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Identification of novel glucocorticoid receptor-regulated genes involved in epidermal homeostasis and hair follicle differentiation

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Abstract

Despite that glucocorticoids (GCs), acting through the glucocorticoid receptor (GR) exert a pivotal role in skin physiopathology, specific genes regulated by GR in this tissue are largely unknown. We have used a transgenic mouse model overexpressing GR in epidermal basal cells and outer root sheath (ORS) of the hair follicle (HF) under the control of the keratin 5 regulatory sequences (K5-GR mice) to identify GR-regulated genes in mouse skin. We analyzed the transcriptomic profile of adult K5-GR skin as compared to non-transgenic adult mice by using oligonucleotide microarrays and identified 173 genes differentially regulated by GR in this tissue. Our data were further validated by semiquantitative RT-PCR and quantitative real-time PCR. We have identified a large subset of hair keratin intermediate filament (*krt*) and hair keratin-associated protein (*krtap*) genes, as well as several *hox* genes as GC-regulated. Since dysregulation of *krt*, *krtaps* and *hox* genes can cause hair disorders, as it occurs in adult K5-GR mice, our findings strongly suggest a role of GR in HF morphogenesis through the coordinated regulation of these hair-specific genes. In addition, we found that GR repressed several genes related to cell growth, such as the immediate early genes *fosb* and *c-fos*, according to the antiproliferative role described for this hormone receptor. By using cultured keratinocytes treated with GR-agonists and -antagonists, we demonstrated that down-regulation of *fosb* is mediated by GR. Identification of novel GR-regulated genes will help us to better understand the role of GCs as physiological modulators and pharmacological agents.
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1. Introduction

Glucocorticoid hormones (GCs) effects are mediated through the glucocorticoid receptor (GR), which acts through the so-called genomic and non-genomic actions exerting pleiotropic roles in many tissues (reviewed in Ref. [1]). Genomic actions of GR occur in the nucleus through binding of the ligand-activated receptor to multicomponent protein–DNA complexes assembled at response elements proximal to target promoters. Transcriptional regulation exerted by GR is dictated by the sequence of a given glucocorticoid response element (GRE), its organization within the chromatin and the activities of regulatory factors in a particular tissue. GR can regulate gene expression through DNA-binding-dependent and -independent mechanisms. The former requires ligand-induced dimerization of GR and binding to specific GREs, whereas the latter does not require DNA-binding of GR, but rather is mediated through interference with other transcription factors, such as NF-κB or AP-1 [2]. Nongenomic actions of GR have been recently demonstrated and are due to physical interaction of the receptor at the plasma membrane with the p85α regulatory subunit of the PI3K which, in turn, modulates AKT activity [3].

In skin, GCs have an enormous physiological and pharmacological relevance; however, specific genes regulated by GR in this tissue are still undeciphered. GCs are potent inhibitors of epidermal proliferation and effective antiinflammatory compounds, which explain that GC analogues are

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the most prescribed agents for many skin disorders undergoing with hyperproliferation and inflammation [4]. However, studying the impact of GR in skin development has been difficult since GR null mice die perinatally [5]; mice carrying a DNA-binding defective mutant of GR (GR dim) are viable and they show no overt skin phenotype [6].

In mammals, the epidermis is a stratified epithelium, able to self renew under both homeostatic and injury conditions by maintaining a population of mitotically active cells in the basal epidermal layer and hair follicles (HF) (reviewed in Ref. [7]). A major function of the epidermis is to act as a barrier that preserves the organism from potentially environmental damage. Acquisition of a competent barrier occurs during embryonic development and it is achieved through a correct balance between proliferation and differentiation. The process of differentiation implies that basal keratinocytes cease to proliferate, lose adherence to the basement membrane and migrate to outer layers called spinous, granular and cornified envelope. Thus, alterations in the processes of keratinocyte proliferation and differentiation may lead to a disturbed epidermal barrier, which will result in dehydration, uncontrolled thermoregulation and augmented susceptibility to infection, often causing skin disorders of keratinization and cornification (reviewed in Ref. [8]). So far, contradicting reports have described either a positive or a negative role of GR in regulating epidermal differentiation and epidermal barrier formation [8]. On one hand, GR has been reported to play a relevant role in the process of barrier acquisition *in utero* since GC injections accelerated barrier formation and, conversely, corticotropin-releasing hormone-deficient newborn mice exhibiting GC deficiency, had delayed maturation of the stratum corneum. In these mice, supplementation of GCs fully rescued skin phenotype [9]. On the other hand, short-term topical and systemic GC treatment to adult mouse skin disturbed epidermal barrier competence [10], mimicking the effects induced by psychological stress, which is likely mediated by increased endogenous GCs [11].

