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Klf4 and corticosteroids activate an overlapping set of transcriptional targets to accelerate *in utero* epidermal barrier acquisition

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Premature infants are at an increased risk for infections and dehydration because of incomplete development of the epidermis, which attains its essential function as a barrier only during the last stages of *in utero* development. When a premature birth is anticipated, antenatal corticosteroids are administered to accelerate lung epithelium differentiation. One pleiotropic, but beneficial, effect of antenatal corticosteroids is acceleration of skin barrier establishment by an unknown mechanism. In mice, the transcription factor Klf4 is both necessary and sufficient, within a developmental field of competence, to establish this skin barrier, as demonstrated by targeted ablation and transgenic expression of Klf4, respectively. Here, we report that Klf4 and corticosteroid treatment coordinately accelerate barrier acquisition *in vivo*. Transcriptional profiling reveals that the genes regulated by corticosteroids and Klf4 during the critical window of epidermal development significantly overlap. KLF4 activates the proximal promoters of a significant subset of these genes. Dissecting the intersection of the genetic and pharmacological pathways, regulated by KLF4 and corticosteroids, respectively, leads to a mechanistic understanding of the normal process of epidermal development *in utero*.

development | transcription factor | skin | glucocorticoid receptor

In the United States, ≈11% of newborns are born prematurely, and in nearly half of all cases, the causes are not fully understood. Prematurely born infants are at an increased risk for life-threatening complications, such as respiratory distress and intraventricular hemorrhage. Transition from the aqueous *in utero* to the terrestrial *ex utero* environment also requires a fully competent epidermal barrier. Located at the interface between the body and the environment, the epidermis prevents both escape of moisture and entry of toxic substances. Because the skin develops its critical barrier function at ≈34 weeks of gestation, premature infants are at a greater risk for percutaneous infection and dehydration. Although the transition to the terrestrial environment *ex utero* accelerates the epidermal differentiation program, an early premature infant requires 2–4 weeks to develop a functional barrier (1).

A transcriptionally regulated program of linear terminal differentiation establishes the barrier within the exterior layers of the epidermis (2). Lipids are proteolytically processed inside lamellar bodies, and structural proteins assemble directly underneath the plasma membrane. As the cell membrane disintegrates, these proteins are cross-linked and serve as the scaffold for lipid extrusion, forming the "bricks and mortar" barrier (3–5). This process of differentiation from a mitotically active basal cell to a terminally differentiated squamous cell is maintained throughout life as part of epidermal regeneration (6).

Our previous studies have shown that the transcription factor *kruppel-like factor* 4 (*Klf4*) is both necessary and sufficient, within a field of competence, to establish a functional barrier (7, 8). Specifically, *Klf4*-deficient mice die perinatally because of dehydration as a direct result of the rapid water loss across their impaired barrier in the terrestrial environment. A whole-mount dye penetration assay reveals that $Klf4^{-/-}$ epidermis never matures *in utero* to exclude passage of small molecules across the skin surface (7). Ectopic expression of *Klf4* in the epidermis from the epidermal cytokeratin (K)5 promoter (K5-*Klf4*) accelerates barrier acquisition by ≈1 day as manifest by epidermal stratification and differentiation and dye impermeability (8). K5-*Klf4* mice demonstrate that the prenatal murine epidermis is competent to respond to a differentiation signal earlier in development and produce a functional barrier similar to the accelerated maturation *ex utero* observed for humans.

In human perinatology, a maternal injection of corticosteroids is standard of care to accelerate lung epithelium differentiation before an anticipated premature delivery (before 34 weeks gestation) (9). Studies in rodents have demonstrated that antenatal corticosteroid injections also accelerate epidermal barrier acquisition (10, 11). Conversely, mice deficient in corticosteroid processing exhibit a developmental delay in barrier acquisition (12). The molecular nature of these corticosteroid targets remains to be elucidated. Corticosteroids signal through the glucocorticoid receptor (GR), which is translocated to the nucleus to act as a transcription factor when bound by the steroid ligand. Intriguingly, early transcriptional studies demonstrated that a glucocorticoid response element (GRE) and a CACCC box, now known to be the element to which KLFs bind, synergistically promote transcription (13, 14). Turner and Crossley (15) revisited these classical experiments with a GRE-CACCC promoter to demonstrate that KLF1 (EKLF) and GR activate transcription from this test promoter with synergistic activation. However, KLF3 (BKLF) repressed both KLF1 and GR activation.

The data presented here coalesces the classical transcription data and the functional role of corticosteroids and KLF4 in barrier development to show coordinate regulation of specific targets during this critical stage of skin development. First, we demonstrate *in vivo* that KLF4 and corticosteroids can cooperatively accelerate barrier acquisition. Second, we determine that KLF4 and GR converge on an overlapping set of transcriptional targets. Finally, we establish that KLF4 regulates expression of a significant subset of these genes by binding to the proximal promoters.

Results

KLF4 and Corticosteroids Cooperatively Activate Barrier Acquisition *in Vivo*. During normal development, barrier acquisition initiates at embryonic day (E) 16.5 on the dorsal surface and spreads laterally to the ventral surface in a patterned fashion. Whole-mount dye penetration assays demonstrate the regions that both have and have not acquired barrier, visualized as white or dye-impermeable and blue or dye-permeable, respectively (11). Fig. 1 shows that barrier

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The authors declare no conflict of interest.

Abbreviations: Dex, dexamethasone; E*n*, embryonic day *n*; GR, glucocorticoid receptor; GRE, glucocorticoid response element; Q-RT-PCR, quantitative RT-PCR; TSS, transcriptional start site.

