but none directly related to the research included in this paper. SC Chen receives royalties from for-profit companies licensing the ItchyQoL instrument. HY and KP state no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: HY, SC Chisolm, SC Chen; Data Curation: HY, SC Chen; Formal Analysis: HY; Funding Acquisition: SC Chen; Investigation: SC Chen; Methodology: HY, SC Chen; Project Administration: SC Chisolm, SC Chen, KP; Resources: SC Chen; Supervision: SC Chen; Visualization: KP; Writing - Original Draft Preparation: HY, SC Chisolm, KP; Writing - Review and Editing: HY, SC Chisolm, SC Chen, KP

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Disclaimer

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Abnormal Glucocorticoid Synthesis in the Lesional Skin of Erythematotelangiectatic Rosacea

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TO THE EDITOR

Rosacea is a chronic inflammatory skin disorder that usually affects the central part of the face. Telangiectasia, vasodilation, inflammatory papules, and, later, fibrosis are common manifestations of rosacea. It is well known that the chronic use of glucocorticoids (GCs) can lead to various side effects, including, for example, the so-called rosacea-like dermatitis, which is a paradoxical inflammatory reaction (Kumrah and Rathi, 2011). Healthy skin has its own cutaneous hypothalamic-pituitary-adrenal axis, producing all the elements for synthesizing and regulating GCs (Nikolakis et al., 2016). On the other hand, studies have shown decreased and dysfunctional local cutaneous GC synthesis in patients with psoriasis and atopic dermatitis (Hannen et al., 2011, 2017). This observation can explain why psoriasis and atopic dermatitis can usually be treated with topical GCs, unlike rosacea. Based on the similarity between rosacea and rosacea-like dermatitis, we hypothesized that the dysregulation of primary cutaneous de novo GC synthesis would play a role in the pathogenesis of rosacea.

To investigate de novo GC synthesis in the lesional skin of patients with erythematotelangiectatic rosacea (ETR), paired lesional and nonlesional facial skin samples were collected from 12 patients with ETR who provided written informed consent. This study was approved by the Institutional Review Board of Seoul National University Boramae Medical Center. Individuals who had used topical GCs were thoroughly excluded. Relative mRNA levels of CYP11A1, CYP21A2, 3β-HSD2, 17β-HSD5, 11β-HSD2, and 5α-reductase 2 were significantly increased in the lesional skin, whereas *StAR*, *CYP17A1*, 3β-HSD1, 11β-HSD1, and 5α-reductase 1 were also increased in the lesional skin of rosacea, albeit nonsignificantly. In addition, the level of GC receptor (GR) α was upregulated in the lesional skin (Figure 1a), suggesting that both de novo synthesis of the GC and the expression level of its receptor are elevated simultaneously. Primer sequences are shown in Supplementary Table S1. Immunohistochemical staining was performed to further confirm the protein expression patterns of involved genes. Of all the elevated genes, *CYP11A1*, which initiates corticosteroid synthesis by converting cholesterol to pregnenolone (Slominski et al., 1996), was increased the most in both the sebaceous gland and the epidermis of rosacea lesions. 3β-HSD2 and 17β-HSD5, which play pivotal roles in GC synthesis, were also increased. Furthermore, not only the expression but also the phosphorylation of GRα was notably increased in the nuclei of sebocytes and epidermal keratinocytes (Figure 1b and c). Characteristics of the primary antibodies are shown in Supplementary Table S2. The aforementioned enzymes and receptor were also increased significantly when ranked by visual grading analysis (see Supplementary Figure S1). Progesterone, which is produced from pregnenolone by 3β-HSD and is also the precursor of cortisol, was found to be significantly increased in the lesional skin of patients with rosacea compared with the nonlesional skin, whereas the level of cortisol was unmeasurable using liquid chromatography-mass spectrometry (Figure 2a). The phosphorylation of GRα induces its translocation into the nucleus where GRα binds to the GC response element of DNA. The binding induces the transcription of IκB, an inhibitor of the NF-κB signaling pathway (Bekhbat et al., 2017). Hence, we checked mRNA levels of IκB as well as NF-κB subunits and its downstream target genes such as *IL-8*, matrix

Abbreviations: ETR, erythematotelangiectatic rosacea; GC, glucocorticoid; GR, glucocorticoid receptor; MMP, matrix metalloproteinase

