Published in final edited form as:

Steroids. 2013 June ; 78(6): 542–547. doi:10.1016/j.steroids.2012.12.014.

The Requirement for p42/p44 MAPK Activity in Progesterone Receptor-Mediated Gene Regulation is Target Gene-Specific

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Abstract

Recent studies have suggested that progestins play a role in the etiology of breast cancer; however, the mechanisms by which progestins promote tumor formation/progression have not been defined. Progestin action, in target tissues such as the breast, is mediated by the progesterone receptor (PR). PR signaling is complex and PR regulates transcription of target genes through a variety of mechanisms. Many cell signaling pathways are activated inappropriately in breast cancer cells and these pathways can regulate PR activity. For example, the p42/p44 MAPK pathway can regulate PR function by altering phosphorylation of PR, as well as its coregulators. We found that inhibition of the p42/p44 MAPK signaling pathway with a MEK inhibitor (U0126) impairs PR-mediated gene induction, but not gene repression. In addition, the effects of U0126 on PR-mediated gene transcription are much greater with long-term versus short-term inhibition and are gene-specific. Finally, treatment with U0126 delays phosphorylation of Ser294, but does not block phosphorylation completely, suggesting that p42/p44 MAPK kinase is not the dominant kinase responsible for phosphorylating this site. Collectively, these studies suggest that in addition to the p42/p44 MAPK pathway, other signaling pathways are also important for PR transcriptional activity in breast cancer cells. The integration of PR transcriptional effects and cell signaling pathways has implications for the initiation or progression of breast cancer. Understanding how these pathways interact may aid in the development of prevention and/or treatment strategies for the disease.

Keywords

Progesterone receptor; breast cancer; p42/p44 MAPK; phosphorylation; U0126; T47D

1. Introduction

Progesterone action, through the progesterone receptor (PR), is important for the regulation of mammary gland development and reproductive function [1-3]. In addition to its defined role in normal target tissues, studies also have suggested that synthetic progestins play a role

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in the development and/or progression of breast cancer [4, 5]. PR is a member of the steroid hormone receptor superfamily that includes the estrogen receptor, androgen receptor and glucocorticoid receptors. Progesterone action in target tissues is mediated by two isoforms of PR, PR-A and PR-B. The two isoforms are encoded by the same gene, but are produced from different mRNAs. They differ only by an additional 164 amino acid sequence unique to the amino-terminus of PR-B [6]. Studies have shown that isoform-specific regulation of gene expression is tissue, cell type and promoter specific [6].

PR can regulate transcription through a variety of mechanisms. In the classical mechanism of PR action, progestins diffuse across the cell membrane and bind PR inducing a conformational change in the receptor that results in dissociation of the receptor from chaperone complexes and receptor dimerization, followed by localization to the nuclear compartment and binding to progesterone-responsive elements [7]. PR regulates transcription through the dynamic and sequential recruitment of primary and secondary co-regulators that alter chromatin structure facilitating transcription. A wide variety of PR coregulators have been identified. These include the p160 steroid receptor coactivators, histone acetylases, DNA helicases, ubiquitin ligases, methylases and kinases that serve to modify histones and remodel chromatin resulting in recruitment and activation of RNA polymerase [8]. PR also can mediate gene repression by binding to promoter regions [9,10] and recruiting corepressors [8, 11, 12] or by physically interfering with the recruitment of other transcription factors [13, 14]. In addition, PR regulates transcription through alternative mechanisms that include PR interacting with and modulating other sequence-specific transcription factors such as AP1 and Sp1 [15], resulting in gene transcription in the absence of direct DNA binding. These tethering interactions may also regulate recruitment of coregulators.

