

Up-regulation of steroid biosynthesis by retinoid signaling: Implications for aging

Abbreviated title: Role of retinoid signaling in aging

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Highlights:

- Retinoids up-regulate StAR expression and steroidogenesis in steroidogenic tissues.
- Retinoid signaling influences steroid coupled physiological functions.
- Retinoids restore steroid biosynthesis in aged epidermal keratinocytes.
- Retinoid signaling may have important implications in healthy aging.

ABSTRACT

Retinoids (vitamin A and its derivatives) are critical for a spectrum of developmental and physiological processes including neurogenesis, reproduction and skin physiology, in which steroid hormones also play indispensable roles. The steroidogenic acute regulatory protein (StAR) predominantly mediates steroid biosynthesis in steroidogenic tissues. We have recently reported that regulation of retinoid, especially all-trans retinoic acid (atRA) and 9-cis RA, responsive StAR transcription is primarily mediated by a liver X receptor (LXR)-retinoid X receptor (RXR)/retinoic acid receptor (RAR) heterodimeric motif in the mouse StAR promoter. In the present study we demonstrate that both atRA and 9-cis RA are capable of enhancing StAR protein expression, (but not its phosphorylation at Ser194, P-StAR), and steroid biosynthesis, in mouse granulosa KK-1, human adrenocortical H295R, astroglial A172, and epidermal keratinocyte HaCaT cells. Activation of cAMP/PKA signaling, by a cAMP analog (Bu)₂cAMP, markedly elevated atRA-induced StAR, P-StAR, and steroid levels. Whereas transient expression of RAR α and RXR α enhanced 9-cis RA treated StAR gene transcription, silencing of RXR α with siRNA, decreased StAR and steroid levels in each of these cell lines. Using EMSAs we showed that an oligonucleotide probe encompassing an LXR-RXR/RAR motif bound to H295R and HaCaT nuclear proteins in a retinoid responsive manner. Chromatin immunoprecipitation studies revealed the association of RAR α and RXR α with the StAR proximal promoter. Further studies demonstrated that StAR mRNA levels decreased in skin tissues of elderly men and women as well as in tissues recovered from various skin diseases and that atRA could restore steroid biosynthesis in epidermal keratinocytes of aged individuals. These findings provide novel insights into the relevance of retinoid signaling in the up-regulation

of steroid biosynthesis in gonadal, adrenal, glial, and epidermal cells, and indicate that retinoid therapy may have important implications in age-related complications and diseases.

1. Introduction

Retinoids, the metabolically active structural derivatives of vitamin A, especially atRA and 9-cis RA, exert a wide range of effects on development, differentiation, reproduction and epidermal homeostasis (Clagett-Dame and Knutson, 2011; Inoue et al., 2012; Jacobs et al., 2006; Vernet et al., 2006). The biological actions of retinoids are principally mediated by the activities of two families of nuclear receptors, the RARs and RXRs, each of which has three subtypes (α , β and γ) with additional isoforms resulting from alternative splicing (Chambon, 2005; Lefebvre et al., 2005). Whereas RARs are activated by both atRA and 9-cis RA, RXRs are induced solely by 9-cis RA. Both RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily of transcription factors that form either hetero- or homo-dimers and bind to a retinoid response element, termed the RARE/RXRE. This element is a direct repeat (DR) of two hexameric half-sites with the consensus sequences 5'-PuG(G/T)TCA-3', their inverted or everted forms being present in the regulatory region of target genes (Chambon, 2005; Kliewer et al., 1992; Lefebvre et al., 2010; Mangelsdorf et al., 1990). However, RXR-RAR heterodimers are the functional units that transduce the retinoid signal (Chambon, 2005; Lefebvre et al., 2010). RARs and RXRs are expressed in varying amounts in steroidogenic tissues, suggesting they may be independently regulated, respond to discrete ligands, and perform distinct cellular functions (Clagett-Dame and Knutson, 2011; Manna et al., 2014). Of note, mice lacking RAR α , RAR γ , RXR α , and RXR β display profound anomalies in gonadal and adrenal functions, including sterility and/or embryonic lethality (Chung et al., 2004; Clagett-Dame and Knutson, 2011; Li and

Clagett-Dame, 2009; Mark et al., 2006), underscoring the importance of retinoid signaling in reproduction as well as steroidogenesis.

The intramitochondrial transport of cholesterol is the rate-limiting and regulated step in steroid biosynthesis, a process that is primarily mediated by the StAR protein, a rapidly synthesized mitochondrial phosphoprotein whose expression, activation, and removal is influenced by PKA, PKC, and a host of other signaling pathways that produce both acute and chronic effects on steroidogenesis (Clark et al., 1994; Manna and Stocco, 2005; Miller and Bose, 2011; Stocco et al., 2005). At the level of the mitochondria, cytochrome P450_{scc} (CYP11A1) cleaves the side chain of cholesterol to form the first steroid, pregnenolone, which is further converted by a series of enzymes to various steroid hormones in pertinent tissues [reviewed in Refs. (Manna et al., 2009; Manna and Stocco, 2005; Miller and Bose, 2011; Stocco et al., 2005)]. Noteworthy, phosphorylation of StAR, especially at Ser194, has been demonstrated to be an indispensable event to obtain the maximal cholesterol transferring activity of StAR for steroid biosynthesis (Jo et al., 2005; Stocco et al., 2005; Manna et al., 2006; Manna et al., 2009a; Manna et al., 2013). In a recent study, we have reported that retinoids, by interacting with an LXR-RXR/RAR motif, synergistically activate cAMP/PKA stimulated StAR expression and steroid biosynthesis in MA-10 mouse Leydig cells (Manna et al., 2014). Additionally, the cooperation/interaction between LXR and RXR/RAR in influencing StAR transcription and steroidogenesis has been shown in gonadal and adrenal cells (Manna et al., 2013; Manna et al., 2014). However, the mechanisms of action of retinoids in the regulation of the steroidogenic response remain elusive.

With aging, multifaceted changes in the neuroendocrine system result in a decline in various hormones, including steroids and, thus, many physiological activities. Age-related

complications and diseases are also frequently associated with decreases in retinoid metabolism and signaling (Mihaly et al., 2011; Olson and Mello, 2010; Ono and Yamada, 2012). Noteworthy, the systemic administration of retinoids, especially RAs, reverses most reproductive and developmental blocks in vitamin A deficient (VAD) rats/mice, demonstrating that retinoid signaling rescues reproductive defects as well as steroidogenesis in VAD animals (Chung and Wolgemuth, 2004; Clagett-Dame and Knutson, 2011; Mark et al., 2006). The involvement of retinoids in skin physiology/pathophysiology has long been known. Human skin possesses endocrine functions that include the capabilities to synthesize cholesterol and express StAR and also house the functional biochemical apparatus for the synthesis of glucocorticoids and sex steroids *de novo* or from precursors of systemic origin (Cirillo and Prime, 2011; Slominski and Wortsman, 2000; Slominski et al., 2013a; Slominski et al., 2014; Zouboulis, 2009). A question remains as to whether retinoids can reverse the decline in steroid biosynthesis in target tissues and thereby maintain steroid requiring physiological activities that become diminished during aging. The experimental approaches utilized here provide evidence that retinoids up-regulate the steroidogenic response in several different endocrine tissues and that retinoid signaling is able to enhance/restore the age associated decline in steroid biosynthesis in epidermal keratinocytes.

