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Combinatorial PX-866 and Raloxifene Decrease Rb Phosphorylation, *Cyclin E2* Transcription, and Proliferation of MCF-7 Breast Cancer Cells

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Abstract

As a potential means to reduce proliferation of breast cancer cells, a multiple-pathway approach with no effect on control cells was explored. The human interactome being constructed by the Center for Cancer Systems Biology will prove indispensable to understanding composite effects of multiple pathways, but its discovered protein–protein interactions require characterization. Accordingly, we explored the effects of regulators of one protein on downstream targets of the other protein. MCF-7 estrogen receptor-positive (ER+) breast cancer cells were treated with raloxifene to upregulate the TGF- β pathway and PX-866 to down-regulate the PI3K/Akt pathway. This resulted in highly significant downstream reduction of cell cycle proliferation in breast cancer cells with no significant proliferation reduction following similar treatment of noncancerous MCF10A breast epithelial cells. Reduced phosphorylation of p107 and substantial reduction of Rb phosphorylation were observed in response. The effects of reduced Rb and p107 phosphorylation were reflected in significant decline in E2F-1 transcriptional activity, which is dependent on pocket protein phosphorylation status. The reduced proliferation was related to decreased expression of cyclins, including E2F-1-regulated *Cyclin E2*, which was also in response to raloxifene and PX-866. All combinations of raloxifene and PX-866 produced significant or highly significant results for reduced MCF-7 cell proliferation, reduced *Cyclin E2* transcription, and reduced Rb phosphorylation. These studies demonstrated that uncontrolled proliferation of ER+ breast cancer cells can be significantly reduced by combinational targeting of two relevant pathways.

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Keywords

PX-866; RALOXIFENE; TGF- β PATHWAY; PI3K/Akt PATHWAY; Rb PHOSPHORYLATION; p107; POCKET PROTEIN; PROLIFERATION; BREAST CANCER

Cancers are dependent on unlimited and unregulated repetition and progression of the mitotic cell cycle, an evolved process essential for multicellular life [Hartman and Fedorov, 2002]. The cell cycle and its regulation are administered by genes, gene products, and pathways of gene products necessary for its establishment, refinement, and maintenance [Tyson and Novak, 2001]. Human genes essential to cell cycle regulation include genes necessary for regulation of transition from G1 to S phase [Qu et al., 2003].

Transition from G1 depends on a vast number of gene products and pathways but most directly on the expression and activity of cyclins and cyclin-dependent kinases (CDKs) in cyclin/CDK complexes, limited by CDK inhibitors [Qu et al., 2003; Cobrinik, 2005]. The “pocket proteins” p107 and Rb are closely related in structure and regulate the transcriptional activity of the E2F family proteins, which have essential roles in regulation of G1 to S transition [Chen et al., 2002; Leng et al., 2002; Cobrinik, 2005]. Pocket proteins are characterized by their conserved pocket structured domains [Singh et al., 2005]. Multiple binding sites, including two sections within the pocket domain, accommodate mutual binding of E2F proteins, cyclins, and histone deacetylases (HDACs) [Sardet et al., 1995; Brehm et al., 1998; Lee et al., 1998; Chen et al., 2002; Singh et al., 2005]. Cell cycle regulation by p107 and Rb is dependent on which proteins bind at the pocket domain, where removal of binding partners may be induced by substitution of phosphate groups donated by complexes of CDK 2 or 4 with cyclin D, E, or A [Harbour et al., 1999; Leng et al., 2002; Chen et al., 2002; Cobrinik, 2005]. Although Rb and p107 activity is suppressed by phosphorylation disassociating E2F proteins from pocket proteins, reduced expression of E2F-1-induced cyclins results from HDACs recruited to the E2F-1 promoter by Rb [Brehm et al., 1998; Leng et al., 2002; Cobrinik, 2005].

As the level of tissue growth and cell reproduction, critical to the event and/or progression of growth-related diseases such as cancer, is regulated by networks of interacting pathways [Stevens et al., 2013], we have investigated the potential down-regulation of cell cycle progression by treatment of cultured human cancer cells with a combination of an activator of an inhibitory pathway (TGF- β), mediated in part by p107, and an inhibitor of an activating pathway (PI3K/Akt) [Testa and Bellacosa, 2001; Chen et al., 2002]. For upregulation of TGF- β pathway activity, we used raloxifene, a selective estrogen receptor modulator (SERM) which competes with 17- β -estradiol (E2) for binding the estrogen receptor ER α and the cytoplasmic estrogen receptor GPR30 and which has been approved by the U.S. Food and Drug Administration (FDA) for use in breast cancer prevention [Cherlet and Murphy, 2007; LeBlanc et al., 2007; Kleuser et al., 2008; Sasaki et al., 2008]. Preliminary gel-based PCR testing in our lab had shown raloxifene to be optimally effective in gene regulation at 1.0 μ M, previously determined to be physiologically relevant [Sasaki et al., 2008]. For down-regulation of the PI3K/Akt pathway we used gradient concentrations of

PX-866, a derivative of wortmannin and an inhibitor of the p110 α unit of phosphoinositide-3-kinase, currently in multiple clinical trials [Ihle et al., 2004].

MATERIALS AND METHODS

CELL CULTURES, REAGENTS, AND PROCEDURES

Human cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and included ER-expressing MCF-7 breast cancer epithelial cells and *hTERT*-expressing but nontumorigenic MCF10A breast epithelial cells. MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 10% APS (amphotericin B, penicillin, and streptomycin) (Mediatech) and treated in phenol red-free DMEM supplemented also with 0.01 μ M β -estradiol (E2) (Sigma-Aldrich, St. Louis, MO) [Cherlet and Murphy, 2007]. MCF10A cells were grown in DMEM/F12 media (Mediatech) with supplementation by 5% horse serum, 0.02 μ g/mL EGF, 10 μ g/mL insulin, 0.001 μ g/mL cholera toxin, 100 μ g/mL hydrocortisone, and 50 μ g/mL penicillin-streptomycin [Muthuswamy et al., 2001]. Charcoal dextran treatment of serum in media can improve the accuracy of comparisons involving induced hormone level differences and improve precision of comparisons with or without known hormone level differences [Lindquist and de Alarcon, 1987; Tee et al., 2004; Zheng et al., 2006]. Experiments using additional cell types will also be needed.

