Intracrine sex steroid synthesis and signaling in human epidermal keratinocytes and dermal fibroblasts

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ABSTRACT **Peripheral intracrine sex steroid synthesis from adrenal precursors dehydroepiandrosterone (DHEA) and DHEA-sulfate has evolved in humans. We sought to establish if there are differences in intracrine, paracrine, and endocrine regulation of sex steroids by primary cultures of human skin epidermal keratinocytes and dermal fibroblasts. Microarray analysis identified multifunctional genes modulated by steroids, quantitative RT-PCR (qRT-PCR) mRNA expression, enzymatic assay aromatase activity, scratch assay cell migration, immunocytochemistry α-smooth muscle actin (α-SMA), and collagen gel fibroblast contraction. All steroidogenic components were present, although only keratinocytes expressed the organic anion organic anion transporter protein (OATP) 2B1 transporter. Both expressed the G-protein-coupled estrogen receptor (GPER1). Steroids modulated multifunctional genes, up-regulating genes important in repair and aging [angiopoietin-like 4 (*ANGPTL4*), chemokine (C-X-C motif) ligand 1 (*CXCL1*), lamin B1 (*LMNB1*), and thioredoxin interacting protein (*TXNIP*)]. DHEA-sulfate (DHEA-S), DHEA, and 17β-estradiol stimulated keratinocyte and fibroblast migration at early (4 h) and late (24–48 h) time points, suggesting involvement of genomic and nongenomic signaling. Migration was blocked by aromatase and steroid sulfatase (STS) inhibitors confirming intracrine synthesis to estrogen. Testosterone had little effect, implying it is not an intermediate. Steroids stimulated fibroblast contraction but not α-SMA expression. Mechanical wounding reduced fibroblast aromatase activity but increased keratinocyte activity, amplifying the bioavailability of intracellular estrogen. Cultured fibroblasts and keratinocytes provide a biologically relevant model system to investigate the complex pathways of sex steroid intracrinology in human skin.—Pomari, E., Valle, L. D., Pertile, P., Colombo, L., and Thornton, M. J. Intracrine sex steroid synthesis and signaling in human epidermal keratinocytes and dermal fibroblasts. *FASEB J.* 29, 508–524 (2015). www.fasebj.org**

Key Words: DHEA · testosterone · estradiol · aromatase · wound healing

MULTIPLE ENDOCRINE CHANGES occur with age, but the best example of programmed aging is demonstrated by aging in the human female reproductive system. Estrogens significantly modulate human skin and insufficiency in postmenopausal women leads to a diminished defense against oxidative stress, resulting in atrophic skin changes, acceleration of skin aging, and higher incidence of nonhealing chronic wounds (1–5). Although estrogen replacement significantly reverses or delays these changes, it carries the risk of breast and uterine cancer (6). Despite its extensive role in skin homeostasis, specifically in aging and wound healing, regulation and transcriptional targets for estrogen signaling in human skin cell populations still remain to be clarified.

Humans and some primates, in addition to very sophisticated endocrine and paracrine systems, have also evolved important intracrine mechanisms for the formation of sex steroids in peripheral tissues via the biosynthesis from DHEA-S and DHEA, which are secreted by the adrenal cortices of both sexes. In an adult woman, DHEA-S is the most abundant steroid, circulating at micromolar concentrations up to 10,000 higher than that of estradiol (7). Following menopause, the peripheral conversion of adrenal DHEA-S, DHEA, and androstenedione (another adrenal androgen) becomes the exclusive and tissue-specific source of sex steroids. However, in aging humans circulating DHEA and DHEA-S levels decline and are reduced to 5–20% of their peak value in individuals 70 to 90 yr of age (8).The role of DHEA-S and DHEA in human physiology continues to be the subject of debate. There are numerous lines of evidence suggesting that high DHEA levels have a beneficial effect on longevity and prevent a number of human pathologic conditions, including heart disease and diabetes (9). DHEA is generally considered to be a weak androgen, although it is also a precursor for the intracrine biosynthesis of potent

Abbreviations: α-SMA, α-smooth muscle actin; *ANGPTL4*, angiopoietin-like 4; AR, androgen receptor; *CXCL-1*, chemokine (C-X-C motif) ligand 1; DHEA (-S), dehydroepiandrosterone (-sulfate); DHT, dihydrotestosterone; 4-DIONE, androstenedione; 5-Diol, androstenediol; E_1, estrone; E_2, estradiol; ER, estrogen receptor; GPER1, G protein-coupled estrogen receptor 1; HSD, hydroxysteroid dehydrogenase; *LMNB1*, lamin B1; OAT, organic anion transporter; OATP, organic anion transporter protein; qRT-PCR, quantitative RT-PCR; SLC, solute carrier; STS, steroid sulfatase; TST, testosterone; *TXNIP*, thioredoxin interacting protein

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TABLE 1. *Primers and conditions used for RT-PCR analysis*

GenBank accession no.	Gene	Primer sequence (sense/antisense)	T_{ann} (°C)	Ext (s)	$MgCl_2$ (mM)
J04964	STS	5′-ACCCTCATCTACTTCACAT-3′ 5′-GTCCATGTTGCTAGTGGGCT-3′	56	20	1.5
M14565	CYP11A1	5′-AAGACTTCACCCCATCTCCGTGAC-3′ 5′-ACCCCAGCCAAAGCCCAAGT-3′	60	25	1.5
NM_000102	CYP17A1	5′-CAATGAGAAGGAGTGGCACCA-3′ 5′-CTTTGAAAGAGTCGATCAGAAAGAC-3′	60	25	1.5
Y07508	CYP19A1	5′-GAATCGGGCTATGTGGACGTGTTG-3′ 5′-AGATGTCTGGTTTGATGAGGAGAG-3′	60	20	1.5
NM_001047	SRD5A1	5′-CATGTTCCTCGTCCACTACG-3′ 5′-GATGCTCTTTTGCTCTACCAG-3′	58	30	1.5
NM_000348	SRD5A2	5′-CCTCTTCTGCCTACATTACTTCC-3′ 5′-CCAGAAACATACGTAAACAAGCC-3′	58	30	1.5
AB026256	OATP2B1	5′-AGCTGTCTGTCGCTACTAC-3′ 5′-CCCAAGACAGCTCACACTC-3′	58	20	2
M12674	ERα	5′-CAGACATGAGAGCTGCCAAC-3′ 5′-CCAAGAGCAAGTTAGGAGCA-3′	60	30	2
AB006589	ERβ	5′-TCCCTGGTGTGAAGCAAGATC-3′ 5′-CGCCGGTTTTTATCGATTGT-3′	60	30	2
BC132975.1	AR	5′-AAGAGGAACAGCAGCCTTCACA-3′ 5′-ATGGGGCAGCTGAGTCATCCT-3′	64	15	1.5
BC013380	ATCB	5′-CACCAACTGGGACGACATGGAG-3′ 5′-GGCCTGGATGGCCACGTACAT-3′	60	15	1.5

STS, steroid sulfatase; *CYP11A1*, P450scc; *CYP17A1*, P450c17; *CYP19A1*, aromatase; *SRD5A1*, 5α-reductase-1; *SRD5A2*, 5α-reductase-2; *ATCB*, β actin; T_{ann}, annealing temperature; Ext, extension time.

androgens and estrogens in peripheral tissues that express the relevant steroidogenic enzymes (10). Although the response of a cell to steroid hormones depends upon the expression of specific receptors, the ratio and activity of these steroidogenic enzymes also has a regulatory function on the availability of specific intracellular steroids (11). DHEA is a weak agonist for both intracellular estrogen receptors (ERα and ERβ), and a weak antagonist for the androgen receptor (AR) (12), but a specific DHEA receptor has not yet been characterized, although it can act via non steroid receptor, nongenomic pathways (13–16).

The biosynthesis of DHEA from cholesterol requires 2 steroidogenic enzymes, cytochrome P450scc, which converts cholesterol to pregnenolone, and cytochrome P450c17, which gives rise to DHEA and the cortisol precursor, 17α-OH-pregnenolone. The half-life of DHEA-S in plasma is significantly longer than that of unconjugated DHEA. However, DHEA-S requires transportation inside the cell by an organic anion transporting polypeptide such as OATP2B1.The enzyme STS removes the sulfate group, and the resulting DHEA can undergo oxidation to androstenedione (4-DIONE) by 3β-hydroxysteroid dehydrogenase type-1/Δ^5-Δ^4-isomerase (3β-HSD-1) (17) or reduction to 5-androstenediol (5-Diol) by 17β-HSD-1 (18). Both steroids can be converted to testosterone, which is metabolized to 17β-estradiol by aromatase, or to 5α-dihydrotestosterone (5α-DHT) by 5α-reductase (19).

Although human skin is considered to be an important target tissue for sex steroids, it is also a primary source of sex steroids. However, the complex pathways of steroid intracrinology and regulation of receptor expression in human skin are still poorly understood. Although the presence of AR, ERα, and ERβ in human skin is well established (11, 19–22), there is increasing evidence that rapid, nongenomic estrogen signaling is mediated by GPR30, also known as GPER1 (23–25), yet its expression in adult human skin has not been reported. The first aim of this study was to determine whether early passage (P3) primary cultures of human skin dermal fibroblasts and epidermal keratinocytes, the key mesenchymal and epithelial cells in the skin, retain the vital steroidogenic machinery required for the conversion of cholesterol to potent androgens and estrogens and therefore would be relevant *in vitro* models for functional studies of steroid hormone action. To establish changes in specific gene expression in response to different steroids, a gene array analysis was performed on human keratinocytes following incubation with DHEA-S, testosterone, and 17β-estradiol and the upregulation of specific genes involved in tissue repair and aging also confirmed by qRT-PCR. Although it has been established that in humans estrogen improves wound healing (26) and testosterone impairs wound healing (27), the role of the adrenal androgen DHEA, precursor to both, and the source of sex steroids in postmenopausal women has not been established. A key aim of this study was to compare the effect of different steroids in the presence and absence of specific aromatase and STS enzyme inhibitors on the migration of primary cultures of both dermal fibroblasts and epidermal keratinocytes in a scratch wound assay. In addition, the effect of estrogen and DHEA on dermal fibroblast differentiation and contraction was investigated and the biologic activity of aromatase, the enzyme required for the conversion of androgens to estrogens, was also measured in both cell types in response to mechanical wounding.

