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# Glucocorticoid Receptor Signaling in Skin Barrier Function

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Lisa M. Sevilla and Paloma Pérez

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## Abstract

Glucocorticoids (GCs) are steroid hormones that regulate the physiology of all tissues and mediate stress responses. Synthetic GCs are commonly prescribed to treat chronic inflammatory conditions including the prevalent skin diseases—psoriasis and atopic dermatitis. GCs act through the GC receptor (GR, NR3C1), a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. In skin, GC therapeutic efficacy is due to the antiproliferative and anti-inflammatory actions of GR; however, in the long term, these benefits are accompanied by adverse profiles including skin atrophy, increased fragility, dehydration, augmented susceptibility to infections, and delayed wound healing. While the therapeutic actions of GC treatments have been extensively studied, only more recently has the physiological role of GR been addressed in skin. *In vivo* and *in vitro* studies in mouse and man have revealed an important function for GR in skin homeostasis. In particular, the characterization of gain- or loss-of-function mouse models has demonstrated relevant roles for GR in skin pathophysiology. The actions of GR are context dependent, and in skin, it regulates different gene subsets and biological processes depending on developmental stage and physiological state. Finally, recent findings emphasize the relevance of local GC biosynthesis and appropriate GR expression in maintaining skin homeostasis.

**Keywords:** glucocorticoids, glucocorticoid receptor, skin barrier, keratins, transcriptional regulation, keratinocyte proliferation and differentiation, inflammatory skin diseases

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## 1. Introduction

Endogenous glucocorticoids (GCs) are steroid hormones that regulate a vast array of biological processes, including development, cellular proliferation and differentiation, metabolism,

immunity, and stress response [1, 2]. In response to physiological cues and stressors, the hypothalamic-pituitary-adrenal (HPA) axis coordinates the systemic production and secretion of GCs from the zona fasciculata of the adrenal glands. Importantly, extra-adrenal, local synthesis of GCs occurs in multiple tissues including the thymus, intestine, brain, and skin, which express functional equivalents of the HPA axis [3, 4]. The tissue availability of these steroid hormones is further regulated by locally expressed  $11\beta$ -hydroxysteroid dehydrogenase type I and II enzymes (HSD11B1 and 2), which catalyze the interconversion between active (cortisol and corticosterone) and inactive (cortisone and 11-dehydrocorticosterone) forms in humans and rodents, respectively [5].

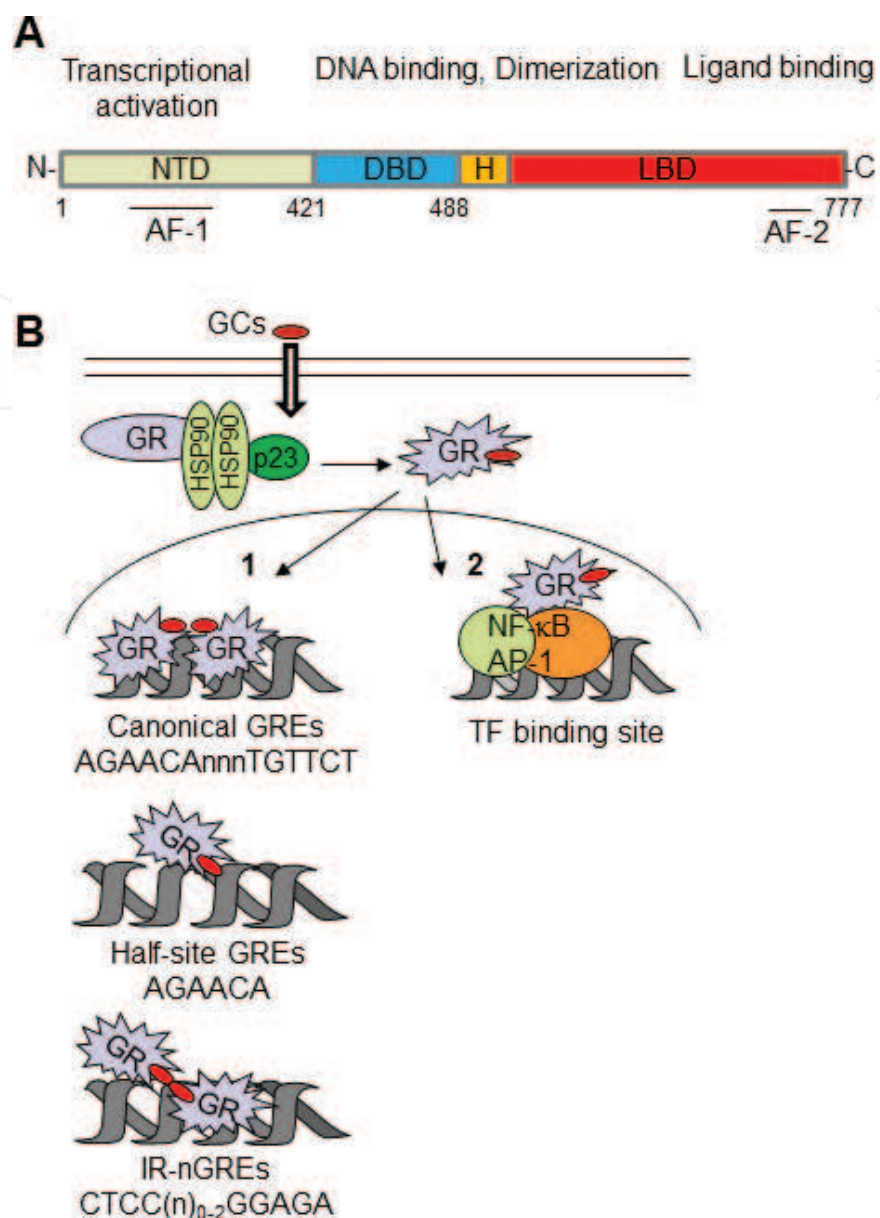
The GC receptor (GR) is a ubiquitously expressed ligand-dependent transcription factor (TF) that belongs to the nuclear receptor (NR) superfamily and mediates the physiological and pharmacological actions of GCs [2, 6]. The actions of GR on transcription are highly context specific, with strikingly different subsets of genes being regulated across different cell types, developmental stages, and pathophysiological states [7].

