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A role for estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) in collagen biosynthesis in mouse skin

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Abstract

Hormonal regulation of the dermal collagenous extracellular matrix plays a key role in maintaining proper tissue homeostasis, however the factors and pathways involved in this process are not fully defined. This study investigated the role of estrogen receptors (ERs) in the regulation of collagen biosynthesis in mice lacking ERα or ERβ. Collagen content was significantly increased in the skin of $ER\alpha^{-/-}$ mice as measured by acetic acid extraction and the hydroxyproline assay and correlated with the decreased levels of MMP-15 and elevated collagen production by $ER\alpha^{-/-}$ fibroblasts. In contrast, collagen content was decreased in the skin of $ER\beta^{-/-}$ mice despite markedly increased collagen production by $ER\beta^{-/-}$ fibroblasts. However, expression of several matrix metalloproteinases (MMPs), including MMP-8 and -15 was significantly elevated suggesting increased degradation of dermal collagen. Furthermore, $ER\beta^{-/-}$ mice were characterized by significantly reduced levels of small leucine proteoglycans (SLRPs), lumican and decorin, leading to the defects in collagen fibrillogenesis and possibly less stable collagen fibrils. $ER\alpha^{-/-}$ mice also exhibited fibrils with irregular structure and size, which correlated with increased levels of lumican and decorin. Together, these results demonstrate distinct functions of estrogen receptors in the regulation of collagen biosynthesis in mouse skin *in vivo*.

Introduction

Collagen biosynthesis and deposition is a multi-phase process, which is tightly regulated to maintain proper skin homeostasis. The synthesis of collagen fibrils can be divided into two stages: intracellular and extracellular. The intracellular phase includes transcription of collagen genes, followed by the synthesis and assembly of procollagen molecules in the endoplasmic reticulum, and secretion into the extracellular space. During the extracellular

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Conflict of interest

The authors state no conflict of interest.

phase the procollagen molecules are converted to collagen forming stable, cross-linked collagen fibrils (Gelse *et al.*, 2003).

Collagen production is regulated by a variety of factors including growth factors, cytokines and hormones. In particular, estrogen regulates many aspects of skin physiology. Decreased levels of estrogen lead to a number of cutaneous changes including loss of elasticity, thinning and wrinkling of the skin (Brincat *et al.*, 1987; Verdier-Sevrain *et al.*, 2006). Estrogen mediates its effects through estrogen receptors, ERα and/or ERβ. Both receptors belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factors (Matthews and Gustafsson, 2003). Furthermore, strong evidence exists for the presence of membrane estrogen receptors with the characteristics of G protein-coupled receptors (GPCRs) (Levin, 2002; Verdier-Sevrain *et al.*, 2006). It has been shown that estrogen receptors contribute to several cell functions such as cell growth and survival, migration, and new blood vessel formation. Previous *in vivo* and *in vitro* studies have demonstrated that human scalp skin and human cultured skin fibroblasts express ERα and ERβ. Expression of both receptors in dermal fibroblasts, which are the major producers of collagen type I, strongly suggest that estrogen directly affects fibroblast biology through the receptor-mediated effects (Haczynski *et al.*, 2002; Thornton, 2002; Thornton *et al.*, 2003) . Consistent with this view, it has been reported that cultured mouse dermal fibroblasts increase collagen synthesis in response to estrogens (Hosokawa *et al.*, 1981; Thornton, 2002). Furthermore, a recent study has demonstrated a cross-talk between profibrotic TGFβ/Fli1 pathway and ERα in human and murine dermal fibroblasts (Hattori et al., 2011). However, the precise mechanisms of collagen regulation by estrogen requires further studies.

Previous experiments using mice lacking either ERα or ERβ have shown that each subtype has overlapping as well as distinct and specific roles in estrogen action *in vivo* (Couse and Korach, 1999; Couse *et al.*, 2003; Matthews and Gustafsson, 2003). The skeleton is one of the major target organs for estrogen. Estrogen regulates the bone growth and remodeling. It has been shown that complete deletion of ERα as well as ERβ leads to a decrease in bone turnover and an increase in the trabecular bone volume of female mice (Sims *et al.*, 2002). New cortical bone production was significantly decreased in $ER\alpha^{-/-}$ mice in response to mechanical stimulus (Lee *et al.*, 2003). Lungs are another organ affected by lack of ERβ. It has been shown that lack of this estrogen receptor leads to underdeveloped alveolar structure and the aberrant extracellular matrix composition in the lungs. These changes induce systemic hypoxia, which may cause cardiac hypertrophy and systemic hypertension (Morani *et al.*, 2006).

In the current study we assessed the biosynthesis of collagen in the skin of ERα and ERβ knockout female mice. Our results demonstrate increased collagen content in the skin of mice deficient in ERα, while in ERβ knockout mice collagen content was reduced. Reduced collagen levels correlated with increased expression of MMPs in $ER\beta^{-/-}$ mice. In addition, expression levels of lumican and decorin were significantly decreased in $ER\beta^{-/-}$ mice, while lumican was increased in $ER\alpha^{-/-}$ mice. Altered levels of SLRPs resulted in structural changes in collagen fibrils, which were observed in both $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice. These findings highlight the significance of estrogen receptors in the complex process of collagen biosynthesis in the skin.

Results

Skin thickness and collagen content are increased in ERα$^{-/-}$ mice but not in ERβ$^{-/-}$ mice

To investigate the effect of the absence of estrogen receptors on skin thickness and collagen deposition, skin samples were stained with hematoxylin-eosin or Masson's Trichrome. Histopathological analyses of skin samples showed that skin thickness in the ERα$^{-/-}$ mice increased compared to control animals (224 ± 14.7 μm *vs* 151.7 ± 10.1 μm, $p < 0.05$) and was about twice that of ERβ$^{-/-}$ mice (108 ± 8.8 μm, $p < 0.05$) (Figure 1a,b,c). In ERβ$^{-/-}$ mice, skin thickness slightly decreased (108 ± 8.8 μm *vs* 151.7 ± 10.1 μm) as compared to controls. Furthermore, ERα$^{-/-}$ animals had more visible collagen as assessed by Trichrome staining compared to ERβ$^{-/-}$ and control mice (Figure 1b).