PR activity is also regulated by cell signaling pathways that alter phosphorylation of PR as well as of coregulators. PR recruits cyclin A/Cdk2 [16, 17], as well as p42/p44 MAPK to chromatin [17], facilitating the phosphorylation of histones and other proteins that regulate transcription. PR also can mediate progestin activation of the Src/Ras/MAPK, PI3K/Akt, and JAK2/Stat3 signaling pathways [18]. These effects are dependent on the interaction of a proline-rich region of the amino terminus of non-nuclear PR with signaling molecules and also can lead to altered transcription of target genes [18]. Deletion of the region prevents rapid activation of kinases and some downstream targets, but does not alter induction of classical PR target genes such as *serum and glucocorticoid induced kinase (SGK)* [18, 19]. Some of the targets that are induced by rapid signaling may also require binding of PR to the target gene whereas others are presumably independent of such binding. In addition to PR's ability to activate rapid signaling pathways, kinases can modulate PR function. For example, PR requires cyclin-dependent kinase 2 (Cdk2) for hormone-dependent activation of some target genes [16, 20]. Although cyclin A/Cdk2, phosphorylates PR and thus can regulate its activity directly, the activity of a PR mutant lacking all consensus Cdk2 sites (measured using a PR responsive reporter) also is enhanced by increased expression of cyclin A [16]. This suggests that there are additional effects of cyclin A/Cdk2 aside from receptor phosphorylation, including phosphorylation of SRC-1 as well as other components of the PR transcription complex [16, 20].

Other kinases such as p42/p44 MAPK have also been shown to regulate PR action. EGF treatment, which activates p42/p44 MAPK, enhances hormone-dependent PR activity [21]. Inhibition of MAP kinase kinase (MEK) upstream of p42/p44 MAPK, results in impaired transcription of reporter [17, 22, 23] and endogenous genes [23-28]. Phosphorylation of PR by p42/p44 MAPK has been proposed to explain the observed effect of inhibition of this signaling pathway on gene transcription of selected MAPK-sensitive endogenous genes [29]. MAPK activation by progestins, however, also leads to activation and recruitment of

kinases that phosphorylate histone H3, ultimately resulting in nucleosome remodeling and target gene induction [17]. These models may not be mutually exclusive and highlight the convergence of genomic and non-genomic mechanisms of PR action to regulate transcriptional activation. Although it is clear that p42/p44 MAPK plays a role in gene induction, there is no information regarding the requirement for p42/p44 MAPK in PR-mediated repression. Moreover, the direct contribution of p42/p44 MAPK versus indirect effects secondary to changes in downstream proteins as a result of long term inhibition of MEK has not been examined. To address these questions, we have studied the effect of inhibition of the p42/p44 MAPK signaling pathway on endogenous target gene induction and repression, as well as on PR phosphorylation in T47D breast cancer cells.

2. Experimental

2.1 Materials

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). The MEK inhibitor U0126 was purchased from Promega (Madison, WI). Dimethyl sulfoxide (DMSO) was obtained from Sigma (St. Louis, MO). R5020 (Promegestone) was from Perkin Elmer (Boston, MA). Epidermal growth factor (EGF) was from Sigma (St. Louis, MO).

2.2 Cell culture

T47D cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and 5 µg/ml insulin at 37°C with 5% CO₂. For gene expression analysis, cells were cultured in serum-free medium for 24 hours before pretreatment with DMSO vehicle or 10 µM U0126 for 1 hour, followed by treatment with ethanol vehicle or 10 nM R5020 for 6 hours or 24 hours. In parallel, samples were harvested for Western blot analysis of total PR levels. For analysis of PR phosphorylation, cells were cultured in serum-free medium for 24 hours before pretreatment with DMSO or 10 µM U0126 for 1 hour or overnight, followed by treatment with ethanol or 10 nM R5020 for 30 minutes or 2 hours. To determine the effect of treatment on p42/p44 MAPK phosphorylation, cells were cultured in serum-free medium for 24 hours before pretreatment with DMSO or 10 µM U0126 for 1 hour, followed by treatment with ethanol or 10 nM R5020 for 5 minutes, 30 minutes, 2 hours, or 24 hours or 50 ng/ml EGF for 10 minutes.

