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Estrogen Receptor Beta is a Novel Therapeutic Target for Photoaging

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RUNNING TITLE

ER β as a therapeutic target for photoaging

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Number of text pages: 19

Number of tables: 1

Number of figures: 5

Number of references: 35

Number of words in Abstract: 221

Number of words in Introduction: 545

Number of words in Discussion: 898

Nonstandard abbreviations: metalloproteinases (MMPs); cyclooxygenase-2 (COX-2);

Estrogen receptors (ER); normal human epidermal keratinocytes (NHEKs); ligand
binding domains (LBDs); normal human dermal fibroblasts (NHDFs).

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ABSTRACT

One of the many harmful factors faced by the skin is solar UV radiation, which damages skin by inducing chronic low grade inflammation through increased expression of pro-inflammatory cytokines, metalloproteinases (MMPs) and cyclooxygenase-2 (COX-2). Estrogen receptors (ER) α and β are ligand-dependent transcription factors that are expressed in skin, and an ER β agonist has previously shown efficacy in vivo in models of pain and inflammation. Since ER β does not carry the breast and uterine proliferation liabilities of ER α , we decided to explore the possibility of using ER β as a target for photoaging. We show that ER β -selective compounds suppressed the expression of cytokines and MMPs in activated keratinocytes and fibroblasts-based in vitro models of photoaging. Further, in activated dermal fibroblasts, ER β -selective compounds also inhibited COX-2. These activities of ER β ligands in skin cells correlated with the expression levels of ER β and showed reversal by treatment with a potent synthetic ER antagonist. Furthermore, the pharmacology of ER β -selective compound was observed in wild-type but not in skin cells obtained from ER β knock-out mice. Finally, we demonstrate that a synthetic ER β agonist inhibited UV-induced photo-damage and skin wrinkle formation in a murine model of photoaging. Therefore, the potential of an ER β ligand to regulate multiple pathways underlying the etiology of photoaging suggests ER β to be a novel therapeutic target for the prevention and treatment of photoaging.

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INTRODUCTION

Photoaging results from the repetitive exposure of skin to damaging effects of solar UV radiation, and is characterized by wrinkles, laxity, dryness and mottled pigmentation. In recent years, some of the molecular mechanisms underlying the etiology of photoaging have been described. The process of photoaging involves three cell types, namely keratinocytes, fibroblasts, and infiltrating neutrophils (Makrantonaki and Zouboulis, 2007; Fisher et al., 2002; Rijken et al., 2005). Within minutes of UVB radiation exposure, epidermal keratinocytes show an increased activation of transcription factors, activator protein 1 (AP-1) and nuclear factor- κ B (NF- κ B), resulting in high expression of matrix metalloproteinases (MMPs) and pro-inflammatory cytokines (Fisher et al., 1996). These cytokines as well as UVA component of the UV radiation in turn activate dermal fibroblasts to secrete MMPs, which damage the collagen component of the dermal extracellular matrix (Fagot et al., 2004). Cytokines and chemotactic factors that are secreted by skin cells also recruit neutrophilic granulocytes to the dermis. These neutrophils further degrade dermal extracellular matrix by secreting MMPs and elastases, thus contributing to UV-mediated dermal collagen and elastin degradation (Rijken et al., 2005). Repetitive UV exposure leads to the accumulation of partially degraded extracellular matrix components in the dermis, resulting in wrinkle appearance. Therefore, a number of pathways and processes involved in photoaging could be targeted by potential therapeutic agents.

Estrogen receptors ($ER\alpha$ /NR3A1 and $ER\beta$ /NR3A2) are ligand-dependent transcription factors that belong to the nuclear receptor superfamily (Robinson-Rechavi et

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al., 2003). Both ER α and β are expressed in human epidermal keratinocytes and dermal fibroblasts and their natural ligand, estrogen, has profound influence on skin (Haczynski et al., 2004; Walker and Korach, 2004). Importantly, estrogen has been shown to decrease aging and wrinkling of the skin when used therapeutically (Wolff et al., 2005). Estrogen prevents aging by increasing skin thickness, dermal collagen content and water holding capacity of post-menopausal skin. Estrogen increased skin moisture by strengthening epidermal barrier and enhancing the production of hydrophilic glycosaminoglycans in the dermis (Quatresooz and Pierard, 2007). Since ER α agonism is associated with unwanted breast and uterine side effects (Jordan, 2002), we decided to examine the possibility of using ER β as a therapeutic target for prevention and treatment of photoaging. Our notion that ER β ligands could be used for photoaging was also strengthened by the in vivo efficacy observed with an ER β compound in pre-clinical models of rheumatoid arthritis, inflammatory bowel disease, and inflammatory/chemical induced pain (Leventhal et al., 2006). ER ligands have been proposed to mediate their anti-inflammatory effects by inhibiting NF-kB-dependent gene expression via involvement of cofactor SRC-2 (Cvoro et al., 2006; Cvoro et al., 2008). In addition, topical estradiol has been shown to increase type I procollagen and decrease MMP1 expression in human epidermis (Rittie et al., 2008). Here, we demonstrate that ER β ligands could interfere with various steps involved in the etiology of photoaging. We show that ER β ligands inhibit the expression of proinflammatory cytokines, and metalloproteinases in cell-based models of UVB-activated normal human epidermal keratinocytes (NHEKs) and cytokine-activated normal human dermal fibroblasts (NHDFs). We also demonstrate the efficacy of a synthetic ER β ligand in a murine model

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of UV-induced photoaging. Our observations indicate that ER β is a potential novel target for photoaging, and could provide a new category of therapeutic agents for the prevention and treatment of this indication.

MATERIALS AND METHODS

ER β selective compounds and other chemical reagents

Test compounds used in this study were supplied by the Wyeth compound library (Princeton, NJ) that were either purchased or synthesized by Wyeth Medicinal Chemistry (Collegeville, PA). All other chemicals and reagents obtained from various vendors and used in this study were reagent grade or better.

