Differential Regulation of the EMT Axis by MEK1/2 and MEK5 in Triple-Negative Breast Cancer

AN ABSTRACT

SUBMITTED ON THE 11th DAY OF NOVEMBER 2016

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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DOCTOR OF PHILOSOPHY

Submitted by

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ABSTRACT

Triple-negative breast cancer (TNBC) presents a clinical challenge due to the aggressive nature of the disease and a lack of targeted therapies. Constitutive activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway has been linked to chemoresistance and metastatic progression through distinct mechanisms, including activation of epithelial-to-mesenchymal transition (EMT) whereby cells adopt a motile and invasive phenotype through loss of epithelial markers, namely Cadherin 1/E-Cadherin (CDH1), and acquisition of mesenchymal markers, such as vimentin (VIM) and Cadherin 2/N-Cadherin (CDH2). While MAPK/ERK1/2 kinase inhibitors (MEKi) have shown promise as antitumor agents in the preclinical setting, application has had limited success clinically. Activation of compensatory signaling, potentially contributing to the emergence of drug resistance, has shifted the therapeutic strategy to combine MEK1/2 inhibitors with agents targeting oncoproteins (RAF) or parallel growth pathways (PI3K).

Conventional MAPK family members have been well-characterized in modulation of cellular processes involved in tumor initiation and progression, yet the role of MEK5-ERK5 in cancer biology is not completely understood. Recent studies have highlighted the importance of the MEK5 pathway in metastatic progression of various cancer types, including those of the prostate, colon, bone and breast. Furthermore, elevated levels of ERK5 expression and activity observed in breast carcinomas are linked to worse prognosis in TNBC patients. The purpose of this work is to explore MEK5 regulation of the EMT axis and to evaluate a novel pan-MEK inhibitor on clinically aggressive TNBC cells.
Our results show a distinction between the MEK1/2 and MEK5 cascades in maintenance of the mesenchymal phenotype, suggesting that the MEK5 pathway may be necessary and sufficient in EMT regulation while MEK1/2 signaling further sustains the mesenchymal state of TNBC cells. Furthermore, additive effects on MET induction are evident through the inhibition of both MEK1/2 and MEK5. Taken together, these data demonstrate the need for a better understanding of the individual roles of MEK1/2 and MEK5 signaling in breast cancer and provide rationale for combined targeting of these pathways to circumvent compensatory signaling and subsequent therapeutic resistance.
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appears interchangeable, I am fortunate to have started and ended this journey with you there every step of the way, and I cannot imagine a day without you, especially on sacred catfish Fridays. From you I have learned that the success of others does not lessen the significance of mine; your celebration of my triumphs and empathy for my failures have created a lasting impression on me. Thank you for firerats, ramen breaks, angry unicorn signs, and conspired pranks on Larrold (tangentially, thanks to Lowry Curley for being a good sport). Thank you to Margarite Matossian for lending a hand and encouragement when I needed it. To Bahia Wahba, thank you for always being on my side, no matter the circumstances. I am greatly appreciative of Thomas “Tom-Tom” Yan for his exceptional diagram-generating skills as well as his creative support in the form of relevant gifs.

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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKT</td>
<td>Cellular Homolog of Murine Thymoma Virus Akt8 Oncogene</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>ca</td>
<td>Constitutively active</td>
</tr>
<tr>
<td>CDH1</td>
<td>Cadherin 1, E-cadherin</td>
</tr>
<tr>
<td>CDH2</td>
<td>Cadherin 2, N-cadherin</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>ECIS</td>
<td>Electrical Cell-Substrate Impedance Sensing</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FOS</td>
<td>FBJ Murine Osteosarcoma Viral Oncogene Homolog</td>
</tr>
<tr>
<td>FRA-1</td>
<td>FOS-Like Antigen 1</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early Growth Response 1</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-radish-peroxidase</td>
</tr>
<tr>
<td>i</td>
<td>Inhibitor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-Terminal Kinase</td>
</tr>
<tr>
<td>JUN</td>
<td>V-Jun Avian Sarcoma Virus 17 Oncogene Homolog</td>
</tr>
<tr>
<td>ko</td>
<td>Knock-out</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF2</td>
<td>Myocyte Enhancer Factor 2</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated extracellular signal-regulated kinase kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-to-epithelial transition</td>
</tr>
<tr>
<td>MFP</td>
<td>Mammary fat pad</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>OS</td>
<td>Overall Survival</td>
</tr>
<tr>
<td>Ovex</td>
<td>Ovariectomized</td>
</tr>
<tr>
<td>p</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived xenograft</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-----------</td>
<td>----------------------------------------------------------------------------</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositol (PI) 3-Kinase</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative reverse transcription real-time polymerase chain reaction</td>
</tr>
<tr>
<td>K-Ras</td>
<td>Kirsten Rat Sarcoma Viral Oncogene Homolog</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal Protein S6 Kinase, 90 kDa</td>
</tr>
<tr>
<td>RFS</td>
<td>Recurrence–free survival</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>Serpin Peptidase Inhibitor, Clade E</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small (or short) hairpin RNA</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>TWIST</td>
<td>Twist Family BHLH Transcription Factor 1</td>
</tr>
<tr>
<td>VIM</td>
<td>Vimentin</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>ZEB</td>
<td>Zinc Finger E-Box Binding Homeobox</td>
</tr>
</tbody>
</table>
INTRODUCTION

PART I: TRIPLE-NEGATIVE BREAST CANCER

1.1 Prognosis and Treatment

Breast cancer is the most frequently diagnosed cancer in women, with a mortality rate second only to lung cancer [1]. Tumors derived from the breast are heterogeneous and classified by molecular subtypes based on hormone receptor status and gene expression profile [2]. Approximately 15% of breast tumors are designated as basal-like, the majority of which are triple-negative, denoted by a lack of estrogen and progesterone receptor expression as well as non-amplification of human epidermal growth factor receptor 2 (HER2). Triple-negative breast cancer (TNBC) is not responsive to endocrine therapy, the mainstay in treatment of estrogen receptor positive (ER+) breast cancer, or other targeted agents, limiting systemic treatment options to cytotoxic chemotherapy [3]. A subset of TNBC tumors exhibit chemosensitivity, even complete pathological response, due to their high proliferation rates, but over half of TNBC patients are resistant to cytotoxic agents [4].

Studies have sought to identify relevant pathways and molecular gene signatures that can predict response to therapies [2]. Through Gene Set Enrichment Analysis, TNBC cell lines were clustered into basal A and basal B categories, comprised of basal-like (BL1 and BL2) and mesenchymal-like (M and MSL) subtypes, respectively [5]. Highly proliferative basal A cells, positive for epithelial markers and associated with breast
cancer 1 (BRCA1) mutations, are distinct from less proliferative basal B cells that display mesenchymal characteristics and enrichment of stem cell-related genes, factors linked to chemo resistance [6, 7]. Targeted agents under evaluation for TNBC demonstrate subtype-specific efficacy. Inactivation of the homologous recombination repair pathway in BRCA1-mutated cancers confers sensitivity to poly ADP-ribose polymerase (PARP) inhibitors, which target key enzymes involved in DNA single strand break repair [6]. In contrast, MSL cell lines, enriched in motility genes, are responsive to inhibition of Src/Abl kinase and mammalian target of rapamycin (mTOR), key downstream effector of the PI3K/AKT pathway [5, 6]. Though several targeted agents are being assessed in clinical trials, to date none have been approved for TNBC. Due to the aggressive nature of the disease and a lack of targeted therapies, triple-negative breast cancer is associated with high rates of distal recurrence and poor outcome compared to other breast cancer subtypes, underlining the need for new treatment options [8].

1.2 Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a transient and reversible process, whereby epithelial cells undergo morphological changes from a rounded epithelial phenotype to a spindle fibroblast-like shape through cytoskeletal rearrangement. Implicated in wound healing, tissue fibrosis, and cancer progression [9], molecular alterations induced by EMT include loss of epithelial markers, namely adherens junction protein Cadherin 1/E-Cadherin (CDH1), and acquisition of mesenchymal markers, such as vimentin (VIM) and Cadherin 2/N-Cadherin (CDH2). Through EMT activation, marked by loss of apicobasal polarity and dissolution of intercellular contacts, cells adopt a motile and invasive phenotype vital to tumor metastasis. Snail family zinc finger 1
(SNAI1), SNAI2 (or SLUG), Twist family BHLH transcription factor 1 (TWIST), and zinc finger E-Box binding homeobox (ZEB) transcription factors pleiotropically modulate EMT, coordinating the repression of epithelial genes and the induction of mesenchymal genes in tandem [10]. Overexpression of these EMT master regulators has been well-documented in invasive cancers [11]. In addition to facilitating cancer cell intravasation and dissemination, the role of EMT has been proposed in metastatic colonization at distant tissues via enrichment of stem cell traits, specifically tumor-initiating capacity [12]. EMT is also associated with chemoresistance, through observations that surviving cancer cells post cytotoxic treatment express elevated levels of mesenchymal genes, as well as resistance to targeted therapies [9]. Given the link between malignant progression and EMT, strategies targeting this process have emerged in cancer therapy.

Various approaches for targeting the EMT axis include: inhibiting EMT-inducing factors, targeting the mesenchymal phenotype, and blocking EMT reversal (or mesenchymal-to-epithelial transition, MET) to prevent formation of metastases [9]. There are limitations associated with these strategies. Due to the variety of signals that are able to induce EMT, such as epidermal growth factor (EGF), transforming growth factor β (TGF-β), and interleukin 6 (IL-6), inhibition of one stimulus would not be sufficient [9]. However, blocking the pathways shared in transducing these signals may be a viable route. Complexity also arises from targeting EMT at different stages of the metastatic cascade. Targeting the mesenchymal state at later stages of the metastatic cascade could promote cell epithelialization and enhanced colonization, while inhibiting MET could support mesenchymalization and drug resistance [9]. Therefore, anti-metastatic therapies
based on EMT-targeting require accurate determination of metastatic burden at diagnosis and development of stage-specific pharmacological agents. Mechanisms of EMT induction must also be elucidated to circumvent or delay the emergence of resistance.
PART II: MAPK SIGNALING IN CANCER

2.1 Overview of MAPK family

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that have well-established roles in cellular processes including proliferation, differentiation, and regulation of cell fate, namely survival and apoptosis [13, 14]. Conventional MAPK family members consist of extracellular signal-regulated kinases (ERKs) 1/2 and 5, p38 MAPKs (α, β, δ, and γ), and c-Jun NH2-terminal kinases/stress-activated protein kinases (JNK/SAPKs) 1/2/3. While these pathways can be activated by various extracellular stimuli, such as growth factors (GFs), cytokines, and environmental stresses such as ultraviolet radiation [15], they share a common three-tiered kinase signaling cascade whereby upstream kinases sequentially catalyze phosphorylation and activation of downstream substrates (Figure 1): signal is transduced from MAPK kinase kinase (MAP3K) to MAPK kinase (MAP2K, also referred to as MEK), which stimulates MAPK, leading to activation an array of downstream effectors, including transcription factors as well as MAPK-activated protein kinases (MKs) [16]. Phosphorylation of MAPKs occurs dually on threonine (T) and tyrosine (Y) residues in a T-X-Y motif located in the activation loop of the kinase domain. ERK1/2 and ERK5 contain a T-glutamic acid (E)-Y sequence, varying from the T-glycine (G)-Y and T-proline (P)-Y motifs found in p38 MAPKs and JNKs, respectively [16]. Phosphorylation of MAPK is reversible through the activation of phosphatases, dephosphorylating proteins serving as negative feedback in the MAPK cascade. The balance of kinase and phosphatase activity is needed to maintain cell sensitivity to stimuli and to prevent prolonged MAPK activation.
Studies have reported redundancies and crosstalk among the MAPK pathways, in part due to positive and negative feedback controls. MEK-ERK1/2 activity can be regulated by other MAPK family members: p38 MAPK stimulates protein phosphatase 2A (PP2A) to dephosphorylate MEK1/2; and sustained activation JNK can attenuate ERK1/2 signaling [17, 18]. Similarly, ERK1/2 activity suppresses the MEK5-ERK5 pathway, where activation of the latter is enhanced by selective MEK1/2 inhibition [19]. Complexity of MAPK signaling is further increased by stimuli- and MAP3K-specific responses. For example, GF-induced activation of ERK1/2 is mediated by the MAP3K c-Raf, but other MAP3Ks may activate ERK1/2 in response to cytokine stimulation [14]. Downstream of MAP2K, overlap in MAPK substrate specificities has been demonstrated in vitro, with MAP kinase-interacting kinase (MNK) 1/2 and MK 2/3 listed among common targets of ERK1/2 and p38 [20, 21]. Moreover, ERK5 can phosphorylate Ets-domain transcription factor (Sap-1a/ELK4), v-Myc avian myelocytomatosis viral oncogene homolog (c-MYC), and ribosomal protein S6 kinase (RSK), substrates previously shown to be regulated by ERK1/2 [22]. Intricacy of the MAPK signaling cascade complicates singular targeting of these pathways.

2.2 Ras and Raf

The Ras family of proteins, encoded by three genes H-Ras, K-Ras, and N-Ras, act as molecular switches to regulate pathways involved in cell proliferation and survival. Ras activation state is dependent on guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs): GEFs promote active guanosine-5’-triphosphate (GTP)-bound Ras by catalyzing the conversion of guanosine 5’-diphosphate (GDP) to GTP while GAPs prevent sustained Ras activation through acceleration of GTP to GDP.
hydrolysis [23]. K-Ras, the most frequently mutated isoform, is prevalent in approximately 30% of solid tumors, with high incidence of activating mutations found in pancreatic, colon, and lung cancers [24]. While K-Ras mutations are not common in breast cancer, its activity is increased in more aggressive breast cancer subtypes, contributing to malignancy and metastatic potential in vivo [25]. Moreover, mutationally activated K-Ras is associated with poor response to cancer therapies [26]. Pharmacological targeting of oncogenic Ras has been largely unsuccessful due to high affinity of Ras for GTP and lack of known allosteric sites. However, a recent study yielded promising results in identification of a targetable allosteric pocket of K-Ras cysteine 12 (G12C) mutant and synthesis of small molecule inhibitors that can irreversibly bind this specific mutant allele to promote Ras association with GDP [27]. Challenges in blocking activated Ras prompted investigation of downstream pathways and development of inhibitors targeting individual MAPK signaling components in cancer therapy.

The main effectors of Ras are the Raf-MEK and Phosphoinositol 3-Kinase (PI3K)-AKT networks. Ras-GTP stimulates protein kinase activity of Raf serine/threonine kinases (A-Raf, B-Raf, and C-Raf/Raf-1) through direct protein interaction. Following formation of the Ras-Raf complex, the sequence of events leading to Raf activation has not been well characterized, though it has been shown that Raf dimerization is essential to its wild-type catalytic activity. While oncogenic potential of C-Raf was discovered prior to that of B-Raf, the latter has garnered attention due to its high frequency of mutation in cancers, with 50% incidence in metastatic melanoma [28, 29]. Furthermore, B-Raf exhibits higher basal kinase activity than C-Raf and only requires one mutation,
commonly V600E, for oncogene activation. Raf inhibitors exert differential effects depending on drug concentration and cell context, particularly Raf mutational and dimerization status [28]. In wild-type B-Raf-expressing cells, where B-Raf is a dimer, treatment with Raf inhibitors induces paradoxical activation of Raf and ERK signaling, whereas in cells expressing monomeric B-Raf (V600E), Raf inhibitors potently inhibit Raf kinase activity and subsequent ERK activation. B-Raf inhibitors, such as vemurafenib and dabrafenib, are effective antitumor agents in patients with B-Raf-mutant melanoma. Even so, acquired resistance to Raf inhibitors emerges around 6 months after the start of treatment, predominantly through MAPK-reactivating mechanisms [30]. To mitigate this obstacle, clinical strategy shifted toward combining B-Raf inhibitors with MEK-targeting agents. Combined B-Raf/MEK inhibition proved to be therapeutically superior in prolonging progression-free survival (PFS) and overall survival (OS) compared to singular inhibition of B-Raf or MEK in patients with metastatic melanoma [31, 32]. Moreover, combination of dabrafenib and trametinib (MEK inhibitor) could be safely administered at full monotherapy doses (150 mg of dabrafenib twice daily and once-daily 2 mg trametinib).

2.3 MEK1/2 Pathway

Structure and function

Of the three Raf isoforms, B-Raf is the most potent MEK kinase, phosphorylating Ser218 and 222 to activate MEK1/2 [29]. ERK1 and ERK2, homologous isoforms that share 85% amino acid identity, are the only known physiological substrates of MEK1/2 [17, 33]. The ERK1/2 signaling pathway is activated mainly by mitogenic factors and
phorbol esters. Ligand binding to receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) leads to formation of active Ras, a small membrane-bound G-protein, initiating signal transduction through effector pathways, notably the Raf/MEK/ERK pathway [16]. ERK signaling induces transcription of genes involved in the regulation of cellular proliferation, survival, EMT, and invasion [17, 34, 35].

Inactive ERK1/2 is detained in the cytoplasm through associations with MEK1/2, phosphoprotein enriched in astrocytes 15 (PEA-15), or phosphatases, which serve as anchor proteins [33, 36]. PEA-15 can also mediate the nuclear export of ERK1/2 [36, 37]. Activated ERK1/2, phosphorylated at threonine and tyrosine residues, dissociates from MEK1/2 and translocates to the nucleus by simple diffusion or interaction with the nuclear pore complex, termed nucleoporins [33]. Upon stimulation, ERK1/2 can phosphorylate an array of nuclear and cytoplasmic substrates, including MNK1/2, MK2, RSK, and MSK1/2, along with transcription factors, such as c-FOS, c-JUN, c-MYC, STAT3, ETS-1, ELK-1, SP-1, and ATF-2, to enhance transcription [15, 17, 33, 38].

