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Steroid synthesis by primary human keratinocytes; implications for skin disease

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ABSTRACT

Cortisol-based therapy is one of the most potent anti-inflammatory treatments available for skin conditions including psoriasis and atopic dermatitis. Previous studies have investigated the steroidogenic capabilities of keratinocytes, though none have demonstrated that these skin cells, which form up to 90% of the epidermis are able to synthesise cortisol. Here we demonstrate that primary human keratinocytes (PHK) express all the elements required for cortisol steroidogenesis and metabolise pregnenolone through each intermediate steroid to cortisol. We show that normal epidermis and cultured PHK express each of the enzymes (CYP11A1, CYP17A1, 3βHSD1, CYP21 and CYP11B1) that are required for cortisol synthesis. These enzymes were shown to be metabolically active for cortisol synthesis since radiometric conversion assays traced the metabolism of [7-^{3}H]-pregnenolone through each steroid intermediate to [7-^{3}H]-cortisol in cultured PHK. Trilostane (a 3βHSD1 inhibitor) and ketoconazole (a CYP17A1 inhibitor) blocked the metabolism of both pregnenolone and progesterone. Finally, we show that normal skin expresses two cholesterol transporters, steroidogenic acute regulatory protein (StAR), regarded as the rate-determining protein for steroid synthesis, and metastatic lymph node 64 (MLN64) whose function has been linked to cholesterol transport in steroidogenesis. The expression of StAR and MLN64 was aberrant in two skin disorders, psoriasis and atopic dermatitis, that are commonly treated with cortisol, suggesting dysregulation of epidermal steroid synthesis in these patients. Collectively these data show that PHK are capable of extra-adrenal cortisol synthesis, which could be a fundamental pathway in skin biology with implications in psoriasis and atopic dermatitis.

1. Introduction

The epidermis forms a barrier acting to prevent dehydration, regulate body temperature and provide protection from biological, chemical and mechanical assaults to the body. Glucocorticoids are essential for maintaining barrier competency, as exemplified in $GR^{-/-}$ mouse, where loss of GR function led to incomplete epidermal stratification, hyperproliferation and abnormal differentiation [1]. In addition, the cortisol analogue dexamethasone has been shown to acutely influence expression of genes regulating cell proliferation, differentiation, apoptosis and inflammation in PHK [2,3]. Accordingly, cortisol (hydrocortisone) is regarded as the most potent therapy for many inflammatory skin conditions including psoriasis and atopic dermatitis.

Whilst cortisol synthesis has not previously been reported in PHK, some key components of the steroid synthesis pathway have been detected in keratinocytes. Keratinocytes contain an abundance of cholesterol, the precursor to all steroids, as they are capable of synthesising cholesterol *de novo* [4]. Additionally, the cholesterol transporter, steroidogenic acute regulatory (StAR) protein has been identified in human epidermis by immunofluorescence histochemistry [5,6]. Furthermore the complete CYP11A1 complex, including ferrodoxin reductase, ferrodoxin and the CYP11A1 enzyme, has been detected in the epidermis and in keratinocytes [5,7], although the functional capacity of these elements has not been reported in PHK. ACTH, CYP11A1, CYP17A1 and CYP21A2 were identified in skin, however CYP11B1, the final enzyme in the sequence of cortisol synthesis, was not detected in this study [8]. Two further studies demonstrated steroid metabolism of oestrogens, androgens and the formation of corticosterone from progesterone in keratinocytes but failed to detect pregnenolone metabolism and cortisol synthesis [9,10].

Since cortisol has such a potent effect on keratinocytes and is extensively used in skin therapy, we examined steroidogenesis

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by PHK to determine if cortisol could be produced locally. Furthermore, we hypothesise that a block in steroidogenesis could be implicated in the pathogenesis of inflammatory skin disorders. Although Slominski et al. [8] found no difference in ACTH, CYP11A1, CYP17A1 and CYP21A2 transcript levels when comparing normal versus psoriatic skin biopsies, a study by Tiala et al. [11] reported reduced StAR mRNA expression in skin of psoriatic patients. StAR is critical for acute steroidogenesis, thus reduced StAR expression could inhibit acute steroidogenesis. Therefore endogenous, extra-adrenal steroidogenesis of cortisol in normal keratinocytes may be essential for maintaining of a healthy epidermis.