For more than half a century, synthetic GCs have been used clinically to manage autoimmune diseases due to their potent anti-inflammatory and immunosuppressive properties [8]. However, their therapeutic use is limited by a host of undesired side effects ranging from osteoporosis, obesity, and muscle wasting to skin atrophy and impaired wound healing [9]. Nevertheless, GCs are still the most effective and widely prescribed therapeutic agent for prevalent inflammatory skin diseases including atopic dermatitis, with a lifetime prevalence of 10–20% in developed countries; and psoriasis, affecting 2% of the European and North American population [10, 11]. Keratins, comprising approximately 30% of epidermal proteins and more than 90% in hair follicles, play key roles in maintaining skin barrier function. In fact, keratin mutations are associated to many genodermatoses and their expression is altered upon inflammation, wounding, or tissue damage. Hormones, and in particular GCs, are major regulators of keratin gene expression in healthy and diseased skin [12].

### 1.1. The glucocorticoid receptor: structure and function

GR was the first identified member of the NR superfamily and characteristic of this group; its domains comprise an N-terminal transactivation domain, a central DNA-binding domain, and a C-terminal ligand-binding domain (**Figure 1A**) [2, 6]. The N-terminal domain is responsible for interactions with the transcriptional machinery as well as coregulators via the activation function (AF)-1 region. The majority of sites for posttranslational modification, including phosphorylation, ubiquitination, and sumoylation are located in this domain, allowing for modulation of receptor function and contributing to context specificity [2, 6]. The highly conserved DNA-binding domain has two zinc finger motifs responsible for recognizing and binding to GC response elements (GREs) as well as a nuclear localization signal. A flexible hinge region connects the DNA-binding domain with the C-terminal ligand-binding domain that contains a hydrophobic pocket for GC binding, an AF-2 region for ligand-dependent interactions with coregulators and a second nuclear localization signal. Receptor dimerization is mediated by sequences in the DNA- and ligand-binding domains [6, 13].

The gene encoding GR, *NR3C1*, contains nine exons with the open reading frame being encoded by exons 2–9. The *NR3C1* transcript can undergo alternative splicing resulting in the



**Figure 1.** Glucocorticoid (GC) signaling through the GC receptor (GR). (A) Functional domains of the GR. NTD, N-terminal transactivation domain; DBD, DNA-binding domain; H, hinge; LBD, ligand-binding domain; AF, activation function. (B) Scheme of GC-regulated transcription. In the absence of ligand, GR is mostly associated to cytoplasmic multiprotein complexes including chaperones. Upon GC binding, activated GR is released from this complex, dimerizes, translocates to the nucleus, and binds directly to DNA (1) or to other TFs via tethering (2) to regulate gene expression. GR-bound genomic regions include canonical GRE elements (imperfect palindrome GREs), half-site GREs, and inverted repeat (IR)-negative (n)GREs.

generation of at least five isoforms [2], with GR $\alpha$  and GR $\beta$ , differing at their C-termini, being the most studied. GR $\alpha$  binds to ligand and carries out classical receptor functions, and will be referred to as simply GR elsewhere in this chapter. On the other hand, GR $\beta$  is incapable of binding to GCs and acts as a dominant-negative inhibitor of GR $\alpha$ . GR $\beta$  is usually expressed at a lower level than GR $\alpha$ , but alterations in the ratio between these isoforms are associated with GC sensitivity and autoimmune disease [2]. The expression of these splice variants in healthy and diseased skin is only beginning to be explored. For example, increased GR $\beta$  was found in patients with severe atopic dermatitis that were unresponsive to GC treatment [14]. However,

a more recent study failed to establish a correlation between isoform ratio and sensitivity to GC therapies in inflammatory dermatoses [15]. Another layer of complexity is added by the discovery that seven GR protein isoforms are generated through alternative translational initiation [2]. These isoforms are differentially expressed across tissues, and while all are capable of binding DNA and ligand, the N-terminal truncations do alter subcellular localization and transcriptional activity. Their precise role in skin pathophysiology remains to be determined.

In the absence of ligand, the majority of GR is sequestered in the cytoplasm in a multiprotein complex that includes chaperones (HSP70, HSP90, and p23) and immunophilins. The classical model of GR activation is that upon binding to GCs, GR dissociates from this complex, dimerizes, translocates to the nucleus, and binds to GREs or to other TFs, regulating gene expression (**Figure 1B**). GR-bound genomic regions are widespread throughout the genome, and are not necessarily found in close proximity to target genes [16, 17]. The canonical GRE sequence is 5'-AGA ACA nnn TGT TCT-3', an imperfect palindrome that contains two half sites and a three base pair spacer. This classical mechanism of transcriptional regulation, which is dependent on DNA-binding and dimerization, was denominated as transactivation. In contrast to other TF-binding sites, GREs show a great deal of variability, with changes in the majority of positions not impeding GR binding [16]. Remarkably, the very sequence of the GRE was demonstrated to affect GR conformation and transcriptional activity, functioning as an allosteric regulator of this TF [18]. Classical GREs are not the only mode for GR chromatin interaction, as GR can also regulate transcription by binding to half site sequences [19], such as those found near the epidermal keratin (K)5, 14, 6, and 17 genes [20]. These keratin response elements allow the simultaneous binding of at least two NRs, which widens the hormone-dependent transcriptional control of keratin expression [21].

