifen and then monitored for colony growth. Ten days later the cells were fixed with 3% glutaraldehyde for 15 minutes. Following fixation, the plates were washed and stained with a 0.4% solution of crystal violet in 20% methanol for 30 minutes, washed with PBS, and dried. Colonies of ≥50 cells were counted as positive. Results were normalized to DMSO (vehicle) treated cells.

RNA Interference

shRNA transfections were performed using Fugene6 as previously described [38]. ERK3-specific shRNA (SureSilencing shRNA) and control shRNA vector plasmids have been previously described [34]. MCF-7-MEK5 cells were grown in a 100 mm dish. Following transfection cells were treated with 300 ng/ml puromycin. Cells were grown in 10% FBS DMEM and treated with 300 ng/ul puromycin every two days for 2 weeks. Colonies were pooled and verification of ERK5 suppression was confirmed using RT-PCR and western blot. Stable pools were maintained in 10% FBS DMEM as described above.

Reverse transcriptase PCR

RT-PCR was performed as previously described [36]. RNA was isolated from cultured cells using RNeasy (Qiagen) and evaluated spectrophotometrically by absorbance (260, 280 nm). Two micrograms of cDNA was transcribed with SuperScript III (Invitrogen, Grand Island, NY) and mRNA was amplified. Primers were used at 20 nmol/L final concentration. Primer sequences are available upon request.

Statistical Analysis

Studies involving more than 2 groups were analyzed by 1-way ANOVA with Tukey’s post-test; all others were subjected to unpaired Student’s t-test (GraphPad Prism V.4) as previously described [36,39,40]. For pathway analysis, data processing and statistics were carried out as we have described [41]. Using Bioconductor, present (P), absent (A) or marginal (M) calls were determined using an MAS5 algorithm. Fraction presence, defined as the average present/absent (P/A) detection call (scores were given as P = 1, M = 0.5 and A = 0) for the groups, was calculated for each microarray probe, and probes with at least one group having a fraction presence of 0.5 were selected. Welch’s t-test was performed for each probe using their log-transformed signals, with
Table 1. Cancer Signaling Pathways Associated with MEKS.

| Pathway Name                  | Impact Factor | Number of Pathway Genes | Genes Altered | Percent Pathway Genes Altered | p-value  |
|-------------------------------|---------------|-------------------------|---------------|-------------------------------|----------|
| Leukocyte Transendothelial Migration | 287.70        | 119                     | 24            | 20.17                         | 2.52E-02 |
| Cell Adhesion Molecules       | 185.12        | 134                     | 32            | 23.88                         | 6.96E-04 |
| Adherens Junction             | 25.78         | 78                      | 23            | 29.49                         | 1.56E-04 |
| Axon Guidance                 | 23.26         | 129                     | 43            | 33.33                         | 5.25E-09 |
| Pathways in Cancer            | 19.12         | 330                     | 81            | 24.55                         | 2.94E-08 |
| Focal Adhesion                | 14.29         | 203                     | 51            | 25.12                         | 4.96E-06 |
| MAPK Signaling                | 13.91         | 272                     | 63            | 23.16                         | 7.53E-06 |
| ECM-Receptor Interaction      | 12.58         | 84                      | 26            | 30.95                         | 2.37E-05 |
| Tight Junction                | 12.56         | 135                     | 35            | 25.93                         | 7.19E-05 |
| Regulation of Actin Cytoskeleton | 12.33       | 217                     | 52            | 23.96                         | 1.69E-05 |
| Small Cell Lung Cancer        | 12.24         | 86                      | 26            | 30.23                         | 3.74E-05 |
| ErbB Signaling                | 10.54         | 87                      | 19            | 21.84                         | 2.02E-02 |
| Melanoma                      | 10.39         | 71                      | 19            | 26.76                         | 2.03E-03 |
| p53 Signaling                 | 10.16         | 69                      | 21            | 30.44                         | 1.82E-04 |
| Pancreatic Cancer             | 9.66          | 72                      | 20            | 27.78                         | 9.50E-04 |
| Non-Small Cell Lung Cancer    | 8.89          | 54                      | 16            | 29.63                         | 1.42E-03 |
| Glioma                        | 8.83          | 65                      | 18            | 27.69                         | 1.74E-03 |
| Apoptosis                     | 8.79          | 89                      | 23            | 25.84                         | 1.22E-03 |
| Phosphatidylinositol Signaling | 7.98          | 76                      | 20            | 26.32                         | 1.95E-03 |
| Colorectal Cancer             | 7.83          | 84                      | 20            | 23.81                         | 6.71E-03 |
| Fc epsilon III Pathway        | 7.57          | 78                      | 20            | 25.64                         | 2.73E-03 |
| Gap Junctions                 | 7.56          | 96                      | 21            | 21.88                         | 1.49E-02 |
| Prostate Cancer               | 7.52          | 90                      | 21            | 23.33                         | 7.10E-03 |
| Chronic Myeloid Leukemia      | 7.12          | 75                      | 18            | 24.00                         | 9.00E-03 |
| Bladder Cancer                | 6.96          | 42                      | 11            | 26.19                         | 1.97E-02 |
| Acute Myeloid Leukemia        | 6.90          | 59                      | 14            | 23.73                         | 2.18E-02 |
| Cisplatin Rhythm              | 6.76          | 13                      | 5             | 38.46                         | 2.18E-02 |
| B cell Receptor Signaling     | 5.98          | 65                      | 14            | 21.54                         | 4.67E-02 |
| VEGF Signaling                | 5.77          | 74                      | 16            | 21.62                         | 3.40E-02 |
| Adipocytokine Signaling       | 5.63          | 67                      | 15            | 22.39                         | 2.96E-02 |
| PPAR Signaling                | 5.42          | 70                      | 17            | 24.29                         | 9.70E-03 |
| Cell Cycle                    | 5.39          | 118                     | 25            | 21.19                         | 1.40E-02 |
| ABC Transporters              | 5.12          | 44                      | 11            | 25.00                         | 2.75E-02 |
| Hedgehog Signaling            | 4.22          | 57                      | 13            | 22.81                         | 3.57E-02 |