TABLE 2. *Primers and conditions used for qRT-PCR analysis*

Primer	Forward sequence	Reverse sequence	Annealing temperature (°C)
ERα	TGGGCTTACTGACCAACCTG	CCTGATCATGGAGGGTCAAA	62.2
ERβ	AGAGTCCCTGGTGTGAAGCAA	GACAGCGCAGAAGTGAGCATC	62.2
GPER1	ATGGATGTGACTTCCCAAGC	GAACAGGCCGATCACGTACT	62.2
Aromatase	ATGAAAGCTCTGTCAGGCCC	TCAACACGTCCACATAGCCC	62.2
GAPDH	TATAAATTGAGCCCGCAGCC	CGACCAAATCCGTTGACTCC	62.2 and 58.6

Each primer was designed using PrimerBlast and the annealing temperature optimized via quantitative PCR optimization.

MATERIALS AND METHODS

Cell culture

Human skin samples were obtained from healthy women following routine plastic surgery procedures [*i.e.,* face-lift surgery (nonhaired facial skin; median age 50) or abdominoplasty (abdominal skin; median age 44)]. For RNA extraction, each sample was transported in RNA later or frozen in liquid nitrogen; for cell culture, biopsies were kept in DMEM (GIBCO, Invitrogen, Milan, Italy, stored at 4°C and processed within 24 h of surgery. Primary cultures of dermal fibroblasts and epidermal keratinocytes were established as described previously (28–30). Briefly, small pieces of skin were washed in PBS containing amphotericin B (750 μl/ml); excess fat and dermis were removed before incubating overnight in dispase (2.4 U/ml) at 4°C, then at 37°C for 1 h. The epidermis was separated from the dermis with forceps, washed, and incubated with 0.05% trypsin/EDTA at 37° for 5–6 min. After briefly vortexing, the cell suspension was transferred into keratinocyte-SFM (K-SFM) medium (Invitrogen) containing 10% fetal calf serum and centrifuged at 1200 $\times$ *g* for 5 min. The cell pellet was resuspended in K-SFM supplemented with epidermal growth factor (0.05 μg/μl), bovine pituitary extract (12.5 mg/ml), L-glutamine (10 mM), and penicillin/streptomycin (100 U/ml and 100 μg/ml) and seeded into a T25 culture flask; the medium was changed every 2–3 d until the cells were 70% confluent. Four or five pieces of the remaining papillary dermis were placed into a T75 culture flask containing DMEM supplemented with 20% fetal bovine serum, L-glutamine (10 mM), and penicillin/streptomycin (100 U/ml and 100 μg/ml) and incubated at 37°C undisturbed for 7–10 d to establish dermal fibroblast explants.

All cells were assayed at passage 3. Additional cultures of dermal fibroblasts were also obtained from female breast skin and male facial skin for the aromatase assay. All human skin samples were used with full consent and ethical approval, conforming to the guidelines contained within the Declaration of Helsinki principles.

Semiquantitative RT-PCR

The cDNA derived from human adrenal gland, liver, ovary, placenta, and human prostate cancer cell lines PC3 and DU145 was used as positive controls to verify the quality and specificity of primers for the genes of interest (**Table 1**). Total RNA isolated from cells using RNeasy Mini Kit Isolation System (Qiagen, Milan, Italy) was measured using NanoDrop (CELBIO, Milan, Italy) and its integrity [RNA integrity number (RIN)] analyzed using Agilent 2100 Bioanalyzer (Agilent Technologies, Milan, Italy). Equal amounts (1 μg) were used as templates for cDNA synthesis using ThermoScript RT-PCR system and random primers (Invitrogen). Primers were designed using Primer3 v. 0.4.0software (*http://primer3.wi.mit.edu/*) and are given along with the conditions in Table 1 (11, 31, 32). All amplifications were performed using the Biotherm Taq polymerase Kit (Società Italiana Chimici, Rome, Italy).

DNA array and qRT-PCR analysis

The microarray analysis was performed using total RNA (RIN $\geq$ 8) extracted from keratinocytes incubated for 24 h with DHEA-S (10 μM), 17β-estradiol (1 nM), testosterone (50 nM), or vehicle control (ethanol 0.0001%). The RNA samples were hybridized on Agilent human whole genome 4 $\times$ 44 K array by Agilent G2565BA scanner (Agilent Technologies). After statistical analysis and initial filtering (MeV 4.7 open-source software, v 4.6; *http://www.tm4.org*), the microarray data were evaluated with Ingenuity Pathway Analysis program version 9.0 (IPA, Ingenuity Systems, Mountain View, CA, USA) using a cutoff of Log2 ratio (sample/vehicle) of $\pm$0.6. The qRT-PCR analysis for the DNA array validation was performed with Hs_TXNIP_1_SG QuantiTect Primer Assay, Hs_LMNB1_1_SG QuantiTect Primer Assay, Hs_ANGPTL4_1_SG QuantiTect Primer Assay, Hs_CXCL1_1_SG QuantiTect Primer Assay and Hs_GAPDH_2_SG QuantiTect Primer Assay. All the QuantiTect Primer Assays were used at 55°C according to the manufacturers instructions (Qiagen, Manchester, United Kingdom). For quantitation of aromatase mRNA, dermal fibroblasts and epidermal keratinocytes were cultured in the presence of 250 nM dexamethasone for 24 h. The primers and annealing temperatures qRT-PCR of aromatase, ERα, ERβ, and GPER1 are given in **Table 2**. Relative qRT-PCR ($2^{-\Delta\Delta Ct}$ method) (33, 34) was performed in triplicate per each gene of interest using QuantiTect SYBR Green PCR Kit (Qiagen, Manchester, United Kingdom) oniQ Real-Time PCR Systems (Bio-Rad, Hemel Hempstead, United Kingdom). The expression of target genes was normalized using β-actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) and ΔCts were calculated by the difference between Ct of genes target and the geometric mean of the 2 housekeeping genes. The *n*-fold expression of a given target gene was calculated as $\mathrm{Log}_2(2^{-\Delta\Delta Ct})$.

Assessment of a range of concentrations of steroids, steroid enzyme inhibitors, and mitomycin C on cultured human dermal fibroblast and epidermal keratinocyte viability

The influence of steroids and enzyme inhibitors at different concentrations on cellular viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (35, 36). Epidermal keratinocytes and dermal fibroblasts were seeded into 96-well plates at a density of 2 $\times$ 10^4 and 1 $\times$ 10^4cells/well, respectively. Cells were treated with 17β-estradiol, testosterone, Arimidex (anastrozole, Sigma-Aldrich, Poole, United Kingdom), or STX64 at concentrations of 1, 10, 50, and 100 nM; DHEA or DHEA-S at concentrations of 1, 10, and 100 μM; and mitomycin C at concentrations of 1, 10, and 100 μg/ml for 24 h. Cells were washed with PBS, 20 μl of MTT reagent (Sigma-Aldrich, Milan, Italy) was added to each well, and cells were incubated for 3 h at 37°C. The mixture in each well was removed, and the formazan crystals were dissolved in 100 μl of DMSO. Absorbance was read at 570 nm with a microplate reader, and the surviving cell

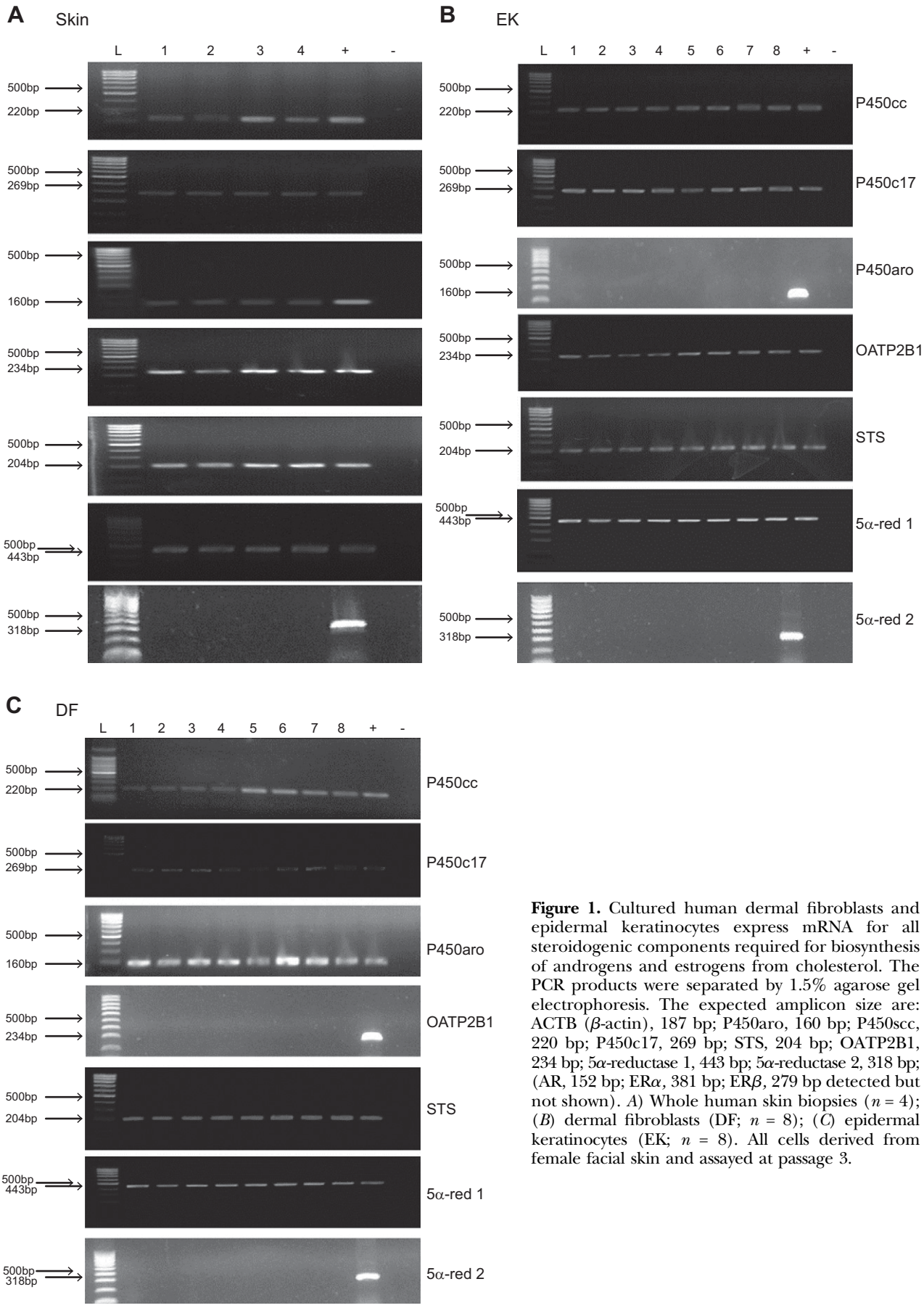


Figure 1. Cultured human dermal fibroblasts and epidermal keratinocytes express mRNA for all steroidogenic components required for biosynthesis of androgens and estrogens from cholesterol. The PCR products were separated by 1.5% agarose gel electrophoresis. The expected amplicon size are: ACTB (β-actin), 187 bp; P450aro, 160 bp; P450scc, 220 bp; P450c17, 269 bp; STS, 204 bp; OATP2B1, 234 bp; 5α-reductase 1, 443 bp; 5α-reductase 2, 318 bp; (AR, 152 bp; ERα, 381 bp; ERβ, 279 bp detected but not shown). *A*) Whole human skin biopsies (n = 4); (*B*) dermal fibroblasts (DF; n = 8); (*C*) epidermal keratinocytes (EK; n = 8). All cells derived from female facial skin and assayed at passage 3.

