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The KRas/MAPK Pathway and Ligand Independent Activation of ERα: Implications for the Treatment of Endometrial Cancer

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A

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DEDICATION

To my husband Zack, and children Kiersten and Jackson. Thank you for supporting me, picking me up when I fall, and making me laugh when I need it the most.

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The KRas/MAPK Pathway and Ligand Independent Activation of ERα: Implications for the Treatment of Endometrial Cancer

Kari L. Ring, M.D.

Thesis Advisor: Karen H. Lu, M.D.

Hormonal therapy remains a first line option for the treatment of recurrent endometrial cancer (EC), however, many tumors demonstrate de novo or acquired resistance. Member kinases of the PI3K/AKT and Ras/MAPK pathways activate estrogen receptor α (ERα) independent of estrogen, however, few studies have evaluated the role of the Ras/MAPK pathway in predicting response to hormonal therapy in EC. The aims of this project were to evaluate the role of ligand independent activation of ERα in EC and to explore therapeutic implications for the treatment of recurrent EC.

A xenograft model for recurrent EC was used to evaluate the effect of treatment with letrozole, everolimus, and metformin *in vivo*. These studies demonstrated that tumors with an activating KRas mutation are resistant to treatment with letrozole even in combination with everolimus. Tumor growth and cellular proliferation were reduced only after the addition of metformin.

To assess signaling through ERα, cells with and without an activating KRas mutation were stimulated with estradiol and phosphorylation at serine 167 (ser167) and serine 118 (ser118) evaluated. KRas mutant cells had decreased expression of ERα and this decrease in expression was mirrored in functional proteomic analysis. KRas mutant cells had no detectable phosphorylation at ser167 and decreased phosphorylation at ser118 in response to estradiol stimulation, which was restored following treatment with a MEK inhibitor.

To address the functional consequence of differential estrogen signaling expression of estrogen induced genes and cell viability assays were evaluated. Following treatment with a MEK inhibitor, mutant KRas cells had increased expression of estrogen-induced genes compared to cells with wild type KRas, mirroring the increase in phosphorylation at ser167 and ser118. Treatment of mutant KRas cells with a MEK inhibitor in the presence of estradiol had no effect, while treatment with a MEK inhibitor in the absence of estradiol resulted in decreased cell viability.

Endometrial cancer cells harboring KRas mutations are functionally ER negative and are resistant to treatment with hormonal therapy. The addition of hormonal therapy to MEK inhibition may provide added benefit for patients with recurrent endometrial cancer compared to either therapy alone, with an improved side effect profile.

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1. Introduction

1.1 Overview

Endometrial cancer remains the most common gynecologic cancer in the United States with an estimated 52,630 new cases and 8,590 deaths in 2014(1). The majority of women who present with endometrial cancer are diagnosed with early stage disease and have a favorable overall survival of approximately 80-90%(2). Unfortunately, women who experience recurrence, specifically recurrence outside of the pelvis, have poor response to current therapies with an overall survival of 12 months(3).

First line treatment for recurrent endometrial cancer includes hormonal therapy or cytotoxic therapy in the form of carboplatin and paclitaxel, however, the addition of targeted therapies based on tumor molecular profiles has recently been explored(4, 5). The most common molecular pathways currently under investigation in the treatment of endometrial cancer include the Phosphatidylinositol 3-Kinase (PI3K)/Protein Kinase B (AKT) and Ras/mitogen-activated protein kinase (MAPK) pathways given the high rates of mutations in these pathways in endometrial cancer(6-13).

In addition, endometrioid endometrial adenocarcinoma is a hormonally driven cancer. Unopposed estrogen exposure as a result of exogenous estrogen, such as post-menopausal hormone replacement therapy, or obesity is one of the most important risk factors for the development of this disease(2). Indeed, while patients with low grade, hormone positive endometrial cancer have a favorable prognosis,

this patient population accounts for the majority of recurrences, given that 80% of endometrial cancers fit into this subtype(2).

There is a significant body of literature supporting the role of the PI3K/AKT pathway and the Ras/MAPK pathway in cancer in general and in the pathogenesis of endometrial cancer specifically. As a result, there are numerous clinical trials currently open using inhibitors of these two pathways alone and in combination. In addition, hormonal therapies alone and in combination with inhibitors of the PI3K/AKT pathway have been investigated in endometrial cancer. However, no studies have addressed the interplay of all three pathways and the implications this interplay may have on the success of targeted therapy in the treatment of endometrial cancer.

1.2 The PI3K/AKT Pathway

The PI3K/AKT pathway plays a key role in cellular growth, metabolism, and survival(14). Endometrial cancer has the highest rate of molecular aberrations in the PI3K pathway compared to other cancer types and the PI3K/AKT pathway is the most commonly altered pathway in endometrial cancer(15, 16).

There are 3 distinct classes of PI3Ks, which are categorized based on structure, substrate specificity, and lipid products. Class IA PI3Ks are the most extensively studied class of PI3Ks and have been implicated in human cancer. Class IA PI3Ks exist as heterodimers made up of a p110 catalytic and p85 regulatory subunit. The PI3K pathway can be activated by a variety of sources including ligand bound receptor tyrosine kinases (RTK), G protein-coupled receptors, and activated Ras(17).

In response to ligand binding, p85 binds to specific phosphotyrosine residues on activated RTKs and releases its inhibition of the p110 catalytic subunit. This causes PI3K to localize to the plasma membrane where it is able to interact with its substrate, phosphatidylinositol 4,5-biphosphate (PIP2). The p110 catalytic subunit can also be directly activated by G-protein coupled receptors and activated Ras(18, 19).

Activated PI3K phosphorylates PIP2, resulting in the conversion of PIP2 to phosphatidylinositol 3,4,5-triphosphate (PIP3). This process is negatively regulated by the tumor suppressor phosphatase and tensin homolog (PTEN). PIP3 binds the pleckstrin homology (PH) domain of phosphoinositide-dependent kinase 1 (PDK1) and AKT, both serine/threonine kinases, and positions the two proteins in close proximity(20). This allows PDK1 to phosphorylate and activate AKT at threonine 308(21, 22). In addition, the mTOR-Rictor (rapamycin insensitive companion of mTOR) complex (mTORC2) phosphorylates AKT at serine 473(23). Phosphorylation at both threonine 308 and serine 473 is necessary for full activation of AKT. Once activated, AKT acts as the central node of this pathway and controls multiple downstream cellular processes.

One of the key downstream targets of AKT is the mammalian target of rapamycin (mTOR) complex. Phosphorylated AKT inactivates the tuberous sclerosis 1/2 (TSC1/2) complex by inhibiting rheb GTPase activity and releases its inhibition on the mTOR-Raptor (mTOR-regulatory-associated protein) complex (mTORC1)(14). This complex then phosphorylates eukaryotic initiation factor 4E-

binding protein (4E-BP) and p70 ribosomal S6 kinase (p70S6K) which promotes protein synthesis and cellular proliferation (Figure 1).

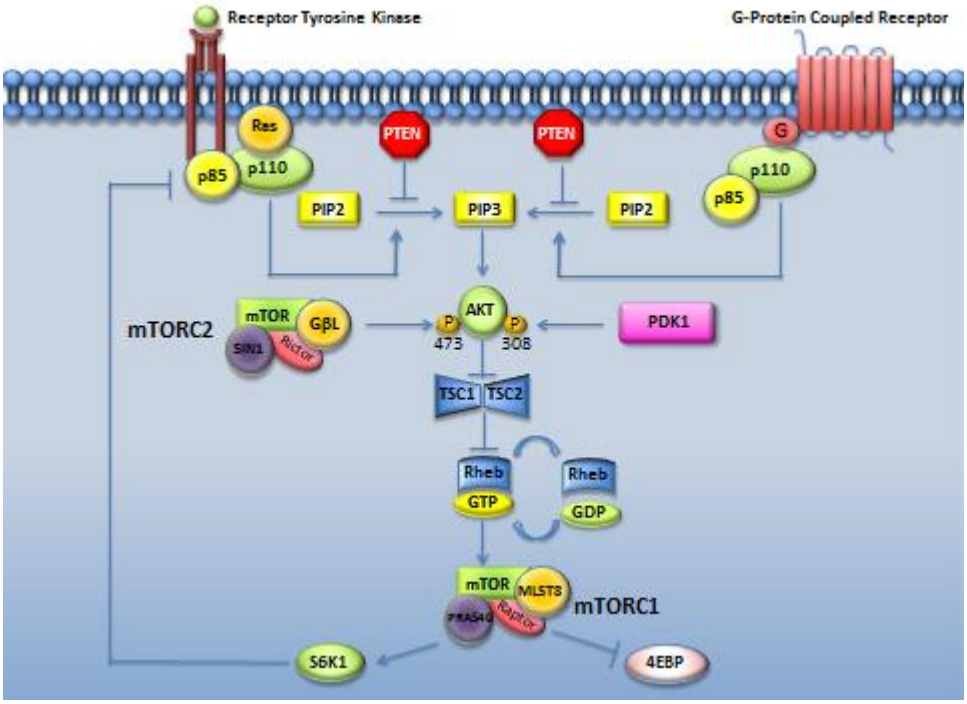


Figure 1. Overview of the PI3K/AKT pathway.

Molecular alterations can occur in multiple effectors in the PI3K/AKT pathway. Activating somatic mutations can be found in the p85 regulatory unit (PIK3R1 and PIK3R2), the p110 catalytic subunit (PIK3CA), and in AKT(6-10). In addition, both mutation and loss of expression of the tumor suppressor PTEN is seen in 34% and 80% of endometrioid endometrial tumors respectively, leading to uncontrolled activation of AKT (11-13).

1.3 The Ras/MAPK Pathway

A second pathway known to play an important role in endometrial cancer is the Ras/MAPK pathway, which mediates cellular survival, proliferation, angiogenesis, and motility(24). Like the PI3K/AKT pathway, the Ras/MAPK pathway can be activated by numerous sources including receptor tyrosine kinases, G protein-coupled receptors, cytokine receptors, and integrins(6, 24, 25).

The Ras family of proteins, including the Kristen murine sarcoma viral oncogene homolog (KRas), NRas, and the Harvey rat sarcoma viral oncogene homolog (HRas) are small GTPases that reside in the cellular plasma membrane. Ras proteins cycle between the inactive guanosine diphosphate (GDP) bound and active guanosine triphosphate (GTP) bound conformation and act as the upstream switch for activation of downstream effectors in the MAPK pathway(26-28). Docking sites for adaptor molecules and signal-relay proteins are created when ligands bind to receptor tyrosine kinases. These activated receptor complexes contain adaptor proteins including SH2-containing protein (SHC), growth factor receptor bound protein 2 (GRB2), and GRB2-associated binding (Gab) protein. This complex recruits SH2 domain-containing protein-tyrosine phosphatase (SHP2) and Son of Sevenless (SOS)1, which activate guanine nucleotide-exchange factors (GNEFs) and displace guanine nucleotides from Ras. This process allows Ras to bind to GTP, resulting in activated Ras(28, 29).

Activated Ras recruits the serine/threonine Raf family of kinases, including A-Raf, B-Raf, and C-Raf to the plasma membrane. Binding of Raf to Ras results in phosphorylation of Raf proteins which in turn phosphorylate and activate MEK1 and

MEK2 at multiple serine residues. MEK1 and MEK2 are dual specificity serine/threonine and tyrosine kinases that activate and phosphorylate extracellular signal regulated-kinase (ERK)1 and ERK2 at both threonine and tyrosine residues. MEK1 and MEK2 have no known downstream effectors other than ERK1 and ERK2. However, ERK1 and ERK2 have multiple downstream effectors, including p90RSK, which control a diverse number of cellular processes including cellular proliferation, apoptosis, and motility (Figure 2)(30-32).

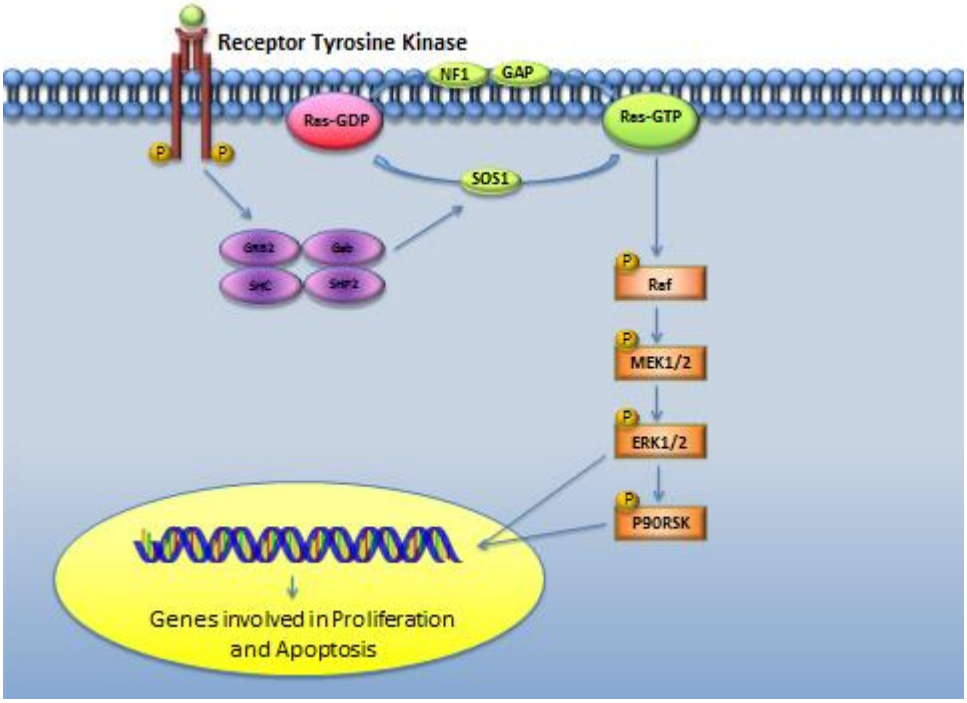


Figure 2. Overview of the Ras/MAPK pathway

Oncogenic alterations in the Ras/MAPK pathway are found in endometrial cancer in the form of activating KRas mutations. Mutant KRas is found in 10-30%

of endometrioid endometrial cancers and frequently co-occurs with alterations in the PI3K/AKT pathway (6, 7, 33-37).

1.4 Estrogen Receptor Alpha (ERα)

It is well established that one of the key risk factors for the development of endometrial cancer is unopposed estrogen(38). In addition, the majority of endometrioid endometrial cancers express ER, emphasizing the role for estrogen in the development and progression of endometrial cancer(39-41). Estrogen signaling was once thought to be a relatively simple signal cascade. Estrogen entered the cell and bound to ER in the cytoplasm, which dimerized and translocated to the nucleus, bound to estrogen response elements (EREs) in the promoters of ER responsive genes and modulated their transcription(42). However, estrogen signaling has proved to be much more complex in recent years with estrogen now known to control both genomic and nongenomic signaling by ligand dependent and ligand independent mechanisms.

Estrogen binds to two isoforms of ER in classical, genomic estrogen signaling: ERα and ERβ. Of these, ERα plays a predominant role in the proliferation of both normal endometrium and endometrial cancer(43). ERα is composed of several functional domains including the N-terminal domain, DNA binding domain (DBD), and C-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2). The ligand-independent activation function (AF1) lies within the N-terminal domain and is the site of phosphorylation and activation of ERα(44). ERα is phosphorylated and activated at several serine residues in AF1 including Serine 118 (Ser118) and Serine 167

(Ser167). This activation can be accomplished both through ligand dependent and ligand independent phosphorylation (Figure 3).