The collagen content in the skin of ERα$^{-/-}$, ERβ$^{-/-}$, and WT mice, was next quantified by a hydroxyproline assay. In agreement with histopathological results ERα$^{-/-}$ mice showed higher hydroxyproline content compared to control mice (18.97 ± 2.1 μg/ml *vs* 9.79 ± 1.0 μg/ml, $p<0.04$), whereas there was a decrease in hydroxyproline content in ERβ$^{-/-}$ mice compared to controls (6.88 ± 1.1 μg/ml *vs* 9.79 ± 1.0 μg/ml, $p<0.05$) (Figure 2a). These data indicate that collagen content in the skin is reduced ~ 30% in the absence of ERβ and significantly increased in the absence of ERα receptor as compared to control animals.

To further investigate the role of estrogen receptors on collagen deposition in the skin, collagen was extracted with acetic acid with the addition of pepsin (Miller and Rhodes, 1982). For the extraction, 8 mm punches from the dorsa of each mouse were used. Equal aliquots from each sample were analyzed by SDS-PAGE. The pattern of collagen bands was similar in all samples, suggesting no qualitative differences in collagen composition (Figure 2b,c). Consistent with the hydroxyproline content results, significantly more collagen was extracted from the skin of ERα$^{-/-}$ mice compared to control mice (3.1 ± 0.3 fold, $p<0.05$) (Figure 2b) while in ERβ$^{-/-}$ mice there was slightly less extracted collagen compared to controls (Figure 2c). These results suggest that ERα and ERβ receptors might have distinct roles in regulating ECM deposition in the mouse skin.

The effects of ER receptor deficiency on collagen synthesis in the skin *in vivo* are not reflected in cultured fibroblasts

To assess the amounts of newly synthesized, non-cross-linked collagens not yet incorporated into large fibrils, collagen was extracted using acetic acid (Bradshaw *et al.*, 2003; Miller and Rhodes, 1982) without the addition of pepsin. 8 mm skin punches were used for extraction. There was a 1.6 ± 0.3 fold, $p<0.05$ increase in acetic acid soluble collagen in ERα$^{-/-}$ females compared to controls (Figure 3a). In contrast to hydroxyproline and pepsin-soluble collagen content, which were significantly decreased, there was no appreciable change in the amount of the acetic acid soluble extracted collagen between controls and ERβ$^{-/-}$ mice (Figure 3b). Next, we analyzed mRNA expression of fibrillar collagens in skin from WT, ERα$^{-/-}$, and ERβ$^{-/-}$ mice by qPCR. The level of pro-Col1a1 increased 5.2 ± 0.2 fold ($p<0.04$), pro-Col1a2 7.7 ± 0.3 fold ($p<0.05$), pro-Col3a1 14.8 ± 1.2 fold ($p<0.01$), pro-Col5a1 1.9 ± 1.2 fold ($p<0.07$) and pro-col5a2 2 ±0.3 fold ($p<0.05$) in skin of ERα$^{-/-}$ mice. The level of pro-Col1a1 decreased 2.4 ± 0.13 fold ($p<0.01$), pro-Col1a2 2.41 ± 0.1 fold ($p<0.02$), pro-Col3a1

3 ± 0.07 fold ($p<0.01$), pro-Col5a1 5.7 ± 0.05 fold ($p<0.01$) and pro-col5a2 3.09 ±0.15 fold ($p<0.05$) in skin of ERβ$^{-/-}$ mice (Figure 3c).

To assess the effects of ER receptor deficiency on collagen production by fibroblasts, collagen mRNA and protein levels were determined in cultured dermal fibroblasts isolated from WT, ERα$^{-/-}$, and ERβ$^{-/-}$ mice without and after treatment with the ligand, 17β-estradiol. As shown in Figure 3d basal pro-Col1a1 mRNA levels were increased in ERα$^{-/-}$ and ERβ$^{-/-}$ mice ~30% and ~60%, respectively, as compared to WT animals suggesting that both receptors have an inhibitory effect on synthesis of collagen gene expression in dermal fibroblasts. Furthermore, 17β-estradiol induced Col1a1 mRNA (~40%) in fibroblasts obtained from WT mice. The response to 17β-estradiol in ERα$^{-/-}$ fibroblast was increased ~30% as compared to untreated cells isolated from ERα$^{-/-}$ mice. However, collagen production did not increase in dermal fibroblasts isolated from ERβ$^{-/-}$ mice after treatment with 17β-estradiol (Figure 3d). Similar responses were also observed at the collagen protein level (Figure 3e). Since fibroblasts obtained from ERβ$^{-/-}$ mice have shown a greater collagen increase at basal level than fibroblasts from ERα$^{-/-}$ mice, their lack of responsiveness to the stimulatory effects of 17β-estradiol may suggest that 17β-estradiol upregulates collagen synthesis by counteracting the inhibitory effect of ERβ. Another possibility is that ERβ, but not ERα, is required for the stimulatory effects of 17β-estradiol on collagen synthesis. These interesting questions require further studies. Together, these results suggest that decreased ECM deposition observed in ERβ$^{-/-}$ mice is not due to the intrinsic properties of ERβ$^{-/-}$ fibroblasts, but is primarily modulated by the exogenous factors in the skin *in vivo*. The reduced responsiveness of ERβ$^{-/-}$ fibroblasts to the stimulatory effects of estrogen may contribute to the decreased deposition of collagen in the skin of ERβ$^{-/-}$ mice.

