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DEVELOPMENT AND DISEASE

Ectopic expression of Kruppel like factor 4 (*Klf4*) accelerates formation of the epidermal permeability barrier

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SUMMARY

Dysfunction of the epidermal permeability barrier can result in dehydration, electrolyte imbalance and poor thermoregulation. Immature skin is a portal of entry for infectious agents and potential toxins in topically applied substances. As the skin is one of the last organs to mature in utero, premature infants born before 34 weeks gestation are at great risk for complications. The transcription factor kruppel-like factor 4 (*Klf4*), has been shown by a targeted ablation, to have an essential function in barrier acquisition. We investigated whether *Klf4* expression in utero is sufficient to establish the epidermal barrier. Specifically, we generated lines of mice that express *Klf4* from a tetracycline inducible promoter when crossed with transgenic mice expressing the tetracycline transactivator tTA from the epidermal keratin 5 promoter. These mice exhibit acceleration in barrier acquisition as manifest by the exclusion of a dye solution one day earlier in development than controls. Underlying this dye impermeability are morphological changes, including an increased number of stratified layers, expression of terminal differentiation markers and assembly of cornified envelopes. By all criteria, *Klf4* ectopic expression accelerates the normal process of terminal differentiation. Premature barrier acquisition in these mice follows the normal pattern rather than the pattern of the transgene promoter, indicating that there are fields of competence in which KLF4 acts. Although other transgenic mice have perturbed barrier acquisition, these mice are the first to accelerate the process of barrier establishment. These studies show that KLF4 regulates barrier acquisition and provides an animal model for studying how to accelerate the process of barrier acquisition for the premature infant.

Key words: KLF4, Epidermis, Permeability barrier, Perinatal mortality, Mouse

INTRODUCTION

Approximately 10% of all births in the US are premature infants. Although sometimes, premature labor can be delayed, other attempts to inhibit premature labor are not successful and thus a ‘cure’ for prematurity remains elusive. Of babies born at 28 weeks gestation, approximately 80% survive. The greatest complication is immaturity of the organs, including lungs and central nervous system. Immaturity of the skin, which only develops its major and necessary function as a barrier at 34 weeks, also places the premature infant at great risk because the poorly developed epidermis is a portal of entry for infection and potential toxins in topically applied substances (Cartlidge, 2000; Hoath and Narendran, 2000; Rutter, 2000; Wilson and Maibach, 1980). Incomplete barrier acquisition of a premature infant can result in dehydration, electrolyte imbalance and poor thermoregulation. Although the transition to the terrestrial environment ex utero accelerates the epidermal differentiation program, an infant born at 25-30 weeks requires 2-4 weeks to develop a functional barrier (Kalia et al., 1998). During this time, the infant requires three-times more liquids because of the rapid loss across the skin surface. Although the lungs have a greater surface area, the ventilator provides humidified temperature-regulated air to minimize water loss across this surface. The infant can be placed in a humidified incubator for a few days to minimize the water loss across the skin surface but this places the infant at risk for infection. Additional complications can arise from placing leads and other monitoring devices on the fragile premature skin.

The major barrier between body and environment is the exterior layer of the epidermis, the stratum corneum, which is sloughed off and repopulated from the inner cells. This process of differentiation is maintained throughout life as part of epidermal regeneration and maturation (Roop, 1995; Steinert, 2000).

Mammalian epidermis, a stratified epithelium, is a self-renewing tissue composed of a population of mitotically active cells in the innermost basal layer and their derivatives that travel upward to the skin surface in a linear program of terminal differentiation (Fuchs and Raghavan, 2002; Niemann and Watt,

2002). This process begins when basal cells concomitantly withdraw from the cell cycle, lose adhesion to the basement membrane and initiate differentiation. In the intermediate spinous layers, the cells remain transcriptionally active, synthesizing and assembling a durable cytoskeletal framework that provides mechanical strength. In the upper granular layer, the cells flatten and the intracellular contents are degraded (including the nuclei). Each cell leaves behind keratin macrofibrils and lipid-containing lamellar bodies that fuse with the plasma membrane. Subsequently, a cornified envelope (CE) is assembled directly underneath the plasma membrane by sequential incorporation of precursor proteins. Finally, in the outermost stratum corneum, the cells become permeable and a calcium influx activates transglutaminase enzymes to irreversibly cross-link the CE proteins, creating a tough, insoluble sac that surrounds the keratin fibers. The CE serves as a scaffold for the lipids extruded from the lamellar bodies, which in turn seal together CEs to create the barrier at the skin surface. The structure that performs the barrier function is analogous to 'bricks and mortar' with the keratin macrofibrils and CEs forming the bricks and extracellular lipids the mortar. Recent experimental results have also demonstrated an essential role for tight junctions in epidermal barrier (Furuse et al., 2002).