Abbreviations: RA, retinoic acid; Retinoids, RA and its derivatives; atRA, all-trans RA; RAR, retinoic acid receptor; RXR, retinoid X receptor; LXR, liver X receptor; PKA, protein kinase A; (Bu)₂cAMP, dibutyryl adenosine 3',5' cyclic monophosphate; StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450_{scc}; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; NE, nuclear extract; ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcription polymerase chain reaction.

2. Materials and methods

2.1. Cells, plasmids, transfections, and luciferase assays

Mouse granulosa KK-1 (Manna et al., 2009b; Manna et al., 2013), human astroglial A172 (Davis and Syapin, 2004), adrenocortical H295R (Clark et al., 1995; Tu et al., 2014) and epidermal keratinocyte HaCaT (Boukamp et al., 1988; Slominski et al., 2013a) cells were respectively cultured in DMEM/F12 medium (Invitrogen Life Technologies, Inc., Grand Island, NY) plus 10% FBS, DMEM/F12 with 1% ITS plus, 2.5% NuSerum, DMEM/F12 with 10% FBS and 1% nonessential amino acids, and F-12K medium with 10% HS, containing antibiotics.

The 5-flanking -254/-1 bp region of the mouse StAR promoter was synthesized using a PCR based cloning strategy and inserted into the *Xho*I and *Hind*III sites of the pGL3 basic vector (Promega, Madison, WI) that contains firefly luciferase as a reporter gene (Manna et al., 2002; Manna et al., 2013; Manna et al., 2014). The -254/-1 bp StAR segment was used for generating mutations in the LXR-RXR/RAR heterodimeric motif 5'-**TGACCCCTGCTTTCCC**-3' (-200/-185 bp region in the mouse StAR promoter, Wt-LXR-RXR/RAR) using the Quikchange site directed mutagenesis kit (Stratagene, La Jolla, CA) (Manna et al., 2014). The sense strand of the oligonucleotide sequence used in mutating the LXR-RXR/RAR site was 5'-CCGTGA**att**CTGCTT**gat**CTATATG-3' (Mut-LXR-RXR/RAR; mutated bases in lowercase boldface letters) and the mutation was verified by *Eco*RI and *Sau*3A1. The pRL-SV40 plasmid containing the Renilla luciferase gene driven by SV40 promoter was obtained from Promega. Expression plasmids for RAR α and RXR α isoforms have been previously described (Manna et al., 2014). All plasmids were confirmed by either restriction endonuclease digestion or sequencing on a PE Biosystems 310 Genetic Analyzer (Perkin-Elmer, Boston, MA).

For transfection studies, different cell types were cultured in either 6- or 12-well plates to ~70% confluence and were transfected using Lipofectamine 2000 transfection reagent (Invitrogen), under optimized conditions (Manna et al., 2011; Manna et al., 2013; Manna et al., 2014). Transfection efficiency was normalized by co-transfecting 10-20 ng pRL-SV40 vector. The amount of DNA used in transfections was equalized with an empty expression vector.

Luciferase activity in the cell lysates was determined by the Dual-luciferase reporter assay (Promega) system (Manna and Stocco, 2007; Manna et al., 2009a; Manna et al., 2014). Following treatments, cells were washed with 0.01 M PBS and 250 μ l of the reporter lysis buffer was added to the cells. Cellular debris was pelleted by centrifugation at 12,000 X g for 10 min at 4 °C, and the supernatant was measured for relative light units (luciferase/Renilla) using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA).

2.2. Immunoblotting

Immunoblotting studies were carried out using total cellular protein (Manna et al., 2011; Manna et al., 2013; Manna et al., 2014). Briefly, equal amounts of protein were loaded onto 10-12% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were electrophoretically transferred onto Immuno-Blot PVDF membranes, which were probed with the specific antibodies (Abs) that recognize StAR (Bose et al., 1999), P-StAR [Ser194; (Manna et al., 2006)], RAR α , RXR α (Cell Signaling Technology, Beverly, MA), CYP11A1 (Chemicon International Inc., Temecula, CA), and β -actin (Applied Biosystems/Ambion, Austin, TX). Following overnight incubation with primary Abs, the membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary Abs for 1h at room temperature. The immunodetection of different proteins was determined using a

Chemiluminescence Imaging Kit (Perkin-Elmer), and the intensity of bands was quantified using a computer-assisted image analyzer (Quantity One Software, Bio-Rad Laboratories).

2.3. Isolation of epidermal keratinocytes from human skin tissues

De-identified human skin tissues of elderly men and women (64-83 years) were obtained from the Department of Dermatology clinic, Texas Tech University Health Sciences Center (TTUHSC; Institutional Review Board (IRB) approval, #L14-085), following various surgeries. Epidermal keratinocytes were isolated from these tissues and grown in DMEM/F12 medium supplemented with 10% FBS, 1% glutamine, epidermal growth factor (10 ng/ml) containing 10000 U/L penicillin and 50 mg/L streptomycin (Hannen et al., 2011; Slominski et al., 2015; Tiala et al., 2007). Briefly, after removing the dermis and connective tissue, the sample was chopped into small pieces, incubated with 0.25% trypsin for 1-2h at 37 °C, and the epidermis was separated from the membrane using needles to release basal keratinocytes. The cell suspension was centrifuged at 1000 X g for 5 min at room temperature, the pellet containing keratinocytes was resuspended in DMEM/F12 growth medium and seeded in 60 X 15 mm tissue culture flasks, in which media were changed every alternate day. When plates were 70-80% confluent, primary keratinocytes were utilized for experiments.

2.4. Extraction of RNA from skin tissues and semi-quantitative RT-PCR

Total RNA was extracted from primary cultures of isolated epidermal keratinocytes of elderly individuals using Trizol reagent (GIBCO-BRL, Grand Island, NY) (Manna et al., 2013; Manna et al., 2014). Extractions of total RNA from formalin fixed, paraffin-embedded, human skin tissues (IRB# L12-084) were carried out with optimized procedures (Hennig et al., 2010; Slominski et al., 2013a; Zhang et al., 2010). Briefly, skin tissues of various ages (14-86 years)

and body locations, obtained from the Dermatology clinic at TTUHSC, were deparaffinized using xylene and then incubated in ethanol. Following aspiration of the ethanol, tissue samples were resuspended in 500 μ l of RNA lysis buffer (10 nmol/L Tris-HCl, pH 8.0, 0.1 mmol/L EDTA, 2% SDS, pH 7.3) containing 50 μ l of 60 mg/ml proteinase K, and incubated overnight at 60-65 °C. Total RNA was purified using two sequential extraction procedures with phenol:chloroform (70:30), and precipitated with isopropanol containing 1/10 volume of 3 mol/L sodium acetate (pH 5.2) and 20 mg/ml of carrier glycogen at -20 °C for 1-2 h. The RNA pellet was washed in 75% ethanol, treated with DNase (10 μ g/ml) at 37 °C for 30 min and purified.

A semi-quantitative RT-PCR procedure was employed for amplifying StAR and L19 cDNAs, utilizing the following primer pairs; StAR sense, 5'-GACCTTGAAAGGCTCAGGAAGAAC-3', StAR antisense, 5'-TAGCTGAAGATGGACAGACTTGC-3', L19 sense, 5'-GAAATCGCCAATGCCAACTC-3', and L19 antisense, 5'-TCTTAGACCTGCGAGCCTCA-3', under optimized conditions (Manna et al., 1999; Manna et al., 2013; Manna et al., 2014). Briefly, RT and PCR were run consecutively in the same assay, which included [α^{32} P]-dCTP (PerkinElmer) in the dNTP mixture. The molecular sizes of StAR (980 bp) and L19 (405 bp) were determined on 1.2% agarose gels, which were vacuum dried and exposed to X-ray films (Phenix Research Products, Candler, NC). Levels of StAR and L19 signals were quantified using an image analyzer (Quantity One Software).

