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Glucocorticoid receptor antagonizes EGFR function to regulate eyelid development

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ABSTRACT The glucocorticoid receptor (GR) plays a crucial role in epidermal morphogenesis during embryonic development, as demonstrated by analyzing genetically modified mouse models of GR gain- and loss-of-function. Eyelid formation constitutes a useful model to study epithelial development, as it requires coordinated regulation of keratinocyte proliferation, apoptosis and migration. We have analyzed this biological process in $GR^{-/-}$ embryos during ontogeny. Our data demonstrate that GR deficiency results in delayed and impaired eyelid closure, as illustrated by increased keratinocyte proliferation and apoptosis along with impaired differentiation in $GR^{-/-}$ eyelid epithelial cells. These defects are due, at least in part, to the lack of antagonism between GR and epidermal growth factor receptor (EGFR) signaling, causing sustained activation of the MAPK/AP-1 pathway and the upregulation of keratin K6 at embryonic stage E18.5. Additionally, we demonstrate that GR regulates epithelial cell migration *in vitro* by interfering with EGFR-mediated signaling. Overall, GR/EGFR antagonism appears as a major mechanism regulating ocular epithelial development.

KEY WORDS: *glucocorticoid receptor, EGFR, genetically modified mice, epithelial cell, eye development*

Introduction

It is known that the glucocorticoid receptor (GR) plays a crucial role during embryogenesis, since this ligand-activated transcription factor is required for maturation of vital organs such as the lung, heart, kidney, gut and epidermis (reviewed in Wintermantel *et al.*, 2004; Revollo and Cidlowski, 2009). GR exerts its biological effects through two different mechanisms that involve DNA binding-dependent and -independent actions, which can be genetically separated and are commonly refered to as transactivation and transrepression functions. In fact, knock-out $GR^{-/-}$ mice die perinatally whereas mice carrying a point mutation which abrogates the dimerization- dependent DNA binding of GR ($GR^{dim/dim}$) are viable (Cole *et al.*, 1995; Reichardt *et al.*, 1998). This distinction, however, is not so clear-cut since GR monomers are also able to bind certain gene promoters and thus, regulate gene transcription (Adams *et al.*, 2003; Rogatsky *et al.*, 2003): In the last years, we have demonstrated that GR is a key player in epithelial development by analyzing genetically modified mouse models of GR gain- and loss-of-function (Pérez *et al.*, 2001; Cascallana *et al.*, 2003; Cascallana *et al.*, 2005; Donet *et al.*, 2008; Bayo *et al.*, 2008). We have recently reported that only the epidermis of $GR^{-/-}$ but not $GR^{dim/dim}$ embryos shows major defects, suggesting that dimerization dependent DNA binding by GR is dispensible for epidermal development (Bayo *et al.*, 2008). In addition, we have shown that transgenic mice with keratinocyte-targeted overexpression of GR (K5-GR mice) featured numerous epithelial defects including epidermal defects and an eyelid opening at birth phenotype (EOB). Overexpression of GR transrepression function in keratinocytes (K5-GR-TR mice) also elicited epithelial alterations that partially overlapped with those found in K5-GR mice (Cascallana *et al.*, 2005; Donet *et al.*, 2008). Remarkably, K5-GR-TR mice featured an EOB phenotype identical to K5-GR mice, indicating that the transrepression function of the GR is sufficient to cause these epithelial ocular anomalies (Donet *et al.*, 2008).

Eyelid formation represents a useful model to study epithelial development since this biological process requires spatio-tempo-

Abbreviations used in this paper: EGFR, epidermal growth factor receptor; GC, glucocorticoid hormone; GR, glucocorticoid receptor.

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ral coordination of cellular proliferation, migration, differentiation and apoptosis. In the mouse embryo, eyelid formation starts at midgestation, when small invaginations of the surface ectoderm begin to grow (E12.5), become distinguishable as protruding edges (E14.5-E15.5) and gradually cover the corneal surface until they fuse at E16.5. At E18.5, eyelids are unequivocally closed and start to separate postnatally (P3-P5) until they reopen around P12 (Kaufman and Bard, 1999). In this work, we analyzed the consequences of GR functional inactivation in ocular development by using $GR^{-/-}$ mice.'Our data show that GR is required to modulate eyelid epithelial morphogenesis through regulation of keratinocyte proliferation, apoptosis, differentiation and migration. It is known that GR suppresses keratin expression of either mitotically-active basal keratinocytes (K5 and K14) or migration-associated keratins K6 and K16. The mechanisms involve binding of GR to AP-1 and interaction of four GR monomers with k6 promoter, respectively (Radoja *et al.*, 2000; De Bosscher and Haegeman, 2009). Accordingly, K5 and K6 expression was altered in $GR^{-/-}$ epithelia. These actions were mediated, at least in part, through interference with EGFR signaling, the loss of which results in increased EGFR and MAPK/AP-1 activation and sustained expression of the keratin K6. *In vitro* wound healing assays demonstrated that constitutive GR overexpression in keratinocytes drastically impairs cell migration and interferes with EGFR function suggesting that lack of cross-talk between GR and EGFR pathway is one of the mechanisms causing delayed eyelid closure in $GR^{-/-}$ embryos.