MMPs are differentially expressed in ERα and ERβ knockout mice

To assess the role of MMPs in the phenotype of the knockout mice, the mRNA expression levels of several MMPs, including MMP-3, -8, -14, -15, and -16, were examined using total RNA isolated from tissue punches taken from the dorsa of WT, ERα$^{-/-}$ and ERβ$^{-/-}$ mice. QPCR was employed to measure mRNA level of MMPs. In ERα$^{-/-}$ mice, the mRNA level of MMP-15 was significantly decreased (2.94 ± 0.3 fold, $p<0.05$), while MMP-8 was only moderately decreased (~20%) (Figure 4a). In agreement with mRNA data, the protein levels of these two MMPs were decreased as well (Figure 4b). These results suggest that decreased degradation of collagen may contribute to the overall increased ECM deposition in ERα$^{-/-}$ mice. The mRNA levels of all examined MMPs were significantly increased in ERβ$^{-/-}$ mice: MMP-3 (16.2 ± 0.5 fold, $p<0.02$), MMP-8 (7.7 ± 0.2 fold, $p<0.05$), MMP-14 (11.1 ± 0.3 fold, $p<0.02$), MMP-15 (8.7 ± 0.2 fold, $p<0.02$), MMP-16 (12.9 ± 0.3 fold, $p<0.01$) (Figure 4c). The protein levels of MMP-8 and MMP-15 were also markedly increased in these mice (Figure 4d). These data strongly suggest that increased collagen degradation may also contribute to the overall decrease in collagen deposition observed in ERβ$^{-/-}$ mice.

Expression of lumican and decorin differ in ERα$^{-/-}$ and ERβ$^{-/-}$ mice

We next determined the expression level of several SLRPs involved in the process of fibrillogenesis (Figure 5a,b,c). The mRNA level of lumican (Lum), fibromodulin (Fmod) and decorin (Dcn) were measured by QPCR. In ERα$^{-/-}$ animals, the mRNA level of Lum

was significantly elevated (9.8 ± 0.2 fold, $p<0.01$), while Dcn was slightly increased (1.7 ± 0.2 fold, $p<0.03$) (Figure 5a). Conversely, in ERβ$^{-/-}$ female mice, a decrease in the mRNA levels of all three SLPRs was observed: Lum (2.36 ± 0.2 fold, $p<0.03$), Dcn (7.72 ± 0.3 fold, $p<0.01$), and Fmod (1.54 ± 0.4 fold, $p<0.07$) (Figure 5b). To further investigate the expression of Lum and Dcn, immunostaining of skin section from WT, ERα$^{-/-}$, and ERβ$^{-/-}$ female mice was performed. High expression levels of Lum and Dcn were observed in control skin fibroblasts and in association with collagen fiber bundles in the ECM (Figure 5c). Consistent with mRNA data the amount of Lum and Dcn were increased in the skin of ERα$^{-/-}$ mice. However, the levels of Lum and Dcn were decreased in the dermis of ERβ$^{-/-}$ mice compared to control skin sections. These findings suggest that ERα may play an inhibitory role in the expression of Lum and Dcn in mice skin, while ERβ may have an opposite role in expression of these proteins. Furthermore, reduced levels of these SLRPs may lead to changes in the assembly of collagen fibrils in ERβ mice.

ERα and ERβ knockout mice have abnormal collagen fibrils

Increased and reduced expression levels of SLRPs in the ERα$^{-/-}$ and ERβ$^{-/-}$ mice, respectively, suggest that collagen fibrillogenesis might be defective in these mice. For that reason, distribution of collagen fibrils was analyzed by transmission electron microscopy (EM). In contrast to control animals, collagen fibrils with irregular sizes and shapes were present in the skin of ERα$^{-/-}$ mice, which may be a result of elevated lumican and decorin levels. Interestingly, fibrils in ERβ$^{-/-}$ mice had regular size, but were distributed more sparsely as compared to fibrils in WT and ERα$^{-/-}$ mice (Figure S_1). Mean fibril diameter was 59 ± 10.4 nm in WT, 84 ± 14.5 nm in ERα$^{-/-}$ and 80 ± 15.5 nm in ERβ$^{-/-}$ mice ($p<0.05$) (Figure S_1).

Discussion

Estrogen regulates a wide variety of physiological processes in the reproductive tract and gonads, as well as in nonreproductive tissues such as cardiovascular and skeletal system and the skin (Couse and Korach, 1999; Thornton, 2002));. ERα and ERβ are known to transduce estrogenic signals however; their role in the skin, specifically in the ECM biosynthesis, is not fully defined.

This study demonstrates that ERα and ERβ play distinct roles in the regulatory pathways involved in synthesis and degradation of ECM in the female mouse skin. In our studies we used ERα $^{-/-}$ mice that express a small truncated form of ERα that contains the ligand independent activation domain. Functionality of this fragment is minimal, as these ERα knockout mice possess hypoplastic uteri as well as ovaries that lacked corpora lutea indicating a lack of an estrogen effect (Lubahn at al., 1993; Couse at al., 1999). Interestingly, adult ERα$^{-/-}$ females are characterized by increased serum levels of estradiol, as well as elevated ovary-derived androgens (Couse at al., 1999; Couse at al., 2003). Using several independent approaches this study demonstrated that overall ECM deposition is increased in the skin of ERα$^{-/-}$ mice, while it is decreased in the skin of ERβ$^{-/-}$ mice.

Several factors may have contributed to the elevated ECM deposition in ERα$^{-/-}$ mice, including elevated collagen synthesis by dermal fibroblasts, as well as decreased expression

of MMP-15 in the mouse skin. Moreover, ERα$^{-/-}$ fibroblasts retained their responsiveness to exogenous estradiol with respect to collagen biosynthesis. Furthermore, testosterone, a known inducer of ECM deposition in mouse skin (Markiewicz *et al.*, 2007; Markova *et al.*, 2004) may have partially contributed to the increased collagen deposition in these animals.