2.5. Silencing of *RXR α*

Knockdown of *RXR α* in different cells was performed with small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen) under optimized conditions (Manna et al., 2013;

Manna et al., 2014; Tu et al., 2014). Silencer negative control and human RXR α (sense, 5'-AGGACUGCCUGAUUGACAAAtt-3'; antisense, 5'-UUGUCAAUCAAGGCAGUCCUtg-3') siRNAs were obtained as annealed oligos from Ambion (Austin, TX). Cells were transfected with RXR α siRNA at 100 nM. Following 36-48h of transfections, cells were utilized in treatments.

2.6. Electrophoretic mobility shift assays (EMSAs)

EMSA experiments were performed using H295R and HaCaT nuclear extracts (NEs) that were prepared following the procedures described previously (Manna et al., 2004; Manna and Stocco, 2007; Manna et al., 2009c; Manna et al., 2014). The sense strands of the oligonucleotide sequences used were: LXR-RXR/RAR, 5'-GGT**GACCCCTGCTTTCCC**-3' (Manna et al., 2014) and RARE-DR5, GGAG**GGTTCACCGAAAGTTCACTCGCA** (de The et al., 1990). The 5'-GG overhangs in the double-stranded oligonucleotides were end-labeled with [$\alpha^{32}\text{P}$]-dCTP (Perkin-Elmer) using Klenow (Promega) fill-in reaction, and protein:DNA binding assays were performed (Manna et al., 2011; Manna et al., 2014). Briefly, NE (10-15 μg) was incubated for 15-20 min at room temperature in a 25 μl reaction buffer (25 mM Tris-HCl, 1 mM EDTA, 4% Ficoll, 10 mM dithiothreitol, 2 μg poly dIdC, 40 ng/ μl BSA, and 12 mM MgCl_2 , pH 7.9) before the addition of a ^{32}P -labeled probe either alone or in the presence of unlabeled oligonucleotide. When Abs were used, the reactions were carried out for an additional 45-60 min on ice. Reactions were then subjected to electrophoresis on 5% PAGE gels in 0.5 X TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3). The gels were dried, exposed to X-ray film (Phenix Research Products), and protein:DNA complexes were analyzed.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out using a kit (Upstate/Chemicon, Temecula, CA) following the manufacturer's instructions, as described previously (Manna et al., 2007; Manna et al., 2011; Manna et al., 2014). Briefly, following treatments, H295R and HaCaT cells were incubated with 1% formaldehyde for 10 min at 37 °C to crosslink DNA and its associated proteins. Cells were washed, scraped, collected in lysis buffer and sonicated for 9-10 cycles of 6 sec pulses using a Tekmar Sonic Disruptor (Fisher Scientific, Pittsburgh, PA). The supernatant containing chromatin was cleared with a protein A agarose/salmon sperm DNA 50% slurry for 30 min at 4 °C with agitation. After centrifugation, the supernatant was immunoprecipitated with 4 µg of Abs specific to RAR α and RXR α for ~16h at 4 °C, followed by incubation with Protein A agarose/salmon sperm DNA for an additional 1h. After washing, protein-DNA complexes were eluted with freshly made elution buffer (1% SDS, 0.1 M NaHCO₃), and the eluate containing 5M NaCl was incubated at 65 °C for 4h to reverse the formaldehyde cross-linking. The resulting samples were treated with 0.5 M EDTA, 1 M Tris-HCl (pH 6.5) and 10 mg/ml proteinase K for 1h at 45 °C, and the purified DNA samples were used for PCR using [α^{32} P]-dCTP in the dNTP mixture. PCR was performed with ~100 ng of DNA and the mouse StAR promoter (forward, 5'-GTCTACTTTAGAGAAGCTAT-3' (bases -255/-227), and reverse, 5'-GAAGGCTGTGCATCATCACTTGAG-3' (bases -62/-39), as described recently (Manna et al., 2014). PCR products were determined on 2% agarose gels, which were vacuum dried, exposed to X-ray film for 1-3h, and the resulting signals were analyzed.

2.8. Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed by ANOVA using Statview (Abacus Concepts Inc., Berkeley, CA) followed by Fisher's protected least significant differences test. Data presented are the mean $\pm$ SE, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Assessment of the role of retinoids in StAR expression and steroidogenesis in classical (gonadal and adrenal) and non-classical (glial and epidermal) steroidogenic cells

The involvement of retinoids in (Bu)₂cAMP and/or type I/II PKA analog stimulated StAR expression and steroid biosynthesis has been recently demonstrated in MA-10 Leydig cells (Manna et al., 2014). The importance of retinoid signaling in the regulation of steroidogenic response was further assessed using a variety of clonal and primary cell culture models (Fig. 1). KK-1 (A) and H295R (B) cells treated with atRA (10 μ M; (Manna et al., 2014)), for 6h, enhanced StAR protein (2.7 ± 0.4 and 2.9 ± 0.3 fold) and progesterone (6 ± 1.1 and 8 ± 1.3 fold) production, over their respective basal levels. Additionally, treatment of A172 (C) and HaCaT (D) cells with atRA, for 24h, resulted in increases in StAR protein (2.4 ± 0.3 and 2.6 ± 0.2 fold) and pregnenolone synthesis (5 ± 0.9 and 7 ± 1.2 fold), respectively. A172 and HaCaT cells were treated in the presence of SU-10603 (20 μ M) and cyanoketone (5 μ M) for blocking the pregnenolone metabolism (Karri et al., 2007). Phosphorylation of StAR at Ser194 was undetected in control and atRA treated cells. A suboptimal concentration of (Bu)₂cAMP (0.1 mM) displayed little to no effects on StAR and steroid levels in these cells. Addition of (Bu)₂cAMP to the atRA incubations significantly enhanced ($p < 0.01$) StAR, P-StAR, and steroid levels in all of the cell types tested. CYP11A1 protein levels were unaltered in KK-1 and

H295R cells in response to atRA, but moderately increased ($p < 0.05$) in A172 and HaCaT keratinocytes, suggesting retinoid-responsive steroid biosynthesis involves differential regulation of this enzyme in classical vs. non-classical steroidogenic tissues.

3.2. Overexpression and silencing of RAR α and/or RXR α on StAR gene transcription and steroidogenesis

The significance of retinoid-induced StAR expression and steroid biosynthesis was next evaluated in conjunction with RAR α and RXR α in different steroidogenic cells. Utilization of these isoforms was based on previous findings (Clagett-Dame and Knutson, 2011; Manna et al., 2014). As illustrated in Fig. 2, KK-1 (A), H295R (B), A172 (C), and HaCaT (D) cells transfected with either pCMX-RAR α (RAR α) or pCMX-RXR α (RXR α) expression plasmid, within the context of the -254/-1 bp StAR-Luc containing wild type LXR-RXR/RAR (Wt-LXR-RXR/RAR), resulted in 2-3 fold increases in StAR promoter activity in response to 10 μ M 9-cis RA, over the responses seen in mock-transfected (pCMX) cells. Conversely, cells transfected with the -254/-1 bp StAR-Luc containing mutations in the LXR-RXR/RAR motif (Mut-LXR-RXR/RAR) decreased basal luciferase responses by 40-66% and coordinately repressed 9-cis RA-induced StAR reporter activity, demonstrating the importance of this putative element in retinoid mediated StAR gene transcription.