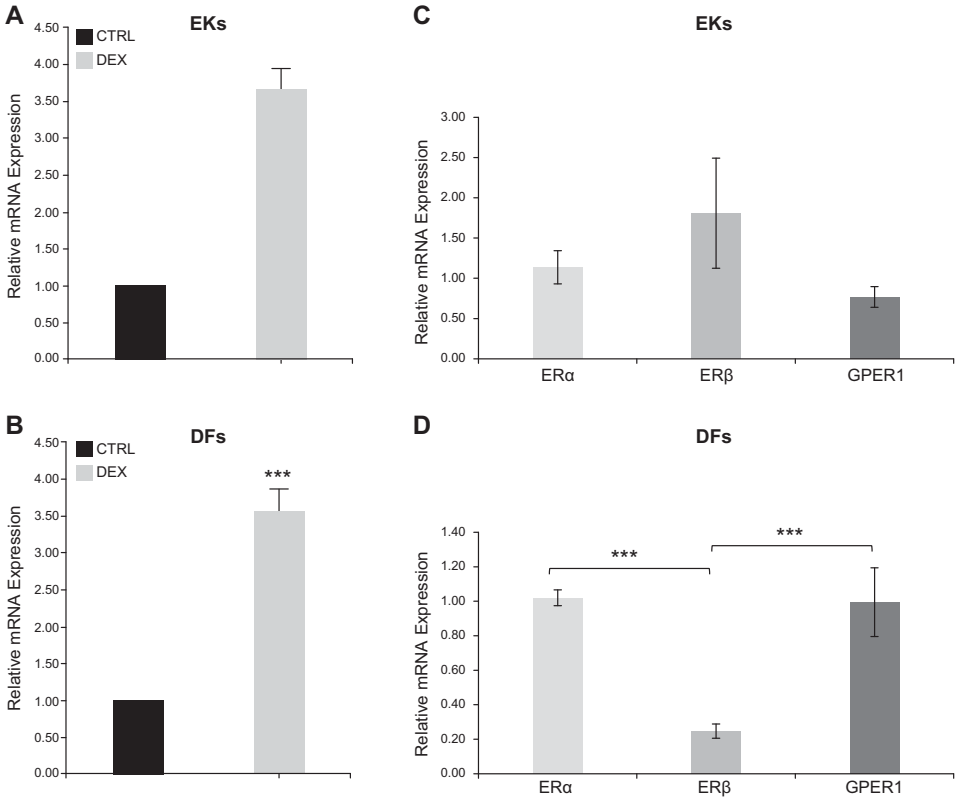


Figure 2. Cultured human dermal fibroblasts and epidermal keratinocytes express GPER1 in addition to ERα and ERβ, and dexamethasone stimulates the expression of aromatase transcripts in both cell types. The relative qRT-PCR assay was used to analyze the mRNA expression of aromatase of cultured human female facial (*A*) epidermal keratinocytes (EK, $n = 3$) and (*B*) dermal fibroblasts (DF, $n = 5$) following 24 h of incubation with 250 nM dexamethasone (DEX) or vehicle control (CRTL). Data presented as donor mean ($2^{-\Delta\Delta Ct}$ method) ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$. *C*, *D*) Relative qRT-PCR mRNA expression of ERα, ERβ, and GPER1 normalized against ERα mRNA expression in (*C*) epidermal keratinocytes and (*D*) dermal fibroblasts (n = 3 donors). $^{***}P < 0.0005$.

fraction was calculated. The cell viability was expressed as a percentage relative to the vehicle control (ethanol 0.0001%).

Scratch wound and cell migration assay

The effect of steroid hormones on fibroblast and keratinocyte migration was assessed using the scratch wound assay as previously described (28, 30, 37). Mechanically wounded cells were assayed in phenol red-free, serum-free medium containing 10 μg/ml mitomycin C (to block proliferation) and the following steroids: DHEA-S (10 μM), DHEA (10 μM), testosterone (50 nM), 17β-estradiol (1 nM), or vehicle control (0.0001% ethanol). Inhibitors of aromatase (Arimidex, 100 nM) and STS (STX64, 100 nM) were included alone or in combination with relevant steroids. Migration was measured at 4, 8, 12, 24, and 48 h in triplicate dishes for each donor. Conditioned medium, collected from dermal fibroblast cultures 24 h after scratching as previously described (37), was also assessed for its effect on the migration of epidermal keratinocytes.

The effect of 17β-estradiol and DHEA on α-SMA expression in human dermal fibroblasts following mechanical wounding *in vitro*

Dermal fibroblasts were grown to confluence in 4-well chamber slides before mechanically wounding by scratching along the length of the chamber well at the midway point using a P200 Gilson pipette tip. After washing with PBS, fibroblasts were incubated in phenol red-free, serum-free DMEM in the presence of vehicle control (0.0001% absolute ethanol), 10 nM 17β-estradiol or 100 nM DHEA; control unwounded intact monolayers were also included. Slides were fixed at 0, 24, and 48 h with cold (−20°C) methanol for 10 min. Cells were rehydrated with PBS for 5 min before blocking endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 30 min. Cells were washed (2 × 5 min) in deionized water followed by PBS Tween 20 (0.05% v/v). Further blocking was performed with 3% bovine serum albumin in PBS (1 h), before incubation with 2% horse serum (Vector Elite ABC kit, Peterborough, United Kingdom) for 1 h. After 3 washes with PBS, cells were incubated with the primary antibody (Smooth Muscle Actin-Clone 1A4; mouse monoclonal- anti-human antibody; DAKO, Glostrup, Denmark) diluted 1:100 in PBS for 18 h at 4°C in a humidified chamber. Negative controls were included where either the primary antibody or secondary antibody was omitted. Cells were washed (3 × 10 min in PBS Tween 20) before incubating with a biotinylated anti-mouse IgG solution (Vector Elite ABC kit, Peterborough, United Kingdom) for 30 min. After 3 × 15 min washes with PBS Tween 20, the chamber slide walls were removed before incubation with horseradish peroxidase avidin-biotin complex (Vector Elite ABC Kit) for 30 min. After 3 × 10 min washes with PBS Tween 20, cells were incubated with 0.05% diaminobenzidine (Sigma-Aldrich, Gillingham, United Kingdom) in 0.05 M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide for up to 10 min. After washing in running tap water for 5 min, slides were submerged in 16 mM $CuSO_4$/123 mM NaCl solution for 5 min and counterstained with hematoxylin for 5 s. After washing, cells were dehydrated through graded alcohol, cleared in histoclear, and mounted with histomount (Agar Scientific, Stansted, Essex, United Kingdom). The percentage of α-SMA-positive cells was quantified by calculating the number of positively stained cells in a population of 100 in 3 different fields by microscopy for each donor ($n = 3$).

Preparation of fibroblast-populated collagen lattices

Type I collagen (4 mg/ml) solution (Upstate Biotechnology, Lake Placid, NY, USA) was neutralized by the addition of 1 N sodium hydroxide. An equal volume of medium containing

TABLE 3. *Top functions of human epidermal keratinocytes following 24 h incubation with steroids, obtained using IPA analysis*

	DHEA-S		Estradiol		Testosterone	
	P value	Number of genes changed	*P* value	Number of genes changed	*P* value	Number of genes changed
Molecular and cellular functions						
Cell-to-cell signaling and interaction	0.000404	14	0.000286	414	0.000158	452
Cellular development	0.0000106	652	0.000343	504	0.000159	552
Cellular movement	0.00000773	476	0.000302	420	0.000158	415
Physiologic system development and function						
Immune cell trafficking	0.00000920	309	0.000297	272	0.000158	271
Tissue morphology	0.00000522	523	0.000297	480	0.000147	573
Diseases and disorder						
Connective tissue disorders	0.00000741	288	0.000272	246	0.0000848	253
Inflammatory disease	0.00000742	379	0.000181	329	0.000158	346

dermal fibroblasts (2×10^5 cells/ml) was added and the solution gently vortexed before 600 μl was added to each well of a 12-well tissue culture plate and incubated at 37°C for 30 min. After the gels had polymerized, 400 μl of medium was added and the plates incubated to allow the fibroblasts to spread throughout the gels. After 48 h, the medium was changed to phenol red-free, serum-free DMEM containing either vehicle control (0.0001% ethanol), DHEA (100 nM), 17β-estradiol (10 nM), Arimidex (100 nM) or a combination of DHEA (100 nM) and Arimidex (100 nM) in triplicate dishes and incubated for 24 h before releasing from the side of the wells by carefully scoring around the circumference using a sterile hypodermic syringe (23.5 G). The gel base was released from the floor of the well by gentle rocking. The medium was changed every 3 d, and the free-floating gels were photographed every other day up to 12 d. The gel area and circumference were analyzed using digital image analysis software (ImageproDiscovery, Media Cybernetics, Silver Spring, MD, USA). Data were quantified from triplicate dishes from 5 different donors.

Aromatase activity in response to mechanical wounding of cell monolayers

Aromatase activity was measured in fibroblasts and keratinocytes using the tritiated water assay as previously described (11, 38). Briefly, cells were seeded into 6-well plates at a density of 1×10^5 per well and grown to confluence, then mechanically wounded as previously described (28, 30, 37). After washing with PBS, they were incubated with serum-free medium containing 0.5 μCi [1β^3H]-androstenedione (NEN Perkin Elmer, Waltham, MA, USA) for 2 or 24 h at 37°C. Cell-conditioned medium was extracted by sequential treatment with chloroform and activated charcoal to remove unmetabolized steroids, and metabolized radiolabeled products in the aqueous phase were measured by scintillation counting. The cell layer was dissolved in RIPA buffer (Sigma-Aldrich, Gillingham, United Kingdom) and the protein content measured using the DC Protein assay (Bio-Rad). Aromatase activity was calculated as mean nmol/mg cell protein in triplicate dishes for each donor.