Kruppel-like factor 4 (*Klf4*, formerly GKLF) is a zinc-finger transcription factor expressed in the differentiated suprabasal cells of the epidermis, crypt cells of the gastrointestinal tract and several other organs (Garrett-Sinha et al., 1996; Katz et al., 2002; Segre et al., 1999; Shields et al., 1996). *Klf4* is necessary for the development of the epidermal barrier, since mice homozygous for a null mutation in *Klf4* die perinatally as a result of water loss across the skin surface (Segre et al., 1999). The goal of this study was to test if *Klf4* is sufficient to accelerate epidermal barrier acquisition with the hope that an understanding of this pathway will lead to better methods to ameliorate this process in the premature infant.

To test if KLF4 is sufficient to establish the epidermal barrier, we specifically overexpressed *Klf4* one stage earlier in development, i.e. in the basal layer of the epidermis. To circumvent perinatal lethality, we utilized the bipartite tetracycline responsive transgenic system. Specifically, we generated 'responder' lines with *Klf4* transcription directed by a minimal promoter with upstream tetracycline response elements (TRE). These mice were crossed with a previously characterized tet-OFF 'transactivator' line expressing the tetracycline transactivating factor (tTA) from the basal keratin 5 (K5) promoter (Diamond et al., 2000). Although both the K5-tTA and TRE- *Klf4* lines were phenotypically normal, crossing them together produced mice with a K5-tTA/TRE- *Klf4* genotype that ectopically expressed *Klf4* in the basal cells of the epidermis earlier in development than wild-type littermates. Such mice formed their barrier in utero one full day earlier than wild-type littermates, providing evidence of the key role played by the transcription factor *Klf4* in the establishment of the barrier function in mice.

MATERIALS AND METHODS

Characterization of the K5-tTA transgene expression in development and detection of β-galactosidase histochemistry

Transgenic mice with the bovine keratin 5 promoter driving the tetracycline transactivator (K5-tTA line 1216) and with the *lacZ* gene downstream of a tetracycline-regulated promoter (TRE-*lacZ*) were gifts from A. Glick. These mice were crossed and maintained without doxycycline to induce expression of tTA in the basal layer of the epidermis throughout development. The morning of vaginal plug detection was taken as embryonic day 0.5 post-coitum (E0.5). Embryos were pre-fixed in 4% paraformaldehyde for 20 minutes (E12.5), 25 minutes (E13.5) and 30 minutes (E14.5). Whole-mount staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) substrate was performed for 4 hours at 37°C. After post-fixing in 4% paraformaldehyde, embryos were photographed under a MZFLIII dissecting microscope (Leica) using a digital Axiocam camera (Zeiss) and images were acquired with Openlab software. Genotyping of embryos was done by PCR on tail DNA using the following primers: K5-tTA transgene: 5′-CCTAGATAACAGAGC-CGCTTTC, 5′-CCATCGCGATGACTTAGT; *lacZ* transgene: 5′-CCAACTTAATCGCCTTGCAG, 5′-GAGCGAGTAACAACCCGT-CG.

Generation of TRE-Klf4 construct and mice

Klf4 cDNA (bp 273-1800 of GenBank accession no. U20344 with CDS bp 311-1762) was amplified by polymerase chain reaction (PCR) from BALB/c newborn skin cDNA, cloned in pBS-KS+ (Promega) and sequence verified. By PCR, *Bam*HI restriction sites were added at both ends of the construct and a Myc tag was added at the amino terminus. This construct was sequence verified and subcloned into the unique *Bam*HI site of pTRE2 vector (Clontech).

The TRE-*Klf4* construct was purified by CsCl gradient (Lofstrand). Thirty micrograms were digested to free the insert with *Xho*I-*Sap*I, purified (Qiagen) and injected into FVB/N one-cell eggs following standard pronuclear injection (Hogan et al., 1986). Positive founders were identified by PCR and maintained on FVB/N background. DNA was isolated by standard techniques. Genotyping was done by PCR on tail DNA using the following primers: TRE-*Klf4* transgene: 5′-CGC-CTGGAGACGCCATCCAC, 5′-CACCTGTGTTGCTGGCAG. Ten founders were identified as positive by PCR and 3 lines were established. TRE-*Klf4* lines were time-mated with the K5-tTA line and supplied with normal water to obtain transactivation. The morning of vaginal plug detection was taken as E0.5.