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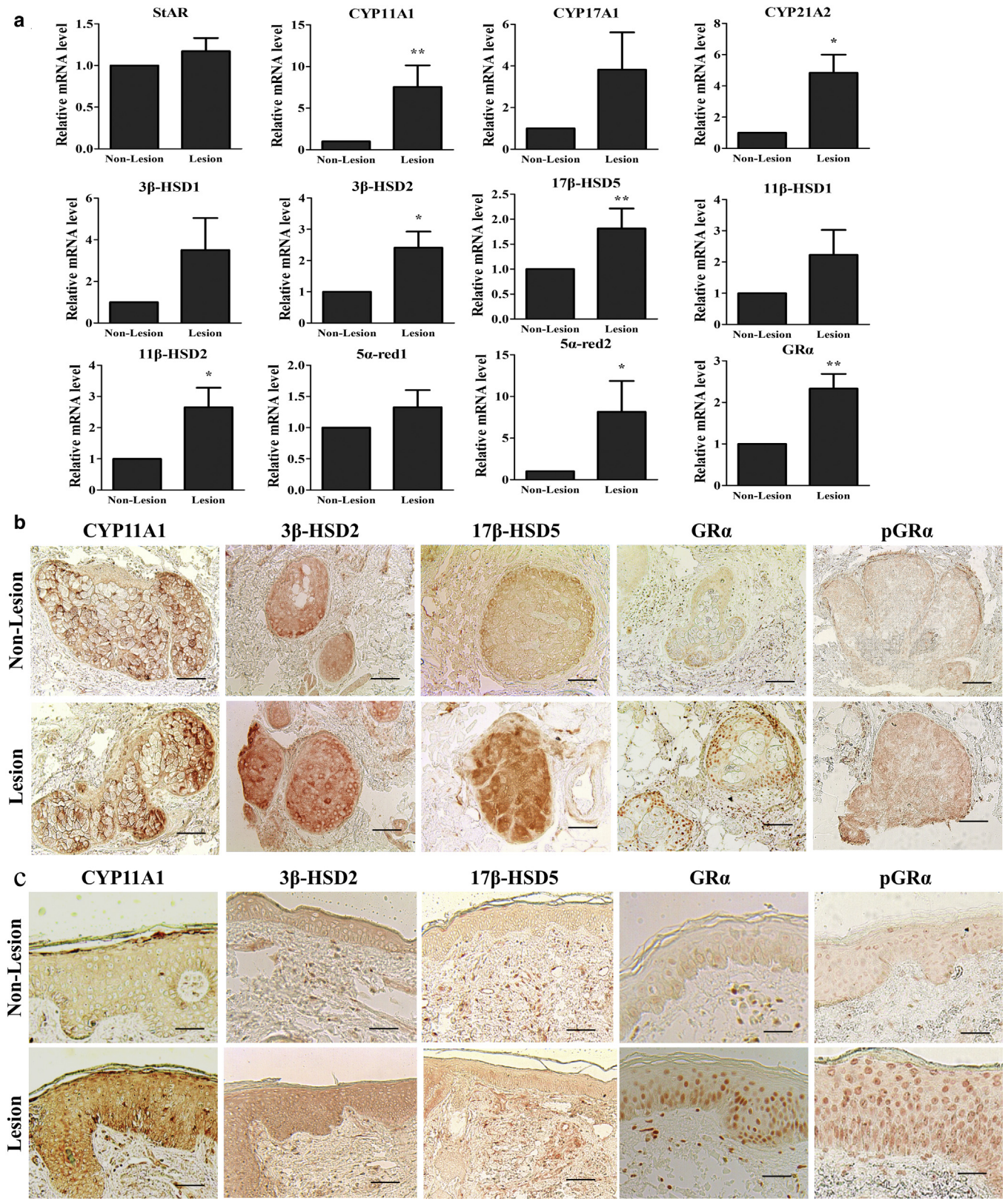

Figure 1. Expression of glucocorticoid-synthesizing enzymes is increased in the lesional skin of patients with rosacea. (**a**) Relative mRNA levels of enzymes synthesizing corticosteroids and GRα were analyzed from patients with rosacea. Nonlesional and lesional skin samples from the same individuals with rosacea were analyzed by quantitative real-time RT-PCR ($n = 12$). Data represent mean ± standard error of relative mRNA expressions, normalized to 36B4. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus nonlesional skin. (**b, c**) The expression patterns of *CYP11A1*, 3β-HSD2, 17β-HSD5, and GRα in the sebaceous gland (**b**) and the epidermis (**c**) were analyzed. Nonlesional and lesional skin samples from the same individuals with rosacea were analyzed by IHC staining (original magnification ×200). Scale bar = 50 μm. The data shown are representative of independent experiments ($n = 12$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus nonlesional skin. GRα, glucocorticoid receptor α; IHC, immunohistochemical; RT-PCR, reverse transcriptase–PCR.