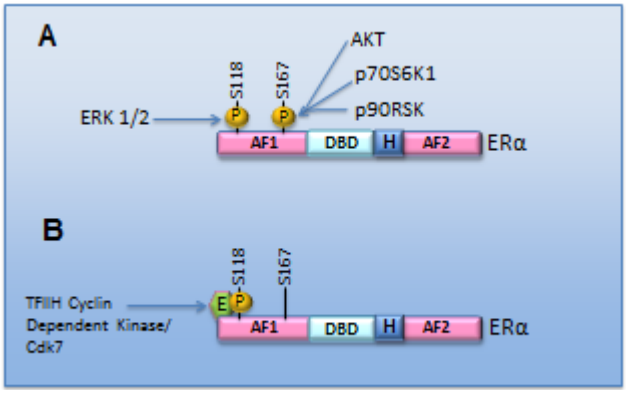


Figure 3. Serine phosphorylation sites on ERα at Ser118 and Ser167 Ligand independent binding sites (A) and ligand dependent binding site (B).

Ligand dependent phosphorylation, as a result of estrogen binding, occurs at Ser118(45). While there is debate regarding the specific kinase that is responsible for phosphorylation at Ser118 as a result of estrogen binding, one candidate is cyclin dependent kinase (Cdk)7(46). Activation of ERα can also be accomplished through ligand independent phosphorylation at both Ser118 and Ser167 by several member kinases of receptor tyrosine kinase pathways, such as the PI3K/AKT and Ras/MAPK pathways, in the absence of estrogen. ERK1/2 phosphorylates Ser118 in the AF1 domain and is independent from the phosphorylation that occurs as a result of estrogen binding(45, 47-49). In addition, AKT, p70S6K, and p90RSK have been found to phosphorylate and activate ERα at Ser167(50-55).

Phosphorylation of serine residues in the AF1 domain of ERα mediates transcription of downstream genes through genomic signaling. Here, phosphorylation of ERα leads to the formation of homo- or heterodimers and transport of the receptor to the nucleus. Dimers bind to EREs which are located in promoter regions of target genes and help to form multi-protein complexes consisting of co-activators, co-repressors, histone acetyltransferases and histone deacetylases, leading to transcription of estrogen induced genes(56, 57).

Nongenomic estrogen signaling results in rapid signaling through multiple protein kinase cascades and is thought to occur when estrogen binds to one of three membrane bound receptors: the G protein-coupled receptor (GPR30), the classic ERα, and a splice variant of ERα, ERα36(58-61). Activation of these kinase cascades then lead to transcription of downstream target genes. Estrogen bound GPR30 stimulates adenylate cyclase, ultimately leading to the release of membrane-tethered epidermal growth factor (EGF) and activation of the Ras/MAPK pathway via EGFR activation(59). Membrane bound ERα trans-activates the EGF, Src, human epidermal growth factor 2 (ErbB2), and insulin growth factor (IGF)-1 receptors, leading to downstream signaling in the Ras/MAPK and PI3K/AKT pathways(62-65). In addition, ligand bound ERα has been shown to bind directly to the p85 regulatory subunit of PI3K, leading to activation of the PI3K/AKT pathway(66). ERα36 is a 36-kDa isoform of ERα that lacks the AF1 and AF2 domains, but retains the DBD, partial dimerization, and ligand binding domains. ERα36 also activates the PI3K/AKT and Ras/MAPK pathway in response to estrogen signaling(67, 68). Figure 4 shows an overview of estrogen signaling.

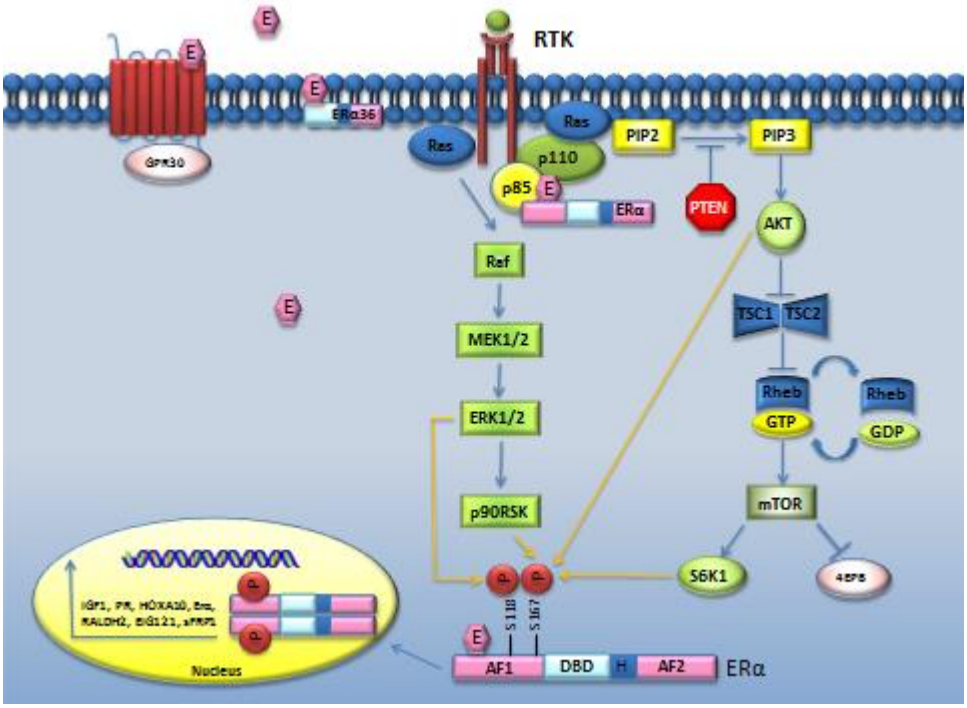


Figure 4. Overview of estrogen signaling

1.5 Pathway Crosstalk

The PI3K/AKT, Ras/MAPK, and estrogen receptor pathways have multiple opportunities for crosstalk that must be considered when approaching the treatment of endometrial cancer. As outlined above, both the PI3K/AKT and Ras/MAPK pathway are able to phosphorylate and activate ERα in the absence of estrogen. In addition, both the Ras/MAPK and estrogen receptor pathways are able to crossactivate the PI3K/AKT pathway at multiple nodes(69). Activated KRas is able to directly bind and activate the p110 catalytic subunit in the absence of ligand binding, leading to activation of AKT(18, 19). ERK1/2 and p90RSK are able to

phosphorylate TSC2 at different sites than AKT, releasing its inhibition on mTORC1, ultimately leading to increased activation of mTORC1(70). ERK 1/2 and p90RSK are also able to directly phosphorylate and activate mTORC1(71, 72). In addition, estrogen binding to membrane bound ERα and ERα36 can lead to nongenomic activation of the PI3K/AKT pathway by direct binding of ERα to the p85 regulatory subunit of PI3K or through increased signaling through RTKs(62, 66). Lastly, genomic estrogen signaling can lead to transcription of ligands that activate receptor tyrosine kinases leading to activation of the PI3K/AKT pathway(65).

Similarly, both the PI3K/AKT and estrogen receptor pathways are able to crossactivate the Ras/MAPK pathway. Conversion of PIP2 to PIP3 by PI3K recruits GAB to the plasma membrane. Phosphorylated GAB interacts with numerous proteins including SHP2 and recruits the GRB2-SOS complex to the plasma membrane. This interaction activates Ras and increases signaling though the MAPK pathway(73-76). This interaction ultimately results in a PI3K/GAB/PI3K feedback loop that further increases PI3K/AKT signaling as well as signaling through the Ras-related C3 botulinium toxin substrate 1(Rac1)/cell division control protein 42 homolog (Cdc42)/p21 activated kinase (PAK) pathway. PAK in turn phosphorylates Raf as well as MEK1(77-79).

Lastly, in addition to cross activation, there are multiple opportunities for crossinhibition between the PI3K/AKT and Ras/MAPK pathways(80, 81). Release of this crossinhibition with single agent inhibition of one pathway could then lead to upregulation of compensatory pathways(81).

1.6 Targeted Therapies in the Treatment of Endometrial Cancer

There are multiple candidates for targeted therapeutics when considering the PI3K/AKT, Ras/MAPK, and ER pathways(5). Unfortunately, thus far, single agent targeted therapies have produced disappointing results, reinforcing the need for combination therapies that target multiple relevant pathways in the treatment of endometrial cancer.

A variety of inhibitors of PI3K, AKT and mTOR are currently available and many of these are currently being utilized in clinical trials. The most extensively studied class of drugs in endometrial cancer includes rapalogs which act as mTORC1 inhibitors. Everolimus (RAD001) is one of these rapalogs that also acts as a macrolide immunosuppressant. A phase II single arm trial of everolimus in recurrent endometrial cancer resulted in an encouraging clinical benefit of 21% at 16 weeks. However, the best response seen in this trial was stable disease, with no complete or partial responses(82). Two other mTOR inhibitors that have been evaluated in endometrial cancer specifically are temsirolimus (CC1-779) and ridaforolimus (AP23573). Trials with these inhibitors, like everolimus, have shown impressive rates of stable disease ranging from 28-69%. However, partial response was only seen in 4-14% of the populations studied, with no complete responses(83, 84).

Several therapeutic agents are currently available that target PI3K specifically or PI3K in combination with mTOR. Investigation of predictive biomarkers for response to treatment with PI3K/AKT/mTOR inhibitors has centered on mutation of, or increased expression or activity of the members of the PI3K/AKT

pathway. A retrospective review of patients enrolled in phase 1 trials with PI3K/AKT/mTOR inhibitors, including patients with endometrial cancer, showed that patients with PIK3CA mutations were more likely to respond with 35% partial response rate in a heavily pretreated group of patients(85). On the other hand, both *in vitro* studies and clinical trials have found that KRas mutations may lead to resistance to PI3K/AKT/mTOR inhibition(85, 86).

While KRas has proven difficult to target, MEK inhibitors are currently being investigated in phase I and II trials in various cancer types. MEK inhibitors bind adjacent to the ATP binding site on MEK, leading to non-competitive interference with MEK function. As a result, MEK inhibitors are highly specific. This specificity is heightened as ERK is the only known downstream effector of MEK(87). In preclinical studies, MEK inhibitors have been found to be cytostatic but not cytotoxic, supporting the notion that additional agents are needed in addition MEK inhibitors to affect tumor regression(88). In addition, while BRAF mutant tumors are sensitive to MEK inhibition, tumors with activating KRas mutations have more variable responses(89, 90). A single arm phase II trial of AZD6244, a MEK1/2 inhibitor was performed by the GOG in patients with advanced or recurrent endometrial cancer. The objective response rate was only 6% with 1 complete and 2 partial responders, however 13 patients had stable disease(91). The authors concluded that AZD6244 was tolerable, but did not meet pretrial specifications for clinical efficacy.

Previous work in our lab elucidated a novel mechanism of action of the oral hypoglycemic agent metformin in the treatment of KRas mutant endometrial

cancers. Endometrial cancer cells with KRas mutations were more sensitive to treatment with metformin compared to cells with wild type KRas and this differential response was due to mislocalization of constitutively active KRas from the plasma membrane(92). These preclinical findings support the use of metformin in KRas mutant endometrial cancers in combination with PI3K/AKT/mTOR inhibition.

Lastly, multiple hormonal therapies have been evaluated in the treatment of recurrent endometrial cancer including progestins, tamoxifen, and aromatase inhibitors with response rates ranging from 10-30%(93-96). Single agent letrozole, an aromatase inhibitor, showed disappointing results with a response rate of only 9.4% in this patient population. Given the extensive crosstalk between the PI3K/AKT, Ras/MAPK, and estrogen receptor pathways, the addition of PI3K/AKT/mTOR inhibitors and MEK inhibitors/metformin to hormonal therapies presents an opportunity for improved response in this patient population.

1.7 Combination Therapy in Breast Cancer

Seventy percent of breast cancers are hormone receptor positive and endocrine therapy remains an integral part of the treatment for these patients(97, 98). However, despite having hormone positive disease, many tumors have de novo or acquired resistance to hormonal therapies(65). Approximately 17- 30% of hormone positive breast cancer patients treated with endocrine therapy experience recurrence(99, 100). This resistance is thought in part to be a result of crosstalk between the PI3K/AKT and ER pathway. As a result, much of the preclinical and clinical investigation to define ligand independent and nongenomic estrogen signaling has been performed in breast cancer.

Multiple clinical trials have been completed in breast cancer utilizing inhibitors of the PI3K/AKT pathway with endocrine therapy. These trials have been performed in the neoadjuvant and recurrent setting with positive results(101). One of these practice changing studies was The Breast Cancer Trials of Oral Everolimus-2 (BOLERO-2) trial. This phase III randomized trial evaluated exemestane, an aromatase inhibitor, and everolimus or exemestane and placebo in hormone positive breast cancer refractory to nonsteroidal aromatase inhibitors. Patients treated with combination therapy had improved progression free survival compared to patients treated with exemestane plus placebo(102). Exemestane and everolimus are now part of the NCCN guidelines for the treatment of patients with hormone refractory recurrent disease(103).

1.8 Combination Therapy in Endometrial Cancer

Breast cancer and endometrial cancer share common molecular aberrations including positive hormone receptor expression and alterations in the PI3K/AKT pathway. Given the success of dual inhibition of the PI3K/AKT and endocrine therapy in breast cancer, a phase II single arm trial of everolimus and letrozole was completed in patients with advanced or recurrent endometrial cancer. Preliminary analysis of 35 evaluable patients revealed a 31% objective response rate with 8 complete responses and 3 partial responses with median response duration of 12.6 months. An additional 6 patients had stable disease with a clinical benefit rate of 49%(104). These positive results contrast the previous single agent phase II trial of everolimus, where there we no objective responses and 6 patients with stable

disease for a clinical benefit rate of 21%(82). Current studies are ongoing to identify predictive biomarkers for response to this treatment regimen.

Activating KRas mutations occur more frequently in endometrial cancer when compared to breast cancer(5, 105, 106). In addition, PTEN, PIK3CA, and PIK3R1 mutations co-occur with KRas mutations in endometrial cancer, while these mutations are mutually exclusive in breast cancer(6, 7, 37, 106, 107).

Differential responses to combination therapy with PI3K/AKT/mTOR inhibitors and endocrine therapy based on KRas mutation status may have important implications in the treatment of endometrial cancer. Characterization of these responses could lead to novel combinations of targeted therapeutics based on tumor molecular profiles in the treatment of endometrial cancer.

Our first aim was to evaluate the addition of metformin to everolimus and letrozole, based on KRas mutation status in preclinical studies. We hypothesized that tumors with an activating KRas mutation will be less responsive to everolimus and letrozole compared to tumors with wild-type KRas. We also hypothesized that the addition of metformin to everolimus and letrozole will improve response in KRas mutant tumors. Our second aim was to evaluate the role of nongenomic and ligand independent activation of ERα in preclinical studies of endometrial cancer. We hypothesized that tumors with an activating KRas mutation have decreased activation of ERα and decreased dependence on the estrogen receptor signaling pathway.

2. Methods

2.1 Cell Culture

Ishikawa, a well differentiated human endometrial carcinoma cell line with loss of PTEN expression, wild type KRas, and positive ERα expression, was purchased from the European Collection of Cell Cultures (EACC, Porton Down, United Kingdom). Ishikawa cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium with L-glutamine supplemented with 10% fetal bovine serum (FBS), penicillin (10,000 U/mL), and streptomycin (10,000 U/mL) (Table 1). Ishikawa cells had previously been transfected with a pMEV-2HA plasmid vector to stably express wild type KRas or mutant KRas (G12V) in our lab by David Iglesias. For the purposes of our *in vitro* studies, these cells were cultured in phenol free Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and supplemented with 10% charcoal stripped FBS, penicillin (10,000 U/mL), and streptomycin (10,000 U/mL). HEC1A, a moderately differentiated human endometrial carcinoma cell line with intact PTEN expression, an activating KRas (G12D) mutation, and positive ERα expression was purchased from the American Type Culture Collection (ATCC, Manassas, VA) (Table 1). Although these cells express ERα, they demonstrate limited response to exogenous estrogen. HEC1A cells were cultured in RPMI 1640 Medium with L-glutamine and supplemented with 10% FBS, penicillin (10,000 U/mL), and streptomycin (10,000 U/mL).