2.3 Real-time quantitative PCR

RNA was isolated using Trizol (Invitrogen, Grand Island, NY) and reverse transcribed using amfiRivert Platinum cDNA Synthesis Master Mix from GenDEPOT (Barker, TX). Real-time quantitative PCR was performed using SYBR Green PCR Master Mix using standard conditions on a StepOnePlus™ real-time PCR machine (Applied Biosystems, Carlsbad, CA). Primers for *18S* (previously described in [30]), *SGK* and *cyclin D1* (*CCND1*) (previously described in [20]), *FK506 binding protein 5* (*FKBP5*) (sense 5'-GGATATACGCCAACATGTTCAA-3', antisense 5'-CCATTGCTTTATTGGCCTCT-3'), *N-myc downstream regulated 1* (*NDRG1*) (sense 5'-CTACCATGACATCGGCATGAA-3', antisense 5'-CGTGGCAGACGGCAAAGT-3'), and *interleukin 1 receptor, type 1* (*IL1R1*) (sense 5'-AATTGATGAAGATGACCCAGTGC-3', antisense 5'-GCAGGATTTTCCACACTGTAATAGTCT-3') were purchased from Sigma-Genosys (The Woodlands, TX). The relative mRNA expression of each gene was determined using a standard curve and normalizing to *18S* mRNA expression.

2.4 Western blot

Cells were lysed in lysis buffer containing 10 mM Tris-HCl pH 7.7 with 1 mM EDTA, 12 mM monothioglycerol and 0.4 M NaCl plus cocktail protease and phosphatase inhibitors purchased from GenDEPOT (Barker, TX) using three freeze-thaw cycles. Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk in TBST (10 mM Tris HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 hour followed by addition of primary antibody and incubation overnight at 4°C. Primary antibodies used were PR 1294 (previously described in [31]), PR phosphorylation-specific Ser294 (previously described in [32]), phospho-p42/p44 and p42/p44 (Cell Signaling, Danvers, MA) and tubulin (Upstate, Lake Placid, NY). Membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (GenDEPOT, Barker, and TX) or HRP-conjugated donkey anti-rabbit IgG (GE Healthcare, Buckinghamshire, England) secondary antibodies in TBST for 1 hour. Proteins were detected using Enhanced Chemiluminescence (ECL) detection reagent (GE Healthcare, Buckinghamshire, England) or ECL 2 Western Blotting Substrate (Pierce, Rockland, IL).

2.5 Statistical Analysis

Experiments were performed at least three times. Representative examples are shown for Western blot analyses. For real-time RT-PCR, bars represent the average $\pm$ standard error of the mean (SEM) of biological triplicates of a representative experiment. Statistical differences between treatment groups were analyzed by two-way ANOVA with Bonferroni's post-hoc test. Tests were performed using GraphPad Prism 5 software (La Jolla, CA) and p-values <0.05 were considered statistically significant.

3. Results

3.1 PR-mediated gene repression is unaffected by U0126 treatment

T47D cells were treated with the indicated combinations of vehicle, 10 μ M U0126, and 10 nM R5020 for 24 hours (Fig. 1). To rule out the possibility that any observed effects of U0126 on PR transcriptional activity were due to loss of PR protein expression, we performed a Western blot analysis and found that U0126 does not decrease PR-B or PR-A expression either in the absence or presence of R5020 (Fig. 1A). Furthermore, there is a reduction in PR mobility upon hormone treatment in the absence or presence of U0126, indicative of total PR phosphorylation occurring in both treatment groups (Fig. 1A). As expected, 24 hours of R5020 treatment down-regulates expression of both receptor isoforms. This hormone-mediated degradation appears to be slightly reduced in the presence of U0126 (Fig. 1A).

Although total PR levels are unchanged in the presence of U0126, R5020-mediated transcription of the PR target gene *SGK* is greatly reduced upon treatment with the inhibitor (Fig. 1B). On the other hand, U0126 did not inhibit R5020-mediated repression of *IL1R1* (Fig. 1B). Note that basal expression of *IL1R1* is increased in the presence of U0126 indicating that U0126 is not an inhibitor of overall transcription. Western blot analysis confirms that p42/p44 MAPK activity is inhibited under these conditions (Fig. 2C).

3.2 Ser294 phosphorylation is not affected by short-term inhibition of p42/p44 MAPK

In order to determine whether the effects of U0126 on PR-mediated gene expression are due to p42/p44-mediated phosphorylation of the receptor we performed Western blot analysis of phosphorylated Ser294 levels. Only PR-B is shown because, as we have shown previously [32], PR-A is a poor substrate for phosphorylation at this site in T47D cells. As expected, there is no Ser294 phosphorylation in the ethanol control. With short-term (1 hour) U0126

pretreatment, there is no decrease in phosphorylated Ser294 expression in the presence of R5020 for 30 minutes or 2 hours (Fig. 2A). In contrast, there is an effect of long-term (overnight) U0126 pretreatment on Ser294 phosphorylation. Ser294 phosphorylation is greatly reduced by U0126 at 30 minutes of R5020 treatment, but is less affected at 2 hours (Fig. 2A).