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p-values less than 0.01 considered significant. To further support the statistical significance of probes having p<0.01, the FDR was also calculated with probe significance defined as an FDR of less than 5%. A moderately stringent fold-change cutoff of ≥2.0 (or ≤−2.0 for down-regulation), which allows for an acceptable balance between false discovery and false-negative rates was applied (in addition to the p-value cutoffs of p<0.001) to determine genes with significant expression alterations.

Results

ERK5 Activation in Clinical Breast Carcinoma and Breast Cancer Cell Lines

While total MEK5 and ERK5 protein expressions in patients has been reported at 50% and 20% respectively, the prevalence of phospho-ERK5 in clinical breast cancer has not yet been investigated by immuno-histochemistry. Therefore, we first determined the relevance of ERK5 activation in clinical breast tumors. To obtain clinical correlation, tissue arrays of 39 unidentified patient samples were analyzed for phospho-state specific antibodies to the Thr218/Tyr220 activation site of ERK5. The array slides were then scored by a pathologist by assessing the intensity of tumor cell staining relative to stromal elements in the same spot. Results demonstrate expression of phospho-ERK5 in 30 of 39 (76.9%) of infiltrating breast cancer biopsy samples (Fig. 1A). Similar ERK5 activation profiles were also found in invasive ovarian cancer tissue arrays using the same scoring system (Fig. S1). These results suggest that ERK5 pathway is a clinically relevant cancer signaling pathway.
Table 2. Pathway Analysis of MEK5 Mediated Gene Expression Changes.