Finally, the most recently identified GR-bound regulatory sequence is the inverted repeat (IR)-negative (n) GRE with the consensus sequence 5'-CTCC (n)<sub>0-2</sub> GGAGA-3' [22]. These sites are termed negative as they promote the assembly of cis-acting corepressor complexes that recruit histone deacetylases resulting in gene repression. Other mechanisms by which GR modulates transcription are by binding composite elements, or juxtaposed binding motifs, with other TFs; and by modulating the transcriptional activity of other TFs by protein-protein interactions, independent of DNA-binding and receptor dimerization, known as tethering (**Figure 1B**) [23]. It was long assumed that tethering, and in particular GR interference with prototypical proinflammatory TFs such as NF- $\kappa$ B and AP-1, mediated the beneficial anti-inflammatory actions of GR, while transactivation was responsible of the adverse side effects. This was mostly based on studies using a GR single mutant (A458T), which impeded dimerization and impaired transactivation of GRE-containing target genes while allowing tethering via AP-1 and NF- $\kappa$ B. This dogma was recently challenged by demonstrating that GR<sup>A458T</sup> could indeed dimerize and bind DNA in a subset of GREs in live cells although with reduced efficiency [13].

More than 20 GR chromatin immunoprecipitation sequencing (ChIP-Seq) studies in different cell/tissue types have been published thus far [7], providing functional insights. For instance, GR largely relies upon other factors to create and maintain open chromatin, contributing to its context specificity [7]. Thus far, the only GR ChIP-Seq experiment performed in keratinocytes evaluated a short treatment with dexamethasone (Dex) [24]. Following a restrictive analysis, 104 GR-bound genomic sites were identified. This small number contrasts with the thousands of targets identified in other cell types [7], however, is in line with transcriptomic data

following 4 h of GC treatment in primary human keratinocytes [25]. Despite the limited number of GR-bound genomic sites detected, their analysis provided important information about functional interactions between GR and other TFs in keratinocyte gene regulation, as several overrepresented TF motifs were identified, including KLF (43%) and AP-1 (28%) [24]. Further experimentation revealed that GR and KLF4 cooperate to regulate the expression of the anti-inflammatory genes *Gilz/Tsc22d3* (GC-induced leucine zipper) and *Zfp36/Tristetraprolin*.

Ligand binding of GR also results in rapid actions, occurring within seconds to minutes, which occur independently of transcription or translation, commonly referred to as nongenomic actions [9]. For instance, ligand-bound GR interferes with the phosphatidylinositol-3-kinase signaling pathway and the downstream kinase AKT, critical for cell proliferation and survival. This interference has been demonstrated in mouse skin and cultured keratinocytes and contributes to the antitumor effects of GCs in this tissue [26].

## 1.2. Adrenal and cutaneous GC production

GC signaling represents a complex homeostatic system that mediates fundamental tissue-specific processes during development as well as adaptive responses to stress. The importance of appropriate GC levels for normal tissue function is clearly illustrated in extreme situations of hormone imbalances where chronic excess or deficiency leads to pathological conditions, such as Cushing's or Addison's disease, respectively [1]. In both scenarios, dysfunctional responses to this hormone can result in differential tissue sensitivity and manifest as clinical GC resistance (e.g., primary generalized glucocorticoid resistance) or hypersensitivity [27]. Remarkably, cutaneous abnormalities in Cushing's patients—skin atrophy, increased fragility and easy bruising, elevated infection risk, and impaired wound healing—are very similar to those found in aging and also after long-term/high-dose GC pharmacological treatments [28, 29].

As GC synthesis and release is tightly controlled by the HPA axis, this neuroendocrine system acts as a major regulator of skin integrity and function. Stress or physiological conditions stimulate the production of corticotropin-releasing hormone receptor (CRH) from the hypothalamus, which in turn induces the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which promotes the synthesis and release of GCs from the adrenal cortex. Under physiological conditions, GC release shows a diurnal pattern controlled by the circadian clock, with peak levels linked to the beginning of daily activity. GCs can inhibit their own production by a feedback mechanism where GR shuts off the secretion of CRH and ACTH [1, 2]. Around 90% of circulating cortisol is bound with high affinity by corticosteroid-binding globulin, while the remaining 10% of circulating GCs consist of roughly equal proportions of cortisol and cortisone. The free circulating cortisone functions as a reservoir of inactive steroid that can be converted into active GCs in a tissue-specific manner.

The discovery that the skin behaves as a local HPA axis analog that is able to produce steroidogenic enzymes and GCs constituted a major breakthrough for understanding alternative mechanisms by which GCs exert their actions in homeostatic and pathological conditions [3, 30]. Since skin is continuously exposed to external perturbations, tissue-specific synthesis of GCs represents an ideal response mechanism and recent studies suggest that systemic and local HPA axes are interconnected [31]. Recent findings emphasize the relevance of the local GC biosynthetic pathway in maintaining skin homeostasis as local GC deficiency and

reduced GR expression in psoriatic lesions contributed to the pathogenesis of the disease. These findings should be considered for designing novel GC-based strategies for treating skin diseases [32, 33].

HSD11B1/HSD11B2 activities maintain appropriate GC levels and constitute a key mechanism to modulate GR function at the prereceptor level both in plasma and in peripheral tissues [5]. HSD11B2 expression and activity is key in the renal and cardiovascular system where GC inactivation is required to avoid the overactivation of the closely related mineralocorticoid receptor, favoring instead binding of the mineralocorticoid aldosterone [34, 35]. In human and mouse skin, HSD11B1 is highly expressed in the epidermis and dermis, with higher levels in differentiating keratinocytes [29, 36]. HSD11B2 has been also detected in the suprabasal epidermis of human and developing mouse skin as well as in sweat glands, an important target for aldosterone-mineralocorticoid receptor regulation [37–40].