Since U0126 is an inhibitor of MEK (upstream of p42/p44 MAPK) we wanted to ensure that 1 hour of treatment is long enough to effectively inhibit p42/p44 MAPK activity. Because phosphorylation of p42/p44 MAPK is required for activity, this phosphorylation can be used as a surrogate for inhibition of activity. Using western blotting, we found that short-term (1 hour) U0126 pretreatment reduced phosphorylation of p42/p44 MAPK under basal (ethanol) or hormone (R5020) conditions greater than 90% relative to pretreatment with DMSO (Fig. 2B). In contrast to short-term treatment (10 minute) with EGF, a known stimulator of p42/p44 phosphorylation, we did not observe increased phosphorylation with short-term (5 minute) R5020 treatment under our culture conditions (Fig. 2B). Even in the presence of EGF, short-term U0126 inhibits activity of p42/p44 MAPK by about 70% (Fig. 2B). In addition, activity was still inhibited after treatment with R5020 for 30 minutes, 2 hours, and 24 hours (Fig. 2C). These results suggest that pretreatment with U0126 for 1 hour is sufficient to effectively inhibit p42/p44 MAPK activity up to at least 24 hours of hormone treatment.

3.3 Effects of MEK inhibition on PR-mediated gene transcription are much greater at 24 hours than at 6 hours

We observed that Ser294 phosphorylation is delayed by long-term, but not short-term, pretreatment with U0126, suggesting that there are indirect effects of p42/p44 MAPK signaling on PR function. To further investigate this, we analyzed PR target gene induction with 6 hours versus 24 hours of R5020 treatment. PR expression levels are not decreased by U0126 pretreatment in the absence or presence of hormone for 6 hours (Fig. 3A) or 24 hours (Fig. 1A). Hormone-mediated *SGK* gene expression is not significantly affected by U0126 with 6 hours of hormone treatment (Fig. 3B). Basal and hormone-mediated *CCND1* expression is modestly decreased upon pretreatment with U0126 followed by 6 hours of hormone treatment (Fig. 3B). In contrast to results obtained after 6 hours of R5020, R5020-mediated *SGK* gene expression is significantly reduced with long-term U0126 treatment (Fig. 3C and Fig. 1B). This dramatic inhibition of PR activity is gene-specific. Similar to *SGK*, induction of *NDRG1* is sensitive to U0126, but induction of *FKBP5* is relatively insensitive (Fig. 3C). This gene-specific effect suggests that the role of p42/p44 and downstream substrates in regulating PR-mediated gene expression is dependent on promoter context.

4. Discussion

PR transcriptional regulation is a complex process involving many proteins and the activity of many signaling pathways that regulate PR phosphorylation as well as the phosphorylation of coregulators. Although the majority of phosphorylation sites in PR contain Ser-Pro motifs suggesting phosphorylation by cyclin dependent kinase and/or p42/p44 MAPK, for the most part the kinases responsible for PR phosphorylation have not been identified. Many of the sites are substrates for Cyclin A/Cdk2 in vitro [33, 34]. Overexpression of this kinase enhances basal phosphorylation of Ser190, but has no effect on hormone-dependent phosphorylation [35]. Ser294 resides in a consensus MAPK phosphorylation site. We have shown previously that activation of p42/p44 MAPK by EGF resulted in phosphorylation of this site and that phosphorylation was blocked by U0126 in T47D cells [36]. However, the same three hour pretreatment with U0126 did not block R5020-induced Ser294 phosphorylation. In an earlier study using T47D Y-B cells, Qiu et al [37] also found that

short term (30 min) pretreatment with U0126 failed to inhibit R5020 induced Ser294 phosphorylation. Ser345 also resides in a consensus MAPK site. Phosphorylation of this site is not induced by EGF in the absence of R5020. [25]. However, R5020-induced phosphorylation is delayed, but not completely blocked by U0126 [25], suggesting that p42/p44 MAPK is one of the kinases that phosphorylates this site, and that PR is a substrate only in the presence of R5020. In this study, we saw no effect of U0126 on Ser294 phosphorylation at early time points, but a substantial delay in the rate of phosphorylation and a modest decrease in extent of phosphorylation after 24 hours of treatment with U0126 (Fig. 2). This suggests that p42/p44 MAPK can contribute to the phosphorylation of Ser294, but is not the dominant kinase.