Cell Line	Cell Line Origin	PI3K/AKT Status	Ras/MAPK Status	Hormone Receptor Status
Ishikawa	Grade 1 Endometrial Adenocarcinoma	Loss of PTEN Expression	KRas WT	ERα +, ERβ +, PRα +, PRβ +
HEC1A	Grade 2 Endometrial Adenocarcinoma	PTEN WT	KRas Mutation (G12D)	ERα +, ERβ +, PRα +, PRβ +

Table 1. Known molecular aberrations in Ishikawa and HEC1A endometrial cancer cell lines

2.2 Reagents and Inhibitors

Everolimus (RAD001), an mTORC1 inhibitor, was provided by the stand up to cancer (SU2C) foundation and was dissolved in sterile dimethyl sulfoxide (DMSO). Metformin (1,1-Dimethylbiguanide hydrochloride), letrozole (a non-steroidal aromatase inhibitor), and β-estradiol were purchased from Sigma (St. Louis, MO) and dissolved in phosphate buffered saline (PBS), DMSO, and ethanol respectively. Trametinib (GSK1120212) was graciously provided by the lab of Dr. Kwong K. Wong at the University of Texas MD Anderson Cancer Center and was dissolved in DMSO. BEZ235, a dual pan-PI3K and mTOR inhibitor, was purchased from Selleck Chemicals (Houston, TX) and dissolved in DMSO.

2.3 In Vivo Xenograft Study

We evaluated the addition of metformin to everolimus and letrozole based on KRas mutation status in an orthotopic model of recurrent endometrial cancer. One hundred and sixty female athymic nude mice were purchased from the Department of Experimental Radiation Oncology at the University of Texas MD Anderson Cancer Center (Houston, TX). Mice were housed with 5 animals per cage and were maintained in accordance with the Institutional Animal and Use Committee

guidelines. At 6 weeks of age, all mice underwent bilateral oophorectomy. Mice were then allowed to recover for 7 days prior to intraperitoneal injection with endometrial cancer cells.

Following recovery, each mouse was injected intraperitoneally with $5x10^6$ early-passage endometrial cancer cells (80 injected with Ishikawa cells, 80 injected with HEC1A cells). Tumors were allowed to progress for 10 days and each group of 80 mice were then placed into 1 of 8 treatment groups, with 10 mice in each treatment group. The treatment groups included, control sesame oil gavage, metformin alone (2.5mg/kg/day), letrozole alone (0.275mg/kg/day), everolimus alone (5mg/kg/day), metformin and letrozole, metformin and everolimus, everolimus and letrozole, and all 3 drugs in combination. Mice were treated by oral gavage of the assigned treatment suspended in 100uL of sesame oil (Figure 4).

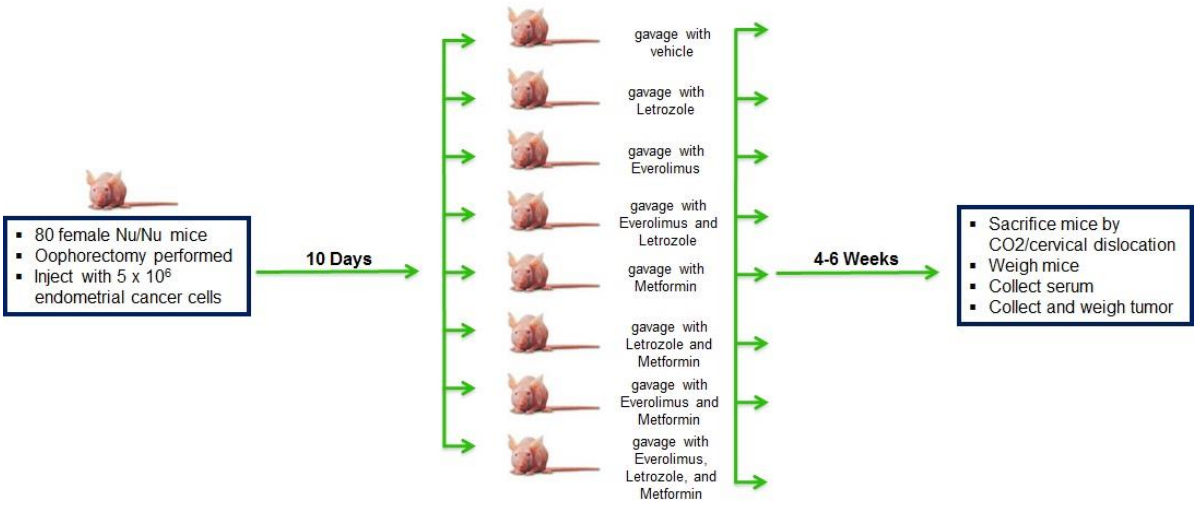


Figure 5. Overview of xenograft model of recurrent endometrial cancer

Mice were treated daily until mice in any one treatment group became moribund at which time all mice in that experiment were euthanized. Mice were weighed prior to necropsy and serum was collected and stored at -80°C. All visible peritoneal tumor was dissected from the abdominal cavity, a portion placed in formalin, and embedded in paraffin. The remainder was flash frozen and stored at -80°C. Serum was analyzed for alanine aminotransferase (ALT) level and along with mouse weight was used as a marker of treatment toxicity.

2.4 Immunohistochemical Analysis of Xenograft Tissues

To evaluate treatment effect on downstream signaling through the PI3K/AKT pathway and cellular proliferation, immunohistochemistry (IHC) was performed on xenograft specimens. Slides were cut from formalin-fixed, paraffin-embedded tissue and IHC for phosphorylated S6 ribosomal protein (pS6rp) and Ki-67 expression were performed. IHC staining was performed using the Lab Vision™ Autostainer 360-2D (Thermo Fisher Scientific, Waltham, MA) utilizing the Division of Surgery IHC Core at the University of Texas MD Anderson Cancer Center (Houston, TX). Primary antibodies utilized were phosphorylated S6 ribosomal protein (S235/236) rabbit monoclonal antibody (Cell Signaling Technology, Beverly, MA) at 1:50 dilution and purified mouse anti-human Ki67 monoclonal antibody (BD Biosciences, San Diego, CA) at 1:50 dilution.

Slides were then scored in triplicate for both pS6rp and Ki-67. Phosphorylated S6rp was scored using a semi-quantitative system of intensity of staining (weak = 1, moderate = 2, strong = 3) and proportion of cells staining

positive (<10%= 0, 10-25% = 1, 26-50% = 2, 51-75% = 3, >75% = 4). Ki-67 was scored as the proportion of nuclei staining positive compared to total nuclei in a high-powered field.

2.5 Western Blot Analysis of ERα Signaling

To evaluate the differential response of Ishikawa and HEC1A tumors to treatment with letrozole, analysis of ERα signaling was performed using western blot. Ishikawa cells stably transfected with wild type KRas and mutant KRas (G12V) were plated in 6 well plates to 70% confluency at 37°C under 5% CO2. Cells were serum starved overnight for 12 hours in phenol free DMEM with L-glutamine containing DMSO alone, 10nM trametinib, or 10nM trametinib and 250nM BEZ235. While initial *in vivo* studies included everolimus and metformin, specific inhibitors of PI3K and MEK were utilized for *in vitro* studies to isolate effects on these pathways, given that both everolimus and metformin can act through inhibition of both the PI3K/AKT and Ras/MAPK pathway. Cells were stimulated with 0.01uM estradiol and whole cell lysates were harvested at 0 minutes, 10 minutes, 30 minutes, 1 hour, and 4 hours. Cells were washed thrice with sterile PBS. Following this, 100uL of urea lysis buffer (3.1mL 1M Tris Hcl, pH6.8, 5mL 20% SDS, 5mL 100% glycerol, 2.5mL 2-mercaptoethanol, 21g urea, bromophnol blue) was added to the culture plate. Cells were harvested with a spatula and placed in 1.5mL centrifuge tubes. Proteins were denatured at 95°C for 5 minutes and cooled on ice. Tubes were then centrifuged at 14,000 rpm for 5 minutes.

Equal amounts of protein (10uL) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel at 30mAmps.

Protein was transferred to an Immobilon polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) over 30 minutes using 25V. Membranes were blocked in 5% nonfat dry milk for 1 hour and washed thrice in non-sterile 1X TBS with 500uL Tween-20 (1xTBST) for 5 minutes each. Membranes were then incubated overnight with the primary antibody at 1:1000-1:2000 dilution in 5% bovine serum albumin (BSA) in TBST with gentle shaking at 4°C. Primary antibodies included phospho-estrogen receptor α (Ser167) (D1A3) Rabbit mAb, phosphor-estrogen receptor α (Ser118) (16J4) Mouse mAb, estrogen receptor α (D8H8) Rabbit mAb, phospho-AKT (Ser473) (D9E) XP Rabbit mAb, AKT Rabbit mAb, phospho p70 S6 Kinase (Thr 389) Rabbit mAb, phospho-p44/42 MAPK (ERK1/2) Rabbit mAb, p44/42 MAPK (ERK1/2) Rabbit mAb, and phospho-p90RSK (Ser 380) Rabbit mAb. All primary and secondary antibodies, excluding β-actin, were purchased from Cell Signaling (Beverly, MA). β-actin levels in each sample were used as a loading control. Blots were stripped and reprobed using anti- β-actin mouse mAb (Sigma, St. Louis, MO).

After overnight incubation in primary antibody, membranes were washed thrice in 1X TBST for 5 minutes each. Membranes were then incubated in 5% nonfat milk in TBST containing the appropriate secondary anti-rabbit or anti-mouse IgG antibody at a 1:2000 dilution (Cell Signaling, Beverly, MA) for 1 hour. Membranes were again washed thrice in 1X TBST for 5 minutes each. Antibody binding was then enhanced using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburgh, PA) and developed.

Relative densities were calculated for each protein of interest using Image J image processing program (National Institutes of Health, Bethesda, Maryland). These were normalized to β-actin controls for graphical depiction of western blot results.

2.6 Quantitative Real Time PCR for Estrogen Induced Genes

To evaluate the downstream effect of differential signaling through ERα in KRas mutant cells, quantitative real time polymerase chain reaction (qRT-PCR) was performed with the aide of the Quantitative Genomics and Microarray Facilities Core at the University of Texas Medical School at Houston(108). Ishikawa cells stably transfected with wild type KRas and mutant KRas (G12V) were plated in 6 well plates to 70% confluency at 37°C under 5% CO2. Cells were serum starved overnight for 12 hours in phenol free DMEM with L-glutamine containing DMSO alone, 10nM trametinib, or 10nM trametinib and 250nM BEZ235. Cells were then stimulated with 0.01uM estradiol and whole cell lysates were harvested at 16 hours. Total RNA was extracted from cell lysates using the RNeasy Mini Kit according to the manufacturer protocol (Qiagen, Valencia, CA) and DNAase treated RNA was diluted to 20ng/uL.

Specific quantitative assays for estrogen-induced gene 121 (EIG121), secreted frizzled-related protein 1 (sFRP1), insulin-like growth factor 1(IGF-1), progesterone receptor (PR), homeobox A10 (HOXA10), ERα, and retinaldehyde dehydrogenase 2 (RALDH2) were developed using Beacon Designer, AlleleID (Premier Biosoft), or RealTimeDesign (Biosearch Technologies) based on the

refseq sequences from the NCBI. Real-time qPCR assay information is provided in Table 2.

Assay No.	Species	Accession No.	Transcript	Primes and Probes
1	Mammal	NR_003286	18SrRNA#2	1335 (+) CGGCTTAATTTGACTCAACAC 1401 (-) ATCAATCTGTCAATCCTGTCC 1359 (+) FAM-AAACCTCACCCGGCCCG-BHQ1 Assay Efficiency = 1.939 Amplicon Length = 68
2	Hum	NM_000675	KIAA1324 (EIG121)	2077 (+) CAACAAGATCCACTCTCTGTG 2136 (-) AAAGCGGAGAAGTTGTAGTTG 2110 (+) FAM-CACCTTCTCACGCAACACTCCGAC-BHQ1 Lowest quantifiable level = 230 Assay efficiency = 1.983 Amplicon Length = 89
3	Hum	NM_003012	hsFRP1	720(+)GAGCCGGTCATGCAGTTCT 786(−)CCTCCGGGAACTTGTCACA 740(+)FAM-CGGCTTCTACTGGCCCGAGATCG-BHQ1 Lowest quantifiable level = 230 Assay efficiency = 2.021 Amplicon Length = 67
4	Hum	NM_001111283	IGF1#2	217(+) CCTGGAGAACTGCACGGTGATCGA 308 (-) GAGGAGGACATGGTGTGCA 289(-) FAM-TCTTCACCTTCAAGAAATCACAAA-BHQ1 Lowest quantifiable level = 230 Assay efficiency = 1.979 Amplicon Length = 92
5	Hum	NM_000926	PRgen#2	3400 (+) GAGCACTGGATGCTGTTGCT 3465 (-) GGCTTAGGGCTTGGCTTTC 3421 (+) FAM-TCCCACAGCCAGTTGGGCGTTC-BHQ1 Lowest quantifiable level = 230 Assay efficiency = 1.996 Amplicon Length = 66
6	Hum	NM_018951	HOXA10#2	1679 (+) CTGAGGTCAATGGTGCAAAG 1758 (-) CCTGTATCCCCTGATTAAACAC 1736 (-) FAM-AGCACAGCACTCCAGGCAGACA-BHQ1 Lowest quantifiable level = 230 Assay efficiency = 2.033 Amplicon Length = 81
7	Hum	NM_000125	ER-alpha#2	1394 (+) TACTGACCAACCTGGCAGACAG 1490 (-) TGGACCTGATCATGGAGGGT 1466 (-) FAM-TCCACAAAGCCTGGCACCCTCTTC-BHQ1 Lowest quantifiable level = 230 Assay efficiency = 1.977 Amplicon Length = 97
8	Hum	NM_003888	hRALDH2	2002(+)AGGCCCTCCTCGCTCAC 2071(−)TCTGCCCCAGAATGAGCTC 2021(+)FAM-ACCCCTCCCTCTCTTCCAAGGAGATC-BHQ1 Lowest quantifiable level = 230 Assay efficiency = 1.972 Amplicon Length = 86

Table 2. PCR primers and probes used in qRT-PCR

cDNA was synthesized in 5 µl (384-well plate) total volume by the addition of 3 µl/well RT master mix consisting of: 400 nM assay-specific reverse primer, 500 µM deoxynucleotides, Superscript II (or Affinityscript) buffer and 1 U/µl Superscript II (or Affinityscript) reverse transcriptase (Invitrogen, Carlsbad, CA), to a 384-well plate (Roche, Nutley, NJ) and followed by a 2 µl volume of sample (25-50 ng/µl). For 96-well plates, 6 µl RT master mix was added to each well followed by 4 µl of RNA sample (25 ng/µl). Each sample was assayed in triplicate plus a control without reverse transcriptase to access DNA contamination levels. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log template concentration range and a no template PCR control. Both were added into RT master mix with reverse transcriptase. Each plate was covered with Biofilm A (Bio-Rad, Hercules, CA) and incubated in a PTC-100 (96) or DYAD (384) thermocycler (Bio-Rad, Hercules, CA) for 30 minutes at 50°C followed by 72˚C for 10 min.

PCR master mix, 15 µl/well, was added directly to the 5 µl RT volume. Final concentrations for the PCR were 400 nM forward and reverse primers (IDT, Coralville, IA), 100 nM fluorogenic probe (Integrated DNA Technologies, San Diego CA), 5 mM MgCl2, and 200 µM deoxynucleotides, PCR buffer, 150 nM SuperROX dye (Biosearch Technologies, Novato, CA) and 0.25 U JumpStart Taq polymerase per reaction (Invitrogen, Carlsbad, CA), final concentrations. RT master mixes and all RNA samples and DNA oligo standards were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan US, Research Triangle Park, NC); PCR master mixes were pipetted utilizing a Biomek 2000 robotic workstation (Beckman,

Fullerton, CA). Each assembled plate was then covered with optically clear film (Applied Biosystems, Foster City, CA) and run in a Roche 480 Light Cycler real-time instrument using the following cycling conditions: 95°C, 2 min; followed by 40 cycles of 95°C, 12 sec and 60°C, 30 sec. The resulting data were analyzed using LightCycler 480 software v 1.5.1.62) with FAM reporter.