Skin cells and cell culture conditions

NHEKs and NHDFs (Cambrex/Lanza, Walkersville, MD), and human dermal fibroblasts cell lines, HFF and BJ-5ta (ATCC), as well as human epidermal keratinocyte cell line KERTr (ATCC) were cultured as per vendors' recommendations. In general, cells were trypsinized and seeded on day 0, compound treatments were done on day 1 with and without UVB (8 mJ/cm², UV Stratalinker 2400, Stratagene, La Jolla, CA) or TNF α (1 ng/ml) activations, and cells were harvested on day 2 with lysis buffer (Applied Biosystems/Ambion, Foster City, CA) directly added to the cultured cells after a PBS wash. Cells were either used for RNA purification using Qiagen RNeasy RNA purification column (Qiagen, Hilden, Germany) as per vendor's protocol or directly processed to cDNA using "Cell-to-cDNA" lysis buffer (Ambion, Foster City, CA).

ER β KO and heterozygote (HET) skin cell preparations

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ER β KO mice were generated at Wyeth Research as previously described (Shughrue et al., 2002) and are not the same as the β ERKO mice (Krege et al., 1998; Harris, 2007).

Animal use was approved by the Institutional Animal Care and Use Committee of Wyeth Research, Collegeville, PA and was conducted in accordance with Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. Heterozygous female were intercrossed to the homozygous KO male to generate HET and KO pups. Genotyping Taqman assays with WT and KO specific primer/probe sets were used to identify HET/KO pups. The primer/probe sequences are: ERBWT forward primer: 5'-CTG GAA GGT GGG CCT GTT C; ERBWT reverse primer: 5'-TGG CAG TGG GTG GCT AAA G; ERBKO forward primer: 5'-ACA TTC TAC AGT CCT GCT GTG ATG A; ERBKO reverse primer: 5'-GGG TGT TGG GTC GTT TGT TC; ERBWT probe, 6FAM-CCAAATGTGCTATGGCCAACTTCTGGAC-TAMRA; ERBKO probe, VIC-TCCCAGCAGCACCGGTAAGCTAAG-TAMRA. Skins from newborn mice (2-3 days old) were isolated and floated on 2.5 mg/ml dispase (Invitrogen/Gibco, Carlsbad, CA) overnight at 4°C and separated into epidermal and dermal layers using small forceps. The epidermal and dermal layers were minced and subjected to several differential centrifugations, fractionations and filtrations as previously described (Zheng et al., 2005). These cells were then cultured in Eagle's minimal essential medium containing fetal bovine serum (8 %) in 24-well culture plates (day 0). Cells were treated with vehicle or ER β -selective compounds on day 2, followed by isolation and purification of RNA on day 3 using RNeasy column (Qiagen, Hilden, Germany). Gene expression profiles were analyzed using Taqman Low Density Array (TLDA) and individual Taqman gene assays (Applied Biosystems, Foster City, CA).

Taqman Low Density Arrays (TLDA) and quantitative RT-PCR

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The RNA or cDNA obtained from the compound treated cells were used in custom-designed TLDA or individual Taqman assays (Applied Biosystems, Foster City, CA) as per vendor's protocols using ABI 7900HT real-time PCR machine. The level of expression was calculated based on the PCR cycle number (Ct), and the relative gene expression level was determined using $\Delta\Delta C_t$ method as described (Livak and Schmittgen, 2001). One TLDA was designed to contain oligonucleotide probe and primer pairs for TNF α (Applied Biosystems Assay ID, Hs00174128_m1), IL1 β (Hs00174097_m1), IL6 (Hs00174131_m1), IL8 (Hs00174103_m1), MMP1 (Hs00233958_m1), MMP3 (Hs00233962_m1), MMP9 (Hs00234579_m1), TIMP1 (Hs00171558_m1), DCN (Hs00754870_s1), COL1A1 (Hs00164004_m1), CCL3 (Hs00234142_m1), CCL4 (Hs99999148_m1), CCL5 (Hs00174575_m1), NOS2A (Hs00167257_m1), PTGS2 (Hs00153133_m1) and 18S (Hs99999901_s1) control gene. Some of these individual gene assays from the list above (identical assay ID) were also purchased from Applied Biosystems and used for confirmation or focused assay purposes. Other gene assays used in this study including human genes ER α and ER β , and mouse genes Mmp13, Ptgs2, Mapk1, Fbn1 etc., were also purchased from Applied Biosystems.

Murine model of photoaging

Five-week-old female albino hairless mice (Hos:HR-1) were obtained from the HOSHINO Laboratory Animals (HOS : Kotoh-cho, Japan). Animals had free access to food and water and were acclimated for 1 week before the study. Eight mice are allocated to each group (total six groups for each test compound). All experimental protocols were approved by IACUC of Clinical Research Institute, Seoul National University Hospital (AAALAC accredited facility). An UV irradiation device that included TL20W/12RS

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UV lamps (Philips, Eindhoven, Netherlands) with an emission spectrum between 275 and 380 nm (peak, 310-315 nm) served as the UV source (Seo et al., 2001). A Kodacel filter (TA401/407; Kodak, Rochester, NY) was mounted 2 cm in front of the UV lamp to remove wavelengths of less than 290nm (UVC). Irradiation intensity at the mouse skin surface was measured using a UV meter (model 585100; Waldmann Co., Germany). The irradiation intensity 20 cm from the light source was 0.5 mW/cm².

Initially, we measured the minimal erythma dose (MED) on dorsal skin of mice. MED can be defined as the minimum amount of radiation exposure required to produce an erythma with sharp margins after 48h. Mice were exposed to UV light 3 times per week (Monday, Wednesday and Friday) for 6 weeks. The irradiation dose was increased weekly by 1 MED (1MED = 100 mJ/cm²) up to 4 MED and then maintained at 4 MED. UV irradiation was stopped after irradiation for the sixth week. ERB-041 or its vehicle (70% ethanol, 30% polyethylene glycol) was topically applied to the dorsal area (50 µl) after each exposure to UV irradiation (5 times a week). Skin wrinkle replica was made with a silicon rubber (Silflo Dental Impression Material, Flexico developments, England) from the backs of unstrained mice. Skin impressions were photographed using a coupling charge system (CCD) video camera and analyzed by Skin-Visiometer SV 600 software (CK electronic GmbH, Germany).