Results

We have anayzed eyelid closure in $GR^{-/-}$ embryos as *in vivo* model to address the impact of GR loss-of-function in epithelial morphogenesis. Macroscopically, $GR^{-/-}$ embryos showed open eyelids at embryonic days E16.5 and E18.5. A detailed histopathological analysis of wt and $GR^{-/-}$ littermates at distinct timepoints showed severe anomalies in the eyelid formation of GR-deficient mice throughout development (Fig. 1A, compare a-d and a´-d´). In a wt developing mouse, eyelid closure is normally completed in 24h, coinciding with the E15.5-E16.5 transition (Fig. 1A; a, b-d; asterisks indicate fused eyelids in $GR^{+/+}$). In contrast,

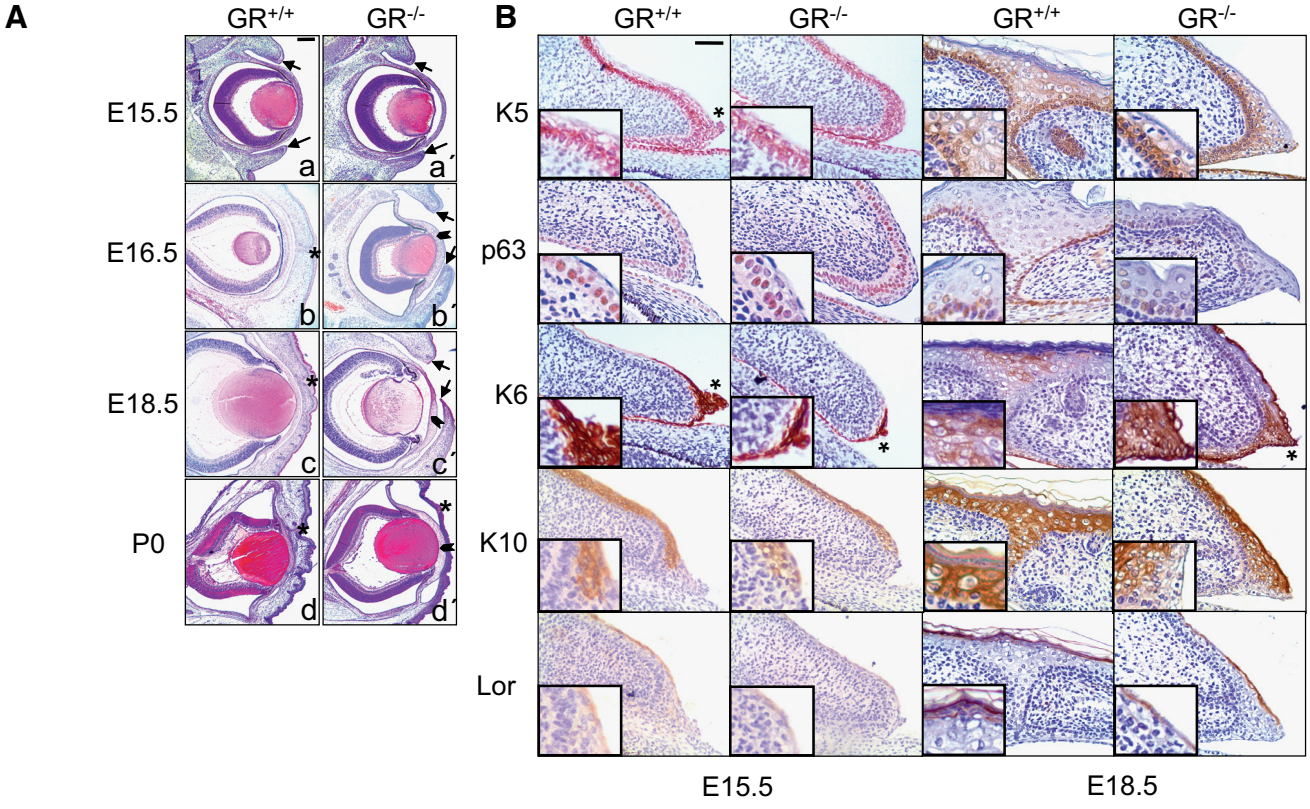

Fig. 1. Delayed and impaired eyelid closure in glucocorticoid receptor (GR) null mice. (A) *Histopatholgical analysis of wt and $GR^{-/-}$ embryos at distinct developmental timepoints. Eyelid closure is normally completed at E15.5-E16.5 transition in wt embryos and eyelids remain fused until postnatal age (a-d). In contrast, $GR^{-/-}$ embryos exhibited unfused eyelids at E16.5 and E18.5 (a´-c´, arrows) along with an abnormal corneal stroma with increased cellularity (b´,c´, arrowheads). $GR^{-/-}$ newborn mice showed closed eyelids with abnormal epithelia and atypical corneal stroma (d´, asterisk and arrowhead, respectively). Bar: 200 μm.* **(B)** *Abnormal morphogenesis of eyelid epithelial cells in $GR^{-/-}$ early (E15.5) and late (E18.5) embryos. Immunostaining for K5, K6, p63, K10 and loricrin was performed using specific antibodies. Note that both K5 and p63 expression stained two-to three suprabasal layers of eyelid epithelia in $GR^{-/-}$ embryos as compared to more restricted labeling at the fused eyelids of $GR^{+/+}$ individuals (K5 and p63; see inset). Increased K6 expression at the eyelids tips of E15.5 (asterisks) was observed in wt mice. Elevated levels of K6 were detected in the unfused $GR^{-/-}$ eyelids at E18.5, as compared with restricted K6 expression at closed eyelids of $GR^{+/+}$ mice. Abnormal K10 and loricrin staining was apparent in $GR^{-/-}$ eyelid epidermis relative to $GR^{+/+}$ mice (K10 and loricrin; see inset). Bar: 100 μm.*