Statistical analysis

Values are presented as means ± SEM. Statistical differences between 2 groups were determined by unpaired *t* test. Comparisons among several groups were performed by ANOVA followed by Bonferroni adjustment. SPSS Statistical Software was used (SPSSx, 2007).

RESULTS

Human dermal fibroblasts and epidermal keratinocytes retain the expression of key steroidogenic genes required for the conversion of cholesterol to potent androgens and estrogens *in vitro*

Human tissues derived from glands or cell lines known to express the genes of interest were used as positive controls. The expression of P450scc (converts cholesterol to pregnenolone) and P450c17 (converts pregnenolone to DHEA) was identified in adrenal gland, OATP2B1 in liver, P450aro (aromatase) in ovary, STS in placenta, 5α-reductase type 1 in PC3, and 5α-reductase type 2 in DU145 cell lines, respectively. Negative controls where the template cDNA was excluded were also included to confirm that there was no DNA contamination. RT-PCR analysis of total RNA extracted from female facial and abdominal whole skin biopsies ($n = 4$) demonstrated the presence of mRNA for AR, ERα, ERβ (data not shown), and P450scc, P450c17, STS, and 5α-reductase type 1 but not 5α-reductase type 2 (**Fig. 1*A***). Primary cultures of dermal fibroblasts ($n = 8$) and epidermal keratinocytes ($n = 8$) derived from female facial skin (all at passage 3), expressed mRNA for AR, ERα, ERβ (data not shown), and P450scc, P450c17, STS, and 5α-reductase type 1. Neither expressed 5α-reductase type 2. All keratinocytes (Fig. 1*B*) but none of the fibroblasts (Fig. 1*C*) expressed OATP2B1, and P450aro was only detected in dermal fibroblasts.

Dexamethasone stimulates aromatase expression in human dermal fibroblasts and epidermal keratinocytes

Although expression of aromatase mRNA was not confirmed in keratinocytes using semiquantitative PCR (Fig. 1*B*), because we have previously demonstrated that the expression of aromatase activity could not be detected in dermal papilla cell cultures unless they were stimulated with dexamethasone (11), we cultured human keratinocytes and fibroblasts in the presence of 250 nM dexamethasone for 24 h and quantitated changes in mRNA

TABLE 4. *Top Log2 ratio (> ±1) up- and down-regulated by 10 μM DHEA-S, 1 nM estradiol, and 50 nM testosterone in EKs derived from female facial skin (n=2)*

Gene	Description	Function	Up or down		
			DHEA-S	Estradiol	Testosterone
TUBA3C/TUBA3D	Tubulin	Cell development	n.s.	n.s.	Down
LMNB1	Lamin B1	Cell development, cell signaling	Up	Up	Up
PPFIA4	Protein tyrosine phosphatase, receptor type, f	Cell interaction	n.s.	Up	Up
SEMA6D	Sema domain, transmembrane domain	Cell interaction	Down	n.s.	n.s.
LOX	Lysyl oxidase	Cell migration	Up	n.s.	n.s.
HSPB7	Heat shock 27 kDa protein family, member 7	Cell signaling	Down	n.s.	n.s.
ABLIM2	Actin-binding LIM protein family, member 2	Cell signaling	n.s.	Down	Down
ARHGAP28	Rho GTPase activating protein 28	Cell signaling	Down	n.s.	n.s.
CABP1	Calcium-binding protein 1	Cell signaling	n.s.	Down	n.s.
DOK2	Docking protein 2	Cell signaling	Down	n.s.	n.s.
MTBP	Transformed 3T3 cell double minute 2	Cell signaling	n.s.	Up	n.s.
SDPR	Serum deprivation response	Cell signaling	n.s.	Up	n.s.
SLN	Sarcolipin	Cell signaling	n.s.	n.s.	Down
STXBP4	Syntaxin binding protein 4	Cell signaling	Up	n.s.	n.s.
TBC1D24	TBC1 domain family 24	Cell signaling	n.s.	Up	n.s.
EGLN3	egl nine homolog 3	Cell signaling	n.s.	n.s.	Up
MNS1	Meiosis-specific nuclear structural 1	Cell signaling, cell development	n.s.	Up	n.s.
OLAH	Oleoyl-ACP hydrolase	Cell signaling, fatty acid biosynthesis	n.s.	n.s.	Up
PHKG1	Phosphorylase kinase, γ 1	connective disorders	n.s.	n.s.	Down
TNNT3	Troponin T type 3	Connective disorders	Down	n.s.	n.s.
CA8	Carbonic anhydrase VIII	Immune cell trafficking	n.s.	n.s.	Up
CFHR3	Complement factor H related 3	Immune cell trafficking	n.s.	n.s.	Down
HLA-G	HLA-G histocompatibility antigen, class I	Immune cell trafficking	n.s.	Down	n.s.
LY9	Lymphocyte antigen 9	Immune cell trafficking	n.s.	n.s.	Down
PIGA	Phosphatidylinositol glycan anchor biosynthesis	Immune cell trafficking	n.s.	Up	Up
SERPINB9	Serpin peptidase inhibitor	Immune cell trafficking	n.s.	Down	n.s.
TMPO	Thymopoietin	Immune cell trafficking	n.s.	n.s.	Up
PTGER2	Prostaglandin E receptor 2	Immune cell trafficking, inflammation	n.s.	Up	Up
RFX4	Regulatory factor X, 4	Immune cell trafficking, inflammation	n.s.	Down	n.s.
CNTFR	Ciliary neurotrophic factor receptor	Inflammation	n.s.	n.s.	Down
CXCL1	Chemokine (C-X-C motif) ligand 1	Inflammation	Up	Up	Up
TXNIP	Thioredoxin interacting protein	Inflammation (acts as a suppressor of tumor cell growth)	Up	Up	Up
ANGPTL4	Angiopoietin-like 4	Migration (secreted protein with a fibrinogen domain)	Up	Up	Up
AMOTL1	Angiomotin-like protein 1	Migration and permeability	n.s.	Up	n.s.

n.s., no significant change.

expression by qRT-PCR (**Fig. 2**). Stimulation of fibroblasts with dexamethasone increased the relative expression of aromatase transcripts (Fig. 2*B*); likewise, dexamethasone stimulated aromatase expression in cultured keratinocytes, confirming not only its expression but also that levels of expression can be induced (Fig. 2*A*).

Cultured human dermal fibroblasts and epidermal keratinocytes express GPER1 in addition to ERα and ERβ

Both cells types expressed all 3 estrogen receptors in culture. The relative level of expression of ERα, ERβ,

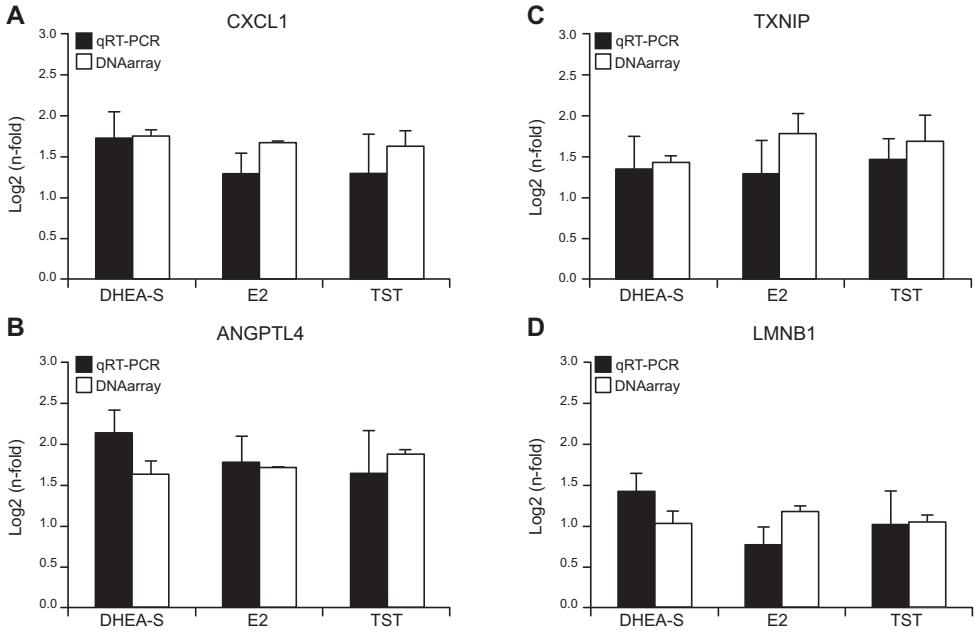


Figure 3. DHEA-S, testosterone, and 17β-estradiol increase gene expression of *ANGPTL4*, *CXCL1*, *LMNB1*, and *TXNIP* in human epidermal keratinocytes. Epidermal keratinocytes (n = 2) derived from female facial skin were incubated with 10 μM DHEA-S, 1 nM 17β-estradiol (E2), 50 nM testosterone (TST), or vehicle control (0.0001% absolute ethanol) for 24 h. Total RNA was isolated and gene expression changes analyzed using microarray technology and qRT-PCR to validate data for (*A*) *CXCL1*, (*B*) *ANGPTL4*, (*C*) *TXNIP*, and (d) *LMNB1*. Data are presented as the mean ± SEM.

and GPER1 was similar in keratinocytes (Fig. 2*C*); however, the expression of ERβ in cultured dermal fibroblasts was ;significantly less than that of ERα and GPER1 (Fig. 2*D*).

DHEA-S, 17β-estradiol, and testosterone differentially modulate gene expression programs in human epidermal keratinocytes

To define changes in gene expression programs triggered by treatment with different steroids, a gene array procedure was carried out on the whole transcriptome of human epidermal keratinocytes. There were 14,616 transcripts significantly and differentially expressed, identified from the ANOVA statistical analysis ($P < 0.05$). After the application of expression value cutoff of ±0.6 Log2 ratio, the differentially expressed (up- and down-regulated) transcripts in keratinocytes treated with DHEA-S, 17β-estradiol, and testosterone were 3246, 3122, and 3099, respectively, by using general setting database of IPA software. Up-regulated genes were 653 (20.1%), 968 (31.0%), and 958 (30.9%), respectively. The differentially expressed genes were annotated in functionality categories using gene ontology terms. Biofunctions related to steroid treatments with a P value < 0.001 are shown in **Table 3**. The top biofunctions where the highest numbers of genes were significantly modulated included cellular development, cell-to-cell signaling, cellular movement, and tissue morphology, and top functions related to disease included inflammatory disease and connective tissue disorders.