MCF-7 cells were treated on 3 consecutive days, with media replacement daily, and harvested 18 h after the latest treatment. The cells were treated with 1.0 μ M raloxifene, with 0.4 μ M PX-866, with 1.0 μ M raloxifene in combination with 0.1, 0.4, or 0.8 μ M PX-866, or only as control/vehicle with 21 μ L DMSO, the median DMSO volume in treatments. With longer treatment, recovery of cell lysates was inconsistent, but testing for time-dependency will, nevertheless, be needed. Raloxifene (Sigma-Aldrich) stock was maintained at room temperature at 54.9 mM concentration. PX-866 (BioVision) was maintained at 190 μ M concentration at temperature -20°C and protected from light. Relevant and physiologically appropriate treatment dosages of raloxifene and PX-866 were chosen based on previous testing [Sasaki et al., 2008; Koul et al., 2010], including gel-based PCR in our laboratory (data not shown).

RNA QUANTIFICATION BY REAL-TIME PCR

Total cellular RNA was harvested from cultured cells using RNeasy Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol. cDNA was synthesized from RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer's instructions. Primers used for real-time PCR were previously tested and validated for the genes *Cdc6* [Wu et al., 2009], *Cyclin E2* [Wu et al., 2009], *E2F-1* [Pulikkan et al., 2010], *IL-11* [Onnis et al., 2013], *p27* [Su et al., 2013], *MAGE-A11* [Minges et al., 2013], and *GAPDH* [Meeran et al., 2010]. RNA expression from three or more experiments for each gene and treatment was measured by real-time PCR using the CFX Connect system (Bio-Rad Laboratories). Gene-specific RNA expression fold change

relative to untreated control cells was determined by the Delta–Delta Ct method [Livak and Schmittgen, 2001], using *GAPDH* as the reference gene.

QUANTIFICATION OF PROTEIN AND MODIFIED PROTEIN BY WESTERN BLOT

Protein was extracted from cultured MCF-7 cells harvested using RIPA Lysis Buffer (Upstate Biotechnology, Charlottesville, VA) in accordance with the manufacturer's instructions. Equal amounts of protein were electrophoresed on Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories) with Precision Plus Protein Kaleidoscope Standards (Bio-Rad Laboratories) as molecular weight marker. Transfer from gel to nitrocellulose membrane was performed using the Trans-Blot Turbo Transfer System, according to the manufacturer's protocol (Bio-Rad Laboratories). Blocking and incubation with primary and secondary antibodies was performed using the SNAP i.d. 2.0 Protein Detection System (EMD Millipore Corporation, Billerica, MA) according to the manufacturer's protocol. Primary antibodies included p107 (C-18): sc-318 (Santa Cruz Biotechnology, Santa Cruz, CA), against the C-terminal region of human protein p107, p-p107 (Ser 975): sc-130209 (Santa Cruz Biotechnology), against human protein p107 phosphorylated at Serine 975, Rb (C-15): sc-50 (Santa Cruz Biotechnology), against the C-terminal region of Rb, p-Rb (Ser 807): sc-293117 (Santa Cruz Biotechnology), against an Rb sequence containing phosphorylated Ser 807, Cyclin E2 (H-140): sc-22777 (Santa Cruz Biotechnology), against the N-terminal region of human protein Cyclin E2 and #4970 (Cell Signaling Technology, Danvers, MA), against the human protein β -actin. Secondary antibodies #AP 182P (Millipore) against rabbit IgG, were conjugated with horseradish peroxidase (HRP), and substrate was provided by Bio-Rad Clarity Western ECL Substrate (Bio-Rad Laboratories). The image was developed with the ChemiDoc XRS+System with Image Lab software (Bio-Rad Laboratories), and the results were quantified and normalized by densitometry using ImageJ.

PROLIFERATION ANALYSIS BY MTT ASSAY

Cells were seeded in a 96-well plate at 10,000 cells and 200 μ L of media per well. After 24 h of incubation, the media was replaced with media containing the same concentrations of vehicle or raloxifene and/or PX-866 as used in treatments for RNA and protein quantification. Following 72 h of treatment, the media in each well was replaced with 50 μ L of MTT solution consisting of 1mg thiazolyl blue tetrazolium bromide (Sigma–Aldrich) per mL phosphate buffered saline (PBS), for an incubation of 4 h. The MTT was next replaced with 150 μ L dimethyl sulfoxide (DMSO) for 10 min incubation and subsequent reading for 595 nm wavelength absorbance on iMark Microplate Absorbance Reader (Bio-Rad). The procedures were repeated for MCF-7 cells and for MCF10A cells.

STATISTICAL ANALYSIS

Three independent experiments were used to determine all numerical values. Statistical significance between control groups was evaluated using Student's *t*-test and the provided values include mean \pm SE. Values of *P* for determining significance were calculated by formula using Microsoft Excel, with *P* < 0.05 considered significant and *P* < 0.01 considered highly significant. *GAPDH* was used as the reference gene for mRNA normalization and β -actin for protein quantified by densitometry.

RESULTS

RALOXIFENE AND PX-866 REDUCED PROLIFERATION OF MCF-7 BUT NOT MCF10A CELLS

MTT was performed to assess the proliferation of both MCF-7 ER+ breast cancer cells and MCF10A hTERT-expressing noncancerous breast epithelial cells treated with raloxifene, PX-866, or combinations of both. As shown in Figure 1A, highly significant reduction of the proliferation of viable MCF-7 cells relative to untreated control cells was observed after 72 h for all cells treated with raloxifene alone or with raloxifene in combination with any concentration of PX-866. The reduction was enhanced significantly as the PX-866 concentration increased. In contrast, Figure 1B shows that no significant reduction was seen in the same time frame for noncancerous MCF10A cells undergoing the same treatments. Having shown highly significant reduction of proliferation with raloxifene-PX-866 combinations, we examined the mechanism of the reduction, including whether it was significantly due to decreased E2F-1-regulated transcription. Among our early experimental findings, we discovered that combinations of raloxifene and PX-866 significantly reduced the transcription level of oncogene *Interleukin-11 (IL-11)* (Fig. 2).