Synthetic, PAGE purified DNA oligos used as standards (sDNA) encompassed at least the entire 5' – 3' PCR amplicon for the assay (Sigma-Genosys, The Woodlands, TX). Each oligo standard was diluted in 100 ng/µl E. coli tRNA-H2O (Roche Diagnostics, Indianapolis, IN) and spanned a 5-log range in 10-fold decrements starting at 0.8 pg/reaction. It has been shown for several assays that in vitro transcribed RNA amplicon standards (sRNA) and sDNA standards have the same PCR efficiency when the reactions are performed as described above with PCR amplicons of less than 100 bases in length. Data are expressed as relative expression normalized to 18S and to DMSO controls. Each treatment group was repeated in triplicate.

2.7 Cell Viability Assays

To evaluate the effect of inhibition of the Ras/MAPK pathway alone and in combination with the PI3K/AKT pathway on endometrial cancer cells with an activating KRas mutation in the presence and absence of estradiol, cell viability assays were performed. Ishikawa endometrial cancer cells stably transfected with wild type KRas and mutant KRas were plated in 96 well plates at a density of $4x10^3$ cells/well in phenol free medium with charcoal stripped FBS. Cells were incubated for 24 hours at 37°C under 5% CO2 prior to treatment. Medium was then replaced

with 100uL of phenol free medium containing DMSO alone, 10nM trametinib, or 10nM trametinib + 250nM BEZ235. All treatment regimens were repeated without estradiol and plus 0.01uM estradiol. Following treatment for 48 hours, cells were incubated for 3.5 hours with 20uL MTT (3-(5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye (Affymetrix, Santa Clara, CA) at 37°C under 5% CO2. The reaction was then halted using 150uL of MTT solvent (2-propanol, 0.1% NP40, 4mM HCl) and plates were gently shaken for 15 minutes. Absorbance at 590nm and 630nm was then recorded and the change in optical density (ΔOD) was calculated. Three independent assays were performed for all assays in triplicate.

2.8 Reverse Phase Protein Array

To further validate our *in vitro* findings and to evaluate relevant pathways involved in ERα signaling, reverse phase protein array (RPPA) was performed with the aide of the Functional Proteomics RPPA Core at the University of Texas MD Anderson Cancer Center. Ishikawa cells stably transfected with wild type KRas and mutant KRas (G12V) were plated in 6 well plates to 70% confluency at 37°C under 5% CO2. Cells were serum starved overnight for 12 hours in phenol free DMEM with L-glutamine containing DMSO alone or 10nM trametinib. Cells were then stimulated with 0.01uM estradiol and whole cell lysates were harvested at 30 minutes.

Cells were washed twice with sterile PBS and 150uL of RPPA lysis buffer, containing 1%Triton X-100, 50mM HEPES, pH7.4, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na_3VO_4, 10% glycerol, containing freshly added protease and phosphatase inhibitors, was added to plates.

Plates were incubated on leveled ice for 20 minutes with occasional shaking every 5 minutes. Cells were scraped off plates with a spatula and cell lysates were collected in 1.5mL microcentrifuge tubes. Lysates were then centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was collected and protein concentration was determined using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit per manufacturer protocol (Thermo Fisher Scientific, Waltham, MA). Protein concentration was adjusted to 1mg/mL and the cell lysate was mixed with 4x SDS sample buffer (40% glycerol, 8% SDS, 0.25M Tris-HCL, pH6.8, 2-mercaptoethanol at 1/10 the volume) without bromophenol blue at a ratio of 3 parts cell lyate to 1 part 4x SDS sample buffer. Samples were boiled for 5 minutes and stored at -80°C.

Cellular proteins were denatured by 1% SDS (with beta-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer (Aushon BioSystems). Total 5808 array spots were arranged on each slide including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively.

Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. Only antibodies with a Pearson correlation coefficient between RPPA and western blotting of greater than 0.7 were used in reverse phase protein array study. Antibodies with a single or dominant band on western blotting were further assessed by direct comparison to RPPA using cell

lines with differential protein expression or modulated with ligands/inhibitors or siRNA for phospho- or structural proteins, respectively.

The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customerized-software Microvigene (VigeneTech Inc.) to generate spot intensity.

Each dilution curve was fitted with a logistic model ("Supercurve Fitting" developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, "http://bioinformatics.mdanderson.org/OOMPA"). This fits a single curve using all the samples (i.e., dilution series) on a slide with the signal intensity as the response variable and the dilution steps are independent variable. The fitted curve is plotted with the signal intensities – both observed and fitted - on the y-axis and the log2-concentration of proteins on the x-axis for diagnostic purposes. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

2.9 Statistical Analysis

Values are expressed as the mean ± standard error of the mean. Data were analyzed with independent t-test for pairwise comparisons. For multiple comparisons, analysis of variance (ANOVA) was used with multiple comparisons determined by the Tukey test. Differences with $p<0.05$ are considered statistically significant.

Results of the RPPA experiment were analyzed with the assistance of the Bioinformatics Core at the University of Texas MD Anderson Cancer Center. For RPPA analysis, the data processing and statistical analyses were performed in R. Median-centered data were used and principal component analysis (PCA) was run to check the grouping of samples. Feature-by-feature two-way ANOVA was used to compare protein expression profiles between different cell types and treatments. Beta-uniform mixture (BUM) models were utilized to fit the resulting p-value distributions in order to adjust for multiple comparisons.

3. Results

3.1 Effect of Treatment Regimen on Tumor Growth

Following intraperitoneal injection of tumor cells, mice were placed into 1 of 8 treatment groups and treated daily by oral gavage. Mice were treated until one mouse in any treatment group became moribund at which point all mice were euthanized and necropsy performed. Time to moribund was 51 days for mice injected with Ishikawa cells and 31 days for mice injected with HEC1A cells. Overall, 84.9% and 97.3% of mice developed tumors in the Ishikawa group and HEC1A group respectively. Mouse weight and serum ALT level were used as a marker of treatment toxicity. There were no differences in mean mouse weight or mean serum ALT among any of the treatment regimens in either the Ishikawa or HEC1A group (Table 3).

Cell Line	Mice (n)	Developed Tumor n(%)	Time to Moribund (days)	Mean Mouse Weight (gm)	p-value	Mean Serum ALT (U/L)	p-value
Ishikawa	80	68 (85%)	51	Control 28.11 M: 28.68 L: 27.92 E: 28.96 M+L: 26.39 M+E: 30.36 E+L: 27.33 M+E+L: 28.60	p=0.054	Control: 51.11 M: 39.75 L: 38.38 E: 60.67 M+L: 28.56 M+E: 28.00 E+L: 41.63 M+E+L: 30.33	p=0.263
Hec1A	80	78 (97%)	31	Control: 27.51 M: 26.55 L: 30.46 E: 27.13 M+L: 27.80 M+E: 27.90 E+L: 27.28 M+E+L: 28.29	p=0.063	Control: 46.00 M: 57.89 L: 30.60 E: 45.44 M+L: 37.40 M+E: 66.71 E+L: 38.20 M+E+L: 41.40	p=0.112

Table 3. Summary of xenograft results The time to moribund for mice in the Ishikawa group was 51 days and 85% of mice developed tumor. The time to moribund for mice in the HEC1A group was 31 days and 97% of mice developed tumor. There were no differences in mean mouse weight or mean serum ALT among any treatment regimens in either the Ishikawa or HEC1A group.

In the Ishikawa group, mice treated with everolimus alone (p=0.006), everolimus and metformin (p=0.0013), everolimus and letrozole (0.0004), and all three drugs in combination (p=0.016) had significantly lower mean tumor weights when compared with control animals. Animals treated with everolimus and letrozole had the lowest mean tumor weight compared to controls. In contrast, only mice treated with all three drugs in combination had significantly lower mean tumor weight compared to control treated animals in the HEC1A group(p=0.0076)(Figure 6).

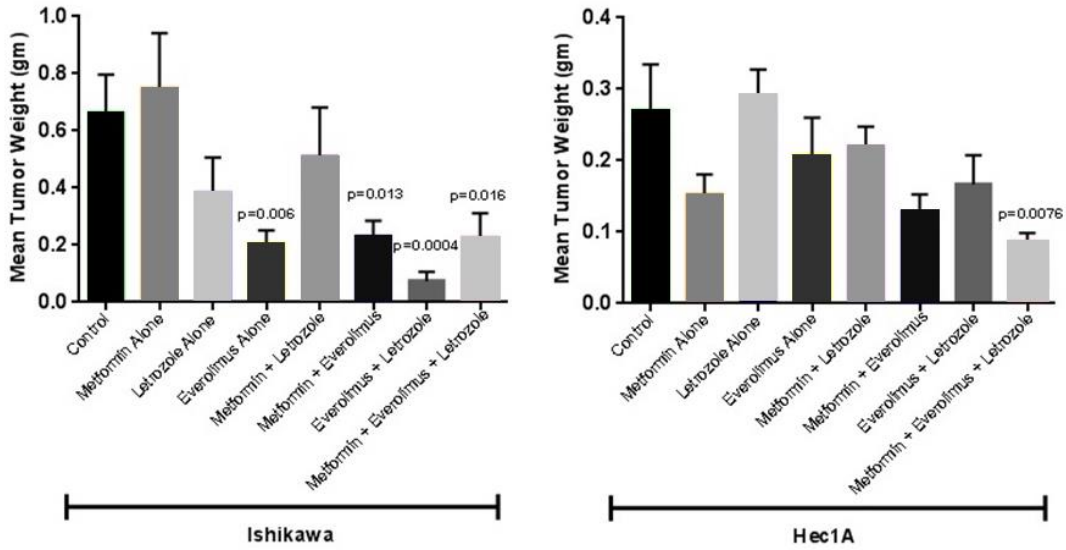


Figure 6. In vivo effect of metformin, everolimus, and letrozole on tumor weight in xenograft model All treatment groups including everolimus resulted in significant reduction in tumor weight in mice injected with Ishikawa cells, with the greatest effect seen in animals treated with everolimus and letrozole. Only the combination of all three drugs resulted in significant reduction in tumor weight in mice injected with HEC1A cells.

Interestingly, when mean tumor weight were normalized to controls, animals in the Ishikawa group had significantly better response to letrozole compared to the HEC1A group (Fold change:0.65 vs 1.23, p=0.035, Figure 7). As a result, we sought to investigate the role of the Ras/MAPK pathway in estrogen signaling in tumors with and without an activating KRas mutation.

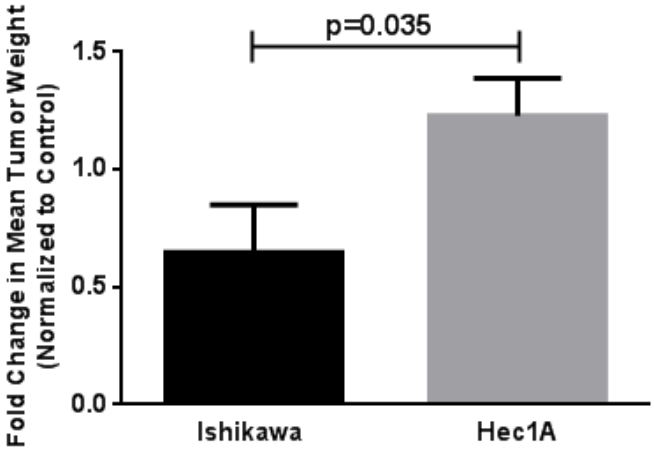


Figure 7. Tumors with an activating KRas mutation are resistant to treatment with letrozole. Tumors in the HEC1A group were resistant to treatment with letrozole compared to tumors in the Ishikawa group. Given the known molecular aberrations in these cell lines, we hypothesized that tumors with activating KRas mutations are resistant to endocrine therapy compared to tumors with wild-type KRas.

3.2 Effect of Treatment Regimen on Expression of pS6rp and Ki67

To evaluate downstream signaling through the PI3K/AKT pathway, orthotopic tumor tissues were stained for pS6rp using IHC. In the Ishikawa group, tumors treated with everolimus (p=0.002) and everolimus and letrozole (p=0.0001) had significantly decreased levels of pS6rp. In the HEC1A group, tumors treated with metformin (p=0.001) and all three drugs in combination (p=0.001) had significantly decreased levels of pS6rp compared to control treated tumors (Figure 8).

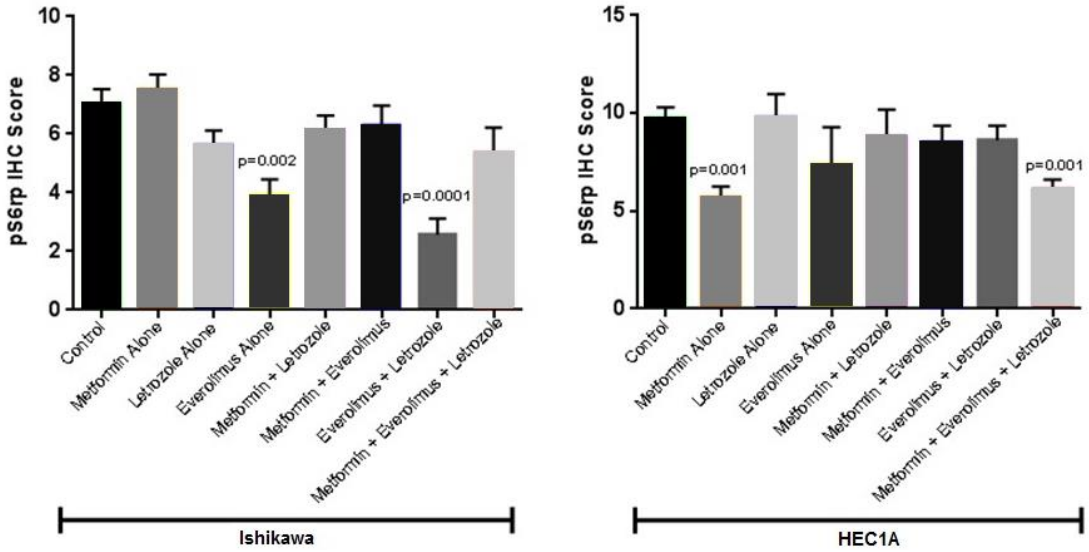


Figure 8. IHC for downstream activation of the PI3K/AKT pathway Levels of pS6rp were significantly decreased in tumors treated with everolimus and everolimus plus letrozole in the Ishikawa group. Conversely, levels of pS6rp were significantly decreased in tumors treated with metformin and all three drugs in combination in the HEC1A group.

Expression of Ki67 by IHC was utilized as a marker of cellular proliferation. In the Ishikawa group, only tumors treated with everolimus and letrozole together had significantly decreased cellular proliferation compared to control treated tumors ($p=0.043$). In the HEC1A group, tumors treated with metformin alone ($p<0.001$), metformin and letrozole ($p=0.016$), everolimus and letrozole ($p=0.035$), and all three drugs in combination ($p=0.001$) had decreased Ki67 expression compared to controls (Figure 9).