The top Log2 ratio ($>\pm1$) up- or down-regulated genes are given in **Table 4**. The 4 genes that were significantly up-regulated by all 3 steroids were *ANGPTL4*, *CXCL1*, *LMNB1*, and *TXNIP*. Their up-regulation was confirmed using qRT-PCR (**Fig. 3**). There was no significant difference in the fold change between treatment of cells with the 3 steroids when measured by either qRT-PCR or DNA array (Fig. 3).

Cell viability following incubation with steroids, enzyme inhibitors, and mitomycin C is reduced at high concentrations

A range of concentrations of DHEA-S, DHEA, 17β-estradiol, testosterone, the aromatase inhibitor Arimidex, the STS inhibitor STX64, and mitomycin C, an inhibitor of cell proliferation, was assessed for their effect on the viability of both epidermal keratinocytes and dermal fibroblasts. With increasing concentrations, there was some reduction in cell viability, but even at the highest concentrations used, viability was not reduced to less than 74% of the control (**Table 5**). This confirmed that the concentrations of steroids, enzyme inhibitors, and mitomycin C used for the migration assay did not reduce cell viability.

DHEA-S, DHEA, and 17β-estradiol but not testosterone stimulates the migration of wounded human epidermal keratinocytes and dermal fibroblasts *in vitro*

A scratch assay as previously described (28, 30) measured epidermal keratinocyte (n = 3) and dermal fibroblast (n = 5) migration in response to 10 μM DHEA-S, 10 μM DHEA, 1 nM 17β-estradiol or 50 nM testosterone at 5 fixed time points (4, 8, 12, 24, and 48 h). Previous dose-response assays using this technique have shown that a range of concentrations of 17β-estradiol (10^{-7}–10^{-9} M) and DHEA (10^{-5}–10^{-8} M) all stimulated cell migration to a similar level (37, 39). The ability of the cells to metabolize these steroids was determined by including 100 nM Arimidex (to block aromatase activity) in the

TABLE 5. *Cell viability of epidermal keratinocytes (EK) and dermal fibroblasts (DF) treated for 24 h with increasing concentrations of 17β-estradiol (E2), testosterone (TST), DHEA and DHEA-S, Arimidex (A), STS inhibitor (STX64), and mitomycin C (MMC)*

	DF		EK	
	%	SEM	%	SEM
E2 (nM)				
1	99.83*	± 0.65	99.92*	± 1.04
10	76.86*	± 2.33	83.64*	± 7.27
50	76.22*	± 2.69	83.28*	± 1.78
100	74.22*	± 4.07	78.31*	± 1.69
TST (nM)				
1	99.04*	± 1.27	92.32*	± 1.64
10	92.98*	± 2.54	92.94*	± 4.59
50	94.43*	± 1.33	93.20*	± 4.15
100	76.39*	± 2.72	75.25*	± 3.70
A (nM)				
1	90.83	± 0.65	91.20	± 2.97
10	89.93	± 0.37	91.88	± 7.47
50	90.00	± 0.98	88.33	± 2.57
100	90.65	± 1.21	90.48	± 2.22
STX64				
1	89.46	± 2.27	90.32	± 3.97
10	87.53	± 1.35	89.06	± 2.05
50	89.72	± 0.94	90.34	± 3.82
100	89.51	± 1.04	90.10	± 3.08
DHEAS (μM)				
1	92.51*	± 1.40	95.54*	± 2.46
10	94.36	± 2.13	94.66*	± 0.68
100	85.41*	± 0.67	85.31*	± 1.59
DHEA (μM)				
1	100.90*	± 0.79	95.64*	± 1.42
10	99.54*	± 1.26	95.50*	± 2.04
100	82.33*	± 0.21	78.62*	± 2.31
MMC (μM)				
1	92.91	± 1.53	90.95*	± 3.22
10	90.69	± 1.65	90.48*	± 2.65
100	82.33*	± 0.21	80.68*	± 4.04

Data are presented as % mean ± SEM (n = 6). *Significantly ($P < 0.001$) different means between doses within each treatment.

presence and absence of DHEA or testosterone, and 100 nM STX64 (to block STS activity) in the presence and absence of DHEA-S. Migration was quantitated as previously described (28, 30). The addition of 10 μg/ml of mitomycin C to block proliferation did not alter the migration of keratinocytes (n = 3) or fibroblasts (n = 5; data not shown). A scratch assay showing the increase in keratinocyte and fibroblast migration in response to DHEA after 24 h and the inhibition of the stimulatory effect in the presence of the aromatase inhibitor is shown in **Fig. 4**.

DHEA and 17β-estradiol had significantly accelerated keratinocyte migration by 4 h, which continued at a similar rate over the 48 h period (**Fig. 5*A–E***).The aromatase inhibitor alone had no effect but consistently abrogated the effect of DHEA and reduced the migratory response to the same levels as that of the control cells. By 12 h, DHEA-S also significantly stimulated keratinocyte migration, which was blocked by the STS inhibitor STX64. There was a partial increase in keratinocyte migration in response to testosterone after 24 and 48 h, which was also completely reversed by the aromatase inhibitor. Although keratinocyte migration was more than twice that of the vehicle control after 24 and 48 h in response to DHEA-S (50.3 and 57.6%, respectively), DHEA (55.2 and 60.5%, respectively), and 17β-estradiol (54.4 and 56.8%, respectively), migration in response to testosterone (26.4 and 26.0%, respectively) was less than half as effective as the other steroids.

After 4 h, 17β-estradiol, DHEA-S, and DHEA had all significantly accelerated fibroblast migration, which continued at a similar rate at all the later time points of 8, 12, 24, and 48 h (Fig. 5*F–J*). After 12 h, although testosterone increased fibroblast migration, it was not as effective as the other steroids (increase in migration by testosterone, 19%; DHEA-S, 39.5%; DHEA, 47.0%; and 17β-estradiol, 38.8%). Coincubation with steroidogenic enzyme inhibitors consistently reduced the stimulation of migration by testosterone, DHEA, and DHEA-S to the same level as seen in the control cells.

A scratch assay was also used to measure epidermal keratinocyte (n = 3) migration in response to conditioned medium collected from dermal fibroblast monolayers (n = 5) 24 h after they had been mechanically wounded. Wounded dermal fibroblasts secreted soluble factors into the culture medium, which stimulated the migration of epidermal keratinocytes by 49% within 4 h (**Fig. 6**).

The expression of α-SMA by human dermal fibroblasts is increased by mechanical wounding, but neither 17β-estradiol or DHEA stimulates any further increase

Immunocytochemical staining for the expression of α-SMA by human dermal fibroblasts (n = 3) demonstrated intact monolayers contained a small number (5 ± 1%) of α-SMA-expressing cells (**Fig. 7*A***). These cells were dispersed throughout the cell monolayer and had a more spread out morphology than the surrounding negatively staining cells. Following mechanical wounding, the number of cells expressing α-SMA significantly increased, representing 18 ± 3% of the total cell population after 24 h and 26 ± 3% after 48 h (Fig. 7*B*). However, no significant difference was demonstrated when fibroblasts were mechanically wounded in the presence of 17β-estradiol (20 ± 2% after 24 h and 25 ± 2% after 48 h) or 100 nM DHEA (20 ± 2% after 24 h and 24 ± 3% after 48 h; Fig. 5*C* and *D*). In all mechanically wounded fibroblast populations, cells expressing α-SMA were found dispersed throughout the cell monolayer, and no particular increase in staining was demonstrated at the edge of the mechanically created wound.

DHEA increases the contractile ability of a human dermal fibroblast-populated lattice via conversion to estrogen

Dermal fibroblasts derived from abdominal skin (n = 5) showed a significant increase in contraction in a fibroblast-populated collagen lattice in response to incubation with

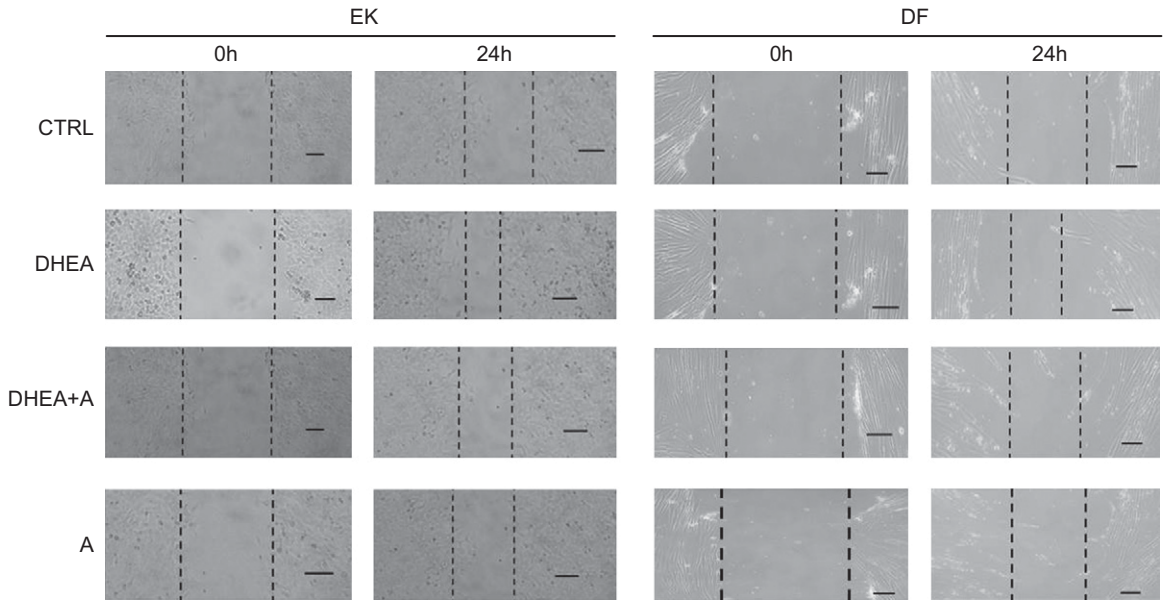


Figure 4. DHEA stimulates migration of epidermal keratinocytes and dermal fibroblasts in a scratch wound assay. A scratch wound assay was used to measure the migration of cultured human epidermal keratinocytes (EK) and human dermal fibroblasts (DF) over time. The distance between the wound edges was measured at fixed points 3 mm apart in each dish according to a standardized template. Immediately following mechanical wounding, the wound edges are straight. After 24 h, the cells can be seen migrating into the wound with a decreased distance between the wound edges. CTRL (vehicle control); DHEA (10 μM); DHEA + A (10 μM DHEA plus 100 nM Arimidex); A (100 nM Arimidex alone). DHEA accelerates migration of both EK and DF, which is blocked by Arimidex; Arimidex alone had no effect on cell migration.