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RESULTS

Identification of ER β as a target for photoaging

In order to evaluate ER β as a target for photoaging, normal human epidermal keratinocytes (NHEKs) were treated either with vehicle or a synthetic ER β -selective ligand, WAY-200070 (Harris, 2007) for 24 hours with or without UV radiation (8mJ/cm²). WAY-200070 is an ER β selective ligand, which bound to ER α and ER β ligand binding domains (LBDs) with IC₅₀ (concentration of the ligand required for 50% displacement of the radiolabeled 17 β -estradiol) values of 155 and 2.3 nM respectively. In addition, it showed EC₅₀ (concentration of the ligand required for 50% increase in luciferase activity) values of 314 and 31 nM, respectively in Gal4-ER α -LBD and Gal4 ER β -LBD transactivation assays (Table 1) (Malamas et al., 2004; Harris, 2007). UV exposure induced the expression of MMPs, cytokines and COX-2 in NHEKs, and inhibited the expression of tissue inhibitor of metalloproteinases 1 (TIMP1) and collagen 1A1 (COL1A1) (Fig. 1). These results indicate that the UV exposure of keratinocytes induces inflammatory and catabolic pathways, thus mimicking the etiology of photoaging. Since UV exposure of epidermal keratinocytes is the first step involved in the etiology of photoaging, we examined the effect of WAY-200070 on UV-induced interleukins, MMPs and COX-2 expression in NHEKs. Interestingly, WAY-200070 decreased the UVB-induced expression of MMP1, MMP3, IL1 β , IL8, TNF α , IL6 and COX-2 in NHEKs (Fig. 2D). Since the etiology of photoaging also involves the activation of dermal fibroblasts by cytokines produced from UV-activated epidermal keratinocytes, we next examined the effect of WAY-200070 on cytokines and MMP expression in TNF α -treated normal human dermal fibroblasts (NHDFs). TNF α induced

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the expression of IL8, IL1 β , COX-2 and MMP1 in NHDFs, which was significantly inhibited after treatment with WAY-200070 (Fig. 2E). Expression of IL6 and MMP3 was also induced in NHDFs, but WAY-200070 did not significantly suppress expression as in NHEKs (Fig. 2E). The difference in the degree of suppression between NHEK and NHDF cells may be because the method of cytokine induction differed (UV vs. TNF α) between the two cell types.

Validation of ER β as a target for photoaging

Since WAY-200070 is selective but not specific for ER β , we next examined whether its potential anti-photoaging in vitro effects were specifically mediated via ER β . To test this, KERTr keratinocytes were treated with WAY-200070 (1 μ M) in the presence or absence of an ER-specific pan-antagonist, ICI182780 (1 μ M) (Escande et al., 2007). Treatment of KERTr cells with WAY-200070 inhibited MMP1 expression in a dose-dependent manner, which was efficiently antagonized by ICI 182780 (Fig. 3A), suggesting that WAY-200070 mediated its effects through ER. To rule out the possibility that WAY-200070 mediated its effect through ER α , we next examined the effect of an ER α -selective ligand, PPT (1 μ M) (Escande et al., 2007), on MMP1 expression in UV-activated NHEKs and TNF α -activated NHDFs. Unlike WAY-200070, PPT failed to inhibit the expression of MMP1 in activated NHEKs and NHDFs (Fig. 3B). In contrast to KERTr, NHEKs and NHDFs, immortalized human fibroblasts cell lines, BJ and HFF, do not show significant expression of ER β (Fig. 3C), and as expected, WAY-200070 did not inhibit the expression of cytokines/MMPs in these cells (data not shown). Finally, we examined the effect of WAY-200070 on responsive gene expression in UV-activated primary skin keratinocytes obtained from ER β heterozygote (HET) and knock-out (KO)

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mice (Shughrue et al., 2002; Ohnemus et al., 2005; Antal et al., 2008; Zhao et al., 2008). WAY-200070 inhibited the expression of Mmp13 (murine counterpart of human MMP1), and another ER-responsive gene, mitogen-activated protein kinase 1 (Mapk1) in ER β heterozygote but not in KO keratinocytes (Fig. 3D, 3E). Basal expression of Mmp13 was increased in the KO cells, suggesting that unliganded ER β may influence Mmp13 expression. For both responsive genes, the loss of repression in the ER β KO cells indicates that the effects of the ligand occur via ER β . Taken together, these results indicate that WAY-200070 mediates its effects on skin cells through ER β .

ERB-041 is as active as WAY-200070 in skin cells

ERB-041 is an ER β -selective compound that bound to ER α and ER β with IC₅₀ value of 1216 and 5.4 nM, respectively, thus demonstrating that it is 226-fold selective for the ER β than ER α (Table 1) (Malamas et al., 2004; Harris, 2007). Therefore, ERB-041 is more ER β -selective than WAY-200070 in binding assays. ERB-041 has shown efficacy in vivo in pre-clinical models of rheumatoid arthritis, inflammatory bowel disease, endometriosis and inflammatory-induced pain (Leventhal et al., 2006). We next compared the effects of ERB-041 (1 μ M) with those of WAY-200070 (1 μ M) on IL1 β and MMP1 expression in skin cells, KERTr cells and NHEKs. KERTr immortalized keratinocytes expressed very high levels of IL1 β and MMP1, which was significantly inhibited by both ERB-041 (3.6-fold) and WAY-200070 (7.8-fold) (Fig. 4A). Further, ERB-041 also significantly inhibited the UV-induced IL1 β and MMP1 expression in NHEKs, although was less efficacious than WAY-200070 in these cells (Fig. 4B). Since ERB-041 showed better ER β selectivity relative to WAY-200070 in binding assay, and it

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has shown efficacy in murine models of inflammation, we decided to use ERB-041 in a murine model of photoaging.