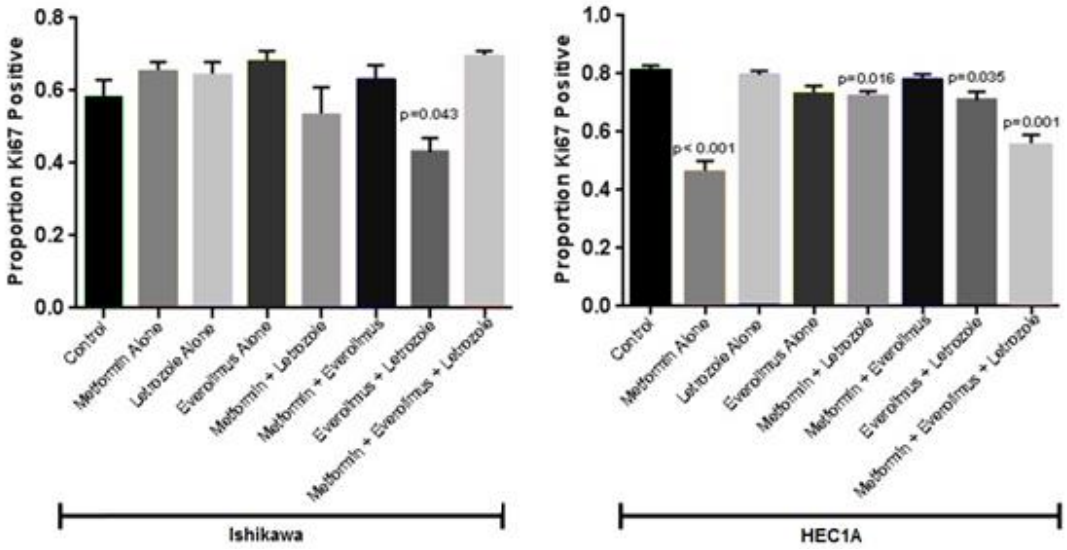


Figure 9. IHC for cellular proliferation Ki67 expression was significantly lower in tumors treated with everolimus and letrozole in combination in the Ishikawa group. In the HEC1A group, tumors treated with metformin, metformin and letrozole, everolimus and letrozole and all three drugs in combination had significantly decreased Ki67 expression compared to controls.

3.3 Effects of Ras/MAPK pathway on ERα Signaling

Given the relative resistance of HEC1A tumors to treatment with letrozole, we evaluated ERα signaling in Ishikawa and HEC1A endometrial cancer cells. Cells were serum starved overnight in phenol free medium and were then stimulated with 0.01uM estradiol. HEC1A cells expressed ERα, however, there was no phosphorylation at ser167 in response to estradiol stimulation, as shown in figure 10. Conversely, Ishikawa cells expressed ERα and had phosphorylation present at ser167 at 10 and 30 minutes respectively.

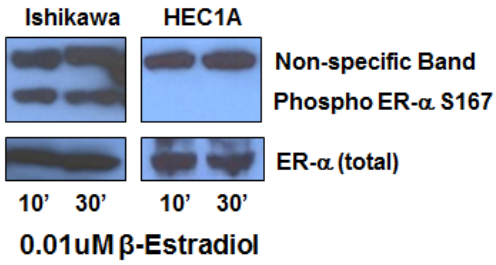


Figure 10. Western blot following estradiol stimulation
Ishikawa cells demonstrated positive ERα expression and phosphorylation of ERα at ser167. Conversely, HEC1A cells expressed ERα, but exhibited no phosphorylation of ERα at ser167.

Given the presence of ERα, but no phosphorylation at ser167 in response to estradiol in HEC1A cells, we utilized Ishikawa cells stably transfected with wild type KRas and mutant KRas (G12V) to further evaluate signaling through ERα. Similar to HEC1A cells, Ishikawa cells transfected with mutant KRas expressed ERα, but had no phosphorylation at ser167. Conversely, Ishikawa cells transfected with wild type KRas expressed ERα and had retained phosphorylation at ser167. Similarly, cells with wild type KRas had increased phosphorylation at ser118 compared to cells with mutant KRas (Figure 11A).

To evaluate the role of the Ras/MAPK pathway in the differential phosphorylation seen at ser167, Ishikawa cells were then treated with 10nM trametinib, a MEK inhibitor, and western blot performed for signaling through ERα. Following treatment with trametinib, both Ishikawa cells with wild type KRas and mutant KRas had signaling through ERα shown by positive phosphorylation at ser167. In addition, treatment with trametinib increased phosphorylation at ser118

in mutant KRas cells, while little effect was seen in cells with wild type KRas (Figure 11A).

The addition of BEZ235, a pan-PI3K/mTOR inhibitor, decreased phosphorylation at ser167 in Ishikawa cells with wild type and mutant KRas. Similarly, treatment with BEZ236 decreased phosphorylation at ser118 in both wild type and mutant KRas cells, however a greater effect was seen in mutant KRas cells (Figure 11A).

Immunoblot for total ERα showed that cells with mutant KRas had decreased total ERα expression compared to cells with wild type KRas (Figure 11A). Figure 11B shows a graphical depiction of relative densities for levels of phospho-ERα ser167, phospho-ERα ser118, and total ERα.

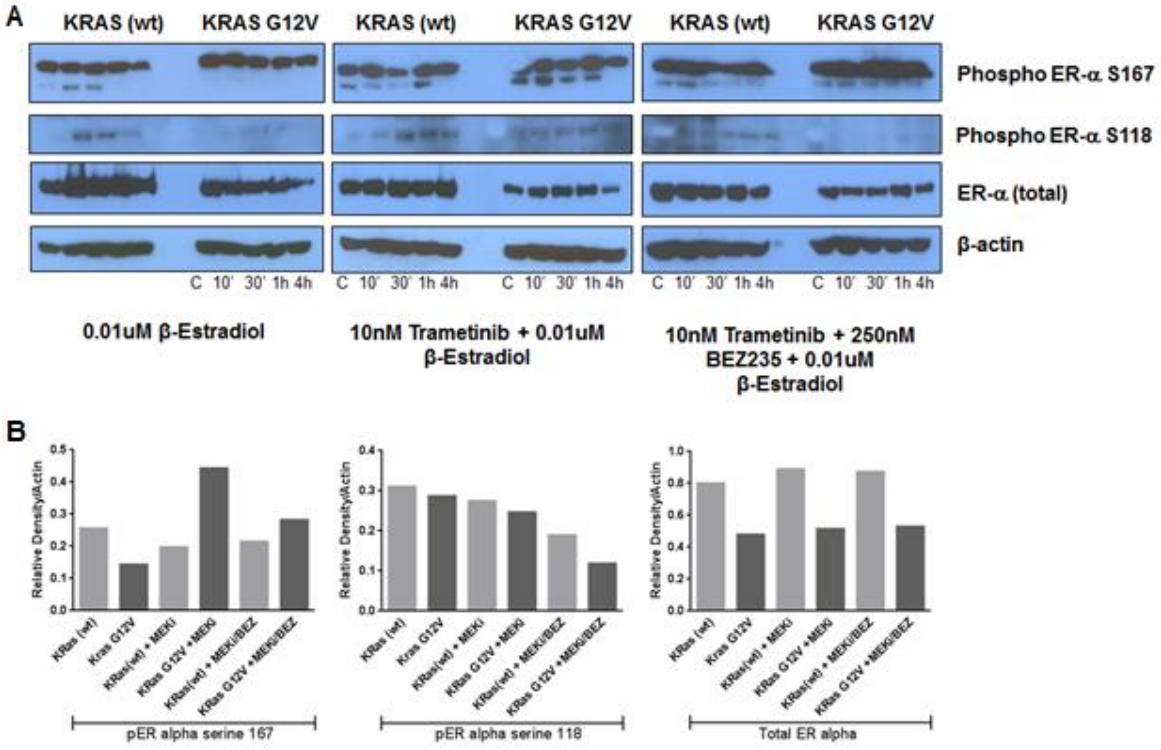


Figure 11. Western blot for ERα signaling A. Cells with mutant KRas express ERα but are not phosphorylated at ser167 in response to estradiol stimulation. Treatment with a MEK inhibitor restored signaling through ser167. Addition of a pan-PI3K/mTOR inhibitor resulted in decreased phosphorylation at ser167. Cells with mutant KRas have decreased phosphorylation at ser118 and phosphorylation increased following treatment with a MEK inhibitor. Addition of a pan-PI3K/mTOR inhibitor decreased phosphorylation at ser118, with a greater effect seen in mutant KRas cells. Lastly, cells with mutant KRas have decreased expression of total ERα compared to cells with wild type KRas. B. Graphical Depiction of immunoblot results expressed as relative density normalized to β-actin control at the 30 minute time point.

3.4 Role of PI3K/AKT and Ras/MAPK member kinases in ERα Signaling

Given that AKT, p70 S6 kinase, ERK1/2, and p90RSK are all able to phosphorylate ERα independent of estrogen, we evaluated phosphorylation at these nodes in the PI3K/AKT and Ras MAPK pathways to evaluate the differential signaling through ERα seen in wild type and mutant KRas Ishikawa cells.

There were no differences in AKT phosphorylation or p70 S6 kinase phosphorylation between wild type and mutant KRas cells. In addition, treatment with trametinib resulted in minimal change in phosphorylation of either AKT or p70 S6 kinase. The addition of BEZ235 decreased phosphorylation of AKT and p70 S6 kinase and in both wild type and mutant KRas cells (Figure 12A). These changes are quantified in figure 12B and are expressed as relative density normalized to β-actin controls.

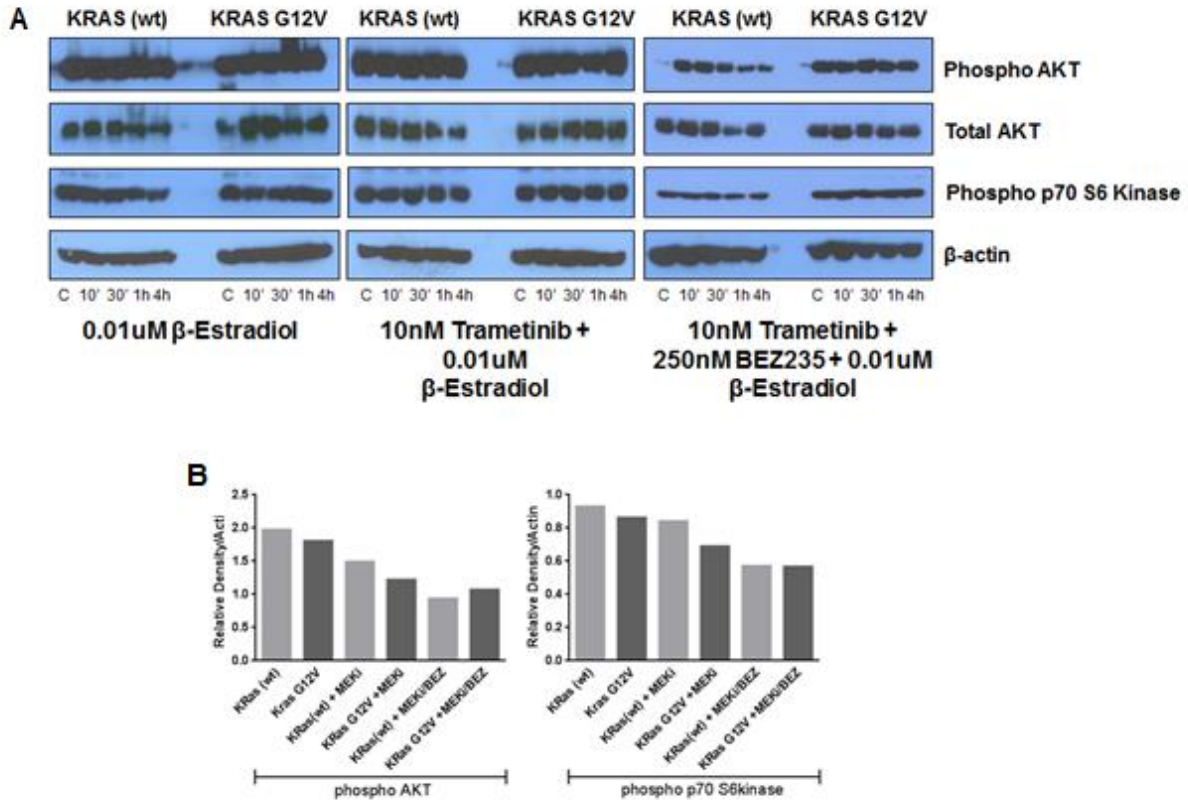


Figure 12. Western blot for PI3K/AKT kinases involved in ERα signaling A. No differences in levels of phospho AKT or phospho p70 S6 kinase were seen in response to estradiol stimulation. Both had minimal decrease in expression following treatment with trametinib. The addition of BEZ235 to trametinib resulted in decreased phosphorylation both kinases. B. Graphical depiction of immunoblot results expressed as relative density normalized to β-actin control at the 30 minute time point.

Following stimulation with estradiol, Ishikawa cells with wild type KRas demonstrated increased phosphorylation of ERK1/2 compared to mutant KRas cells. Following treatment with trametinib, phosphorylation at ERK1/2 was absent in wild type KRas cells but increased in mutant KRas cells. The addition of BEZ235

resulted in a relative decrease in phosphorylation of ERK1/2 in both wild type and mutant KRas cells (Figure 13C shows a positive control).

Ishikawa cells with wild type KRas demonstrated increased phosphorylation of p90RSK compared to mutant KRas cells following estradiol stimulation. Treatment of cells with trametinib led to a relative decrease in phosphorylation of p90RSK in wild type cells and increased phosphorylation p90RSK in KRas mutant cells. The addition of BEZ235 lead to decreased phosphorylation of p90RSK in both wild type and mutant KRas Ishikawa cells (Figure13A). This is depicted graphically in figure 13B.

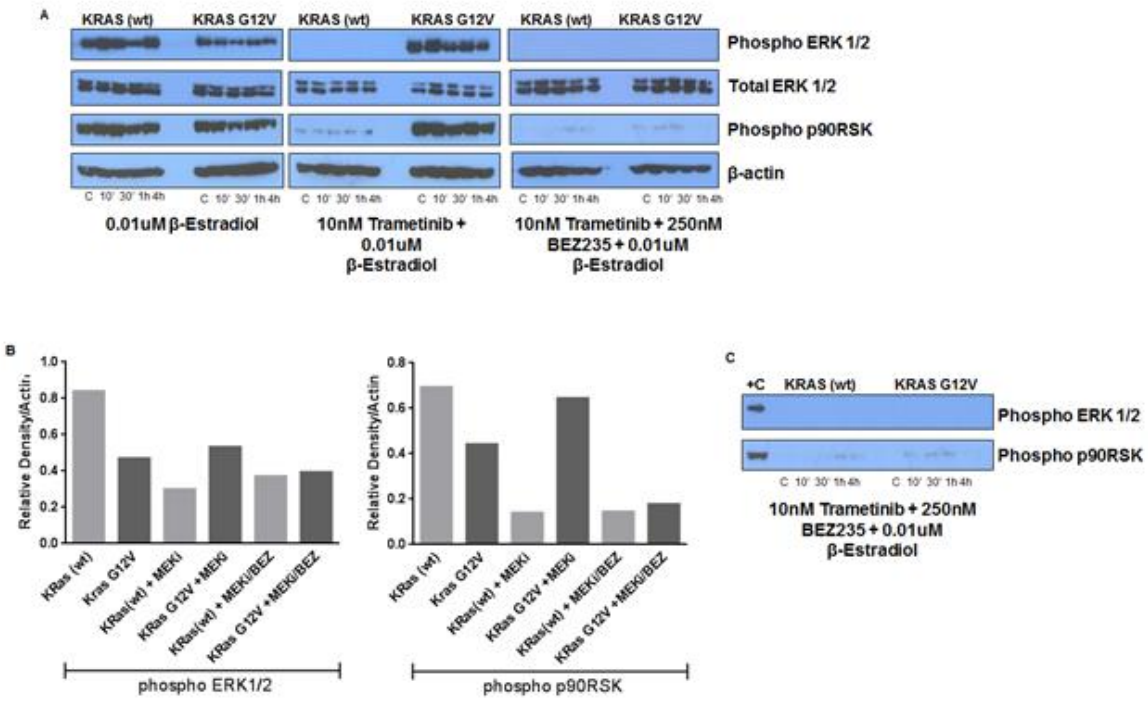


Figure 13. Western blot for Ras/MAPK kinases involved in ERα signaling A. Wild type KRas cells had increased phosphorylation of ERK1/2 and p90RSK in response to estradiol stimulation compared to mutant KRas cells. Following treatment with trametinib, wild type cells exhibited decreased phosphorylation at ERK1/2 and p90RSK, while KRas mutant cells had increased phosphorylation of ERK1/2 and p90RSK. The addition of BEZ235 to trametinib resulted in decreased phosphorylation of both kinases in wild type and KRAS mutant cells. B. Graphical Depiction of immunoblot results expressed as relative density normalized to β-actin control at the 30 minute time point. C. Western blot for phosphorylated ERK1/2 and p90RSK expression following treatment with trametinib and BEZ235 with positive control.