## 2. GR function in skin development

### 2.1. Development of the epidermis and its appendages

Barrier formation begins with epidermal commitment around E10.5 when surface ectoderm cells begin to express the keratinocyte-specific intermediate filament proteins K5 and 14 [41]. By E14.5, keratinocytes stratify, express K1 and 10, and begin terminal differentiation forming the postmitotic spinous and granular layers and the outermost SC [42]. The SC is composed of fully differentiated dead keratinocytes, or corneocytes (described as bricks) surrounded by specialized extracellular lipids (or mortar), extruded by lamellar bodies at the granular layer-SC interface [43]. Elegant studies subjecting mouse embryos to whole mount dye exclusion assays revealed that the epidermal permeability barrier acquisition is patterned, beginning at initiation sites at ~E16.5 and spreading in moving fronts until completion by ~E17.5 [44]. Hair follicle patterning and morphogenesis begins at E14.5 when placodes, or clusters of basal keratinocytes, form stimulated by inductive signals from the dermis [45, 46]. Sebaceous glands begin to form toward the end of gestation, and pilosebaceous units continue maturation post-natally. Eccrine sweat glands begin to form late in mouse development and are restricted to paw pads. All epidermal appendages contribute to skin function, as sweat glands and hair contribute to thermoregulation and sebaceous glands secrete lipid-rich sebum that waterproofs the skin and has antimicrobial activities. Importantly, defects in epidermal differentiation during development can lead to inflammatory skin disease later in life [45].

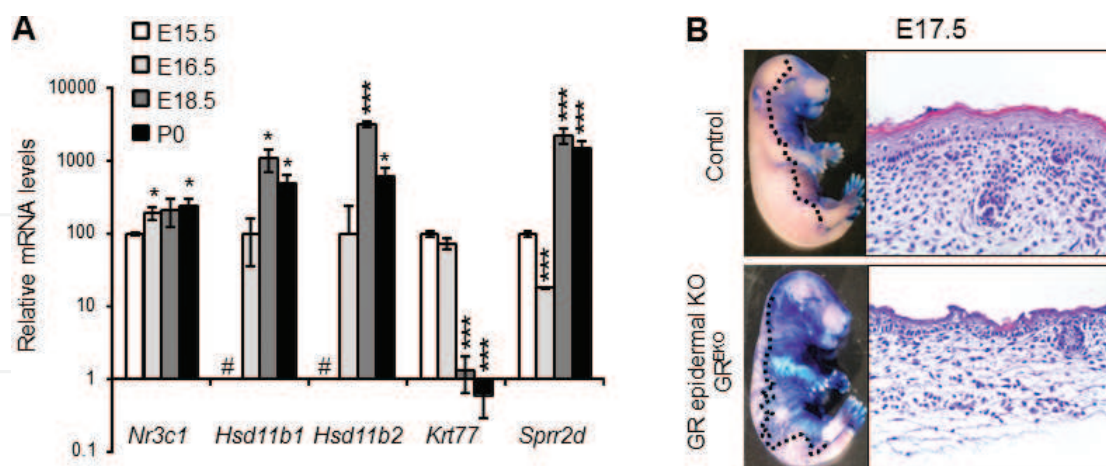
The signaling pathways orchestrating epidermal development and keratinocyte terminal differentiation have been extensively studied [41, 42, 46]. The master regulator TF p63 is crucial for early epidermal specification and differentiation, but its expression must decrease in keratinocyte terminal differentiation, a process regulated by functional interactions between more than 50 TFs, including GR [41, 47, 48]. A keratinocyte cell line derived from mice deficient in epidermal GR (see Section 3.2) showed defects in terminal differentiation, with an increased expression of the predominant isoform  $\Delta Np63$  [24]. Further experimentation showed that GR inhibits p63 expression, fitting with its proposed role as an inhibitor of the early stages of

keratinocyte differentiation [24, 25]. Heterozygous mutations are present in the *TRP63* gene in patients with different ectodermal dysplasias, developmental disorders in which the epidermis and its appendages fail to develop normally. Mouse models with reduced expression of p63 mimic features of the human disease [47]. Strikingly, transgenic mice with ectodermal overexpression of GR also exhibit features of ectodermal dysplasia, strong evidence of functional interactions between these TFs [49, 50].

## 2.2. GCs and skin barrier formation

During development, GCs are provided maternally as well as by the embryo; in mice, systemic synthesis begins around E14 and peaks around birth [51]. Embryonic *Nr3c1* expression is already detected at E10.5 and negative regulation of HPA axis components POMC and CRH occurs by E16.5 and is dependent upon GR [51]. Whether GCs are synthesized locally during skin development is not known, and is a subject for future investigation. We have evaluated and detected *Nr3c1* expression in mouse skin starting at E14.5, though it may be present at earlier stages. Interestingly, epidermal GR transcript and protein expression peaks at E16.5, the critical period for epidermal barrier acquisition, and decreases thereafter [52]. Importantly at E16.5, the skin levels of *Hsd11b1* and *Hsd11b2* are relatively low, compared to E18.5 when expression increases by more than 10- and 30-fold, respectively [38]. These data indicate that during barrier acquisition there is abundant receptor and a supply of active GCs in the skin (**Figure 2A**).

The first direct evidence that GCs regulate epidermal development was that antenatal exposure of rats to pharmacological doses of GCs accelerated permeability barrier acquisition, assessed functionally by measurements of transepidermal water loss (TEWL), and supported



**Figure 2.** Relative gene expression of GR and enzymes modulating GC availability during embryonic skin development. (A) Relative mRNA levels of *Nr3c1*/GR, *Hsd11b1*, *Hsd11b2*, *Krt77*, and *Sprr2d* in embryonic (E15.5-E18.5) and newborn (P0) mouse skin. *Krt77* and *Sprr2d* are shown as markers of earlier and later epidermal development. #, not assessed. Statistically significant differences relative to E15.5 are indicated (n = 4 per age; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001). (B) Epidermal barrier is impaired in GR epidermal KO ( $GR^{EKO}$ ) mice. Left: Toluidine blue staining of control and  $GR^{EKO}$  mice (E17.5) demonstrated delayed and altered epidermal barrier formation in  $GR^{EKO}$  mice. Dotted lines point to the dorsoventral and anteroposterior patterns of epidermal maturation. Right panel: hematoxylin and eosin-stained skin sections (E17.5) show immature thinner epidermis with abnormal differentiation of suprabasal layers in  $GR^{EKO}$  relative to control mice.