ER β ligand inhibits wrinkle formation in vivo

We next examined whether the anti-inflammatory/anti-catabolic molecular changes observed with an ER β compound in vitro translate into efficacy in a murine model of photoaging. Therefore, the effect of ERB-041 was examined on UV-induced wrinkle formation in a hairless albino mouse model (Chang et al., 2008). The experimental plan included two groups of mice. One group was mock exposed [UV(-) group] and the second group of mice was exposed 3 times/week to UV treatment [UV(+) group]. The UV(-) group was further divided into two subgroups, wherein animals were treated topically with either vehicle or ERB-041 (10 mM) 5 times/week for 6 weeks. Similarly, the UV(+) group was divided into 4 subgroups, wherein mice were treated topically with vehicle or 0.1, 1.0 or 10 mM ERB-041 (Fig. 5A). UV exposure of hairless mice for 6 weeks resulted in the appearance of prominent wrinkles in the UV(+) vehicle-treated group in comparison to mock-exposed groups, as shown by silicon rubber replicas of the mouse dorsal skin (Fig. 5A). Replica scans showed a quantitative increase in wrinkle score in the UV(+), vehicle-treated group relative to UV(-), mock-exposed group (Fig. 5B). Remarkably, a statistically significant decrease in wrinkle score was observed at all three doses of the ER β ligand as compared to UV(+), vehicle-treated animals (Figs. 5A and B). At 0.1 and 1 mM concentrations of ERB-041, wrinkle scores in the UV(+) groups were not significantly different ($P < 0.05$) from those of the UV(-) groups (Fig. 5B). With 10 mM of ERB-041, wrinkling was reduced to a lesser extent; this high dose may have

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caused an off target effect that limited its efficacy. These results indicate that the ER β ligand inhibits the signs of photodamage in a murine model of photoaging.

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DISCUSSION

An explosion in the population of aged individuals has resulted in an increase in demand for products that can diminish wrinkles and maintain the youthful appearance of the skin. Currently, natural (retinoic acid, RA) and synthetic (tazarotene, Avage) retinoids are the only class of topical prescription agents that are approved by Food and Drug Administration (FDA) for the treatment of photoaged skin (Goldfarb et al., 1990). However, retinoid usage is associated with local skin side effects of irritation, erythema, burning, pruritus and scaling (McGuire et al., 1988; Effendy et al., 1996). Other treatments, like botulinum toxins and hyaluronic acid fillers are injectable and painful, and they do not modulate the underlying etiological process of photoaging. Therefore, there is a need for safe and efficacious agents for the prevention and treatment of photoaging.

This manuscript provides the first evidence demonstrating ER β as a novel target for the prevention and potentially the treatment of photoaging. In this study we demonstrate that ER β -selective ligands exhibit molecular signatures of a potential photoaging therapeutic, since a synthetic specific agonist inhibited the expression of pro-inflammatory cytokines, MMPs and COX-2 in activated skin cells (Fig. 2). The broad anti-inflammatory potential of an ER β -selective compound did translate into efficacy in vivo, since it inhibited UV-induced wrinkle formation in a murine model of photoaging (Fig. 5).

Natural and synthetic estrogens have long been recognized for their ability to rejuvenate human skin. These estrogens were included in many skin care products, however no clear mechanisms of action were defined. A recent study showed that

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estrogen cream failed to protect sun damaged skin in a clinic (Rittie et al., 2008). In this study, no significant changes in collagen production were observed in women or men after estradiol treatment of photoaged forearm or face skin. This is consistent with our unpublished results using estradiol in our in vitro photoaging model, wherein we found no significant reversal of the cytokine and MMP gene expression induced by UV, as well as no significant induction of procollagen synthesis. Similarly, in our in vitro assays most ER β ligands did not significantly increase procollagen expression. However, the suppression of MMPs by ER β ligands, especially MMP1, is likely to translate into the reduction of collagen degradation induced by UV exposure. The positive effect of ERB-041 in the in vivo murine photoaging model (Fig. 5) supports the idea that suppression of MMPs may lead to a reduction in collagen degradation and eventually the prevention of wrinkle formation. Furthermore, the inability of estradiol to suppress MMP1 in our in vitro photoaging model may explain why it did not improve sun-exposed human skin in the cited study (Rittie et al., 2008). The data presented in this manuscript point to ER β and not ER α as the receptor responsible for mediating the anti-photoaging effect.

As ER β ligands suppress several cytokines and MMP1, their anti-inflammatory effect is likely to involve NF κ B pathways. Since retinoic acid, an FDA approved anti-photoaging retinoid, mainly suppresses AP1-responsive metalloproteinases, collagenase, stromelysin-1 and 92 Kd gelatinase (Fisher et al., 1996) and not NF κ B-responsive cytokines (unpublished observations), retinoids may exert their anti-photoaging effects via inhibition of AP1-dependent gene expression (Herrlich and Ponta, 1994; Nagpal et al., 1995; Boehm et al., 1995). Therefore, if successfully developed and approved, ER β ligands will become a novel class of anti-photoaging agent. It has been postulated that

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the anti-inflammation potential of ER α /ER β is tissue/cell type specific (Brandenberger et al., 1997). ER α plays a more important role in the uterus, whereas ER β appears to play important roles in ovary, cardiovascular system and brain (Brandenberger et al., 1997). Although much evidence points to the clear involvement of an NF- κ B mediated mechanism for the anti-inflammatory activities of ERs, most of this evidence also suggests the involvement of other co-factors (Guzeloglu-Kayisli et al., 2008). For example, ER ligands have been proposed to mediate their anti-inflammatory effects by inhibiting NF- κ B-dependent gene expression via involvement of cofactor SRC-2 (Cvoro et al., 2006; Cvoro et al., 2008). Binding of our ER β ligands may allow more effective interaction of ER β with SRC-2, thus providing more potent ER β -mediated anti-inflammatory activity than estradiol. Based on our findings, we conclude that ER β , but not ER α , plays a major role in reversing the process of photoaging in skin, and its anti-photoaging activity could potentially involve cofactor recruitment or NF- κ B mediated mechanism or both.

Our recent publication showed that treatment with an LXR ligand significantly prevented UV-induced skin thickening as well as reducing skin wrinkling (Chang et al., 2008). In the current study, although the ER β ligand was more efficacious than an LXR agonist in suppressing MMP1 and cytokine gene expression in vitro, it did not show a similar effect on skin thickness (data not shown). The efficacy of LXR ligands in reducing UV-induced skin thickening may lie in their ability to promote lipid synthesis and transport, consequently supporting barrier function. The potent anti-inflammatory activity of ER β ligands, together with the lipogenicity of LXR ligands, suggests that the

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combination of these two nuclear receptor ligands might be most efficacious for preventing and treating photoaging.

In summary, we have identified ER β as a target for photoaging, and shown that selective ligands could reverse the in vitro photoaging process at least at the molecular level. A proof of concept study using ERB-041 showed statistically significant reversal of wrinkle formation in a murine photoaging model (Fig. 5) warranting further development of this target for the prevention and treatment of photoaging.