Our recent work has allowed the study of GR function in skin physiopathology by analyzing transgenic mice that overexpress GR in epidermal basal cells, outer root sheath (ORS) of the HF, and other stratified epithelia, under the control of the keratin K5 regulatory sequences (K5-GR mice) [12]. Adult K5-GR mice showed epidermal hypoplasia and an average decrease of 50% in the number of HFs, as compared with *wt* mice, as well as dysplastic HFs and orphan hyperplasic sebaceous glands [13]. This mouse phenotype is in agreement with the reported skin atrophy and HF dysplasia that occurs in human skin upon topical application of GC analogues, thus representing an excellent animal model for studying the underlying mechanisms of GR action. In this work, we aimed to identify gene regulated by GR in skin by comparing the gene expression profile of adult K5-GR transgenic mice and non-transgenic mice. We have found 173 genes that are differentially regulated in K5-GR skin, of which some are known targets of GCs and, importantly, we have identified for the first time a large number of novel GR-regulated genes involved in keratinocyte proliferation and differentiation as well as HF development. Altogether, the identification of several hair keratins (*krts*), hair keratin-associated proteins (*krtaps*) and *hox* as GR-regulated genes, among others, strongly suggests a role of GR in HF morphogenesis through the coordinated regulation of these hair-specific genes. Concomitant with enhanced differentiation, GR inhibited keratinocyte proliferation through repression of genes, such as the immediate early genes *fosb* and *c-fos*. We also demonstrated that down-regulation of *fosb* is receptor-mediated in cultured keratinocytes and *in vivo*.

2. Materials and methods

2.1. Animals and treatments

Animal experimentation was always conducted with accepted standards of humane animal care in our registered animal facility (CV-46007, Centro de Investigación Príncipe Felipe) in accordance with current Spanish and European normative which governs research with animals (Real Decreto 1201/2005, B.O.E. #252, 10 of October, 2005 and Convenio Europeo 1-2-3 del 18/3/1986).

K5-GR hemizygous mice of line 285 (C57Bl/6J × DBA) F1 mixed genetic background) have been previously reported [12]. Mice were housed in micro isolated boxes in which the air is filtered in both directions, allowing maximum isolation. Transgenic mice for these studies were generated by mating heterozygous K5-GR mice with *wild-type* (*wt*) mice, thus obtaining 50% of transgenic and 50% of non-transgenic progeny.

For gene expression profiling experiments, dorsal skin from K5-GR transgenic ($n = 6$) and non-transgenic male ($n = 6$) adult littermates (10-week-old) was used. Collection of mouse skin (1 cm × 1 cm) was performed in anesthetized mice that had been shaved 48 h before with a razor blade to avoid skin irritation. The piece of skin was then rapidly frozen in liquid nitrogen and kept at −80 °C until RNA extraction. Three animals of each genotype were pooled and RNA samples were used for the microarrays by duplicate. For semi-quantitative RT-PCR, an independent experiment was performed using RNA from dorsal skin of K5-GR transgenic ($n = 4$) and non-transgenic male ($n = 4$) adult littermates (10-week-old).

2.2. Cell culture and treatments

PB keratinocytes were grown in Dulbecco's modified Eagle's medium (BioWhitakker, Inc., Walkersville, MD) supplemented with 10% fetal calf serum (FCS, BioWhitakker, Inc.). For experiments, cells were starved in 0.1% FCS for 16 h, then treated for 8 h with either vehicle, Dex (Sigma, St. Louis, MO) 10 nM or 1 μM, or with Dex 1 μM plus Ru486 (BioMol) 10 μM. After that time, cells were stimulated with 20% FCS for 30 min. Experi-

ments were performed in triplicate and mean value ± S.D. estimated.

2.3. RNA preparation

Total RNA was isolated by using Trizol reagent (Invitrogen, Molecular Probes, Eugene, Oregon), following manufacturer's recommendations and its quality was determined using the Nano 6000 Chip in the Bioanalyzer from Agilent Technologies, Inc. (Palo Alto, CA).

2.4. Gene expression profiling, identification of regulated genes and statistical analysis

Experiments were performed by duplicate by using pooled RNA from K5-GR transgenic mice and *wt* littermates. Total RNA (10 μg) was used for cDNA synthesis according to the Affymetrix manual. Hybridization to GeneChips MOE 430 v2.0 arrays representing 45,101 transcripts and Expressed Sequence Tags (EST) followed by probing and scanning was performed according to the Affymetrix manual. The background subtraction and normalization of probe set intensities was performed using the method of Robust Multiarray Analysis (RMA) described by Irizarry et al. [14]. To identify differentially expressed genes, gene expression intensity was compared using a moderated *t*-test and a Bayes smoothing approach developed for a low number of replicates [15]. To correct for the effect of multiple testing, the false discovery rate, was estimated from *p* values derived from the moderated *t*-test statistics [16]. The analysis was performed using the affylmGUI Graphical User Interface for the limma microarray package [17]. Genes were considered to be significantly differentially expressed if *p*-values were <0.05 (controlling the expected false discovery rate to no more than 5%). Under these conditions, a minimal mean ratio of 1.4-fold was used as threshold for GR-dependent induced or repressed genes.