2. Materials and methods

2.1. Skin specimens

Normal human fronto-temporal scalp skin specimens were obtained with informed consent during routine face-lift surgery (Caucasian females over 45 years) and snap frozen and cut into sections for immunofluorescence histochemistry analysis or used to isolate primary keratinocytes. The East London and City Health Authority Research Ethics Committee approved the use of redundant human skin.

2.2. Antibodies

Mouse anti-StAR and mouse anti-3BHSD1 were purchased from Abcam (Cambridge, UK). Goat anti-CYP11A1, goat anti-CYP17A1, goat anti-CYP21 and goat anti-CYP11B1 were purchased from Santa-Cruz Biotechnology (CA, USA). Alexa Fluor 488 donkey anti-goat IgG and Alexa Fluor 488 goat anti-mouse IgG secondary antibodies were purchased from Molecular Probes (Carlsbad, CA, USA). Rabbit anti-goat and goat anti-mouse HRP conjugated secondary antibodies were from Dako Cytomation (Glostrup, Denmark).

2.3. Immunofluorescence histochemistry

Frozen sections of skin were thawed for 20 min at room temperature then fixed and permeabilised in ice-cold 1:1 (v/v) methanol:acetone for 10 min. All tissue was blocked in 10% (v/v) serum for 1 h at room temperature. Negative controls consisted of sections incubated with non-immune serum, PBS-Tween 20 with 1.5% (v/v) serum (Sigma–Aldrich, Poole, UK), in place of primary antibody. All other sections were incubated for 1 h at room temperature with mouse anti-StAR (1 μg/ml) or overnight at 4 °C with mouse anti-3βHSD1 (1 μg/ml), goat anti-CYP11A1 (4 μg/ml), goat anti-CYP17A1 (2 μg/ml), goat anti-CYP21 (4 μg/ml) or goat anti-CYP11B1 (4 μg/ml) antibodies. Primary antibody binding was detected by applying Alexa Fluor 488 goat anti-mouse or donkey anti-goat IgG (each at 2 μg/ml) for 1 h at room temperature. The DNA-binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma–Aldrich, Poole, UK) was used to visualise the cell nuclei in skin sections. Sections were mounted using immunofluorescent mounting medium (DakoCytomation, Glostrup, Denmark). Fluorescent signals were observed using a Leica DM 5000B fluorescence microscope and images were captured using a Leica DFC 350FX camera and Leica QWin Pro software.

2.4. Cell culture

PHK were isolated from redundant face-lift skin as described by Rheinwald and Green [12] and expanded with the support of mitomycin C-treated 3T3 mouse fibroblast cells in DMEM:Hams F12 3:1 (v/v) media supplemented with 10% (v/v) foetal calf serum (BioWest, Paris, France), 1% (v/v) glutamine, cortisol (0.4 μg/ml), insulin (5 μg/ml), epidermal growth factor (10 ng/ml) and cholera toxin (1 nmol/L) (all Sigma–Aldrich, Poole, UK). At 70% confluency, 3T3 fibroblasts were removed with versene (PAA, Pasching, Austria) and the keratinocytes trypsinised and seeded in the absence of 3T3 cells for 1–2 passages in keratinocyte-SFM (Invitrogen, Paisley, UK) prior to using for experiments. No keratinocytes were used for experiments beyond passage 3.

H295R cells (ATCC, Manassas, USA) were cultured at 37 °C under a humidified atmosphere of 5% (v/v) CO_2 in air in DMEM:Ham's F12 medium (Sigma–Aldrich, Poole, UK) supplemented with 2.5% (v/v) Nu-Serum I and 1x ITS+ premix (both BD Biosciences, Belgium). JEG-3 human choriocarcinoma cells (Professor V.K. Chatterjee, Department of Medicine, University of Cambridge, Cambridge, UK), were similarly cultured at 37 °C under a humidified atmosphere of 5% (v/v) CO_2 in air in DMEM (Sigma–Aldrich, Poole, UK) supplemented with 10% (v/v) FBS (Biosera, East Sussex, UK) and 1% (v/v) L-glutamine. The cells were grown to 70% confluency before use.