In additional studies, transfection of KK-1 (A), H295R (B), A172 (C), and HaCaT (D) cells with 100 nM of specific human RXR α siRNA resulted in 72-86% decreases in endogenous RXR α protein levels in these cells when compared with scrambled siRNAs (Fig. 3). Treatment with 9-cis RA had no apparent effects in RXR α protein levels in all of these cell lines tested. Depletion of RXR α attenuated basal and 9-cis RA-induced StAR and steroid levels between 43

and 68%. Expression of the CYP11A1 protein was unaltered, ruling out nonspecific silencing. Altogether, these results suggest that retinoid signaling enhances StAR protein and steroid biosynthesis by activating transcription of the StAR gene in granulosa, adrenal, glial and epidermal cells.

3.3. Functional relevance of the LXR-RXR/RAR element in StAR gene expression

To better understand the involvement of the LXR-RXR/RAR heterodimeric motif in retinoid responsive StAR gene transcription, protein:DNA binding studies were carried out using EMSAs. The results presented in Fig. 4A show that a ³²P-labeled oligonucleotide probe corresponding to the LXR-RXR/RAR region (a DR4-like sequence; (Manna et al., 2014)) in the mouse StAR promoter resulted in a major protein:DNA complex with H295R NE (lanes 1-7). Protein:DNA binding was increased with atRA (10 μ M, 6h) treated NE (compare lanes 1 and 2), which was competitively inhibited with an unlabeled DR5 RARE consensus (de The et al., 1990) sequence (lane 3). NE obtained from (Bu)₂cAMP (0.1 mM) stimulated H295R cells displayed no effect on protein:DNA binding (lane 4). The combined treatments with atRA and (Bu)₂cAMP further elevated protein:DNA complex (lane 5), and the binding was affected by both RAR α (lane 6) and RXR α (lane 7) Abs. Protein:DNA binding was markedly inhibited with RXR α Ab, suggesting NE binding to the LXR-RXR/RAR motif are largely RXR members. The LXR-RXR/RAR element was previously found to bind *in vitro* transcribed/translated RAR α and RXR α proteins in EMSAs, as well as MA-10 NE, and protein:DNA binding was affected by its unlabeled and mutant sequences (Manna et al., 2014). Additionally, a ³²P-labeled LXR-RXR/RAR probe displayed qualitatively similar results with HaCaT NE (Fig. 4B, lanes 1-7) in a

retinoid response manner, suggesting the ability of this motif to bind the RAR and RXR family proteins.

To obtain more insight into these mechanisms, ChIP analyses were performed. As illustrated in Fig. 4, H295R (C) and HaCaT (D) cells treated with 9-cis RA (10 μ M), which activates both RARs and RXRs, did not affect the association of RAR α and RXR α isoforms with the proximal StAR promoter when compared with untreated controls. Association of these RAR and RXR isoforms was observed with neither distal StAR promoter nor IgG (data not shown), observations in agreement with our recent findings (Manna et al., 2014). Taken together, these results indicate that regulation of retinoid mediated StAR gene transcription is predominantly mediated by the LXR-RXR/RAR heterodimeric motif.

3.4. Expression of StAR mRNA in aging and diseased skin tissues, and the influence of retinoid signaling on the steroidogenic response in aged epidermal keratinocytes

Aberrant skin cholesterol synthesis, resulting in a global reduction in steroids, is associated with many skin disorders (Elias et al., 2011; Slominski et al., 2013a; Slominski et al., 2013b); thus, it was of interest to study cutaneous steroidogenesis. The results summarized on a scatter plot revealed an inverse correlation between StAR mRNA expression and age (Fig. 5). StAR mRNA levels were decreased in skin tissues of elderly men and women (37-86 years) when compared with younger (14-35 years) individuals, suggesting an attenuation of steroid biosynthesis in aging skin of both sexes.

The levels of StAR mRNA were also reduced in many inflammatory skin diseases, including psoriasis, intertrigo, eczema, and atopic dermatitis, and warts (human papillomavirus

etiology), when compared to anatomic site-, age- and sex-matched non-diseased samples (Fig. 6). These data indicate that steroid biosynthesis is decreased in these skin disorders.

In additional studies, the hypothesis that retinoid signaling enhances the steroidogenic response in epidermal keratinocytes, and thereby can rescue steroid dependent functions in maintaining skin biological systems, was examined. Primary cultures of isolated epidermal keratinocytes of elderly men treated with 9-cis RA (10 μ M), for 24h, in the presence of SU-10603 and cyanoketone, demonstrated increases in StAR mRNA (3.1 ± 0.4 fold), StAR protein (2.5 ± 0.3 fold), and pregnenolone (6 ± 1.2 fold) levels, over their respective basal values (Fig. 7). P-StAR was undetected in response to 9-cis RA. Whereas (Bu)₂cAMP had no effects on these parameters, it markedly enhanced 9-cis RA-induced StAR, P-StAR and pregnenolone levels. Studies, albeit limited, on isolated epidermal keratinocytes from elderly women treated with 9-cis RA reflect qualitatively similar phenomena on StAR and pregnenolone levels (data not shown). These results indicate that retinoid signaling is capable of restoring steroid biosynthesis in aged skin keratinocytes that may play an important role in epidermal homeostasis.

4. Discussion

A hallmark of healthy physiological function is the ability to sense, respond to, and control innumerable processes, which require several regulatory systems to interact with one another. Complex and delicate endocrine changes affecting the structure and function of a multitude of organs, occur as life progresses from adulthood into senescence. This aging process results in a decline in various hormones and, as a consequence, affects many physiological functions (Chahal and Drake, 2007; Huhtaniemi and Forti, 2011; Makrantonaki and Zouboulis, 2007; Manor and Lipsitz, 2013). The occurrence of hormone deficiencies (or

endocrinosenescence) is constituted to be an important cause of human senescence. Endocrinosenescence includes growth hormone/insulin-like growth factor-1 axis (somatopause), hypothalamic-pituitary gonadal axis (hypogonadism), testosterone (andropause), estradiol (menopause), and dehydroepiandrosterone (adrenopause) (Huhtaniemi and Forti, 2011; Janovick et al., 2013; Manor and Lipsitz, 2013; Shaw et al., 2009; Traub and Santoro, 2010; Veldhuis, 2013). The manifestations of these deficiencies include, but are not limited to, inefficient hypothalamic-pituitary-thyroidal-adrenal-gonadal (HPTAG) axes, decreased steroid biosynthesis, sexual dysfunction, depression, osteoporosis, increased risk of cardiovascular disease, and skin disorders. Preservation of hormonal balance is the key to proper functioning of various physiological activities during the course of healthy aging. A large body of epidemiological evidence indicates that eating a diet rich in vitamins has numerous health benefits, as well as protective effects on the development of diseases, and results in increased longevity (Hammar and Ostgren, 2013; Park et al., 2012; Thomas, 2006). One such category of vitamins, vitamin A (retinol) and its derivatives, notably RAs (collectively referred to as retinoids), have antioxidant properties, and play unique modulatory and integrative roles across multiple metabolic and physiological processes (Bikle, 2012; Clagett-Dame and Knutson, 2011; Das et al., 2014; Manna et al., 2014). The present findings expand our understanding of the action of retinoids by elucidating the events in which retinoid signaling up-regulates steroid biosynthesis in gonadal, adrenocortical, glial and epidermal cells, namely by increasing StAR expression, and that retinoids can reverse the decline in steroid biosynthesis in aged epidermal keratinocytes through this same mechanism.