Immunohistochemistry

Backskin samples from double transgenic and wild-type littermates embryos were taken at different embryonic stages laid down on a paper towel, cut in half and either frozen on dry-ice in OCT (Tissue-Tek) or fixed overnight in 4% PFA in PBS (later embedded in paraffin wax). Frozen sections were hybridized using mouse monoclonal anti-Myc antibody (1:100 from Upstate Biotechnology Inc.), and the MOM peroxidase kit or rabbit polyclonal KLF4 antibody (1:100) and the Vectastain ABC kit with peroxidase DAB substrate (Vector Laboratories). Paraffin sections were hybridized using rabbit polyclonal antibodies (Covance): K1 (1:500), K14 (1:1000), loricrin (1:500) and filaggrin (1:1000). Biotinylated secondary antibodies against rabbit (Vector Laboratories) were used at a 1:200 dilution in combination with Vectastain ABC kit and peroxidase DAB substrate kit (Vector Laboratories).

For the proliferation assay, pregnant females were injected intraperitoneally with 50 μg/g body weight BrdU (Sigma) and skin samples from embryos were recovered 1.5 hours after injection and processed as described above. Paraffin sections were hybridized using mouse monoclonal BrdU antibody (clone BRD.2, Neomarkers) following the manufacturer's recommendations (HCl and trypsin treatments, antibody diluted at 1:500), the MOM peroxidase and DAB substrate kit (Vector Laboratories).

RNA isolation and northern blot analyses

Sample isolation: at E10.5 and E11.5, heads of the embryos were removed and the bodies were used for tissue sampling; at E12.5, E13.5

and E14.5, heads and inner organs were removed prior to tissue processing; at E16.5, skin was isolated by dissection. All samples were snap frozen in liquid nitrogen. After genotyping, skin samples from 2-3 embryos from the same litter (same genotype) were combined if necessary. Tissue was pulverized, homogenized in Trizol (Invitrogen) and RNA was extracted following the manufacturer's recommendations. Approximately 15 μg RNA was loaded in every lane and visualized by ethidium bromide for integrity of the samples. Blots were hybridized for 2 hours at room temperature (ExpressHyb, Clontech) with *Klf4* antisense probe (bp. 537-1471 GenBank accession no. U20344) or with G3PDH probe as loading control. Signals were quantified using Molecular Dynamics Phosphorimager and IQ analysis software.

Immunoblotting

Embryonic skin was isolated and snap frozen in liquid nitrogen. After genotyping, skin samples from 2-3 embryos (same genotype) were pulverized, placed in 250 μl boiling LDS sample buffer (without dye) and boiled for 5 minutes. Protein concentration was determined using the Lowry method (DC protein assay, Biorad). Equivalent amounts of protein (~10 μg) were resolved using SDS-PAGE (4-12% Bis-Tris gels, Invitrogen) and electrophoresed onto nitrocellulose (Invitrogen). Equal transfer was assayed with Ponceau S staining (Sigma). The blots were incubated for 1 hour at room temperature with each primary antibody: polyclonal rabbit anti-KLF4 (1:2,000), involucrin (Covance, 1:10,000), loricrin (Covance, 1:10,000), filaggrin (Covance, 1:10,000) and mouse monoclonal anti-p84 (GeneTex, 1:10,000). This was followed by a 1 hour incubation with HRP-conjugated secondary antibodies and detection with ECL reagents (Amersham).

Barrier function assays

We performed dye penetration assay with X-gal at pH 4.5 for approximately 8 hours at 37°C as described previously (Hardman et al., 1998). The tail tips were removed for genotyping purposes. After staining, embryos were photographed as described above.

Isolation of cornified envelopes

Skin of E15.5 and E18.5 embryos was minced and placed in 1 ml of boiling extraction buffer (0.1 M Tris pH 8.5, 2% SDS, 20 mM DTT, 5 mM EDTA pH 7.5) and boiled for 5 minutes (Hohl et al., 1991). Samples were centrifuged for 10 minutes and cornified envelopes (CEs) were resuspended in their own volume of extraction buffer. CEs were directly put onto a slide and photographed in phase contrast under an Axiophot microscope (Zeiss).