Deregulation of the ERK pathway is evident in approximately one-third of all cancers. ERK1/2 signaling mediates processes essential to cancer progression, promoting cell proliferation and survival through transcriptional induction of cell cycle regulators, namely cyclin D1, c-FOS, and ELK1, as well as post-translational modification, thereby inactivation, of pro-apoptotic proteins, including FOXO3a, Bim, and Bad [36, 39]. Furthermore, ERK1/2 regulates expression of proteins associated with EMT and metastatic progression (c-JUN, FRA-1, ZEB1/2, SNAI2, TWIST1, MMP-1, and CDH1) [40-43]. These studies extensively outlining the role of MAPK in oncogenesis have led to the synthesis of inhibitors for this signaling cascade.
Pharmacological Inhibitors

*Mechanism of action*

Aberrations in MAPK signaling occur at several levels, mainly around the Ras-Raf axis. Activating mutations in Ras and Raf lead to persistent ERK1/2 signaling and stimuli-independent, uncontrolled cell proliferation, providing rationale for development of MEK1/2 inhibitors (MEK1/2is). Indeed, pharmacological inhibition of MEK1/2 through PD184532 (CI-1040) or selumetinib (AZD6244) has been shown to suppress *in vitro* cancer cell proliferation and *in vivo* xenograft growth [44, 45]. The majority of MEK1/2 inhibitors are non ATP-competitive and bind at allosteric sites on MEK1/2, altering its 3-dimensional structure [46]. The catalytically inactive conformation of MEK1/2, stabilized by MEK1/2i binding, prevents its phosphorylation and activation of downstream effector ERK1/2.

Response to MEK1/2is is dependent on cellular context. Following MEK1/2 inhibition, loss of ERK1/2-dependent negative feedback results in C-Raf activation, which enhances phosphorylation of MEK1/2 and restores ERK1/2 activity in cells with wild-type B-Raf but not in B-Raf mutant cells. Monomeric mutant B-Raf, in contrast to its dimeric wild-type counterpart, is not subjected to ERK1/2-induced inhibitory phosphorylation, supporting the use of MEK1/2is in B-Raf-mutated cancers [47, 48]. Notably, more novel MEK1/2is, such as trametinib (GSK1120212), mitigate this C-Raf-driven feedback relief through regulation of Raf-MEK1/2 interactions and disruption of MEK1/2 phosphorylation, thereby, exerting sustained effects on ERK inhibition [46, 48]. At approximately 4 days, the average effective half-life of trametinib is reported to be longer than that of other MEK1/2is [49]. Potent MEK1/2 inhibition by trametinib is also
underlined by its IC₅₀ of 0.7 nM against purified MEK1 compared to concentrations of 17 nM and 14 nM for PD184352 and AZD6244, respectively [46]. Therefore, distinctions in mechanisms of drug activity dictate therapeutic efficacy.

Clinical assessment in cancer therapy

MEK1/2is have been investigated mainly in the K-Ras or B-Raf mutant cancer setting. To date, trametinib is the only MEK1/2i that has received regulatory approval as a monotherapy for B-Raf-mutated unresectable or metastatic melanoma based on improvement in progression-free and overall survival compared to chemotherapy [50]. While some MEK1/2is, such as PD184352 and its structural analogue PD0325901, have been discontinued from clinical development due to poor response rate or drug-associated toxicity [51], others have demonstrated promising clinical activity as well as tolerability, especially AZD6244 in phase II trials for treatment of metastatic biliary cancer and low-grade serous ovarian cancer, warranting further investigation [52, 53]. AZD6244 has also shown encouraging results in low-grade pediatric glioma and plexiform neurofibromatosis [46].

Nevertheless, efforts to improve therapeutic efficacy and, importantly, prevent emergence of resistance led to rational combination of intrapathway inhibitors. In phase III studies, dual inhibition of B-Raf and MEK1/2 was superior to B-Raf monotherapy, as determined by tumor response, duration of response, risk of progression, and progression-free as well as overall survival, in patients with B-Raf mutant advanced stage melanoma [54, 55]. Furthermore, trametinib monotherapy was not clinically effective in B-Raf mutant melanoma patients previously treated with B-Raf inhibitors, suggesting that mechanisms of resistance to B-Raf-targeted therapy may also mediate sensitivity to
MEK1/2s [56]. Dual targeting strategies extend beyond constituents of the Ras-Raf-MEK cascade; in combination with docetaxel, MEK1/2/s selumetinib and trametinib exhibited robust activity in non-small cell lung cancer (NSCLC) patients [46].

Preclinical studies have shown that activation of compensatory signaling pathways contribute to primary and acquired MEK1/2i resistance [57]. Downstream of Ras and parallel to the MAPK cascade, oncogenic PI3K signaling stimulated by mutations in the PI3KCA gene or loss of tumor suppressor phosphatase and tensin homolog (PTEN) function can confer resistance to MEK1/2/s [58]. Concurrent inhibition of MEK1/2 and PI3K pathways enhanced antitumor efficacy in B-Raf and K-Ras mutated xenograft models, prompting clinical analysis of combination therapy in patients with advanced tumors, but progress has been hindered due to cumulative toxicities [58]. Accordingly, ongoing trials are focused on dose modifications to ensure safety while optimizing activity.

Growth factor pathways, especially within the MAPK signaling network, exhibit a high degree of crosstalk and redundancy, enabling cancer cells to adapt and become refractory to single pathway inhibition. Convergence of the MEK1/2 and MEK5 cascades on downstream targets, including cyclin D1, highlights the importance of this understudied pathway in cancer progression (Figure 1) [59]. Moreover, as overexpression of cyclin D1 has been shown to promote resistance to inhibitors of ERK1/2 signaling, inhibition of MEK5 combined with MEK1/2/s has therapeutic potential surpassing that of MEK1/2 monotherapy [60, 61].
MEKK2/3

MEKK2/3 signal transduction can be activated by environmental stress, GFs, and cytokines [63]. In response to these extracellular stimuli, MEKK2 or MEKK3 bind to the N-terminal domain of MEK5 and phosphorylate Ser311 and Thr315; however, the mechanisms of MEKK2/3 activation by external stimuli have not been fully elucidated [77]. Compared to MEKK3, MEKK2 has a higher binding affinity for MEK5, but both MEKKs can also activate other conventional MAPK pathways, including JNK and p38 MAPK, via phosphorylation of their respective upstream MAP2Ks [78, 79].

Figure 1. Downstream substrates of the MEK1/2 and MEK5 pathways. Emerging studies have shown that MEK1/2 and MEK5 signaling regulate common gene targets. However, the MEK5 cascade has non-redundant functions separate from that of the MEK1/2 pathway. [15, 17, 33, 36, 38-43, 59, 62-76]
Overexpression of MEKK2 has been detected in prostate and colorectal cancers, while elevated MEKK3 expression has been identified in breast, cervical, lung, kidney, and esophageal cancers [80-83].

MEKK2 is necessary for epidermal growth factor receptor (EGFR)- and human epidermal growth factor receptor 2 (HER2)-dependent activation of ERK5. Knockdown of MEKK2 inhibited tumor growth of triple-negative MDA-MB-231 and HER2-positive BT474 breast cancer xenografts and diminished metastasis of the TNBC cells [84]. MEKK2 has been shown to regulate breast cancer cell migration by inducing focal adhesion turnover, specifically ubiquitylation and consequent removal of paxillin from focal adhesion complexes [85, 86]. To date, there are no selective MEKK2 inhibitors, though six compounds with potent in vitro MEKK2 inhibitory activity have recently been reported. Ponatinib (AP24534, Iclusig), among this list of kinase inhibitors, is an FDA-approved drug indicated for BCR-ABL-targeting in treatment of chronic myeloid leukemia, suggesting its potential both as a preclinical research tool to elucidate the role of MEKK2 in cancer and as a drug repurposed for MEKK2-dependent cancers in the clinical setting [87].

The role of MEKK3 as a regulator of NF-κB signaling is well-documented [88, 89]. Overexpression of MEKK3 in glioma and ovarian cancer cells enhanced NF-κB activation and increased expression of cell survival factors to confer resistance to cytotoxic effects of chemotherapeutic agents [82, 90]. Conversely, silencing of MEKK3 by RNAi sensitized breast cancer cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) cytotoxicity through suppression of nuclear factor κB (NF-κB) transcriptional activity [91]. Furthermore, MEKK3 depletion induced cell death in renal
cancer cells and reduced tumor growth of breast cancer cells but did not significantly affect the frequency of metastasis [81, 84]. Despite their involvement in processes essential to tumorigenesis and malignancy, MEKK2/3 are understudied kinases. Instead, efforts have been focused on parallel MEKKs and downstream effectors of MEKK2/3.

2.5 MEK5 Pathway

Structure and function

In the MAPK signaling network, MEK5 most resembles MEK1/2 by sequence alignment but remains the only known MEK activator of ERK5 [16]. Isoforms of MEK5 (50 kDa α and 40 kDa β) differ in the N-terminus, which accounts for their relative binding affinities for ERK5. MEK5α has a distinct docking site in its N-terminal extension, a phox and Bem1p (PB1) domain, crucial to ERK5 activation and transcriptional induction via myocyte enhancer factor 2C (MEF2C), therefore, it is a stronger activator of ERK5 than MEK5β, which lacks this consensus motif [92]. Moreover, the PB1 domain, present in all three components of this signaling cascade, acts as a scaffold to facilitate and maintain specificity of MEKK2–MEK5–ERK5 interaction and signaling [93].

Due to its extended C-terminus containing a nuclear localization signal (NLS), two proline-rich regions, and a transcriptional activation domain (TAD), ERK5, or big MAP kinase 1 (BMK1), is more than twice the molecular weight of other MAPKs (110 kDa). This structural distinction enables active ERK5 to undergo autophosphorylation of its C-terminal TAD, an ability unique to ERK5, thereby exerting direct control over gene transcription [94]. In the unphosphorylated state, ERK5 presents an inactive
conformation, where its N- and C-terminal domains are bound together in the cytosol. Activation by MEK5 induces an open configuration of ERK5, exposing the NLS, to relieve the autoinhibitory effects and facilitate ERK5 translocation to the nucleus [22, 95, 96]. ERK5 activity is also regulated by splice variants (a, b, and c) [97]. While ERK5a is the most highly expressed isoform, ERK5b and c, both deficient in protein kinase activity, can inhibit MEK5-mediated ERK5a stimulation. Additionally, protein tyrosine phosphatase STEP-like (PTP-SL) is capable of dephosphorylating ERK5 to prevent its nuclear translocation [95].

Known substrates of ERK5 include transcription factors Sap-1a (also known as ELK4), c-FOS, c-MYC and MEF2 (A, C, and D) and kinases, such as RSK and serum/glucocorticoid-regulated kinase (SGK) [63-67]. Similar to other proline (Pro)-directed MAPKs, ERK5 substrate recognition and subsequent phosphorylation occurs on amino acids Ser or Thr adjacent to a Pro residue (-X-Ser/Thr-Pro-X- sequence). Additionally, ERK5 protein kinase activity can be non-Pro-directed, as in the case of ERK5 autophosphorylation and ERK5-mediated MEK5 phosphorylation on Ser/Thr sites not directly preceding Pro residues [98]. These findings further distinguish ERK5 from other conventional MAPK family members.

**Pharmacological Inhibitors**

Interest in the MEK5 pathway has emerged in cancer research partly due to its overlap with the MEK1/2 pathway, and the discovery that first-generation MEK1/2 inhibitors PD98059, U0126, and PD184352 also exhibit activity toward MEK5 provided impetus for the development of MEK5 selective inhibitors to parse the role of these pathways in cancer progression [22, 99]. The indolinone-6-carboxamides BIX02188 and
BIX02189 (Boehringer Ingelheim Pharmaceuticals) were the first selective small-molecule inhibitors of MEK5 signaling to be described, inhibiting MEK5 catalytic function with IC\textsubscript{50} 4.3 and 1.5 nM, respectively [100]. BIX02189 also displayed more potent suppression of ERK5 kinase activity with IC\textsubscript{50} 59 nM compared to that of BIX02188 (810 nM). Both compounds also inhibited transcriptional activity of MEF2, a downstream substrate of the MEK5 signaling cascade, in a dose-dependent manner. These MEK5 inhibitors blocked ERK5 phosphorylation without affecting activation of ERK1/2, p38 MAPK, or JNK [100].

Synthesis of XMD8-92 stemmed incidentally from a screen of analogs of BI-2536, a highly selective, ATP-competitive polo kinase inhibitor [101]. XMD8-92 selectivity for ERK5 was validated through profiling first against a diverse panel of 402 kinases and then against all detectable kinases in HeLa cell lysates, identifying ERK5 as most potently inhibited target with IC\textsubscript{50} of 1.5 µM. MEK5 and ERK1/2 were not inhibited by XMD8-92, but the compound did significantly reduce ERK5-dependent MEF2C-driven gene expression. Pharmacokinetics and tolerability of XMD8-92 was also evaluated in Sprague-Dawley rats. A single intravenous or oral dose of XMD8-92 was found to have a 2-hour half-life clearance of 26 mL/min/kg and high oral bioavailability with 69% dose absorption. After a single oral dose of 2 mg/kg, maximal plasma concentrations reached 500 nM within 30 minutes, with 34 nM remaining 8 hr post drug administration. To assess tolerability, plasma concentrations of XMD8-92 were maintained at high levels, 10 µM following IP dosing of 50 mg/kg, for 2 weeks. Animals did not show signs of morbidity or mortality [101].
TG02, an oral pyrimidine-based multi-kinase inhibitor, blocks CDKs 1, 2, 3, 5, and 9 with IC$_{50}$ values below 10 nM in addition to janus kinase 2 (JAK2), p38δ, and ERK5 with IC$_{50}$ values of 19, 56, and 43 nM, respectively [102, 103]. The pharmacokinetic profile showed drug levels retained in tumors were above the IC$_{50}$ for 8 and 24 hours after a single oral dose of 30 or 60 mg/kg, respectively [103]. TG02 treatment was well-tolerated in mice, even at maximum oral dosing of 40 mg/kg daily, with no body weight loss at endpoint. This novel anti-cancer agent has recently completed phase I of clinical trials for treatment of leukemia and multiple myeloma patients, the results of which may unveil the potential for MEK5 signaling inhibitors in cancer therapy.

**Role in drug resistance**

*Cytotoxic therapy*

Drug resistance, both primary (intrinsic) and acquired, is a major obstacle in cancer therapeutics, indicative of more clinically aggressive tumor cells contributing to disease progression. The efficacy of cytotoxic agents used in chemotherapy, the standard-of-care for various cancer types, is mitigated by activation of signaling pathways, such as MEK5, that confer drug resistance [74]. MEK5 signaling has been shown to promote epithelial-to-mesenchymal transition (EMT), cell survival, and evasion of apoptosis – mechanisms linked to adaptive resistance [74, 104, 105].

Expression profiling revealed MEK5 upregulation in apoptotically resistant (APO−) MCF-7 breast cancer cell variants compared to apoptotically sensitive (APO+) cells. Transfection of dominant-negative (DN) ERK5 plasmid into APO− cells reduced cell viability in a dose-dependent manner versus vector control, and the cytotoxic effects
of DN-ERK5 expression were augmented by treatment with apoptotic-inducing agents etoposide, tumor necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL). Furthermore, phorbol ester (PMA) stimulation failed to rescue cell viability of DN-ERK5-transfected cells treated with TRAIL [106]. In basal-like breast cancer subtypes, overexpression of MEK5 in conjunction with ERK5 was associated with poor relapse- and metastasis-free survival in patients who received chemotherapy compared to patients not treated with chemotherapy, which suggests that MEK5-ERK5 expression could serve as a predictive marker for patient benefit from systemic treatments in the ER-negative breast cancer setting [107]. Moreover, in MDA-MB-231 cells ERK5 inhibition by TG02 augmented anti-cancer effects of chemotherapeutic agents conventionally used in TNBC treatment, including taxotere, vinorelbine, and cisplatin [108]. These results support the role of MEK5 signaling in regulation of survival and apoptosis and implicate MEK5 pathway involvement in chemoresistance [106].

The pyrimidine analog 5-fluorouracil (5-FU), a widely used chemotherapeutic agent, is the standard treatment for colon cancer [109]. While clinical efficacy of 5-FU exceeds that of other drugs, only 30% of colon cancer patients initially respond to therapy and the majority of which will develop resistance [110]. In vitro treatment of colon cancer cells HCT116 and SW620 with 5-FU reduced activation of both MEK5 and ERK5. Constitutive activation of MEK5 conferred a survival advantage to HCT116 cells exposed to 5-FU compared to empty vector cells, whereas downregulation of MEK5 signaling, either by transfection of dominant-negative ERK5 construct or treatment with a highly-selective ERK5 inhibitor XMD8-92, enhanced sensitivity of HCT116 cells to 5-FU-induced cytotoxicity through stimulation of p53-dependent transcriptional activation
of p21 and Puma. The anti-apoptotic effects of 5-FU treatment in conjunction with ERK5 inhibition were recapitulated in vivo using an HCT116 xenograft model. Combination therapy using 5-FU and XMD8-92 significantly increased apoptosis and reduced tumor burden in comparison to monotherapy of each compound [111]. Consistent with this study, ERK5 inhibition via XMD8-92 treatment combined with doxorubicin, another chemotherapeutic agent, demonstrated synergistic induction of p53 and promoted significant tumor regression in both HeLa cervical cancer cells and A549 lung cancer cells [112]. Furthermore, small hairpin RNA (shRNA)-mediated knockdown of ERK5, as a mirror of ERK5 pharmacological inhibition, sensitized HMESO malignant mesothelioma cells to doxorubicin in vitro and synergized with doxorubicin in enhancing anti-tumor activity compared to vector control [74]. These findings provide rationale for the application of MEK5 pathway inhibitors coupled with 5-FU- or doxorubicin-based chemotherapy to enhance therapeutic efficacy and potentially delay the onset of drug resistance.

**Targeted therapy**

Pursuit of mechanism-based, individualized therapeutics has led to the development of small-molecule inhibitors and monoclonal antibodies targeting key signaling molecules or networks that drive cancer progression. Targeted therapies, though diverse in their mechanisms of action, have not overcome the hurdle of drug resistance. MEK5 signal transduction has been implicated as a critical factor in mediating sensitivity to several targeted therapies.