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Acknowledgments

The authors would like to thank Ms. Wei Wang for her expertise in isolating skin cells from new bourn mouse skin tissue. We also thank Dr. Heather Harris for her expert consultation in the field of estrogen receptors.

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REFERENCES

- Antal MC, Krust A, Chambon P, and Mark M (2008) Sterility and absence of histopathological defects in nonreproductive organs of mouse ER β -null mutant. *Proc Natl Acad Sci USA* **105**:2433-2438.
- Boehm MF, Heyman RA, Patel S, Stein RB, and Nagpal S (1995) Retinoids: biological function and use in the treatment of dermatological diseases. *Exp Opin Invest Drugs* **4**:593-612.
- Brandenberger AW, Tee MK, Lee JY, Chao V, and Jaffe RB (1997) Tissue distribution of estrogen receptors alpha and beta mRNA in the midgestational human fetus. *J Clin Endo Metabo* **82**:3509-3512.
- Chang KCN, Shen Q, Oh IG, Jelinsky SA, Jenkins SF, Wang W, Wang Y, LaCava M, Yudt MR, Thompson CC, Freedman LP, Chung JH, and Nagpal S (2008) Liver X receptor is a therapeutic target for photoaging and chronological skin aging. *Molecular Endocrinology* **22**:2407-2419.
- Cvoro A, Tzagarakis-Foster C, Tatomer D, Paruthiyil S, Fox MS, and Leitman DC (2006) Distinct roles of unliganded and liganded estrogen receptors in transcriptional repression. *Molecular Cell* **21**:555-564.
- Cvoro A, Tatomer D, Tee MK, Zogovic T, Harris HA, and Leitman DC (2008) Selective estrogen receptor- β agonists repress transcription of proinflammatory genes. *The Journal of Immunology* **180**:630-636.
- Effendy I, Weltfriend S, Patil S, and Maibach HI (1996) Differential irritant skin responses to topical retinoic acid and sodium lauryl sulfate: alone and in crossover design. *British Journal of Dermatology* **134**:424-430.

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- Escande A, Pillon A, Servant N, Cravedi JP, Larrea F, Muhn P, Nicolas JC, Cavaillès V, and Balaguer P (2006) Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. *Biochemical Pharmacology* **71**:1459-1469.
- Fagot D, Asselineau D, and Bernard F (2004) Matrix metalloproteinase-1 production observed after solar-simulated radiation exposure is assumed by dermal fibroblasts but involves a paracrine activation through epidermal keratinocytes. *Photochem Photobiol* **79**:499-505.
- Fisher GJ, Datta S, Talwar HS, Wang ZQ, Varani J, Kang S, and Voorhees JJ (1996) Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* **379**:335-339.
- Fisher GJ, Kang S, Varani J, Bata-Sorgo Z, Wan Y, Datta S, and Voorhees JJ (2002) Mechanisms of photoaging and chronological skin aging. *Arch Dermatol* **138**:1462-1470.
- Goldfarb MT, Ellis CN, and Voorhees JJ (1990) Topical tretinoin: its use in daily practice to reverse photoageing. *British Journal of Dermatology* **122**:87-91.
- Guzeloglu-Kayisli O, Halis G, Taskiran S, Kayisli U, and Arici A (2008) DNA-binding ability of NF- κ B is affected differently by ER α and ER β and its activation results in inhibition of estrogen responsiveness. *Reproductive Sciences* **15**:493-505.
- Haczynski J, Tarkowski R, Jarzabek K, Wolczynski S, Magoffin DA, Czarnocki KJ, Ziegert M, Jakowicki J, and Jakimiuk AJ (2004) Differential effects of estradiol, raloxifene and tamoxifen on estrogen receptor expression in cultured human skin fibroblasts. *Int J Mol Med* **13**:903-908.

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Harris H (2007) Estrogen receptor- β : Recent lessons from in vivo studies. *Molecular Endocrinology* **21**:1-13.

Herrlich P and Ponta H (1994) Mutual cross-modulation of steroid/retinoic acid receptor and AP-1 transcription factor activities : A novel property with practical implications. *Trends Endocrinol Metab* **5**:341-346.

Jordan VC (2002) The secrets of selective estrogen receptor modulation: Cell-specific coregulation. *Cancer Cell* April:215-217.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor β . *Proc Natl Acad Sci USA* **95**:15677-15682

Leventhal L, Brandt MR, Cummons TA, Piesla MJ, Rogers KE, and Harris HA (2006) An estrogen receptor- β agonist is active in models of inflammatory and chemical-induced pain. *European Journal of Pharmacology* **553**:146-148.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Methods* **25**:402-408.

Makrantonaki E and Zouboulis CC (2007) Molecular mechanisms of skin aging: state of the art. *Ann N Y Acad Sci* **1119**:40-50.

Malamas MS, Manas ES, McDevitt RE, Gunawan I, Xu ZB, Collini MD, Miller CP, Dinh T, Henderson RA, Keith Jr JC, Harris HA (2004) Design and synthesis of aryl diphenolic azoles as potent and selective estrogen receptor- β ligands. *J Med Chem* **47**:5021-5040

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McGuire J, Langdon R, Birchall N, and Kupper T (1988) Interleukin-1 alpha mRNA induced by cycloheximide PMA, and retinoic acid is reduced by dexamethasone in PAM-212 keratinocytes. *Ann N Y Acad Sci* **548**:283-290.

Nagpal S, Athanikar J, and Chandraratna RAS (1995) Separation of Transactivation and AP1 Antagonism Functions of Retinoic Acid Receptor. *J Biol Chem* **270**:923-927.

Ohnemus U, Uenalan M, Conrad F, Handjiski B, Mecklenburg L, Nakamura M, Inzunza J, Gustafsson JA, and Paus R (2005) Hair cycle control by estrogens: catagen induction via estrogen receptor (ER)- α is checked by ER β signaling. *Endocrinology* **146**:1214-1225.

Quatresooz P and Pierard GE (2007) Downgrading skin climacteric aging by hormone replacement therapy. *Expert Rev Dermatol* **2**:373-376.