Our current data demonstrate that RAs enhanced StAR expression and steroid levels, without altering StAR phosphorylation, in classical and non-classical steroidogenic cells. The

activation of cAMP/PKA signaling, by a suboptimal dose of (Bu)₂cAMP (0.1 mM), markedly elevated not only retinoid-induced StAR expression, but also its phosphorylation, concomitant with increased steroid production, demonstrating that a low level of PKA activity is critical in the modulation of steroidogenesis (Stocco et al., 2005; Manna et al., 2006; Manna et al., 2009a; Manna et al., 2013). The magnitude of induction on the steroidogenic response mediated by retinoids and (Bu)₂cAMP is similar to that achieved with a maximally stimulating dose of (Bu)₂cAMP (1.0 mM) (Manna et al., 2014; Slominski et al., 2015). Noteworthy, however, that steroidogenesis in glial and epidermal cells through retinoid signaling is quite modest when compared to cAMP/PKA stimulated StAR expression and steroid synthesis in gonadal and adrenal cells. As well, in contrast to the rapid induction of cAMP-responsive steroidogenesis in classical endocrine tissues, elevation in the steroidogenic potential requires a considerably longer period of time in non-classical target tissues. The latter event reinforces the notion that the chronic effect of retinoid-mediated steroidogenesis involves increased transcription/translation of the CYP11A1 enzyme (Wickenheisser et al., 2005). Hence, it is conceivable that regulation of retinoid dependent StAR expression and steroid production in gonadal and adrenal vs. glial and epidermal cells involve different mechanisms. We have reported that steroid biosynthesis in response to retinoids is influenced by hormone-sensitive lipase (HSL) in gonadal and adrenal cells (Manna et al., 2013; Manna et al., 2014); as such, the involvement of HSL in controlling retinoid-regulated steroidogenesis in non-classical steroidogenic tissues cannot be excluded. Nonetheless, it is worth mentioning (based on our preliminary data) that retinoid signaling increases StAR and LHβ mRNA levels in mouse pituitary gonadotrope LβT2 cells (Turgeon et al., 1996) (Manna PR. et al., unpublished observations). While there is yet no information demonstrating that the pituitary gland secretes steroid(s), this gland expresses cytochrome P450

17 α -hydroxylase/C17, 20-lyase, 3 β -HSD, and cytochrome P450 aromatase and has been proposed to synthesize androgens (Do Rego et al., 2007; Galmiche et al., 2006).

The contribution of retinoid signaling in the regulation of the steroidogenic response was elucidated by overexpression and silencing studies, utilizing RAR α and/or RXR α , the functionally predominant retinoid isoforms in steroidogenic tissues (Chung et al., 2004; Clagett-Dame and Knutson, 2011; Manna et al., 2014; Mark et al., 2006). The present results document that an increase in either RAR α or RXR α levels resulted in elevated retinoid responsive StAR gene transcription in KK-1, H295R, A172, and HaCaT cells, observations that are in agreement with our recent findings in MA-10 Leydig cells (Manna et al., 2014). In contrast, substantial knockdown of endogenous RXR α protein resulted in ~50% decreases in StAR expression and steroid biosynthesis, suggesting other isoforms may play permissive roles in steroidogenesis. These results are reminiscent of previous findings that demonstrated that disruption of RAR α , RAR γ , RXR α , and RXR β isoforms exhibits abnormalities in gonadal and adrenal steroidogenic functions (Chung and Wolgemuth, 2004; Clagett-Dame and Knutson, 2011; Mark et al., 2006).

Regulation of StAR gene transcription has been shown to be coordinated by multiple trans-regulatory factors, which bind directly or indirectly to sequence-specific DNA elements located within the 5'-flanking ~250 bp region (that is highly conserved among different species) of the mouse StAR promoter, including an LXR-RXR/RAR motif (Clem et al., 2005; Cummins et al., 2006; Manna et al., 2003; Manna et al., 2009b; Manna et al., 2009c; Manna et al., 2014; Manna and Stocco, 2007). Transcriptional synergy requires the simultaneous interaction of multiple transcription factors with CREB binding protein (CBP) and its homolog p300 (CBP/p300) or additional relevant co-activators (Vo and Goodman, 2001; Manna and Stocco, 2007; Manna et al., 2014). In the present study, the functional relevance of the LXR-RXR/RAR

element was assessed by different approaches demonstrating that alteration/inhibition of RAR α and RXR α markedly affected the steroidogenic response. In addition, EMSA and ChIP analyses revealed the specificity of the LXR-RXR/RAR heterodimeric motif in retinoid-mediated regulation of StAR gene transcription. However, association of both RAR α and RXR α was unaffected by 9-cis RA, suggesting promoter occupancy involves post-translational modification of these receptors. We have recently reported on the activation of CREB signaling in retinoid responsive steroidogenesis as well as recruitment of P-CREB (and not CREB) and CBP to the StAR promoter (Manna et al., 2014). CBP/p300 act as integrators among diverse signaling pathways (Vo and Goodman, 2001; Manna et al., 2009a; Manna and Stocco, 2007) and it is likely that retinoids phosphorylate Fos/Jun, CCAAT-enhancer binding protein β , and GATA-4 (which have been demonstrated to be involved in StAR gene expression) at different Ser and Thr residues and recruit CBP/p300 in retinoid regulated transcription of the StAR gene (Clem et al., 2005; Hiroi et al., 2004; Manna et al., 2009a; Manna and Stocco, 2008; Yivgi-Ohana et al., 2009). Studies have demonstrated that retinoid activity is influenced by phosphorylation of RAR and RXR at several Ser/Thr residues by cdk7/cyclin H, which is associated with the general transcription factor TFIIH and MAPKs (Bastien et al., 2002; Gianni et al., 2003; Macoritto et al., 2008). This suggests that retinoids phosphorylate several Ser and Thr residues via receptor-dependent and receptor-independent events, underlying the signaling cross talk between nuclear and cell-surface receptors. It should also be noted that RXR/RAR heterodimerizes with several factors including LXRs, peroxisome proliferator-activated receptors, vitamin D receptors and thyroid hormone receptors which bind to hexameric half-sites (Chambon, 2005; Kliewer et al., 1992; Lefebvre et al., 2010). Thus, these heterodimers can recognize the LXR-RXR/RAR motif

in the StAR promoter and result in a large array of combinatorial actions that underlie the pleiotropic effects of retinoids in transcriptional regulation of the StAR gene.

Age-related changes affect the functional properties of all endocrine glands with many being so intertwined that a reduction in function in one gland can adversely affect the biological activities of others (Chahal and Drake, 2007; Makrantonaki and Zouboulis, 2007; Manor and Lipsitz, 2013). A failure in HPG axes results in hypogonadism, a common phenomenon in aging, which affects both testicular and ovarian functions (Huhtaniemi and Forti, 2011; Janovick et al., 2013; Traub and Santoro, 2010). Endocrinosenescence, involving the HPTAG axis, also affects feedback regulatory mechanisms and causes an array of physiological abnormalities. An intriguing aspect of the present study is that retinoids, in particular RAs, are capable of restoring/enhancing StAR expression and steroid biosynthesis in various target tissues including aged epidermal keratinocytes. This implies that retinoid signaling is capable of reversing the declines in steroid dependent age-associated impaired biological activities. Our present data also provide evidence that expression of StAR mRNA decreased not only in skin tissues of elderly men and women but also in several inflammatory skin diseases, suggesting that steroid biosynthesis is frequently attenuated and/or dysregulated during skin aging and in many skin disorders. These results are in support of previous findings that demonstrated that StAR expression is decreased or absent in a number of skin diseases (Hannen et al., 2011; Slominski et al., 2013a; Slominski et al., 2015; Suomela et al., 2009). In a recent report (Manna PR et al., 2015), we have demonstrated that retinoid signaling effectively enhances cholesterol efflux in mouse macrophages, an event is tightly connected with LXR pathways, especially the induction of the ATP-binding cassette transporter A1 that is a key regulatory molecule in cellular lipid transport and atherosclerosis. It was also found that mouse macrophages express relatively

higher levels of RAR α than that of RXR α , implicating that the majority of retinoid responsive macrophage cholesterol efflux is effected by RARs. Studies have demonstrated that removal of excess cholesterol from macrophage foam cells is critical in limiting plaque stability and progression of atherosclerosis (Ning et al., 2009; Taylor et al., 2010; Tazoe et al., 2008). Therefore, retinoid mediated macrophage cholesterol efflux may have important implications in regressing atherosclerotic cardiovascular disease.