Endocrine resistance, either de novo or acquired, is evident in up to 50% of patients on an antiestrogen regimen, the mainstay in treatment of estrogen receptor alpha (ER-α)-
positive breast cancer. ER-α signaling is an integral component of breast cancer biology as well as an important molecular mechanism perverted in endocrine therapy resistance [113]. Overexpression of MEK5 in the antiestrogen-sensitive, ER-α-positive (ER+) MCF-7 cell line downregulated ER-α expression and transcriptional activity in an ERK5-dependent manner and increased clonogenic survival following endocrine treatment [114]. These results delineate the role of MEK5-ERK5 signaling in progression to a more malignant estrogen-independent phenotype.

In breast tumors positive for human epidermal growth factor receptor 2 (HER2) expression, anti-HER2 therapy, such as trastuzumab, has demonstrated clinical efficacy in the adjuvant setting, yet approximately 20% of patients experience relapse [115]. High ERK5 expression in patients with HER2-positive breast cancer was associated with worse disease-free survival [116]. HER2-enriched breast cancer cell lines SKBR3 and BT-474 have been shown to express constitutively active ERK5 [117]. Downregulation of ERK5 expression or activation potentiated anti-proliferative effects of trastuzumab in BT-474 cells, indicating that pharmacological inhibition of ERK5 may enhance anti-cancer action of trastuzumab [116]. In another cancer model, expression of dominant-negative ERK5 increased sensitivity of myeloma cells to apoptosis induced by the proteasome inhibitor PS341. Furthermore, overexpression of ERK5 in these cells abrogated the effects of PS341 on cell death [118]. Taken together, these studies implicate the MEK5-ERK5 pathway as a fundamental component of drug resistance in cancer therapy. Defining the mechanisms by which MEK5 promotes a therapeutically resistant phenotype may provide insight for the design of next generation anti-cancer agents.
Role in tumorigenesis

The MEK5/ERK5 cascade has been emerging as an important mediator of cell proliferation through induction of cell cycle regulators, including cyclin D1, c-MYC, SGK, RSK2, and NF-κB [59, 65, 68-71]. Through phosphorylation of MEF2 transcription factors, MEK5 has been shown to regulate the expression of c-JUN, a proto-oncogene vital to cell growth [64, 72]; moreover, the ERK5-MEF2 axis has been reported in activation of survival signaling [119]. It has also been demonstrated that ERK5 can phosphorylate S403 and T409 of tumor suppressor promyelocytic leukemia protein (PML) and inhibit its activity, thereby downregulating the induction of p21 expression and enabling cells to overcome the G1-S phase [101, 120]. Constitutive activation of MEK5 in prostate and colon cancer cell lines accelerated cell cycle progression and increased proliferation [111, 121, 122]. Similarly, ERK5 knockdown studies using RNA interference (RNAi) or pharmacological inhibition by XMD8-92 treatment delayed cell cycle progression and decreased proliferation in various cancer types (Table 1). There are, however, conflicting reports showing that in cell lines harboring K-Ras or B-Raf mutations neither MEK5 inhibition, via BIX02189 or dominant-negative (DN) construct, nor siRNA-mediated downregulation of ERK5 affected cell growth, suggesting that in this cell context ERK5 is a dispensable proliferative signal [19, 111]. Interestingly, these results were also shown in ERK5-amplified SNU449 and KYSE30 cells [19], directly contradicting previous research demonstrating that knockdown of ERK5 resulted in cell growth inhibition in the ERK5-dysregulated hepatocellular and esophageal cancer cells, respectively [123, 124].
Using a conditional ERK5 knockout mouse model, Hayashi et al. demonstrated that tumor cells inoculated subcutaneously into the right flank region of the animals exhibited impaired vasculature development and reduced tumor growth, suggesting the involvement of ERK5 in the regulation of tumor-associated angiogenesis as well as tumor formation [68]. Studies since then have supported the involvement of the MEK5/ERK5 pathway in cancer progression (Table 2). Hyperactivation of MEK5 in ER+ breast cancer cells enhanced estrogen-independent tumorigenesis [114], and ERK5 overexpression supported prostate tumor growth [125]. The role of ERK5 in tumor formation was further established as its silencing by shRNA impaired growth of malignant mesothelioma, T cell leukemia, and hepatocellular carcinoma xenografts through regulation of pro-inflammatory cytokines or NF-κB signaling [70, 72, 74]. Moreover, XMD8-92 treatment decreased tumor volume of various cancer types [72, 101, 111]. TG02, a multi-kinase inhibitor that targets ERK5, has also been shown to be an efficacious anti-tumor agent in the multiple myeloma and breast cancer settings [102, 108]. Based on studies demonstrating that shRNA-mediated knockdown of ERK5 did not alter growth dynamics of triple-negative breast cancer xenografts [84, 126], the anti-proliferative effects have been proposed as an artifact of TG02 activity against CDK targets. However, partial silencing of ERK5 may not be sufficient to exert anti-tumor effects in certain cell lines. For example, 70% ERK5 inhibition in SNU449 cells decreased proliferation while not affecting apoptosis, whereas 90% reduction of ERK5 expression in KYSE30 cells resulted in suppression of cell growth and significant induction of cell death [124]. As the Clustered Regularly Interspaced Short Palindromic Repeats/CRIOSPR-associated protein-9 (CRISPR/Cas9) knockout system has been widely adopted for precision genome editing,
it would be a beneficial tool in delineating the involvement of MEK5/ERK5 in tumorigenesis. Despite the controversy surrounding this research arena, MEK5 signaling remains a viable therapeutic target and elucidation of this pathway is needed to stratify the anti-cancer armamentarium.

**Role in metastatic progression**

Dysregulated MEK5 signaling is associated with metastatic risk in prostate, breast, colon, kidney, bone, and oral cancers and less favorable survival outcome [73, 114, 122, 127-130]. Molecular inhibition of ERK5 *in vitro* suppressed cell motility and invasion of liver, breast, and prostate cancer cells [72, 131] and decreased metastasis of breast cancer xenografts *in vivo* [84, 126]. Conversely, cancer cells overexpressing MEK5 or ERK5 exhibited a migratory and invasive phenotype [121, 125], denoted by increase in tumor metastases [73, 122].

Metastasis, a complex process in which malignant cells originating from the primary tumor infiltrate and colonize distal organs, is organized into simplified steps: local invasion, intravasation of cells into the circulation, dissemination, extravasation of cells at distant sites, and colonization. Epithelial-to-mesenchymal transition (EMT) is an integral part of metastatic progression whereby cells adopt motile and invasive capabilities through loss of epithelial markers, namely Cadherin 1/E-Cadherin (CDH1), and acquisition of mesenchymal markers, such as vimentin (VIM) and Cadherin 2/N-Cadherin (CDH2). MEK5 signaling has been implicated in the activation of EMT and transcription factors linked to EMT induction, including NF-κB and FOS-Like Antigen 1 (FRA-1) [66, 114, 122]. Furthermore, ERK5 signaling has been shown to regulate the expression of matrix metalloproteinase (MMP) family members (MMP-1, 2, 9, 12, and
known for their role in degradation of the extracellular matrix (ECM) to potentiate cancer cell dissemination [73, 74, 121, 132], and other proteins involved in migration and invasion, such as tissue inhibitor of metalloproteinases 2 (TIMP2) and bone morphogenic protein 5 (BMP5) [73, 74]. While many studies have presented a positive correlation between ERK5 expression and EMT induction, dissenting observations have been reported. Knockdown of ERK5 in A549 metastatic lung cancer cells resulted in reduced protein expression of CDH1 and ZO-1, upregulation of snail family zinc finger 1 (SNAI1), CDH2, and VIM, and enhanced cell migration [133]. Contrary to these findings, another study using A549 cells cited EMT suppressive, or MET inducing, effects of ERK5 depletion, including increased levels of CDH1 and reduction in cell migration, through regulation of SNAI2 with no change observed in SNAI1 levels [126]. These morphogenetic changes were recapitulated in a highly aggressive mesenchymal breast cancer model where suppression of ERK5 induced an epithelial phenotype and decreased intravascular invasion, leading to significantly fewer circulating tumor cells (CTCs) derived from primary orthotopic xenografts and reduction of metastatic lesions [126].

In addition to tumor cell intravasation, EMT has been linked to enrichment of the cancer stem cell (CSC)-like phenotype, further cementing its role in the metastatic cascade [12]. CSCs exhibit tumor-initiating potential, vital for metastatic colonization, attributed to their ability to self-renew and generate differentiated progeny that do not bear CSC cell-surface markers. From the multitude of studies establishing connections between MEK5 signaling and EMT, it follows that ERK5 would be involved in regulation of CSCs. Indeed, ERK5 activation was associated with enhanced CSC tumor
sphere formation and tumor-initiating capacity [134]. Inhibition of ERK5 abrogated the effects of MEK5 activity on tumorigenicity of A549 spheres through hypoxia-inducible factor 1α (HIF-1α)-mediated upregulation of apoptosis-associated genes BCL2 interacting protein 3 (BNIP3) and BNIP3 like (BNIP3L).

Involvement of the MEK5 pathway has also been described in disruption of actin dynamics leading to alterations in cell migration/invasion potential and metastatic dissemination. For instance, transfection of ERK5 expression construct in prostate cancer cells promoted formation of invadopodia, actin-rich protrusions associated with invasiveness of cancer cells [73]. Additionally, novel roles of ERK5 have been demonstrated in cytoskeletal remodeling pathways. PMA-stimulated ERK5 activity was implicated in regulation of cell morphology through phosphorylation of focal adhesion kinase (FAK) on S910 [135]. Moreover, integrin-mediated FAK signaling was linked to ERK5 activation in prostate and breast cancer cells, resulting in enhanced cell motility [131]. The ER-α/ERK5/cofilin (CFL1) network is another regulatory pathway of actin organization. While it has previously been shown that MEK5 signaling represses ER-α expression in breast cancer cells thus promoting a more malignant hormone-independent phenotype, the role of ER-α was recently discovered in nuclear recruitment of ERK5 and CFL1, restricting their colocalization to cytoplasmic regions of actin remodeling, to suppress metastatic capacity [114, 136]. Notably, in ER-negative cell lines introduction of ER-α or ERK5 inhibition using XMD8-92 impaired cell motility and invasiveness [136].

Cell division cycle 42 (Cdc42), a member of the Rho GTPase family was shown to exert breast cancer cell line-specific effects on metastatic potential in part through
regulation of the ERK5 pathway. It was reported that knockdown of Cdc42 increased ERK5 phosphorylation and suppressed cell motility and invasion in moderately metastatic Hs-578T breast cancer cells, suggesting that ERK5 signaling negatively correlates with metastatic progression [137, 138]. However, Cdc42 depletion enhanced cell migration and invasion in highly aggressive MDA-MB-231 breast cancer cells [137]. If activation of ERK5 associated with Cdc42 silencing, then ERK5 would exert pro-metastatic effects in these highly invasive cells; yet ERK5 activity was shown to decrease the invasive potential of MDA-MB-231 cells [138]. These conflicting results highlight the nuanced and cellular context-dependence of ERK5 function in modulating the metastatic phenotype and further supports continued investigation of the MEK/ERK5 signaling in regulation of invasive/metastatic potential.
Table 1. Effects of MEK5/ERK5 signaling on *in vitro* cancer cell proliferation.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cell line</th>
<th>Targeted Approach</th>
<th>Effects (compared to control)</th>
<th>Mechanism</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>prostate carcinoma</td>
<td>LNCaP</td>
<td>CA-MEK5</td>
<td>increased proliferative index</td>
<td>CA-MEK5 expression increased percentage of cell in S phase.</td>
<td>[121]</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>overexpression</td>
<td>increased proliferative index</td>
<td>High levels of ERK5 is associated with accelerated cell cycle progression.</td>
<td>[125]</td>
</tr>
<tr>
<td>T cell leukemia</td>
<td>Jurkat T</td>
<td>shRNA</td>
<td>did not affect cell cycle progression, sensitized cells to TNF-α</td>
<td>ERK5 activates NF-κB signaling and promotes nuclear localization and transcriptional activity of p65.</td>
<td>[70]</td>
</tr>
<tr>
<td>breast carcinoma</td>
<td>BT-549</td>
<td>shRNA</td>
<td>decreased cell proliferation</td>
<td>TG02 decreased expression of antiapoptotic proteins MCL1 and BCL2 and triggered cell death through caspase-dependent and -independent mechanisms.</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>Hs-578T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCC1187</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>TG02</td>
<td>delayed cell cycle progression, induced apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCC1187</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>multiple myeloma</td>
<td>MM1S</td>
<td>TG02</td>
<td>suppressed cell cycle progression and induced apoptosis</td>
<td>TG02 activated apoptosis through intrinsic and extrinsic pathways.</td>
<td>[102]</td>
</tr>
<tr>
<td>cervical adenocarcinoma</td>
<td>HeLa</td>
<td>DN-ERK5 (Ala-Glu-Phe [AEF])</td>
<td>inhibited proliferation</td>
<td>ERK5 inhibited PML function and p21 expression to regulate cell proliferation.</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>XMD8-92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lung carcinoma</td>
<td>NCI-H1793</td>
<td>siRNA</td>
<td>did not affect cell proliferation or death</td>
<td></td>
<td></td>
</tr>
<tr>
<td>esophageal carcinoma</td>
<td>KYSE30</td>
<td>siRNA</td>
<td>decreased proliferation, increased cell death</td>
<td>Dysregulated ERK5 signaling drives cell proliferation.</td>
<td>[124]</td>
</tr>
<tr>
<td>hepatocellular carcinoma</td>
<td>SNU449</td>
<td>siRNA</td>
<td>decreased proliferation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: ERK5 inhibition; MEK5 inhibition
Table 1. (cont.) Effects of MEK5/ERK5 signaling on *in vitro* cancer cell proliferation.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cell line</th>
<th>Targeted Approach</th>
<th>Effects (compared to control)</th>
<th>Mechanism</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hepatocellular carcinoma</td>
<td>HepG2</td>
<td>siRNA</td>
<td>decrease in proliferation</td>
<td>XMD8-92 treatment decreased expression of cyclin D1.</td>
<td>[72]</td>
</tr>
<tr>
<td></td>
<td>Huh-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HepG2</td>
<td>XMD8-92</td>
<td>decrease in proliferation, reduction of cells in S phase and increased percentage of cells in G0/G1, no indications of apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Huh-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNU449</td>
<td>siRNA</td>
<td>inhibited cell growth</td>
<td>RNAi knockdown of ERK5 decreased mitotic index, implicating ERK5 involvement in regulation of mitotic entry.</td>
<td>[123]</td>
</tr>
<tr>
<td></td>
<td>SNU449</td>
<td>siRNA BIX02189</td>
<td>inhibition of ERK5 expression or activity did not decrease proliferation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>colon carcinoma</td>
<td>HCT116</td>
<td>siRNA</td>
<td>did not affect proliferation</td>
<td>K-Ras/B-Raf-mutated or ERK5 amplified cancer cells are not dependent on MEK5 pathway for proliferation.</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>HCT116</td>
<td>BIX02189</td>
<td>inhibited proliferation at high doses (&gt;10 µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT29</td>
<td>BIX02189</td>
<td>inhibited proliferation at high doses (&gt;10 µM), did not affect proliferation in 3D culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCT116</td>
<td>DN-MEK5</td>
<td>did not affect proliferation index (however, ERK5 overactivation by CA-MEK5 increased cell proliferation)</td>
<td>ERK5 inhibition was associated with increased p53 transcriptional activity, upregulating p21 and Puma.</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td>SW620</td>
<td>DN-MEK5</td>
<td>did not affect proliferation index</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SW620</td>
<td>CA-MEK5</td>
<td>accelerated cell cycle progression in CA-MEK5 cells, which was abolished by XMD8-92 treatment</td>
<td>MEK5 signaling promotes cell cycle progression through degradation of IkB leading to NF-κB activation.</td>
<td>[122]</td>
</tr>
</tbody>
</table>

Note: ERK5 inhibition; MEK5 inhibition
Table 2. Effects of MEK5/ERK5 signaling on *in vivo* tumorigenesis.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cell line</th>
<th>Targeted Approach</th>
<th>Effects (compared to control)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>lung carcinoma</td>
<td>LL/2</td>
<td>deletion of host gene</td>
<td>delayed tumor development, reduced tumor vasculature</td>
<td>[68]</td>
</tr>
<tr>
<td>melanoma</td>
<td>B16F10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prostate carcinoma</td>
<td>PC3</td>
<td>overexpression</td>
<td>enhanced tumor formation</td>
<td>[125]</td>
</tr>
<tr>
<td>breast adenocarcinoma</td>
<td>MCF7</td>
<td>CA-MEKS</td>
<td>enhanced tumor growth independent of estrogen, shRNA-downregulation of ERK5 decreased MCF7-MEK5 tumor growth</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>TG02</td>
<td>delayed tumor growth</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>shRNA</td>
<td>did not significantly affect tumor growth</td>
<td>[84]</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>shRNA</td>
<td>did not significantly affect tumor growth</td>
<td>[126]</td>
</tr>
<tr>
<td>multiple myeloma</td>
<td>MM1S</td>
<td>TG02</td>
<td>inhibited tumor growth</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td>OPM2</td>
<td></td>
<td>inhibited tumor growth</td>
<td></td>
</tr>
<tr>
<td>malignant mesothelioma</td>
<td>HMMESO</td>
<td>shRNA</td>
<td>impaired tumor formation</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td>H2373</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cell leukemia</td>
<td>EL-4</td>
<td>shRNA</td>
<td>impaired tumor formation</td>
<td>[70]</td>
</tr>
<tr>
<td>hepatocellular carcinoma</td>
<td>Huh-7</td>
<td>shRNA</td>
<td>suppressed tumor growth by 100-fold</td>
<td>[72]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XMD8-92</td>
<td>suppressed tumor growth due to reduction in cell proliferation, no change in levels of apoptosis</td>
<td></td>
</tr>
<tr>
<td>colon carcinoma</td>
<td>HCT116</td>
<td>XMD8-92</td>
<td>inhibited tumor growth by 46%</td>
<td>[111]</td>
</tr>
<tr>
<td>pancreatic adenocarcinoma</td>
<td>AsPC-1</td>
<td>XMD8-92</td>
<td>inhibited tumor growth and decreased tumor volume</td>
<td>[139]</td>
</tr>
<tr>
<td>cervical adenocarcinoma</td>
<td>HeLa</td>
<td>XMD8-92</td>
<td>inhibited tumor growth, blocked tumor cell proliferation</td>
<td>[101]</td>
</tr>
<tr>
<td>lung carcinoma</td>
<td>LL/2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: ERK5 inhibition; MEK5 inhibition *(4175 TGL variant)*
PROBLEM STATEMENT

Triple-negative breast cancer (TNBC) exhibits increased metastatic potential and high rates of distal recurrence compared to other breast cancer subtypes, and there are no targeted agents approved for treatment of this aggressive disease [3]. As epithelial-to-mesenchymal transition (EMT) is an integral part of metastatic progression, characteristic of more invasive cells capable of disseminating to distant sites, strategies targeting this process have emerged in cancer therapy [9]. While molecular mechanisms of EMT are not fully elucidated, many signaling pathways are known to activate the EMT program, including the MAPK/ERK kinase (MEK) 1/2 and MEK5 pathways [114]. However, there are conflicting reports on the role of the MEK5 cascade in modulating the metastatic phenotype [108, 137]. The purpose of this work is to elucidate MEK5 signaling in regulation of the EMT axis and breast cancer progression. Furthermore because MEK1/2 and MEK5 share common effector pathways and gene targets, we propose that dual inhibition of these kinases will be more efficacious than current inhibitors targeting only MEK1/2.
SPECIFIC AIMS

Specific Aim 1: Elucidate the role of MEK5-ERK5 signaling in EMT and breast cancer progression.