The phenotype of the ERβ$^{-/-}$ mice with respect to collagen biosynthesis appears to be more complex. ERβ$^{-/-}$ mice showed an overall decrease of ECM deposition in the skin, whereas collagen production by dermal fibroblasts was notably increased. However, ERβ$^{-/-}$ fibroblasts were no longer responsive to exogenous estradiol with respect to collagen biosynthesis, which may have contributed to the overall lower levels of collagen mRNA and protein in the skin of these mice. Currently, we cannot fully explain the differences between collagen levels *in vitro* and *in vivo* in ERβ$^{-/-}$ mice, suggesting that hormonal and/or other paracrine factors may influence the phenotype of these mice *in vivo*. In addition, our results suggest that unstable collagen fibrils due to the reduced levels of lumican and decorin as well as elevated levels of MMPs may have contributed to the enhanced degradation of the newly synthesized collagen. The normal range of soluble collagen extracted by acetic acid digestion from ERβ$^{-/-}$ mouse skin is consistent with this idea. Testosterone levels in ERβ$^{-/-}$ mice were comparable to that of control animals (Couse *et al.*, 2003), suggesting that this factor does not play a role in the phenotype of ERβ$^{-/-}$ mice. Our results are consistent with a recent study by (Campell *et al.*, 2010) who investigated the role of ER receptor subtypes in wound healing. These authors showed delayed wound repair in ERβKO mice, which was attributed to the elevated levels of MMP-2 and -9. We have observed elevated activity of these gelatinases (Figure S_2), as well as elevated mRNA levels of several membrane type MMPs (MT-MMPs). Protein levels of MMP-8 and -15 were also increased. MMPs, a family of zinc-dependent endopeptidases, are responsible for the degradation of multiple ECM components, including collagens (Birkedal-Hansen *et al.*, 1993). For example, MMP-8 cleaves the α1 chain and α2 chain of native type 1 collagen, as well as α-chains of type 2 and type 3 collagens (Song *et al.*, 2006). The direct role of MMP-15 in collagen biosynthesis is not known. However, it has been shown that MMP-15 can activate MMP-2, which is known to share the same cleavage sites in type 1 collagen with MMP-8 (Aimes and Quigley, 1995; Song *et al.*, 2006; Visse and Nagase, 2003).

We have previously demonstrated that Lum, Dcn, as well as Fmod were downregulated in the skin of ovariectomized mice (Markiewicz *et al.*, 2007). In this study, we also observed reduced levels of Lum and Dcn in mice lacking ERβ, suggesting a primary role of ERβ in regulating expression of these two SLRPs. Furthermore, in mice lacking ERα, Lum and, to a lesser degree Dcn, were increased, suggesting an antagonistic role of the two ER receptors in regulation of these genes. While Fmod was significantly downregulated in ovariectomized mice, the absence of either ER receptor did not significantly altered its expression, suggesting that ER receptors may not directly contribute to the Fmod regulation by estrogen. SLRPs are associated with collagen fibrils and have been shown to play important roles in collagen fibrillogenesis (Kalamajski and Oldberg, 2010). Initial analysis of collagen structure in the skin of the ERα$^{-/-}$ and ERβ$^{-/-}$ mice has revealed abnormalities in both shape and diameter of collagen fibrils, consistent with changes in expression levels of selected SLRPs. Accordingly, previous studies have shown that collagen fibrils in Lum mice

have a bigger diameter, irregular contour and some appear to be laterally fused, resulting in loose and fragile skin in these mice (Chakravarti, 2002; Kalamajski and Oldberg, 2009). Likewise, irregular collagen fibrils were observed in $Dcn^{-/-}$ mice resulting in a weakened skin architecture (Kalamajski and Oldberg, 2010; Reed and Iozzo, 2002). Furthermore, recent studies revealed a complex role of SLRPs in various physiological and pathological conditions through a cross-talk with cellular signaling pathways (Iozzo and Schaefer, 2010) further underscoring the complexity of ER receptor signaling in skin biology.

Taken together, this study strongly supports the key role of ERα and ERβ in the process of collagen biosynthesis in the skin. Importantly, each receptor has a distinct regulatory role in this process, strongly suggesting that receptor ratio is important in maintaining proper tissue homeostasis. While more studies are needed to evaluate the expression of each receptor during aging and in pathological skin conditions, such knowledge may open a new avenue in utilizing receptor-specific ligands as therapeutic agents. Interestingly, a recent study has shown that an ERβ-specific compound suppressed expression of MMPs and inhibited UV-induced photodamage and skin wrinkle formation in a mouse model (Chang *et al*., 2010). On the other hand, inhibition of ERβ function by a selective antagonist may be useful in treating fibrotic skin conditions by promoting collagen degradation. We are just beginning to understand the regulatory role of estrogen receptors during collagen fibrillogenesis. Further studies are warranted to identify the mechanisms by which estrogen receptor pathways regulate this complex process.

Materials and Methods

Mice genotyping

10-12 week old control C57BL/6 (WT), ERα KO and ERβ KO (Couse and Korach, 1999) female mice were used for the study (8-10 animals per group). All experiment mice were sacrificed under a general anesthesia (Tribromoethanol) (Gelse *et al*., 2003). The Institutional Animal Care and Use Committee approved all animal procedures.

Genomic DNA was isolated from mouse tails using the Puregene Core Kit A (Qiagen,Inc) according to the manufacturer's instruction. Mice were genotyped by PCR analysis – ERα was amplified for 30 cycles each at 95°C/30s, 63.2°C/45s, and 72°C/45s. The primer sequences used were: 5’-CGGTCTACGGCCAGTCGGGCATC-3’, 5’-CAGGCCTTACACAGCGGCCACCC -3’, 5’-GTCCTGATAGCGGTCCGCCA-3’, 5’-GTGTTCCGGCTGTCAGCGCA-3’

The ERα knockout results in a 555bp fragment while the ERα controls result in a 281bp fragment. ERβ was amplified for 35 cycles each at 95°C/30s, 58°C/60s, and 72°/60s.The primer sequences used were: 5’-GCAGCCTCTGTTCCACATACAC-3’, 5’-CATCCTTCACAGCAGACAC-3’, 5’-TGGACTCACCACGTAGGCTC-3’.

The ERβ knockout results in a 404bp fragment and ERβ controls result in a 356bp fragment.

Cell culture

Mouse dermal fibroblast cultures were established from skin taken from the back of WT, ERαKO and ERβKO according to protocol describing by Asano et al.,(Asano *et al.*, 2009) .Since phenol red has been shown to have estrogenic effects in certain tissues (Walsh-Reitz and Toback, 1992) fibroblasts were cultured in phenol red-free DMEM (Gibco) containing 20% charcoal/dextran treated fetal bovine serum (Hyclone).

Histology

Skin samples were removed from the dorsal side of mice and fixed in 4% paraformaldehyde. Samples were embedded in paraffin and sectioned perpendicular to the epidermal surface. Paraffin was removed and slides were stained with hematoxylin-eosin or Masson Trichrome according to the routine histologic protocol.