by ultrastructural data showing mature SC and lipid lamellar bodies and by increases in total SC lipid content [53]. Later, study of the GC-deficient *CRH*<sup>-/-</sup> mice revealed delays in epidermal development and barrier formation, with structurally immature lipid lamellar bodies and decreased lipid deposition and expression of SC proteins involucrin (IVL), loricrin (LOR), and filaggrin (FGN) [54]. These delays were evident at E17.5 when barrier should be fully functional and could be rescued by supplementation with GCs. Importantly, by birth *CRH*<sup>-/-</sup> mice had a structurally normal epidermis and SC, though its functionality was not assessed through TEWL measurements, suggesting a transient role of GCs in skin development. These initial studies were validated by whole mount dye exclusion assays with mouse embryos that had been exposed to pharmacological doses of GCs [55], which developed the permeability barrier earlier than controls (~1/2 day). A later study used gene profiling to determine that the critical time window for transcriptional responsiveness of skin to maternal GC-treatment is from E15.5 to E16.5 [55]. Genes found to be upregulated in GC-exposed skin included those encoding FGN and late cornified envelope proteins mapping to the epidermal differentiation complex, found on chromosome 3q in mice, which contains over 55 genes involved in keratinocyte terminal differentiation.

### 2.3. Mouse models for studying GR function in skin development

Normal skin development requires GCs and can be accelerated by maternal exposure to this hormone; however, it was necessary to evaluate specific gain- and loss-of-function GR mouse models to pinpoint the role of this NR in the skin (Table 1). Gain-of-function was assessed using

Mouse model	Description	Skin development	Adult skin homeostasis	Refs
<i>GR</i> <sup>-/-</sup>	Global GR KO	Perinatal death Impaired epidermal barrier SC virtually absent Increased KC proliferation and apoptosis	--	[60]
<i>GR</i> <sup>A458T/A458T</sup>	Global GR knock-in: transcription defective mutant A458T	Viable No skin phenotype	No skin phenotype	[60]
<i>K5-GR</i>	Overexpression of GR targeted to epidermis and derivatives (constitutive)	Viable (perinatal death at high doses of the transgene) Epidermal thinning; reduced HF number; dysplastic HF	Focal alopecia Delayed skin wound repair Reduced cutaneous inflammation and non melanoma skin cancer	[49,50, 84, 91]
<i>K5-GR-TR</i>	Overexpression of the transcription defective mutant GR (P493R, A494S) targeted to epidermis and derivatives (constitutive)	Viable No skin phenotype	Focal alopecia Delayed skin wound repair Partial reduction of cutaneous inflammation	[59, 91]
<i>K5-cre//GR</i> <sup>loxP/loxP</sup> ( <i>GR</i> <sup>EKO</sup> )	GR epidermal KO (constitutive)	Impaired KC differentiation Increased KC proliferation and apoptosis Skin inflammation	Increased cutaneous inflammation, non-melanoma skin cancer, and melanocytic foci	[66, 83]
<i>K14-cre-ER</i> <sup>T2//GR</sup> <sup>loxP/loxP</sup>	GR epidermal KO (induced in adult)	--	Thickened skin Reduced KC differentiation Increased dermal inflammation	[52]

**Table 1.** Summary of the skin phenotypes of genetically modified mice with GR gain- and loss-of-function. *Abbreviations:* GR, glucocorticoid receptor; SC, stratum corneum; KC, keratinocyte; HF, hair follicle; K, keratin.

transgenic mice (K5-GR) that express GR under the control of the K5 promoter that is active in all stratified epithelia, including the basal layer of the epidermis, hair follicles, and sebaceous glands [49, 56]. The effects of high levels of GR overexpression during development were deleterious, causing perinatal lethality and lesions lacking epidermis and/or skin (**Table 1**). Transgenic mice overexpressing lower levels of GR survived to adulthood and were fertile, but also showed developmental abnormalities in morphogenesis of the epidermis and hair [49, 50]. During development and at birth, K5-GR epidermis is thinner than littermate controls, with drastic reductions in K5 expression [50], consistent with the antiproliferative effects of pharmacological GC treatments on keratinocytes [57, 58]. In addition, keratinocyte-specific GR overexpression caused accelerated epidermal differentiation as seen by increased LOR staining (our unpublished data). To evaluate the respective contributions of GR transactivation/transrepression in the phenotype of the K5-GR transgenic mice, another transgenic mouse model (K5-GR-TR) was generated with epidermal overexpression of GR carrying a double mutation (P493R and A494S) in the second half of the second zinc finger which impairs transcriptional activation but not transrepression of AP-1 and NF- $\kappa$ B [59]. The K5-GR-TR mice showed normal skin development without abnormalities in histology or in the expression of K5, K10, FGN, LOR, and IVL ([59] and our unpublished data) suggesting that dysregulation of epidermal markers in developing skin due to GR overexpression depends upon its ability to activate transcription (**Table 1**).

The analysis of the complete loss of function GR<sup>-/-</sup> mice revealed phenotypes complementary to the K5-GR model. These mice die upon birth due to defects in lung maturation, so analysis was unable to go beyond this stage [51]; however, a pronounced skin phenotype was observed in developing and newborn GR<sup>-/-</sup> animals featuring abnormal K5 expression in suprabasal layers, almost negligible levels of FGN, LOR, and IVL, and increased apoptosis. An increase in phosphorylated ERK was observed in GR<sup>-/-</sup> keratinocytes *in vivo* as well as *in vitro* where it was shown to contribute to the increased apoptosis [60]. Dye exclusion assays confirmed that the formation of the permeability barrier was delayed relative to control littermates (**Table 1**). The defective epidermal differentiation in the GR<sup>-/-</sup> mice correlated with the altered expression of genes in the epidermal differentiation complex, with strong repression of members of the *Small proline repeat rich* family and *Corneodesmosin* (*Cdsn*) and upregulation of early differentiation genes such as the epithelial-specific gene transcripts E74-like factor 5 (*Elf5*) and keratin 77 (*Krt77*) [52]. In contrast, GR<sup>A458T/A458T</sup> knock-in mice were viable and fertile, and had normal histological appearance and expression of K5, K10, and LOR, suggesting that the transactivation function is not required for survival or skin development [60, 61]. It is worth noting that newborn mice with complete loss of the cytoplasmic chaperone p23 showed striking similarities in defects in skin development to those observed in GR<sup>-/-</sup> mice [60, 62], including defective keratinocyte differentiation, proliferation, and increased apoptosis. Indeed, GR-controlled target genes such as *Elf5*, *Krt77*, and *Cdsn* were also dysregulated in p23<sup>-/-</sup> skin and GR nuclear translocation upon GC treatment was defective in p23<sup>-/-</sup> cultured keratinocytes, indicating cell autonomous defects [62].