2.5. Semiquantitative RT-PCR

Reverse transcription was performed by using 1 μg of RNA and oligo-dT (Fermentas Inc., Burlington, Canada) followed by PCR using specific oligonucleotides for *krtap14* (forward: 5′-GAT GTG GTA CCC CTA CTT TCT-3′; reverse: 5′-GTT TTG CCA ATT ACA GGA ACT C-3′), *krtap16-7* (forward: 5′-TGT GCT GCA ACT ACT ACG G-3′; reverse: 5′-GCC ATA TCC ACA GCC ATA T-3′), *krtap16-8* (forward: 5′-CTA TGG CTG TGG CTA CCG-3′; reverse: 5′-ACA TGC AGT TCA GAA TTG GAG-3′), *Gprc5d* (forward: 5′-CAA ACT GCC CCT GTT CGC TAC TTC-3′; reverse: 5′-GAC CTG TAG AGT ATG CTC AGC TCA-3′) and *fosb* (forward: 5′-AGC TGT TGA CCC TTA TGA CAT G-3′; reverse: 5′-CGC CGA GGA CTT GAA CTT CAC TGT-3′). To achieve semiquantitative amplification, 25 cycles were used; bands corresponding to specific PCR products were quantitated by using Image Gauge Software (Fuji Photo Film, USA, Inc., Edison, NJ).

2.6. Quantitative RT-PCR

Oligoprimer pairs were designed by GeneTools software (Biotools Inc., Edmonton, Alberta, Canada) and their specificity was verified by blast in the GenBank database. Briefly, after Dnase1 treatment of total RNA and purification (Rneasy MinEluate Cleanup, QIAGEN) to remove contamination of genomic DNA, RNA quality was assessed (Bioanalyzer, AGILENT) and cDNA synthesis performed by reverse transcriptase.

cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based Realtime PCR quantification using the LightCycler Realtime PCR apparatus (Roche Inc. (Nutley, NJ)). Reagents were obtained from the same company and were used as described by the manufacturer. The conditions for PCR reactions were: denaturation at 95 °C for 10 s, annealing at 56–66 °C for 5 s and elongation at 72 °C for 7–13 s. The reaction was then heated for 3 s at 2 °C lower than the melting temperature of the DNA fragment. Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signal.

Data calculation and normalization was performed using second derivative and double correction method [18] and using the housekeeping genes hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) and ATP synthase O subunit (Atp5o). Hprt1 and Atp5o have shown to be genes having stable expression levels from embryonic life through adulthood in various tissues [18]. mRNA expression levels are expressed as ratio of $(\text{gene/housekeeping})_{\text{transgenic}}/(\text{gene/housekeeping})_{\text{wt}}$.

The following genes were evaluated by q-RT-PCR: *c-fos* (FBJ osteosarcoma oncogene, 1423100_at); *fosb* (FBJ osteosarcoma oncogene B, 1422134_at); *cyr 61* (cysteine rich protein 61, 1442340_x_at); krt2-10 (keratin complex 2, basic, gene 10, 1427365_at) and S100a3 (S100 calcium binding protein A3, 1421856_at).

3. Results

Despite the pivotal role of GCs in skin physiopathology, specific genes regulated by of GR in this tissue are largely unknown. To identify GR-regulated genes in mouse skin, we have compared the transcriptome of a transgenic mouse model overexpressing GR under the control of the keratin 5 regulatory sequences (K5-GR mice, [12]) *versus* non-transgenic mice. We isolated total RNA from dorsal skin of K5-GR transgenic and non-transgenic adult mice (10-week-old) and, after reverse transcription, the cDNAs were hybridized to GeneChips MOE 430 v2.0 oligonucleotide arrays (Affymetrix, www.affymetrix.com) which represent over 39,000 transcripts and EST. Microarray data revealed 173 genes or EST sequences that were significantly up- (50.9%) or down-regulated (49.1%) in K5-GR skin. Of all regulated genes, 31.8% were either not annotated or clones

Table 1
Classification of up- and down-regulated genes in transgenic skin overexpressing GR by functional categories ($n = 173$)