Hydroxyproline assay

Measurement of hydroxyproline content in the skin was carried out as described by Markiewicz et al. (Bradshaw *et al.*, 2002; Markiewicz *et al.*, 2007).

Extraction of collagen from skin by acetic acid method with or without addition of pepsin

The acetic acid extraction of collagen was performed according to the previously described protocol by Markiewicz et al. (Markiewicz *et al.*, 2007).

Quantitative real-time RT-PCR analysis

Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method according to the previously described protocol (Chomczynski and Sacchi, 1987; Markiewicz *et al.*, 2007). The primers used for qPCR are listed in Supplemental Table 1.

Western blot analysis

Skin tissue was homogenized and lysed in radioimmunoprecipitation assay (RIPA) buffer. Fifty micrograms of protein was separated via SDS-polyacrylamide gel electrophoresis (under reducing conditions) and transferred to a nitrocellulose membrane (Bio-Rad,CA). The blots were probed overnight with a 1:500 dilution of antibody directed against the MMP-8 or MMP-15 (Santa Cruz Biotechnology, Inc) followed by incubation with the secondary antibody and developed with ECL kit (Pierce). As a control for equal protein loading β-actin antibody was used (Sigma,Inc).

Fibroblasts were grown in phenol red- free 20% DMEM, and for the last 24 hours cultured cells were kept in serum free medium with or without addition of estrogen (5nM). The next day cells were lysed in RIPA buffer, protein concentration was measured and samples were processed as described above. For detection of collagen type 1 anti-mouse collagen type 1 antibody (Santa Cruz Biotechnology, Inc) at dilution of 1:4000 was used.

Gelatin Zymography

Protein samples were separated on 8% SDS–polyacrylamide gels containing 0.1% gelatin under nonreducing conditions. Next, gels were washed with 2.5% Triton X-100 and

incubated overnight at 37 °C in 50 mM Tris (pH 8.0)/ 5 mM $CaCl_2$. The gel was then stained with Coomassie blue G-250 (Bio-Rad).

Immunohistochemistry

Immunohistochemistry was performed according to the previously described protocol (Markiewicz et al.,2007). The slides were deparaffinized and rehydrated through a graded series of ethanol. To expose core proteins, sections were treated with appropriate enzymes (chondroitinase ABC or beta-endogalactosidase). The sections were then incubated with 1:100 dilution of antibody directed against Dcn or Lum (Santa Cruz Biotechnology, Inc) followed by incubation with the secondary antibody. The immunoreactivity was visualized with diaminobenzidine and the sections were counterstained with hematoxylin.

Electron Microscopy

Sections of skin were generated from five WT, five $ER\alpha^{-/-}$ and five $ER\beta^{-/-}$ female mice at 3 months of age and analyzed in the EM Core Facility using a JEOL 1210 EM at the MUSC. Collagen fibril diameters were measured in scanned images generated from electron micrographs with NIH Image software.

Statistics

Statistical differences were determined by either Student's t-test or Anova analysis of variance followed by Bonferroni post hoc test. Values of less than or equal to 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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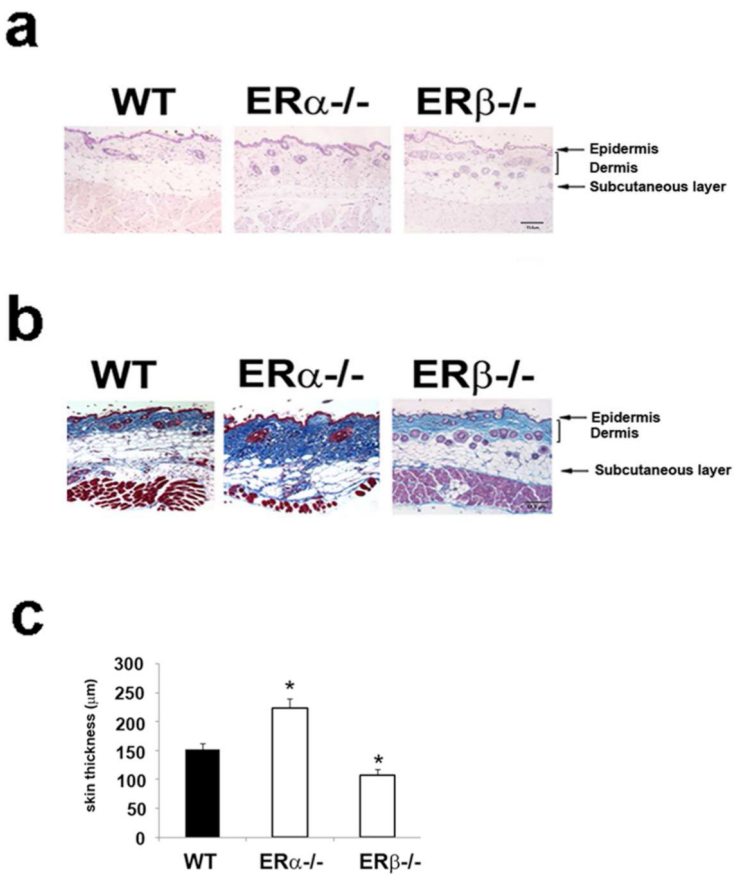
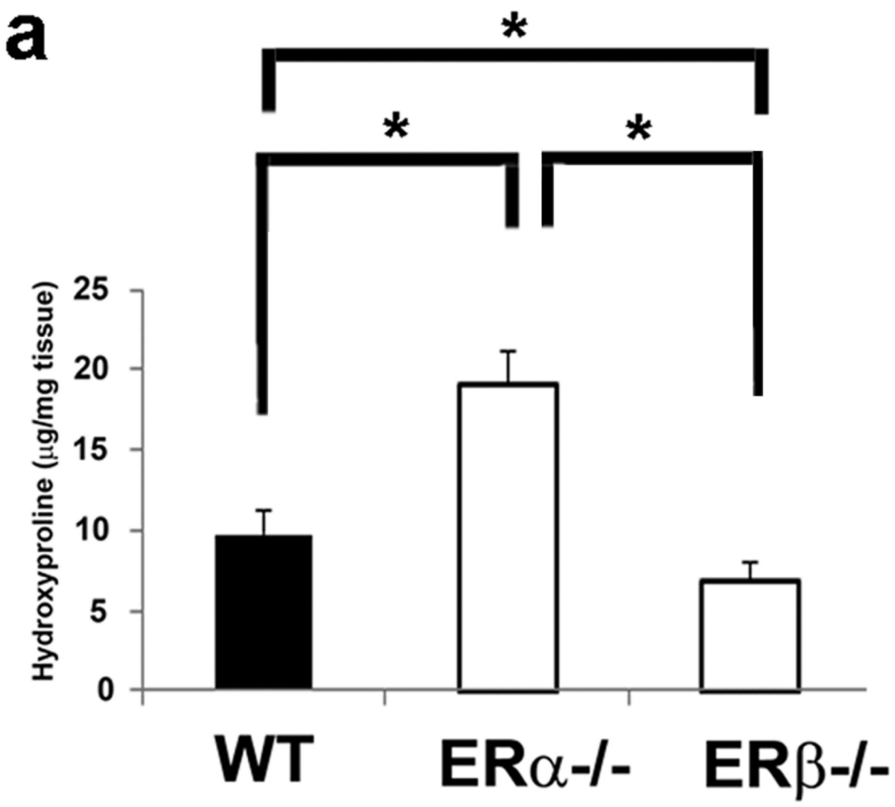