Taken together, retinoid signaling plays a vital role in the up-regulation of StAR expression and steroid biosynthesis in gonadal, adrenal, glial, and epidermal cells. Retinoid mediated regulation of the steroidogenic response, *in vivo*, may influence multiple effects that serve to i) restore cholesterol/steroid coupled physiological activities, ii) reverse defects linked with vitamin A deficiency including reproductive capacity, iii) ameliorate abnormalities connected with hypogonadism, iv) alleviate problems associated with skin complications and diseases, v) regress/stabilize atherosclerotic cardiovascular disease. It is tempting to speculate that retinoid signaling is capable of preventing and/or restoring various deficiencies that occur as a result of the attenuation of cholesterol/steroid requiring physiological processes. These processes are especially paramount for geriatric populations to live longer and healthier.

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Figure legends

Fig. 1. Effect of atRA on (Bu)₂cAMP stimulated StAR, P-StAR, CYP11A1, and steroid levels in KK-1, H295R, and A172 cells, and in HaCaT keratinocytes. These cells were treated without (Basal) or with atRA (10 μ M), (Bu)₂cAMP (0.1 mM), or their combination, for either 6h (A and B) or 24h (C and D), as indicated, and subjected to cellular protein preparation. A172 and HaCaT cells were treated in the presence of SU-10603 (20 μ M) and cyanoketone (5 μ M). Representative immunoblots illustrate StAR, P-StAR, and CYP11A1 levels using 20-30 μ g (A and B) or 60-70 μ g (C and D) of total protein. Immunoblots shown are representative of 4-6 independent experiments. β -actin expression was assessed for loading controls. Accumulation of progesterone (A and B; bottom panels) and pregnenolone (C and D; bottom panels) in the media was determined from different groups and expressed as ng/mg protein. Data represent the mean $\pm$ SE of 4 independent experiments. Different letters above the bars indicate that these groups differ significantly from each other at least at $p < 0.05$. Note the different scales on the graphs.

Fig. 2. Overexpression of RAR α and RXR α in 9-cis RA induced StAR promoter activity. KK-1 (A), H295R (B), A172 (C), and HaCaT (C) cells were transfected with pCMX, pCMX-RAR α (RAR α), and pCMX-RXR α (RXR α), within the context of either wild type -254/-1 bp StAR-Luc (Wt-LXR-RXR/RAR) or mutant (Mut-LXR-RXR/RAR) plasmid, as indicated, in the presence of pRL-SV40. Following 36h of transfection, cells were treated without (Basal) or with 9-cis RA (10 μ M), for either an additional 6h (A and B) or 24h (C and D). Luciferase activity in the cell lysates was determined and expressed as StAR promoter activity, RLU (luciferase/Renilla). Data represent the mean $\pm$ SE of 3-5 independent experiments.

Fig. 3. Silencing of RXR α in 9-cis RA induced StAR protein and steroid levels. KK-1 (A), H295R (B), A172 (C), and HaCaT (D) cells were transfected with either 100 nM of negative control (scrambled) or specific human RXR α (RXR α) siRNA, as indicated. Following 36-48h of transfection, cells were treated without or with 9-cis RA (10 μ M) for either an additional 6h (A and B) or 24h (C and D), and subjected to cellular protein preparation. Representative immunoblots illustrate expression of RXR α , StAR, and CYP11A1 in different treatment groups. Immunoblots are representative of 4-6 independent experiments. Accumulation of progesterone (A and B; bottom panels) and pregnenolone (C and D; bottom panels) in the media was determined and expressed as ng/mg protein (n = 4, $\pm$ SE). Different letters above the bars indicate that these groups differ significantly from each other at least at p < 0.05. Note the different scales on the graphs.

Fig. 4. Binding of the LXR-RXR/RAR motif in the StAR promoter to H295R and HaCaT NE in EMSAs, and association of RAR α and RXR α with the StAR promoter by ChIP analyses. NE (10-15 μ g) obtained from control, atRA (10 μ M), (Bu)₂cAMP (0.1 mM), and atRA plus (Bu)₂cAMP treated H295R (A) and HaCaT (B) cells were incubated with the ³²P-labeled LXR-RXR/RAR probe. A major protein:DNA complex was observed with the LXR-RXR/RAR probe and H295R (A) and HaCaT (B) NE (lanes 1-7 in both cases). Protein:DNA binding was challenged with unlabeled DR5 RARE consensus sequence (lane 3) and, RAR α (lane 6) and RXR α (lane 7) Abs (A and B). Cold competitor was used at 100-fold molar excess. Migration of free probes is shown in panels A and B. Data are representative of 3-4 independent experiments. ChIP assays were carried out as described under Materials and Methods (C and D). Crosslinked sheared chromatin

obtained from control (None) and 9-cis RA treated groups was immunoprecipitated (IP) without or with anti-RAR α and anti-RXR α Abs. Recovered chromatin was subjected to PCR analysis using primers specific to the proximal -255/-39 bp region of the StAR promoter, as specified under Materials and Methods. Representative autoradiograms illustrate the association of RAR α and RXR α with the StAR promoter. Data shown are representative of 3 independent experiments.

Fig. 5. Expression of StAR mRNA in men and women skin tissues. De-identified, formalin fixed, paraffin-embedded skin specimens of various ages (14-86 years) and body locations were obtained from the Dermatology clinic at TTUHSC. Total RNA from these skin specimens was extracted and purified using the procedures described under Materials and Methods. StAR mRNA levels in different samples were determined by a semi-quantitative RT-PCR. A total of 106 skin specimens were analyzed and were made up of the following samples: elderly (37-86 years; 25 men and 24 women) and young (14-35 years; 26 men and 31 women).

Fig. 6. Relative levels of StAR mRNA in various inflammatory skin diseases. De-identified, formalin fixed, paraffin-embedded diseased and non-diseased men and women skin specimens were obtained from the Dermatology clinic at TTUHSC. Total RNA from these skin tissues was extracted and purified using procedures as described in the legend of Fig. 5 and under Materials and Methods. StAR mRNA levels in these skin specimens (a total of 87 samples were analyzed; 46 men and 41 women) were determined by a semi-quantitative RT-PCR.