Rijken F, Kiekens RC, and Bruijnzeel PL (2005) Skin-infiltrating neutrophils following exposure to solar-simulated radiation could play an important role in photoageing of human skin. *Br J Dermatol* **152**:321-328.

Rittie L, Kang S, Voorhees JJ, and Fisher GJ (2008) Induction of collagen by estradiol-Difference between sun-protected and photodamaged human skin in vivo. *Archives of Dermatology* **144**:1129-1140.

Robinson-Rechavi M, Garcia HE, and Laudet V (2003) The nuclear receptor superfamily. *J Cell Science* **116**:585-586.

Seo JY, Lee SH, Youn CS, Choi HR, Rhie GE, Cho KH, Kim KH, Park KC, Eun HC, and Chung JH (2001) Ultraviolet radiation increases tropoelastin mRNA expression in the epidermis of human skin in vivo. *J Invest Dermatol* **116**:915-919.

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Shughrue PJ, Askew GR, Dellovade TL, and Merchenthaler I (2002) Estrogen-binding sites and their functional capacity in estrogen receptor double knockout mouse brain.

Endocrinology **143**:1643-1650.

Walker VR and Korach KS (2004) Estrogen receptor knockout mice as a model for endocrine research. *ILAR Journal* **45**:455-461.

Wolff EF, Narayan D, and Taylor HS (2005) Long-term effects of hormone therapy on skin rigidity and wrinkles. *Fertility and Sterility* **84**:285-288.

Zhao C, Dahlman-Wright K, and Gustafsson JA (2008) Estrogen receptor β : an overview and update. *Nuclear Receptor Signaling* **6**:e003.

Zheng Y, Du X, Wang W, Boucher M, Parimoo S, Stenn KS (2005) Organogenesis from dissociated cells: Generation of mature cycling hair follicles from skin-derived cells. .

J Invest Dermatol **124**:867-876.

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FIGURE LEGENDS

Figure 1. UV induces cytokines and MMPs gene expression in human primary keratinocytes

Relative gene expression levels of cytokines, MMPs, and other genes of interest in vehicle- (open bars) and UV-treated (8 mJ/cm², black bars) NHEKs were measured using quantitative RT-PCR. Specifically, the expression of these genes was measured by using custom-made Taqman Low Density Arrays (TLDA), and the results were normalized to 18S RNA expression. p-Values were determined by Student's *t*-test.

Figure 2. ER β ligand shows efficacy in keratinocyte and fibroblast-based in vitro models of photoaging

Structure of key compounds used in this study. A, Estradiol. B, WAY-200070. C, ERB-041. WAY-200070 reverses UV effect in the cell-based model of photoaging. The relative expression level of vehicle (open bars) and WAY-200070, 1 μ M (black bars) treated UV-activated NHEK cells (D), and TNF α -treated NHDF cells (E), was measured by quantitative RT-PCR. p-Values were determined by Student's *t*-test.

Figure 3. Validation of ER β as a target of photoaging

A, ICI blocks ER β ligand effect on MMP1 gene expression in keratinocyte cell line, KERTr. The effect of WAY-200070 (0 nM, 25 nM, 50 nM, and 100 nM) on MMP1 gene expression in the absence (open bars) and presence (black bars) of ICI (1 μ M) in KERTr cells is shown. The relative expression was measured by quantitative RT-PCR and the results were normalized to 18S RNA expression. B, The effect of PPT in TNF α -treated NHDF and UV-treated NHEK. A potent ER α agonist, PPT (1 μ M), did not repress MMP1 expression in NHEKs and NHDFs. Cells were treated with PPT for 24 hours.

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RNA was prepared and MMP1 expression was analyzed by qRT-PCR. C, Relative gene expression of ER β in human skin cells. ER β expression was analyzed in keratinocytes KERTr, NHEK, and dermal fibroblasts NHDF, HFF and BJ cells by qRT-PCR. D-E, ER β KO abolishes the suppression effect of ER β ligands on Mmp13 and Mapk1 gene expression respectively. ER β KO and HET mouse epidermal keratinocytes were isolated as described in the Materials and Methods. These *ex vivo*-cultured keratinocytes were activated by UV and incubated in the presence and absence of WAY-200070 (1 μ M) (black bars) for 24 hours. The relative gene expression levels for Mmp13 (D), Mapk1 (E) were measured using quantitative RT-PCR. p-Values were determined by Student's *t*-test.

Figure 4. Inhibition of IL1 β and MMP1 gene expression in human keratinocytes by ERB-041 and WAY-200070

Human keratinocyte cell line KERTr cells (A) or NHEKs (B) were treated with vehicle (black bars), WAY-200070, 1 μ M (open bars) or ERB-041 (1 μ M) (grey bars) for 24 hours. The relative gene expression levels for IL1 β and MMP1 were measured using quantitative RT-PCR as described in Materials and Methods. p-Values were determined by Student's *t*-test.

Figure 5. Topical application of ERB-041 shows efficacy in a murine model of photoaging. UV radiation effect on hairless mouse skin of various concentrations (0.1, 1 and 10 mM) of ERB-041 after 6 weeks of topical treatment is shown. The mice were either mock irradiated [UV (-)] or exposed to UV radiation [UV (+)] three times a week. The UV dose was increased weekly by 1MED (1MED = 130 mJ/cm²) to a maximum of 4MED and subsequently maintained at this level for the remainder of the study period.

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Wrinkle formation was assessed after 6 weeks of UV exposure by preparing skin replicas (A), and measured quantitatively by computer scanning of the replicas (B). Representative photographs taken at week 6 are shown. Values represent the mean \pm SEM (n=8). The bars with letter “a” represent groups that were statistically significantly different ($P<0.05$) than those with letter “b”. The bars with letter “b” represent groups that were not significantly different ($P<0.05$) from each other.