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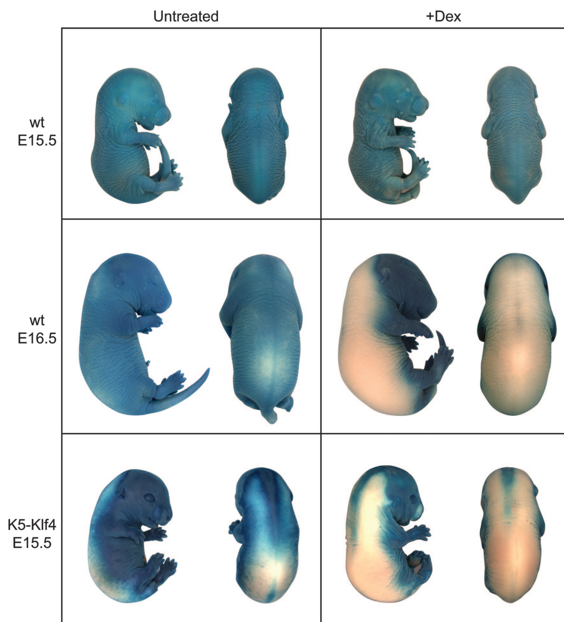

Fig. 1. Ectopic expression of Klf4 and levels of corticosteroids coordinately accelerate developmental barrier acquisition. As visualized with a whole-mount dye penetration assay on E15.5 and E16.5 embryos, corticosteroid injections accelerate barrier acquisition of wild-type (wt) embryos by one-half day. Corticosteroid-treated E15.5 K5-*Klf4* mice show greater barrier acceleration than either transgenic untreated littermates or wt corticosteroid-treated embryos.

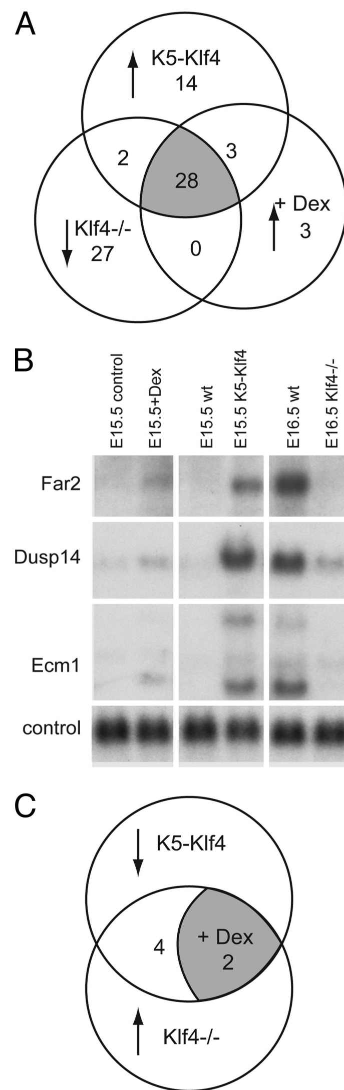

Fig. 2. Potential targets of corticosteroids and Klf4 in epidermal development. (*A*) Venn diagram of genes potentially activated by KLF4 and corticosteroid treatment. Transcriptional profiling identified genes misregulated in corticosteroid-treated, Klf4$^{-/-}$, and K5-Klf4 embryonic skin during the critical developmental period of barrier acquisition (E15.5-E16.5). Twenty-eight genes are found up-regulated >2-fold in K5-Klf4, down-regulated >2-fold in Klf4$^{-/-}$ embryos, and up-regulated >2-fold in corticosteroid-treated mice. (*B*) Northern blot analysis of representative genes: Fatty acetyl coA reductase 2 (*Far2*), Dual specificity phosphatase 14 (*Dusp14*), and Extracellular matrix 1 (*Ecm1*). (*C*) Six genes are identified as 2-fold down-regulated in K5-*Klf4* and 2-fold up-regulated in *Klf4*$^{-/-}$ embryos, and two of these are repressed in corticosteroid-treated mice.

acquisition has not yet initiated in wild-type embryos by E15.5, but by E16.5, barrier has initiated on the dorsal surface. Antenatal maternal injections of corticosteroids, dexamethasone (Dex) or β-methasone, can accelerate barrier acquisition *in utero* by ≈0.5 days in mice, evident at E16.5 (Fig. 1). Ectopic expression of Klf4 also accelerates barrier acquisition *in utero* by ≈1.0 day in K5-*Klf4* transgenics. To investigate the possible coordinate action of Klf4 and corticosteroids *in vivo*, we analyzed compound mouse models. Antenatal maternal injections of Dex further accelerate barrier acquisition of K5-*Klf4* embryos (Fig. 1). These data suggest a possible coordinate action of these pharmacologic and genetic pathways.

To investigate further the interactions of KLF4 and corticosteroids, we performed two additional experiments. Quantitative RT-PCR (Q-RT-PCR) and Northern blot analysis demonstrated that antenatal maternal injections of Dex do not alter the levels of *Klf4* expression during development (data not shown). Dye impermeability and transepidermal water loss studies showed that antenatal injections of Dex do not rescue the barrier defect in *Klf4*$^{-/-}$ mice (data not shown). These results are consistent with coordinate action of corticosteroids and KLF4.

Identification of Developmental Targets of Corticosteroids and KLF4. To identify the pathways and downstream targets regulated by corticosteroids and KLF4 in epidermal development, we performed transcriptional profiling of dorsal skin from *Klf4*$^{-/-}$, K5-*Klf4* transgenic, and Dex-treated mice at E15.5 and E16.5, the critical stages of barrier acquisition (7, 8). We also compared the transcriptional profile of normal E15.5 and E16.5 dorsal skin to determine the changes in epidermal gene expression during this developmental time. The gene expression data were analyzed to identify genes that are either up- or down-regulated in test samples compared with age-matched controls. Significant changes are observed in E16.5 *Klf4*$^{-/-}$, E15.5 corticosteroid-treated, and E15.5 K5-*Klf4* mouse skin compared with controls. In contrast, the expression profiles of E16.5 corticosteroid-treated and E16.5 K5-*Klf4* mouse skin were not significantly different from controls. Differences in gene expression profiles of the pharmacologically altered embryonic skin precede the observable manifestation of dye impermeability (i.e., comparing E15.5 corticosteroid-treated mouse skin with controls). When the skin has achieved dye impermeability, the transcriptional profiles are similar to the controls (e.g., E16.5 Dex-treated vs. -untreated or E16.5 K5-*Klf4* vs. control skin).