RALOXIFENE AND COMBINATIONS WITH PX-866 REDUCE THE PHOSPHORYLATION AND EXPRESSION OF POCKET PROTEIN p107

We examined whether the treatments altered the level of phosphorylation of p107. As shown in Figure 3B, a highly significant reduction of phosphorylated p107 was observed in MCF-7 cells treated with 1.0 μ M raloxifene in combination with 0.1 or 0.4 μ M PX-866, measured at 58.5% and 61.8%, respectively, of the level for untreated control cells, according to ImageJ computations. To determine whether the treatments reduced the phosphorylation or simply reduced the expression of p107 protein, we evaluated the level of total p107 protein (both phosphorylated and unphosphorylated) by Western blot. As shown by Figure 3C, a significant level of reduction of total p107 protein was seen for treatments with 0.4 μ M PX-866 used alone or with raloxifene in combination with either 0.1 or 0.4 μ M PX-866. To determine whether the reduction of phosphorylated p107 indicated a reduction of phosphorylation, we measured phosphorylated p107 normalized in ImageJ to total p107 instead of normalized to the reference protein β -actin. As shown in Figure 3D, the normalization to total p107 indicated that an actual reduction of phosphorylation resulted only in cells treated with raloxifene alone.

RALOXIFENE-PX-866 COMBINATIONS REDUCED THE TRANSCRIPTION OF E2F-1-REGULATED GENES *E2F-1*, *CDC6*, AND *CYCLIN E2*, ESSENTIAL TO G1-S TRANSITION

We next used real-time PCR to measure whether the mRNA expression levels of four targets of E2F-1 transcriptional activity were affected by the treatments and whether the level of MAGE-A11 itself might, in fact, be affected. As shown in Figure 4A, there was no significant change in the level of *MAGE-A11* expression, at least not at the level of transcription, due to treatment with raloxifene and/or PX-866.

Significant mRNA reduction was observed for three of the E2F-1-regulated genes examined. Figure 4B shows no significant treatment-induced transcription change of the *p27* CDK

inhibitor. A significant transcription decrease for *E2F-1* was observed when MCF-7 cells were treated with 1.0 μM raloxifene in combination with 0.4 μM PX-866, as shown in Figure 4C. As shown in Figure 4D, significant decreases in the level of transcription of *Cdc6* were observed when MCF-7 cells were treated with raloxifene in combination with 0.1 or 0.4 μM PX-866 or with 0.4 μM PX-866 alone. In contrast to the more limited effects on other E2F-1-regulated genes, Figure 5A shows that there was highly significant reduction of *Cyclin E2* following treatment with PI3K inhibitor PX-866 either alone at 0.4 μM or at any concentration (0.1, 0.4, or 0.8 μM) in combination with raloxifene.

We performed Western blot analysis to confirm the highly significant down-regulation of Cyclin E2 following treatment with raloxifene and/or PX-866. As shown by Figure 5C, highly significant reduction of Cyclin E2 protein was exhibited but was observed following treatment with raloxifene alone and not following treatment with PX-866 either alone or in combination.

RALOXIFENE AND PX-866 COMBINATIONS REDUCED THE EXPRESSION AND PHOSPHORYLATION OF THE Rb POCKET PROTEIN ESSENTIAL TO E2F-1 MEDIATED G1-S TRANSITION

We extended our analysis of treatment effects on phosphorylation of p107 to the related pocket protein Rb. As shown in Figure 6B, phosphorylated Rb was significantly or highly significantly decreased in cells treated with 0.4 μM PX-866 alone or with 1.0 μM raloxifene in combination with any concentration of PX-866, 0.1, 0.4, or 0.8 μM . Phosphorylated Rb in cells treated with raloxifene in combination with 0.1 or 0.8 μM PX-866 was reduced to 41% or 48% of control cell concentrations, respectively. As shown by Figure 6C, total protein expression, as with p107, was also reduced significantly with some treatments, which included raloxifene alone or in combination with 0.1 or 0.8 μM PX-866. When phosphorylated Rb was normalized to total Rb protein expression, as shown in Figure 6D, a significant decrease in phosphorylated protein relative to total protein was exhibited for all cells treated with a combination of raloxifene and PX-866.

DISCUSSION

Using the MTT assay, we showed that viable MCF-7 breast cancer cells were highly significantly reduced, relative to untreated control cells, after treatment with raloxifene alone or with raloxifene combined with tested concentrations of PX-866. In contrast, no significant reduction was observed after similar treatments of noncancerous MCF10A breast epithelial cells. We investigated whether the decrease in proliferation of viable MCF-7 cells was the result of a mechanism significantly decreasing E2F-1-mediated transcription regulation.

Both the phosphorylation and expression levels of p107 and Rb proteins, which mediate transcription regulation by E2F proteins [Dyson, 1998], were reduced by raloxifene and/or combinations with PX-866. The transcription levels of four genes transcriptionally regulated by E2F-1 were evaluated in treated and untreated cells using real-time PCR. The genes included *Cdc6* and *Cyclin E2*, both essential to progression from G1 to S phase, the self-regulated *E2F-1* gene, which provides cyclic positive feedback, and the CDK inhibitor *p27* that serves in cyclic negative feedback [Johnson et al., 1994; Dyson, 1998; Yan et al., 1998;

involves an effect by IL-11 upon MAGE-A11, then treatment with raloxifene and PX-866 should increase or decrease the MAGE-A11-mediated interference with p107 repressive activity.

Without more global reduction of E2F-1-mediated transcription, the reduced E2F-1 transcriptional activity did not appear to fully account for the reduced MCF-7 cell proliferation or the full extent of *Cyclin E2* transcription decrease. We found that raloxifene and PX-866 in combination reduced the transcription level of *IL-11* in MCF-7 cells, and CCSB discovered a protein–protein interaction between IL-11 and MAGE-A11, which impacts the repressive activity of TGF- β pathway component p107 [Su et al., 2013]. Effects, if any, of raloxifene and PX-866 on p107 phosphorylation, essential to most changes in the activity of E2F proteins regulated by p107 [Dyson, 1998; Leng et al., 2002], could profoundly impact TGF- β pathway activity, given that MAGE-A11 has been reported to alter the p107 binding partners, recruit AR, and convert p107 from a repressor to an activator of transcription [Su et al., 2013].