3.5 Quantitative RT-PCR for Estrogen-Induced Genes

To evaluate the functional consequence of differential estrogen signaling in wild type and mutant KRas endometrial cancer cells, qRT-PCR was performed for 7 estrogen induced genes: EIG121, sFRP1, IGF1, PR, HOXA10, ERα, and RALDH2.

Cells were treated with DMSO alone, trametinib, or trametinib and BEZ235 and stimulated with estradiol for 16 hours.

sFRP1 (p=0.002) and RALDH2 (p=0.007) had significantly lower expression in mutant KRas cells. IGF1, PR, HOXA10, and PR also showed a trend toward decreased expression (Figure 14).

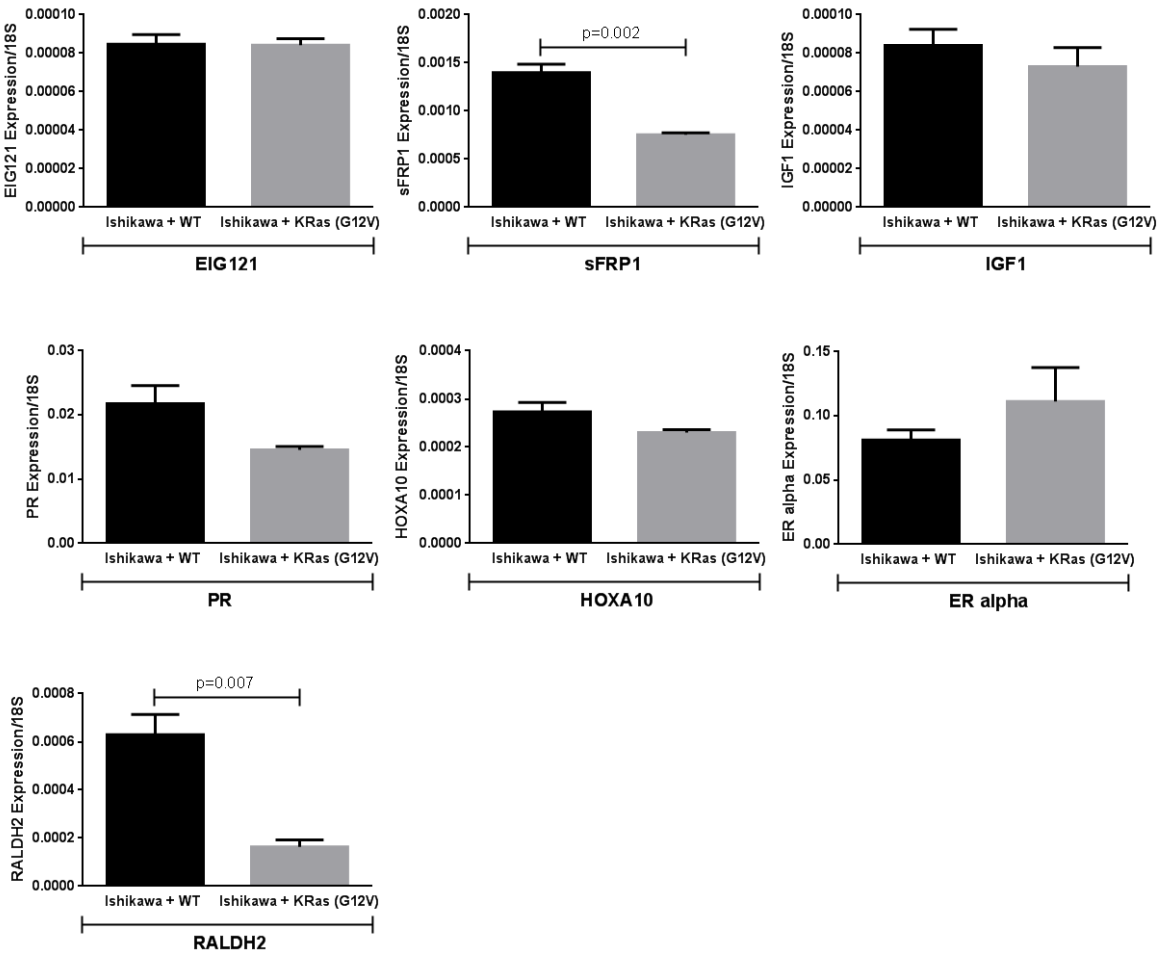


Figure 14. Quantitative RT-PCR for estrogen-induced genes following estradiol stimulation sFRP1 and RALDH2 had decreased expression in mutant KRas cells following estradiol stimulation. All experiments were performed in triplicate.

All 7 genes showed a trend towards increased relative expression in mutant KRas cells following treatment with trametinib. In contrast, relative expression was either decreased or unchanged when cells with wild type KRas were treated with trametinib. Four of the genes, EIG121 ($p=0.001$), sFRP1 ($p<0.0001$), PR ($p=0.0003$), and HOXA10 ($p=0.002$) had significantly increased relative expression in KRas mutant Ishikawa cells following treatment with trametinib compared with wild type cells (Figure 15).

The addition of BEZ235 to trametinib resulted in significantly decreased expression of EIG121 ($p<0.0001$), sFRP1 ($p<0.0001$), IGF1 ($p=0.003$), PR ($p<0.0001$), HOXA10 ($p<0.0001$), and RALDH2 ($p=0.035$) in mutant KRas cells and EIG121 ($p=0.0003$), sFRP1 ($p=0.0003$), PR ($p=0.006$), and HOXA10 ($p=0.008$) in wild type cells (Figure 15).

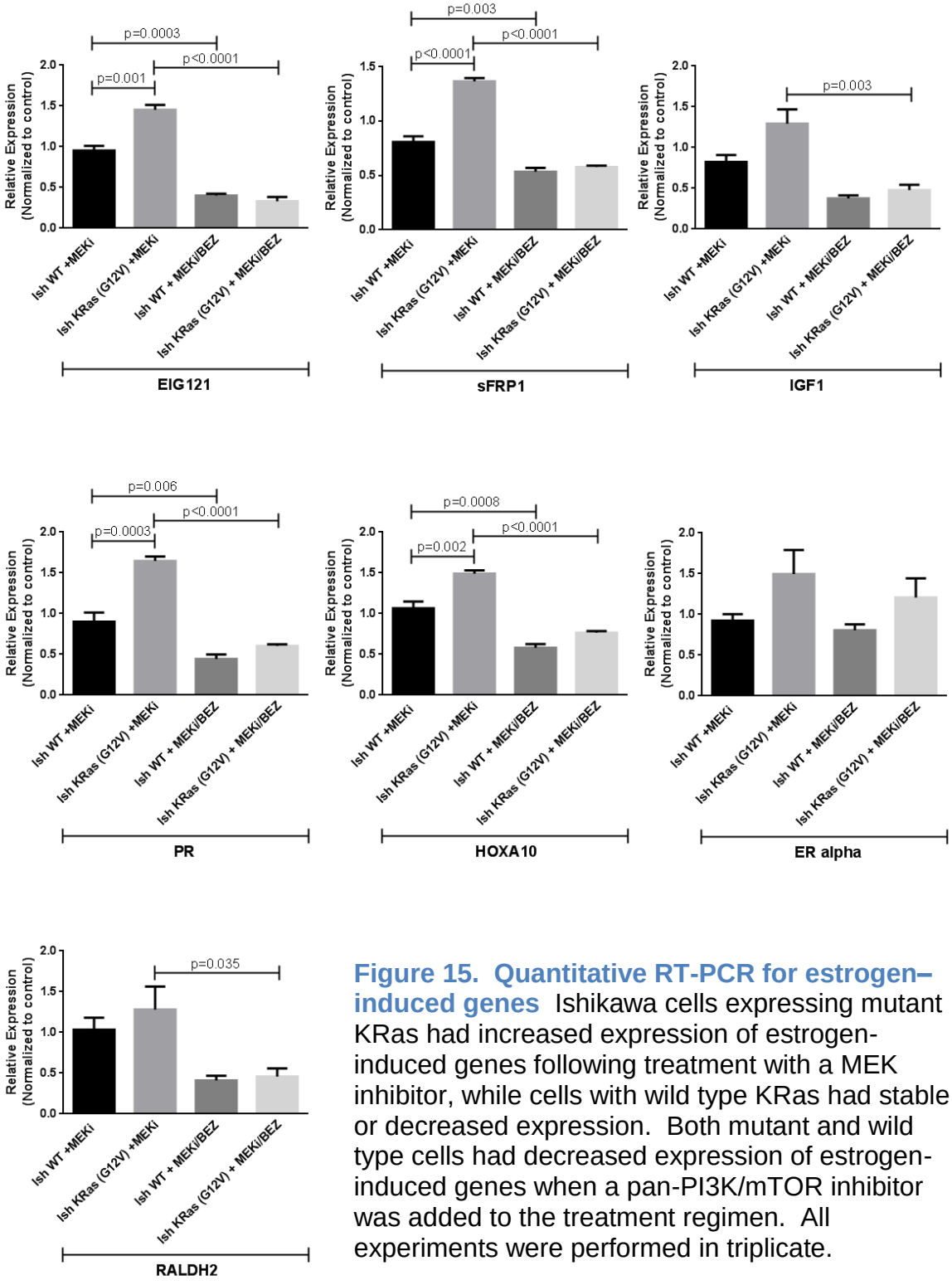


Figure 15. Quantitative RT-PCR for estrogen–induced genes Ishikawa cells expressing mutant KRas had increased expression of estrogen-induced genes following treatment with a MEK inhibitor, while cells with wild type KRas had stable or decreased expression. Both mutant and wild type cells had decreased expression of estrogen-induced genes when a pan-PI3K/mTOR inhibitor was added to the treatment regimen. All experiments were performed in triplicate.

3.6 Cell Viability Studies

To evaluate the effect of differential signaling on tumor growth, cell viability assays were performed with wild type and mutant KRas Ishikawa cells. Cells were treated with DMSO alone, 10nM trametinib, and 10nM trametinib with 250nM BEZ235 for 48 hours in the presence and absence of estradiol. Ishikawa cells with mutant KRas had significantly higher cell viability at 48 hours compared to cells with wild type KRas at baseline ($p=0.0012$).

Treatment with trametinib alone did not decrease relative cell viability in the presence of estradiol in cells with either wild type or mutant KRas. In Ishikawa cells with wild type KRas, there was a significant reduction in relative cell viability compared to DMSO treated controls in cells treated with trametinib in the absence of estradiol compared to those treated in the presence of estradiol ($p=0.001$). This significant reduction was also seen in Ishikawa cell with mutant KRas, where cells treated with trametinib in the absence of estradiol had decreased viability ($p=0.0008$). In addition, cells treated with the combination of trametinib and BEZ235 in the presence of estrogen had significantly decreased viability when compared to cells treated with trametinib alone in both the wild type ($p=0.0001$) and mutant KRas groups ($p<0.0001$, Figure 16).

There was no difference in relative cell viability in mutant KRas cells treated with trametinib alone or trametinib and BEZ235 in the absence of estrogen. Conversely, in wild type KRas cells, the combination of trametinib and BEZ235 resulted in significantly reduced cell viability compared to trametinib alone ($p=0.0003$, Figure 16.)

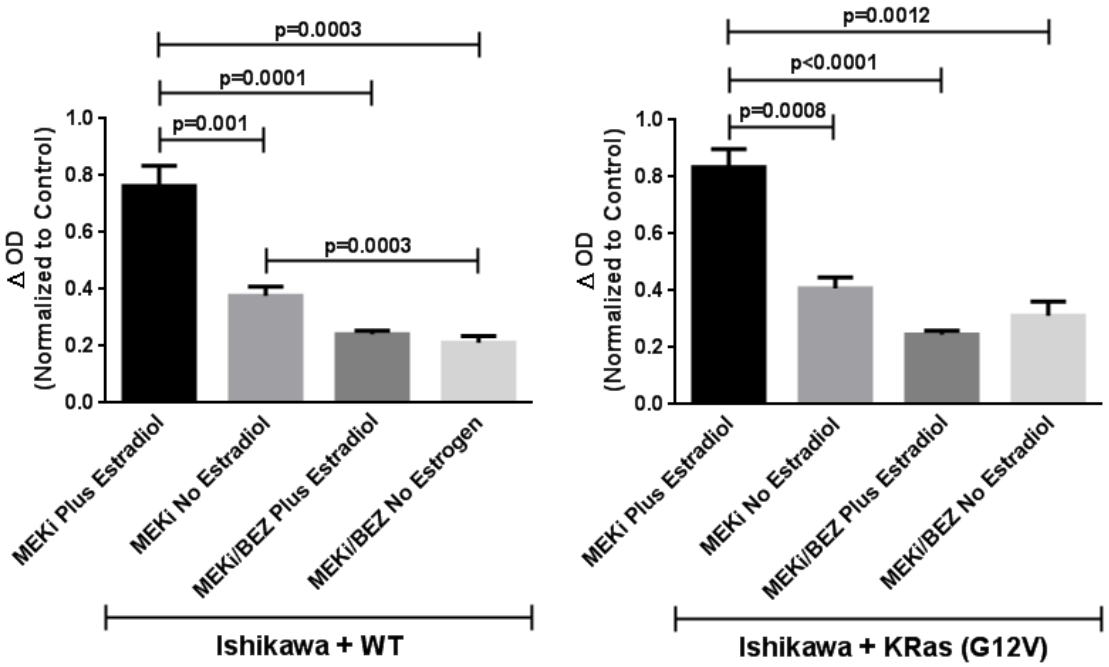


Figure 16. Cell viability assays in Ishikawa cells with wild type and mutant KRas Treatment with trametinib alone had no effect on cell viability in either wild type or mutant KRas cells in the presence of estradiol. However, both wild type and mutant KRas cells had decreased cell viability after treatment with trametinib in the absence of estradiol compared to cells treated in the presence of estradiol. Both wild type and mutant KRas cells had decreased relative cell viability when treated with trametinib and BEZ235 in the presence of estradiol compared to trametinib alone in the presence of estradiol. Ishikawa cells with wild type KRas had significantly decreased relative cell viability when treated with trametinib and BEZ235 together compared to trametinib alone in the absence of estrogen. There was no difference in relative cell viability seen in mutant KRas cells treated with trametinib alone or trametinib plus BEZ235 in the absence of estrogen.

3.7 RPPA Analysis

RPPA analysis was performed to validate our *in vitro* findings of differential ERα signaling and to investigate alternate pathways that may contribute to this differential signaling. Ishikawa cells with wild type KRas and mutant KRas were treated with DMSO control and 10nM trametinib. They were then stimulated with estradiol and whole cell lysates collected at 30 minutes. These lysates were analyzed using the RPPA platform. Analyses of protein expression were performed based on cell type, defined as the presence of wild type or mutant KRas, treatment type, defined as DMSO control or trametinib, or the interaction of cell type and treatment type. Principal component analysis revealed grouping of samples by KRas status and treatment group (control or 10nM trametinib, Figure 17).