| Gene Symbol | Genbank | Basal Expression (vs MCF-7) | Description |
|-------------|---------|-----------------------------|-------------|
| **Cell Adhesion Molecules** | | | |
| CDH3 | NM_001793.4 | −6.16 | cadherin 3, type 1, P-cadherin |
| CLDN3 | NM_001306.3 | −6.27 | claudin 3 |
| CLDN4 | NM_001305.3 | −9.78 | claudin 4 |
| CLDN7 | NM_001185022.1 | −21.86 | claudin 7 |
| CNTN1 | NM_001256063.1 | 25.80 | contactin 1 |
| HLA-DPA1 | NM_001245225.1 | 20.11 | major histocompatibility complex, class II, DP alpha 1 |
| ITGA4 | NM_000885.4 | 22.64 | integrin, alpha 4 |
| ITGA8 | NM_003638.1 | 15.13 | ITGA8 integrin, alpha 8 |
| JAM3 | NM_001205329.1 | 15.21 | JAM3 junctional adhesion molecule 3 |
| NCAM1 | NM_000615.6 | 25.80 | neural cell adhesion molecule 1 |
| NCAM2 | NM_004540.3 | −5.79 | neural cell adhesion molecule 2 |
| NLGN1 | NM_014932.2 | 15.25 | neuroligin 1 |
| PTPRM | NM_001105244.1 | 9.04 | protein tyrosine phosphatase, receptor type, M |
| PVRL3 | NM_001243286.1 | 18.30 | poliovirus receptor-related protein 3 |
| SDC4 | NM_002999.3 | 5.61 | syndecan 4 |
| VCAN | NM_001126336.2 | 54.86 | ersican |
| **Cell Cycle** | | | |
| CCND1 | NM_053056.2 | −7.60 | cyclin D1 |
| CDC7 | NM_001134419.1 | 3.30 | cell division cycle 7 homolog |
| SFN | NM_006142.3 | −10.89 | Stat5b |
| SKP2 | NM_001243120.1 | 5.16 | S-phase kinase-associated protein 2 (p45) |
| TGFB2 | NM_001135599.2 | −4.55 | transforming growth factor, beta 2 |
| **Epithelial-to-Mesenchymal Transition** | | | |
| CDH1 | NM_004360.3 | −46.38 | cadherin 1, type 1, E-cadherin (epithelial) |
| CDH2 | NM_001792.3 | 48.25 | cadherin 2, type 1, N-cadherin (neuronal) |
| LEF-1 | NM_001130713.2 | 8.68 | lymphoid enhancer-binding factor 1 |
| SDC1 | NM_0006946.1 | −3.60 | syndecan 1 |
| SNAI2 | NM_003068.4 | 11.10 | snail homolog 2 |
| TWIST1 | NM_000474.3 | 2.77 | twist homolog 1 |
| VIM | NM_003380.3 | 33.99 | vimentin |
| ZEB1 | NM_001128128.2 | 12.29 | zinc finger E-box binding homeobox |
| ZEB2 | NM_001171653.1 | 8.74 | zinc finger E-box binding homeobox 2 |
| **ErbB Signaling** | | | |
| AREG | NM_001657.2 | −134.28 | amphiregulin |
| BTC | NM_001729.2 | −3.65 | betacellulin |
| CAMK2D | NM_001221.3 | 3.47 | calcium/calmodulin-dependent protein kinase II delta |
| ERBB3 | NM_000505915.1 | −5.17 | v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 |
| ERBB4 | NM_001042599.1 | 3.04 | ERBB4 v-erb-a erythroblastic leukemia viral oncogene homolog 4 |
| PAK1 | NM_00128620.1 | 5.16 | p21 protein (Cdc42/Rac)-activated kinase 1 |
| RPS6KB1 | NM_003161.2 | −3.75 | ribosomal protein S6 kinase |
| SHC4 | NM_0012349.3 | −3.80 | SHC (Src homology 2 domain containing) family, member 4 |
| TGFA | NM_001099691.2 | −4.91 | transforming growth factor, alpha |
| **Estrogen Receptor** | | | |
| TFAP2C | NM_003222.3 | −13.56 | transcription factor AP-2 gamma |
| BCAS1 | NM_003657.2 | −12.13 | breast carcinoma amplified sequence 1 |
| CXCL12 | NM_000609.5 | −5.37 | chemokine (C-X-C motif) ligand 12 |
| ESR1 | NM_000125.3 | −31.25 | estrogen receptor isoform 1 |
| GREB1 | NM_014668.3 | −24.88 | growth regulation by estrogen in breast cancer 1 |
We next determined if well established breast cancer cell lines also exhibited activation of ERK5. Phosphorylated ERK5 levels at the Thr218/Tyr220 activation site were examined by Western blot analysis in human breast cancer cell lines with varying degrees of ERα expression. The MCF-7 cells, T47D and ZR-75 cells are models of ERα(+) breast carcinoma whereas SKBR3 and MDA-MB-231 cells are well-established ERα(−) breast cancer models. Variants of MDA-MB-361 exist, and here we use MDA-MB-361 cells that are ERα(+) [42]. Analysis revealed increased phospho-ERK5 protein levels in the ERα(−) SKBR3, MDA-MB-231 and MDA-MB-361 cell lines (Fig. 1B). These results were in stark contrast to the ERα(+) cell lines MCF-7, T47D and ZR-75, which displayed markedly low phospho-ERK5 levels, demonstrating an inverse correlation between phospho-ERK5 protein expression and ERα levels.