The number of genes 2-fold higher in K5-*Klf4* transgenic mouse skin, 2-fold higher in corticosteroid treated mouse skin, and 2-fold lower in Klf4$^{-/-}$ mouse skin than controls is presented in a Venn diagram in Fig. 2*A*. Forty-seven genes are up-regulated >2-fold in K5-*Klf4* embryonic skin as compared with controls. Thirty-four genes are up-regulated >2-fold in Dex-treated mouse skin as

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Table 1. Table of genes up-regulated in corticosteroid-treated and K5-Klf4 and down-regulated in Klf4$^{-/-}$ embryos with fold changes determined by Q-RT-PCR

Gene	+Dex	K5-Klf4	Klf4$^{-/-}$	E16.5 E15.5
Alox12b	6.1	5.7	−1.7	3.1
Cdsn	>10	>10	>−10	>10
Dusp14	5.2	6.3	−1.9	4.4
Ecm1	5.0	5.5	−3.0	4.3
Ephb6	1.7	6.1	−4.0	2.3
Far2	3.5	6.5	−2.5	5.5
Fmo2	>10	>10	−3.5	5.9
Gm2a	4.0	8.0	−2.8	2.2
Idb4	3.7	4.9	−2.8	2.5
IL-18	>10	>10	−1.6	5.6
Klf3	1.4	5.7	−5.7	1.9
Klk7	>10	>10	>−10	>10
Mtap2	8.6	8.6	−4.0	2.0
Nalp10	>10	4.6	−7.5	>10
Ptgs1	3.5	2.8	−2.1	2.5
Serpina12	>10	>10	>−10	>10
Smpd3	>10	>10	−2.3	4.9
Spink5	5.7	3.2	>−10	3.1
Tesc	4.3	4.6	−3.7	1.0

All samples are normalized to β2-microglobulin.

compared with controls. Also, 95% of these genes are up-regulated from E15.5 to E16.5 during wild-type epidermal development, underscoring the observation that ectopic expression of *Klf4* or Dex treatment accelerates the normal process of differentiation. Fifty-seven genes are down-regulated >2-fold in E16.5 *Klf4*$^{-/-}$ embryonic skin as compared with controls. Twenty-eight genes are in the overlap of all three categories. The two genes up-regulated in K5-*Klf4* and down-regulated in *Klf4*$^{-/-}$ samples, whose expression is not altered in corticosteroid-treated mouse skin are *Klf4* and *Klf3*. As described above, *Klf4* expression levels do not change with Dex treatment, and the levels of *Klf3* may directly depend on levels of *Klf4* expression, as has been observed for KLF3 in *Klf1* (*Eklf*$^{-/-}$) erythroid cells (16). Of the 27 genes down-regulated in only *Klf4*$^{-/-}$ embryos, only 9 are up-regulated during normal epidermal development (E15.5–E16.5). These other 18 genes represent either earlier defects in *Klf4*$^{-/-}$ epidermal specification or genes down-regulated in response to an impaired barrier.

For representative samples, expression levels were confirmed on Northern blots with three examples shown in Fig. 2*B*. *Fatty acetyl co-A reductase 2* (*Far2*) and *Dual specificity phosphatase 14* (*Dusp14*) are up-regulated between E15.5 and E16.5 with higher expression in corticosteroid-treated and K5-*Klf4* mouse skin than controls and lower expression in *Klf4*$^{-/-}$ mouse skin. *Extracellular matrix 1* (*Ecm1*) is alternatively spliced with three isoforms detected in the skin. Quantifying the two major forms of *Ecm1* (both upper and lower bands), *Ecm1* is dramatically up-regulated in corticosteroid-treated and K5-*Klf4* mouse skin and down-regulated in *Klf4*$^{-/-}$ mouse skin. Interestingly, the intermediate-sized splice form of *Ecm1* is not down-regulated as significantly in the *Klf4*$^{-/-}$ mouse skin. To confirm the expression levels of all genes identified in this analysis, Q-RT-PCR was performed on independently isolated samples (Table 1). Of the 28 genes identified as up-regulated in K5-Klf4 and Dex-treated mouse skin and down-regulated in *Klf4*$^{-/-}$ mouse skin, 5 (Filaggrin, Lce2, Lce3, Lce5, and Lce7) map to the epidermal differentiation complex, a tandem array of genes encoding proteins that are cross-linked to form the proteinaceous component of the barrier (17). We have studied the coordinate gene regulation of the epidermal differentiation complex in *Klf4*$^{-/-}$ mice (18). We also identified targets that map to two other clusters of genes: (*i*) epiregulin and betacellulin are small EGF-like ligands and (*ii*) Il1f5, Il1f6, and Il1f8 are IL-1 family members. We did not

Table 2. Table of genes identified as down-regulated in corticosteroid-treated and K5-*Klf4* skin and up-regulated in *Klf4*$^{-/-}$ skin with fold changes determined by Q-RT-PCR

Gene	+Dex	K5-Klf4	Klf4$^{-/-}$	E16.5 E15.5
Clca1	N/C	>−10	3.2	−1.4
Clca2	N/C	−6.1	4.9	−1.4
Cx26	−4.0	−1.6	9.2	−1.2
Dsc2	N/C	−2.0	7.5	−3.2
Rgs5	N/C	−2.0	1.6	−1.0
Upk1b	>−10	−4.9	>10	−5.6

All samples are normalized to β2-microglobulin.

pursue analysis of the 10 genes, which map to these 3 clusters, because their regulation is apt to be more complex. Instead, we focused on 18 unique putative targets activated by KLF4 and Dex treatment during late stages of embryonic epidermal differentiation. These 18 genes are involved in diverse pathways in epidermal regulation, including lipid synthesis (Alox12b and Far2) and transcriptional regulation (Idb4 and Klf3), which will be discussed below.