Although raloxifene in two combinations with PX-866 highly significantly reduced the level of p107 phosphorylated at Ser 975, PX-866 alone or in combinations with raloxifene reduced the level of total p107 protein. Since MAGE-A11 has also been reported to protect p107 protein from ubiquitin-mediated degradation [Su et al., 2013], a treatment-associated decrease in p107 protein, as we observed, would be consistent with a treatment-associated reduction of MAGE-A11-p107 interaction and with protection of the role of p107 in negative regulation of transcription and cell cycle progression.

Because of the similarity between p107 and Rb in structure and function, we extended the phosphorylation assessment to Rb and found that the treatment-associated reduction of phosphorylation was greater for Rb than for p107. The role of p107 in limiting S phase entry is mostly as a backup for Rb [Herrera et al., 1996; Hurford et al., 1997; Dyson, 1998]. Phosphorylated Rb protein was reduced significantly by all combinational treatments and by PX-866 alone. Total Rb protein, like total p107, was significantly reduced by three of the treatments but the reason, whether or not related to ubiquitin-mediated protein degradation, is a subject for future exploration. When the treatment effects on actual phosphorylation were evaluated, a significant reduction in Rb phosphorylation was indicated for all treatments with combination of raloxifene and PX-866.

Transcription of *Cyclin E2*, in particular, is regulated more by Rb than by p107. *Cyclin E2* has been shown to be overexpressed in Rb double-knockout cells despite a compensatory increase in p107 expression [Herrera et al., 1996; Hurford et al., 1997]. While E2F-1, E2F-2, and E2F-3 are able to bind the activating site on the *Cyclin E2* promoter, E2F-4 and E2F-5 are unable to bind [Sardet et al., 1995; Karlseder et al., 1996; Le Cam et al., 1999]. Since p107 preferentially binds E2F-4 or E2F-5, it is a poor substitute for Rb at the *Cyclin E2* promoter unless its E2F binding partner is stably changed to E2F-1 [Sardet et al., 1995; Hurford et al., 1997; Dyson, 1998; Le Cam et al., 1999; Chen et al., 2002; Su et al., 2013].

Reduced E2F-1-mediated transcription and reduced proliferation resulted more from reduction of Rb phosphorylation than p107 phosphorylation, but the proliferation reduction

was the result of reduced *Cyclin E2* expression, which can also be achieved by mechanisms other than a decline in E2F-1 transcriptional activity. Down-regulation of Rb phosphorylation likely contributed a redundant effect on *Cyclin E2* expression, while an additional contribution was likely provided by a decrease in *Cyclin D1* expression and activity, which is essential to *Cyclin E2* down-regulation [Caldon et al., 2009; Caldon and Musgrove, 2010]. Decreased Cyclin D1 expression has been demonstrated in glioblastoma cells as the result of treatment with PX-866 [Koul et al., 2010]. We will test for raloxifene and PX-866 effects on Cyclin D1 expression in MCF-7 cells. Raloxifene and PX-866 produced decreases in proliferation of MCF-7 breast cancer cells, likely through redundant mechanisms. While some important results followed treatments with raloxifene or PX-866 alone, highly significant reduction of MCF-7 cell proliferation, highly significant reduction of *Cyclin E2* transcription and significant or highly significant reduction of Rb phosphorylation followed combinations of raloxifene and PX-866. The value of joint pathway modification was demonstrated.

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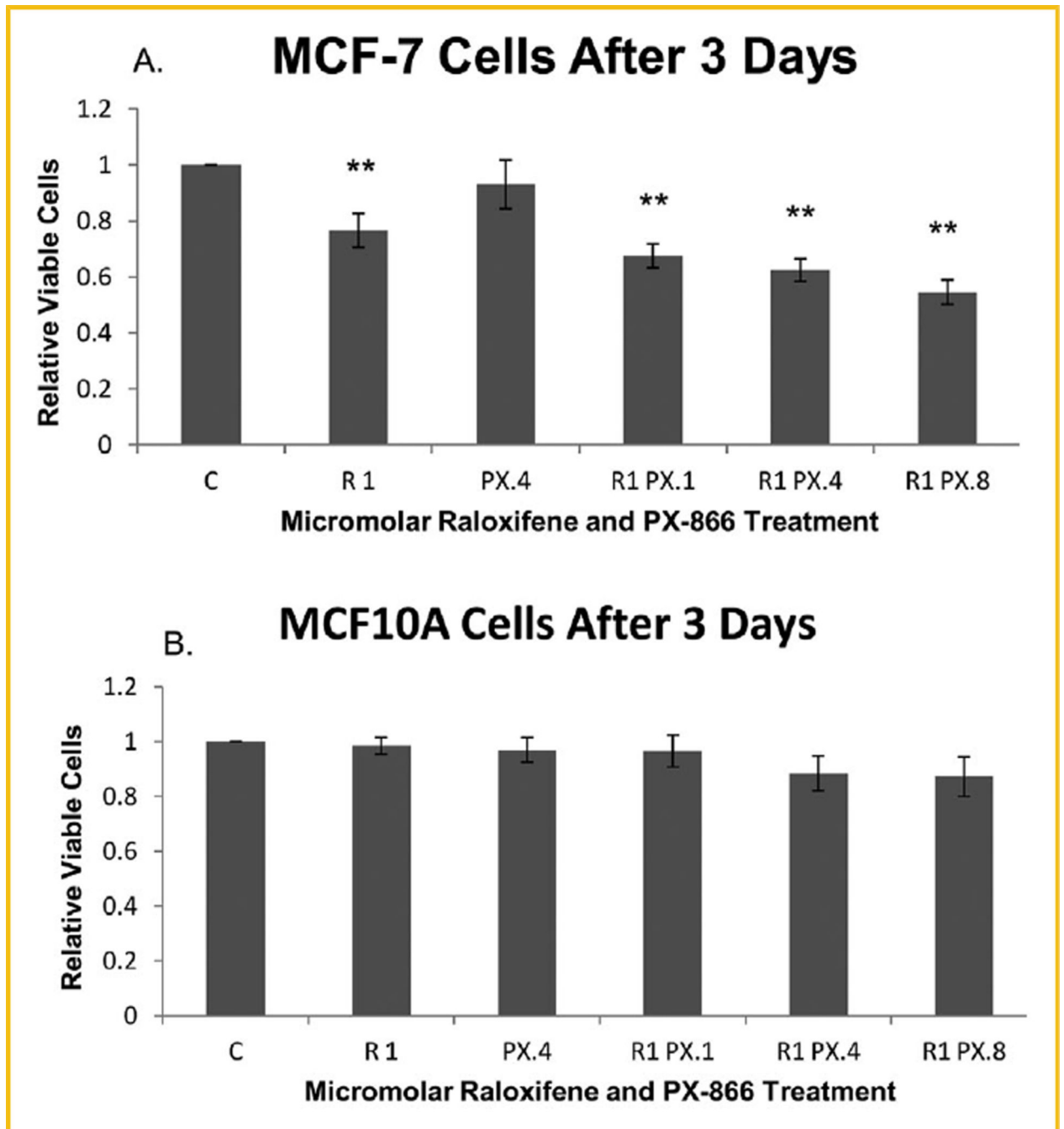