2.5. Western blot analysis of steroidogenic enzymes

PHK were seeded at 5×10^5 cells per well of six well plates in 3 ml K-SFM with supplements and allowed to adhere overnight. The cells were then washed three times and incubated in K-SFM without supplements for 24 h prior to protein extraction in 300 μl urea buffer (8 mol/l urea, 1 mol/l thiourea, 0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mmol/l dithiothreitol (DTT) and 24 mM spermine (all Sigma–Aldrich, Poole, UK). H295R and JEG-3 cells were grown to 70% confluency in six well plates, washed three times in PBS before protein extraction in 300 μl urea buffer. Protein lysates (2–20 μg) were separated on a 10% (w/v) bis–tris NuPAGE gel at 200 V for 50 min in MOPS running buffer (all Invitrogen, Paisley, UK). Proteins were transferred to a hybond-C membrane (GE healthcare, Buckinghamshire, UK) at 30 mAmps for 2 h. Blots were washed three times and incubated overnight at 4 °C in blocking buffer (5% (w/v) BSA-TBS-T), and subsequently incubated for 1 h at room temperature in primary-antibody diluted in relevant blocking buffer. Blots were washed three times for 5 min then incubated in goat anti-mouse or rabbit anti-goat HRP conjugated secondary antibody for 1 h at room temperature. The protein of interest was detected with enhanced chemiluminescence (ECL)-plus (GE Healthcare, Buckinghamshire, UK).

2.6. Radiometric conversion assays

PHK (passage 2) were plated at 2×10^5 cells/ml in 24 well plates in K-SFM (1 ml/well) and incubated overnight. Cells were washed three times and incubated in 1 ml/well EpiLife with no supplements (Invitrogen, Paisley, UK) since this medium does not contain steroids. Cells to be treated with 100 μmol/l trilostane (Dr. J.R. Puddefoot, Queen Mary, University of London) and 10 μmol/l ketoconazole (Sigma–Aldrich, Poole, UK) were pre-incubated for 2 h with these compounds. All keratinocytes were washed three times then incubated for 24 h in triplicate wells (1 ml/well) with 90.0 pmol [7-^{3}H]-pregnenolone (Perkin Elmer, Boston, MA, USA) ± 100 μmol/l trilostane and 10 μmol/l ketoconazole in EpiLife with no additional supplements. Assay blanks were set up and incubated as described above but in the absence of cells.

Steroid metabolites released by keratinocytes were extracted by transferring the aqueous culture medium from each well to a screw cap borosilicate culture tube and vigorously mixing with two volumes of chloroform (HPLC grade, Fisher, Leicestershire, UK). After centrifugation at 1000*g* for 30 min at 4 °C, the aqueous phase was removed and the remaining chloroform was evaporated

under nitrogen gas at 45 °C. Steroid extracts were reconstituted in up to 50 μl ethyl acetate, from which 20 μl was transferred onto a thin layer chromatography (TLC) plate (Merck, Damstadt, Germany). The remaining 30 μl was reserved to be run with authentic standards (10 mmol/l each of pregnenolone, progesterone or cortisol; all Sigma–Aldrich, Poole, UK). Migration of reference steroids was monitored by spiking aliquots of each sample with 0.37 kBq [^{3}H]-cortisol, [^{3}H]-progesterone or [^{3}H]-pregnenolone. TLC plates were developed in a pre-equilibrated atmosphere of 92:8 (v/v) chloroform:95% (v/v) ethanol. Resolution of [^{3}H]-steroid metabolites was quantified using an AR2000 Bioscan radiochromatogramme scanner (Lablogic Ltd., Sheffield, UK) and Laura Lite software (version 3.0; Lablogic Ltd., UK).

2.7. Cortisol enzyme-linked immunosorbent assay (ELISA)

PHK were seeded at 2×10^5 cells/ml in 12 well plates in EpiLife complete culture medium (Gibco, Invitrogen Corporation, Paisley, UK) (1 ml/well) and allowed to adhere overnight. Cells were then washed three times before 24 h incubation in EpiLife without any supplements. An assay blank was set up and incubated as described above but in the absence of cells.