Figure 1. Histopathological analyses of collagen in female mouse skin tissue

(a) Representative examples of skin sections stained with H/E **(b)** Skin samples were processed and stained with Masson's trichrome as described in the Methods section. Collagen staining appears blue. Original magnification, x100;scale bar,15.0 m). **(c)** Graphical presentation of skin thickness measured from the top of the granular layer to the junction between the dermis and subcutaneous fat using Sot Advanced Image Software. Values are presented as means ± SD. *$p<0.05$



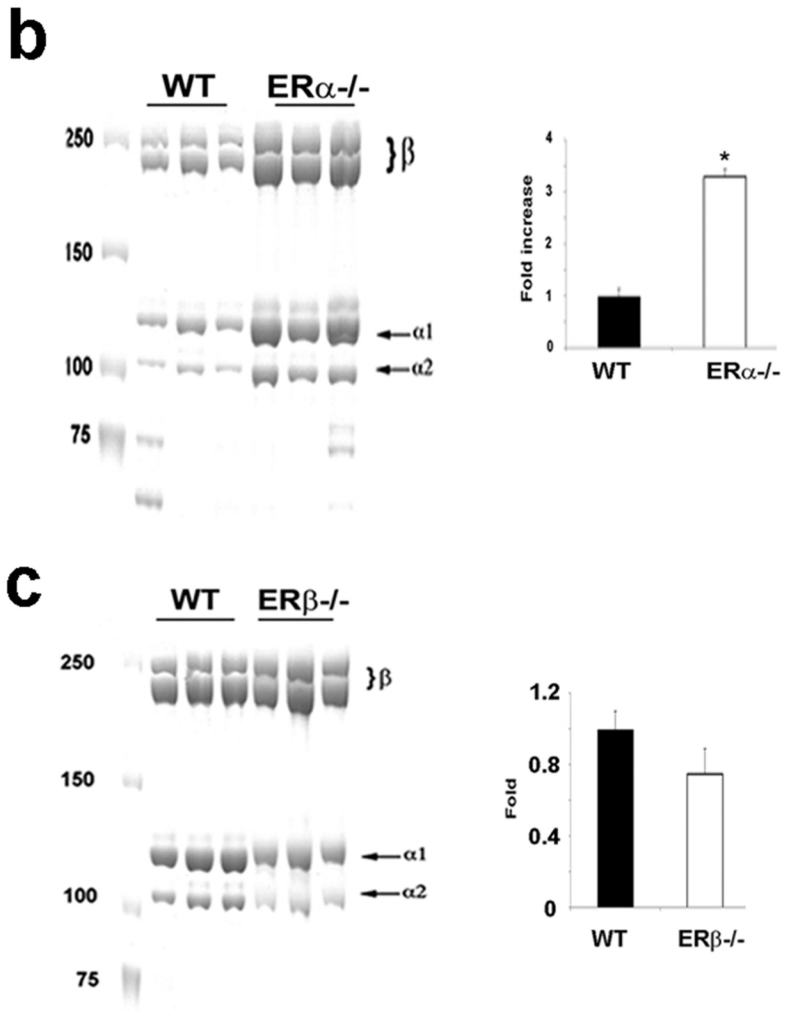
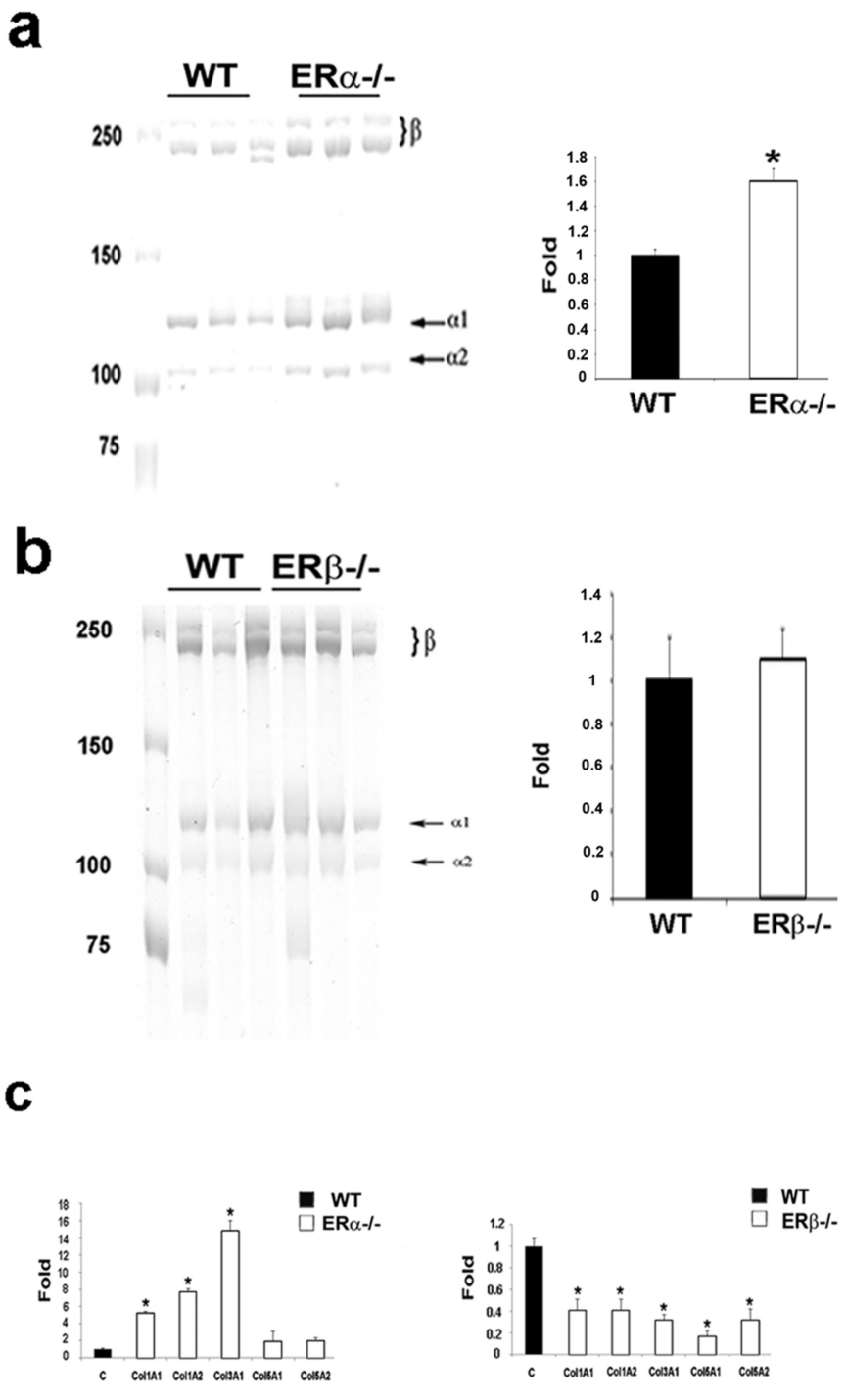