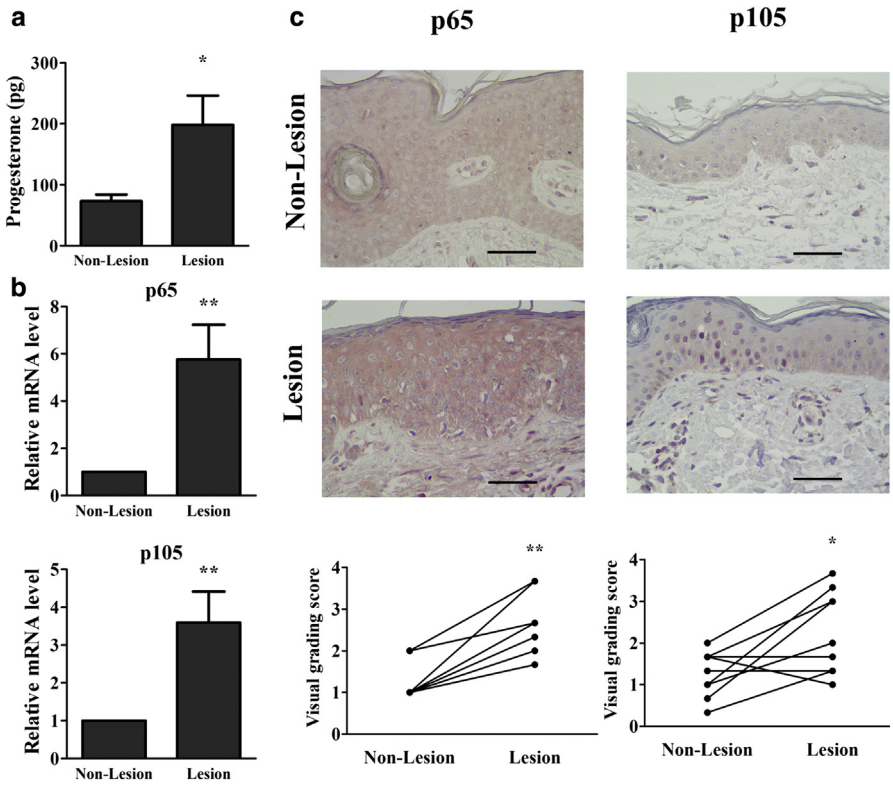

Figure 2. Expression of NF-κB subunits and the level of progesterone are increased in the lesional skin of patients with rosacea. (**a**) The amount of progesterone was analyzed from patients with rosacea. Nonlesional and lesional skin samples from the same individuals with rosacea were analyzed by LC-MS ($n = 10$). Data represent mean ± standard error of progesterone level. *$P < 0.05$ versus nonlesional skin. (**b**) Relative mRNA levels of NF-κB subunits were analyzed from patients with rosacea. Nonlesional and lesional skin samples from the same individuals with rosacea were analyzed by quantitative real-time RT-PCR ($n = 12$). Data represent mean ± standard error of relative mRNA expressions, normalized to 36B4. **$P < 0.01$ versus nonlesional skin. (**c**) The expression patterns of p65 and p105 in the epidermis were analyzed. Nonlesional and lesional skin samples from the same individuals with rosacea were analyzed by IHC staining (original magnification ×400). Scale bar = 100 μm. The data shown are representative of independent experiments ($n = 12$). Staining intensity of the sebaceous gland was graded using a visual grading score (0–5). The scores are presented as dot plots. *$P < 0.05$ and **$P < 0.01$ versus nonlesional skin. IHC, immunohistochemical; LC-MS, liquid chromatography-mass spectrometry; RT-PCR, reverse transcriptase–PCR.