Up-regulated genes

Genbank	Description/annotation	Gene symbol/Alias	Fold change
Cytoskeleton organization and biogenesis			
NM_010666	Keratin complex-1, acidic, gene C29/keratin 27	krt1-c29/krt27	3.09
AK01464	4733401L19Rik	krt28	1.8
NM_010659	Keratin complex 1, acidic, gene 1	krt1-1/Ha1/krt31	2.04
NM_027983	Keratin complex 1, acidic, gene 3	krt1-3/Ha3/Ha4/krt33a	2.02
NM_027563	Keratin complex 1, acidic, gene 4	krt1-4/Ha2/krt342.99	
NM_016880	Keratin complex 1, acidic, gene 24	krt1-24/Ha5/krt35	1.93
NM_008476	Keratin complex 2, basic, gene 6a	krt2-6a/mK6a/krt6a	1.41
NM_019956	Keratin complex 2, basic, gene 6g	krt2-6g/mK6irs/krt71	3.08
NM_212485	Type II keratin Kb36	kb36/krt73	3.24
AF312018	Keratin complex 2, basic, gene 19	krt2-19/krt81	1.56
X99143	Keratin complex 2, basic, gene 10	krt2-10/MHb4/krt86	2.74
NM_016879	Keratin complex 2, basic, gene 18	krt2-18/Hb5/krt85	2.74
AK017442	Keratin associated protein 2-4	krtap2-4	3.48
BC060278	Keratin associated protein 3-1	krtap3-1	2.29
NM_025524	Keratin associated protein 3-3	krtap3-3	4.43
NM_001037822	Keratin associated protein 5-5	krtap5-5	1.48
NM_010672	Keratin associated protein 6-1	krtap6-1/HGTpII.1, HGTpII.2	4.1
NM_010673	Keratin associated protein 6-2	krtap6-2/HGTpII.4	2.51
D89901	Keratin associated protein 6-3	krtap6-3/HGTpII.3	3.01
AA727386	Keratin associated protein 8-1	krtap8-1	4.41
NM_010676	Keratin associated protein 8-2	krtap8-2/HGTpI alpha	1.82
NM_015741	Keratin associated protein 9-1	krtap9-1	2.44
NM_183189	Keratin associated protein 13-1	krtap13-1	1.66
NM_013707	Keratin associated protein 14	krtap14/Pmg-1/mKAP13	2.53
NM_013713	Keratin associated protein 15	krtap15/Pmg-2	5.08
NM_130872 AF345293	Keratin associated protein 16-3	krtap16-3	1.87
AV294513	Keratin associated protein 16-6	krtap16-6	2.61
NM_130875	Keratin associated protein 16-7	krtap16-7	2.28
AF345298	Keratin associated protein 16-8	krtap16-8	4.25
NM_010855	Myosin, heavy polipeptide 4, skeletal muscle	Myh4	1.5
Cell growth, differentiation and adhesion			
NM_008606	Matrix metalloproteinase 11	Mmp11	1.69
AK017438	RIKEN cDNA 5530401N06 gene	5530401N06Rik	1.62
NM_008125	Gap junction membrane channel protein beta 2	Gjb2	1.56
NM_009263	Secreted phosphoprotein 1	Spp1	1.47
NM_007400	A disintegrin and metallopeptidase domain 12	Adam12	1.4
Development/morphogenesis			
NM_010923	Neuronatin	Nnat	2.48
NM_016669	Crystallin, mu	Crym	1.77
Lipid metabolism			
NM_053080	Aldehyde dehydrogenase family 1, subfamily A3	Aldh1a3	1.71
NM_011044	Phosphoenolpyruvate carboxykinase 1, cytosolic	Pck1	1.67
NM_008256	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	1.65
NM_010174	Fatty acid binding protein 3, muscle and heart	Fabp3	1.56
NM_009605	Adiponectin, C1Q and collagen domain containing	Adipoq	1.56
Immune and defense response			
NM_153108	Beta defensin 8	Defb8	3.07
NM_152839	Immunoglobulin joining chain	Igj	1.54
NM_011315	Serum amyloid A3	Saa3	1.42
Signal transduction			
NM_053118	G protein-coupled receptor, family C, group 5, member D	Gprc5d	1.87
Apoptosis			
NM_020581	Angiopoietin-like 4	Angptl4	1.67
NM_207680	BCL2-like 11 (apoptosis facilitator)	Bcl2l11	1.46
Miscellaneous			
NM_011310	S100 calcium binding protein A3	S100a3	2.8

Table 1 (*Continued*)