A similar analysis was undertaken to identify genes repressed by KLF4 and corticosteroid treatment; i.e., genes down-regulated >2-fold in K5-*Klf4* and Dex-treated skin and up-regulated in *Klf4*$^{-/-}$ skin compared with controls (Fig. 2*C*). A very small number of epidermal genes are down-regulated during normal epidermal development from E15.5 to E16.5 (<10). We identified six genes as potentially repressed by KLF4, with the gene names and fold changes given in Table 2. Only *Cx26* and *Upk1b* are down-regulated in corticosteroid-treated mouse skin and *Dsc2* and *Upk1b* in wild-type embryos from E15.5 to E16.5. Stemming from this observation that KLF4 modulates *Connexin 26* (*Cx26*), we have recently published a study (19) demonstrating the role of this gap junction protein in epidermal barrier establishment.

Regulation of Proximal Promoter Regions by KLF4. Transcriptional profiling does not address whether KLF4 or Dex directly regulate these genes or whether the misregulation is a read-out of a downstream effect. To determine whether any of these genes are direct targets, we first examined whether Klf4 regulated the sequences upstream of the transcriptional start site (TSS). To define the start of transcription, we used a combination of published results, data mining of spliced ESTs, and 5′ RACE.

For the 19 genes activated by KLF4 (Table 1) and 5 genes repressed by KLF4 (Table 2), we cloned ≈1.1 kb proximal promoter fragments (≈1 kb upstream and ≈0.1 kb downstream of the TSS) into a promoterless luciferase construct and transiently transfected each individually into a mouse keratinocyte (epidermal) cell line. Significantly shorter promoters were cloned for *Ecm1* and *Fmo2* because another gene and a large repetitive element maps proximal to the TSS, respectively.

As shown in Fig. 3, the basal level of activity for 21 of the 24 (19 + 5) constructs in mouse keratinocytes was 3-fold greater than the basic luciferase construct, suggesting that these are bona fide promoters. Of the 19 constructs made for genes activated by KLF4, 12 showed >2-fold activation when cotransfected with Klf4, suggesting that they are direct targets of KLF4: *Alox12b* (2.0-fold), *Cdsn* (2.2-fold), *Dusp* 14 (6.2-fold), *Ephb6* (4.7-fold), *Far2* (21.8-fold), *Gm2a* (21.9-fold), *Idb4* (3.3-fold), *Klf3* (9.2-fold), *Klk7* (7.0-fold), *Mtap2* (3.5-fold), *Smpd3* (6.0-fold), and *Tesc* (13.7-fold) (Fig. 3*A*). Of the seven clones not activated by Klf4, two were the smaller fragments described above and two did not exhibit basal promoter activity. Additionally, KLF4 may activate regulatory sequences further upstream of the transcription start site or contained within the introns. In summary, KLF4 activates the proximal promoters of

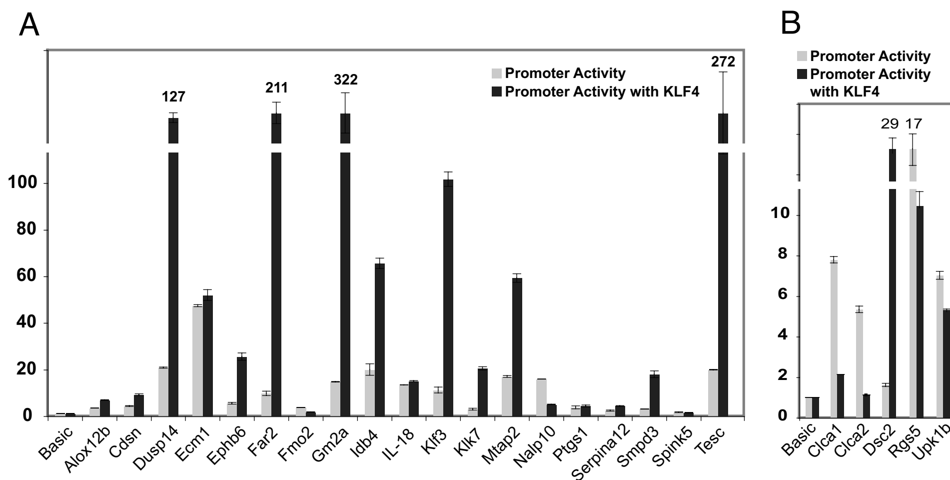

Fig. 3. Klf4 regulates proximal promoter of target genes. Constructs with proximal promoter (≈1 kb of sequence upstream of TSS) regions cloned upstream of a promoterless luciferase gene are transfected in keratinocytes in the absence or presence of *Klf4* and then normalized to vector control. (*A*) Twelve of the 19 promoters of genes induced by *Klf4* show >2-fold activation when cotransfected with *Klf4*. If promoter level is >100, the value is given above the bar. (*B*) Two of the five promoters of genes repressed by *Klf4* expression show >2-fold repression when cotransfected with *Klf4*. If promoter level is >10, the value is given above the bar.

a significant number of the genes identified as up-regulated in K5-*Klf4* and down-regulated in *Klf4*$^{-/-}$ transgenics.