metalloproteinase (*MMP*)-2, and *MMP-9*. We observed that the mRNA level of IκB was significantly increased; however, p65, p105, *IL-8*, *MMP-2*, and *MMP-9* were also increased concurrently (Figure 2b and as shown in Supplementary Figure S2a). In correlation with the mRNA levels, p65 and p105 protein levels were significantly increased (Figure 2c). To investigate the relationship between corticosteroid-synthesizing enzymes and *MMP-2*, which plays a role in the pathogenesis of rosacea by triggering cutaneous angiogenesis (de Medeiros et al., 2017), Pearson correlation analysis was performed using the mRNA ratio (lesion:nonlesion) of each corticosteroid synthesis–related gene shown in Figure 1a. *CYP11A1* showed positive correlation with *MMP-2* (Supplementary Figure S2b). This suggests that de novo synthesis of corticosteroid may be related to the upregulation of genes that contribute to the pathogenesis of rosacea.

In this study, the increment in the synthesis of steroid enzymes and the simultaneous increase in the expression of GRα were observed in patients with rosacea. On the contrary, when the ex vivo skin was irradiated with UV, the production of cortisol was increased and GRα was downregulated simultaneously (Skobowiat et al., 2013). The homeostatic function, that is, downregulation of GRα, observed in normal skin may not work properly in the lesional skin of patients with rosacea. Moreover, considering that the major site affected in rosacea is usually the convex area of the face, and it is the most UV-irradiated area, we speculate that long-term exposure to UV plays a cardinal role in increasing the steroidogenesis observed in patients with rosacea. It was observed in this study that NF-κB pathway–related molecules were increased in the lesional skin of patients with rosacea. Because this study included only patients with ETR, we can confirm that inflammatory reactions mediated by the NF-κB pathway exist even in the ETR subtype without papules and pustules. *MMP-2* and *MMP-9* are known to be involved in the angiogenesis of cutaneous wounds and in neovascularization of tumors (de Medeiros et al., 2017; Deryugina et al., 2014). Our research shows that the increase of *CYP11A1* has a strong correlation with the increase of *MMP-2*. These results support the hypothesis that the activation of the GC system is responsible for the increased inflammation of rosacea and the development of telangiectasia. When the GR is activated, it usually inhibits NF-κB and its associated pathway through IκB, which will act in the direction to lower inflammation (Widén et al., 2003). However, in this study, to the contrary, we observed cases in which the steroids acted paradoxically in the direction of increasing inflammation. The use of GCs in poststroke edema patients can increase inflammation and exacerbate the patient's condition (Gomes et al., 2005). Activation of GR and increase of the p65 subunit of NF-κB were at the same time observed in myeloid cells under acute injury condition (Sorrells et al., 2013). Although it is unknown under which circumstances GCs produce proinflammatory effects, the timing of GC release may be an important determinant factor. GC release after immune activation has an anti-inflammatory effect, whereas the presence of GCs before immune activation has a stimulatory effect on inflammation (Frank et al., 2012). GC synthesis before immune activation may show a proinflammatory effect in ETR. Our results suggest that ETR is an inherently inflammatory disorder mediated by NF-κB and that localized GC synthesis and GR expression are increased in ETR unlike in other cutaneous inflammatory diseases, thereby impairing the crosstalk

between GR and NF-κB and thus contributing to ETR pathogenesis.

Data availability statement

Information about primers and antibodies can be found in Supplementary Tables S1 and S2.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.02.036.

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Major Differences in Expression of Inflammatory Pathways in Skin from Different Body Sites of Healthy Individuals

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TO THE EDITOR

Human skin is a site of constant dialogue between the immune system and the external environment (Romani et al., 2010). Antigen-presenting cells and T cells play a crucial role in the cutaneous immune microenvironment, creating a defense against external stimuli. Regulatory T cells (Tregs), tolerance-promoting BDCA3$^+$ dendritic cells (DCs), and Langerhans cells (LCs) have been proposed to constrain excess immune activation, thus promoting immune homeostasis or tolerance in normal skin (Jongbloed et al., 2010). Cutaneous microenvironments associated with hair follicles (Scharschmidt et al., 2015) or sebaceous glands may also promote tolerance via Tregs or IL-10–expressing T helper type (Th) 17 T cells (Béke et al., 2018). Understanding the diversity of immune cells across different body locations in healthy skin is highly relevant for the emerging epicutaneous immunotherapy approach tested for food allergy (Shreffler et al.,

Abbreviations: DC, dendritic cell; LC, Langerhans cell; Th, T helper type; Treg, regulatory T cell

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SUPPLEMENTARY MATERIALS AND METHODS

Human skin samples

To investigate de novo glucocorticoid synthesis in the lesional skin of patients with erythematotelangiectatic rosacea (ETR), both lesional and nonlesional facial skin samples were collected from the same 12 volunteers with ETR (mean age, 40.1 years; range, 30–55 years). The volunteers who had used topical glucocorticoids were excluded by scrupulous review of past history. Skin biopsy samples were immediately stored in −80 °C to analyze for mRNA expression levels or fixed in 10% formalin and stored as a paraffin block at room temperature for staining.