Fig. 1. Raloxifene and PX-866 reduced MCF-7 but not MCF10A cell proliferation following three consecutive days of treatments. C, control; R1, raloxifene 1.0 μ M; PX.1, PX-866 0.1 μ M; PX.4, PX-866 0.4 μ M; PX.8, PX-866 0.8 μ M. Proliferation and viability of treated MCF-7 and MCF10A cells in MTT assay. (A) MCF-7 cells treated with raloxifene alone were highly significantly reduced after 72 h to 76.6% of the level of untreated control cells. After the same 72 h, MCF-7 cells treated with raloxifene in combination with 0.1, 0.4, or 0.8 μ M PX-866 were highly significantly reduced to 67.5%, 62.4% or 54.5%, respectively, with the

reduction of viable cell proliferation increasing with PX-866 dose increase. (B) No significant reduction of proliferation of viable MCF10A cells was observed with the same treatments for the same 72 h duration. Values represent averages from three independent experiments \pm SEM. * $P < 0.05$. ** $P < 0.01$.

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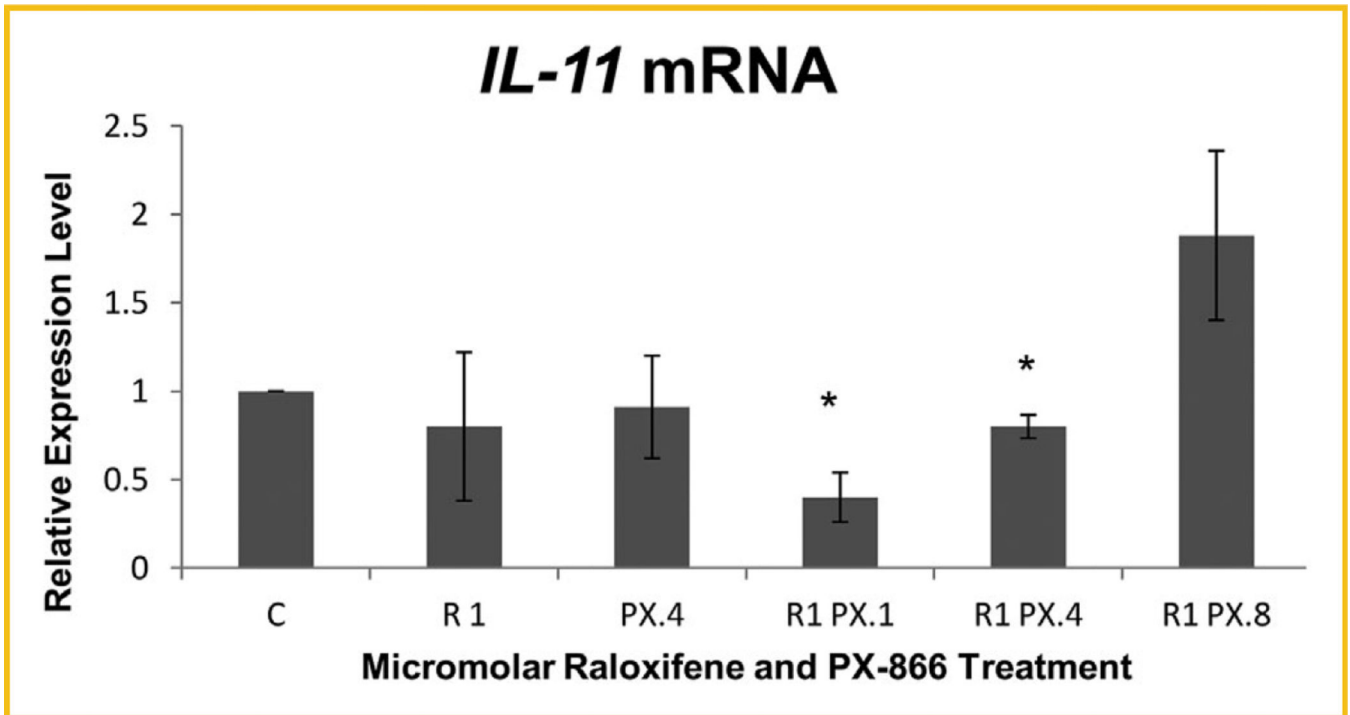
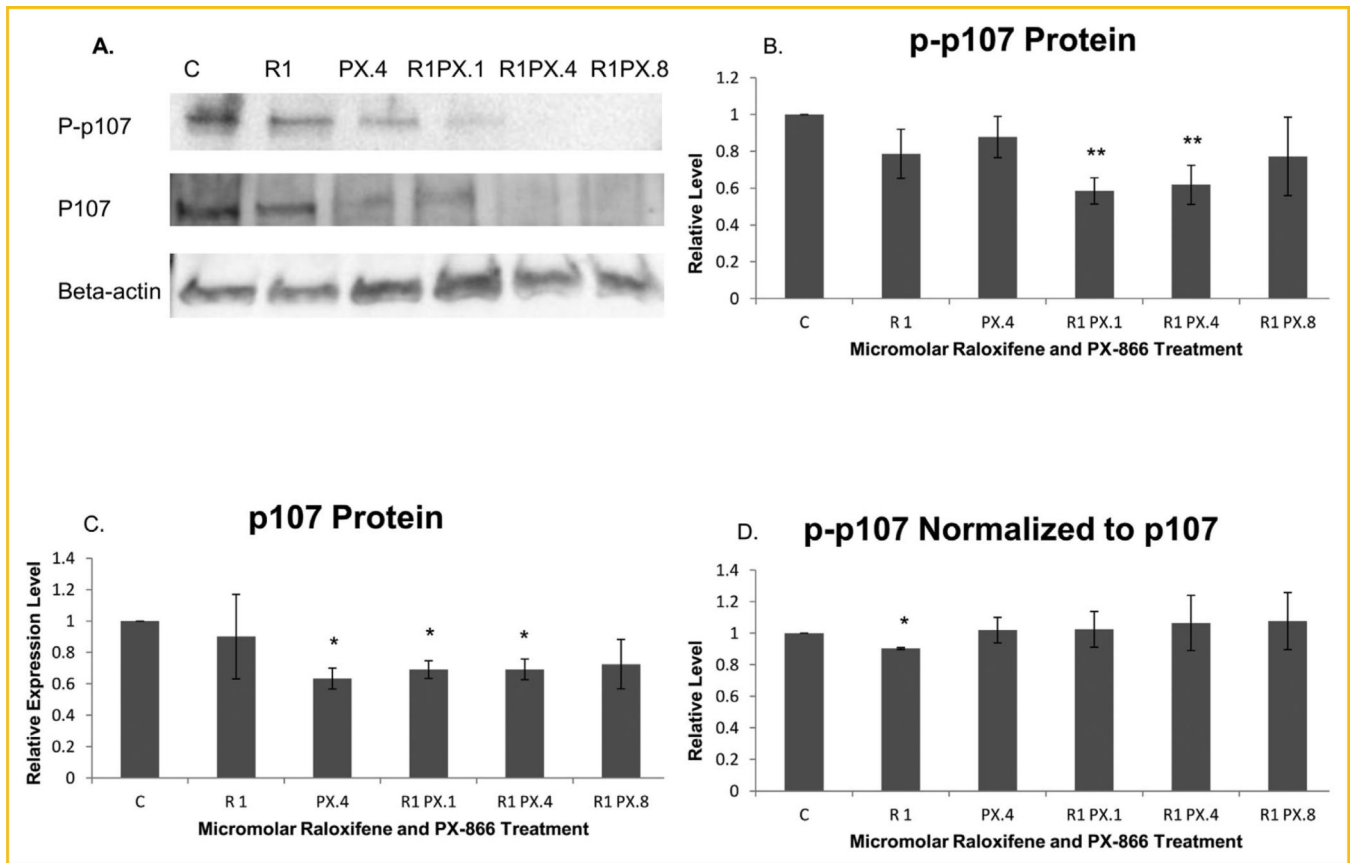
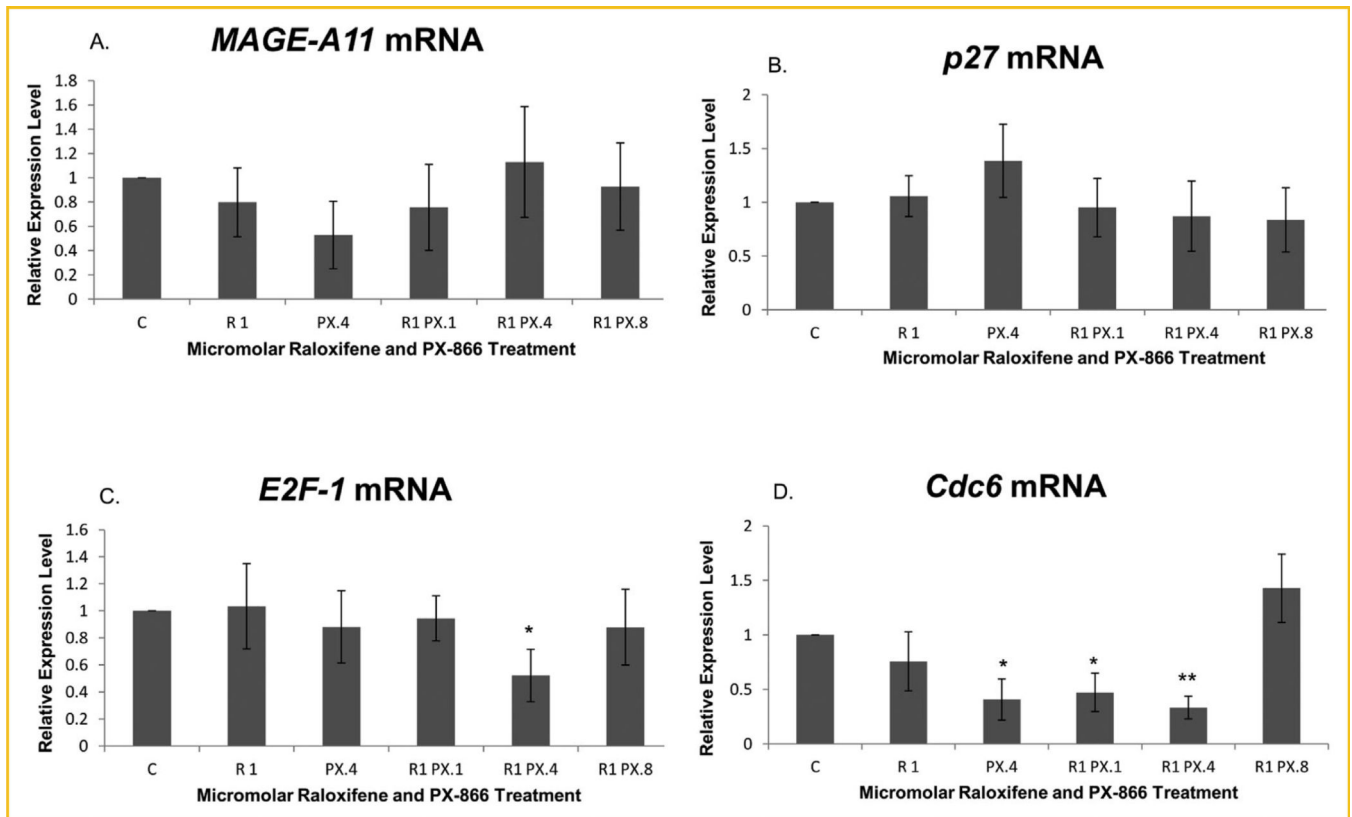