Fig. 7. Effect of 9-cis RA on (Bu)₂cAMP-stimulated StAR mRNA, StAR protein, P-StAR, and pregnenolone levels in isolated epidermal keratinocytes. De-identified fresh skin tissues from

elderly men (64-83 years; 5 different specimens) were obtained from the Dermatology clinic at TTUHSC upon surgery. Epidermal keratinocytes were isolated from these aged skin tissues following the procedures described under Materials and Methods. Primary cultures of isolated keratinocytes were treated without (Basal) or with 9-cis RA (10 μ M), (Bu)₂cAMP (0.1 mM), or their combination, for 24h, as indicated, in the presence of SU-10603 (20 μ M) and cyanoketone (5 μ M). Following treatments, cells were processed for either total RNA extraction or cellular protein preparation. A representative autoradiogram illustrates expression of StAR mRNA in different treatment groups using a semi-quantitative RT-PCR (A). Integrated optical density (IOD) values of each StAR band was quantified and normalized with the corresponding L19 bands and presented below the autoradiogram. Representative immunoblots illustrate StAR, P-StAR, and CYP11A1 levels in different treatment groups using 60-70 μ g of total protein in each lane (B). Autoradiogram and immunoblots are representative of 3-5 independent experiments. β -actin expression was assessed as a loading control. Accumulation of pregnenolone in the media was determined from different treatment groups and expressed as ng/mg protein C). Data represent the mean $\pm$ SE of 4 independent experiments. *, $p < 0.05$; ***, $p < 0.01$; vs. basal.