This study was approved by the Institutional Review Board of Seoul National University Boramae Medical Center, and all human subjects gave written informed consent according to the Declaration of Helsinki Principles.

Quantitative real-time reverse transcriptase–PCR

Total RNA was isolated from the skin samples using RNAiso (Takara Bio, Shiga, Japan) and converted to cDNA using First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. To quantitatively analyze mRNA expression, PCR was performed on a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) according to the manufacturer's instructions, with the human primer pairs in Supplementary Table S1.

The PCR conditions were 50 °C for 2 minutes, 95 °C for 2 minutes, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The data were analyzed using the comparative ΔΔCt method and presented as mean ± standard error of relative mRNA expressions against corresponding controls and normalized to 36B4.

Immunohistochemical staining

Paraffin-embedded skin samples were sectioned 6 μm thick and mounted onto silane-coated slides (Dako, Glostrup, Denmark). The slides were dewaxed in xylene substitutes (Thermo Fisher Scientific, Waltham, MA), rehydrated in ethanol, and washed with water. Then, samples were autoclaved in Target Retrieval Solution (Dako, Glostrup, Denmark) for antigen retrieval. Endogenous blocking by 3% hydrogen peroxide and exogenous blocking by preblocking solution (GBI Labs, Mukilteo, WA) were performed to lessen the nonspecific staining. Primary antibodies were diluted in Diluent Buffer (Dako, Glostrup, Denmark) and used to probe each antigen (Supplementary Table S2). After washing with phosphate buffered saline, the slides were incubated with secondary antibody and streptavidin-horse radish peroxidase consecutively for 15 minutes each and visualized using AEC kit (GBI Labs, Bothell, WA).

Liquid chromatography-mass spectrometry

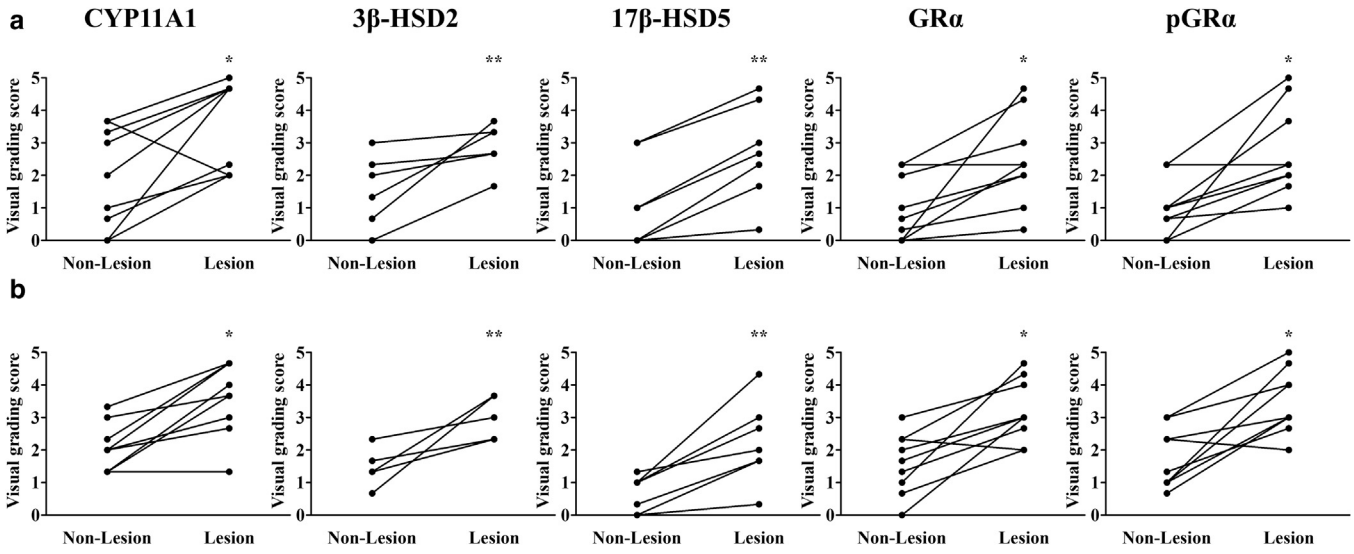
Measurement of cortisol and its precursors and metabolites in paraffin-embedded skin tissues was performed using liquid chromatography-mass spectrometry (LC-MS). Briefly, tissue samples were washed with 2 ml of *n*-hexane at 60 °C three times and pulverized with 2 ml phosphate buffer (pH 7.2) twice using a TissueLyser (Qiagen, Hilden, Germany). Homogenized sample was purified with a solid-phase extraction cartridge after enzymatic hydrolysis with β-glucuronidase, and the sample was quantitatively estimated by LC-MS. The LC-MS system (LC/MS 8050, Shimadzu, Japan) was composed of a Shimadzu Nexera ultra-performance liquid chromatograph and 8050 triple quadrupole mass spectrometer. All steroids were separated through a 1.9 μm particle C18 column (50 × 2.1 mm) and gradient elution with a mobile phase consisting of 0.1% formic acid in 5% acetonitrile (solvent A) and 0.1% formic acid in 95% acetonitrile (solvent B) at a flow rate of 250 μl/minute. In method validation, the linearity (r^2) was >0.992 within 0.1–20 ng/ml dynamic range, which were defined as the lowest concentrations with accuracy and precision of less than 20%.