Fig. 2. Raloxifene- and PX-866-reduced IL-11 transcription levels following 3 consecutive days of treatments. Significant decrease in *IL-11* transcription was seen following 3 days of treatment with 1.0 μM raloxifene in combination with 0.1 or 0.4 μM PX-866 in MCF-7 cells, with reduction to 40.3% and 80.2%, respectively, of the level of control cells. Values represent averages from three independent experiments \pm SEM. * $P < 0.05$. ** $P < 0.01$.

**Fig. 3.**

Immunoblotting analyses show reduced p107 expression and phosphorylation following three day treatments. (A) Western blot images of phosphorylated p107 (P-p107), total p107 protein without regard for phosphorylation (P107) and β -actin. Representative images shown are from the gels used to derive the densitometry averages represented in B–D. (B) Densitometry showed that phosphorylated p107, normalized to β -actin, was highly significantly reduced following 3 days of treatment with 1.0 μ M raloxifene in combination with 0.1 or 0.4 μ M PX-866. (C) Total p107 protein normalized to β -actin was significantly reduced following three days of treatment with 0.4 μ M PX-866 alone or with 0.1 or 0.4 μ M PX-866 in combination with 1.0 μ M raloxifene. (D) The decrease in phosphorylated p107 was compared to the decrease in total p107 by normalizing p-p107 to total p107, rather than to β -actin. The calculated results showed that only cells treated with raloxifene alone specifically exhibited significant reduction of p107 phosphorylation. Values represent averages from three independent experiments \pm SEM. * $P < 0.05$. ** $P < 0.01$.

**Fig. 4.**

Real-time PCR revealed reduced transcription levels of some E2F-1 regulated genes in treated MCF-7 cells. (A) *MAGE-A11* and (B) *p27* were not significantly altered by treatments with raloxifene and/or PX-866. The transcription level of (C) *E2F-1* was significantly reduced only by treatment with 1.0 μ M raloxifene in combination with 0.4 μ M PX-866, to 56.0% of the level of control cells. The (D) *Cdc6* transcription level relative to control cells was significantly reduced by 0.4 μ M PX-866 alone and by 0.1 μ M PX-866 in combination with raloxifene to 40.7% and 47.3%, respectively, and highly significantly reduced by 0.4 μ M PX-866 in combination with raloxifene to 33.4% of the level of control cells. Values represent averages from three independent experiments \pm SEM. * $P < 0.05$. ** $P < 0.01$.