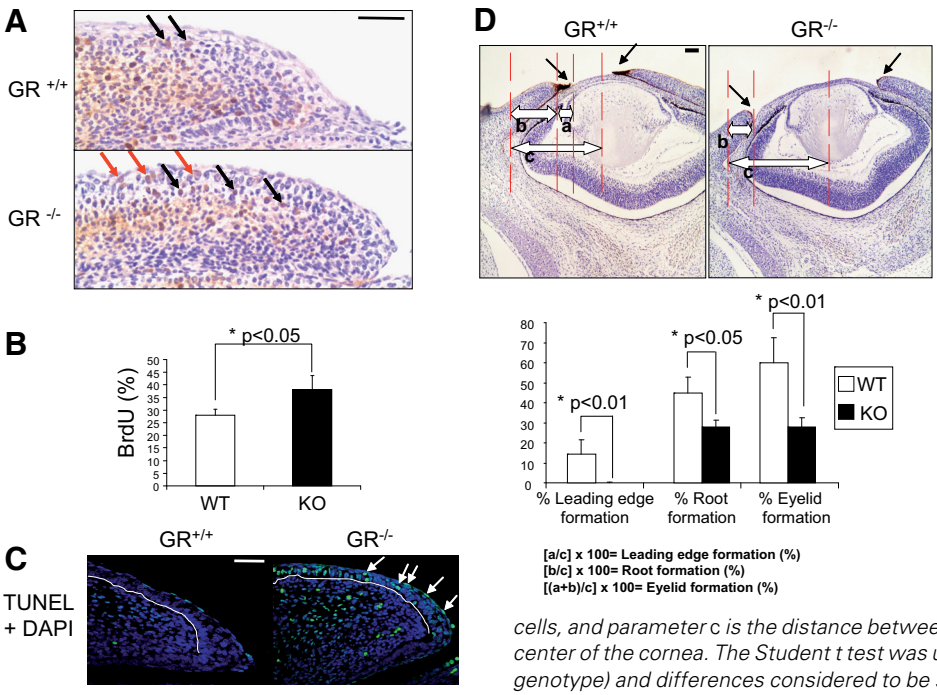

Fig. 2. Increased proliferation and apoptosis in GR$^{-/-}$ eyelid epithelial cells and impaired eyelid formation in GR$^{-/-}$ mice. (A) In vivo *BrdU labeling showed increased epithelial proliferation in GR$^{-/-}$ relative to GR$^{+/+}$ E15.5 embryos. Note that augmented keratinocyte proliferation was detected in eyelid basal (black arrows) and suprabasal (red arrows) cells.* **(B)** *Quantitative differences were statistically significant, p<0.05.* **(C)** TUNEL *staining shows augmented apoptosis in all eyelid epithelial layers of GR$^{-/-}$ relative to GR$^{+/+}$ E15.5 embryos. Arrows point to TUNEL-positive cells and the line denotes the basal membrane of the eyelid epithelium.* **(D)** *Quantitation of formation of eyelid, leading edge and root in GR$^{-/-}$* vs *wt E15.5 littermates (five of each genotype). Measurements were done as summarized in Materials and Methods. Briefly, the migrating epithelial cells were identified by K6 immunostaining. Parameter* a *is the distance of migration; parameter* b *the distance between the axis delimitating the conjunctival sac and the first K6-stained cells, and parameter* c *is the distance between the axis delimitating the conjunctival sac and the center of the cornea. The Student t test was used to calculate statistical significance (n= 5, each genotype) and differences considered to be statistically significant when p<0.05. Bar: 100 μm.*

GR$^{-/-}$ embryos revealed unfused eyelids at E16.5 and even at E18.5 (Fig. 1A; b´, c´, arrows) along with an abnormal corneal stroma with increased cellularity (Fig. 1A; b´, c´, arrowheads). The eyelids of GR$^{-/-}$ newborn mice were closed but the epithelia appeared abnormally enlarged and undifferentiated (Fig. 1A; compare d and d´) and an atypical corneal stroma persisted. Penetrance of the reported ocular phenotype in GR$^{-/-}$ mice was 89% (n=43), with either one or two eyes affected (54.55% and 45.45%, respectively).

As an attempt to understand how loss of GR is causing these defects, we analyzed the expression of markers of keratinocyte proliferation, migration and differentiation in the eyelid epithelia of early (E15.5) and late (E18.5) embryos by immunostaining (Fig. 1B). In wt embryos, K5 expression was apparent in all epithelia basal cells of the eyelids, cornea and conjunctiva (Fig. 1B, K5). In GR$^{-/-}$ littermates, labeling of these epithelial cell layers was similar, although additional K5 expression was detected suprabasally in the eyelid epithelia (Fig. 1B, K5, see inset). The epithelial-specific marker p63 was detected in keratinocytes of GR$^{+/+}$ and GR$^{-/-}$ embryo eyelids and cornea. However, p63 immunostaining at the leading edge of early and late GR$^{-/-}$ embryo eyelids was more suprabasal as compared to the more restricted p63 labeling at wt eyelid tips (Fig. 1B, p63).

In E15.5 wt mice, K6 was strongly expressed at the eyelids tips, defining the migrating cells at the leading edge of this epithelium (Fig. 1B, K6, asterisk). The conjuctival epithelium was also K6-positive (not shown). Notably, at E18.5, where cell migration is no longer required since eyelids are already fused, K6 immunostaining was restricted to the closed eyelids (Fig. 1B). In contrast, GR$^{-/-}$ embryos stained weakly for K6 at the eyelid borders at E15.5 whereas an abnormally strong K6 signal was detected at E18.5, both at the tip of unfused GR$^{-/-}$ eyelids and in conjuctival epithelium (Fig. 1B, K6, asterisk and data not shown). The observed K6 expression pattern indicates a delay in the ocular epithelia development of GR$^{-/-}$ mice.

Previous reports described that eyelid fusion precedes epithelial differentiation, since positive K10 staining was detected in epithelial cells of wt embryos only after eyelid closure (Zhang *et al.*, 2005). We examined K10 and loricrin expression and found that in E15.5 wt embryos, both markers were present in suprabasal eyelid epithelia although absent at the leading edge. In E18.5 wt embryos, only suprabasal cells of the fused eyelids stained positive for both markers. In contrast, reduced staining of K10 and loricrin was apparent in GR$^{-/-}$ eyelid epidermis of early embryos whereas abnormal expression of these proteins persisted in the unfused eyelids from GR$^{-/-}$ E18.5 embryos (Fig. 1B, K10 and loricrin).