Specific Aim 2: Evaluate effects of dual MEK1/2 and MEK5 inhibition on breast cancer cells.

Specific Aim 3: Determine the global effects of pan-MEK inhibition in triple-negative breast cancer cell lines.
MATERIALS AND METHODS

Cells and reagents

Breast cancer cell lines MDA-MB-231, BT-549, Hs-578T, MDA-MB-157, MDA-MB-468, BT-20, ZR75, T47D, and SKBR3 were acquired from American Type Culture Collection (ATCC). MCF-7N cell variant (subclone of MCF-7 human breast adenocarcinoma line from ATCC was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN) in 1996. Liquid nitrogen stocks were made upon receipt and maintained until the start of each study. Cells were used for no more than 6 months after being thawed. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; pH 7.4; Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, Salt Lake City, UT), 1% non-essential amino acids, minimal essential amino acids, sodium pyruvate, antibiotic/anti-mycotic and insulin under mycoplasma-free conditions at 37°C in humidified 5% CO₂ and 95% air. For charcoal-stripped experiments, cells were maintained for 48 hours in phenol red-free DMEM without glutamine (Invitrogen, Carlsbad, CA) supplemented with 5% charcoal-stripped FBS (Atlanta Biologicals, Flowery Branch, GA), 1% non-essential amino acids, minimal essential amino acids, sodium pyruvate, pen strep and GlutaMAX. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (Waltham, MA). Dosing for SC compounds inhibiting the MEK1/2 and/or MEK5 pathway(s), kindly provided by Patrick Flaherty (Duquesne University, Pittsburgh, PA), was 1 μM unless otherwise indicated.
**Plasmids**

The constitutively active pcDNA3-MEK5(DD) expression plasmid, graciously donated by Marcus Buschbeck (Max-Planck-Institute of Biochemistry, Martinsried, Germany), was produced by site-directed mutagenesis replacing S311 and T315 by aspartate (D). PCIN4-hFRA1 construct was a generous gift from Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA). Using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), a phospho-mimic FRA-1 construct with S265 mutated to D and a kinase-dead construct with S265 mutated to A were kindly generated by Melyssa Bratton (Xavier University, New Orleans, LA). Additionally, FRA-1 wild-type, S265D, S265D-T223D-T230D, and S252A/S265A constructs were generously provided by Dany Chalbos (Institut de Recherche en Cancérologie, Montpellier, France) and Marc Piechaczyk (Institut de Génétique Moléculaire de Montpellier, Montpellier, France).

**Generation of stable knockout cell lines**

Using a pU6-driven guide strand with dual expression cassettes for Cas9/EGFP plasmids based approach (Horizon, Cambridge, UK), TNBC (MDA-MB-231 and HS578T) cells were transfected with 5 individual guide strands targeting exons 3 and 5 of the MAPK7 gene. After 24 hours, cells were sorted for GFP expression using the Becton Dickinson FACSVantage. Flow profiles were analyzed by FACSDiVa software (BD Biosciences).

**Generation of stable cell lines**

TNBC cells were plated in 10 cm dishes and allowed to adhere overnight at 37°C. Cells were transfected with 5 µg of plasmid in 300 µL Opti-MEM. Transfection was
accomplished using 15 µL Attractene per manufacturer’s instructions (Qiagen, Valencia, CA). Media was changed the following day and cells were treated with selectable marker every two days. Once stable cells were obtained, viable colonies were cloned or pooled. Stable expression or knock-out was assessed by qPCR and WB. Transient transfections of pCDNA3-MEK5DD and HA-ERK5 were carried out as follows: MDA-MB-231- or Hs-578T-ERK5-ko cells were plated at 1 x 10^6 cells per dish in a 10 cm dish in 10% DMEM, cells were allowed to adhere at 37°C. After 24 hours, plates were transfected with 5 µg of plasmid using Attractene as per manufacturers’ protocol (Qiagen, Valencia, CA). After 48 hours, cells were collected for total RNA extraction and qPCR was performed.

**qPCR**

Cells were grown in phenol red-free DMEM supplemented with 5% charcoal-stripped (CS) fetal bovine serum (5% CS-DMEM) for 48 hours and treated with compounds. After 24 hours, cells were collected and total RNA was extracted using the Quick RNA Mini Prep Kit in accordance with the manufacturer’s protocol (Zymo Research, Irvine, CA). The quality and concentration of RNA were determined spectrophotometrically by absorbance at 260 and 280 nm using the NanoDrop ND-1000. Total RNA (1 µg) was reverse-transcribed using the iScript kit (BioRad, Hercules, CA) and qPCR was performed using SYBR-green (Bio-Rad Laboratories, Hercules, CA). Primer sequences are listed in Table 3. Cycle number was normalized to β-actin and vehicle-treated cells scaled to 1, n = 3. For patient-derived xenografts, RNA was isolated from tumor pieces using QIAzol Lysis Reagent (Qiagen, Valencia, CA) and Quick RNA Mini Prep Kit (Zymo Research, Irvine, CA).
Table 3. qPCR primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence F:</th>
<th>Sequence R:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>GGCACCCAGCACAGAACATGAAGA</td>
<td>ACTCTCTGCTTTGCTGATCCAC</td>
</tr>
<tr>
<td>ANGPT1</td>
<td>GCCCTAAGCCATCAGCAATC</td>
<td>AGGGTGCAATCCACAGCCCAA</td>
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<tr>
<td>ALDH1A1</td>
<td>CGCAAGAAGGGTTTCAG</td>
<td>TAATAGTCCGCCCCTCTCGG</td>
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<td>CD24</td>
<td>TGCTCTACCCACGCGAGATT</td>
<td>GCCAACCAGAGGTTGGAA</td>
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<td>CD31</td>
<td>CCGTTGAGAAAGAGGACCGC</td>
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<td>CD44</td>
<td>ATCTTGACCTCCTTGGCC</td>
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<tr>
<td>CDH1</td>
<td>CCTGCCATTCTGGGATCTCT</td>
<td>CCGAAGAAGCGAGAGCAGCAG</td>
</tr>
<tr>
<td>CDH2</td>
<td>GCCCCTCAAAGTTACCCTCAA</td>
<td>AGCCCAAGTATGGTCAATTT</td>
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<tr>
<td>Cyclin D1</td>
<td>GCCGGAGAGGGTGTGAGGAG</td>
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<td>EGR1</td>
<td>GCAAGCGTAAGGGCGTCG</td>
<td>GCTCATCAAACCCAGCGCCA</td>
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<td>ELK4</td>
<td>CCACTGGGAATAGTGAGGCG</td>
<td>GCCATCAAAGGCTTACG</td>
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<td>ER-α</td>
<td>GCCATGGGAGATCTTCGA</td>
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<td>ERK5</td>
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<td>FOS</td>
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<td>FOXA1</td>
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<td>FRA-1</td>
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<td>KLF2</td>
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Table 3. (cont.) qPCR primer sequences.

<table>
<thead>
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<th>Gene</th>
<th>Sequence</th>
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<tr>
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<tr>
<td></td>
<td>R: CTTCCAGCAACAAAGAAGGAC</td>
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<td>MEF2C</td>
<td>F: TCTCCCATCCAGTGTCAGG</td>
</tr>
<tr>
<td></td>
<td>R: TCTCCACCCATCAAGTCCAC</td>
</tr>
<tr>
<td>MEF2D</td>
<td>F: CCCAGGCAGGAAAGGGGTGA</td>
</tr>
<tr>
<td></td>
<td>R: GGTTGGTGAGGGAATGAGT</td>
</tr>
<tr>
<td>MEK1</td>
<td>F: TGTCGCCAGAAAGACTCCAG</td>
</tr>
<tr>
<td></td>
<td>R: GATACTTCGGAACCAGCATC</td>
</tr>
<tr>
<td>MEK5</td>
<td>F: GGACCTCTCAACACAGCAG</td>
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<tr>
<td></td>
<td>R: TGTGACTGTGCTCCTGTTG</td>
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<tr>
<td>MMP1</td>
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<tr>
<td></td>
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<tr>
<td>MMP2</td>
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<td>MMP9</td>
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<td></td>
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<td></td>
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<tr>
<td>MYC</td>
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<td></td>
<td>R: AGAGAAGGCGGATCCTGGCA</td>
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<tr>
<td>PLAU</td>
<td>F: GGAACACCTCCTACACAAGGA</td>
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<tr>
<td></td>
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<tr>
<td>PLAUR</td>
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<td></td>
<td>R: CCCACTGCGGTACTGGGACAT</td>
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<td>RSK2</td>
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<td></td>
<td>R: ATTATTCCAGGGTGATGAG</td>
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<tr>
<td>SERPINE1</td>
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<td></td>
<td>R: ATCAGTTGCGCAGAAGAGAG</td>
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<td>SNAI1</td>
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<tr>
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<td>R: GACAGCTTGTGCTTGAGGAC</td>
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<td>SNAI2</td>
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<td></td>
<td>R: GACCCTGGTCTCAGAAGGA</td>
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<td>SOX4</td>
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<tr>
<td>TIMP1</td>
<td>F: GCGGATCTTCACAGGCCATCC</td>
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<td></td>
<td>R: GTAGGTTGTTGGAAGCCC</td>
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<tr>
<td>TIMP3</td>
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<tr>
<td>TWIST</td>
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<td></td>
<td>R: TGTCCATTTTCTCCTTCTCGGA</td>
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<tr>
<td>VEGFA</td>
<td>F: ACGAAAGCGCAAGAAATCCC</td>
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<td></td>
<td>R: CTCCAGGCGATGCAACAGCA</td>
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<td>VIM</td>
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<td></td>
<td>R: GCTTTGCTTAGGGCAATC</td>
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<tr>
<td>ZEB1</td>
<td>F: TGCACTGAGTGGGAAAAGC</td>
</tr>
<tr>
<td></td>
<td>R: TGTTGATGCTGAAAGGACGA</td>
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<tr>
<td>ZEB2</td>
<td>F: CGCTTGAACATCAGAAGGA</td>
</tr>
<tr>
<td></td>
<td>R: CTGGGCACACTCTGAGCTT</td>
</tr>
</tbody>
</table>
**Western blot**

Cells were cultured in 10% FBS-supplemented DMEM. At confluence or post 24-hour treatment, cells were collected in PBS, pelleted, and lysed with mammalian protein extraction reagent (MPER) supplemented with 1% protease inhibitor and 1% phosphatase inhibitors (I/II) (Invitrogen, Grand Isles, NY). Samples were centrifuged at 12,000 RPM for ten minutes at 4°C to obtain supernatant containing protein extracts. NanoDrop ND-1000 was used to determine protein concentration of samples by absorbance at 260 and 280 nm. After proteins were heat-denatured at 100°C on a heating block, 40 µg of protein was loaded per lane on Bis-Tris-nuPAGE gel (Invitrogen, Grand Isles NY). Protein was then transferred to nitrocellulose membranes using iBlot and iBlot transfer stacks per manufacturer’s instructions (Invitrogen, Grand Isles, NY). Membrane was incubated at room temperature with 5% bovine serum albumin (BSA) in 1% Tris-buffered saline, 0.1% Tween 20 (TBS-T) for 1 hour to block non-specific binding followed by 4°C incubation overnight with primary antibodies (listed in Table 4). After three 15-minute washes in 1% TBS-T, membranes were incubated with appropriate secondary antibodies for at least one hour. IR-tagged secondary antibodies were purchased from LiCor Biosciences (Lincoln, NE) and used at a 1:10,000 dilution in 5% BSA. Following incubation with secondary antibodies, membranes were washed three times for 15 minutes per wash in 1% TBS-T, and blots were analyzed by the Odyssey Infrared Imaging System (LiCor Biosciences). Band density was quantified by LiCor gel imager. Data were normalized to Rho GDI-α (Santa Cruz Biotechnology, Santa Cruz, CA), serving as loading control. Experiments were conducted in triplicate with representative blots shown.
Table 4. Western blot antibodies.

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Phospho Site</th>
<th>Concentration (WB)</th>
<th>Vendor</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt</td>
<td>Rabbit</td>
<td>S473</td>
<td>1:1,000 1:10,000</td>
<td>Cell Signaling</td>
<td>9271</td>
</tr>
<tr>
<td>CDH1</td>
<td>Rabbit</td>
<td>-</td>
<td>1:700 1:5,000</td>
<td>Cell Signaling</td>
<td>3195</td>
</tr>
<tr>
<td>p-c-Jun</td>
<td>Rabbit</td>
<td>S73</td>
<td>1:700 1:10,000</td>
<td>Cell Signaling</td>
<td>3270</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Mouse</td>
<td>-</td>
<td>1:1,000 1:20,000</td>
<td>Cell Signaling</td>
<td>9107</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td>Rabbit</td>
<td>T202/Y204</td>
<td>1:1,000 1:20,000</td>
<td>Cell Signaling</td>
<td>4377</td>
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<tr>
<td>ERK5</td>
<td>Rabbit</td>
<td>-</td>
<td>1:700 1:10,000</td>
<td>Cell Signaling</td>
<td>3552</td>
</tr>
<tr>
<td>p-ERK5</td>
<td>Mouse</td>
<td>T218/Y220</td>
<td>1:200 1:10,000</td>
<td>Santa Cruz</td>
<td>135761</td>
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<tr>
<td>FRA-1</td>
<td>Rabbit</td>
<td>-</td>
<td>1:1,000 1:10,000</td>
<td>Cell Signaling</td>
<td>5281</td>
</tr>
<tr>
<td>p-FRA-1</td>
<td>Rabbit</td>
<td>S265</td>
<td>1:1,000 1:10,000</td>
<td>Cell Signaling</td>
<td>3880</td>
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<td>LC3B</td>
<td>Rabbit</td>
<td>-</td>
<td>1:1,000 1:10,000</td>
<td>Cell Signaling</td>
<td>3868</td>
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<td>Cell Signaling</td>
<td>2352</td>
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<tr>
<td>p-MEK1/2</td>
<td>Rabbit</td>
<td>S217/221</td>
<td>1:700 1:10,000</td>
<td>Cell Signaling</td>
<td>9121</td>
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<td>MEK5</td>
<td>Rabbit</td>
<td>-</td>
<td>1:200 1:10,000</td>
<td>Santa Cruz</td>
<td>10795</td>
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<td>p-MEK5</td>
<td>Rabbit</td>
<td>-</td>
<td>1:200 1:10,000</td>
<td>Santa Cruz</td>
<td>135702</td>
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<td>p-p38 MAPK</td>
<td>Rabbit</td>
<td>T180/Y182</td>
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<td>Rho GDI-α</td>
<td>Rabbit</td>
<td>-</td>
<td>1:2,000 1:20,000</td>
<td>Santa Cruz</td>
<td>360</td>
</tr>
</tbody>
</table>

Senescence assay

Cellular senescence was detected using the Senescence β-Galactosidase Staining Kit according to the manufacturer’s protocol (Cell Signaling). Representative images of treated cells are shown at 100x.

Apoptosis analysis

Cells were grown in 5% CS-DMEM for 48 hours, seeded (2 x 10^5 cells/well) in 12-well plates, and allowed to attach overnight. After 48 hours of treatment (DMSO, SC-79, -151, -181, -79+181), cells were pelleted and analysis of apoptosis was carried out using the Annexin V-FITC Apoptosis Detection Kit in accordance with the manufacturer’s protocol (Sigma, St. Louis, MO, USA). Apoptotic cells were detected by flow cytometry using the Gallios flow cytometer with Gallios software (Beckman Coulter). Results are
represented as cell apoptosis index normalized to vehicle control ± SEM of triplicate experiments.

**Crystal violet viability assay**

Cells were plated at a density of 5,000-10,000 cells, depending on average cell size, per well in a 96-well plate in 5% charcoal-dextran stripped media and allowed to adhere overnight at 37 °C in humidified 5% CO₂. The following day (day 0), cells were treated with drug or vehicle. Plates were harvested on days 0, 3, and 5, fixed with glutaraldehyde, and stained with 1% crystal violet in 10% methanol solution. After morphological changes were observed under an inverted microscope, cells were lysed with 33% acetic acid, and the absorbance was read at 570 nm in a Synergy plate reader (BioTek Instruments, Winooski, VT). Data are represented as mean cell viability normalized to vehicle treatment ± SEM of triplicate experiments with internal duplicates.