Supernatant was collected and cortisol measured via ELISA (R&D Systems, Minneapolis, USA). The ELISA was conducted according to the manufacturer's instructions with the exception that all samples were assayed neat without being diluted in assay buffer. Standards and non-specific binding controls for the ELISA were therefore prepared in EpiLife without any supplements to account for this modification to the assay. The working range of this ELISA was 0.156–10 ng/ml with an assay sensitivity of 0.071 ng/ml and cross reactivity of prednisolone: 4.4%, Reichstein's substance S: 3.4%, progesterone: 1.7% and cortisone: 0.2%.

3. Results and discussion

3.1. Detection of steroid enzymes in PHK and human epidermis

To identify if keratinocytes had the potential to synthesise cortisol, protein expression of steroid enzymes required for cortisol steroidogenesis was determined. CYP11A1, CYP17A1, 3βHSD1, CYP21 and CYP11B1 were all detected in protein lysates from keratinocytes (Fig. 1A). There are two isoforms of 3βHSD; 3βHSD2, expressed in the adrenal glands and gonads, and 3βHSD1, which is expressed in peripheral tissue [13]. Western analysis indicated that the antibody was specific for 3βHSD1 since 3βHSD1 was detected in PHK and JEG3 cells (positive control) but was absent in H295R immortalised adrenal cells (negative control) that express 3βHSD2.

Steroid enzyme expression was also examined by immunofluorescence histochemistry in epidermis (Fig. 1B). CYP11A1, CYP17A1, 3βHSD1 and CYP11B1 were expressed in a perinuclear location throughout all the epidermal layers. This is in agreement with a previous study that identified CYP11A1, CYP17A1 and ferredoxin reductase expression throughout the epidermis [7]. CYP21 was also identified in each stratum of the epidermis however a stronger signal was detected in the basal layer where stem cells and proliferating keratinocytes reside.