We further assessed altered proliferation in GR$^{-/-}$ eyelid epithelial cells by measuring *in vivo* BrdU incorporation in GR$^{-/-}$ *vs* wt E15.5 embryos, and found qualitative and quantitative differences among them (Fig. 2A, B). In wt embryos, most BrdU-positive keratinocytes were detected in the basal cell of the eyelid epithelia (black arrows). In contrast, augmented keratinocyte proliferation was detected in basal (black arrows) and suprabasal (red arrows) cells of GR$^{-/-}$ eyelids. When comparing the proliferation of eyelid basal cells, differences were found statistically significant (Fig. 2A, 38.1% *vs* 28%, respectively, p<0.05). At later developmental stages (E18.5), we could not detect increased proliferation of the eyelid basal keratinocytes of GR$^{-/-}$ relative to wt (data not shown). However, we found BrdU-positive nuclei in the suprabasal layers of E18.5GR$^{-/-}$ eyelids, as occurred in E15.5 GR-deficient embryos. Additionally, we detected augmented apoptosis in all eyelid epithelial layers of GR-deficient embryos by *TUNEL* staining, in contrast with scarce apoptotic cells in wt littermates (Fig. 2B, arrows). Overall, our results demonstrate that GR is required for proper proliferation, apoptosis, migration and differentiation of the eyelid epithelial cells of GR$^{-/-}$ embryos.

To further characterize the alterations in GR$^{-/-}$ eyelid closure, we quantitated several parameters to estimate the percentage of formation of the eyelid, leading edge and root, following the

methodology described by Mine and co-workers (Mine *et al.*, 2005). For these measurements, we performed K6 immunostaining to compare eyelid closure in $GR^{-/-}$ and wt E15.5 littermates, using K6-positive cells to define the migrating epithelial cells (Fig. 2C). In wt embryos, extension of both leading edge and the root was already evident at E15.5 with estimated percentage of formation of 14.5% and 45%, respectively (Fig. 2C). In sharp contrast, the leading edge of GR null mice was not formed at this stage and it was not apparent until E18.5 (Fig. 1B and data not shown). In addition, root formation was reduced almost two-fold, as compared to wt embryos. These determinations allowed us to quantitate the overall completion in $GR^{-/-}$ eyelid formation at E15.5 as of 28% relative to 60% in wt (Fig. 2C).

It is well known that the epidermal growth factor (EGF) and its receptor (EGFR) play a crucial role in epithelial development (Zenz *et al.*, 2003; Xia and Karin, 2004). The antagonism between GR and EGFR signaling has been demonstrated in different pathophysiological processes. However, this cross-talk has not been examined in eyelid formation. In wt embryos, EGFR was detected at the tip of eyelid epithelial cells around E15.5 but decreased at E18.5, when eyelid closure had completed. In contrast, EGFR protein expression was evident at E15.5 in $GR^{-/-}$ embryos and remained abnormally high at E18.5, as compared to wt (Fig. 3A, EGFR). The observed elevation in total EGFR levels correlated with increased phosphorylated (p-)EGFR immunostaining in $GR^{-/-}$ open eyelids at E18.5 (Fig. 3A, p-EGFR). To ascertain whether GR could also regulate EGFR at the transcriptional level, we examined the skin of E18.5 $GR^{-/-}$ embryos as compared to wt littermates by quantitative RT-PCR (Fig. 3B). Our data demonstrated increased *egfr* mRNA levels in $GR^{-/-}$ embryos, thus indicating additional mechanisms of GR/EGFR biological antagonism.

We next examined whether increased EGFR signaling would cause augmented ERK phosphorylation in the developing eyelids of $GR^{-/-}$embryos (Fig. 3C, p-ERK). Despite unchanged total ERK levels in wt and $GR^{-/-}$embryos (not shown), we detected p-ERK at E15.5 only in GR-deficient eyelids. In E18.5 wt embryos, p-ERK was restricted to the granular layer of closed eyelids whereas high levels were still detected at the eyelid tip of $GR^{-/-}$ embryos. Overall, our results suggest that proper (and transient) regulation of EGFR signaling and its downstream effectors, such as ERK and K6, are required during ocular epithelia development. Abnormally constitutive activation and/or expression of these proteins, as in GR-deficient embryos, likely results in impaired eyelid closure.

The formation of the mouse eyelid during the embryogenesis is similar to the wound healing process since it requires coordinated proliferation, migration and differentiation of keratinocytes (reviewed in Martin and Parkhurst, 2004). We thus examined the role of GR and its interference with EGFR function in epithelial cell migration by *in vitro* wound healing assays using primary culture keratinocytes (MPKs). Since cell confluence is necessary for these experiments, and given that $GR^{-/-}$ MPKs exhibit abnormal cell growth and apoptosis (Bayo *et al.*, 2008), we evaluated MPKs isolated from K5-GR transgenic mice, in which GR is constitutively active (Pérez *et al.*, 2001). This system allows us to evaluate the role of GR in keratinocyte migration without adding exogenous ligands, which can elicit different actions depending on dosing and kinetics. Fig. 4 summarizes three independent wound scratch