Genbank	Description/annotation	Gene symbol/Alias	Fold change
Up-regulated genes			
NM_009532	X-ray repair complementing defective repair in Chinese hamster cells 1	Xrcc1	1.95
NM_009984	Cathepsin L	Ctsl	1.43
Down-regulated genes			
Cytoskeleton organization and biogenesis			
NM_080285	Cortactin binding protein 2	Cttnbp2	1.62
AK029836	Myosin IXa	4732465J09Rik	1.46
Cell growth, differentiation and adhesion			
NM_008036	FBJ osteosarcoma oncogene B	fosb	2.30
NM_010516 BB533736	Cysteine rich protein 61	cyr61	2.23
NM_177259	Disabled homolog 1 (Drosophila)	Dab1	1.72
NM_008973	Pleiotrophin	Ptn	1.59
NM_007595	Calcium/calmodulin-dependent protein kinase II, beta	Camk2b	1.58
NM_176930	RIKEN cDNA C130076O07 gene	C130076O07Rik	1.57
NM_145741	Growth differentiation factor 10	Gdf10	1.55
NM_010234 AV026617	FBJ osteosarcoma oncogene	fos	1.46
NM_013642	Dual specificity phosphatase 1	Dusp1	1.42
Development/morphogenesis			
NM_010456 AA98718	Homeo box A9	Hoxa9	1.8
NM_010462	Homeo box C10	Hoxc10	1.77
NM_013869	Tumor necrosis factor receptor superfamily, member 19	Tnfrsf19	1.58
NM_013834	Secreted frizzled-related sequence protein 1	Sfrp1	1.48
NM_010453	Homeo boxA5	Hoxa5	1.47
NM_028882	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	Sema3d	1.46
Lipid metabolism			
NM_024450 BE133651	Stearoyl-coenzyme A desaturase 3	Scd3	1.85
NM_001004146	RIKEN cDNA 4933439C20 gene	4933439C20Rik	1.64
NM_001042451	Synuclein, alpha	Snca	1.57
NM_146006	Lanosterol synthase	Lss	1.52
NM_001033599	Acyl-CoA synthetase long-chain family member 6	Acsl6	1.49
Immune and defense response			
NM_183257	Hepcidin antimicrobial peptide 2	Hamp2	1.88
NM_009244	Serine (or cysteine) peptidase inhibitor, clade A, member 1b	Serpin A1b	1.53
Signal transduction			
NM_008137	Guanine nucleotide binding protein, alpha 14	Gna14	1.57
NM_022420 BC02000	G protein-coupled receptor, family C, group 5, member B	Gprc5b	1.62
Apoptosis			
NM_007870	Deoxyribonuclease 1-like 3	Dnase1l3	1.87
NM_153407	cDNA sequence BC035295	BC035295	1.49

The 173 genes induced or repressed 1.4-fold or greater by GC were categorized based on protein function; however, many of these genes likely fit into more than one category.

that represented the same gene. We have focused this study on mRNAs coding for known genes. The complete list of genes is under Supplemental information. Table 1 shows the classification of up- or down-regulated genes into seven functional categories, according to their Gene Ontology annotation. Although genes were categorized based on protein function, many of these genes likely fit into more than one category.

Given that microarray data are not really quantitative, we aimed to validate our results by performing both semi-quantitative PCR and quantitative real-time-PCR (q-RT-PCR, Table 2). For this purpose, we performed an independent experiment using RNA obtained from a different set of non-transgenic and transgenic animals ($n = 4$ of each genotype) sex- and age-matched to reproduce the experimental groups used for the microarrays. Table 2 shows duplicate samples of up- and down-regulated genes analyzed by semi-quantitative PCR using specific oligonucleotides for *krtap 14*, *krtap16-7*, *krtap16-8*, *Gprc5d* and *fosb*. In addition, we performed q-RT-PCR to quantitate the transcript levels of another subset of positive- and negative-regulated genes in K5-GR skin. Our data confirmed that *krt2-10*/*krt86* and *S100a3* were up-regulated by GR whereas *c-fos*, *fosb* and *cyr61* were validated as down-regulated by GR (Table 2). Overall, our results demonstrate an excellent correlation between data obtained by the microarray experiment and semi-quantitative PCR and q-RT-PCR methods performed in an independent experiment.

Table 2
Quantitation of several genes up- and down-regulated in transgenic skin over expresing GR vs. *wt* obtained by microarray analysis, by semi-quantitative PCR (Sq PCR) and quantitative real-time PCR (q-RT-PCR)

Fold regulation	Microarrays data	Sq PCR[a]	q-RT-PCR[b]
Up-regulated genes			
krtap14	2.41	2.0	nd
krtap16-7	2.22	2.6	nd
krtap16-8	4.37	3.3	nd
Gprc5d	1.90	3.4	nd
krt2-10	4.36	4.9	31
S100a3	2.75	1.4	3.0
Down-regulated genes			
c-fos	1.63	nd	2.9
fosb	2.30	3.2	6.1
cyr61	2.25	1.4	1.7

RNA was obtained from *wt* and K5-GR skin in an independent experiment and Sq PCR and qRT-PCR were performed as indicated under Section 2. Mean value of duplicates is shown in all cases. nd means not determined.

[a] For Sq PCR, specific oligonucleotides corresponding to the indicated genes were used and normalized to β-actin levels.

[b] For q-RT-PCR, indicated transcript levels were quantitated using two housekeeping genes and expressed as a ratio of $(\text{gene/housekeeping})_{\text{transgenic}}/(\text{gene/housekeeping})_{\text{wt}}$.