Of the five constructs made for genes repressed by KLF4, two showed >2-fold repression when cotransfected with Klf4, suggesting that they are direct targets of KLF4 (Fig. 3*B*). *Clca1* and *Clca2* were repressed by KLF4 to almost basal levels of promoter activation, 3.6- and 4.8-fold, respectively. We have shown that the *Cx26* promoter is repressed 2.1-fold by KLF4 cotransfection (19). The *Dsc2* construct did not exhibit basal promoter activity and was, in fact, activated by *Klf4* cotransfection.

Direct Binding of KLF4 to Promoter Region. To test whether KLF4 activates the promoters by direct binding, we focused on *Far2*. The original *Far2* construct (1 kb upstream and 0.1 kb downstream of the TSS) is 19.4-fold activated by Klf4. First, we deletion-mapped the promoter and determined that a construct with –0.4 kb upstream of the TSS retains 9.8-fold Klf4 activation, but a construct with −0.1 kb upstream of TSS is not activated by KLF4 (Fig. 4*A*). To refine further the binding sites and to test whether KLF4 binds directly, we performed EMSA with probes spanning the Far2 promoter from −0.4 to −0.1 kb. Two probes (5 and 6) bound KLF4 with high specificity (Fig. 4*A*). Based on the previously published KLF4 binding sequence (RCRCCYY), probe 5 contains one site with 7 of 7 matches (GCGCCCT) that, when mutated, abolished KLF4 binding (data not shown) (20). Probe 6 contained three possible KLF4-binding sites. To refine the binding specificity, each possible KLF4 binding site in fragment 6 was individually and in pairs mutated to reveal that only the third site (ACACCCg) binds KLF4 (Fig. 4*B*). To determine whether KLF4 activation requires the sites identified by EMSA, we individually mutated the sites from fragments 5 and 6 in the 0.4-kb Far2 promoter (0.4Far2 × 5, 0.4Far2 × 6). Whereas 0.4Far2 × 5 retained full KLF4 activation, 0.4Far2 × 6 reduced KLF4 activation from 9.8-fold to 5.2-fold (Fig. 4*B*). These data demonstrate that the KLF4 site in fragment 6 is partially responsible for the KLF4 activation of the Far2 promoter. The residual activity observed in 0.4Far2 × 6 may reflect KLF4 indirectly interacting with DNA sequences in the *Far2* promoter or binding not detected by EMSA.

Regulation of Target Genes by Corticosteroids. Corticosteroids effect their function by binding to the GR, which then is translocated to the nucleus to act as a transcription factor. To identify GREs within regulatory sequences of these target genes, we used a genomic approach. We used the transcription factor binding prediction program TRANSFAC with the consensus GRE sequence of "ANRACAnnnTGT" to identify GRE elements in the DNA sequence from 5 kb proximal of the TSS through the second exon of the target genes (21, 22). Sequences with >90% similarity to the core GRE consensus sequence were identified at the predicted rate of approximately every 6 kb of nonrepetitive sequence. To discriminate whether these predicted GREs might be functional, we assessed whether they are conserved among vertebrate species by using both MultiPipMaker and the MultiZ alignment tracks at the

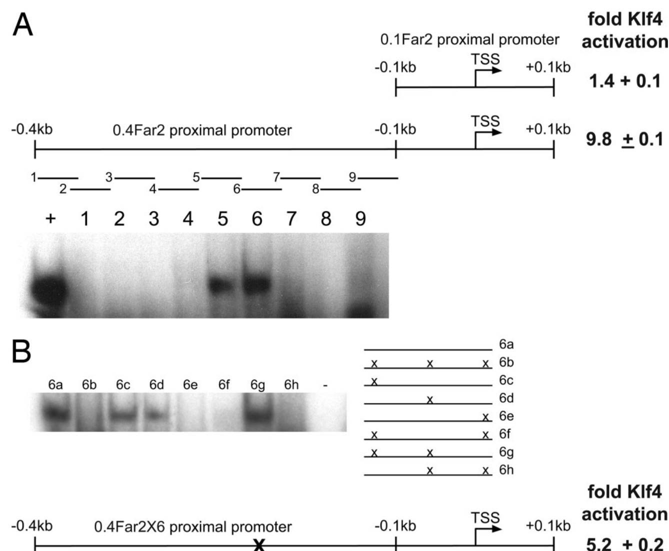

Fig. 4. KLF4 directly binds the proximal promoter of the *Far2* gene. (*A*) Fold activation by Klf4 of the *Far2* −0.4 and −0.1 deletion constructs. Location of probes used for EMSA that tile across the Far2 promoter. KLF4 binds to probes 5 and 6. (*B*) Mutational analysis of possible KLF4-binding sites to determine specificity of KLF4 binding. Probe 6 has three possible KLF4-binding sites, which are all mutated individually and in pairs to demonstrate that only the most 3′ site is required for KLF4 binding to this probe. Mutation of the KLF4-binding site 6 in the 0.4Far2 promoter (0.4Far2 × 6) reduces KLF4 activation from 9.8- to 5.2-fold.

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University of California, Santa Cruz genome web browser (23–25). Intriguingly, we found that many of these predicted GRE elements localize to the most highly conserved sequences in the gene. For example, the intronic sequence of Dusp14, spanning 17.5 kb, contains a single region of 125 bp that shows strong sequence conservation (67%) across human, mouse, dog, and opossum (Fig. 5, which is published as supporting information on the PNAS web site); this region contains a highly conserved GRE (AgA ACA gat TGT) (Fig. 5). Similarly, the intron of Mtap2 contains two regions that are highly conserved across all eutherian mammals and the metatherian opossum; these regions contain highly conserved GREs. (Fig. 5). Examples in five additional genes described are in Fig. 6, which is published as supporting information on the PNAS web site. Thus, many of the genes that are induced by corticosteroids *in utero* contain GREs embedded within sequences that have been highly conserved across 185 million years of evolution (26). This suggests that the genes may be directly regulated by corticosteroids.