DHEA (100 nM) or 17β-estradiol (10 nM) over a 12 d period (**Fig. 8**). Incubation with Arimidex alone did not alter fibroblast contraction, but significantly inhibited DHEA-stimulated contraction.

Opposing effects of mechanical wounding on aromatase activity in cultured human dermal fibroblasts and epidermal keratinocytes

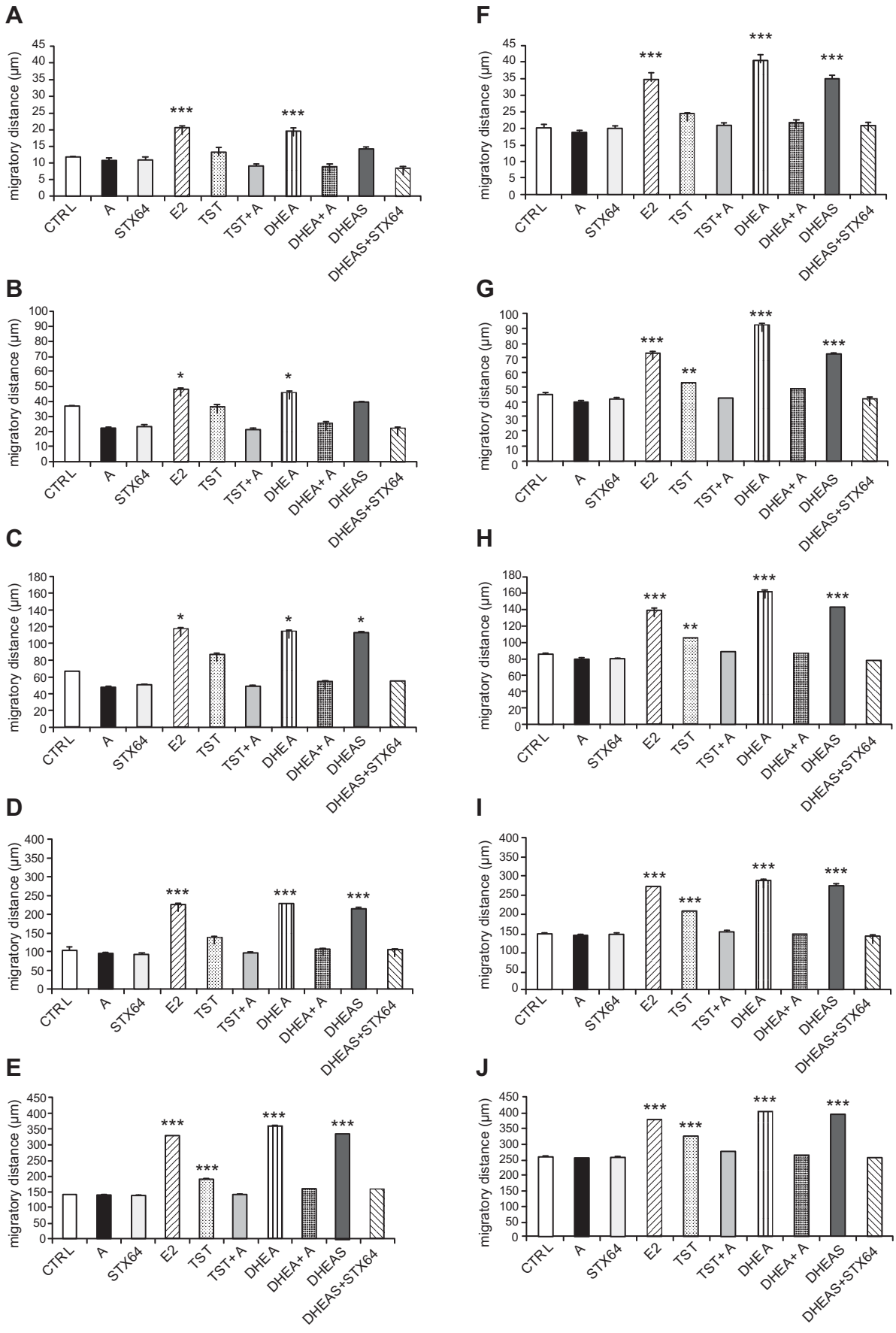
To clarify further the role of aromatase in wound healing, we measured changes in aromatase activity in response to mechanical wounding using the tritiated water assay to quantify the enzymatic activity of aromatase (11, 38), by comparing parallel dishes of intact and mechanically wounded keratinocytes and fibroblasts. Mechanically wounding keratinocytes increased activity by as much as 400%, and mechanically wounding fibroblasts consistently resulted in a decrease in activity (reduced by 38–67%; **Table 6**). Three additional fibroblasts cultures derived from male facial skin and female abdominal and breast skin also showed a reduction in aromatase activity by 53, 38, and 74%, respectively.

DISCUSSION

Although the ovaries and testes are the exclusive sources of androgens and estrogens in lower mammals, in man and higher primates, active sex steroids are synthesized locally in peripheral tissues from circulating precursors, thus providing target tissues with controls that adjust the metabolism of sex steroids to local requirements. This study is not only consistent with previous work describing human skin as both a hormone target and an endocrine gland (20–22, 40), but establishes that early passage (P3) primary cultures of human dermal fibroblasts and epidermal keratinocytes retain in culture the key steroidogenic proteins seen in whole skin biopsies that are required for the conversion of cholesterol to potent androgens and estrogens (Fig. 1). Although expression of aromatase in keratinocytes was not confirmed using semiquantitative PCR (Fig. 1), in additional studies with dexamethasone stimulation, as previously described for dermal papilla cells (11), and real-time PCR, expression of aromatase mRNA was demonstrated in keratinocytes (Fig. 2*A*).

In addition to the intracellular estrogen receptors ERα and ERβ, the G protein-coupled receptor 30 (GPER1) has been introduced as an estrogen receptor responsible for nongenomic estrogen signaling (23–25). To date, there are no studies on the distribution of GPER1 in human skin, but this study has demonstrated the presence of mRNA for all 3 receptors in cultured keratinocytes and fibroblasts (Fig. 2*C–D*). The relative level of ERβ expression in fibroblasts was lower than that of GPER1and ERα, and in keratinocytes the relative expression of the 3 estrogen receptors was similar.

Human organic anion and cation transporters are classified within 2 solute carrier (SLC) transporter superfamilies, *SLCO* and *SLCO22A*. OATPs are encoded by genes in the *SLCO* superfamily and mediate the transmembrane uptake of a wide range of organic compounds (41). Eleven different OATPs have been identified in human tissues, and although many have broad substrate specificity, some are highly conserved with a narrow spectrum of transport substrates (42). Notwithstanding,



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information on the expression of OATP transporters in human skin is limited. DHEA-S and estrone-sulfate are the predominant substrates for OATP2B1 (42); therefore, expression of STS and OATP2B1 in human keratinocytes supports the correlation between the transport of DHEA-S and its intracrine conversion into DHEA. However, DHEA-S has also been identified as a substrate for other members of this superfamily including OATP1A2, OATP1B1, and OATP1B3 (43), as well as OAT4, which is encoded by the *SLCO22A* gene superfamily and specific to humans (41). Although fibroblasts also expressed STS, their lack of OATP2B1 suggests that they may rely on one of these other transporters, although paracrine steroid exchanges with keratinocytes are also a possibility (**Fig. 9**).

Gene array expression revealed significant changes in a number of genes in keratinocytes that were differentially modulated by DHEA-S, estradiol, or testosterone (Tables 3 and 4). For example, only estradiol specifically up-regulated 5 genes and down-regulated 3 genes that were not modulated by DHEA-S or testosterone (Table 4), and DHEA-S specifically up-regulated 2 genes and down-regulated 5 genes that were not modulated by either estradiol or testosterone. Likewise, testosterone was able to up-regulate an additional 4 genes and down-regulate an additional 5 genes that were not influenced by either DHEA-S or estradiol (Table 4).This suggests all 3 steroids may have individual specific roles in the modulation of the human keratinocyte transcriptome, and further studies into the role of these genes in human keratinocyte biology are required. Although a significant number of genes were modulated by the 3 different steroids, surprisingly only 4 genes (*ANGPTL4*, *CXCL1*, *TXNIP*, and *LMNB1*) were significantly up-regulated by all 3 steroids. This was also confirmed with qRT-PCR (Fig. 3). The ANGPTL4 protein and CXCL1 receptor have previously been shown to have an important role in re-epithelialization during wound healing (44, 45), and TXNIP is up-regulated during peripheral nerve injury (46), while decreased protein levels of LMNB1 are associated with skin aging (47). Therefore, these may all be important target genes for the protective action of estrogen against keratinocyte apoptosis and epidermal atrophy with aging, and the stimulation of keratinocyte migration following injury.

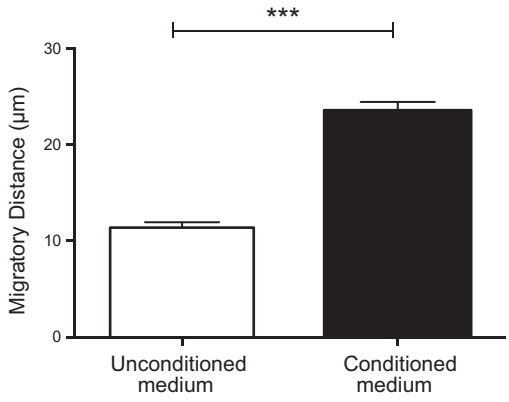

Figure 6. Mechanical wounding of cultured dermal fibroblasts stimulates the secretion of soluble factors that stimulate the migration of epidermal keratinocytes. A scratch wound assay was used to measure the migration of cultured epidermal keratinocytes ($n = 3$) after 4 h in response to conditioned medium from mechanically wounded monolayers of dermal fibroblasts (pooled from 5 donors). Six points per dish were assessed and each point was performed in triplicate dishes. White bar, unconditioned medium; black bar, dermal fibroblast-conditioned medium. Data presented as donor mean ± SEM. $***P < 0.001$.