A large proportion of genes identified in this microarray analysis (26.6% of total and 52.3% of the up-regulated genes), belong to the hair keratin intermediate filament (*krt*), which are the major structural proteins of hard epithelial tissues, and hair keratin-associated protein (*krtap*) families, specifically involved in differentiation of the HF and or sweat/sebaceous glands [19]. Of these, we have listed only the annotated genes encoding for known proteins (Table 1) and followed the new consensus nomenclature for mammalian keratins as described in Schweizer et al. [19]. All identified keratins were either hair keratins (type I or II) or epithelial keratins specifically expressed in the HF. Genes encoding for hair acidic keratins (type I) mapped on chromosome 11, basic keratins (type II) mapped on chromosome 15 whereas krtaps localized on gene clusters mapping in chromosomes 11 and 16. KRT and KRTAP families comprise more than 70 different proteins which are the main components of hair fibres produced by HFs [20]. KRTAPs are essential for the formation of a rigid and resistant hair shaft through their extensive disulfide bond cross-linking with abundant cysteine residues of hair keratins. KRTAPs include the high-sulfur/ultra-sulfur and the high-glycine-tyrosine proteins, each group following a precise spatio-temporal expression pattern in the hair follicle, either in the proliferative matrix cells, the cells of the cortex or cuticle. Note that, among the hair keratin up-regulated genes (Table 1), *krt1-1*, *krt1-3*, *krt1-4*, *krt2-10*, *krtap8-1* and *krtap14* localized to the hair cortex, as previously shown by *in situ* hybridization. Moreover, all GR-regulated hair acidic keratins and most of the krtaps were involved in terminal differentiation of follicles in the growth stage or anagenic phase [21,22]. This relatively high abundance of transcripts that are normally expressed in anagenic HFs is most likely due to the fact that skin samples used for this microarray analysis were collected from 10-week-old mice depilated 48 h before, a procedure normally inducing the hair growth stage.

The identification of several *hox* genes (*hoxa5*, *hoxa9*, *hoxc10*) as regulated by GR (Table 1) deserves particular attention since they constitute the largest group of patterning molecules in HF development and cycling and have been involved in transcriptional regulation of *krtaps* (reviewed in Ref. [23]). The positive role of GR in HF differentiation was further supported by the up-regulation of the signal transduction G-protein coupled receptor (*Gprc5d*) and peptidyl arginine deiminase, type III (*Padi3*) genes, both expressed in the anagen HFs [24,25]. Altogether, the identification of several hair *krts*, *krtaps* and *hox* as GC-regulated genes, among others, strongly suggests a role of GR in HF morphogenesis through the coordinated regulation of these hair-specific genes.

Since GR exerted antiproliferative effects in K5-GR mice, concomitant reduced expression of genes related to cell growth was expected. Accordingly, we detected down-regulation of several immediate early genes, such as *fosb* and *c-fos* (Tables 1 and 2), which appears as a mechanism for keratinocyte growth inhibition mediated by GR *in vivo*. Moreover, GR negatively regulated other markers known to be involved in regulation of cell growth and cell cycle, including *cyr61*, *Ptn*, *Camk2b* and *Gdf10*. The identified genes are candidates to mediate the reported growth inhibitory role of GR in keratinocytes, thus contributing to the epidermal hypoplasia exhibited by adult K5-GR mice.

Our results confirmed GR-dependent repression of *c-fos in vivo* and identified *fosb* as a novel gene regulated by GR in skin. Given that *fosb* was originally identified as an immediate early gene and since no specific function has been assigned to FosB in keratinocytes so far, we aimed to study whether *fosb* was specifically regulated by GC action in keratinocytes. To this, we examined *fosb* transcript levels in response to the synthetic GC analogue dexamethasone (Dex) in the non-transformed keratinocyte cell line PB (Fig. 1). Cells were starved in 0.1% serum for 16 h and either left untreated or treated with the indicated concentrations of Dex for 8 h before

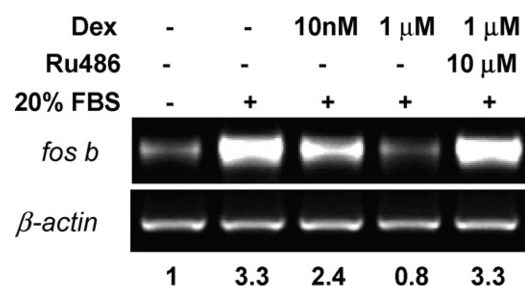

Fig. 1. Regulation of *fosb* by activated GR. Cultured cells of the non-transformed keratinocyte line PB were starved in 0.1% serum for 16 h and either left untreated or treated with Dex (10 nM or 1 μM) for 8 h or Dex 1 μM plus the GR antagonist Ru486 (10 μM) before being stimulated with 20% serum for 30 min. Cells were collected, RNA extracted and *fosb* transcript levels analyzed. Basal transcript levels of *fosb* were arbitrarily set as 1 and values expressed as relative to untreated cells.