Visual grading analysis

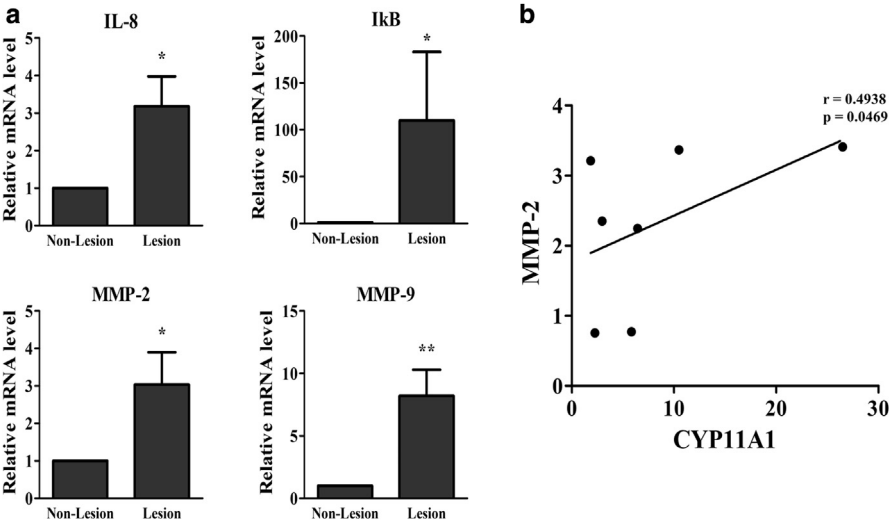
The degree of immunohistochemical staining intensities was evaluated using a 6-point scale, from 0 (unstained) to 6 (very intensively stained), by three independent dermatologists. The significance was evaluated using this score.

Statistical analysis

Data are presented as mean ± standard error. Significance was determined using the Wilcoxon matched-pairs signed rank test. *P*-values < 0.05 were considered statistically significant. The correlations between genes were determined by Pearson correlation analysis.



Supplementary Figure S1. Scoring of IHC staining intensity. (**a, b**) Immunohistochemical staining intensity of the sebaceous gland (a) and the epidermis (b) was graded using a visual grading score, from 0 (unstained) to 6 (very intensively stained) by three independent dermatologists. The scores are presented as dot plots. *$P < 0.05$ and **$P < 0.01$ versus nonlesional skin. IHC, immunohistochemical.