We were unable to directly test the functional role of these highly conserved GREs in epidermal development, because we lack a relevant biological system in which to assess their potential. Embryonic keratinocytes are difficult to use directly, because they commit irreversibly to differentiation when placed in culture (27). Moreover, multiple established lines of postnatally derived mouse keratinocytes do not provide a suitable model system: Genes identified as corticosteroid-responsive *in utero* are not up-regulated in keratinocytes transfected with GR and treated with Dex. This result suggests either that corticosteroids act in a nonkeratinocyte autonomous manner *in utero* or that the target genes are developmentally regulated. As discussed below, a biological rationale does support the suggestion that corticosteroids have distinct effects depending on the developmental window. In any case, direct proof of whether these genes are directly regulated by corticosteroids will require a suitable model system that recapitulates the regulation seen *in vivo*.

Discussion

Our previous studies have demonstrated that *Klf4* is both necessary and sufficient, within a field of competence, to achieve maturation of the epidermal permeability barrier *in utero* (7, 8). Here, we examine the targets of KLF4 during this developmental window and identify genes that are directly regulated by KLF4. We find a significant overlap between the genetic and pharmacological pathways, regulated by KLF4 and antenatal corticosteroid treatment, respectively. The *in vivo* studies (Fig. 1) show that coordinate activation of both of these pathways can accelerate the epidermal maturity of an E15.5 embryo to resemble an E17 embryo. Compared with human skin development, E15.5 is ≈26 weeks and E17 is ≈32 weeks, which is a critical window for *ex utero* development of premature babies. Regulating either KLF4 expression or the downstream pathways activated by both KLF4 and corticosteroids in the skin ultimately may lead to more selective treatments to accelerate this process *ex utero* for prematurely born infants.

For these experiments, we used the K5-*Klf4* transgenic line that expresses physiologic levels of *Klf4*, although earlier in development. Lines that expressed higher than physiologic levels of *Klf4* exhibited specific defects in outgrowth of the limbs, craniofacial abnormalities, and omphacoele (8). Ectopic expression of GR from the K5 promoter results in the same developmental manifestations, but the mice were not specifically tested for barrier acceleration (28). Corticosteroid injections further accelerate the barrier acquisition of the K5-*Klf4* embryos *in vivo* without these additional deleterious side effects, demonstrating that stimulating with lower levels of both corticosteroids and Klf4 is more beneficial than increasing just one stimulus to higher levels. Classical studies with an explant model of fetal skin development, which closely parallels *in utero* development, demonstrated that both glucocorticoid and thyroid hormone induce expression of differentiation proteins and accelerate barrier formation (29, 30). Interestingly, KLF4 and thyroid hormone have been shown to synergistically activate expression of an enterocyte differentiation promoter (31). Future studies should be performed to address how the thyroid hormone pathway integrates with corticosteroids and KLF4 to regulate epidermal barrier development.

Transcriptional profiling demonstrates that corticosteroids and KLF4 regulate an overlapping set of targets. KLF4 can both activate and repress the proximal promoters of target genes, up- and down-regulated by KLF4 *in vivo*, respectively. KLF4 contains activation and repression domains, both of which appear to function in regulating gene expression during keratinocyte differentiation (20). In contrast, we find no evidence that corticosteroid treatment either regulates the endogenous gene expression or directly regulates the promoters of these target genes in established mouse keratinocyte cells. These experiments suggest either that the effect of Dex *in vivo* is nonkeratinocyte autonomous or that these are developmentally regulated targets of Dex. Corticosteroid deficiency delays epidermal maturation until E17.5, but by birth, the epidermis is mature by all physiological criteria, defining this critical window of corticosteroid activity (12). Moreover, although corticosteroid treatment accelerates barrier acquisition *in utero*, either topical or systemic glucocorticoid treatment of adult skin results in an inhibition of lipid synthesis and delayed barrier recovery (32).

Some of these genes identified in these screens already have been implicated in human skin and epidermal barrier disorders. 12(R)-lipoxygenase (ALOX12B), an epidermal lipoxygenase that catalyzes the oxygenation of arachidonic acid, is mutated in nonbullous congenital ichthyosiform erythroderma (33). SPINK5 is a serine protease inhibitor mutated in Netherton's syndrome, a congenital ichthyosis with atopic features (34). Corneodesmosin (*Cdsn*) is proteolysed prematurely in Spink5-deficient mice, and also maps proximal to the HLA-C region associated with psoriasis susceptibility (35, 36). Fatty acyl-CoA reductase 2 (FAR2) reduces fatty acids to fatty alcohols, a key step in lipid biosynthesis (37). IL-18 is an inflammatory cytokine that plays a role in atopic dermatitis by enhancing IL-4 and IL-13 production and stimulating the synthesis of IgE (38). The function in skin of the proteins encoded by additional target genes remains to be elucidated.

Although this study focuses on barrier acquisition during the *in utero* developmental stages, barrier must be maintained throughout life and reestablished after a breach. Reestablishment of the barrier is a key trigger in the wound repair process, signaling the transition from increased proliferation to reestablishment of the homeostatic balance (19). Impaired epidermal barrier function is a hallmark feature of two of the most common inflammatory skin disorders, psoriasis and atopic dermatitis (39). Very recent genetic findings of commonly occurring mutations in the epidermal cornification protein filaggrin underlying susceptibility to both atopic dermatitis and asthma underscore the clinical need to understand better how barrier establishment is regulated (40). Analysis of the sensitive *in utero* development should help to elucidate the pathways necessary to reestablish the barrier of chronic skin diseases after injury.