Our laboratory recently generated a stable constitutively active MEK5 overexpressing cell line (MCF-7-MEK5) from parental ERα(+) MCF-7 cells [34]. We utilized this cell model to examine the effect of MEK5 signaling on breast cancer tumor growth. Using a NOD/SCID murine model of in vivo tumorigenesis, tumor formation of MCF-7-MEK5 overexpressed xenografts was compared to MCF-7-vector cells in the presence of estrogen. Results demonstrate earlier tumor initiation in the MEK5 expressing cells (p<0.05) and greater tumor growth (p<0.05) compared to MCF-7-vector tumors (Fig. 1C).

### Table 2. Cont.

| Gene Symbol | Genbank | Basal Expression (vs MCF-7) | Description |
|-------------|---------|-----------------------------|-------------|
| NCOA3       | NM_001174087.1 | −10.07 | nuclear receptor coactivator 3 |
| PGR         | NM_0009264.6 | −11.56 | progesterone receptor |
| PRLR        | NM_000949.5 | −32.25 | prolactin receptor |
| TFF1        | NM_003225.2 | −192.11 | trefoil factor 1 |
| AKT3        | NM_001206729.1 | 86.28 | v-akt murine thymoma viral oncogene homolog 3 |
| CACNA2D1    | NM_000722.2 | 28.01 | calcium channel, voltage-dependent, alpha 2/delta subunit 1 |
| CACNA2D3    | NM_018398.2 | 9.17 | calcium channel, voltage-dependent, alpha 2/delta subunit 3 |
| CACNB2      | NM_000724.3 | 4.23 | calcium channel, voltage-dependent, beta 2 subunit |
| FGF9        | NM_002010.2 | 7.09 | fibroblast growth factor 9 (gla-activating factor) |
| MAP2K6      | NM_002758.3 | 4.73 | mitogen-activated protein kinase kinase 6 |
| PLA2G4A     | NM_024420.2 | 14.51 | phospholipase A2, group IVA (cytosolic, calcium-dependent) |
| RASGRF2     | NM_006909.2 | 7.97 | Ras protein-specific guanine nucleotide-releasing factor 2 |
| DUSP4       | NM_001394.6 | −5.56 | dual specificity phosphatase 4 |
| HSPB1       | NM_001540.3 | −6.39 | heat shock 27 kDa protein 1 |
| CACNA1D     | NM_000720.2 | −5.87 | CACNA1D calcium channel, voltage-dependent, L type, alpha 1D subunit |
| CACNG4      | NM_014405.3 | −8.68 | calcium channel, voltage-dependent, gamma subunit 4 |
| CCNG1       | NM_004060.3 | 3.08 | cyclin G1 |
| CDK6        | NM_001145306.1 | 6.74 | cyclin-dependent kinase 6 |
| CDKN2A      | NM_000077.4 | 10.62 | cyclin-dependent kinase inhibitor 2A |
| PMAIP1      | NM_021127.2 | 6.66 | phorbol-12-myristate-13-acetate-induced protein 1 |
| PPM1D       | NM_003620.3 | −3.20 | protein phosphatase, Mg2+/Mn2+ dependent, 1D |
| SESN1       | NM_00199933.1 | 4.08 | sestrin 1 |
| SESN3       | NM_144665.2 | 7.29 | sestrin 3 |
| THBS1       | NM_003246.2 | −45.05 | thrombospondin 1 |
| TFAP2C      | NM_003222.3 | −13.5 | transcription factor AP-2 gamma |
| FOS         | NM_005252.3 | −12.63 | FBJ murine osteosarcoma viral oncogene homolog |
| FOSB        | NM_001114717.1 | −1.71 | FBJ murine osteosarcoma viral oncogene homolog B |
| TFAP2A      | NM_001032280.2 | 1.87 | transcription factor AP-2 alpha |
| FOSL2       | NM_005253.3 | 2.66 | FOS-like antigen 2 |
| JUN         | NM_002228.3 | 2.66 | jun proto-oncogene |
| JUNB        | NM_002229.2 | 8.84 | jun B proto-oncogene |
| MEF2C       | NM_001131005.2 | 21.70 | myocyte enhancer factor 2C |