Figure 2. Absence of ERα or ERβ differentially modulate collagen content in female skin tissue

(a) Hydroxyproline levels were measured as described in Materials and Methods. The amount of hydroxyproline in each sample was calculated by comparison to a hydroxyproline standard curve and expressed as μg of hydroxyproline/ml. Values are presented as means ± SD. **(b,c)** The acetic extraction of collagen was performed with addition of pepsin (see Methods). Arrows indicate collagen α1(I) and α2(I) subunits. Slower migrating bands represent cross-linked α-chain dimers (also termed β-components). The molecular weight marker for protein (kd) is shown on the left. A graphical representation of collagen levels quantified using NIH Image densitometry software is shown on the right. Values are presented as means ± SD. *$p<0.05$



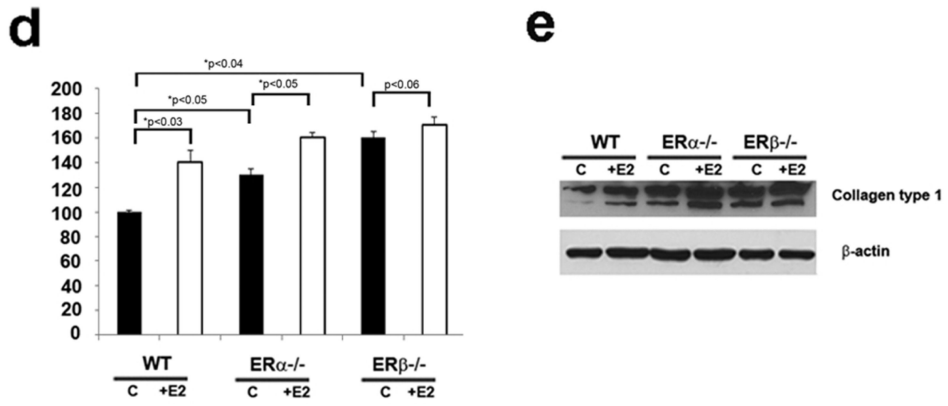


Figure 3. Absence of ERα and ERβ differetially modulate collagen synthesis in the skin and cultured fibroblasts

(a,b) Skin samples were taken with an 8 mm biopsy punch from the dorsal side of WT, ERα$^{-/-}$ or ERβ$^{-/-}$ mice. Acetic acid extraction of collagen was performed as described in Materials and Methods. Arrows indicate collagen α1(I) and α2(I) subunits. β-components represent cross-linked α-chain dimers. The molecular weight marker for proteins (kd) is shown on the left. A graphical representation of collagen level quantified using NIH Image densitometry software is shown on the right. Values are presented as means ± SD. *p<0.05. **(c)** qPCR analysis of fibril forming collagens in skin isolated from control (black bars), ERα$^{-/-}$ and ERβ$^{-/-}$ mice (white bars). Fold-change shown in the graphs is normalized to mRNA expression by one of the control mice. Values are presented as means ± SD.*p<0.05 **(d)** Dermal fibroblasts obtained from ERα$^{-/-}$ and ERβ$^{-/-}$ mice were treated for 24 hours with 17β-estradiol (10nM). Collagen a1(1) mRNA levels were determined by qPCR. Values were normalized to control (arbitrarily set at 100) and presented as means ± SD.*p<0.05. Representative Western blot of collagen protein levels is shown **(e).**