In order to study the effects of GR loss beyond birth as well as to assess cell-type specific contributions to skin development and homeostasis, knockouts were generated using GR<sup>flox/flox</sup> mice, which have the third exon, encoding part of the GR DNA binding domain, flanked by loxP sites reviewed in [63]. When crossed with transgenic mice that express the Cre recombinase, exon 3 is disrupted and the GR gene inactivated due to out-of-frame splicing and premature translational termination [64]. Newborn mice lacking mesenchymal expression of

GR (Dermo1-Cre//GR<sup>flox/flox</sup>) had defects in dermal collagen and elastin production as well as histological abnormalities in the epidermis, which included rounded suprabasal keratinocytes and a dense SC [65]. These results indicate that dermal defects due to the lack of GR in fibroblasts impact the adjacent epidermis; however, as more detailed analysis was not performed, the exact mechanisms remain unclear. Mice lacking epidermal GR (K5-Cre//GR<sup>flox/flox</sup> or GR<sup>EKO</sup>) showed defective skin development with delayed epidermal barrier formation, abnormal keratinocyte differentiation, hyperproliferation, and SC fragility [66] (**Table 1**). Toluidine blue dye exclusion assays revealed patchy disorganized barrier initiation sites and irregularities in barrier fronts, more evidence that GR regulates this process (**Figure 2B**). The mechanism is not entirely clear but may be related to negative regulation by GR of the kinase AKT and/or the AP-1 member Jun in the barrier front, as the activity of both must be spatiotemporally controlled for proper barrier acquisition [67]. Consistent with this phenotype was abnormal interfollicular K6 expression, decreased levels of LOR, FGN, and CDSN, and alterations in epidermal lipids. In addition to the barrier defects, GR<sup>EKO</sup> mice had an inflammatory phenotype with increases in epidermal STAT3, AKT, and ERK activities and with dermal infiltrates containing macrophages and degranulated mast cells. Gene expression profiling data identified upregulation of *Elf5* and *Krt77* as well as the keratins *Krt6a*, *Krt6b*, and *Krt16*, which are induced in the context of hyperproliferative/inflammatory skin diseases, and many other genes such as *Tslp* and *S100a8/9* commonly induced in inflammatory skin diseases [66]. These data indicated that newborn GR<sup>EKO</sup> mice suffer skin disease with features of atopic dermatitis and psoriasis. Loss-of-function mutation in epidermal barrier genes has been linked to atopic dermatitis and psoriasis, indicating that defects in keratinocyte terminal differentiation can be a predisposing factor for these diseases [11].

### 3. GR function in adult skin homeostasis

Research on the molecular mechanisms underlying GC actions has been put forward—at least to a great extent—because of the wide and efficacious use of GC-derived compounds for the treatment of chronic inflammatory diseases including those affecting skin. Contrary to the perinatal period, in which GCs accelerate skin barrier formation, GC treatment of adult animals perturbs permeability barrier homeostasis, suggesting unique roles for the GR in development and adulthood [29, 68].

#### 3.1. Endogenous GCs affect skin integrity in aging and stress

Intrinsic or chronological skin aging affects nonexposed areas, and is mainly attributed to genetic factors and endocrine alterations. In contrast, extrinsic or pathological aging is principally due to repeated exposure to UV irradiation. In skin that is sun-exposed, both types of aging are superimposed [69]. Aging skin is characterized by gradual loss of the structural and functional characteristics of the tissue, which becomes more prone to damage, infections, and retarded wound healing, with consequent increases in the susceptibility of individuals to cutaneous disorders including those associated with inflammation and/or cancer [70]. Moreover, increased age in humans and mice correlates with abnormal skin barrier function with augmented TEWL and impaired mechanical properties partly due to a marked reduction in SC

lipids resulting in decreased lipid layers, or mortar (see Section 2.1), between corneocytes [69]. Also, aged humans and mice typically have increased serum cytokines and markers of inflammation. Remarkably, correction of skin barrier defects in older mice by application of petrolatum or glycerol significantly reduced serum cytokines opening the attractive possibility that enhancing epidermal functions could ameliorate or prevent inflammation-associated disorders in elderly humans [71].

Another prominent feature of aged skin is atrophy, reduced epidermal, and dermal thickness caused by decreased keratinocyte proliferation, and profound alterations in extracellular matrix proteins of the dermis such as collagen, elastin, and proteoglycans, contributing to the formation of wrinkles and increased fragility [72]. GCs exert antiproliferative effects in skin inhibiting the proliferation of keratinocytes and fibroblasts. Transcriptomic analyses in human-cultured keratinocytes demonstrated that GCs regulate numerous genes participating in cytoskeletal rearrangements and ECM remodeling including *Actin* and *Krt6*, whose repression is consistent with the inhibition of keratinocyte migration and wound healing by GCs [23]. GCs also regulated numerous genes related to keratinocyte differentiation including *FLG* and *CDSN*. Additional studies in GC-treated adult mouse epidermis also identified numerous target genes related to cell cycle or DNA synthesis [73]. The induction of the stress-inducible mTOR inhibitor REDD1 contributed to the atrophogenic GC effects. As GCs elicited similar anti-inflammatory responses in control and *Redd1*<sup>-/-</sup> mice, the use of REDD1 inhibitors may have therapeutic implications [73].