Elegant work from Beato's group showed that PR interacts with p42/p44 MAPK and the downstream kinase Msk1 and can recruit these kinases to promoters resulting in phosphorylation of serine 10 in histone H3 of nucleosome B of the MMTV promoter in T47D cells containing an integrated MMTV promoter/reporter [17]. Moreover treatment with PD98059, a MEK inhibitor, reduced hormone dependent induction of MMTV-luciferase and a subset of PR responsive genes exhibited reduced activity at 6 hours. Our studies show very little, if any, effect on *SGK* induction at 6 hours, but as previously reported [22, 38] basal and hormone-mediated *CCND1* induction is sensitive to U0126 (Fig. 3B). The effects of MEK inhibition are more substantial after 24 hours. Hormone-mediated induction of *SGK* and *NDRG1* is sensitive to U0126, but induction of *FKBP5* is minimally affected (Fig. 3D). In contrast, *IL1R1* was strongly repressed by R5020 at 24 hours, both in the absence and in the presence of U0126 (Fig. 1B). A previous study demonstrated that *CCND1* is not induced with longer hormone treatment [19]; therefore, we did not analyze expression of this gene with 24 hours of hormone treatment. Similarly, since there were only modest effects of U0126 on *NDRG1* and *FKBP5* gene induction with 24 hours of hormone, we did not analyze the effect of U0126 on the expression of these genes with the shorter (6 hour) hormone treatment. These time-dependent and gene-specific effects suggest that the role of p42/p44 MAPK in regulating PR activity is complex and that many of the observed effects are secondary to actions of downstream substrates of the kinases or to other changes as a result of long term inhibition. Although PR can recruit p42/p44 MAPK to promoters, induction of *SGK* at 6 hours apparently does not require recruitment of the active kinase. The variation in sensitivity of PR mediated gene induction to U0126 treatment at 24 hours suggests that the requirements for PR mediated gene expression differ extensively and that the contribution of proteins regulated by MEK/MAPK signaling to PR mediated gene regulation also differ substantially. The p42/p44 MAPK signaling cascade has been implicated in a variety of processes that can affect gene transcription, including regulation of chromatin modification and of nuclear import machinery [39]. The reduction in the rate and extent of Ser294 phosphorylation at 24 hours of treatment suggests that the activity of other kinases also may be reduced. Impairment of PR shuttling has been suggested to explain the effect of p42/p44 MAPK inhibition on PR-mediated gene transcription in T47D-Y cells that stably express PR-B [37]. In our study in T47D cells, the effects on PR activity are gene specific and thus presumably include changes in other proteins required for regulation of specific target genes as well as any changes in PR shuttling caused by inhibition of MAPK.

Recent studies have illustrated the integration of PR and cell signaling pathways. Progestin/PR signaling has been implicated in breast cancer development and/or progression [4, 5]. Although the exact mechanisms have yet to be defined, PR can drive breast cancer cell proliferation in early stages of the disease [40] and also can regulate mammary stem cell function [41]. Protein kinases have been reported to be upregulated and inappropriately activated in breast cancer [29]. Thus, the integration of these signaling pathways has implications for the initiation or progression of breast cancer and highlights the need to

understand how these pathways interact in order to develop prevention and/or treatment strategies for the disease.

Acknowledgments

The authors would like to thank the Baylor College of Medicine Department of Molecular and Cellular Biology Tissue Culture Core for technical assistance and the Proteomics Shared Resource of the NCI supported Dan L. Duncan Cancer Center (P30CA125123) for production of the monoclonal antibodies to PR. This study was supported by NIH grants R01CA57539 (NLW, DPE) T32CA090221 (LST) and F32GM103080 (LST).

Abbreviations

PR	progesterone receptor
MAPK	mitogen-activated protein kinase
Cdk2	cyclin-dependent kinase 2
EGF	epidermal growth factor
DMSO	dimethyl sulfoxide

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Highlights

- Progesterone receptor-mediated gene repression is not affected by MEK inhibition.
- Ser294 phosphorylation is not affected by short-term MEK inhibition.
- Effects of long-term MEK inhibition on gene transcription are gene-specific.