**Transwell migration assay**

Breast cancer cells were cultured in 5% DMEM for 48 hours and treated with SC-151 or vehicle for 3 days. 2.5 x 10⁴ cells in 500 µL phenol red-free Opti-MEM were then seeded in the upper chamber of a 24-well transwell chamber. 5% DMEM was used as a chemoattractant in the lower wells. Phenol red-free Opti-MEM was used in one well as a negative control to assess basal migration rates. After 24 hours, inner membranes were scrubbed to remove non-migrated cells. Cells on the outer membranes were fixed in formalin and stained with crystal violet. Membranes were excised from the transwell insert and mounted on glass slides. Number of migrated cells were visualized by
microscopy and counted. Bars represent percent control migrated cells per 200x field of view ± SEM for triplicate experiments.

**Electrical Cell-Substrate Impedance Sensing (ECIS)**

ECIS Model 1600R (Applied Biophysics, Troy, NY) was utilized to monitor cell behavior in real-time (Δt = 160-seconds). Cells were grown on 8W10E+ arrays (Applied Biophysics) containing 40 interdigitated surface electrodes per well. Dispersion of surface electrodes allowed for averaging of the electrical impedance measurements (Z(t,f), frequency = 62.5 Hz to 64 kHz) across the cell monolayer. A small, constant AC current (1 µA, 4kHz) applied across the 250-µm diameter electrodes at the bottom of the ECIS arrays provided live-monitoring capability without causing damage to cell plasma membrane, and the resulting potential across the electrodes was measured by the ECIS instrument, with calculated capacitance and resistance values reported in the ECIS ZΘ software.

**Immunofluorescent staining**

Cells were seeded in 96-well plates at a density of 2,000 cells per well and treated with vehicle or SC-151. For morphometric analysis, cells were fixed in formalin 3 days after drug treatment and permeabilized with Triton X-100 (Sigma, St. Louis, MO, USA). The cytoskeleton was identified with Alexa Fluor® 555-conjugated antibody against phalloidin (1:200; Cell Signaling). Cells were counterstained with DAPI (1:1000; Invitrogen). ApoTome fluorescent images were taken on an inverted microscope (Zeiss, Thornwood, NY) and digitally filtered to obtain optical slices. For Ki-67 analysis, cells were fixed and stained as previously described (Rhodes et al., Molecular Cancer 2010). 5
images per well were captured at 400x, n = 3. Results are represented as percent positive Ki-67 staining (red) of total number of cells visualized by DAPI (blue).

**Image-based morphometric analysis**

Polygonal outline and length measurements tools provided in the AxioVision software (Zeiss) formed the basis for morphometric analysis. Based on information obtained from these tools, four metrics for cellular morphology were identified and defined. Aspect ratio was determined through the use of the length measurement tool. Cellular areal coverage, nucleus to cytoplasm area ratio, and circularity assessments were determined from area and perimeter measurements obtained by the polygonal outline tool. A total of 20 images were taken and analyzed, 10 from each duplicate well. Selection criteria for cells included the following: (1) well-defined border (eliminates most cells in aggregates or dim cells) and (2) must contain only one nucleus (eliminates dividing cells and cells out of plane of focus).

**Activator Protein-1 (AP-1) luciferase assay**

Cells were seeded in 24-well plates at a density of 50,000 cells per well in 5% DMEM and allowed to attach. After 24 hours, cells were transfected with 50 ng pLuc-AP1 plasmid, using 6 µL Effectene (Qiagen) per µg of DNA. After 5-8 hours, cells were treated with vehicle, PMA, or compounds and incubated at 37 °C. After 18 hours, the medium was gently aspirated, and 100 µL lysis buffer was added per well. Cells were lysed on the shaker at room temperature for an hour. 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).
**Immunohistochemical staining**

Tumors were fixed in 10% buffered formalin for 24 to 36 hours. Paraffin-embedded sections (4 µm thickness) mounted on slides were manually deparaffinized in xylene, rehydrated in a series of graded ethanol solutions, steamed in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes before 5-minute incubation with 3% hydrogen peroxide for antigen retrieval. Sections were washed with PBS, blocked for 30 minutes in 10% normal goat serum (Invitrogen), and incubated overnight in primary antibody. The following antibodies were used for IHC: Ki67 (prediluted) (NeoMarkers, Fremont, CA), E-cadherin (1:400), and β-catenin (1:800). After incubation with primary antibody, slides were rinsed in PBS, incubated with biotinylated secondary antibody (Vector labs) for 30 minutes, washed with PBS followed by incubation with ABC reagent (Vector labs) for 30 minutes. Staining was visualized through incubation in 3, 3-diaminobenzidine (DAB) and counterstaining with Harris hematoxylin. As negative control, samples were incubated with either 10% goat serum or non-specific rabbit IgG. After dehydration, slides were mounted with Permount (Fisher) and visualized using a Nikon OPTIPHOT microscope. Bright-field images (200X magnification) were captured by Nikon Digital Sight High-Definition color camera (DS-Fi1) using NIS-Elements BR software.

**Whole genome sequencing and pathway analysis**

BT-549 and MDA-MB-231 cells were treated with vehicle or SC-151 and extracted for total RNA. Changes in gene expression were determined using next generation sequencing as described [140]. Genes significantly up-regulated in both cell lines were pooled and uploaded into the online pathway interaction database (PID).
[http://www.cancer.gov], followed by analysis of significantly down-regulated genes. Based on -log(p-value) calculated from output data, top regulated pathways were determined.

**Animal xenograft studies**

Immune-compromised SCID/beige female mice (4-6 weeks 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*. Breast cancer cells were collected and viable cells in suspension with 50 µL of sterile PBS mixed with 100 µL Matrigel (BD Biosciences, San Jose, CA). Injections were made bilaterally into the inguinal mammary fat pads on day 0 (n = 5 animals/group) using 27 ½ gauge sterile syringes. All the procedures in animals were carried out under anesthesia using a mix of isoflurane and oxygen delivered by mask. For SC-151 studies, animals were treated on day 0 or when tumors were palpable (day 7-11 post cell injection) with either DMSO or SC-151 (25mg/kg). Treatments were administered once daily or only on weekdays (5 days on/2 days off schedule) for approximately 30 days. Details of each mouse study were outlined in **Table 5**. Tumor size was measured biweekly for 30 days using a digital caliper. The volume of the tumor was calculated using the following formula: $4/3 \pi L S^2$, where $L$ is the larger radius and $S$ is the smaller radius. At necropsy, animals were sacrificed by CO$_2$ exposure followed by cervical dislocation. All procedures involving these 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.

Table 5. Outline of in vivo studies.

<table>
<thead>
<tr>
<th>Cell Injection Date</th>
<th>Cell Line</th>
<th>Cells/Injection</th>
<th>Treatment</th>
<th>Schedule of Administration</th>
<th>Treatment Start</th>
<th>Tumor Resection</th>
<th>Necropsy</th>
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<td>11.05.2011</td>
<td>MDA-MB-231</td>
<td>5x10^6</td>
<td>vehicle</td>
<td>once daily</td>
<td>Day 7</td>
<td>Day 29</td>
<td>Day 29</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>SC-151</td>
<td></td>
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<td></td>
</tr>
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<tr>
<td>05.07.2013</td>
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<td>vehicle</td>
<td>once daily</td>
<td>Day 0</td>
<td>Day 30</td>
<td>Day 45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-151</td>
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<tr>
<td>08.13.2014</td>
<td>BT-549</td>
<td>3.5x10^6</td>
<td>vehicle</td>
<td>once daily - weekdays</td>
<td>Day 0</td>
<td>Day 23 - end of treatment (no tumor take)</td>
<td>Day 63</td>
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<td>SC-151</td>
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<td>08.28.2014</td>
<td>MCF7</td>
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<td>Day 11</td>
<td>Day 28</td>
<td>Day 28</td>
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<td>Day 28</td>
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<td>04.12.2016</td>
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<td>Hs-578T-ERK5-ko</td>
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<td>06.20.2016</td>
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<td>Hs-578T-MEK5-ca</td>
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</tbody>
</table>

Patient-derived tumor xenograft

Tissues, procured through the Louisiana Cancer Research Consortium Biospecimen Core, were processed following NIH regulations and institutional guidelines of Tulane University. Triple-negative lumpectomy sample ~15 mm^3 was excised from patient and cut into 4 equal pieces of approximately 3-5 mm^3. Samples were coated with 100 µL of Matrigel (BD Biosciences) and implanted orthotopically into the mammary fat pads of severe combined immune deficient (SCID)/beige mice. After ostensible tumor take was established, tumors were measured using a digital caliper and calculated using the formula 4/3πLS^2 (L = larger radius, S = smaller radius). When tumor size reached 1000 mm^3 in size, tumors were excised, sectioned into pieces approximately 3-5 mm^3, and
transplanted into new SCID/beige mice. Following the first implantation of patient-derived sample, tumor transplants were numbered consecutively, beginning with T1. Tumor samples 2-3 mm³ in size were collected for drug treatment ex vivo. At necropsy, animals were euthanized by exposure to CO₂ followed by cervical dislocation. Tumors, livers, and lungs were removed and snap frozen or fixed in 10% formalin for future analysis. All animal procedures 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 animals programs of Tulane University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

**Statistical analysis**

Statistical analyses were performed using Graphpad Prism software (Graph-Pad Software, Inc., San Diego, CA). Data were subjected to unpaired Student’s t-test, with p-value < 0.05 considered statistically significant. Studies involving more than two groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparison tests. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
CHAPTER ONE: ROLE OF MEK5-ERK5 IN EMT AND BREAST CANCER PROGRESSION

Introduction

The mitogen-activated protein kinase (MAPK) pathway has well-established roles in cellular processes vital to tumor initiation and progression including proliferation, differentiation, and regulation of cell fate, namely survival and apoptosis [15]. Conventional MAPK family members, including c-Jun N-terminal protein kinases (JNK), p38, and MAPK/extracellular signal-regulated kinases (MEK) 1/2, have been extensively characterized in cancer with outlined mechanisms of signal transduction and regulation of key processes, yet the role MEK5-ERK5 in cancer pathophysiology is poorly defined. Recent studies have highlighted the importance of the MEK5 pathway in metastatic progression of prostate cancer [73], colon cancer [122], and bone cancer [127].

Previous studies in our lab have implicated MEK5 signaling in the activation of epithelial-to-mesenchymal transition (EMT) and progression to hormone independence in estrogen receptor positive breast cancer cells [114]. Furthermore, elevated levels of ERK5 expression and activity have been shown in breast carcinomas [114] and have been linked to worse prognosis in triple-negative breast cancer (TNBC) patients [108]. In this report, we used the CRISPR-Cas9 approach to knock out ERK5 expression in clinically aggressive TNBC cells and examined the effects on tumorigenic potential at the primary and metastatic sites.
Results

*ERK5 is involved in maintenance of the EMT phenotype in TNBC cells.*

To assess the role of ERK5 in EMT regulation, we used the CRISPR/Cas9 knock-out approach in phenotypically mesenchymal triple-negative breast cancer cell lines. Expression of ERK5, examined by western blot, was significantly ablated in both MDA-MB-231- and Hs-578T-ERK5-ko cells (0.027-fold and 0.063-fold, respectively, p < 0.001) compared to parental cells (Figure 2).

![Western Blot](image)

**Figure 2. Confirmation of ERK5-ko cells.** ERK5 expression was knocked out in MDA-MB-231 and Hs-578T cells using the CRISPR/Cas9 system (Horizon). Total protein was extracted from TNBC-parental and -ERK5-ko cells and western blot was performed for total ERK5 expression. Rho-GDIα served as a loading control. Bars represent normalized protein density ± SEM with parental control cells set to 1, n ≥ 3. *** p < 0.001.
Morphological changes, from a spindle fibroblast-like shape to a more rounded epithelial phenotype, induced by ERK5-knockout and upregulation of CDH1 transcript (6.13-fold, p < 0.05) and protein (4.37-fold, p < 0.05) expression in MDA-MB-231 cells suggested a partial reversal of EMT (Figure 3). While phenotypic alterations were less apparent in Hs-578T-ERK5-ko cells, analysis of gene expression revealed upregulation of CDH1 (3.28-fold, p < 0.05) and downregulation of EMT-associated genes, including matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM) degradation, in both ERK5-ko cell lines compared to parental cells (Figure 4).

**Figure 3. Loss of ERK5 expression in TNBC cells promotes an epithelial phenotype.** (A) Cell morphology of parental and ERK5-ko cells, viewed at 200x magnification. (B) TNBC cells were plated in dextran stripped phenol free DMEM for 48 hours prior to RNA extraction for gene expression analysis by qPCR. (C) Western blot of CDH1 protein expression in ERK5-ko cells compared to parental MDA-MB-231 cells. * p < 0.05.
Figure 4. ERK5-ko alters expression of EMT-associated genes. (A) MDA-MB-231- or (B) Hs-578T-parental and -ERK5-ko cells were cultured in dextran stripped phenol free DMEM for 48 hours prior to RNA extraction for gene expression analysis by qPCR. * p < 0.05; ** p < 0.01.

Consistent with induction of mesenchymal-to-epithelial transition (MET), loss of ERK5 expression hindered transwell motility in vitro by 30.2% and 79.4% in MDA-MB-231 and Hs-578T cells, respectively, compared to parental control (Figure 5). Proliferation of TNBC cells, assessed by Ki-67 immunofluorescent staining, was not significantly affected by ERK5 knock-out, therefore anti-migratory effects observed were not convoluted by differences in proliferative rates (Figure 6).

Figure 5. ERK5-ko cells exhibit decreased migration potential. Migration assay for MDA-MB-231- and Hs-578T-parental and -ERK5-ko cells. 2.5x10⁴ cells were seeded in transwell inserts. After 24 hours, migrated cells were fixed, stained with crystal violet, and quantified. Bars represent average number of migrated cells normalized to parental cells ± SEM of triplicate experiments. * p < 0.05; ** p < 0.01.
MEK5 signaling promotes mesenchymal features.

To further elucidate MEK5 signaling involvement in maintenance of the EMT axis, we stably transfected MEK5 using a phosphomimetic mutant plasmid in TNBC cell lines. Constitutive activation of MEK5, confirmed by qPCR and western blot (Figure 7), downregulated CDH1 levels and promoted expression of EMT-related genes (Figure 8A-C). Moreover, MEK5 constitutively active (MEK5-ca) cells showed greater migration potential, 3.25-fold (p < 0.01) of MDA-MB-231- and 1.24-fold (p < 0.05) of Hs-578T-
vector cells (Figure 8D). Cell proliferation was not altered by enhanced MEK5 activity (Figure 9).

Figure 7. Confirmation of MEK5-ca cells. MDA-MB-231 and Hs-578T cells were transfected with vector or MEK5DD plasmid. Cells were treated with selectable marker (puromycin). Viable colonies were cloned and pooled for analysis. qPCR was performed on (A) MDA-MB-231- and (B) Hs-578T-vector and -MEK5-ca cells for MEK5 expression. (C) Total protein was extracted from TNBC-MEK5-ca cells and western blot was performed for total MEK5 expression. Rho-GDIα served as a loading control. Bars represent normalized protein density ± SEM and vector-control cells set to 1, n ≥ 3. * p < 0.05; ** p < 0.01.
Figure 8. Constitutive activation of MEK5 downregulates CDH1 expression and enhances TNBC cell migration. (A) qPCR analysis of CDH1 expression in TNBC-vector and -MEK5-ca cells. (B) MDA-MB-231- or (C) Hs-578T-vector and -MEK5-ca cells were cultured in dextran stripped phenol free DMEM for 48 hours prior to RNA extraction for gene expression analysis by qPCR. (D) Transwell migration assay for MDA-MB-231- or Hs-578T-parental and -ERK5-ko cells. 2.5x10⁴ cells were seeded in an insert (8 µm pore). After 24 hours, migrated cells were fixed, stained with crystal violet, and quantified. Bars represent average number of migrated cells normalized to parental cells (set to 100%) ± SEM of triplicate experiments. * p < 0.05; ** p < 0.01; *** p < 0.001.
As proof of principle, ERK5-knockout cells were transiently transfected with MEK5-ca, ERK5, or vector constructs. Re-expression of MEK5 family members did not significantly alter expression of CDH1 or select MEK5-regulated genes (MEF2C and MEF2D) compared to vector control (Figure 10). These results suggest that concurrent overactivation of MEK5 and expression of ERK5 is necessary to induce changes in gene transcription.

Figure 9. Effects of MEK5 constitutive activation on cell proliferation. (A) IF staining of Ki-67 in MDA-MB-231-vector and -MEK5-ca cells, viewed at 200x. (B) 5 representative images were taken per well and percentage of Ki-67-positive cells relative to total (Dapi-positive) cells was calculated. Bars represent mean % of Ki-67 positive cells ± SEM of triplicate experiments.
ERK5 ablation impairs tumor formation in vivo.

ERK5 has been implicated in MEK5-induced hormone-independent tumor formation in luminal breast cancer cells [114], yet shRNA-mediated knockdown of ERK5 did not significantly affect primary tumor growth of MDA-MB-231 TNBC cells [84, 126]. To elucidate the effects of complete CRISPR-Cas9-mediated ERK5 ablation on tumorigenesis, we used an orthotopic xenograft model. SCID/beige animals were inoculated with TNBC-parental and -ERK5-ko cells into the mammary fat pad and monitored for tumor formation. Primary tumor growth of ERK5-ko cells was significantly impaired, with tumor weight 6.2 times lower than that of MDA-MB-231 parental xenografts (p < 0.001) on day 28 post cell injection (Figure 11), supporting a role for ERK5 in cancer progression.
Figure 11. ERK5 is essential for MDA-MB-231 tumorigenesis in vivo. (A) Female SCID/beige mice (n = 5/group) were bilaterally injected into the MFP with 5x10^6 MDA-MB-231-parental and -ERK5-ko cells. Tumor size was measured biweekly using a digital caliper, and tumor volume was calculated with the formula \(4/3 \pi Ls^2\), where \(L\) = long radius and \(s\) = short radius. Data points represent mean tumor volume ± SEM. (B) At day 28 post cell injection, primary tumors were excised and weighed. Animals were allowed to continue to monitor for distant metastasis. *** p < 0.001.