Materials and Methods

Generation of Mice. Mice were time-mated and the morning of vaginal plug detection was called E0.5. The pregnant female was injected intramuscularly at day 13.5 and 14.5 of pregnancy with 1 mg/kg body mass of dexamethasone Solution (Phoenix Pharmaceutical, St. Joseph, MO) or with vehicle 0.9% saline. Genotyping of K5-*Klf4* line 2 and *Klf4*$^{-/-}$ mice was done as published in refs. 7 and 8.

Barrier Function Assays. Whole-mount dye penetration assays with X-Gal substrate at pH 4.5 were performed for 4 h as described in ref. 11. After fixing in 4% paraformaldehyde, embryos were photographed under a MZFLIII dissecting scope (Leica, Bannockburn, IL) by using a digital Axiocam camera (Zeiss, Thornwood,

NY), and images were acquired with Openlab software. When necessary, tail tips were removed for genotyping.

mRNA Analysis. Dorsal skin (1 cm^2) from three E15.5 or E16.5 mouse embryos were collected, snap-frozen in liquid nitrogen, pulverized, and homogenized in TRIzol to isolate RNA (Invitrogen, Carlsbad, CA). For microarray studies, these mRNAs were purified with an RNeasy kit (Qiagen, Valencia, CA) and cDNA, labeled with Cy3 or Cy5 dUTP (GE Healthcare Biosciences, Piscataway, NJ), was made from 30 μg of total RNA. Affymetrix (MU 430 A+B 2.0) cDNA microarray slides contain 45,000 probe sets, which represents 34,000 well substantiated mouse genes. We identified ≈20,000 probes as present in mouse skin during the developmental window analyzed in these experiments. Slides were analyzed on an Agilent scanner and evaluated with IPLab software. After normalization to control for hybridization, multiple pairwise testing was carrier out to identify genes with 2-fold or greater changes in expression with $P < 0.001$. For Northern blot analysis, 10 μg of skin mRNA was loaded per lane and visualized by ethidium bromide for integrity of the samples. Blots were hybridized with antisense probe against *Ecm1*, *Dusp14*, and *Far2* or with G3PDH probe as loading control. For Q-RT-PCR, unique primers spanning intron boundaries were generated and resulting amplicons were sequenced verified. Primer sequences are provided as Table 3, which is published as supporting information on the PNAS web site. Reactions were carried out with SybrGreen labeling by using the Q-PCR mix (Invitrogen) and run on the ABI Prism 7500 sequence detector (PE Applied Biosystems, Foster City, CA). PCRs were run on agarose gels to ensure that correct size product was generated. A cDNA dilution series was run in triplicate to ensure amplification was in the linear range. cDNA synthesis was normalized to amplification of β-2microglobulin.

DNA Constructs and Transfections. Promoter regions were amplified by PCR from BAC DNA with the Advantage-HF 2 PCR Kit or Advantage-GC 2 PCR Kit by following manufacturer's instructions (BD Biosciences, San Jose, CA). Amplicons were digested with restriction enzymes contained uniquely in primer sequences and cloned directly into pGL3 Basic luciferase reporter vector (Promega, Madison, WI). Alternatively, chimeric primers were used, and the amplicon cloned directly into the vector with BD-In fusion. Exact nucleotide positions of the clones are given in Table 4, which is published as supporting information on the PNAS web site. *Far2* promoter deletion constructs were generated by cutting at the 5′ SpeI site brought in from the chimeric primer and PstI and StuI in the promoter sequences, blunting with T4 DNA polymerase and relegating to form the −0.4 kb- and −0.1-kb constructs, respectively. Mutations in the *Far2* promoter were created by PCR amplifying with mismatched oligos and then recloning into the pGL3 Basic promoter.

Cell Culture and Transfections. The SP-1 mouse keratinocyte cell line was cultured under the standard conditions of S-MEM media (Invitrogen) with 8% chelex-treated FBS (Gemini, West Sacramento, CA) at 0.05 mM Ca^{2+} (41). Cells were seeded at 2–3 × 10^5 cells per well and transfected with Lipofectamine Plus (Invitrogen) under optimized conditions. Full-length Klf4 cDNA was amplified by RT-PCR from newborn skin and cloned into pcDNA3 and sequence-verified. Empty pcDNA3 vector was used as a control for DNA concentration. Transfections include a control *Renilla* luciferase plasmid (phRL-null) for normalization, and dual luciferase measurements were made (Promega, Madison, WI).

EMSA. Klf4 cDNA encoding the zinc finger portion of the protein (amino acid 308–474 of S405921) was cloned into TOPO His-6 pET100 (Invitrogen), sequence-verified, and transformed into BL21 Star cells (HIS-KLF4Zn). Expression was induced during a 1-h growth at 30°C with 0.5 mM IPTG in the presence of 2% ethanol. Protein was purified on a Nickel Pro-Bond column (Invitrogen) under native conditions at pH 8.0. For EMSA, double-stranded oligonucleotides, provided in Table 5, which is published as supporting information on the PNAS web site, were labeled with T4 kinase and [γ-^{32}P]ATP. Probe (40,000 cpm) was incubated with 0.2 μg of recombinant HIS-Klf4Zn protein and then run on a 6% DNA retardation gel in 0.5× TBE.