Supplementary Figure S2. Expression of NF-κB–related genes and correlation between *CYP11A1* and *MMP-2*. (**a**) Relative mRNA levels of IκB and related genes were analyzed from patients with rosacea. Nonlesional and lesional skin samples from the same individuals with rosacea were analyzed by quantitative real-time RT-PCR ($n = 10$). Data represent mean ± standard error of relative mRNA expressions, normalized to 36B4. *$P < 0.05$ versus nonlesional skin. (**b**) Correlation between *CYP11A1* and matrix metalloproteinase (*MMP*)-2 was analyzed by Pearson correlation analysis. The mRNA ratios (lesion:nonlesion) of genes obtained from quantitative real-time RT-PCR were used as variants ($n = 10$). Coefficient and *P*-value are presented in the graph. *MMP*, matrix metalloproteinase; RT-PCR, reverse transcriptase–PCR.

Supplementary Table S1. Human and Mouse Primer Sequences for Quantitative Real-Time RT-PCR

Gene Symbol	5′ Primer Sequence	3′ Primer Sequence
StAR	GAGAAGTCTTGCTTTATGGGCTCAAGAATG	GGTGCCTATGAAAGCAATAGGGAAACATGT
CYP11A1	GAGATGGCACGCAACCTGAAG	CTTAGTGTCTCCTTGATGCTGGC
CYP17A1	TGAGTTTGCTGTGGACAAGG	TCCGAAGGGCAAATAGCTTA
CYP21A2	TCCCAGCACTCAACCAACCT	CAGCTCAGAATTAAGCCTCAATCC
3β-HSD1	AGAAGAGCCTCTGGAAAACACATG	TAAGGCACAAGTGTACAGGGTGC
3β-HSD2	AGAAGAGCCTCTGGAAAACACATG	CGCACAAGTGTACAAGGTATCACCA
17β-HSD5	GTCATCCGTATTTCAACCGGAGTAAATTGC	CATCGTTTGTCTCGTTGAGATCCCAGA
11β-HSD1	TTGCTTTGGATGGGTTCTTC	AGAGCTCCCCCTTTGATGAT
11β-HSD2	GACCTGACCAAACCAGGAGA	GCCAAAGAAATTCACCTCCA
5α-red 1	CCAATGGCGCTTCTCTATGGACTTTGTAAA	CCCAGAGCTTGAAATTCTGACCTGTTACA
5α-red 2	GGCGACAGTTGCTTCTTAGCTATTGCT	GTGGGTATGAAGCCACACATGTACTTGGAT
GRα	CATTGTCAAG AGGGAAGGAA ACTC	GATTTTCAAC CACTTCATGC ATAGAA
IκB	GCAAAATCCTGACCTGGTGT	GCTCGTCCTCTGTGAACTCC
p65	GCGAGAGGAGCACAGATACC	CTGATAGCCTGCTCCAGGTC
p105	CCTGGATGACTCTTGGGAAA	TCAGCCAGCTGTTTCATGTC
p38	GCCCCAGTAGTCAGAAGCAG	TAGGGGCTGAAGAGAGGTGA
MMP-2	GGCCAAGTGGTCCGTGTG	GAGGCCCCATAGAGCTCC
MMP-9	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCGTCCTTAT
36B4	TGGGCTCCAAGCAGATGC	GGCTTCGCTGGCTCCCAC

Abbreviations: GRα, glucocorticoid receptor α; *MMP*, matrix metalloproteinase; RT-PCR, reverse transcriptase–PCR.

Supplementary Table 2. Characteristics of the Primary Antibodies Used in IHC Staining

Primary Antibody	Source	Dilution	Incubation Time	Antigen Retrieval
CYP11A1	Abcam	400:1	1 h 30 min	Autoclaved in Target Retrieval Solution (pH 6.0)
3β-HSD2	Abcam	100:1	2 h	Autoclaved in Target Retrieval Solution (pH 9.0)
17β-HSD5	Abcam	100:1	2 h	Autoclaved in Target Retrieval Solution (pH 9.0)
GRα	Cell Signaling Technology	100:1	1 h 30 min	Autoclaved in Target Retrieval Solution (pH 6.0)
pGRα	Cell Signaling Technology	100:1	2 h	Autoclaved in Target Retrieval Solution (pH 9.0)
p65	Cell Signaling Technology	100:1	20 h at 4 °C	Autoclaved in Target Retrieval Solution (pH 6.0)
p105	Abcam	100:1	20 h at 4 °C	Autoclaved in Target Retrieval Solution (pH 6.0)

Abbreviations: GRα, glucocorticoid receptor α; h, hours; IHC, immunohistochemical; min, minutes.