being stimulated with 20% fetal bovine serum (FBS) for 30 min. Cells were collected, RNA extracted and *fosb* transcript levels analyzed. As expected, the mRNA corresponding to *fosb* was induced (3.3-fold) by FBS. Dex treatment inhibited this induction in a dose-dependent manner; at 1 μM Dex, *fosb* transcript levels had reached basal levels. In addition, combined treatment of Dex with the GR-antagonist Ru486 completely reversed the effect of the ligand, thus reinforcing the idea that activated GR negatively regulates *fosb* (Fig. 1).

By using a transgenic mouse model of gain-of-function, we have identified novel GR-regulated genes in skin, which highlight that GR plays a major role in HF differentiation and epidermal homeostasis. These studies may contribute to better understand the role of GCs as physiological modulators in this tissue.

4. Discussion

One of the most relevant findings of this study is that a great percentage of the GR-regulated genes identified have been previously reported as specifically expressed in the HF following a precise spatio-temporally controlled pattern. The mature HF consists of an outer root sheath contiguous with the basal layer of the epidermis, an inner root sheath serving as the channel from which the hair exits the skin surface, and the hair shaft itself [7]. Remarkably, the HF is the only organ in the mammalian body which undergoes cyclic transformations and regeneration during its entire life-time. In these periods, a new follicle replaces the hair that degenerated and was released, through stages of rapid growth (anagen), apoptosis-driven regression (catagen), and quiescence (telogen). It is known that different hormones, including GCs, govern growth and development of the HF along with the sebaceous and apocrine glands, and the arrector pili muscle, which form the pilosebaceous unit (PSU). Recently, it was demonstrated in organ-cultured human scalp HFs that PSUs behave as independent peripheral neuro-endocrine organs, resembling the hypothalamic-pituitary–adrenal (HPA) axis [26]. These findings constituted a breakthrough for understanding how steroid hormones modulate hair growth and differentiation, even in the absence of endocrine, neural, or vascular systemic connections.

Our data provide evidences at the molecular level supporting a prominent role of GR in HF morphogenesis through regulation of numerous hair-specific genes, including *krts*, *krtaps* and *hox* family genes (Tables 1 and 2). Given that nearly all members of the *hox* gene family have been described in either skin or hair, or both, in mouse and human, the fact that only a subset of *hox* genes (*hoxa5*, *hoxa9*, and *hoxc10*) is down-regulated in K5-GR mice supports a great specificity of GR action in modulating hair development through these transcription factors. To date, it is not clear whether or not KRTAP mutations are involved in human hair disorders, although several transgenic mouse models with hair defects exhibited changes in their KRTAP gene expression patterns, being Hoxc13 the best characterized. Hoxc13 expression was detected in developing hair follicles, tongue and nail, all typical regions of hair keratin and possibly KRTAP expression. Interestingly, both Hoxc13-deficient mice and Hoxc13-overexpressing mice showed a fragile hair phenotype [27,28]. Microarray analysis revealed that Hoxc13 overexpression resulted in the down-regulation of a great number of high sulphur and high glycine tyrosine *krtap* genes, including *krtap14*, *krtap15* and several members of the *krtap16* family [27]. Remarkably, all these genes have also been found differentially expressed in K5-GR mice, as shown in Tables 1 and 2, suggesting that they may be useful markers of fragile hair disorders. However, additional studies in patients would be necessary to draw any conclusion about the role of these genes in human hair disorders.

It is still a matter of debate whether GR favors or disturbs epidermal barrier formation [8]. This is most likely due to the different model systems that have been used to approach the issue. Previous evidences supporting that GR accelerate epidermal barrier formation come from studies in fetal mouse skin using physiologic concentrations of GCs whereas a negative role of GR has been reported in mouse adult skin topically treated with pharmacological doses of GCs for a short period [9,10]. Our transgenic model represents a more direct approach to study GR function since the receptor is targeted at proliferating basal keratinocytes, from which all epidermis originates, starting at the earlier stages of epithelial development (12.5 dpc), before stratification occurs [29]. We previously demonstrated that constitutive targeting of GR transgene elicited epidermal hypoplasia and reduced HF number at late embryo stages and newborn mice as well as in adult mice [12,13]. However, whereas the expression of loricrin, an epidermal marker of terminal differentiation, was increased at the transcript and protein level in K5-GR newborn mice, we found no changes in adult individuals (data not shown). These data suggest that GR-mediated regulation may be differential in the perinatal period *versus* the adult age.