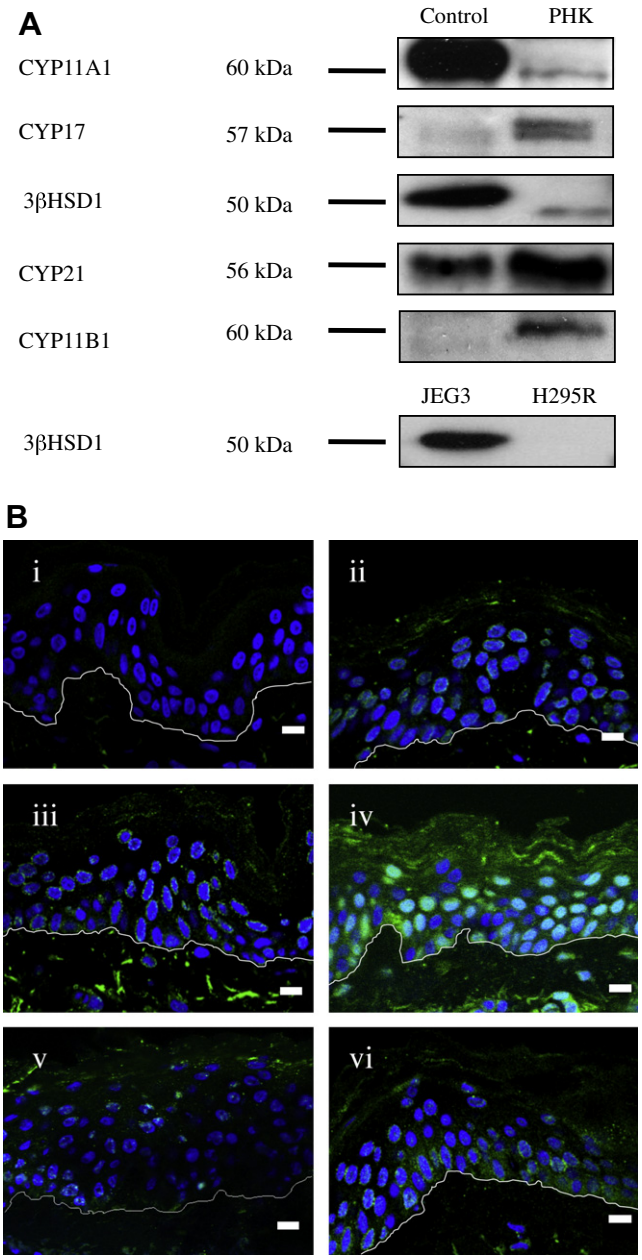

Fig. 1. (A) Western blots of steroid enzyme expression in cultured primary human keratinocytes. H295R cell lysates were used as a positive control for all proteins except for 3βHSD1 where JEG-3 cell lysates were used. Steroidogenic enzymes were detected with protein loading levels as follows: CYP11A1 (H295R = 5 μg/lane, keratinocytes = 20 μg/lane), CYP17A1 (H295R = 10 μg/lane, keratinocytes = 10 μg/lane), 3βHSD1 (JEG-3 = 2 μg/lane, keratinocytes = 2 μg/lane), CYP21 (H295R = 10 μg/lane, keratinocytes = 10 μg/lane) and CYP11B1 (H295R = 10 μg/lane, keratinocytes = 10 μg /lane). (B) Immunofluorescence histochemistry shows the expression pattern steroid enzymes required for cortisol synthesis in normal human skin from Caucasian females (aged between 50 and 65 years). Skin sections were probed with DAPI (blue nuclear stain), (i) non-specific anti-goat IgG (negative control), (ii) CYP11A1, (iii) CYP17A1, (iv) 3βHSD1, (v) CYP21 and (vi) CYP11B1. In each panel, the binding of primary antibodies was detected with Alexafluor 488 secondary antibody (green). Images are representative of at least four observations, each made in skin samples from a different individual. Scale bar = 10 μm.

3.2. PHK metabolise pregnenolone to cortisol

The metabolism of [^{3}H]-pregnenolone by PHK over 24 h was then examined to determine whether the observed steroid enzymes were metabolically active. The products of [^{3}H]-pregnenolone metabolism co-migrated with progesterone, 17α-hyroxyprogesterone, 17α-hydroxypregnenolone, corticosterone, cortisone and cortisol (Fig. 2A). Production of cortisol from pregnenolone was confirmed by comigration of pregnenolone metabolites with an authentic [1,2,6,7-^{3}H]-cortisol standard. Other steroid intermediates were also detected but could not be resolved completely by thin layer chromatography. To further confirm the pregnenolone metabolic pathway, trilostane (a 3βHSD1 inhibitor) and/or