Previous studies have demonstrated that estrogen improves wound healing in elderly patients of both sexes (26); in contrast, testosterone has a detrimental effect (27), and the role of DHEA has not been established. In this study, DHEA-S, DHEA, and 17β-estradiol all significantly increased keratinocyte and fibroblast migration in a scratch wound assay, and testosterone only had a partial effect at later time points (Fig. 5). Although the best-described mechanism of estrogen signaling is mediated via the genomic intracellular nuclear receptor proteins ERα and ERβ, it is now apparent that many cells also express membrane estrogen receptors coupled to cytosolic signal transduction proteins that direct nongenomic signaling cascades via conventional second messengers producing rapid responses to estrogen (23–25). This is the first study to demonstrate that in

Figure 5. Migration of human dermal fibroblasts and epidermal keratinocytes following mechanical wounding *in vitro* is accelerated by 17β-estradiol, and DHEA-S and DHEA following aromatization, but not testosterone. A scratch wound assay was used to measure the migration of cultured human dermal fibroblasts (DFs; $n = 5$) and epidermal keratinocytes (EKs; $n = 3$) derived from female facial skin, in response to vehicle control (CTRL), 100 nM aromatase inhibitor (A), 100 nM STS inhibitor (STX64), 1 nM 17β-estradiol (E2), 50 nM testosterone (TST), 10 μM DHEA, or 10 μM DHEA-S. Cells were also incubated with TST and DHEA in the presence of A, and DHEA-S in the presence of STX64. Six points per dish were assessed and each point was performed in triplicate dishes for each cell line. Migration was measured at 4, 8, 12, 24, and 48 h. Data are presented as donor mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. *A*) EK migration 4 h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A); (*B*) EK migration 8 h after wounding (*E2 vs. control; DHEA vs. control and DHEA + A); (*C*) EK migration 12 h after wounding (*E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64); (*D*) EK migration 24 h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64; TST vs. control and TST + A); (*E*) EK migration 48 h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64; TST vs. control and TST + A); (*F*) DF migration 4h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64); (*G*) DF migration 8 h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64; **TST vs. TST + A only); (*H*) DF migration 12 h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64; **TST vs. control and TST + A); (*I*) DF migration 24 h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64; TST vs. control and TST + A); (*J*) DF migration 48 h after wounding (***E2 vs. control; DHEA vs. control and DHEA + A; DHEA-S vs. control and DHEA-S + STX64; TST vs. control and TST + A).

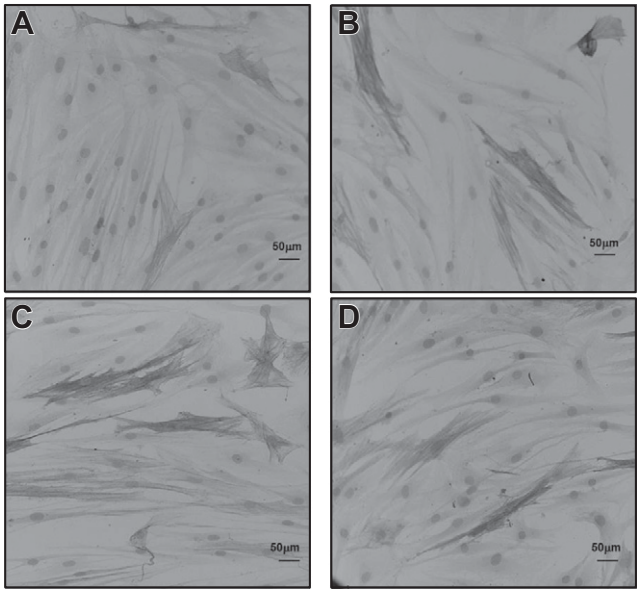

Figure 7. Mechanical wounding increases the expression of α-SMA by cultured human dermal fibroblasts. Confluent cell monolayers of dermal fibroblasts ($n = 3$) were stained for α-SMA using a monoclonal primary antibody. *A*) Positive-staining cells were detected dispersed within the intact cell monolayer. *B*) Mechanical wounding led to an increase in the number of positively staining cells dispersed throughout the monolayer after 24 h. The presence of 10 nM 17β-estradiol (*C*) or 100 nM DHEA (*D*) did not alter the expression of α-SMA in mechanically wounded cells. Expression was dispersed throughout the monolayer and not concentrated near the wound margins.

addition to ERα and ERβ, human dermal fibroblasts and epidermal keratinocytes also express GPER1 (Fig. 2*C–D*). The rapid migratory response after only 4 h is probably mediated via GPER1, and additional signaling via ERα and ERβ may be involved in sustaining the response. Although it has been reported that DHEA prevents keratinocyte apoptosis via membrane binding sites (16), coincubation with steroidogenic enzyme inhibitors reversed the effect of DHEA (aromatase inhibitor) and DHEA-S (STS inhibitor), confirming that conversion to estrogen is required to stimulate cell migration in keratinocytes and fibroblasts (Figs. 4 and 5).

In humans, serum concentrations of DHEA-S and DHEA are significantly higher than other steroids and they are generally considered to be precursors to more potent androgens and estrogens. Labrie *et al.* (8) postulated that more than 30% of total androgen in men and over 90% of estrogen in postmenopausal women comes from their synthesis in peripheral tissues. Although the principle source of DHEA-S and DHEA is considered to be the adrenal cortices, the present study confirms human skin also has a potential role in the synthesis of DHEA-S and DHEA (Figs. 1 and 2). Because the skin is highly vascularized, how much is released into the circulation and how much is used principally in the metabolic activity of the skin is unknown, but it is clearly an important function of human skin.

Although a specific DHEA receptor has not been identified, Webb *et al.* (48) demonstrated that DHEA was similar to 17β-estradiol in activating ERβ, less effective at modulating ERα, but could activate G-protein membrane receptors that modulate a variety of signaling cascades. However, in the present study the effect of DHEA was reversed in the presence of an aromatase inhibitor (Figs. 4, 5, and 7), suggesting aromatization is required. The effect of DHEA-S was also impaired in the presence of a STS inhibitor, indicating conversion of DHEA-S to the unconjugated form, providing further evidence for a role for DHEA as an estrogen precursor in the wound-induced migration of human keratinocytes and fibroblasts. Because DHEA declines significantly with age, this further amplifies estrogen deficiency in postmenopausal women and may be an additional contributing factor to the chronic wounds seen in the elderly population.

A novel observation of this study was that testosterone (typically an intermediate steroid in the conversion of DHEA to 17β-estradiol in endocrine tissues) only had a partial effect on migration at the later time points (Fig. 5). Testosterone is either aromatized to estrogen or reduced to 5α-DHT by the relevant enzymes. Because both cell types express 5α-reductase type 1 (Fig. 1), this suggests 5α-DHT is the predominant metabolite rather than estradiol, especially at the early time points. This supports the concept that in human peripheral tissues, testosterone is not an intermediate in estrogen production and DHEA is metabolized to 17β-estradiol via an alternative pathway. This would corroborate with other studies that propose DHEA is metabolized to 17β-estradiol via androstenedione and estrone in peripheral tissues, rather than testosterone (49, 50) (see Fig. 9). Because 5α-DHT can retard keratinocyte migration (51) and decrease fibroblast viability (52), avoidance of this pathway would limit the production of 5α-DHT. However, further reduction of 5α-DHT by 3β-HSD to 5α-androstane-3β, 17β-diol may also be a possibility, because this metabolite is also a ligand for ERβ; likewise, 5-Diol, the intermediate in the metabolism of testosterone from DHEA (see Fig. 9), is also a ligand for ERβ (19, 53, 54) and binding to ERβ may be another mechanism to reduce the intracellular availability of testosterone. Therefore, in addition to aromatase, specific human

TABLE 6. *Aromatase activity increases in epidermal keratinocytes (EK) and decreases in dermal fibroblasts (DF) 24 h after mechanical wounding*

Cell type	Site	Sex	% change in aromatase activity
EK	Face	Female	414
EK	Face	Female	299
EK	Face	Female	93
DF	Face	Female	67
DF	Face	Female	53
DF	Face	Female	50
DF	Face	Female	65
DF	Face	Female	55
DF	Face	Male	53
DF	Abdomen	Female	38
DF	Breast	Female	74

Each figure is derived as % aromatase activity 24 h after wounding (10^2 nmol/mg protein) *vs.* equivalent nonwounded cells.

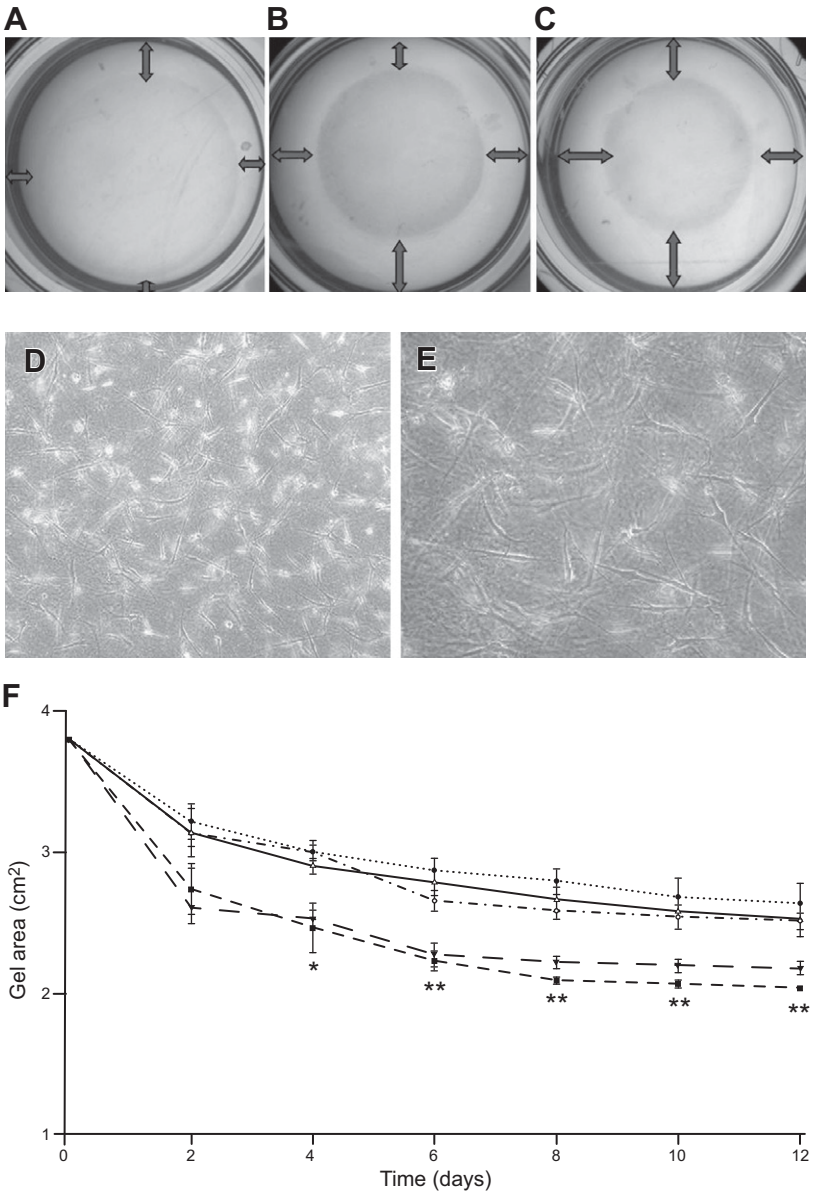

Figure 8. Conversion of DHEA to estrogen is required for the increased contractile properties of human dermal fibroblasts in a collagen lattice. Fibroblast-populated collagen lattices were used to assess the contractile ability of cultured human female abdominal dermal fibroblasts. The area of individual gels was calculated from 15 separate points and fibroblasts from each donor ($n = 5$), performed in triplicate dishes. The arrows indicate the distance of the gel edge from the outer wall of the culture dish at 0, 90, 270, and 360 degrees on day 2 (*A*), day 6 (*B*), and day 12 (*C*). Image of fibroblast-populated collagen lattice gel showing fibroblasts at ×200 (*D*) and × 400 (*E*) magnification, with fibroblasts distributed in different layers within the gel. (*F*) The amount of contraction of fibroblast-populated collagen lattices in response to incubation with; dotted line (vehicle control 0.0001% ethanol), small dashed line (100 nM DHEA), large dashed line (10 nM 17β-estradiol), solid line (100 nM Arimidex), and dotted and dashed line (DHEA in combination with Arimidex) over a 12 d period. The original area of the gels at day 0 was 3.8 cm^2. Data presented as donor mean ($n = 5$) ± SEM. *$P < 0.05$ and **$P < 0.01$ for DHEA and estradiol vs. control.