Interestingly, the present study has revealed that some genes previously reported to be essential in epidermal barrier formation were regulated in the skin of transgenic mice overexpressing GR. Among them, there are genes encoding for enzymes with protease or anti-protease activity as well as several enzymes involved in steroid and lipid metabolism (Table 1). We highlight here 3-hydroxy-3-methylglutaryl-CoA synthase (Hgmcs2), an enzyme specifically involved in steroid and lipid biosynthesis and previously described as a transcriptional target of GCs in other tissues [30]. *Hmgcs2* is a transcriptional target of members of the nuclear receptor superfamily PPAR/RXR in the epidermis, as shown by various epidermis-specific mouse models [31]. We hypothesize that the positive regulation of *Hmgcs2* in GR-overexpresing skin would argue in favor of a protective role of GR in regulating the epidermal barrier homeostasis. Given the key role of epidermal lipids in barrier formation, the identification of the regulatory mechanisms modulating the expression of

genes involved in lipid biosynthesis is of enormous importance to understand the molecular basis of several human skin disorders of keratinization and cornification.

Collectively, our study approaching the identification of GR-regulated genes in skin by using an *in vivo* model appears as very reliable since we have used complementary approaches to the microarray experiment, such as and semi-quantitative PCR and q-RT-PCR methods. Anyhow, we have found some discrepancies respect to previous reports [32], such as the negative-instead of positive-regulation of the dual specificity MAPK phosphatase *Dusp1* by GR. This is most likely due to cell type differences and, to our knowledge, neither the expression pattern of *Dusp1* nor its regulation by GCs have been previously described in keratinocytes. Similarly, and despite NF-κB repression has been involved in the antiproliferative effect of GCs in other cell types [33], we did not find changes in *ikbα* transcript levels neither in the microarray studies nor by Sq PCR (data not shown). Although such a role for NF-κB in keratinocytes can not be ruled out, our data strongly support that GR-mediated growth inhibition is partially exerted through interference of the hormone receptor with the activator protein (AP)-1 signaling pathway, which plays a key role in cell growth, differentiation and oncogenesis in different tissues [34].

In skin, GR/AP-1 cross-talk is particularly relevant since AP-1 is crucial for many processes in skin physiopathology including epithelial development, skin inflammation and cancer [35,36]. The AP-1 family of transcription factors is composed of the *fos* (*c-fos*, *fosb*, *fra-1*, *fra-2*) and *jun* (*c-jun*, *junb*, *junD*) gene families. Different hetero- and homo-dimers combination is possible to form AP-1 complexes, with which GR physically interacts resulting in inhibition of the AP-1 transactivating function [36]. As an additional mechanism, GCs also play a role in repressing the action of MAPKs, such as extracellular regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), further reducing AP-1 activity. Most of the genes belonging to the *fos* and *jun* families contain AP-1 binding sites in their promoters, therefore, diminished AP-1 transactivation results in their reduced transcription. This has been described for *c-fos*, which is transcriptionally down-regulated by GR through GR/AP-1 negative interference in many cell types, including keratinocytes [6].

Although the expression of FosB in mouse and human epidermis has been reported as undetectable for several groups [32,37], other reports have shown *fosb* expression in mouse epidermal tissue [38]. Our results suggest that *c-fos* and *fosb* are dispensable for the expression of terminal differentiation-associated proteins and the maintenance of differentiation in K5-GR mice, in agreement with the lack of skin phenotype of *c-fos*- and *fosb*-deficient mice (reviewed in [39]). However, c-fos plays a determinant role in skin responses such as tumor progression as the absence of c-fos caused malignant conversion of skin tumors [40]. Contrary to *c-fos*, *c-jun* transcript was induced by Dex in *wt* and GRdim mouse skin, likely mediated by the DNA-binding independent function of GR on Jun/Jun homodimers [6]. We did not find variation in the levels of c-jun in K5-GR skin as compared to *wt* mice (not shown). Since c-Jun is continuously available whereas c-Fos and FosB are decreased in GR-overexpressing skin, formation of Jun/Fos heterodimers, the most potent activators, is unfavored. That implies that other AP-1 complexes, such as Jun homodimers are more feasible and might differentially regulate another subset of AP-1-target genes. This might be an explanation for the observed up-regulation of matrix metalloproteinase (MMP)-11/stromelysin-3 (Table 1), which contains AP-1 binding sites in its promoter. It has been reported that its positive regulation is mediated through increased Jun expression and binding of Jun/AP-1 complexes [32].

Our previous work has shown that GR strikingly altered epidermal and HF development [12,13]. By using a genomic approach, we here report the identification of numerous genes crucial in epidermal homeostasis and HF morphogenesis as GR-regulated genes. Our findings may contribute to elucidate the precise mechanisms by which GC hormones modulate skin physiopathology.

Conflict of interest

We here declare that there is no conflict of interest of any of the participant institutions.

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