In contrast, knockout of ERK5 in Hs-578T cells did not significantly alter tumor volume of xenografts compared to that of parental tumors at endpoint (Figure 12A). However, ERK5-ko cells formed smaller tumors than parental cells days 10-18 post inoculation, suggesting the involvement of ERK5 in tumor growth. To further explore the role of MEK5 in Hs-578T tumorigenesis, we examined tumor formation of MEK5-ca cells and observed no significant differences in growth between Hs-578T-vector and -MEK5-ca xenografts (Figure 12B).
In culture TNBC-ERK5-ko cells exhibited a shift in morphology and gene expression signature to a more epithelial phenotype from their mesenchymal parental counterparts. To determine impact of ERK5 knock-out on the EMT phenotype in vivo, parental and -ERK5-ko xenograft sections were examined using immunohistochemistry (IHC). While edges of ERK5-ko tumors stained more positively for CDH1 than parental control tumors, no significant change was observed in CDH1 expression between ERK5-ko and parental control tumors overall (Figure 13).

Figure 12. Hs-578T tumor formation in vivo. Female SCID/beige mice (n = 5/group) were bilaterally injected into the MFP with (A) 2x10^6 Hs-578T-parental and -ERK5-ko cells or (B) 4x10^6 Hs-578T-vector and -MEK5-ca cells. Tumor size was measured biweekly using a digital caliper, and tumor volume was calculated with the formula 4/3 π L*s^2, where L = long radius and s = short radius. Data points represent mean tumor volume ± SEM.
Loss of ERK5 suppresses metastatic colonization.

Given the prevalence of ERK5 expression in metastatic malignancies [73], [122], [127], we then examined the effects of ERK5 knock-out on metastatic progression following surgical resection of the primary tumor on day 28 post inoculation. Animals were monitored for 4 weeks, then lungs were harvested for analysis at necropsy. Metastatic burden was visibly and quantifiably reduced in lung sections of MDA-MB-231-ERK5-ko-injected mice. Greater area of metastasis and number of metastatic lesions was evident in animals injected with parental TNBC cells compared to ERK5-ko cells (Figure 14). There was no observed difference in metastatic potential between Hs-578T control and ERK5-ko tumors (Figure 15).
Figure 14. Loss of ERK5 suppresses MDA-MB-231 lung metastasis in vivo. At necropsy 30 days post survival surgery, lungs were harvested and fixed in formalin. (A) Hematoxylin and eosin (H&E) staining of representative lungs from each group, visualized using Aperio ScanScope slide scanner, demonstrate decreased metastasis in the ERK5-knockout injected animals. Magnified regions were viewed at 200x. (B) Total area of metastases was quantified using Aperio ImageScope software (Leica Biosystems). Points represent total area of metastases per lung section ± SEM. Parental, n = 3; ERK5-ko, n = 4. (C) Number of metastatic lesions was determined. * p < 0.05, *** p < 0.001.
MEK1/2 inhibition augments effects of ERK5 knock-out.

While the MEK1/2 and MEK5 cascades may be distinct in structure and stimuli, they share common effector pathways and gene targets [62]. Combined gene expression signature of ERK1/2 and ERK5 is strongly correlated with decreased overall and metastasis-free survival (hazard ratio 13.31, p < 0.05 and 3.99, p < 0.01, respectively) in breast cancer patients (Figure 16). To explore potential additive effects of dual MEK blockade, we employed commercially available MEK1/2 inhibitors in our ERK5-knockout cell lines. CDH1 induction was amplified in drug-treated ERK5-ko cells compared to parental control (Figure 17A), in concordance with a significant repression of cell motility observed in the former (Figure 17B). Together, these findings suggest

**Figure 15. Metastatic potential of Hs-578T-parental and -ERK5-ko cells in vivo.** At necropsy 30 days post survival surgery, lungs were harvested and fixed in formalin. Hematoxylin and eosin (H&E) staining of representative lungs from each group, visualized using Aperio ScanScope slide scanner, depict low metastatic potential of Hs-578T tumors. Scale bar = 4 mm; magnified regions were viewed at 200x.
that both MEK1/2 and MEK5 are involved in regulation of the mesenchymal phenotype and present a need to dually target the MEK pathways to fully inhibit the EMT axis.
Figure 16. High expression of ERK1/2 and ERK5 in BC patients is associated with decreased survival. Using combined gene expression of MAPK3/1 and MAPK7 encoding ERK1/2 and ERK5 proteins, respectively, plots of (A) metastasis-free survival (HR: 13.31) and (B) overall survival (HR: 3.99) in breast cancer patients were generated. Goswami, CP. *BMC Cancer* 2014.

Figure 17. MEK1/2 inhibition augments effects of ERK5-k0. (A) qPCR analysis of CDH1 expression in MDA-MB-231-parental and -ERK5-k0 cells following 24-hr treatment with vehicle (DMSO) or MEK1/2 inhibitors (1 μM). Bars represent fold change of CDH1 expression normalized to vehicle-treated parental control cells (set to 1) ± SEM of triplicate experiments. (B) Transwell migration assay for MDA-MB-231-parental and -ERK5-k0 cells after 3-day treatment with vehicle or MEK1/2 inhibitors (1 μM). 2.5x10^4 cells were seeded in an insert (8 μm pore). After 24 hours, migrated cells were fixed, stained with crystal violet, and quantified. Bars represent average number of migrated cells normalized to parental vehicle-treated cells (set to 100%) ± SEM of triplicate experiments. * p < 0.05; ** p < 0.01.
Discussion

Metastasis accounts for the majority of cancer-related deaths. Epithelial-to-mesenchymal transition (EMT) is a transient and reversible process characterized by morphological and molecular changes that enable epithelial cells to adopt a motile and invasive phenotype vital to cancer metastasis [9]. Dysregulated MEK5 signaling is associated with metastatic risk in prostate, breast, colon, kidney, bone, and oral cancers and less favorable survival outcome [73, 114, 122, 127-130]. However, studies have described conflicting roles of ERK5 in modulating the metastatic phenotype [126, 138], prompting our investigation of MEK5/ERK5 signaling in regulation of the EMT axis in highly aggressive breast cancer cells.

In this study, we used the CRISPR-Cas9 approach successfully knock out ERK5 in two triple-negative breast cancer cell lines MDA-MB-231 and Hs-578T. In concordance with other published work [73, 74, 126], we observed that stable depletion of ERK5 induced the reversal of EMT, marked by suppression of EMT-associated gene signature and upregulation of CDH1 expression. Moreover, migration potential was significantly reduced in ERK5-ko cells compared to that of parental control, supporting other results previously reported through use of a dominant-negative ERK5 construct [131], while constitutive activation of MEK5 enhanced TNBC cell migration. Our data suggest that ERK5 signaling promotes a motile phenotype. These in vitro findings were recapitulated in our in vivo MDA-MB-231 xenograft model demonstrating the vital role of ERK5 expression and activity in formation of metastases. Consistent with current literature [84], Hs-578T-parental and -derived cells were minimally invasive in vivo, attributing to the lack of contrast in metastatic potential between control and ERK5-ko tumors. Additional
ERK5-knockout cell lines are needed to further define the complex role of MEK5 signaling in regulation of metastasis. To this end, characterization of MEK5-ca tumor metastasis would contribute to elucidating MEK5 involvement in breast cancer malignancy, as overexpression of MEK5 or ERK5 has previously been shown to promote a migratory and invasive phenotype in prostate and colon cancer cells [121, 125], evidenced by increase in tumor metastases [73, 122].

The MEK5/ERK5 cascade has been emerging as an important mediator of cell proliferation through induction of cell cycle regulators, including cyclin D1, c-MYC, SGK, RSK2, and NF-κB [59, 65, 68-71]. Constitutive activation of MEK5 in prostate and colon cancer cell lines accelerated cell cycle progression and increased proliferation [111, 121, 122]. Similarly, ERK5 knockdown studies using RNA interference (RNAi) or pharmacological inhibition by XMD8-92 treatment delayed cell cycle progression and decreased proliferation in various cancer types, including the breast cancer cell lines MDA-MB-231, Hs-578T, and BT549 [108]. Our results showed that neither MEK5 constitutive activation nor ERK5 ablation altered in vitro proliferation of MDA-MB-231 cells. Interestingly, loss of ERK5 expression significantly impaired tumor formation in vivo, contrary to studies showing that shRNA-mediated knockdown of ERK5 did not affect tumor growth of MDA-MB-231 TNBC cells [84, 126]. This discrepancy suggests that complete knockout of ERK5 expression is necessary to suppress tumorigenesis. Indeed, it has been shown that the degree of ERK5 silencing exerts differential effects based on cell line specificity. For example, 70% ERK5 inhibition in liver cancer cells (SNU449) decreased proliferation while not affecting apoptosis, whereas 90% reduction of ERK5 expression in esophageal cancer cells (KYSE30) resulted in suppression of cell
growth and significant induction of cell death [124]. Preclinical research has consistently confirmed potent anti-tumor activity of ERK5-specific inhibitor XMD8-92 in several cancer cell lines [72, 101, 111]. Here, we have provided a molecular basis for continued analysis of ERK5 as a viable therapeutic target.

MEK1/2 inhibitors (MEK1/2i) have been investigated mainly in the K-Ras or B-Raf mutant cancer setting, where cells display addiction to MEK1/2 activity [47, 48]. As a monotherapy, MEK1/2i efficacy is hindered by resistance, due in part to activation of compensatory growth pathways [57]. Within the MAPK signaling network, the MEK1/2 and MEK5 pathways share common downstream targets [59]; therefore, MEK5 has been proposed as a contributor to MEK1/2i resistance [60, 61]. We found that elevated expression of ERK1/2 and ERK5 in BC patients is associated with lower overall and metastasis-free survival. In ERK5-k0 cells, treatment with pharmacological inhibitors of MEK1/2 exerted additive effects on induction of MET and repression of cell migration, supporting therapeutic potential of combined MEK1/2,5 inhibition. Collectively, our data implicate MEK5 signaling as a driver of cell motility and a pro-metastatic factor, laying groundwork for anti-ERK5 therapy to prevent cancer progression.
CHAPTER TWO: EVALUATION OF SINGLE-AGENT DUAL TARGETING MEK1/2 AND MEK5 IN TRIPLE-NEGATIVE BREAST CANCER

Introduction

Breast cancer is a heterogeneous disease, stratified into molecular subtypes based on hormone receptor status and gene expression profile [2]. Approximately 15% of breast tumors are triple-negative, denoted by a lack of estrogen and progesterone receptor expression as well as non-amplification of human epidermal growth factor receptor 2 (HER2). Triple-negative breast cancer (TNBC) does not respond to endocrine therapy, the mainstay in treatment of estrogen receptor positive (ER+) breast cancer, or other targeted agents, limiting systemic treatment options to cytotoxic chemotherapy [3]. Correlated with enhanced metastatic potential and higher mortality rate, TNBC presents a clinical challenge due to the aggressive nature of the disease and lack of targetable markers [8]. Although TNBC constitutes a lower percentage of diagnosed cases, there is an increased need for therapy.

Constitutive activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway has been linked to chemoresistance and metastatic progression through distinct mechanisms, including activation of epithelial-to-mesenchymal transition (EMT) whereby cells adopt a motile and invasive phenotype through loss of epithelial markers, namely Cadherin 1/E-Cadherin (CDH1),
and acquisition of mesenchymal markers, such as vimentin (VIM) and Cadherin 2/N-Cadherin (CDH2) [10, 141, 142]. While MAPK/ERK1/2 kinase inhibitors (MEK1/2is) have shown promise as antitumor agents in the preclinical setting, application has had limited success clinically [50-53]. Activation of compensatory signaling, potentially contributing to the emergence of drug resistance, has shifted the therapeutic strategy to combine MEK1/2 inhibitors with agents targeting upstream oncoproteins (RAF) or parallel growth pathways (PI3K) [54, 55, 57, 58].

Within the MAPK signaling cascades, convergence of the MEK1/2 and MEK5 networks on downstream targets highlights the importance of the latter understudied pathway in cancer progression [59]. Previous studies in our lab have demonstrated the role of ERK5 in maintenance of the mesenchymal phenotype in TNBC cells. Moreover, concurrent inhibition of MEK1/2 and MEK5 signaling through pharmacological and genetic approaches, respectively, exerted additive effects on mesenchymal-to-epithelial transition (MET) induction and suppression of cell migration. In this report, we evaluated the effects of a novel allosteric/non-ATP competitive MEK1/2 and MEK5 inhibitor (pan-MEKi) SC-151 (Table 6), developed and synthesized by our collaborators at Duquesne University, on a panel of mesenchymal and highly metastatic breast cancer cell lines.
Table 6. Allosteric MEK1/2 and/or MEK5 inhibitors.

<table>
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<tr>
<th>Structure</th>
<th>Compound ID</th>
<th>Chemical Class</th>
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<td>diphenyl aniline</td>
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<tr>
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<td>diphenyl aniline</td>
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<tr>
<td>(C_{18}H_{19}F_2N_3O)</td>
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Results

*Dual inhibition of MEK1/2 and MEK5 exerts molecular subtype-specific effects on breast cancer cell lines.*

Duquesne compounds with MEK1/2,5-selective inhibition activity, measured by relative phosphorylation of corresponding ERKs, were screened in the TNBC cell line MDA-MB-231 (*Figure 18A*). Compared to other compounds, pan-MEK inhibitor SC-151 exhibited the most robust effects in suppression of cell viability and migration as well as induction of epithelial marker CDH1, suggesting a role for dual MEK1/2,5 inhibition in targeting the EMT axis (*Figure 18B-D*). Furthermore, FITC-Annexin V/PI flow cytometry analysis revealed that pan-MEKi treatment of MDA-MB-231 cells significantly increased apoptotic index, normalized to vehicle control, in comparison to singular MEK1/2 and MEK5 inhibitors SC-79 and SC-181, respectively (*Figure 19*).

*In vitro,* SC-151 treatment yielded differential biological effects based on the molecular subtype of breast cancer cell lines. Specifically, pan-MEK inhibition reduced cell viability (*Figure 20A*) and migration in more aggressive TN cell lines (MDA-MB-231, BT-549, MDA-MB-157) but not estrogen receptor positive (ER+) cell lines (MCF-7, ZR-75, T47D) (*Figure 21A*). Furthermore, SC-151 treatment suppressed MDA-MB-231 cell invasion compared to vehicle control (*Figure 21B*). To determine if the pro-apoptotic and anti-proliferative effects observed *in vitro* (*Figure 19* and 20B&C, respectively) translated to the *in vivo* setting, we used an orthotopic xenograft model. SCID/beige mice were inoculated with either MCF-7 or MDA-MB-231 cells and given SC-151 treatment. There was no observed difference between MCF-7 xenografts treated with SC-151 and vehicle (*Figure 22A&B*). In contrast, treatment with pan-MEKi
significantly reduced MDA-MB-231 average tumor volume compared to the vehicle-treated group (Figure 22C).

To further evaluate the anti-tumor efficacy of the pan-MEK inhibitor in a more translational setting, we established a TN patient-derived xenograft (PDX) model 2O0. Animals were implanted with 2-3 mm$^3$ tumor pieces and randomized into treatment groups once palpable tumors formed. SC-151 treatment markedly inhibited tumorigenesis of PDX-2O0 xenograft compared to vehicle control (Figure 22D), consistent with results using the TN cell line MDA-MB-231 model. Together, these data demonstrate a role for pan-MEK inhibition in TNBC subtypes.
Figure 18. Effects of MEK inhibition on MDA-MB-231 cells. (A) Cells were treated with MEK inhibitors and phosphorylation of ERK1/2 and ERK5 was measured. (B) Cells were treated with vehicle (DMSO) or SC-151 (10 μM) for crystal violet viability assay. Data are represented as mean cell viability normalized to vehicle treatment ± SEM of triplicate experiments with internal duplicates. (C) qPCR analysis of CDH1 expression in MDA-MB-231 cells following treatment with MEK inhibitors. (D) Cells were cultured in charcoal-stripped DMEM and treated with MEK inhibitors (1 μM) or vehicle control. After 3 days, 2.5x10^9 cells were seeded in a transwell insert (8 μm pore). After 24 hours, migrated cells were fixed, stained with crystal violet, and quantified. Bars represent average number of migrated cells normalized to vehicle-treated cells (set to 100%) ± SEM of triplicate experiments. * p < 0.05; ** p < 0.01.
Figure 19. Pan-MEK inhibitor induces apoptosis. MDA-MB-231 were grown in 5% CS-DMEM for 48 hours, seeded (2 x 10^5 cells/well) in 12-well plates, and allowed to attach overnight. After 48 hours of treatment (DMSO, SC-79, -151, -181), cells were pelleted and analysis of apoptosis was carried out using the Annexin V-FITC Apoptosis Detection Kit. Apoptotic cells were detected by flow cytometry using the Gallios flow cytometer with Gallios software. Results are represented as cell apoptosis index normalized to vehicle control ± SEM of triplicate experiments. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 20. Pan-MEKi downregulates proliferation of TNBC cells in vitro. (A) Cells from TNBC cell lines (MDA-MB-231, Hs-578T, BT-549), ER-positive cell lines (MCF-7, ZR-75), and ER-negative cell line (BT-474) were treated with vehicle or SC-151 (1 µM, 10 µM, 100 µM) for crystal violet proliferation/viability assay. Data are represented as mean cell viability normalized to vehicle treatment ± SEM of triplicate experiments with internal duplicates. (B) Representative images (> 5) were taken per well and percentage of Ki-67-positive cells relative to total (Dapi-positive) cells was calculated. Bars represent mean % of Ki-67 positive cells ± SEM of triplicate experiments. (C) Immunofluorescent staining of Ki-67 in MDA-MB-231 cells treated with vehicle or SC-151 for 24 hours, viewed at 200x. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 21. SC-151 suppresses TNBC migration and invasion. (A) Transwell migration assay of breast cancer cells following 3-day treatment of SC-151. Bars represent average number of migrated cells normalized to vehicle-treated cells (set to 100%) ± SEM of triplicate experiments. (B) Transwell invasion assay of MDA-MB-231 cells following 3-day treatment with pan-MEK inhibitor. Bars represent average number of invaded cells normalized to vehicle-treated cells (set to 100%) ± SEM of triplicate experiments. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 22. Pan-MEKi inhibits TNBC tumorigenesis in vivo. (A) Female SCID/beige mice (n = 5/group) were bilaterally injected into the MFP with 5x10^6 MCF-7 cells. Beginning on day 11 post cell injection, mice were treated IP with vehicle or SC-151 (25 mg/kg) 5 days/week for 18 days. Bars represent normalized average tumor volume ± SEM. (B) MCF-7 tumor weight (g) of vehicle and SC-151-treated groups at day 28 post cell injection. (C) Female SCID/beige mice (n = 5/group) were bilaterally injected into the mammary fat pad (MFP) (day 0) with 1x10^6 MDA-MB-231 cells. Beginning on day 0, mice were treated intraperitoneally (IP) with vehicle or SC-151 (25 mg/kg) once daily for 31 days. Results represent average tumor volume ± SEM. (D) Female SCID/beige mice (n = 5/group) were bilaterally implanted into the mammary fat pad (MFP) with 2-3 mm^3 pieces of 200 PDX. On day 1, mice were randomized into treatment groups and were treated, beginning on day 4, intraperitoneally with vehicle or SC-151 (25 mg/kg) once daily for 25 days. Results represent average tumor volume ± SEM. * p < 0.05, *** p < 0.001.
Pan-MEKi induces a phenotypic shift in TNBC cells.