Figure 1

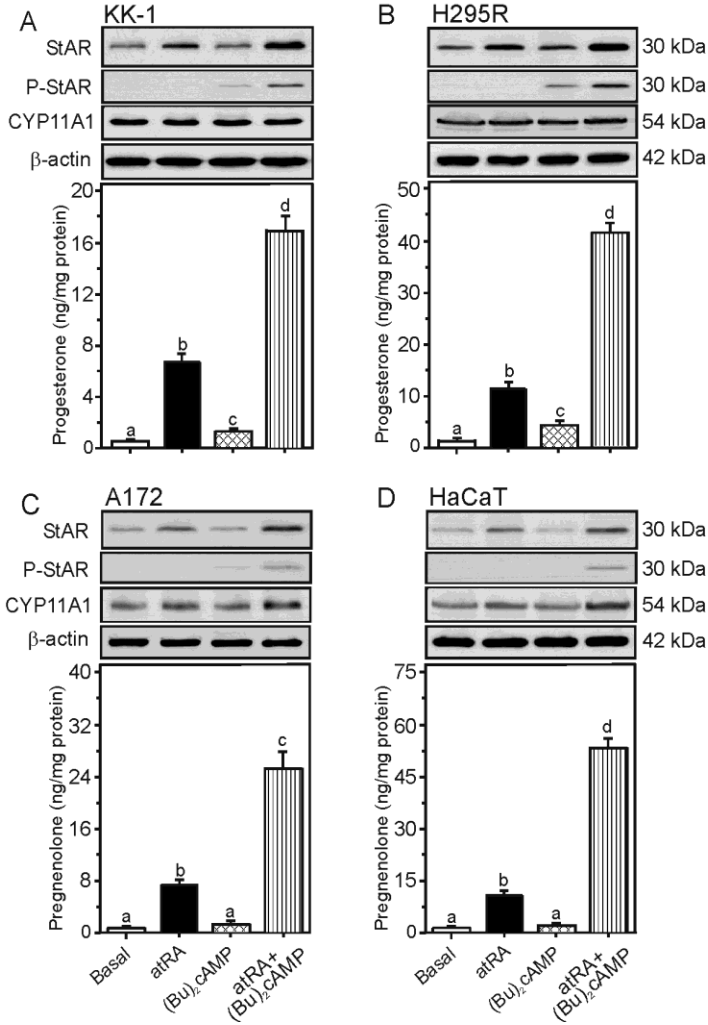


Figure 2

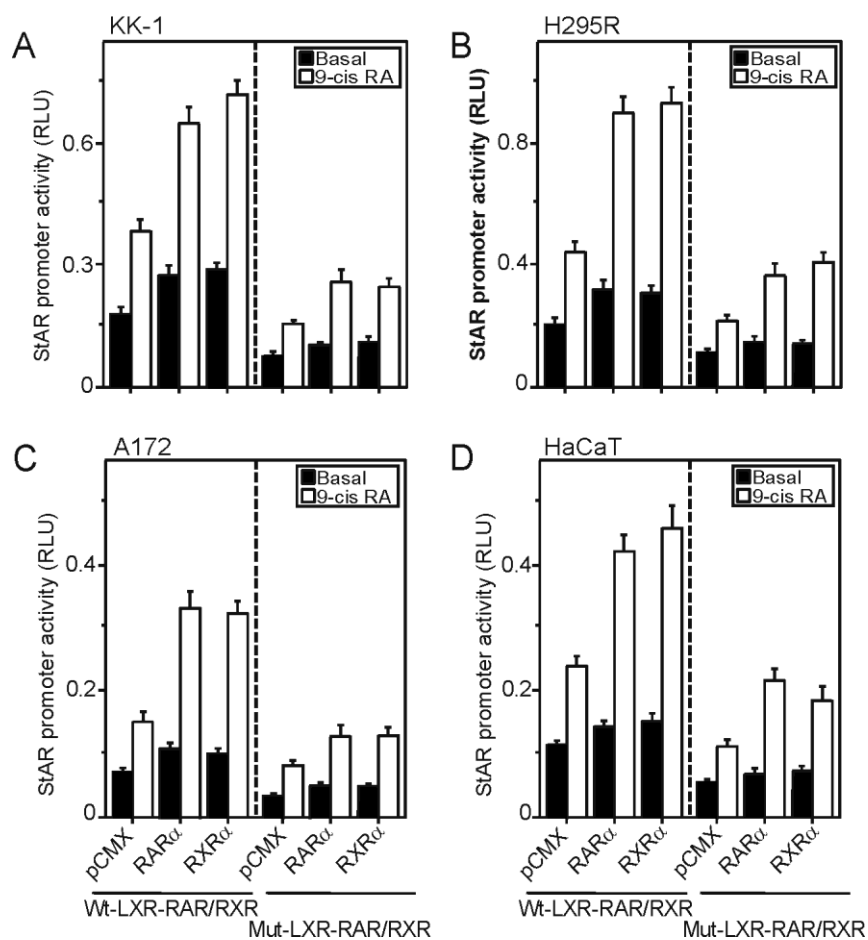


Figure 3

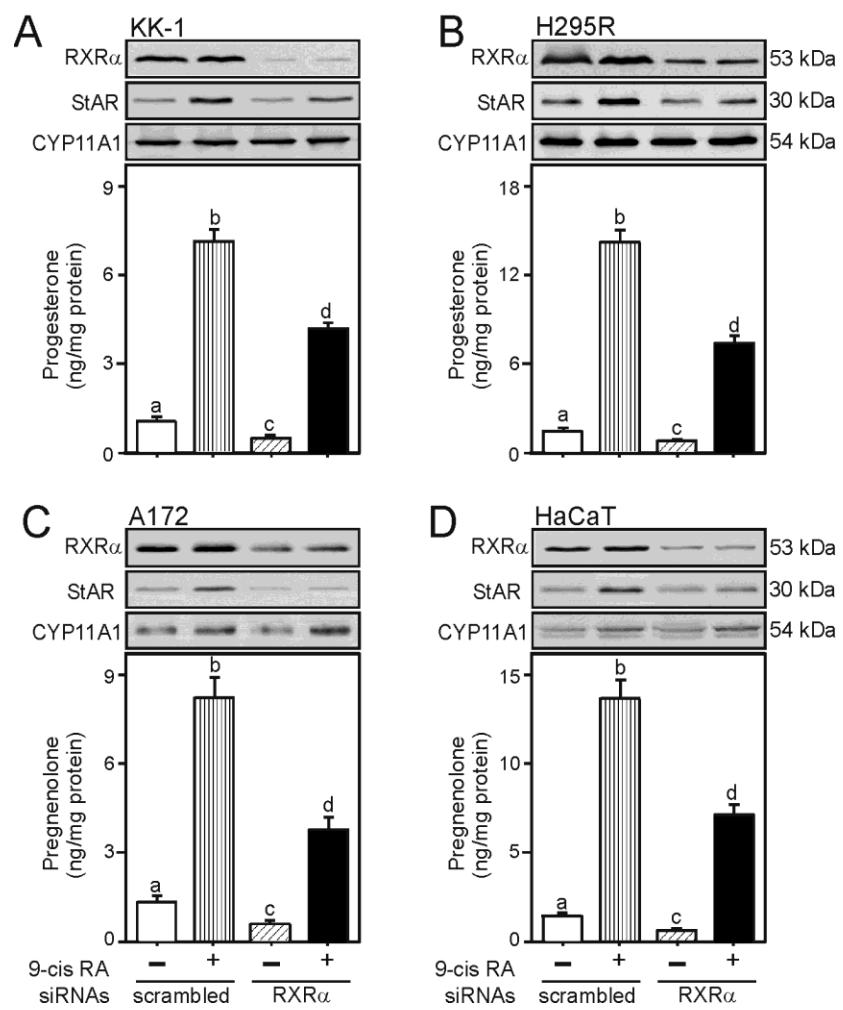


Figure 4

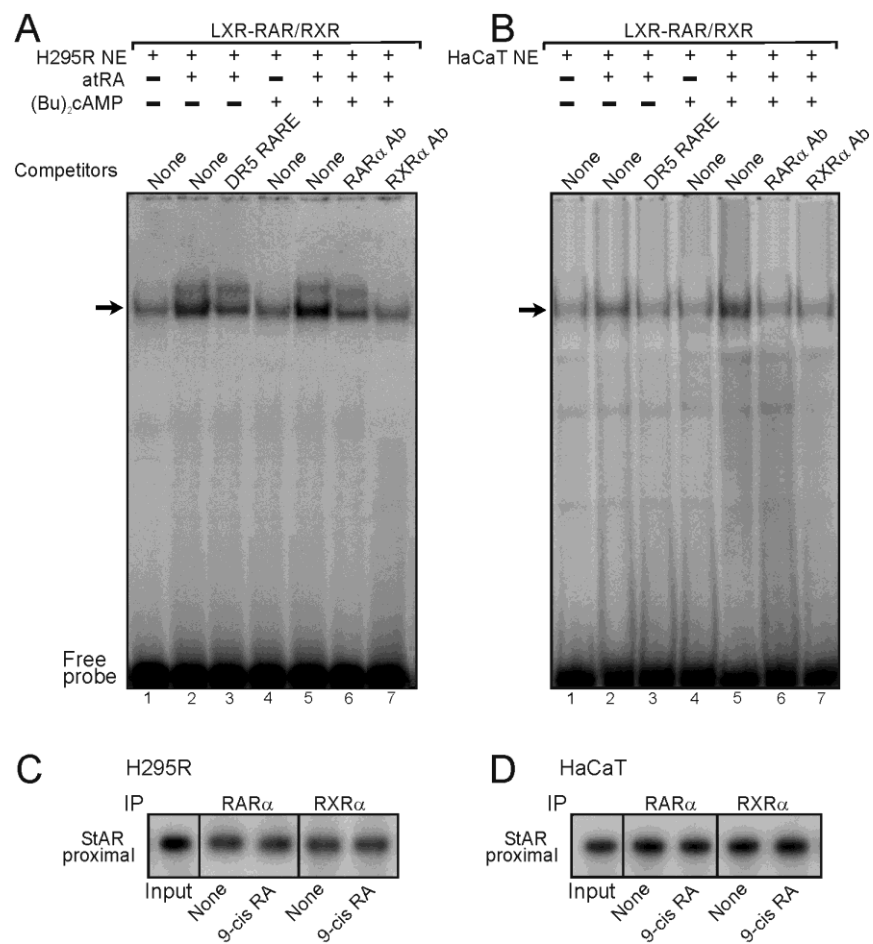


Figure 5

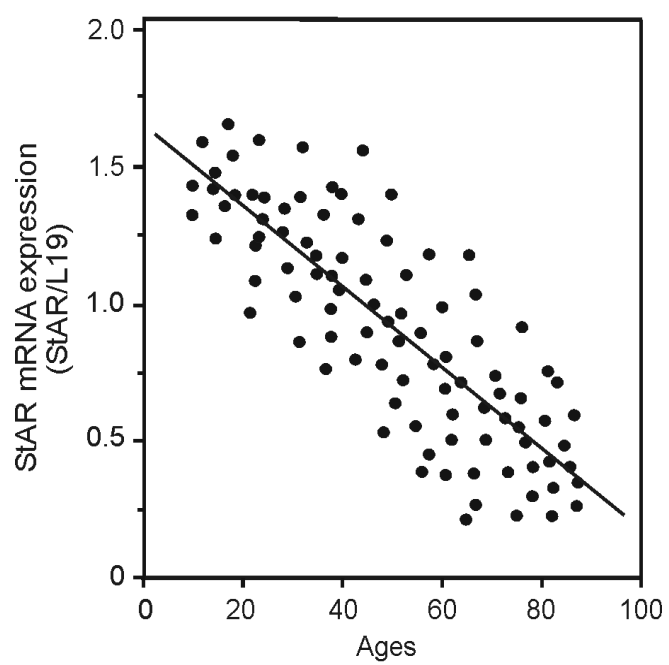


Figure 6

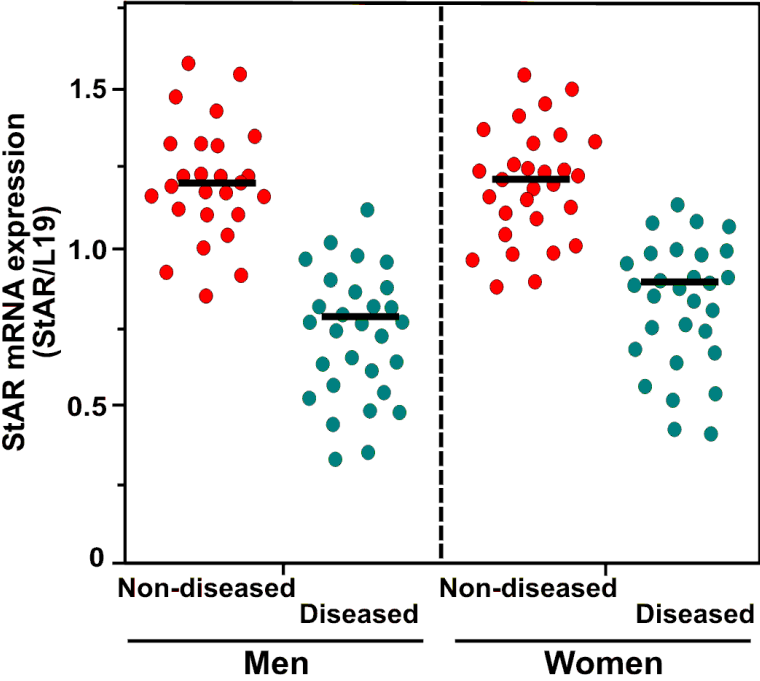


Figure 7