The activity of HSD11B1 was increased in human and mouse skin samples from old relative to young subjects as well as in photodamaged versus nonexposed human skin biopsies, suggesting that local conversion of inactive to active GCs contributes to intrinsic and extrinsic aging of this tissue [74]. *Hsd11b1* KO mice were partially protected against age-induced skin damage showing increased collagen density as well as improved wound healing relative to controls [74]. Furthermore, topical treatment with a HSD11B1 inhibitor accelerated cutaneous wound healing in aged mice [74]. Altogether, HSD11B1 targeting appears as a promising pharmacological target to ameliorate cutaneous GC adverse side effects.

As endogenous GC production is also increased in stress conditions due to HPA reactivity, there is a link between pathologies with chronic-elevated GC levels and altered epidermal function. In particular, psychological stress is known to exacerbate features of skin diseases such as psoriasis and AD through increased GC production [75, 76]. In psychologically stressed mice, elevated GC levels inhibited epidermal lipid synthesis and downregulated the expression of antimicrobial peptides leading to decreased SC integrity and increased risk of infection [76, 77]. These defects could be reversed by reducing GC production through administration of an inhibitor of CRH or the GR antagonist RU486 and also by topical treatment with exogenous lipids [75, 76]. However, and paradoxically, it has also been reported that the stress-induced production of endogenous GCs exerted beneficial effects in cutaneous function in three different murine models of dermatoses, likely due to the anti-inflammatory effects of acute increases in endogenous GCs [78].

### 3.2. Skin alterations in adult transgenic mice with GR gain and loss of function

It is well known that GC treatment may cause marked epidermal thinning as well as retarded growth of hair follicles and hair loss, which may result in alopecia. Consistent with this, adult

epidermis from K5-GR and K5-GR-TR mice showed pronounced epidermal hypoplasia with flattened keratinocytes and discontinuous K5 staining as well as reduction in the number of hair follicles (50 or 25% decrease, respectively) [49, 50, 59]. These data indicate that although GR-dependent transcriptional activation was partially impaired in K5-GR-TR mice, the overexpression of this GR mutant was sufficient to inhibit keratinocyte proliferation and alter hair follicle growth in adulthood but not during development [59]. Microarray studies in skin of K5-GR mice identified upregulation of a large subset of hair keratins, keratin-associated proteins, and downregulation of several *Hox* genes indicating a role of GR in hair follicle homeostasis through the control of keratin genes [79].

In addition, adult K5-GR mice exhibited abnormalities affecting other ectodermal derivatives, including exocrine glands such as the sweat glands, the ocular secretory Meibomian glands, and the preputial glands [49, 50]. In fact, the phenotype of K5-GR mice recapitulated the triad of clinical symptoms that defines the human syndrome hypohidrotic ectodermal dysplasia (hair, teeth, and exocrine glands) [50]. Although the exact mechanisms underlying these defects have not been characterized, the overexpression of GR impaired the expression and/or activity of NF- $\kappa$ B and p63 in several epithelia [50]. The fact that neither GR<sup>EKO</sup> nor K5-GR-TR adult mice exhibited the ectodermal defects observed in adult K5-GR mice suggests that these abnormalities depend on elevated levels of transcriptionally competent GR.

The severe skin phenotype of newborn GR<sup>EKO</sup> mice featuring barrier defects and inflammation resolved spontaneously around postnatal day 5. Adult mice showed only a mild phenotype of increased keratinocyte proliferation and patches of impaired epidermal differentiation indicating that barrier function is largely intact (**Table 1**). However, as in other models with impaired skin barrier development, epidermal GR loss resulted in increased susceptibility in adulthood to inflammatory triggers such as PMA, with elevated levels of K6 consistent with its upregulation in GR<sup>EKO</sup> skin during development [66].

Also, the tamoxifen-inducible epidermal deletion of GR in adult mice (K14-Cre-ER<sup>T</sup>//GR<sup>lox/lox</sup> mice) resulted in skin alterations with thickened epidermis, abnormal expression of K6 in the interfollicular epidermis, K10 localization restricted to the most suprabasal epidermal layer, reduced and patchy expression of LOR and CDSN, and the presence of dermal infiltrates [52]. After acute PMA treatment, K14-Cre-ER<sup>T</sup>//GR<sup>lox/lox</sup> mice showed significantly increased keratinocyte proliferation and skin inflammation, with pronounced recruitment of polymorphonuclear cells [52]. An independently generated mouse model with tamoxifen-inducible GR epidermal deletion in adulthood also showed increased induction of TSLP, a key marker of atopic dermatitis, which could not be inhibited by GCs [22].

The posttranslational modifications of GR play an important role in the susceptibility to PMA-induced skin inflammation. It was recently demonstrated that GR sumoylation at K310 in mice (K293 in humans) is required for the formation of a repressing complex, which is involved in both GR-mediated IR nGRE gene repression and transrepression of NF- $\kappa$ B/AP-1-driven transcription. Mice harboring mutations that impaired GR sumoylation at this site showed more severe responses to PMA-induced skin inflammation, which could not efficiently be suppressed by Dex [80, 81]. Experiments in mice with keratinocyte-specific inactivation of the components of the repressing complex NCoR1/SMRT or HDAC3 showed the lack of Dex-induced transrepression as tethering of the complex on DNA-bound NF- $\kappa$ B/AP1 was impaired [80, 81].