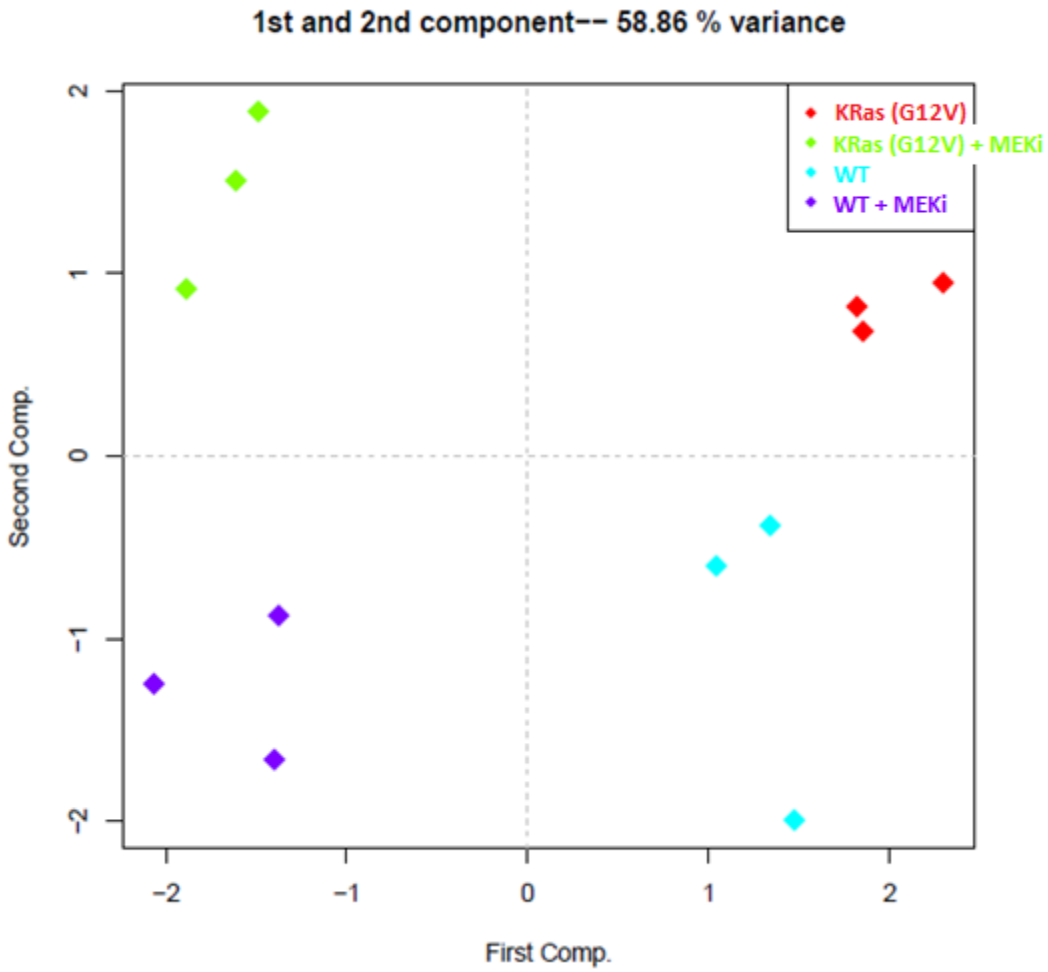
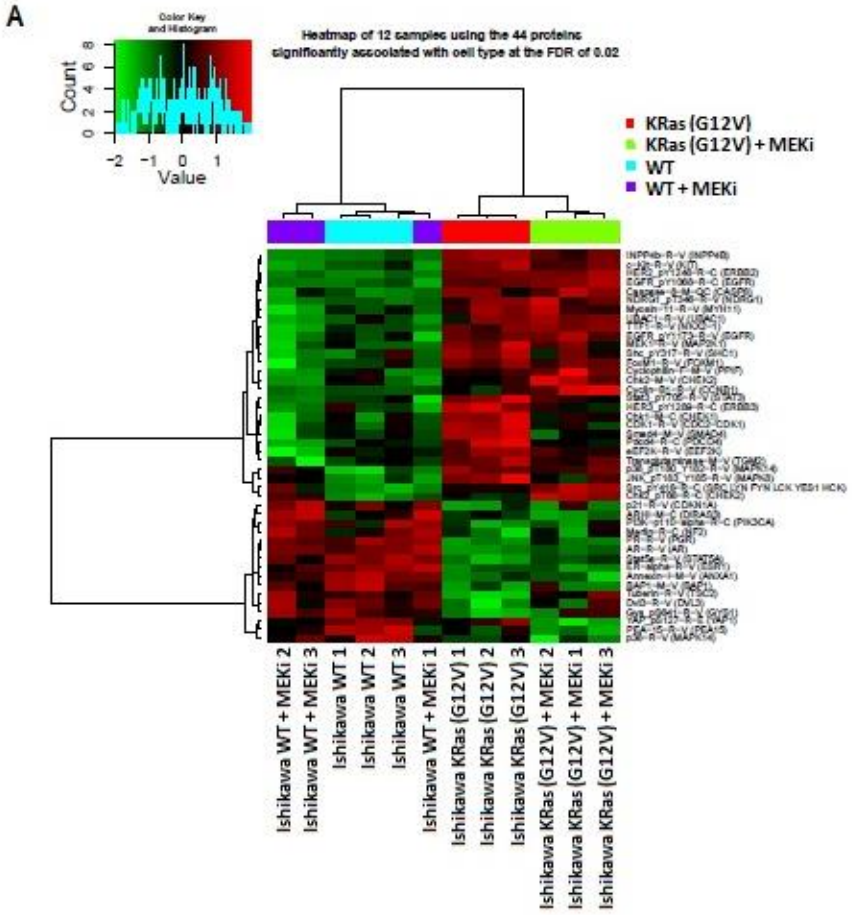
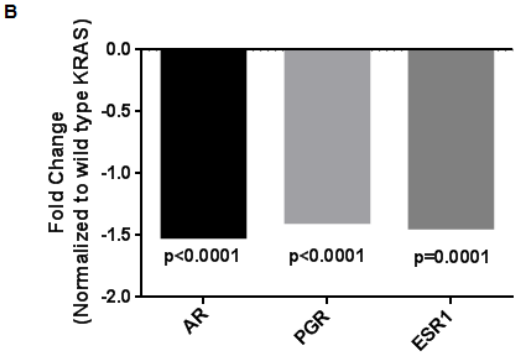


Figure 17. Principal component analysis of normalized RPPA data The first principal component (vertical line) splits the samples by treatment status. The second principal component (horizontal line) splits samples by cell line involved.

After adjusting for multiple comparisons, 38, 44, and 70 proteins were differentially expressed between Ishikawa cells with wild type and mutant KRas at false discovery rate levels of 0.01, 0.02, and 0.05 respectively. Overall, Ishikawa cells with mutant KRas had increased phosphorylation of proteins involved in the Ras/MAPK pathway including EGFR (1.46 fold, $p<0.001$), ERBB2 (1.37 fold, $p<0.0001$), MAPK14 (1.42 fold, $p=0.001$), SHC (1.21 fold, $p=0.002$), and MAPK8

(1.25 fold, p=0.004) (Figure 18A, C). Furthermore, consistent with the previous *in vitro* studies, Ishikawa cells harboring a KRas mutation had decreased expression of ERα (ESR1, p=0.0001). KRas mutant cells also had decreased expression of the androgen receptor (AR, p<0.0001) and the progesterone receptor (PGR, p<0.0001) (Figure 18B).





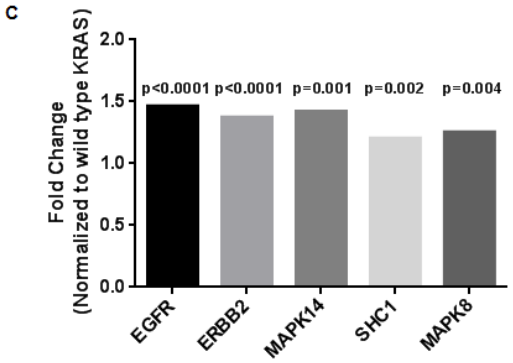
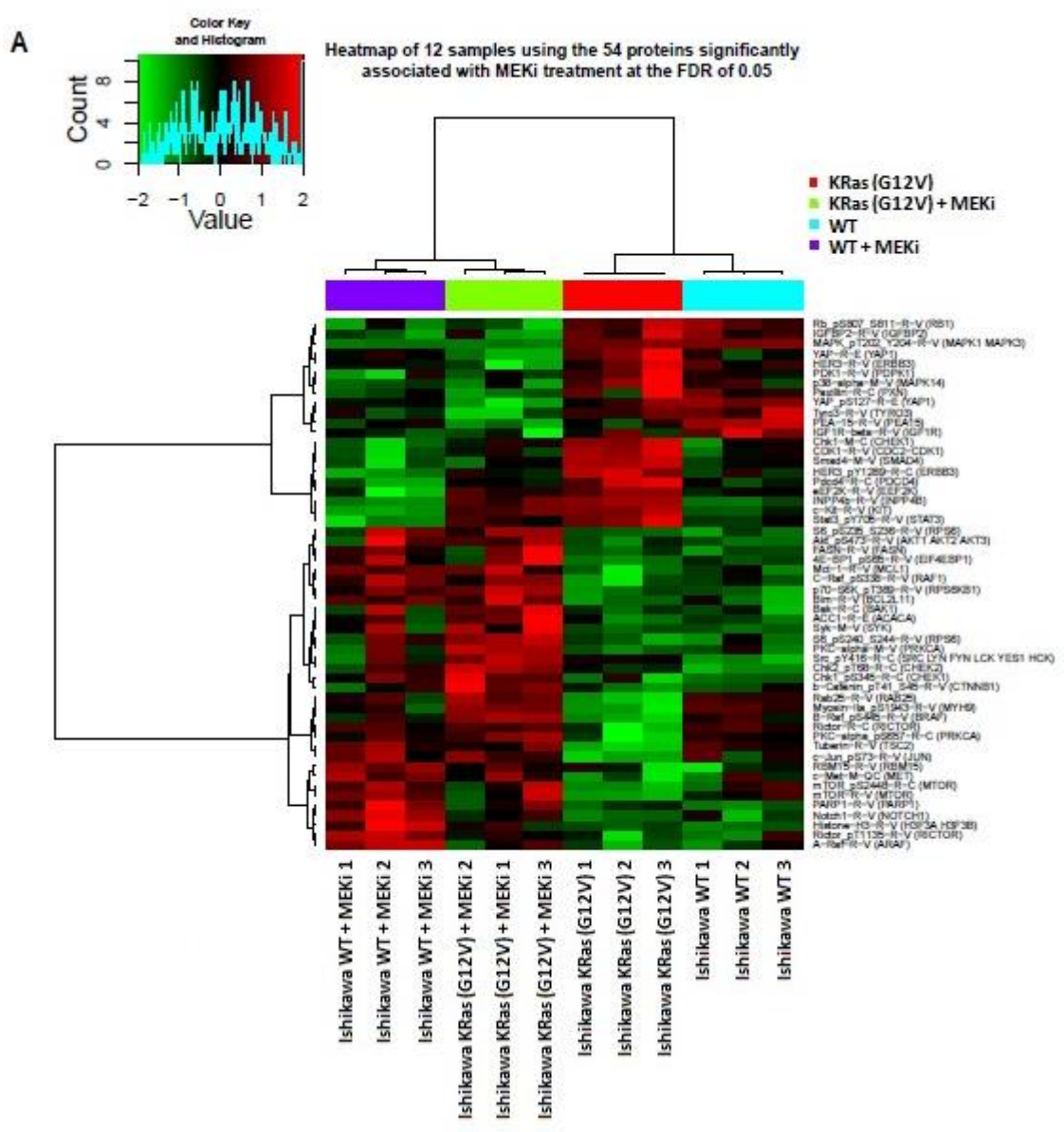


Figure 18. RPPA analysis of samples by KRas mutation status
A. A total of 44 proteins were associated with cell type at a false discovery rate of 0.02. Pearson correlation was used to compute distance and Ward's linkage was used as the clustering method. B. Androgen receptor, progesterone receptor, and ERα all had decreased expression in Kras mutant Ishikawa cells compared to cells with wild type KRas. C. Mutant KRas cells had increased phosphorylation of members of the Ras/MAPK pathway.

In addition, differences in protein expression profiles were seen in 17, 33, and 54 proteins due to treatment regimen at false discovery levels of 0.01, 0.02, and 0.05 (Figure 19). Consistent with MEK inhibition, there was a 6.5 fold decrease in MAPK1/MAPK3 expression ($p<0.0001$) in cells treated with a MEK inhibitor.



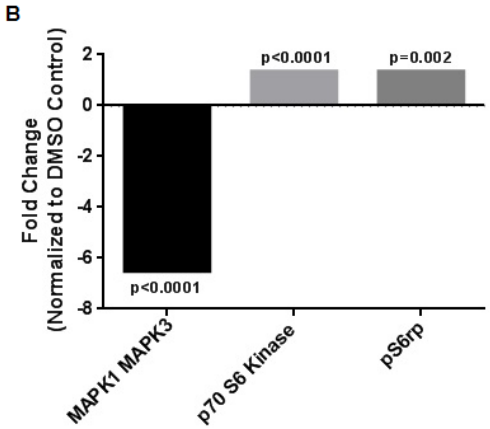


Figure 19. RPPA analysis by treatment group A. A total of 54 proteins were associated with treatment type at a false discovery rate of 0.05. Pearson correlation was used to compute distance and Ward's linkage was used as the clustering method. B. Cells treated with trametinib had a 6.5 fold decrease in MAPK1/MAPK3 expression. Conversely, phosphorylation of p70 S6 kinase and s6 ribosomal protein increased following treatment with trametinib.

We then evaluated the interaction between cell type and treatment type. While there was clustering of groups based on this interaction with significant differences in expression, there were no specific pathways that as a whole were up or down regulated by MEK inhibition based on KRas mutational status (Figure 20).

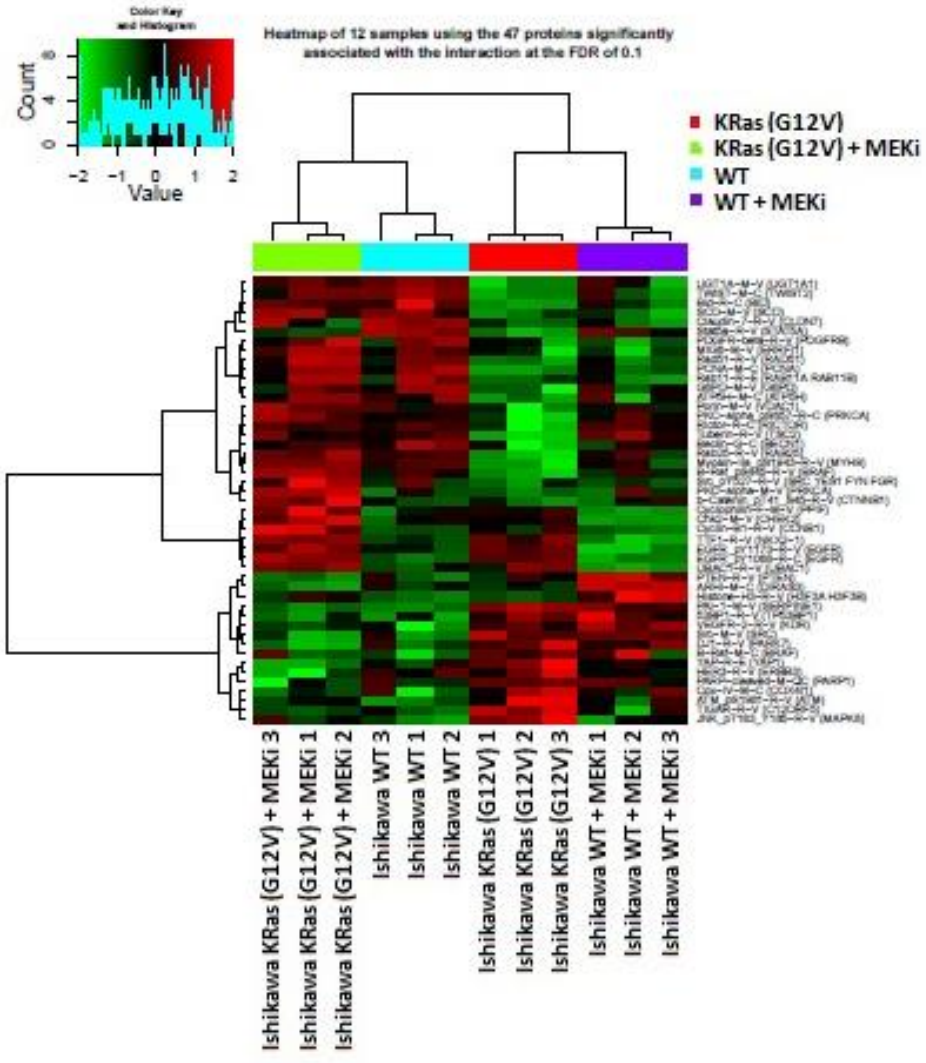


Figure 20. RPPA analysis by the interaction of cell type and treatment type A total of 47 proteins were associated with the interaction of cell type and treatment type at a false discovery rate of 0.1. Pearson correlation was used to compute distance and Ward's linkage was used as the clustering method.