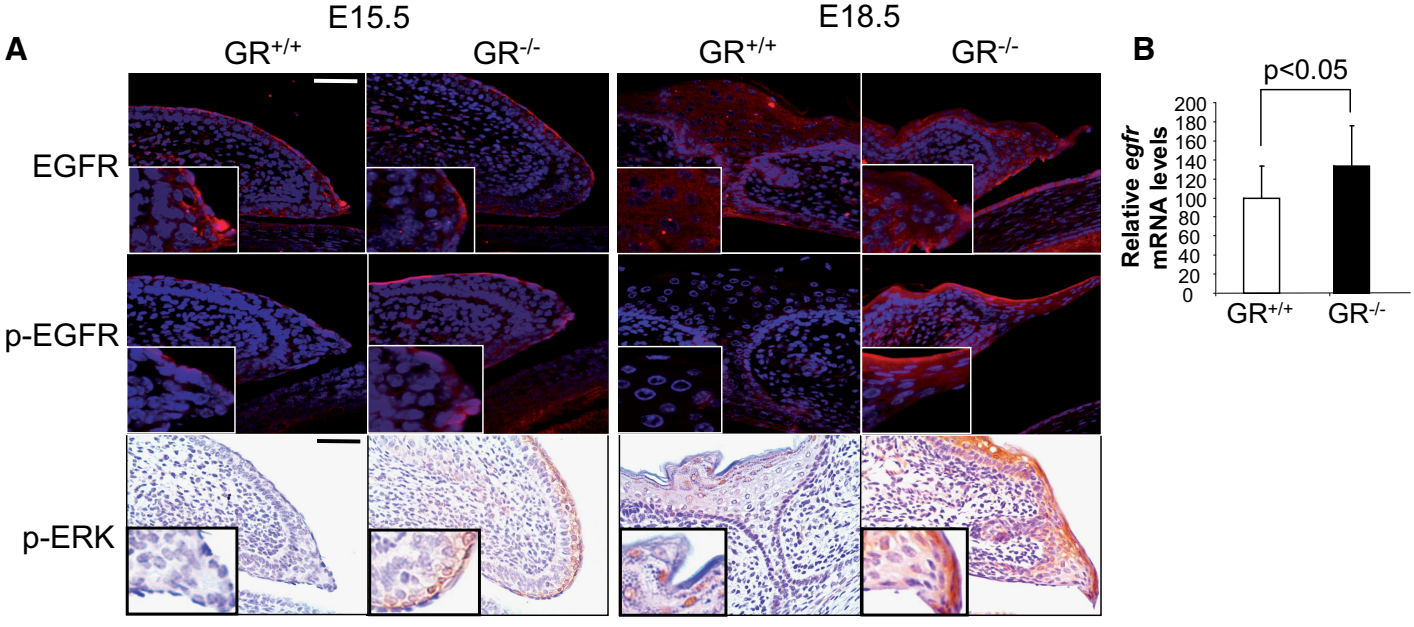

Fig. 3. Glucocorticoid receptor (GR) interferes with EGFR-mediated signaling during eyelid development. (A) *Immunofluorescences were performed in $GR^{-/-}$ and $GR^{+/+}$ embryos at E15.5 and E18.5 with specific antibodies against total EGFR and phosphorylated EGFR (p-EGFR). EGFR expression was slightly induced in $GR^{-/-}$ relative to $GR^{+/+}$ E18.5 embryos. Moreover, increased p-EGFR immunostaining was also detected in $GR^{-/-}$ E15.5 and E18.5 eyes.* **(B)** *Transcript levels of* egfr *were examined in skin of $GR^{-/-}$ and $GR^{+/+}$ E18.5 embryos by quantitative RT-PCR. Asterisk denote that differences in four individuals of each genotype were statistically significant; (student´s t test, $p<0.05$).* **(C)** *Immunostaining in $GR^{-/-}$ and $GR^{+/+}$ embryos at E15.5 and E18.5 for phosphorylated (p)-ERK. p-ERK was detected at E15.5 only in GR-deficient eyelids and the phosphorylation was sustained until E18.5, in contrast to wt embryos, where p-ERK was restricted to the granular layer of closed eyelids. Bar: 100 μm.*