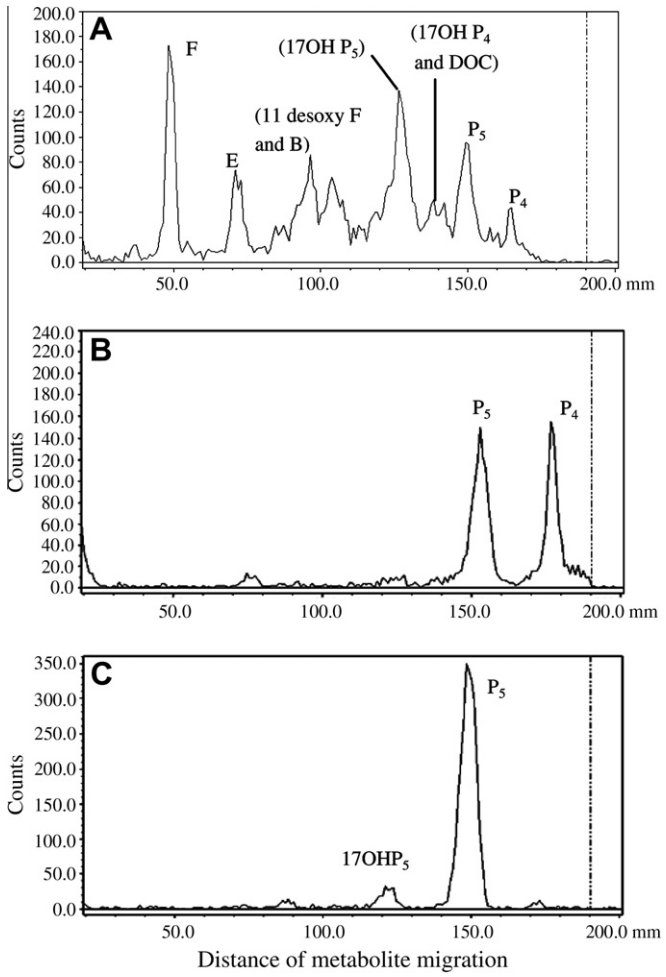

Fig. 2. Normal human facelift keratinocyte metabolism of [7-^{3}H] pregnenolone to cortisol. Cells were plated at a density of 2.5×10^5 cells/ml in 24 well plates in serum free medium (1 ml/well). After 24 h, cells were washed three times and incubated for 24 h in 1 ml of fresh serum-free medium containing 0.037 MBq of (A) [^{3}H]-pregnenolone (90.0 pmol), (B) [^{3}H]-pregnenolone (90.0 pmol) with ketoconazole (10 μmol/l) or (C) [^{3}H]-pregnenolone (90.0 pmol) with trilostane (100 μmol/l). Steroid metabolites were extracted from the culture medium into chloroform, concentrated by evaporation and then resolved by thin layer chromatography. Each panel shows a representative chromatogramme for three separate assays (each performed with triplicate wells) using keratinocytes isolated from a different individual. F, cortisol; E, cortisone; 11-desoxy-F, 11-desoxycortisol; B, corticosterone; 17OHP$_5$, 17α-hydroxypregnenolone; 17OHP$_4$, 17α-hydroxyprogesterone; DOC, 11-deoxycorticosterone; P$_5$, pregnenolone and P$_4$, progesterone.

ketoconazole (a CYP17A1 inhibitor) were used to block pregnenolone metabolism. In the presence of ketoconazole the only major product of [^{3}H]-pregnenolone metabolism was progesterone (Fig. 2B), indicating that CYP17A1 was effectively inhibited. Ketoconazole blocks CYP17 activity and will prevent metabolism of progesterone to 17-hydroxyprogesterone and ultimately cortisol. Ketoconazole is not known to inhibit CYP21, thus, theoretically, progesterone metabolism to deoxycorticosterone followed by corticosterone production could still progress. However, progesterone metabolism to corticosterone was not detected in the presence of ketoconazole in this system. This could be for two reasons, firstly this is a human, whole cell system and thus the predominant metabolic glucocorticoid pathway favours the synthesis of cortisol (the predominant glucocorticoid in humans) rather than corticosterone (the predominant glucocorticoid in rodents); secondly, the amount of progesterone metabolised from pregnenolone may be insufficient to overcome the energy of activation for CYP21 deoxycorticosterone production. Trilostane also effectively blocked the pathway by inhibiting 3βHSD1 [^{3}H]-pregnenolone metabolism so that the predominant steroid species were pregnenolone and to a lesser extent 17-hydroxypregnenolone (Fig. 2C). These data demonstrate that the keratinocytes are capable of metabolising pregnenolone via 3βHSD1 and CYP17A1 to form cortisol. A highly sensitive cortisol ELISA further confirmed the production of cortisol by PHK. PHK synthesised 0.37 ± 0.09 ng cortisol/ml, equivalent to 1.0 ± 0.2 nmol/L over a 24 h period. This is physiologically relevant since 1–10 nmol/L cortisol is sufficient to activate the glucocorticoid receptor [14,15].

A previous radiometric study failed to identify pregnenolone or progesterone metabolism but rather androgen and oestrogen metabolism was detected in primary human keratinocytes [10]. The apparent differences between the findings of the current and preceding studies could reflect the impact of specific cell culture conditions. Milewich et al. [10] incubated keratinocytes with [^{3}H]-pregnenolone in either simple buffers (Tris buffer or Krebs–Ringer phosphate solution) or in RPMI each supplemented with glucose. Hence the choice of buffer system or culture medium may have inhibited pregnenolone metabolism. Slominski et al. [9] also attempted to detect cortisol synthesis from progesterone but were only able to measure metabolism to DOC. However Slominski et al. [9] used HaCaT immortalised keratinocytes rather than primary cells which could account for differences in steroid metabolism. In contrast, other extra-adrenal tissue has been shown to synthesise steroids including cortisol. The hair follicle has been reported to express elements of the hypothalamic–pituitary–adrenal (HPA) axis and to synthesise cortisol [16], sebocytes demonstrate the transformation of 22R-hydroxycholesterol to 17-hydroxypregnenolone [7], whilst melanocytes and fibroblasts have also been shown to produce corticosterone and cortisol [17–19].