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1. Kalia YN, Nonato LB, Lund CH, Guy RH (1998) *J Invest Dermatol* 111:320–326.
2. Dai X, Segre JA (2004) *Curr Opin Genet Dev* 14:485–491.
3. Elias PM (2005) *J Invest Dermatol* 125:183–200.
4. Kalinin AE, Kajava AV, Steinert PM (2002) *BioEssays* 24:789–800.
5. Segre J (2003) *Curr Opin Cell Biol* 15:776–782.
6. Fuchs E, Raghavan S (2002) *Nat Rev Genet* 3:199–209.
7. Segre JA, Bauer C, Fuchs E (1999) *Nat Genet* 22:356–360.
8. Jaubert J, Cheng J, Segre JA (2003) *Development (Cambridge, UK)* 130:2767–2777.
9. NIH Consensus Development Panel (1995) *J Am Med Assoc* 273:413–418.
10. Aszterbaum M, Feingold KR, Menon GK, Williams ML (1993) *J Clin Invest* 91:2703–2708.
11. Hardman MJ, Sisi P, Banbury DN, Byrne C (1998) *Development (Cambridge, UK)* 125:1541–1552.
12. Hanley K, Feingold KR, Komuves LG, Elias PM, Muglia LJ, Majzoub JA, Williams ML (1998) *J Invest Dermatol* 111:440–444.
13. Schule R, Muller M, Otsuka-Murakami H, Renkawitz R (1988) *Nature* 332:87–90.
14. Strahle U, Schmid W, Schutz G (1988) *EMBO J* 7:3389–3395.
15. Turner J, Crossley M (1998) *EMBO J* 17:5129–5140.
16. Crossley M, Whitelaw E, Perkins A, Williams G, Fujiwara Y, Orkin SH (1996) *Mol Cell Biol* 16:1695–1705.
17. Marshall D, Hardman MJ, Nield KM, Byrne C (2001) *Proc Natl Acad Sci USA* 98:13031–13036.
18. Patel S, Kartasova T, Segre JA (2003) *Mamm Genome* 14:140–148.
19. Djalilian AR, McGaughey D, Patel S, Seo EY, Yang C, Cheng J, Tomic M, Sinha S, Ishida-Yamamoto A, Segre JA (2006) *J Clin Invest* 116:1243–1253.
20. Yet SF, McA'Nulty MM, Folta SC, Yen HW, Yoshizumi M, Hsieh CM, Layne MD, Chin MT, Wang H, Perrella MA, *et al.* (1998) *J Biol Chem* 273:1026–1031.
21. Chen L, Finnerty C, Gustafson WC, Bush CR, Chi P, Guo H, Luxon B, Fields AP, Thompson EA (2003) *Recent Prog Horm Res* 58:155–174.
22. Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, Meinhardt T, Pruss M, Reuter I, Schacherer F (2000) *Nucleic Acids Res* 28:316–319.
23. Blanchette M, Kent WJ, Riemer C, Elnitski L, Smit AF, Roskin KM, Baertsch R, Rosenbloom K, Clawson H, Green ED, *et al.* (2004) *Genome Res* 14:708–715.
24. Schwartz S, Elnitski L, Li M, Weirauch M, Riemer C, Smit A, Green ED, Hardison RC, Miller W (2003) *Nucleic Acids Res* 31:3518–3524.
25. Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, Hou M, Rosenbloom K, Clawson H, Spieth J, Hillier LW, Richards S, *et al.* (2005) *Genome Res* 15:1034–1050.
26. Margulies EH, NISC Comparative Sequencing Program, Maduro VVB, Thomas PJ, Tomkins JP, Amemiya CT, Luo M, Green ED (2005) *Proc Natl Acad Sci USA* 102:3354–3359.
27. Okuyama R, Nguyen BC, Talora C, Ogawa E, Tommasi di Vignano A, Lioumi M, Chiorino G, Tagami H, Woo M, Dotto GP (2004) *Dev Cell* 6:551–562.
28. Perez P, Page A, Bravo A, Del Rio M, Gimenez-Conti I, Budunova I, Slaga TJ, Jorcano JL (2001) *FASEB J* 15:2030–2032.
29. Hanley K, Rassner U, Elias PM, Williams ML, Feingold KR (1996) *J Invest Dermatol* 106:404–411.
30. Komuves LG, Hanley K, Jiang Y, Elias PM, Williams ML, Feingold KR (1998) *J Invest Dermatol* 111:429–433.
31. Siddique A, Malo MS, Ocuin LM, Hinnebusch BF, Abedrapo MA, Henderson JW, Zhang W, Mozumder M, Yang VW, Hodin RA (2003) *J Gastrointest Surg* 7:1053–1061, discussion 1061.
32. Kao JS, Fluhr JW, Man MQ, Fowler AJ, Hachem JP, Crumrine D, Ahn SK, Brown BE, Elias PM, Feingold KR (2003) *J Invest Dermatol* 120:456–464.
33. Jobard F, Lefevre C, Karaduman A, Blanchet-Bardon C, Emre S, Weissenbach J, Ozguc M, Lathrop M, Prud'homme JF, Fischer J (2002) *Hum Mol Genet* 11:107–113.
34. Chavanas S, Bodemer C, Rochat A, Hamel-Teillac D, Ali M, Irvine AD, Bonafe JL, Wilkinson J, Taieb A, Barrandon Y, *et al.* (2000) *Nat Genet* 25:141–142.
35. Nair RP, Stuart PE, Nistor I, Hiremagalore R, Chia NV, Jenisch S, Weichenthal M, Abecasis GR, Lim HW, Christophers E, *et al.* (2006) *Am J Hum Genet* 78:827–851.
36. Yang T, Liang D, Koch PJ, Hohl D, Kheradmand F, Overbeek PA (2004) *Genes Dev* 18:2354–2358.
37. Cheng JB, Russell DW (2004) *J Biol Chem* 279:37789–37797.
38. Tsutsui H, Yoshimoto T, Hayashi N, Mizutani H, Nakanishi K (2004) *Immunol Rev* 202:115–138.
39. Segre JA (2006) *J Clin Invest* 116:1150–1158.
40. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, Goudie DR, Sandilands A, Campbell LE, Smith FJ, *et al.* (2006) *Nat Genet* 38:441–446.
41. Strickland JE, Greenhalgh DA, Koceva-Chyla A, Hennings H, Restrepo C, Balaschak M, Yuspa SH (1988) *Cancer Res* 48:165–169.