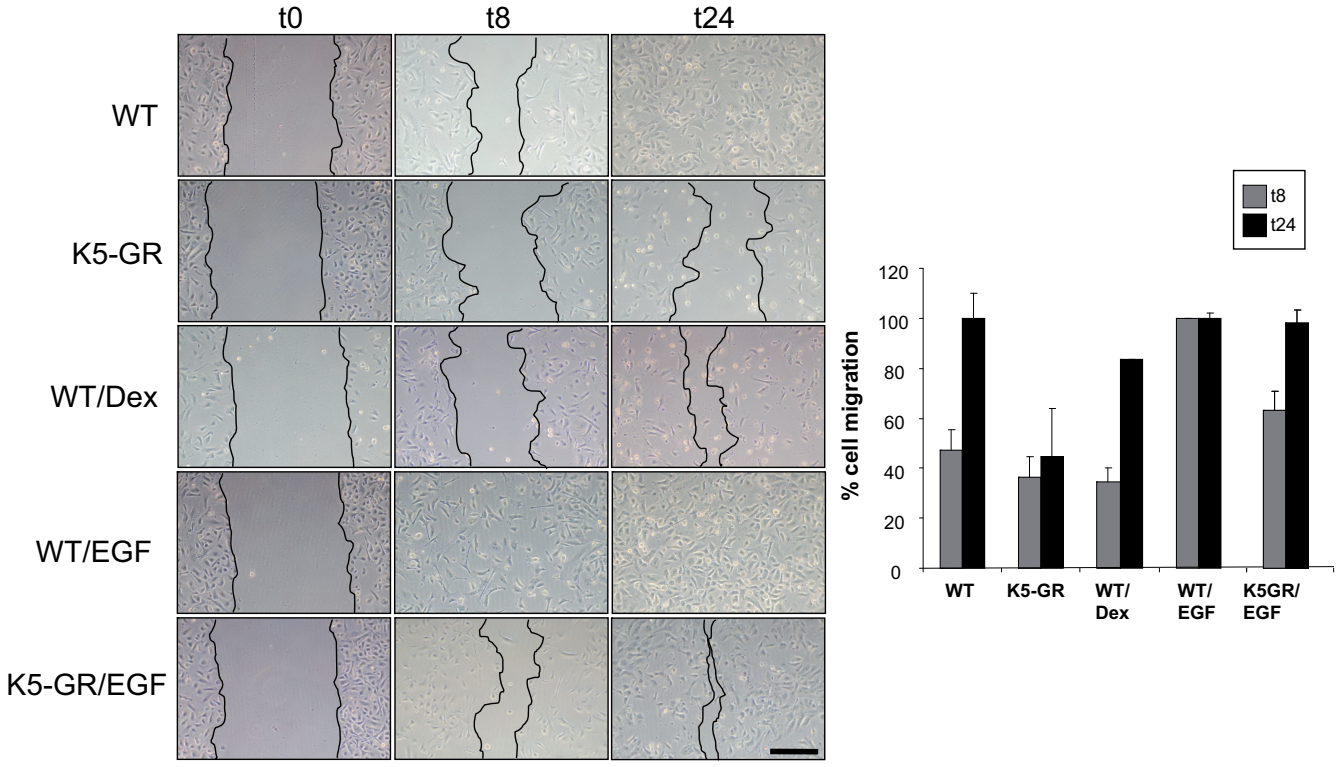

Fig. 4. Glucocorticoid receptor (GR) delays keratinocyte migration in *in vitro* wound scratch assays. *Wound scratch experiments were performed by using MPKs from wt and K5-GR mice and wound closure was evaluated at 8h (t8) and 24h (t24). Cells were treated with vehicle, Dex (100 nM) or EGF (25 ng/ml), as indicated. Note that the delayed keratinocyte migration in K5-GR MPKs was similar to that elicited by Dex treatment of wt cells. EGF accelerates keratinocyte migration in both genotypes although to a lesser extent in K5-GR cells. Microphotographs are representative of three independent experiments. The graph shows the average of three independent experiments. Quantitation of cell migration percentage was estimated as indicated in Materials and Methods. Bar: 100 μm.*

experiments that elicited consistent results. In wt cells, keratinocyte migration was 47.3% at 8h after scratching (t8) and was completed at 24h. In contrast, K5-GR MPKs exhibited 33.5% migration at t8, a marked delay that was still very prononunced at t24 (44.2%). For comparative purposes, we evaluated the response of wt MPKs to the GC-analogue Dex and EGF (Fig. 4). As previously described (Lee *et al.,* 2005), Dex delayed and EGF accelerated wt keratinocyte migration at t8 (35.2% and 100%, respectively). At t24, retarded migration elicited by Dex treatment was approximately 83.6%. Note that constitutive GR overexpression in K5-GR MPKs elicited effects similar to Dex treatment in wt cells. The retarded migration of K5-GR MPKs was only slightly potentiated by Dex treatment (data not shown). K5-GR keratinocytes also responded to EGF although with delayed kinetics as compared to wt cells (64.7% and 97.3% at t8 and t24, respectively).

Discussion

During mammalian embryogenesis, the surface ectoderm gives rise to the corneal and conjunctival epithelia and the epidermis of the eyelid. Eyelid formation takes place during mouse embryonic days E15.5 to E16.5 and requires the proliferation and migration of epithelial cells to cover the ocular surface, acting as a protective barrier for normal eye development (Xia and Karin, 2004). This process is similar to epidermal formation which results in the acquisition of a competent barrier necessary to protect the organism from environmental damage (Segre, 2006). Our recent work has shown that GR is required for epidermal formation since its absence results in an immature epidermal barrier (Bayo *et al.*, 2008). Analogously, histological evaluation of $GR^{-/-}$ embryos showed delayed and impaired eyelid closure, which took around 72-96h to complete instead of 24h, as occurs in wt mice (Fig. 1). Delayed progression in total eyelid formation was around 50% in $GR^{-/-}$ E15.5 embryos and approximately 78.3% in E18.5 individuals relative to their wt counterparts (Fig. 2 and data not shown). The fact that GR modulates proliferation and apoptosis of eyelid epithelial cells (Fig. 2 A,B) adds to our previous findings in epidermis showing that this nuclear receptor regulates these processes in a cell-autonomous manner (Bayo *et al.*, 2008) and highlights a general role of GR in epithelial morphogenesis. Our data also illustrate that these processes need to be temporally coordinated in order to complete proper eyelid formation. We found increased expression of markers of keratinocyte proliferation (K5, p63) in the eyelid epithelial cells of $GR^{-/-}$ embryos relative to wt, at a time where these markers should be restricted to the basal cell layer and a few suprabasal cells at the site of fusion (Fig. 1). We also observed incomplete differentiation of epithelial cells in $GR^{-/-}$ embryos (Fig. 1, K10 and loricrin), most likely due to the absence of eyelid fusion, which should precede terminal differentiation (Zhang *et al.*, 2005).