3.3. Steroid regulators in normal and diseased epidermis

Immunofluorescent histochemistry showed that StAR was almost exclusively expressed in the basal layer of the epidermis, suggesting the site for acute steroid production is limited to the stem and proliferating keratinocytes as opposed to the differentiating cells (Fig. 3). StAR was also reported to be predominantly expressed in stem and proliferating cells of other steroid-producing extra-adrenal tissue, such as sebocytes [20]. MLN64, another (putative) cholesterol transporter that shares sequence homology with StAR [21], was only identified in the supra-basal layers of the epidermis. Although the precise role of MLN64 has yet to be determined, MNL64 reportedly enhances cholesterol transport for steroid synthesis in reconstituted systems and has been shown to control steroid transport in steroidogenic cells that do not express StAR [21]. MLN64 was previously identified in whole-skin extracts by western blot [5]. However our study demonstrates the suprabasal localisation of MLN64 in normal epidermis, suggestive of differential regulation of cholesterol delivery for steroidogenesis, depending on the level of keratinocyte proliferation and differentiation. Overall, the results demonstrate that keratinocytes express regulators of steroid synthesis and each of the steroidogenic enzymes required for cortisol synthesis.

Since steroids have been shown to manipulate keratinocyte biology and cortisol analogues have been used to treat inflammatory skin conditions, we wanted to determine if proteins that regulate the steroid pathway were aberrantly expressed in psoriasis and eczema. Previously published research has reported StAR expression to be down regulated in lesional psoriatic skin [11]. However, this study did not investigate StAR expression in eczema. We now show that in contrast to normal skin, StAR was not detected in the basal layer of both eczema and uninvolved psoriatic