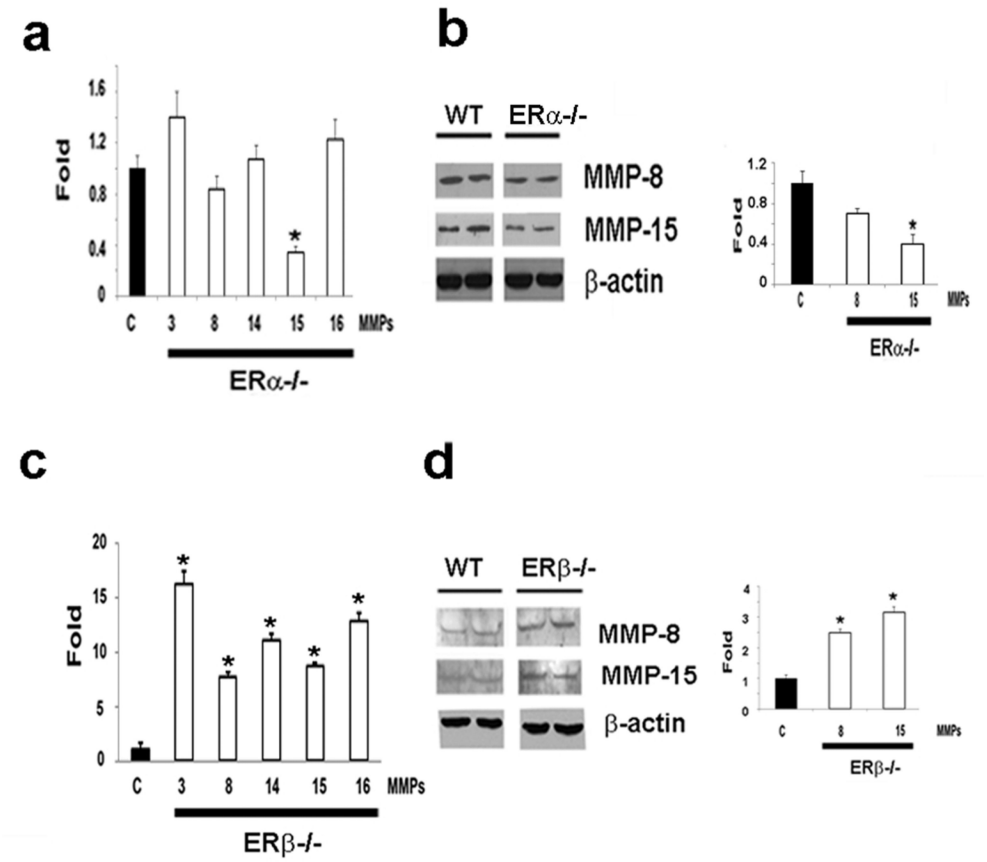


Figure 4. Absence of ERα and ERβ modulate mRNA and protein expression of MMPs in female mouse skin

Quantitative real-time PCR analyses of gene expression of indicated MMPs in WT (black bars), ERα$^{-/-}$ **(a)** and ERβ$^{-/-}$ **(b)** (white bars) mice. Fold-change shown in the graphs is normalized to mRNA expression by one of the control mice. Values are presented as means ± SD. *p<0.05 **(c,d)** Representative Western blots of MMP-8 and MMP-15 in skin samples from WT, ERα$^{-/-}$ and ERβ$^{-/-}$ mice. Immunodetection of β-actin is shown as loading control. *p<0.05

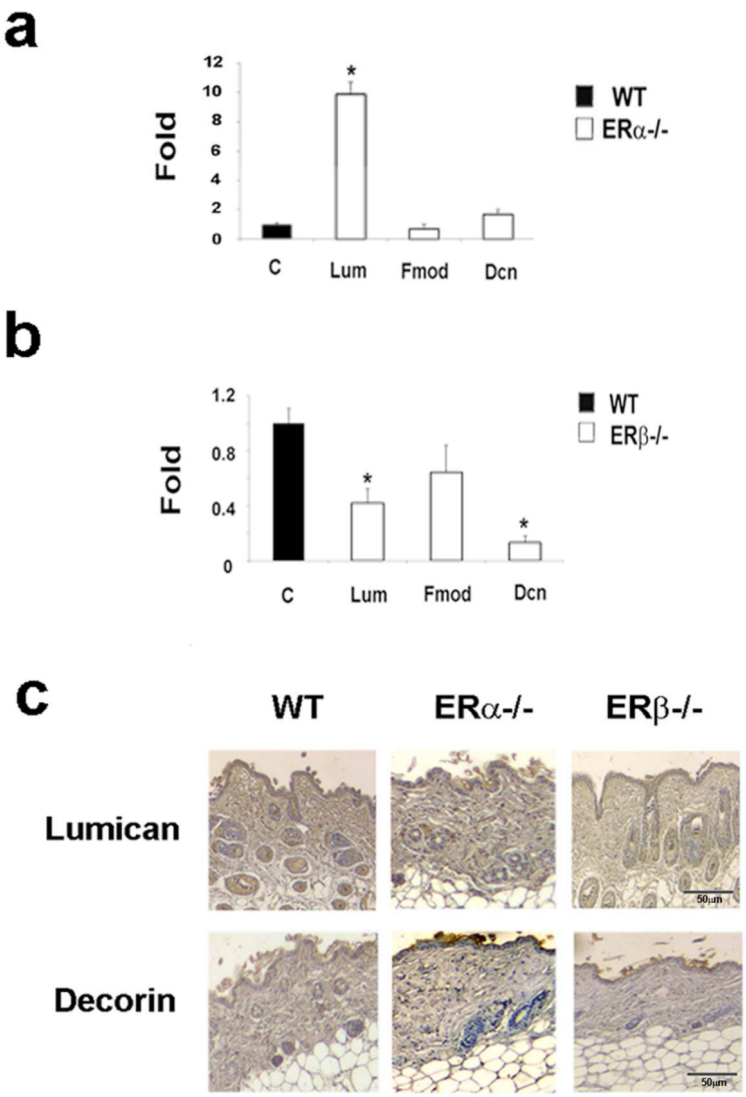


Figure 5. Quantitative real-time PCR and protein analyses of SLRPs in WT, ERα$^{-/-}$ and ERβ$^{-/-}$ mice

(a,b) qPCR analysis of gene expression of Lum, Fmod, and Dcn in skin isolated from control (black bars), ERα$^{-/-}$ and ERβ$^{-/-}$ mice (white bars). Fold-change shown in the graphs is normalized to mRNA expression by one of the control mice. Values are presented as means ± SD.*$p<0.05$ **(c)** Immunostaining was performed on paraffin embedded, formalin fixed skin tissue from control, ERα$^{-/-}$ and ERβ$^{-/-}$ female mice (original magnification,x200;scale bar,50 m). To expose core proteins, sections were treated with appropriate enzymes(see Methods).