Our previous studies reported an EOB phenotype in K5-GR

and K5-GR-TR embryos that was indeed the most consistent epithelial defect found in both transgenic mouse models (Cascallana *et al.*, 2003; Donet *et al.*, 2008). In addition, our data demonstrated that the transrepression function of the GR is sufficient to cause these epithelial anomalies (Donet *et al.*, 2008). It was indeed surprising to find open eyelids in GR gain- and loss-of-function mouse models although, undoubtedly, it further supports a key role for this transcription factor in ocular epithelial morphogenesis. Since $GR^{-/-}$ embryos exhibit increased corticosterone levels due to a feedback mechanism mediated by the hypothalamus-pituitary-adrenal axis (Cole *et al.*, 1995 and our unpublished data), it is possible that elevated hormone levels may be acting through the mineralocorticoid receptor (MR) in $GR^{-/-}$ ocular epithelial cells and thus mimick increased GC-signaling, even in the absence of GR. Supporting this hypothesis, mice overexpressing the MR in keratinocytes (K5-MR) showed an EOB phenotype virtually identical to that of K5-GR and K5-GR-TR embryos (Sainte Marie *et al.*, 2007). The similarities between K5-GR and K5-MR mouse models extend to epidermal defects, including abnormal hair follicle formation and hypoplastic epidermis (Sainte Marie *et al.*, 2007). GR and MR recognize the same hexameric DNA response elements and, theoretically, could transactivate the same gene promoters (Kumar and Litwack, 2009). A subset of epithelial-specific genes may be regulated in common by both nuclear receptors. In this regard, our data show that GR represses *egfr* transcription in skin (Fig. 3B). However, MR has been reported to upregulate *egfr* mRNA expression in other cell types (Grossman *et al.*, 2007).

GR exerts many of its biological actions through interactions with distinct signaling pathways, including MAPK/AP-1 and NF-kappaB (reviewed in De Bosscher and Haegeman, 2009). Two main pathways have been involved in eyelid formation, the MEKK1/JNK/c-Jun pathway which transductes TGF-β and activin signals and the TGF-α/EGFR/ERK pathway; both result in AP-1 activation (revised in Xia and Karin, 2004). EGFR plays a key role in eye morphogenesis and, accordingly, disruption of the EGFR locus resulted in an EOB phenotype as well as immature development of skin epithelial cells, teeth, lung and gastrointestinal tract (reviewed in Sibilia *et al.*, 2007). We analyzed whether EGFR was abnormally expressed and/or activated in $GR^{-/-}$ embryos, and also investigated alterations in its downstream effector ERK. The expression pattern of both phosphorylated EGFR and ERK demonstrated sustained activation in $GR^{-/-}$ E18.5 embryos, a timepoint at which these proteins should be normally restricted to the more differentiated epithelial cells at the fused eyelids (Fig. 3). Sustained expression of ERK and K6 in the eyelids of $GR^{-/-}$ E18.5 mice is consistent with previous reports *in vitro* showing that EGF-signaling can induce the expression of K6 through AP-1 sites in its promoter (Lee *et al.*, 2005).

The process of eyelid closure parallels skin wound healing, which also requires proliferation and migration to complete re-epithelization across the wound (reviewed in Martin and Parkhurst, 2004; Barrientos *et al.*, 2008). The expression of the migration-associated keratin K6 increases during wound healing in adult skin and is required for normal re-epithelialization (Wong and Coulombe, 2003; Wojcik *et al.*, 2000). Activated GR can inhibit the expression of specific keratins through several mechanisms involving transcriptional and non-transcriptional events (Radoja *et al.*, 2000). GCs were shown to cause cytoskeleton remodeling by repressing K6/K16 expression and thus, inhibiting keratinocyte migration and causing deregulated growth and differentiation (Stojadinovic *et al.*, 2005). These GR actions are relevant during the wound healing process, whereby K6 expression in suprabasal keratinocytes at the wound´s edge is repressed once the epidermis covers the wound. This occurs through antagonism between GR and EGFR and involves activation of β-catenin and c-myc and blockade of EGF effects through the formation of a complex consisting in four GR monomers, β-catenin and coactivator-associated-arginine-methyltransferase-1 (Lee *et al.*, 2005; Stojadinovic *et al.*, 2005).

So far, the *in vivo* cross-talk between GR/EGFR in eyelid formation had not been previously investigated. Our data *in vivo* show that GR is a master regulator required for the spatio-temporal control of the EGFR/MAPK/AP-1 signaling at the leading edge of eyelid keratinocytes and suggest that this interference is required for normal eyelid development.

Materials and Methods

Animals

All mice were handled in accordance with the 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).

$GR^{-/-}$ and K5-GR mice have been previously reported (Cole *et al.*, 1995; Pérez *et al.*, 2001). $GR^{+/-}$ hemizygous mice (B6D2/F1) intercrosses were programmed to obtain $GR^{-/-}$, $GR^{+/-}$ and $GR^{+/+}$ mice. Embryos were obtained by cesarean derivation at the indicated day post-conception (dpc; the morning of the day that the vaginal plug was seen was considered as day 0.5 pc.). For histopathological evaluation, we analyzed embryos of different timepoints from $GR^{-/-}$, $GR^{+/-}$ and $GR^{+/+}$ genotypes (n=43). For preparation of mouse primary keratinocytes (MPKs) and wound scratch assays, skin from K5-GR mice and wt newborn littermates was excised and processed (n=38).

Antibodies

The antibodies used included rabbit polyclonal antibodies against keratin K5 (PRB-160P), K6 (PRB-169P), K10 (PRB-159P) and loricrin (PRB-145P) from Covance (Babco, Berkeley, CA). Antibodies against p63 (sc-404), p-c-jun (sc-822) and EGFR (sc-03) were from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA) and anti-p-EGFR (53A5), anti-p-ERK (Thr202/Tyr204) (#4376) and p-JNK (Thr183/Tyr185) (# 9251) were purchased from Cell Signaling (Cell Signaling Technology Inc., Beverley, MA). Secondary biotin-conjugated anti-rabbit or anti-mouse antibodies were from Jackson ImmunoResearch (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA).