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TABLES

Table 1. Binding affinity and the relative transactivation potency of selected skin active ER β ligands

Data extracted mainly from recent publications (Malamas et al., 2004; Harris, 2007) and confirmed with our unpublished internal testing

	ER Binding Affinity			ER Transactivation Assay		Base Structure
	ER β nM	ER α nM	Ratio	ER β EC ₅₀ nM	ER α EC ₅₀ nM	
17 β -estradiol	2	2	1	9	3	
WAY-200070	2.3	155	68	31	314	Benzoxazole
ERB-041	5.4	1216	226	290	>3000	Benzoxazole

Figure 1

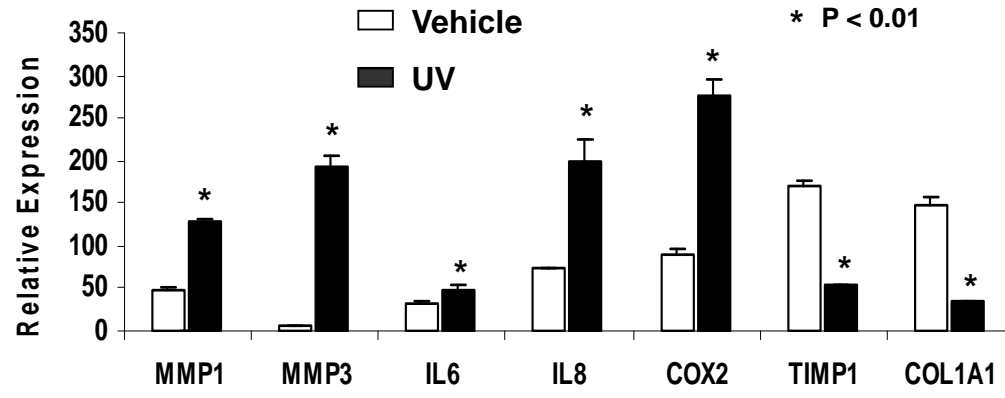


Figure 2

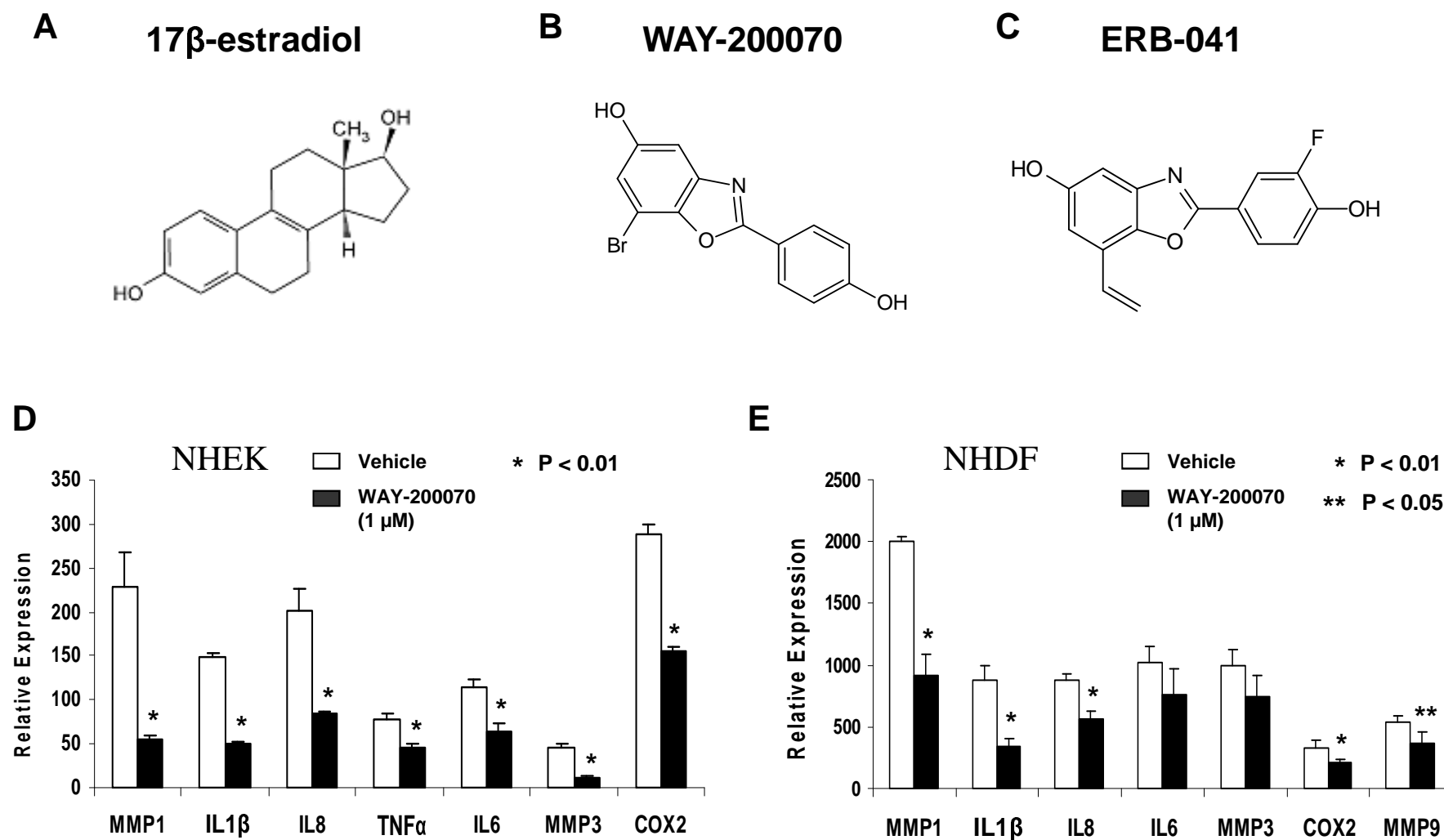


Figure 3

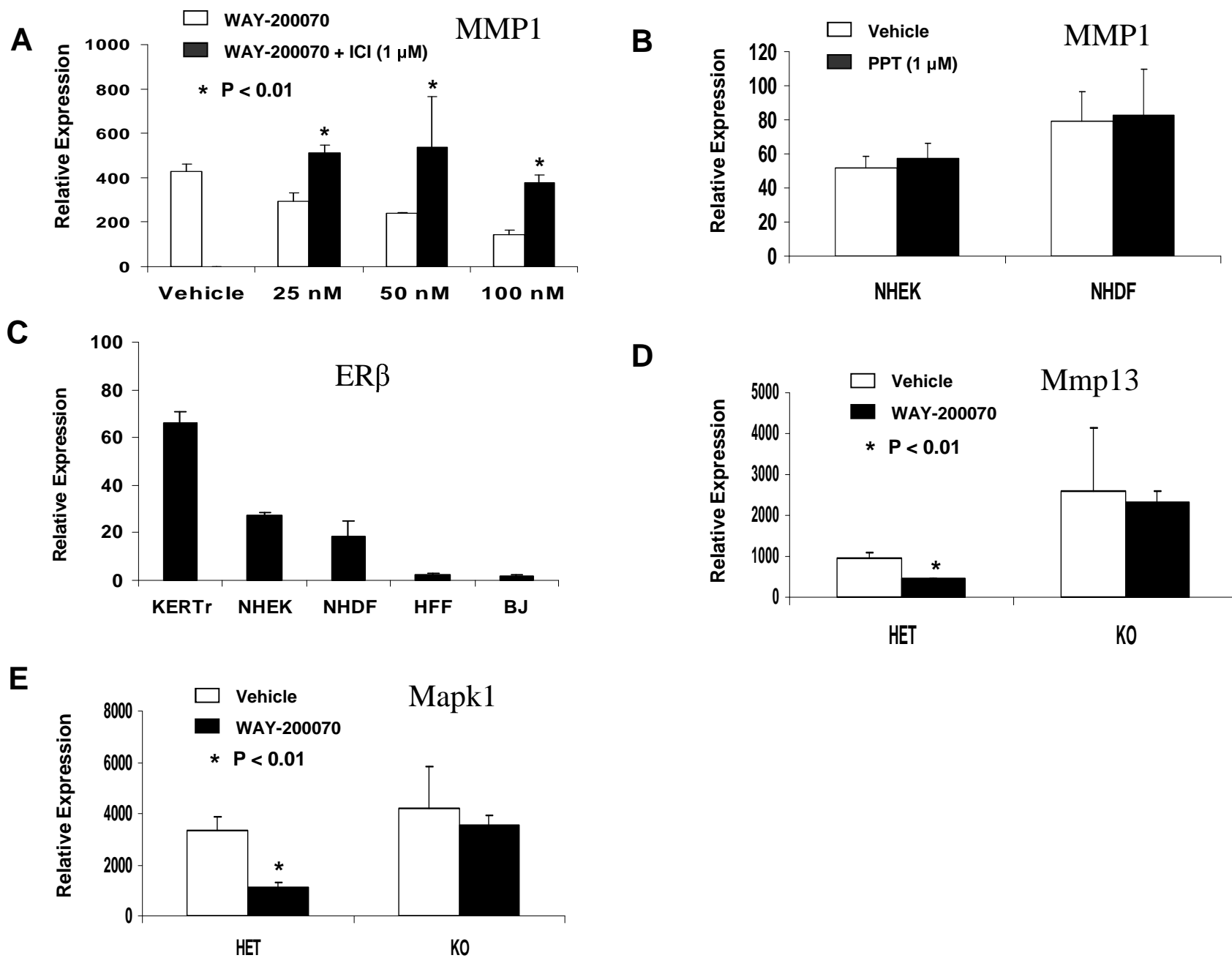


Figure 4

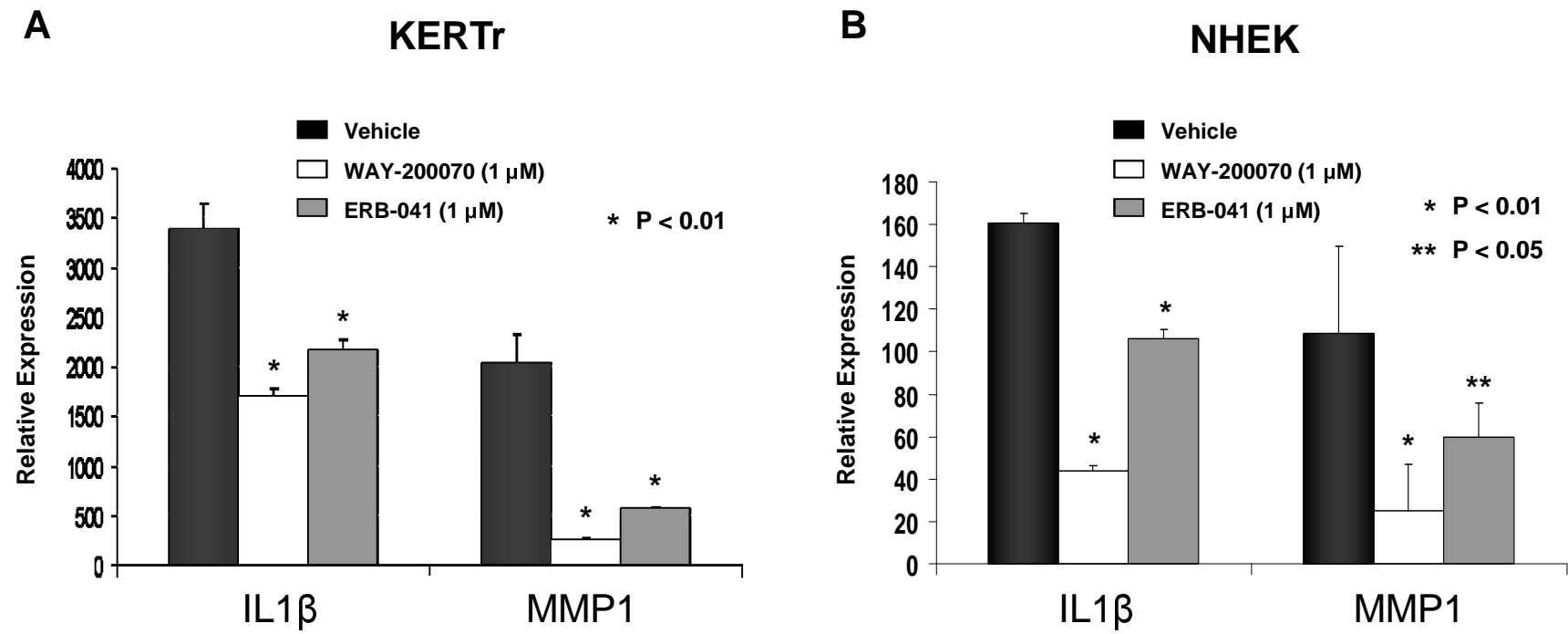


Figure 5