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We next sought to investigate the mechanism of the observed MEK5 mediated tumorigenesis. Global gene expression profiling was performed on MCF-7-MEK5 cells and compared to MCF-7-vector cells (Fig. 2). The above analysis identified 3404 significantly altered genes: 1883 up-regulated and 1521 down-regulated transcripts. Although the altered genetic profile in MEK5 overexpression was diverse, it could be organized into functional signaling categories using the Kyoto Encyclopedia of Genes and Genomes database and Gene Ontology algorithms. Analysis revealed several pathways significantly altered in the MCF-7-MEK5 overexpressing cells. Interestingly, some of these pathways are known to regulate cancer signaling in other tumor types, including leukemia, lymphoma, melanoma, and prostate (Table 1). These results suggest that the MEK5 pathway is a relevant signaling pathway in various cancers in addition to breast.

The microarray findings were then analyzed to determine the effect of MEK5 activity on breast cancer signaling pathways...
MEK5 Expression Alters Estrogen Receptor Signaling and Promotes Endocrine Therapy Resistance

EMT has been associated with the loss of ER-α expression [43,44]. Given the enhanced EMT-associated changes found in our array data and our findings of an inverse correlation between ERK5 activation and ER-α expression (Fig. 1B), we further investigated the ER-α signaling changes associated with MEK5 overexpression. To investigate ER-α genomic activity, clustering analysis was performed on 89 known ER-α mediated genes from our microarray data. Results of this analysis were similar to clustering using the whole mRNA profiles (Fig. S3). MCF-7-MEK5 expressing cells displayed a marked decrease in ER-α gene expression, as well as downstream ER-α regulated gene expression, compared to MCF-7-vector cells (Table S4). This was accompanied by the loss of the critical ER-α cofactors NCOA3 and GATA3 and suppression of classical estrogen responsive genes, including PgR, SDF-1/CXCL12, GREB1, and prolactin receptor (PRLR) (Table 2 and Table S4).

The above findings of decreased ER-α expression were confirmed using qRT-PCR analysis of select genes in the ER signaling pathway. We found significantly decreased ER-α mRNA expression in MEK5 cells compared to vector cells (Fig. 3A). Down-regulation of ER-α in these cells was confirmed using analysis of estrogen response element (ERE) luciferase. MCF-7-MEK5 cells exhibited a diminished estrogen induced ERE transcriptional activity compared to vector cells both at the basal level and following stimulation with E2 (Fig. 3B). To further validate these findings, we determined whether the differential ER-α signaling in MEK5 cells translated to changes in estrogen-induced gene expression. Consistent with the loss of ER-α expression, the MCF-7-MEK5 cells displayed a loss of estrogen-stimulated mRNA expression of PgR, SDF-1, c-Myc, and Cathepsin-D compared to MCF-7-vector cells (Fig. 3C).

The loss of ER-α expression in MCF-7-MEK5 expressing cells parallels observations in clinical breast carcinoma progression to therapeutic resistance [2]. Given the above inverse correlation of phospho-ERK5 activation and decreased ER-α expression in breast cancer cell lines (Fig. 1B) we set out to determine if MEK5-ERK5 signaling could play a role in the progression to endocrine therapy resistance. MCF-7-MEK5 and MCF-7-vector cells were treated with the clinical endocrine therapeutics fulvestrant (ICI 182,780) and tamoxifen and analyzed for cell viability. As seen in Fig. 3D, MTT analysis demonstrates MEK5 overexpression led to an endocrine therapy resistant phenotype, with MCF-7-MEK5 cells exhibiting increased resistance to both ICI 182,780 and tamoxifen compared to MCF-7-vector. MEK5-induced endocrine resistance was confirmed using long-term colony formation assays. Expression of MEK5 increased clonogenic survival following treatment with endocrine therapy (Fig. 3E). Taken together, these results confirmed our microarray findings that MEK5 expression was associated with loss of ER-α activity and suggest progression towards endocrine resistance.
MEK5-ERK5 Signaling Promotes Progression to an ER-Negative and Hormone Independent Phenotype In Vivo