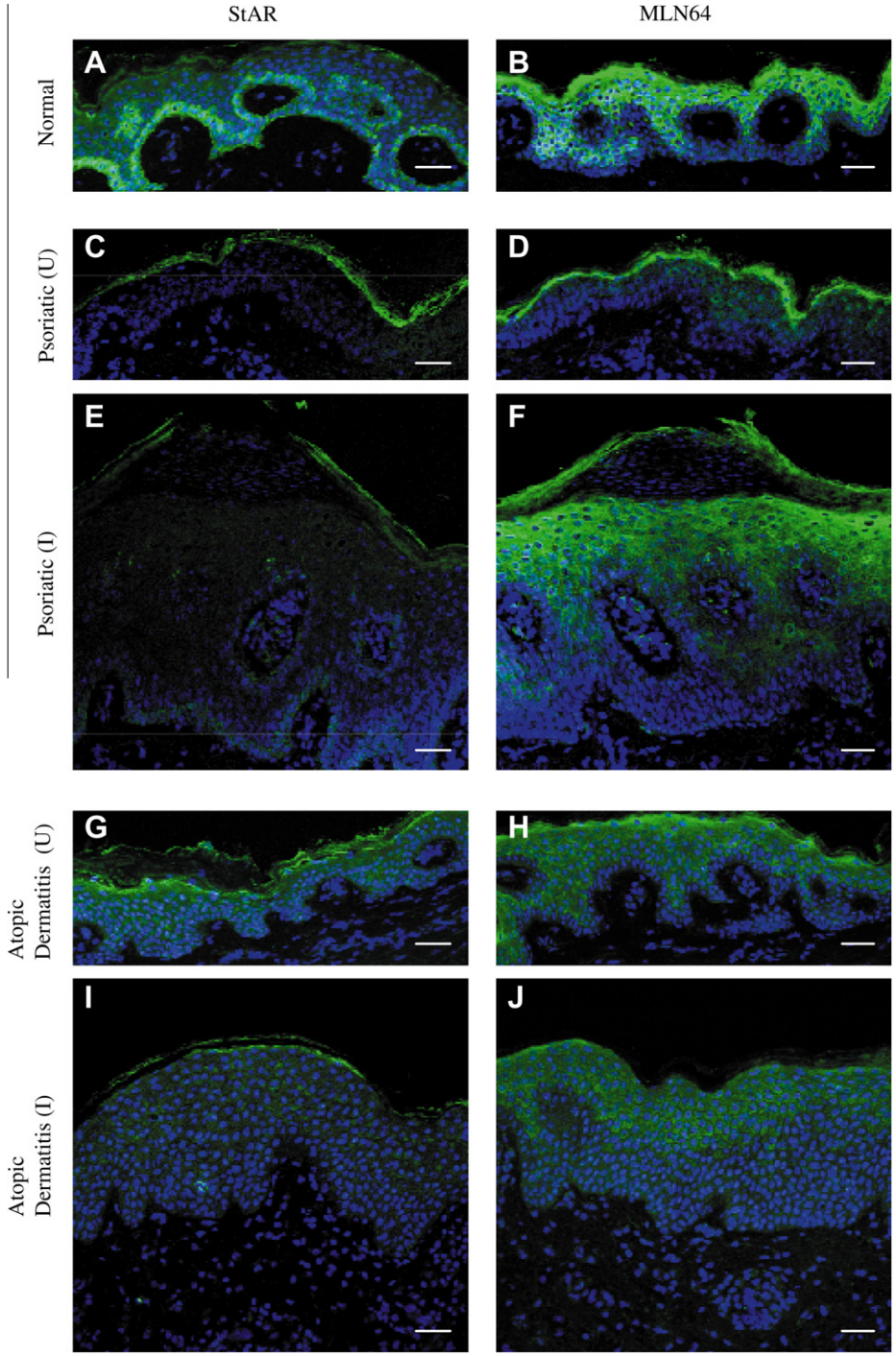

Fig. 3. Immunofluorescence histochemistry shows the expression pattern of StAR and MLN64 psoriatic and atopic dermatitis human skin. Representative images of panel (A and B): normal whole skin facelift sections from Caucasian females (aged between 50 and 65 years); panel (C and D) non-lesional and panel (E and F) lesional from patient matched psoriatic biopsies; panel (G and H) uninvolved and panel (I and J) involved areas of skin from patient-matched atopic dermatitis biopsies. Skin sections were probed with DAPI (blue nuclear stain) and rabbit polyclonal StAR antibody (A, C, E, G and I) or rabbit polyclonal MLN64 antibody (B, D, F, H and J) that was detected with Alexafluor 488 secondary antibody (green). (A–C) Depicts a representative image of at least 15 observations, each made in skin samples from a different individual. Images (D–I) and (J–O) depict a representative image of up to four observations each made in paired biopsies from different patients. Scale bar = 50 μm, U, uninvolved; I, involved.

skin and that low levels of StAR expression were detected in lesional sections of psoriatic skin only.

In uninvolved regions of diseased skin, MLN64 expression was unchanged relative to normal skin, however expression was delayed in more supra-basal layers of lesional psoriatic and atopic dermatitis epidermis. Thus it is possible that the late onset of MLN64 expression in lesional regions of psoriatic and atopic dermatitis skin is related to the poor keratinocyte differentiation and dysregulated lipid dynamics that is associated with these two disorders. Taken together, the data suggests that key regulators of steroidogenesis are aberrantly expressed in psoriasis and atopic dermatitis.

Here we show that epidermis and PHK possess each of the components required for synthesising steroids, including regulators and enzymes of the pathway. Furthermore, we report that PHK can metabolise pregnenolone through each intermediate steroid

to cortisol, suggesting that these cells are capable of extra-adrenal cortisol synthesis. Finally, we found that there was an aberrant expression pattern of StAR and MLN64 in psoriatic and atopic dermatitis skin, two conditions that are commonly treated with cortisol. Down regulation or absence of these steroid synthesis regulators suggests that the steroidogenesis pathway could be restricted in psoriasis and eczema. Since cortisol has been shown to influence keratinocyte proliferation, differentiation, inflammation and apoptosis, local cortisol synthesis in keratinocytes could represent a new pathway that is critical for maintaining a functional, healthy epidermis.

Declarations of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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