Figure 1. PR-mediated gene repression is unaffected by U0126 treatment

A) T47D cells were cultured in serum-free medium for 24 hours before pretreatment with DMSO or U0126 (10 μ M) for 1 hour, followed by treatment with ethanol or R5020 (10 nM) for an additional 24 hours. PR and tubulin protein expression levels were determined by Western blot analysis. A representative experiment is presented. U0126 does not decrease PR expression levels in the absence or presence of R5020, but may inhibit ligand-dependent downregulation of PR. B). In parallel, relative *SGK* and *IL1R1* mRNA expression levels were determined by real-time RT-PCR and normalized to *18S* rRNA levels. *SGK* gene induction was robustly decreased with U0126, but *IL1R1* gene repression was not affected by U0126. **** $p < 0.0001$ compared to DMSO R5020, * $p < 0.05$ compared to DMSO ETOH and *** $p < 0.001$ compared to U0126 ETOH. All bars represent the average $\pm$ SEM of triplicate samples from a representative experiment.

Figure 2. Ser294 phosphorylation is not affected by short-term inhibition of p42/p44 MAPK
 A) T47D cells were cultured in serum-free medium for 24 hours before pretreatment with DMSO or U0126 (10 μ M) for 1 hour or overnight, followed by treatment with ethanol or R5020 for an additional 30 minutes or 2 hours. Phosphorylated Ser294 and tubulin expression levels were determined by Western blot analysis. A representative experiment is presented. Densitometry was performed on scanned films using ImageJ software and numerical data is presented. Pretreatment with U0126 for 1 hour does not significantly affect Ser294 phosphorylation at either hormone time point. Overnight pretreatment with U0126 decreases phosphorylation of Ser294, especially after 30 minutes of hormone treatment. B) T47D cells were cultured in serum-free medium for 24 hours before pretreatment with DMSO or U0126 (10 μ M) for 1 hour, followed by treatment with ethanol or R5020 (10nM) for 5 minutes or EGF (50 ng/mL) for 10 minutes. Phosphorylated p42/p44 MAPK and total p42/p44 MAPK levels were assessed by Western blot analysis. A representative experiment is shown. U0126 pretreatment for 1 hour decreases phosphorylation of p42/p44 MAPK under basal, R5020 treatment, and EGF treatment conditions. C) T47D cells were cultured and pretreated as in panel A, followed by treatment with ethanol or R5020 for an additional

30 minutes, 2 hours, or 24 hours. Phosphorylated p42/p44 MAPK, total p42/p44 MAPK and tubulin levels were assessed by Western blot analysis. A representative experiment is shown. Inhibition of p42/p44 MAPK phosphorylation is sustained up to 24 hours of R5020 treatment.

Figure 3. Effects of MEK inhibition on PR-mediated gene transcription are much greater at 24 hours than at 6 hours

A) T47D cells were cultured in serum-free medium for 24 hours before pretreatment with DMSO or U0126 (10 μ M) for 1 hour, followed by treatment with ethanol or R5020 (10 nM) for 6 hours. PR and tubulin protein expression levels were determined by Western blot analysis. A representative experiment is shown. U0126 does not affect PR expression levels in the absence or presence of R5020. B) In parallel to the experiments in panel A, relative *SGK* and *CCND1* mRNA expression was determined using real-time qPCR and normalized to *18S* rRNA expression. *SGK* gene induction was insensitive to U0126, while *CCND1* basal and hormone-mediated gene expression was decreased with U0126. * $p < 0.05$ compared to respective treatment in DMSO group. C) T47D cells were cultured and pretreated as in panel A, followed by treatment with ethanol or R5020 (10 nM) for 24 hours. Relative *SGK*, *NDRG1* and *FKBP5* mRNA expression was determined using real-time qPCR and normalized to *18S* rRNA expression. *SGK* gene induction was abrogated by U0126, while *NDRG1* gene induction was robustly decreased by U0126. In contrast, *FKBP5* gene induction was relatively insensitive to U0126. * $p < 0.05$ compared to DMSO R5020. **** $p < 0.0001$ compared to DMSO R5020.