The above findings of decreased ER-α signaling (Fig. 3) and increased ER-α independent growth signaling (Table S2) were indicative of a hormone independent phenotype in the MCF-7-MEK5 cell line. Therefore, we next sought to confirm this finding of MCF-7-MEK5 hormone-independence in xenograft animal models. MCF-7-vector and MCF-7-MEK5 cells were implanted into the mammary fat pad of Nu/Nu mice in the absence of estrogen and monitored for tumor formation. MEK5 overexpressing cells were capable of tumor formation while MCF-7-vector cells were unable to form tumors without estrogen as far out as 50 days (Fig. 4A). This lack of MCF-7-vector tumor formation in the absence of estrogen is consistent with previously published studies [36]. To further validate the mechanism of MEK5 mediated hormone independent tumor-igenesis, tumors from the initial mouse study (Figure 1C) with estrogen treated animals were processed for H&E and IHC staining for ER-α and PgR expression (Fig. 4B). These data confirm that ERK5 is essential for the increased tumor growth found in our previous in vivo experiments, as the ability of MEK5 expression to enhance tumor formation in immuno-compromised mice was suppressed by RNAi-mediated ablation of ERK5 (Fig. 5C).

MEK5 Drives Hormone Independent Tumorigenesis

Knockdown of ERK5 Reverses Hormone Independence and Restores ER-α expression

To confirm a role for the MEK5-ERK5 signaling pathway in the hormone independent phenotype of MEK5 overexpressing cells, shRNA ERK5 or vector constructs were stably transfected into MCF-7-MEK5 cells. Significant downregulation of ERK5 was observed in MCF-7-MEK5-ERK5-shRNA transfected cells compared to empty-shRNA vector (Fig. 5A and 5B). In vivo experiments further validated that ERK5 was essential for the increased tumor growth found in our previous in vivo experiments, as the ability of MEK5 expression to enhance tumor formation in immuno-compromised mice was suppressed by RNAi-mediated ablation of ERK5 (Fig. 5C).

We next used shRNA-targeted to ERK5 to investigate whether suppression of ERK5 signaling drives the loss of ER-α expression. MCF-7-vector, MCF-7-MEK5-(shRNA-empty) and MCF-7-MEK5-ERK5-shRNA cells were analyzed using RT-PCR and Western Blot for expression of ER-α. These data show that in the MEK5 overexpressing cells, ER-α is downregulated at both the mRNA (Fig. 5D) and protein (Fig. 5E) levels. Conversely, blockade of ERK5 by shRNA-ERK5 restored ER-α expression. Interest-
ingly, ER-β expression and ER-α expression demonstrated an inverse relationship in MEK5 overexpressing cells and MEK5-ERK5-shRNA cells. Expression of ERK5 directly correlated with mRNA and protein expression of ER-β while shRNA knockdown of ERK5 inhibited expression of ER-β (Fig. 5D–E).

We next sought to investigate the mechanism of MEK5 mediated ER-α suppression. The above microarray findings revealed an inverse correlation between AP2 gamma (γ) and MEK5 expression, with MCF-7-MEK5 cells exhibiting markedly decreased AP2γ transcription compared to MCF-7-vector (Table 2). The transcription factor AP2γ regulates expression of ER and is known to increase expression ER-α and decrease expression of ER-β [45–50]. Therefore, we investigated whether there was an association between MEK5-ERK5 signaling pathway and AP2γ expression. As seen in Figure 5, we demonstrate an inverse correlation between ERK5 expression and the transcription factor AP2γ at both the mRNA (Fig. 5D) and protein (Fig. 5E) levels. This decrease in AP2γ expression in MCF-7-MEK5 cells is consistent with the above microarray analysis (Table 2). shRNA knockdown of ERK5 reversed the AP2γ suppression and restored ER-α expression in the MEK5 overexpressing cells (Figure 5D–E). Taken together, these results suggest that AP2γ may be involved in MEK5 mediated ER-α suppression.