4. Discussion

Hormonal therapy remains a first line option for the treatment of recurrent endometrial cancer. Patients with low grade, hormone receptor positive disease are felt to be optimal candidates for these therapies, however, not all patients respond, with response rates ranging from 10-30% with various regimens(91-94). Given this variability in response, a better understanding of the crosstalk with pathways involved in estrogen signaling, including the PI3K/AKT and Ras/MAPK pathway, is needed to help predict response to endocrine therapies. There is extensive literature supporting the role of ligand independent activation of ERα by member kinases of both the PI3K/AKT and Ras/MAPK pathways(44). In addition, combination therapies targeting the estrogen receptor pathway and the PI3K/AKT pathway have been evaluated in numerous cancers, including recurrent endometrial cancer(98, 104). However, no studies to date have evaluated the role of the Ras/MAPK pathway, and KRas mutation status specifically, in predicting response to hormonal therapy in endometrial cancer. Our *in vivo* model demonstrated that tumors with an activating KRas mutation are resistant to hormonal therapy, even in combination with an mTOR inhibitor. Tumors responded only following the addition of metformin, which has previously been shown to have activity in KRas mutant endometrial cancer cells(92).

Other studies in breast cancer have similarly found that activation of the Ras/MAPK pathway is associated with resistance to hormonal therapy(107-109). Guitierrez et al. showed that increased expression of phosphorylated ERK, but not phosphorylated AKT was associated with resistance to tamoxifen therapy(109).

However, these studies have mainly been correlative and have not focused on cancers with KRas mutations specifically.

Given the differential response to endocrine therapy in our *in vivo* model, we investigated ERα signaling, downstream expression of estrogen-induced genes, protein expression, and cell viability *in vitro* to further dissect the role of the Ras/MAPK pathway in relation to the estrogen receptor pathway in endometrial cancer. First, we demonstrated that KRas mutant cells have decreased expression of ERα compared to cells with wild type KRas. This decrease in expression was mirrored in functional proteomic analysis. In addition, KRas mutant cells had no detectable phosphorylation at ser167 and decreased phosphorylation at ser118 in response to estradiol stimulation. These findings suggest that KRas mutant cells are functionally ER negative, and may be unresponsive to estrogen even when the estrogen receptor is expressed. Furthermore, Ishikawa cells with wild type KRas actually demonstrated increased phosphorylation of ERK1/2 in response to estradiol stimulation compared to mutant KRas cells. Taken in concert, the decreased phosphorylation of ERK1/2 and decreased phosphorylation at ser167 and ser118 suggest that mutant KRas cells are resistant to nongenomic activation of the Ras/MAPK pathway by estradiol compared to wild type cells. This hypothesis shifts the paradigm of estrogen induced tumorigenesis in endometrial cancer. While ligand dependent ERα activation certainly plays a role in downstream transcriptional activation of genes including growth factors, nongenomic activation of the PI3K/AKT and Ras/MAPK pathway may also be a highly relevant event leading to proliferation in endometrial cancer. Given that KRas mutant cells may not be as susceptible to

nongenomic activation, hormonal therapy will not be as effective in the treatment of these tumors.

In our *in vitro* studies, KRas mutant cells had decreased activation of ERα. As a result, we sought to investigate if inhibition of the Ras/MAPK pathway could restore phosphorylation at relevant serine residues. Interestingly, treatment with a MEK inhibitor restored signaling at ser167, increased signaling at ser118 and lead to increased expression of downstream estrogen-induced genes. This in turn suggests that KRas mutant endometrial cancer cells treated with a MEK inhibitor may have enhanced nongenomic activation of the Ras/MAPK pathway in response to estradiol stimulation.

Hou et al. investigated the use of MEK inhibition in ER positive ovarian cancers and found an increase in ERα expression following treatment with a MEK inhibitor. Similar to our study, they found increased expression of ERα and downstream estrogen-induced genes following treatment with a MEK inhibitor that was not associated with increased signaling through AKT(109). However, there are important differences that need to be explored when drawing comparisons with the current study. The study performed by Hou et al. found an increase in expression of phosphorylation at ser118, but not ser167 following treatment with a MEK inhibitor. This is in contrast to the present study, where we demonstrated that treatment with a MEK inhibitor resulted in increased phosphorylation at both ser167 and ser118. These discordant findings could be the result of multiple methodological differences. First, we utilized Ishikawa endometrial cancer cells with and without an activating KRas mutation, while Hou et al based their studies on ERα expression alone in

SKOV3 ovarian cancer cells. Second, we evaluated phosphorylation at ser167 and ser118 at time points ranging from 10 minutes to 4 hours, the rapid time frame in which nongenomic estrogen signaling occurs, while Hou et al. evaluated phosphorylation at 24 hours. Despite these differences, this study also showed that combination treatment with a MEK inhibitor and an anti-estrogen decreased cell viability.

In order to investigate the differential estrogen signaling seen in cells with and without an activating KRas mutation, we evaluated activity of 4 of the downstream kinases in the PI3K/AKT and Ras/MAPK pathways known to phosphorylate ERα in a ligand independent fashion. There were no differences in phosphorylation at AKT or p70 S6 kinase, both members of the PI3K/AKT pathway. This suggests that restoration of phosphorylation at ser167 and ser118 are not the result of upregulation of the PI3K/AKT pathway following treatment with a MEK inhibitor. However, there were differences in phosphorylation of ERK1/2 and p90 RSK, members of the Ras/MAPK pathway. Cells with wild type KRas had decreased phosphorylation of ERK1/2 and p90RSK in response to treatment with a MEK inhibitor, while cells expressing mutant KRas had increased phosphorylation of ERK1/2 and p90RSK in response to treatment with a MEK inhibitor. These findings support the hypothesis that treatment of KRas cells with MEK inhibition leads to resensitization and nongenomic activation of the Ras/MAPK pathway in response to estradiol stimulation.

Our findings could have important implications for the treatment of endometrial cancer, where as many as 30% of cancers harbor activating KRas

mutations. It is clear that single agent therapy, with hormonal therapy or MEK inhibition, is not effective in the treatment of recurrent disease. As a result rational combinations of targeted therapeutics are needed to improve survival in this patient population. Given the extensive crosstalk between the ERα, PI3K/AKT, and Ras/MAPK pathways, it seems prudent to address all three when approaching the design of clinical trials. Current trials include the combination of MEK inhibitors and inhibitors of the PI3K/AKT pathway. These inhibitors in combination should decrease downstream activation of growth signals associated with their own pathways, but should also decrease downstream activation of estrogen-induced growth signals by decreasing ligand independent activation of ERα. While this combination addresses all three relevant pathways, these regimens have already shown significant toxicity in this group of patients in phase I and II clinical trials. Indeed, the safety lead in group for a phase II trial at our institution combining a MEK inhibitor and AKT inhibitor has recently been suspended due to significant toxicities including rash, diarrhea with electrolyte abnormalities, hypertension, and a possible cerebrovascular accident(110). This further highlights the needs for combination therapies with improved side effect profiles.

To evaluate possible combinations of targeted therapeutics, we evaluated cell viability *in vitro* in cells with and without KRas mutation. Treatment of KRas mutant cells with a MEK inhibitor in the presence of estrogen did not decrease cell viability, while treatment with a MEK inhibitor in the absence of estrogen significantly decreased cell viability. Interestingly, the addition of a pan-PI3K/mTOR inhibitor did not decrease cell viability further when added to the MEK inhibitor in the

absence of estrogen in KRas mutant cells. Conversely, the addition of PI3K inhibition to MEK inhibition did significantly decrease cell viability in endometrial cancer cells with wild type KRas. While the addition of BEZ235 significantly decreased cell viability when added to trametinib in the absence of estradiol in wild type cells, the actual difference in relative change was small. This raises the question of whether the addition of a PI3K/mTOR inhibitor would have clinically meaningful results in practice.

Given these findings, three possible future trial designs could be proposed. The possible design is a single arm trial of a MEK inhibitor in combination with an anti-hormonal therapy for patients with ER positive recurrent endometrial cancer and an activating KRas mutation (Figure 21A). The combination of a MEK inhibitor with a hormonal agent could offer similar efficacy with an improvement in side effect profile over combination therapies currently in phase II clinical trials. This combination has also been suggested by other authors in the treatment of ER positive ovarian cancer(57). A second design would again start with a single arm trial consisting of a MEK inhibitor in combination with an anti-hormonal therapy, but patients would then be randomized following 8-12 weeks of therapy if they had stable disease to continue therapy with a MEK inhibitor and anti-hormonal or to add a PI3K/mTOR inhibitor to a MEK inhibitor in place of the anti-hormonal therapy. Patients with an objective response at 8-12 weeks would continue with a MEK inhibitor and anti-hormonal and patients with progressive disease would then be switched to a MEK inhibitor in combination with a PI3K/mTOR inhibitor (Figure 21B). A third, more comprehensive design, would include all patients with ER

positive recurrent disease and would address the question raised by our *in vitro* cell viability studies of whether the addition of a PI3K/mTOR inhibitor would add clinically meaningful benefit for patients with wild type KRas. This design would stratify patients by KRas mutation status and patients would be randomized to receive a MEK inhibitor plus an anti-hormonal therapy or a MEK inhibitor in combination with a PI3K/mTOR inhibitor (Figure 21C).

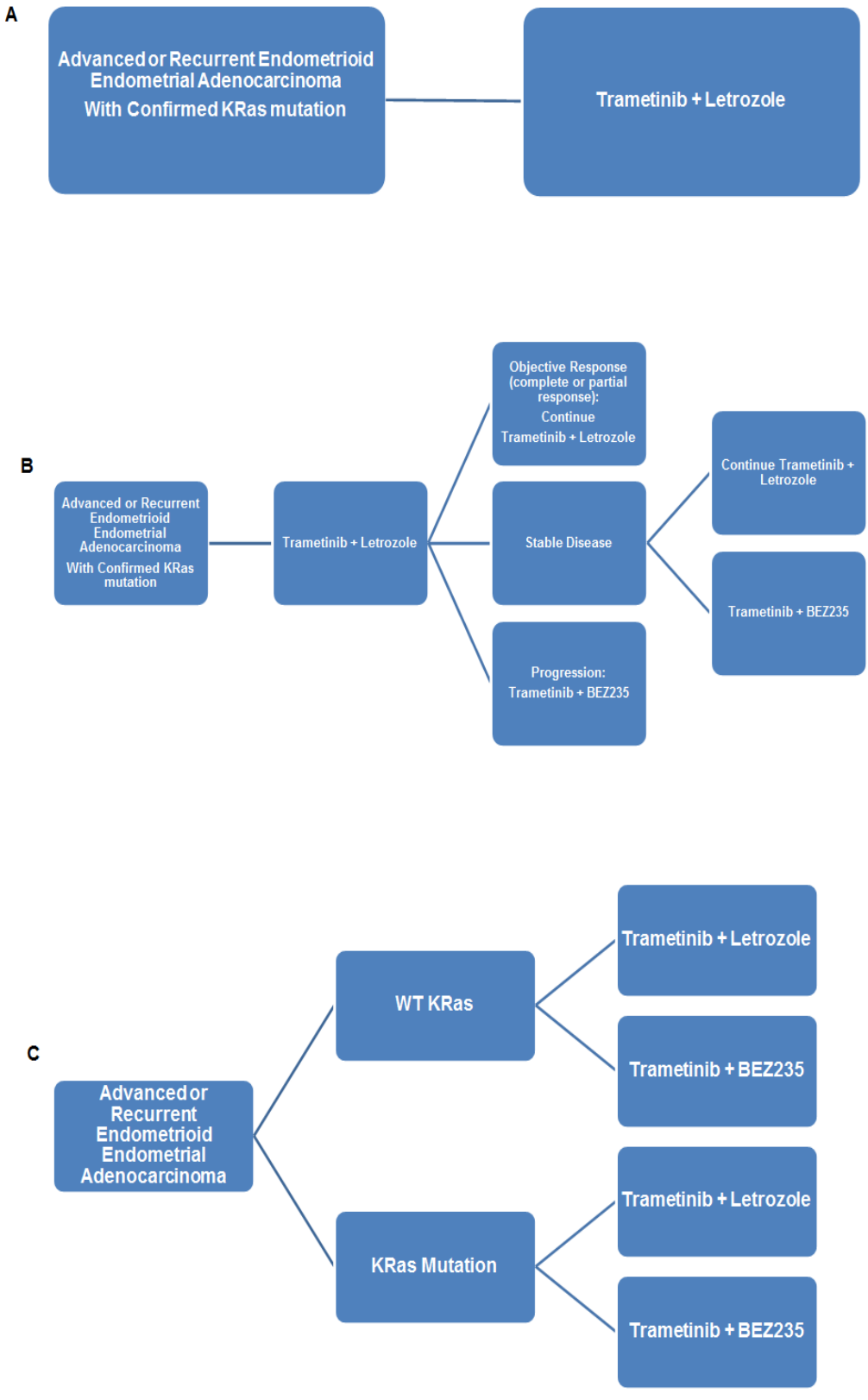


Figure 21. Possible future clinical trial designs

One limitation of our study is that we used Ishikawa cells to transfect wild type and mutant KRas for our *in vitro* models, as Ishikawa cells exhibit loss of PTEN expression at baseline. Indeed, activation of the PI3K/AKT pathway itself has been shown to be a predictive marker for nonresponse to MEK inhibition in KRas mutant cancers(89). However, even in the presence of baseline loss of PTEN expression, cells transfected with mutant KRas had decreased cell viability when treated with trametinib in the absence of estradiol, and the addition of BEZ235 did not increase response. In addition, as both the wild type and mutant KRas cells exhibited loss of PTEN expression, this molecular aberration was controlled for in our *in vitro* model. Lastly, activation of the PI3K/AKT and Ras/MAPK pathways co-occurs in a proportion of endometrial tumors and allows these preclinical findings to be applied to a broader group of endometrial tumors(6, 7, 37, 106, 107).

By stimulating cells with estradiol and assessing signaling at rapid time points, we essentially evaluated nongenomic activation of the PI3K/AKT and Ras/MAPK pathways and the resultant effect on ligand independent phosphorylation at ERα. This leaves us with the inability to dissect out the individual roles of each type of signaling. However, this does present a more complete model for estrogen signaling in aggregate and allows for accurate assessment of the sensitivity of these tumors to treatment with various therapeutic combinations. This is ultimately what is important in designing future clinical trials using these agents.

5. Conclusions

In conclusion, endometrial cancer cells harboring KRas mutations are functionally ER negative in response to estradiol stimulation, despite expressing ERα, and are resistant to treatment with letrozole compared to cells with wild type KRas *in vivo*. Treatment with a MEK inhibitor *in vitro* restores phosphorylation of ser167 and ser118 and in turn leads to increased expression of estrogen-induced genes. Treatment of KRas mutant cells with MEK inhibition in the absence of estradiol leads to significantly reduced cell viability and the addition of PI3K inhibition does not increase response. The addition of an anti-hormonal therapy to MEK inhibition may provide added benefit for patients with recurrent endometrial cancer compared to either therapy alone, with an improved side effect profile. These findings provide preclinical support for the combination of endocrine therapy and MEK inhibition in the treatment of KRas mutant endometrial tumors.

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