We employed our lead pan-MEKi SC-151 as a pharmacological approach to determine the combined role of MEK1/2 and MEK5 in regulation of the EMT axis. Through immunofluorescence staining of actin filaments, we performed semi-quantitative, image-based analysis to measure alterations in MDA-MB-231 cell morphology in response to pan-MEK inhibition. Following SC-151 treatment, TNBC cells transformed from a mesenchymal phenotype to a more epithelial-like state, denoted by a significant increase in areal coverage (quantification of cell spread) and circularity compared to vehicle treatment (Figure 23A&B), suggesting the reversal of EMT.

To validate qPCR data from Figure 18C, SC-151-induced upregulation of CDH1 expression in MDA-MB-231 cells was analyzed by western blot (Figure 23C). Triple-negative PDX tumors treated ex vivo with our lead pan-MEK inhibitor also exhibited increased levels of CDH1 and CD24 expression compared to vehicle-treated tumors (Figure 23D). In TNBC cell lines, EMT-related gene transcripts were downregulated with pan-MEK inhibition (Figure 24A). Using combined gene expression analysis, plot of overall survival in breast cancer patients was generated, demonstrating a 3-fold increase in hazard ratio associated with high expression of this EMT gene signature (Figure 24B). Additionally, pan-MEKi treatment suppressed expression of genes associated with invasion (MMP1 and MMP9), cell cycle progression (FOS, MYC, and cyclin D1), and angiogenesis (CD31) (Figure 25A). We also tested effects of MEK1/2,5 inhibition in MCF-7-derived cell lines FR (fulvestrant resistant) and with truncated CXCR4 (ΔCTD) as models of mesenchymal-phenotype breast cancer cell, more aggressive and less differentiated than their parental counterparts, and observed reduction
in transcript levels of genes linked to EMT induction (Figure 25B). We further verified that morphological changes induced by SC-151 were indicative of MET, not senescence or autophagy (Figure 26). Together, these data show that pan-MEK inhibition targets the mesenchymal phenotype and EMT axis.
Figure 23. Dual inhibition of MEK1/2.5 promotes epithelialization of TNBC models. 
(A) MDA-MB-231 cells were seeded in 96-well plates at a density of 5,000 cells/well and treated with vehicle (DMSO) or SC-151. The cytoskeleton was identified with AlexaFluor 555-conjugated antibody against phalloidin. Cells were counterstained with DAPI. ApoTome fluorescent images were taken on an inverted microscope (Zeiss) and digitally filtered to obtain optical slices. Polygonal outline and length measurements tools provided in the AxioVision software (Zeiss) formed the basis for morphometric analysis. 
(B) Based on information obtained from these tools, four metrics for cellular morphology were identified and defined. (C) Western blot analysis of CDH1 expression of MDA-MB-231 cells following 24-hour treatment with vehicle or SC-151 (1 μM). (D) qPCR analysis of CDH1 and CD24 expression in PDX tumors following ex vivo 3-day treatment with pan-MEK inhibitor (1 μM). * p < 0.05.
**Figure 24. Inhibitor of MEK1/2,5 targets the EMT axis.** (A) Gene expression changes of TNBC cells were analyzed by qPCR following 24-hour SC-151 treatment. Bars represent average fold change normalized to vehicle ± SEM of triplicate experiments. * p < 0.05; ** p < 0.01; *** p < 0.001. (B) Using combined gene expression of selected EMT genes, plot of overall survival in breast cancer patients was generated. Goswami, CP. *BMC Cancer* 2014.
Figure 25. SC-151 alters gene expression in mesenchymal-phenotype cells. (A) Triple-negative or (B) MCF7-derived cells were cultured in dextran stripped phenol free DMEM for 48 hours and treated with SC-151 (1 µM). After 24 hours, cells were pelleted and extracted for RNA. qPCR analysis was performed for EMT- and MEK-associated gene expression. Bars represent average fold change normalized to vehicle (set to 1) ± SEM of triplicate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 26. MEKi treatment does not affect cellular senescence or autophagy in MDA-MB-231 cells. (A) Cellular senescence was detected using the Senescence β-Galactosidase Staining Kit according to the manufacturer’s protocol. Representative images of treated cells are shown at 100x. Senescent cells are flagged by arrows. (B) Western blot of LC3B in MDA-MB-231 cells. Image shown is representative of triplicate experiments.
Effects of lead pan-MEKi SC-151 are both rapid and durable.

Electrical cell-substrate impedance sensing (ECIS) was used to characterize the onset of effects exerted by pan-MEKi on TNBC cell morphology and movement, measured by capacitance and resistance-$\Omega$. SC-151 treatment enhanced formation of cell-cell interactions and monolayer development, marked by an increase in resistance-$\Omega$ and decrease in capacitance (Figure 27), respectively. Capacitance and resistance values were statistically different between vehicle- and drug-treated cells at 13 hours following treatment, suggesting a rapid cellular transformation from a mesenchymal to epithelial phenotype. As cells become confluent on top of the electrode, the capacitance and resistance-$\Omega$ values of vehicle- and drug-treated cells converge.

To evaluate the durability of the effects exerted by pan-MEKi, we treated MDA-MB-231 cells with SC-151 and measured transcript levels of CDH1 by qPCR at indicated times (Figure 28A). Expression of CDH1 in SC-151-treated cells steadily increased from the 3-day to 10-day timepoints and was significantly upregulated at day 10 compared to vehicle control cells (Figure 28B). Cells cultured in regular media for 4 days post 3-day treatment with SC-151 maintained CDH1 levels comparable to transcript expression detected on day 3.

To determine if this pan-MEKi-induced epithelial phenotype was recapitulated in vivo, we quantified CDH1 staining of MDA-MB-231 xenografts treated with vehicle or SC-151. Pan-MEKi-treated tumors exhibited a 4.37-fold increase in percent of cells positive for CDH1 expression compared to that of vehicle-treated tumors (Figure 29). Lung tissues were also analyzed for p-ERK1/2 and p-ERK5 in control group and animals treated with pan-MEKi. Expression levels of p-ERK1/2 and p-ERK5 were reduced in the
SC-151-treated tumors compared with control tumors, confirming SC-151 suppression of ERK1/2,5 activity in TNBC xenografts (Figure 30).

Figure 27. Pan-MEKi alters ECIS Profile of TNBC cells. 2.5x10^5 MDA-MB-231 cells were seeded in 8-well chamber 8W10E+ slides. (A,B) Resistance (4 kHz) and (C,D) capacitance of MDA-MB-231 cells was measured in real-time using ECIS system. 67 hours after seeding, cells were treated and monitored for 24 hours. Data points represent mean ± SD, n = 5. One-way ANOVA followed by Dunnett post hoc test was used for statistical comparison. * p < 0.05.
### Figure 28. SC-151 exerts durable effects on CDH1 induction in MDA-MB-231 cells.

(A) Schematic of experiment. (B) At time points indicated, cells were collected and pellets were extracted for total RNA. qPCR was performed for CDH1 expression. Bars represent fold change normalized to vehicle-treated cells (set to 1). * p < 0.05.

### Figure 29. Pan-MEKi increases CDH1 expression in MDA-MB-231 xenografts.

Tumors were sectioned and stained by IHC, viewed at 200x. Bars represent percentage of stained cells per view, n = 28. *** p < 0.001.
Inhibition of MEK1/2,5 decreases metastasis of PDX model.

Animals inoculated with MDA-MB-231 cells were sacrificed and lungs harvested on day 29 post cell-injection. Analysis of lung sections revealed no significant change in metastatic burden between SC-151- and vehicle-treated animals (Figure 31). Using our TN patient-derived xenograft model, we observed represssion of metastasis in the SC-151

Figure 30. IHC staining of phosphorylated ERK1/2,5 in MDA-MB-231 tumors following SC-151 in vivo treatment. Stained tumor sections were visualized using Aperio ScanScope slide scanner, viewed at 40x and 100x with scale bars = 500 μm and 200 μm, respectively.

_inhibition of MEK1/2,5 decreases metastasis of PDX model._
treatment group compared to vehicle control group (Figure 32). Variance in duration of study could account for these discordant results, as differences in MDA-MB-231 metastatic colonization altered by pan-MEK inhibition may not be detectable by day 29 while the PDX experiment was carried out to day 60.

Figure 31. SC-151 treatment does not significantly affect MDA-MB-231 lung metastasis in vivo at day 29 post cell injection. At necropsy, lungs were harvested and fixed in formalin. (A) Hematoxylin and eosin (H&E) staining of representative lungs from each treatment group, visualized using Aperio ScanScope slide scanner. Scale bar = 4 mm; magnified regions were viewed at 200x. (B) Total area of metastases was quantified using Aperio ImageScope software (Leica Biosystems). Points represent total area of metastases per lung section ± SEM, n = 5 per group. (C) Number of metastatic lesions was determined.
upregulated CDH1 expression in vector cells, this effect was abrogated by constitutive

**Figure 32. Pan-MEK inhibition decreases metastasis of 2O0 patient-derived xenograft.** At necropsy, lungs were harvested and fixed in formalin. (A) Hematoxylin and eosin (H&E) staining of representative lungs from each group, visualized using Aperio ScanScope slide scanner, demonstrating reduced metastatic lesions in the SC-151-treated animals compared to control group. Scale bar = 4 mm; magnified regions were viewed at 100x with scale bar = 100 µm. (B) Total area of metastases was quantified using Aperio ImageScope software (Leica Biosystems). Points represent total area of metastases per lung section ± SEM, n = 3 per group. (C) Number of metastatic lesions was determined. * p < 0.05.

**Hyperactivation of MEK5 abolishes SC-151-mediated MET**

To investigate the requirement for inhibition of the MEK5 pathway in the induction of MET, response to SC-151 was determined. While pan-MEK inhibition strongly upregulated CDH1 expression in vector cells, this effect was abrogated by constitutive
activation of MEK5 (Figure 33). Moreover, levels of CDH1 in MEK5-ca cells were not restored to baseline expression of vector cells even with SC-151 treatment. To analyze biological effects of MEK5 constitutive activation in pan-MEKi-treated TNBC cells, we examined differences in proliferation using IF staining of Ki-67. In vector cells pan-MEKi treatment decreases Ki-67-positive cells by 28.1% compared to vehicle control (p < 0.05), but in MEK5-ca cells treated with SC-151 the change in cell proliferation is not statistically significant from vehicle treatment (Figure 34), confirming that effects of the compound are contingent on dual inhibition of the MEK1/2 and MEK5 pathways.

Figure 33. Constitutive activation of MEK5 abrogates pan-MEKi-induced CDH1 expression. qPCR was performed for CDH1 expression in MDA-MB-231 cells treated with SC-151 for 24 hours. Bars represent fold change normalized to vehicle-treated cells, set to 1. ** p < 0.01; *** p < 0.001.
Figure 34. MEK5 constitutive activation alters SC-151 suppression of cell proliferation. (A) IF staining of Ki-67 in MDA-MB-231-vector and -MEK5-ca cells treated with vehicle or SC-151 for 24 hours, viewed at 200x. (B) Bars represent mean % of Ki-67 positive cells ± SEM of triplicate experiments. * p < 0.05.
MEK1/2,5 inhibition promotes epithelialization of ERK5 knock-out cells

The importance of both the MEK1/2 and MEK5 pathways have been shown in regulation of the EMT axis. Because our lead pan-MEKi does not fully block MEK5 signaling (Figure 18), we treated MDA-MB-231-ERK5-ko cells with SC-151 to determine if MET-inducing effects would be amplified in the absence of ERK5. Inhibition of MEK1/2,5 resulted in escalation of ECIS-resistance values (Figure 35), indicative of increased cell-to-cell contact, in ERK5-ko cells and further drove them towards a more epithelial and less motile phenotype (Figure 36A&B). While expression of cell migration-associated gene early growth response 1 (EGR1) was significantly downregulated in MDA-MB-231 cells treated with SC-151 compared to vehicle treatment, pan-MEK inhibition did not significantly affect EGR1 transcript levels in ERK5-ko cells due to already low basal expression of this gene (Figure 36D). This suggests that the MEK5 pathway is the principal mediator of EGR1. In contrast, cyclin D1 and MYC transcript levels were not altered by ERK5-ko in MDA-MB-231 cells, but expression of these genes was significantly reduced following SC-151 treatment (Figure 36C), denoting the importance of MEK1/2 inhibition in targeting these cell-cycle regulators.
Figure 35. Pan-MEK\textsubscript{i} promotes formation of intercellular interactions of ERK5-ko cells. 2.5\times 10\textsuperscript{5} MDA-MB-231-parental or -ERK5-ko cells were seeded in 8-well chamber 8W10E\textsuperscript{+} slides. (A,B) ECIS plot and graph demonstrating changes in resistance-Ohm with time. Media was changed at \sim 24 hours to allow cells to grow to confluence prior to treatment with SC-151 (1 \mu M), applied at \sim 24 hours. Values represent mean \pm SD, n = 3. ** p < 0.01; *** p < 0.001.
Figure 36. SC-151 drives ERK5-ko cells to a more epithelial phenotype. (A) Western blot of CDH1 protein expression in ERK5-ko cells compared to parental MDA-MB-231 cells. (B) Migration assay of MDA-MB-231-ERK5-ko cells pretreated for 3 days with SC-151. (C-D) qPCR analysis of MDA-MB-231-parental and -ERK5-ko cells treated with vehicle (DMSO) or SC-151. Bars represent raw cycle numbers normalized to actin. * p < 0.05; ** p < 0.01; *** p < 0.001.
Discussion

The emergence of drug resistance has hindered the success of monotherapies, shifting the therapeutic strategy to combination anti-cancer treatments and highlighting the use of multi-kinase inhibitors [54, 55, 57, 58, 103]. Activation of MEK5 signaling has been proposed as a mechanism of drug resistance to MEK1/2 inhibitors, owing to the high degree of overlap in downstream substrates [60, 61]. Accordingly, preclinical data have shown compensatory signaling by MEK5 to rescue cancer cell growth as a result of MEK1/2 inhibition [143]. At another point of convergence, the MEK1/2 and MEK5 pathways have both been implicated in regulation of epithelial-to-mesenchymal transition, an integral part of metastasis, contributing to disease progression: constitutively active MEK1 has been shown to promote EMT and cancer cell invasion [141], and the role of MEK5 in induction of a mesenchymal and malignant phenotype has been documented by our lab as well as others [114, 121, 125]. Furthermore, ERK1/2 and ERK5 activity is associated with disruption of the actin cytoskeleton, resulting in enhanced cell motility, and inhibition of both kinase pathways is necessary to restore actin dynamics [144].

In this study, we evaluated the biological effects of single-pathway inhibitors and novel dual inhibitors of MEK1/2 and MEK5 (pan-MEKis), developed and synthesized by our collaborators at Duquesne University, on highly invasive triple-negative breast cancer cells MDA-MB-231. We found that pan-MEKi SC-151 was more effective at repressing the EMT phenotype, evidenced by downregulation of cell migration and induction of CDH1 expression, than singular targeting of the MEK pathway. Treatment of SC-151 also decreased cell proliferation and induced apoptosis. We expanded our analysis of
pan-MEK inhibition to other BC molecular subtypes and observed cell type-specific effects, as TNBC cell lines were more sensitive to SC-151-mediated effects on cell viability and migration compared to responses seen in ER-positive or HER2-amplified cells. In accordance with these *in vitro* data, pan-MEK inhibition potently suppressed tumor growth both in the MDA-MB-231 cell line and 200 patient-derived xenograft models of triple-negative breast cancer, whereas tumorigenesis of ER+ MCF-7 xenograft was not affected by drug treatment. Cell-line specificity of SC-151 was anticipated based on lower incidence of MEK5 amplification and activation in ER+ compared to TN breast cancer subtypes, and in using an unbiased screen, basal-type cell lines were found to be more sensitive to MEK1/2 inhibitors than their luminal-type counterparts [108, 114, 145]. Based on clinical practices to evaluate drug inhibition of MEK activity [146], we assessed efficacy of our compound through IHC staining of MDA-MB-231 primary tumors for phosphorylated ERK1/2 and ERK5 and confirmed dual pathway suppression.