Histological and Immunohistochemical analysis

Embryo heads were fixed in 4% paraformaldehyde (PFA) or 70% ethanol and embedded in paraffin. Consecutive 3 to 4 μm-thick sections were obtained. For histopathology, sections were stained with hematoxylin/eosin. Prior to immunostaining, paraffin sections were dewaxed and microwaved in 10 mM citrate solution. For immunohistochemistry, paraffin sections were blocked with 5% fetal bovine serum, and then incubated with the primary antibody for at least one hour. Slides were washed three times with PBS, and then incubated with conjugated secondary antibodies for 1 h. Finally, the reaction was visualized with the Avidin-Biotin-Complex (ABC) kit from DAKO (Vectastain Elite, Vector Laboratories, Inc, Burlingame, CA) using diamino-benzidine as chromogenic substrate for peroxidase. Slides were mounted and analyzed by light microscopy (Leica DM RXA2), and microphotographs were taken at the indicated magnification.

In vivo *epithelial BrdU labeling*

Epithelial cell proliferation was measured by i.p. injection of BrdU (130 μg/g of body weight, Roche) into pregnant female mice 1 h before sacrifice. BrdU incorporation was detected by immunohistochemistry of paraffin-embedded sections using a mouse anti-BrdU monoclonal antibody (biotest, Roche) followed by hematoxylin counterstaining. The number of BrdU-positive cells and the number of total cells was determined per 200 μm of interfollicular epithelium in each section. Experiments were performed at least in five individuals of each genotype and differences were assessed by using the t test, with statistical significance when $p < 0.05$.

Analysis of apoptosis in tissue sections

To detect individual apoptotic cells in paraffin-embedded tissue sections, the *In situ Cell Death Detection kit* (Roche) was used, following manufacturer´s recommendation. Paraffin sections immersed in 0.1 M citrate buffer, pH 6 were microwave-irradiated for 5 min, and then rinsed with PBS prior to the TUNEL reaction. Four 15.5 dpc embryos of each genotype were examined.

Measurements of eyelid formation, leading edge formation and root formation

We have quantitated the differences in eyelid formation of $GR^{-/-}$ *vs* wt E15.5 littermates, following the methodology described by Mine *et al*. These measurements were done in slides that were stained with K6, in order to delimitate the migrating epitheliall cells as K6-positive cells. This migration distance was considered as parameter *a*; the distance between the axis delimitating the conjunctival sac and the first K6-stained cells was denominated as *b*; the distance between the axis delimitating the conjunctival sac and the center of the cornea was denominated as *c*. The percentage of root formation was calculated as [b/c] x 100; percentage of leading edge formation was calculated as [a/c] x 100, and the percentage of eyelid formation was [(a+b)/c] x 100. Calculations were done in five individuals of each genotype and differences considered to be statistically significant when $p<0.05$.

MPK isolation, culture and wound scratch

MPK isolation was performed as previously described (Bayo *et al*., 2008). Briefly, skin was peeled off, incubated in 0.25% trypsin to separate the epidermis from the dermis and homogenized. MPKs were pooled (using at least two mice per point) and 10^6 cells were plated into one 35 mm diameter collagen coated petri dish (BD Biosciences) and cultured at 37°C in standard medium. After 24 h, the medium was replaced with complete low calcium medium and cells were grown until confluency. The composition of standard medium was: Essential modified Eagle´s medium EMEM (BioWhitakker, Inc., Walkersville, MD), supplemented with 4% fetal calf serum (FCS, BioWhitakker, Inc.) plus 0.6 mM $CaCl_2$ and antibiotics. To prepare low-calcium medium, FCS was depleted of divalent cations by treatment with Chelex deionizing resin (BioRad, Hempstead, UK) and supplemented with $CaCl_2$ to a final concentration of approximately 0.05 mM. EGF (Sigma, St. Louis, MO) (10 ng/ml) and antibiotics were added to growth medium.

For wound scratch assays, MPKs were incubated in EMEM/1% FBS O/N, and then treated with mitomycin (10μg/ml) for 1h. Next, cells were wounded with a yellow tip, treated as indicated and cell migration followed up for 8-24h. Experiments were performed in triplicate and mean value ± SD estimated. Vehicle, Dexamethasone (Dex, Sigma, St. Louis, MO, 100 nM) or EGF (25 ng/ml) were added for the indicated times to confluent wt MPKs.

For each wound scratch experiment, the surface area that remained uncovered by the cells for each time-point and condition was quantitated (Adobe Photoshop 8.1.0). These measurements were expressed as a percentage of distance coverage by cells moving into the scratch wound area 8 h and 24 h after wounding. Six images were analyzed per condition and time-point; then, averages and standard deviations were calculated.

RNA preparation and quantitative RT-PCR

Total RNA was isolated from back skin of $GR^{-/-}$ and control littermates (four animals of each genotype) by using Trizol reagent (Invitrogen, Molecular Probes, Eugene, Oregon), following manufacturer´s recommendations. Reverse transcription was performed by using 1 μg of RNA and oligo-dT (Fermentas Inc., Burlington, Canada) followed by qPCR using specific oligonucleotides for *egfr*

Forward, 5´-CAAAGTGATGTCTGGAGCTAT-3';

Reverse, 5´CTTGCTGGGATTCCATCATAAG-3'. Technical triplicates were performed and mean value ± SD estimated.

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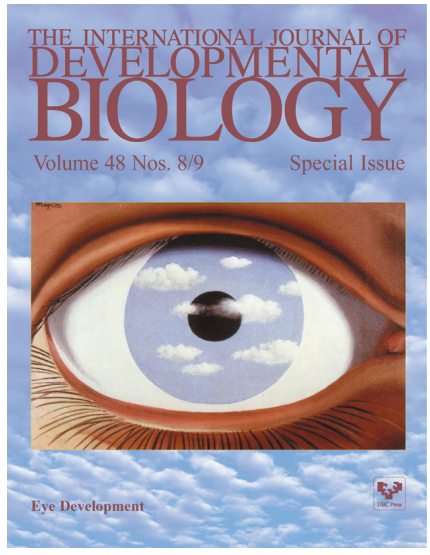

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