3β- and 17β-hydroxysteroid dehydrogenases (HSDs) may also have an important role in the control of estrogen synthesis (55) in human skin during the wound-healing process.

The complex autocrine and paracrine relationships between dermal fibroblasts and epidermal keratinocytes are well established (56). During re-epithelialization, keratinocyte migration is predominately mediated by soluble diffusible factors produced by the underlying fibroblasts. We have shown that conditioned medium taken from monolayers of dermal fibroblasts scratched in culture to produce a wounding response stimulate migration of keratinocytes by 49% after only 4 h (Fig. 6). Because we have previously demonstrated that mechanically wounding human dermal fibroblast cultures stimulates the release of activated TGF-β by 37% (37) and TGF-β induces an epithelial-mesenchymal transition in keratinocytes (57), this may be a mechanism by which keratinocyte migration is enhanced.

A key component of dermal wound healing to reduce the size of the wound is contraction by the myofibroblastic phenotype of wound-healing fibroblasts (58). Although mechanically wounding the fibroblast monolayers increases α-SMA expression, a myofibroblast marker (Fig. 7), incubation with 17β-estradiol or DHEA did not augment this further. Although murine fibroblast migration in a scratch assay was stimulated by 17β-estradiol and an ERβ agonist, in collagen gels, estrogens inhibited mouse fibroblast contraction (59). In contrast, the present study demonstrated that both 17β-estradiol and DHEA stimulate gel contraction, with the requirement that DHEA is aromatized to estrogen (Fig. 8). However, there are significant differences in the anatomy and physiology of loose murine skin and the more firmly attached human skin (60). Murine skin has a panniculus carnosus layer, which produces rapid wound contraction following injury, which is not present in human skin; instead the dermal fibroblasts play a key role in the production of granulation tissue that

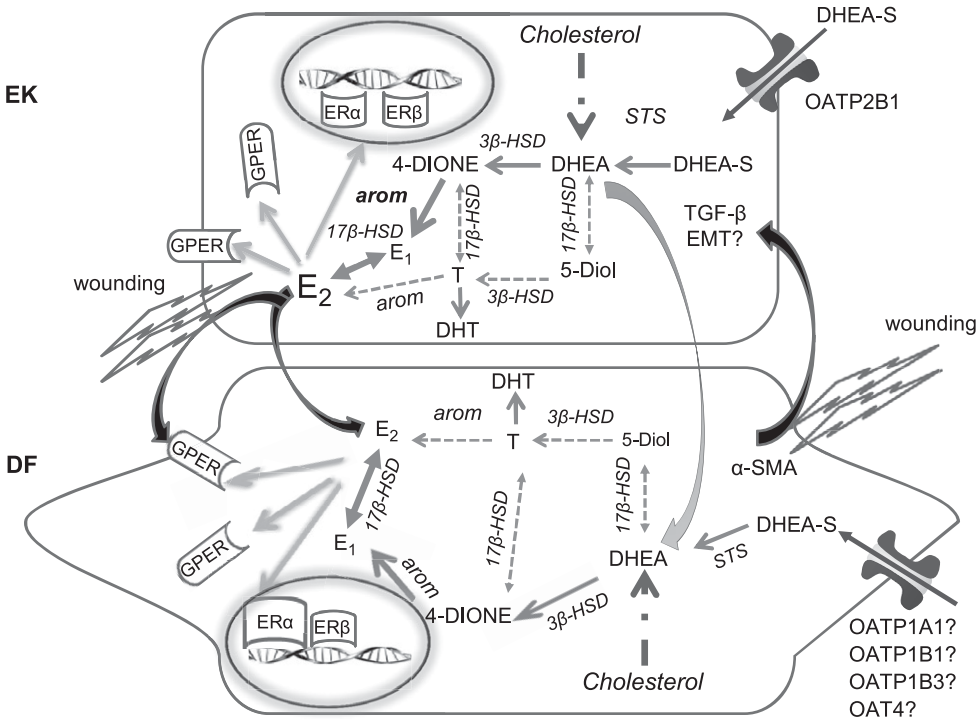

Figure 9. Circulating DHEA-S is transported into keratinocytes (EK) via a specific transporter (OATP2B1), where steroid sulfatase (STS) removes the sulfate and DHEA is oxidized to androstenedione (4-DIONE). This may also provide a source of DHEA to neighboring dermal fibroblasts (DF; gray curly arrow), although DFs may also use alternate DHEA-S transporters (*e.g.,* OAT4?). Additional DHEA is metabolized within both cell types from cholesterol. In both cell types 4-DIONE is aromatized to estrone (E_1), which is converted to estradiol (E_2) by 17β-hydroxysteroid dehydrogenase (17β-HSD). Estradiol binds to ERα, ERβ, and GPER1, although in DFs ERβ is not as highly expressed. Mechanically wounding (jagged lines) EKs up-regulate aromatase (arom) activity, which will increase the production of E_2; this may increase receptor activation and also provide an additional and immediate source of estrogen to neighboring dermal fibroblasts (black curly arrows). Both cell types express the nuclear receptors ERα and ERβ, which modulate the genomic effects of estrogen and GPER1, which modulates rapid cell-signaling pathways. GPER1 can be located at the plasma membrane, as well as in the cytoplasm, where it is thought to move along the intermediate filaments (24). Mechanically wounding DFs increases expression of αSMA and stimulates the release of TGF-β (black curly arrow), which most likely accelerates EK migration by inducing an epithelial-mesenchymal transition (EMT). This is probably not activated by estrogen because aromatase activity is down-regulated in DFs following mechanical wounding. Broken gray arrows represent steroidogenic pathway in classic endocrine tissues, which does not appear to be the predominant metabolic pathway in EKs and DFs. testosterone (T), 5α-dihydrotestosterone (DHT).

promotes re-epithelialization by the keratinocytes. Although, estrogen stimulates gel contraction, it does not up-regulate the expression of α-SMA (Fig. 7), which suggests that rather than contraction of myofibroblasts, the reduction in gel size is probably due to the migration of the fibroblasts through the gel.

This is the first study to establish whether mechanical wounding leads to changes in aromatase activity in fibroblasts and keratinocytes; increased activity by keratinocytes supports the concept of a mechanism by which local concentrations of estradiol can alter quickly following acute stress or damage. This is in line with studies demonstrating that aromatase is up-regulated in glial cells following injury and that the subsequent increase in estrogen promotes neuroprotection (61). The decrease in activity by fibroblasts is in contrast to these studies and suggests an increase in activity may not be desirable. A recent study has reported that 17β-estradiol and an ERβ agonist prevent the differentiation of cardiac fibroblasts into myofibroblasts (62). Furthermore, the production of key proteins such as α-SMA, fibronectin, vimentin, and collagen I and III, implicated in fibroblast transition and fibrosis, were all inhibited by estrogen acting through ERβ (62). Although estrogen has positive effects on fibroblast migration (Fig. 5), it may have detrimental effects on differentiation. These results further highlight the intricacy of steroid biosynthesis in human skin, required to maintain the optimal local availability of steroid hormones under different physiologic conditions.

Menopause highlights 3 important features that distinguish humans from lower mammals [*i.e.,* termination of ovarian function and estrogen secretion, secretion of DHEA by the adrenal cortex, and the presence of intracrine enzymes in peripheral tissues that convert DHEA into active sex steroids, as reviewed by Labrie and Labrie (63)]. Estrogens and their receptors are directly involved in various age-related diseases and the impact of estrogen deficiency in accelerating skin aging is well documented (3, 5, 64–66). Furthermore, recent studies have suggested that human skin cells provide a useful *in vitro* model that will allow us to gain a better understanding of the global pathogenesis of human hormonal aging, particularly in tissues that share the same embryonic origin (66).

In summary, this study verifies that intracrine conversion of DHEA is the source of estrogen in keratinocytes and fibroblasts without producing testosterone as an intermediate and also highlights the existence of mesenchymal-epithelial interactions in the control of the bioavailability of estradiol when cells are subjected to physical trauma (Fig. 9). DHEA is widely available as an antiaging dietary supplement and although estrogen replacement has potential side effects, DHEA is only converted into sex steroids in target tissues such as the skin, which contain the relevant physiologic enzymatic machinery. A 1 yr double-blind, placebo-controlled study showed that 50 mg/d DHEA significantly improved effects of skin aging,

without creating any harmful consequences (67); hence DHEA may provide a safer therapy for impaired wound healing, particularly in the postmenopausal woman who now spends a significant proportion of her life in a state of estrogen deficiency. Primary cultures of human dermal fibroblasts and epidermal keratinocytes provide a biologically relevant *in vitro* model system to investigate the complex pathways and intricacies of steroid intracrinology in human skin. Now that we have established that they retain their steroidogenic properties in culture, we can start to develop more relevant 3-dimensional model systems. Because there are significant differences between human and animal skin in terms of steroidogenesis and wound repair, the development of relevant 3-dimensional *in vitro* human skin models is essential to our understanding how steroid intracrinology regulates gene expression involved in inflammation, aging, and repair and will help develop better treatments for chronic wounds, particularly in the elderly. FJ

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