The recent finding that GR haploinsufficiency in mice and reduced GR expression in human biopsies correlates with increased incidence of tumor formation provides a causal role for this TF in tumorigenesis, reinforcing the denomination of GR as a tumor suppressor gene [82]. In fact, the analyses of mouse models with epidermal-specific GR overexpression or inactivation demonstrated that GR exerts tumor suppressor actions during skin carcinogenesis [80, 84]. GR<sup>EKO</sup> mice subjected to the classical two-stage protocol—consisting in a single low-dose application of the mutagen 12-dimethylbenz(a) anthracene (DMBA) followed by repeated PMA treatments—exhibited earlier papilloma formation with higher incidence and multiplicity, as well as increased tumor size relative to controls [83]. Also, papillomas in GR<sup>EKO</sup> mice displayed signs of early malignization, including delocalized expression of laminin A, dermal K5-positive cells, abnormal expression of K13, and focal loss of E-cadherin. Consistent with the keratinocyte atypia *in vivo*, cultured GR<sup>EKO</sup> keratinocytes showed abnormal spindle-like morphology, loss of E-cadherin, and upregulation of smooth muscle actin and SNAIL, overall suggesting epithelial-mesenchymal transition [83]. Conversely, transgenic K5-GR//Ha-ras+ mice showed resistance to PMA-induced skin carcinogenesis with delayed onset of papilloma appearance, reduced tumor burden, and significant decrease of papilloma size (eightfold) relative to WT//Ha-ras+ controls [84]. Mechanistically, GR function in mouse skin tumorigenesis was mediated through negative interference with the NF-κB, AKT, and STAT3 pathways [83, 84]. It has also been postulated that the antitumor effects of GR in K5-GR//Ha-ras+ mice were exerted by decreasing the number of follicular stem cells as well as their proliferative potential, with associated changes in their transcriptional signature [85]. However, recent work suggests that increases in the local concentration of bioactive GCs can also exert tumor-promoting effects in solid tumors of epithelial origin [86]. Overall, additional studies are required to understand the apparently controversial data on the role of GC signaling on epithelial tumor development and progression.

Another major adverse effect associated with pharmacological GC treatment is delayed wound healing. Wound healing is a complex process comprising inflammatory, proliferative, and remodeling phases, which requires coordinated interactions among keratinocytes, immune cells, and fibroblasts to repair tissue damage and restore skin homeostasis [87]. Although inflammation is required for skin barrier restoration, alterations in levels or kinetics of expression of inflammatory mediators can be detrimental and result in chronic wounds or delayed healing [88]. Secretion of growth factors and cytokines including FGFs, EGF, IL-1, and IL-6 stimulates keratinocyte proliferation and migration, a process called re-epithelialization, normally accompanied by collagen deposition, formation of new granulation tissue, and wound contraction [87, 88].

Endogenous GC excess, for instance, in diabetic patients, results in chronic nonhealing wounds and often in lower limb amputations. GC-activated GR inhibits wound closure by blocking EGF-induced keratinocyte migration. The mechanism involves the formation of a repressor complex together with β-catenin to inhibit K6 and K16 expression at the wound edge [88]. Importantly, farnesyl pyrophosphate (FPP), an intermediate in the pathway of cholesterol biosynthesis, acts as GR agonist and also suppresses the *Krt6* gene and inhibits keratinocyte migration. The activation of GR by cortisol or FPP caused nuclear translocation of β-catenin, leading to induction of *c-myc*, a hallmark of chronic nonhealing wounds. This led to the proposal to use statins to restore epidermal homeostasis as targeting the cholesterol pathway interferes with the production of FPP and cortisol, resulting in substantial reduction of GR

activation, and c-myc downregulation [89]. It has also been shown that GCs can also inhibit keratinocyte migration and wound healing by activation of nongenomic signaling pathways involving membrane GR regulation of phospholipase C/protein kinase C ultimately activating  $\beta$ -catenin and c-myc [90].

The use of K5-GR and K5-GR-TR mice allowed us to demonstrate that keratinocyte-targeted GR overexpression delayed skin wound healing [91]. This delay resulted from reducing the inflammatory response and decreasing keratinocyte migration *in vitro* and *in vivo*, consistent with the impaired skin healing observed with GC treatment [91]. While in K5-GR mice, cutaneous healing was delayed at days 4 and 8 after wounding, there was only a delay at day 4 in K5-GR-TR mice. These changes correlated with reduced K6 staining in both mouse models at day 4, which only persisted at day 8 in K5-GR, along with discontinuous K10 expression indicating an incomplete restoration of the epidermal barrier. These animals showed normal healing by day 8, concomitant with decreased repression of proinflammatory cytokines and growth factors relative to K5-GR mice. In wound healing experiments with both transgenic mouse models, keratinocyte proliferation was inhibited correlating with reduced ERK activity, *in vitro* and *in vivo*, and collagen deposition was reduced to a similar extent [91]. These data suggest that the early stages of wound closure are negatively regulated by GR independently of transcription, while GR transcriptional actions are necessary for delaying later stages of healing.

Finally, cutaneous production of GCs upon acute wounding is a major regulator of inflammatory responses as locally produced cortisol acts as a negative feedback to shut off the synthesis of the proinflammatory cytokine IL-1 [4, 25, 92]. The inhibition of skin-specific GC synthesis by using the  $11\beta$ -hydroxylase inhibitor metyrapone or targeting HSD11B1 accelerated wound closure *in vivo*. These findings illustrate the relevance of local GCs to achieve a proper balance between pro- and anti-inflammatory signals upon injury, and thus modulate skin homeostasis. It has been also suggested that the interaction between cutaneous and systemic production of GCs has an impact on wound healing [31].

## 4. Conclusions

*In vivo* and *in vitro* studies, and in particular, the characterization of mouse models with gain- or loss-of-function of epidermal GR has highlighted a previously unrecognized role for this TF in skin development. Transcriptomic studies demonstrated a central role for GR in the regulation of epidermal genes, and specifically keratins with key roles in proliferation and differentiation including *Krt6* and *Krt77*. As in other models with impaired skin barrier development, epidermal GR loss resulted in increased susceptibility to inflammatory triggers in adulthood. The regulation of different biological processes and gene subsets by GR in skin was dependent on the developmental stage and physiological state, consistent with the context-specific actions of this TF. The identification of more specific downstream mediators of GC action with reduced adverse side effects remains a central objective in dermatological research. Also, the interactions between systemic and local HPA axes and GC production must be taken into consideration for developing novel strategies for treating cutaneous diseases.

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## Conflict of interest

The authors declare no conflict of interest.

## Author details

Lisa M. Sevilla and Paloma Pérez\*

\*Address all correspondence to: [pperez@ibv.csic.es](mailto:pperez@ibv.csic.es)

Instituto de Biomedicina de Valencia (IBV)-CSIC, Valencia, Spain

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