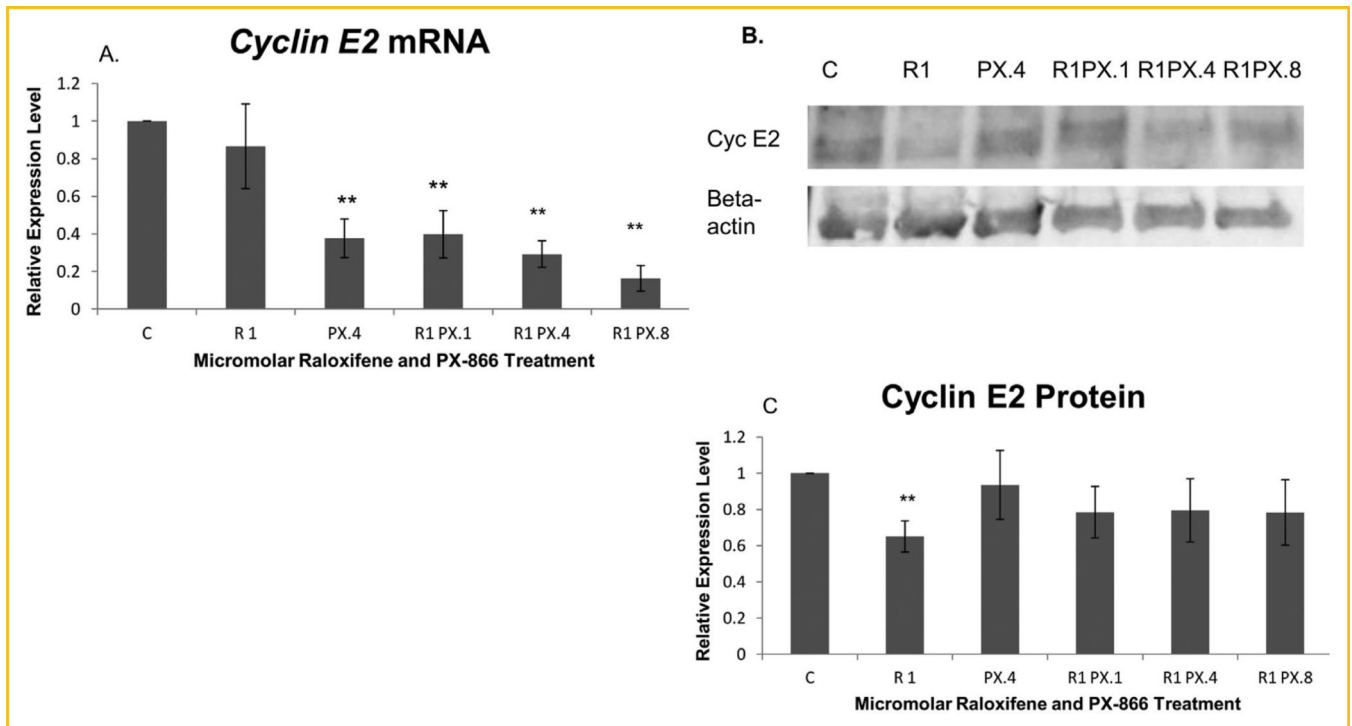
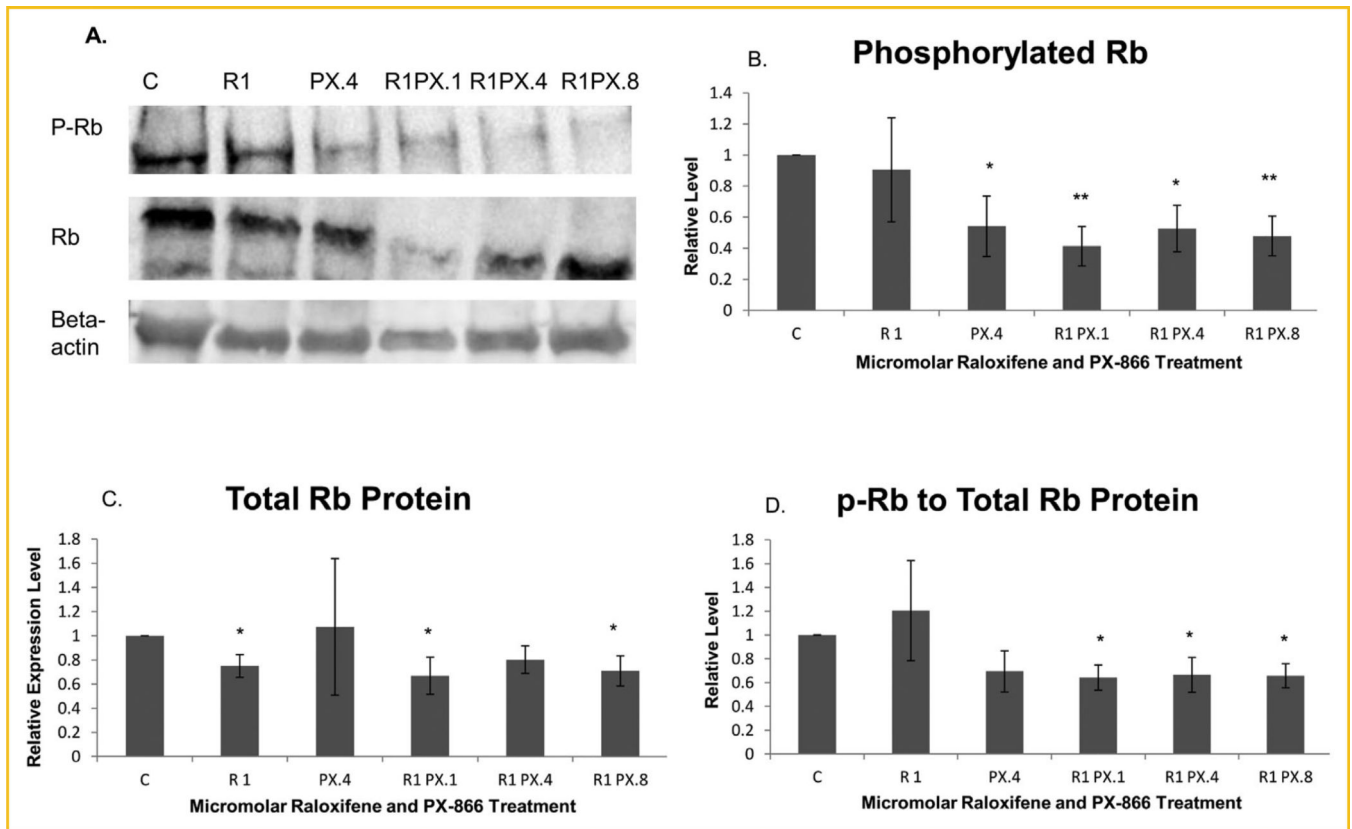


Fig. 5. PX-866-reduced Cyclin E2 expression at the mRNA level and raloxifene at the protein level. (A) The *Cyclin E2* mRNA level in treated MCF-7 cells was highly significantly decreased to 37.7% of the level in untreated control cells by treatment with PX-866 alone and to 40.0%, 29.3%, and 16.4% by treatment with 0.1, 0.4 and 0.8 μ M PX-866, respectively, in combination with 1.0 μ M raloxifene. (B) Western blot images of Cyclin E2 (Cyc E2) and β -actin. (C) Significant reduction of Cyclin E2 protein in treated MCF-7 cells, like the reduction of p107 phosphorylation, was seen only in samples treated with raloxifene alone, in spite of highly significant transcription reduction using PX-866 alone or in combination. A highly significant reduction of Cyclin E2 to 65.1% relative to the β -actin reference protein was observed using 1.0 μ M raloxifene. The effects of raloxifene were more notable at the protein level while the effects of PX-866 were more notable at the transcription level. Values represent averages from three independent experiments \pm SEM. * $P < 0.05$. ** $P < 0.01$.

**Fig. 6.**

PX-866- and-raloxifene reduced Rb expression and phosphorylation. (A) Western blot images of phosphorylated Rb (P-Rb), total Rb (Rb) and β -actin. (B) Western blot showed that the phosphorylated Rb protein level was significantly reduced after treatment with PX-866 alone or with 0.4 μ M PX-866 in combination with 1.0 μ M raloxifene and highly significantly reduced following treatment with 0.1 or 0.8 μ M PX-866 in combination with raloxifene. (C) Total Rb protein was significantly reduced following treatment with raloxifene alone or in combination with 0.1 or 0.8 μ M PX-866. (D) Phosphorylated Rb relative to total Rb protein was significantly reduced following each combination of PX-866 and raloxifene treatment, to 64.2%, 66.5%, and 65.8% of the level of control cells by use of 0.1, 0.4 and 0.8 μ M PX-866, respectively. Values represent averages from three independent experiments \pm SEM. * $P < 0.05$. ** $P < 0.01$.