To better understand the correlation between MEK5 induced repression of ER-α in the MCF-7 breast cancer cell line we next transiently overexpressed the AP2γ transcription factor in the MCF-7-MEK5 cell line and performed qRT-PCR to determine ER-α expression levels. While a significant increase in expression of AP2γ was observed in MCF-7-MEK5 cells transiently transfected with AP2γ there was no observed increase in ER-α gene expression (Figure S4A) despite an increase in AP2γ (Figure S4B). This data suggests that while MEK5 may alter AP2γ gene expression, there is no significant correlation between AP2γ and ER-α expression in this cell line.

**Discussion**

While recent evidence indicates a role for MAPK signaling in cancer oncogenesis and metastasis, the role of the MEK5-ERK5 pathway in breast cancer progression remains poorly understood. Currently, there is little data on the activation status of the MEK5-ERK5 pathway among breast cancer patients [13]. In this study we show clinical relevance of the MEK5-ERK5 signaling pathway through measurement of activated ERK5 in clinical tumor samples. We demonstrate activated ERK5 in 76.9% of breast biopsy samples as well as differences in pERK5 levels across breast cancer cell lines. Interestingly, ERK5 correlated with decreased ER-α protein expression in these breast cancer cells. Recent evidence suggests that MAPKs, namely ERK1/2, may regulate ER-α expression [8,9,10]. To our knowledge, this connection between ERK5 and ER-α expression has yet to be described in the literature.

We further elucidated the relationship between MEK5-ERK5 signaling and the ER regulated pathways. Expression of MEK5 suppressed ER-α, but not ER-β protein levels, and abrogated downstream ERE transcriptional activity and E2 induced gene transcription. ER-α expression in MCF-7-MEK5 cells could be restored using shRNA knockdown of ERK5. MEK5 mediated ER-α suppression conferred resistance to the endocrine therapies fulvestrant and tamoxifen in vitro and promoted hormone independent tumor growth in vivo. This is the first study to link MEK5-ERK5 signaling with ER expression and endocrine therapy resistance. As seen in Figure 6, we propose a model for MEK5/ERK5 signaling crosstalk with ER-α signaling to give
rise to endocrine resistance and increased EMT. Through global gene expression profiling, we demonstrated MEK5 expression not only abrogated ER-α signaling, but also enhanced alternative mitogenic and survival signaling pathways to promote tumor growth. Pathway analysis of MEK5 cells revealed increased activation of ER independent growth signaling pathways, including MAPK, ERBB and PI3K-AKT, which may contribute to the hormone independent tumor growth of these cells.

There are several potential mechanisms for MEK5-induced repression of ER-α including regulation of downstream transcription factors. We found increased expression of the transcription factor TWIST1, which is known to decrease protein levels of ER-α in order to promote endocrine therapy resistance [51]. MEK5 cells also exhibited increased NF-kB mediated gene expression (data not shown), which is associated with the loss of ER-α and increased hormone independent survival [52]. The MEK5-ERK5 pathway is less understood than other MAPK pathways and the downstream targets of MEK5-ERK5 may not be fully identified.

Pathway analysis of our microarray data further revealed enhanced EMT-associated gene changes associated with MEK5. We and others have reported increased expression of MEK5-ERK5 in mesenchymal breast cancer cells, however, the mechanism of this correlation has yet to be determined [34]. Here, we identified several markers involved in MEK-induced EMT-associated gene changes, including increased expression of the ZEB family of zinc proteins and the TWIST1, SNAI1 and SNAI2 transcription factors. ZEB1/2 and SNAIs are known to alter E-cadherin mediated cellular adhesion whereas TWIST1 increases tumor seeding resulting in distant metastases [53,54]. The EMT gene expression changes associated with MEK5 identified in this study corroborate previously published findings of MEK5 induced progression of breast cancer to a mesenchymal phenotype.

Taken together, the findings presented here provide new insight into the signaling mechanisms that regulation progression of breast cancer to hormone independence. Our data demonstrate a critical role for MEK5-ERK5 signaling in the progression to a hormone independent and EMT phenotype of breast carcinoma. Further studies are needed to better our understanding of the potential activators of MEK5-ERK5 and subsequent downstream signaling. However, our findings demonstrate the clinical relevance and therapeutic potential of targeting the MEK5-ERK5 pathway in the treatment of endocrine resistant breast cancer.

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