Given the link between malignant progression and EMT, strategies targeting this process have emerged in cancer therapy [9, 10]. Here, we demonstrate that MEK1/2,5 inhibition induces morphological and molecular changes indicative of EMT reversal; these effects are both rapid and durable with measurable alterations in cell behavior 13 hours post treatment and persisting for 10 days. Overexpression of EMT master regulators such as SNAI1 and TWIST as well as loss of CDH1 has been well-documented in invasive cancers [11]. In addition to targeting the mesenchymal phenotype, pan-MEKi suppressed cancer stem cell-like characteristics. The combined EMT and CSC signature is associated with increased hazard ratio and lower overall survival in BC patients, suggesting the therapeutic benefit of pan-MEK inhibition.
Consistent with *in vitro* findings on induction of CDH1 expression, SC-151-treated TNBC tumors stained strongly for membranous CDH1 at the tumor periphery. While CDH1 expression at the invasive front has been correlated with lower metastatic potential [147], pan-MEK inhibition did not decrease MDA-MB-231 formation of lung metastases at day 29 post cell injection, potentially due to insufficient length of the study to allow for development of metastases. In foreign tissue micrometastases may persist in a state of dormancy until they are able to adapt and restore their growth mechanisms [148]. In a follow-up 60-day study using our established TN patient-derived xenograft model, SC-151 treatment significantly reduced metastatic burden, highlighting the potential clinical utility of MEK1/2,5 inhibitors as PDXs closely retain molecular characteristics of the original tumor.

The role of MEK1/2 in cancer progression is well-established, and development of its inhibitors has far surpassed that of the MEK5 pathway. In this report, we have shown that both pathways regulate the EMT axis. In particular, pan-MEK1-induced CDH1 expression is dependent on inhibition of MEK5. We have also identified ERK5, and not ERK1/2, as a regulator of EGR1, a transcription factor known to promote cancer cell growth and metastasis through direct regulation of target genes such as TGFβ1, fibronectin, as well as tumor suppressors PTEN and p53 [149]. While studies have demonstrated transcriptional activation of EGR1 by MEK1/2 [62, 150], here we present evidence to the contrary and support a link between MEK5 and EGR1. Collectively, our data advocate dual targeting of MEK1/2 and MEK5 in phenotypically mesenchymal and aggressive breast cancer cells to hinder tumor progression.
CHAPTER THREE: IDENTIFICATION OF FRA-1 AS A POTENTIAL TARGET OF PAN-MEK INHIBITION

Introduction

The transcription factor Activator protein 1 (AP-1) regulates a broad spectrum of cellular processes, many of which are vital to oncogenic transformation such as proliferation, differentiation, migration, and apoptosis [151]. The heterodimeric AP-1 complex is composed of members of the Fos (c-Fos, FosB, FRA-1, and FRA-2) and Jun (c-Jun, JunB, and JunD) family (Figure 37). In breast cancer, Fos-related antigen-1 (FRA-1) expression is associated with an invasive phenotype, as higher transcript levels of FRA-1 are observed in more aggressive triple-negative tumors compared with luminal subtypes, and consequently poor prognosis [152, 153]. FRA-1 stimulates expression of pro-motility factors, including those involved in focal adhesion, extracellular matrix degradation, and epithelial-to-mesenchymal transition (EMT) [154-156]. The role of FRA-1 in malignant progression is further substantiated by in vivo xenograft studies demonstrating that FRA-1 depletion in highly invasive breast cancer and colon cancer cell lines impairs metastatic spread [154, 157].

Growth factor-induced activation of AP-1 components is predominantly mediated by the MAPK pathway [158]. Activated ERK1/2 increases FRA-1 expression at the transcriptional level and also phosphorylates the protein, thereby stabilizing FRA-1 to prevent its proteolytic degradation as well as promoting AP-1 function [151, 159].
MEK5-ERK5 signaling is also involved in regulation of FRA-1 activity by an alternative undefined mechanism [66]. In the present study, we evaluated FRA-1 as a downstream target of our novel pan-MEK inhibitor SC-151. Additionally, we investigated the contribution of MEK5 signaling to expression and activation of FRA-1.

**Figure 37. AP-1 activation by MEK1/2 and MEK5.** Fos and Jun family members heterodimerize to form the AP-1 complex, which regulates a variety of cellular processes.
**Results**

*Whole-transcriptome analysis of pan-MEK inhibition in TNBC cells*

To identify the mechanism by which pan-MEK inhibition suppresses migratory capacity of mesenchymal cells, we used RNA-seq to analyze global gene expression changes induced by SC-151 treatment in TNBC cell lines. Of the 436 and 542 genes significantly altered in MDA-MB-231 and BT-549 cells, respectively, following pan-MEK inhibition, expression levels of 4 genes were upregulated and 26 genes were downregulated across both cell lines (Figure 38). Analysis generated by the Pathway Interaction Database demonstrated that AP-1-regulated transcription was the top regulated pathway and implicated FRA-1 as a pan-MEKi target (Figure 39). To validate these results, we expanded to other triple-negative breast cancer cell lines and observed significant reduction of FRA-1 expression with SC-151 treatment compared to vehicle control (Figure 40A). Furthermore, pan-MEK inhibition decreased protein levels of total and phosphorylated FRA-1 (p-FRA-1) in MDA-MB-231 cells (Figure 40B&C). Downregulation of FRA-1 functional activity was further validated using AP-1 luciferase assay following SC-151 treatment (Figure 40D). Through qPCR analysis, we observed that pan-MEKi-mediated suppression of FRA-1 persisted for 10 days post treatment in MDA-MB-231 cells (Figure 41). While FRA-1 levels were steadily restored after removal of drug, FRA-1 was still significantly repressed at day 7 post drug removal (7dΔ) compared to vehicle control (Figure 41).

Our previous results demonstrated that anti-migratory effects induced by pan-MEKi SC-151 were molecular subtype-specific (Figure 21). To determine the role of FRA-1 in MEK1/2- and MEK5-mediated migration, we examined effects of pan-MEK
inhibition on FRA-1 expression and activation across a subset of HER2+ (BT-474 and SKBR3) and ER+ (MCF-7, ZR-75, and T47D) breast cancer cell lines using western blot analysis. SC-151 treatment did not alter total or phosphorylated FRA-1 protein levels compared to vehicle control, consistent with our results showing that pan-MEKi did not inhibit migratory potential of ER+ or HER2+ breast cancer cells.
Figure 38. Next Generation Sequencing Analysis of TNBC cell lines treated with SC-151. BT-549 and MDA-MB-231 cells were treated with SC-151 or vehicle for 24 hours and extracted for total RNA. Changes in gene expression were determined using next generation sequencing as described (Miller et al. 2014). Venn diagrams illustrating (A) increased or (B) decreased gene expression across the cell lines following SC-151 treatment. (C) Summary of gene changes induced by SC-151. p < 0.01
**Figure 39. Whole transcriptomic analysis of SC-151 treatment on TNBC cell lines.**

(A) Fold change of genes altered by pan-MEK inhibition in BT-549 and MDA-MB-231 cells. (B) Genes significantly up-regulated in both cell lines were pooled and uploaded into the online pathway interaction database (PID) [http://www.cancer.gov], followed by analysis of significantly down-regulated genes. Based on -log(p-value) calculated from output data, top regulated pathways were determined.
Figure 40. Pan-MEK inhibition downregulates FRA-1 expression and activity in TNBC cells. (A) qPCR analysis of FRA-1 expression in TNBC cell lines treated with SC-151. Bars represent average fold change normalized to vehicle-treated cells ± SEM of triplicate experiments. BT-549 cells were treated with 10 µM of compound. (B) WB of total and (C) phosphorylated FRA-1 expression in MDA-MB-231 cells following 24-hour SC-151 treatment. (D) MDA-MB-231 cells were transfected with the AP-1 luciferase reporter construct and treated with SC-151 for 8 hours before cell lysis. Bars represent percent relative luciferase units (RLUs) ± SEM, n = 3 with internal duplicates. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 41. Durable suppression of FRA-1 in MDA-MB-231 cells treated with SC-151. At time points indicated on the x-axis, cells were collected and pellets were extracted for total RNA. qPCR was performed for FRA-1 expression. Bars represent fold change normalized to vehicle-treated cells, set to 1. ** p < 0.01; *** p < 0.001.
Figure 42. FRA-1 protein levels of ER+ and HER2+ cells are not altered by pan-MEK inhibition. Western blot analysis of total and phosphorylated FRA-1 protein following 24-hour SC-151 treatment (1 μM) in breast cancer cells. Images are representative of at least two independent experiments.
Effects of FRA-1 overexpression or constitutive activation on pan-MEKi response

To elucidate FRA-1 regulation by pan-MEK inhibition, we stably transfected MDA-MB-231 cells with FRA-1 or vector control plasmids. FRA-1 stable clones were confirmed through qPCR and western blot (Figure 43). SC-151 response was evaluated following overexpression of FRA-1. While total FRA-1 protein expression was similar in vehicle and SC-151-treated FRA-1-overexpressing cells, phosphorylated and activated FRA-1 was diminished with SC-151 treatment (Figure 44A&B). As expected, FRA-1 overexpression did not abrogate the anti-migratory effects of pan-MEK inhibition compared to vehicle control (Figure 44C).

![Figure 43](image)

**Figure 43. Confirmation of FRA-1 overexpression in MDA-MB-231 cells.** (A) qPCR and (B) Western blot confirmation of FRA-1 expression in pooled populations of MDA-MB-231 cells transfected with vector control or FRA-1 plasmid. (C) phosphorylated FRA-1 protein expression. Rho-GDIα served as a loading control. * p < 0.05.
Figure 44. SC-151 overcomes effects of FRA-1 overexpression in MDA-MB-231 cells. (A) Western blot of FRA-1 total and (B) phospho protein expression in FRA-1-overexpressing cells treated with SC-151 for 24 hours. (C) Transwell migration assay of FRA-1 overexpressing cells following 3-day treatment with SC-151. Bars represent average number of migrated cells normalized to vehicle-treated cells (set to 100%) \pm \text{SEM}, n = 3. ** p < 0.01; *** p < 0.001.
Stable lines expressing myc-tagged, phospho-mutants of FRA-1 or vector plasmid were derived from MDA-MB-231 cells (Figure 45). Generously provided by Dany Chalbos and Marc Piechaczyk, S265D and S265D-T223D-T230D constructs were used as a model of FRA-1 constitutive activation, where serine/threonine were replaced with aspartic acid residues, and the S252A/S265A plasmid, with serine mutated to alanine, as a kinase-dead mimic [160]. Ectopically expressed Fra-1 and endogenous Fra-1 was distinguished by the myc-tag. As shown in Figure 46A, MEK1/2,5 inhibition did not significantly affect the expression of the myc-tagged Fra-1 protein while decreasing total FRA-1 levels in FRA-1(D) cells. Cell migration was reduced by 72.1% in vector cells treated with SC-151 compared to 38.6% decrease in SC-151-treated FRA-1(D) cells versus vehicle control, indicating that constitutive activation of FRA-1 antagonized the anti-migratory effects induced by MEK1/2,5 inhibition (Figure 46B).

![Figure 45. Confirmation of FRA-1 constitutive active and kinase dead mutants.](image_url)

Western blot validation of FRA-1 expression in pooled populations of MDA-MB-231 cells transfected with vector control or FRA-1 (D, DDD, or AA) mutant plasmids. Representative image is shown, n = 3.
Figure 46. Effects of FRA-1 mutations on SC-151 response. (A) Western blot analysis for Myc and FRA-1 expression in MDA-MB-231-derived cells treated with vehicle or SC-151 (1 μM) for 24 hours, n ≥ 3. (B) Transwell migration assay of vector or constitutively active FRA-1 cells following 3-day treatment with SC-151. Images are viewed at 40x, with scale bar = 200 μm. Bars represent average number of migrated cells normalized to vehicle-treated cells (set to 100%) ± SEM, n = 3. * p < 0.05; ** p < 0.01; *** p < 0.001. ⊙ denotes dataset compared to SC-151-treated vector cells.
MEK5 signaling in regulation of FRA-1

Both the MEK1/2 and MEK5 cascades have been shown to regulate expression and activity of FRA-1. To discern individual contributions of each pathway in mediating the effects of our lead pan-MEKi, we examined FRA-1 expression in the TNBC-ERK5-ko cell lines previously generated. Levels of total FRA-1 protein were comparable between parental and ERK5-ko cells, but in MDA-MB-231 cells, loss of ERK5 decreased phosphorylated FRA-1 expression by over half of p-FRA-1 levels detected in parental cells (Figure 47). Conversely, constitutive activation of MEK5 stimulated expression of total and phosphorylated FRA-1 protein in both TNBC cell lines compared to that of parental (Figure 48A&B). To determine the requirement of MEK5 signaling in response to pan-MEK inhibition, we treated MEK5-ca cells with SC-151 and observed significant repression on FRA-1 expression and activation in comparison to vehicle-treated cells (Figure 48C&D).
Figure 47. Expression of FRA-1 in ERK5-ko cells. Western blot analysis of (A) total and (B) phosphorylated FRA-1 expression in MDA-MB-231- and Hs-578T- parental and ERK5-ko cells. Representative image is shown, n ≥ 2. ** p < 0.01.
Figure 48. MEK5 regulation of FRA-1 expression. Western blot analysis of (A) FRA-1 and (B) p-FRA-1 expression in TNBC cells, n ≥ 2. Western blot analysis for (C) FRA-1 and (D) p-FRA-1 expression in Hs-578T-parental and -MEK5-ca cells treated with vehicle or SC-151 (1 µM) for 24 hours, n = 3. * p < 0.05; ** p < 0.01.
Discussion

Our previous findings demonstrate that MEK1/2 and MEK5 inhibition via treatment with pan-MEKi SC-151 induced reversal of EMT and decreased cell migration, promoting a less aggressive phenotype in triple-negative breast cancer cells. Here we used whole transcriptomic analysis to identify FRA-1 as a potential target of pan-MEK inhibition based on its significant downregulation in both MDA-MB-231 and BT-549 cells treated with SC-151, and analysis through Pathway Interaction Database confirmed AP-1 as the top regulated pathway. Given MEK1/2 and MEK5 involvement in its modulation [66, 151, 159], FRA-1 is a viable target of the dual inhibitor. Although downregulation of FRA-1 has been shown to impair EMT and promote epithelial characteristics [152, 161], interestingly, in our system the MET effects appeared to be separate from anti-migratory effects: SC-151 suppressed FRA-1 in TNBC cells, but induction of CDH1 was only detected in cell lines MDA-MB-231 and MDA-MB-468 (Figure 25A). Furthermore, FRA-1 activation of EMT occurs through upregulation of ZEB1/2 [160], while pan-MEKi-mediated EMT reversal involves targeting expression of SNAI1 and TWIST but not ZEB1/2, suggesting a FRA-1-independent mechanism (Figure 24).

In accordance with our results showing breast subtype-specific effects of pan-MEK inhibition (Figures 20&21), SC-151 repression of FRA-1 was observed only in TNBC cells, not ER+ or HER2 amplified cell lines. Because pan-MEKi acted on both FRA-1 expression and activation, we used molecular approaches to determine which was crucial in regulating cell migration. Pan-MEKi-mediated downregulation of phosphorylated FRA-1 and subsequent cell migration was not affected by FRA-1 overexpression in
MDA-MB-231 cells. In contrast, constitutive activation of FRA-1 using a phosphomimetic mutant partially negated anti-migratory effects exerted by MEK1/2,5 inhibition, indicating that post-translational modification of FRA-1 is important to migratory capacity. Depletion of endogenous FRA-1 protein expression by pan-MEKi may account for suppression of cell migration still evident in FRA-1 constitutively active cells treated with SC-151. While the role of FRA-1 has been well-established in migration and invasion, denoting its potential as a therapeutic target, FRA-1 is difficult to target pharmacologically by small molecule inhibitors [155]. Our data demonstrate pan-MEKi-mediated suppression of FRA-1 activity, providing preclinical efficacy to support further investigation of targeting MEK1/2,5 in migratory and aggressive cancer cells.
CONCLUDING REMARKS

Growth factor pathways, especially within the MAPK signaling network, exhibit a high degree of crosstalk and redundancy, enabling cancer cells to adapt and become refractory to single pathway inhibition. Indeed, MEK1/2 inhibitor efficacy as a monotherapy is hindered by resistance, due in part to activation of compensatory growth pathways such as MEK5 [57, 60, 61, 143]. Here, we described the role of MEK5 signaling in regulation of the mesenchymal phenotype and tumor progression. We also evaluated the effects of a novel pan-MEK inhibitor and provided strong preclinical evidence of its efficacy in triple negative breast cancer cell lines as well as patient-derived xenograft models. As targeted therapy for triple-negative breast cancer is still in its infancy, these data present a viable therapeutic strategy, warranting further investigation into mechanisms by which MEK1/2 and MEK5 individually modulate the EMT axis. To this end, our lab is continuing to analyze the transcriptional network altered by kinase interactions in EMT regulation. Additionally, as MEK inhibition has been shown to sensitize resistant cancer cells to targeted therapies, synergistic and sensitizing effects of SC-151 combined with inhibitors of alternative signaling pathways as well as kinases upstream of MEK must be examined.
APPENDIX

List of Publications

Van T. Hoang, Thomas J. Yan, Jane E. Cavanaugh, Patrick T. Flaherty, Barbara S. Beckman, Matthew E. Burow. MEK5-ERK5 Signaling in Cancer: Implications for Targeted Therapy.


REFERENCES


143. de Jong, P.R., et al., ERK5 signalling rescues intestinal epithelial turnover and tumour cell proliferation upon ERK1/2 abrogation. Nat Commun, 2016. 7: p. 11551.


BIOGRAPHY

Van T. Hoang was born in Saigon, Vietnam as the youngest child of Nguyen Thi Tuyet Nga and Hoang Van De. Duc is her only sibling. In 2005 she graduated from Orange Park High School, where she served as flute leader in marching band. She completed her undergraduate training at University of Florida with a B.S. in Chemical Engineering in May 2010. Following graduation Van worked as an intern in the Product Development sector at Bacardi until March 2011. In the fall she enrolled in the Biomedical Sciences Graduate Program at Tulane University School of Medicine in New Orleans, LA. Subsequently, she joined the laboratory of Dr. Matthew E. Burow, who supported her doctoral studies in conjunction with the Biomedical Sciences Program. She has accepted a post-doctoral position at the National Cancer Institute and will be working under the mentorship of Dr. John Brognard in January 2017.