RESULTS

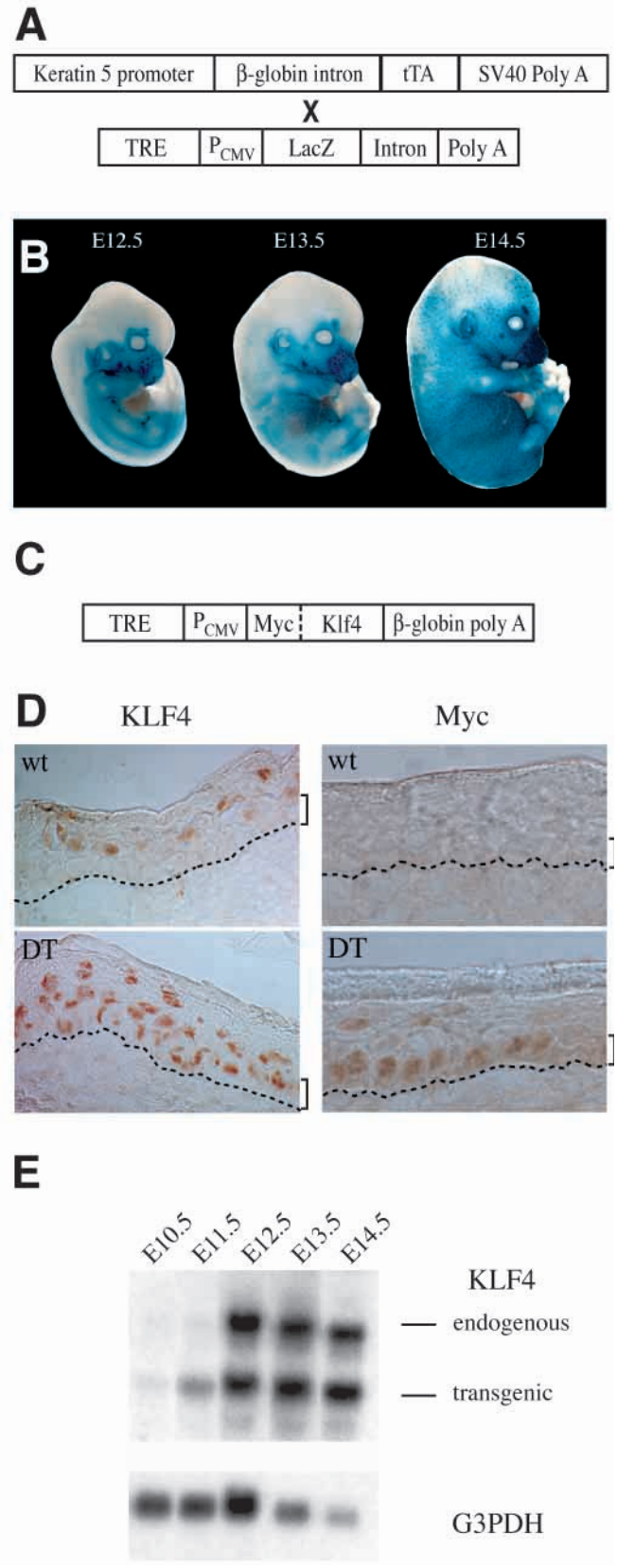
We previously demonstrated that the transcription factor *Klf4* is necessary to establish the epidermal barrier in utero. *Klf4*-deficient mice are unable to prevent the penetration of dyes through their skin and suffer a perinatal death as a result of rapid water loss across the skin surface (Segre et al., 1999). To assess if *Klf4* is also sufficient to regulate the processes necessary to establish the epidermal barrier, we wanted to examine the phenotype of transgenic mice that ectopically express *Klf4* earlier in development. *Klf4* is normally expressed in the suprabasal cells of the epidermis once it stratifies (Garrett-Sinha et al., 1996; Segre et al., 1999). Initially, we generated transgenic mice that express *Klf4* earlier in development in the basal cells of the epidermis from the keratin14 (K14) promoter. These K14-*Klf4* transgenics failed to nurse and died within 24 hours, probably as a result of cleft palate (see below). To circumvent the perinatal lethality, we utilized the bipartite tetracycline activator system. Turning promoters ON and OFF in development has proved to be a powerful tool to dissect the necessary window of protein expression in development (Lewandoski, 2001). However, we used the system in its most basic form without modulating the levels of doxycycline by mating two phenotypically normal mice and analyzing double transgenics which ectopically express *Klf4*.

Characterization of the transgenic lines K5-tTA and TRE-*Klf4*

A 'tet-OFF' transgenic line expressing the tetracycline transactivator tTA from the keratin 5 promoter (K5-tTA) has been previously characterized and shown to express tTA in the basal layer of the epidermis and in the outer root sheath of the hair follicles of adult mice (Diamond et al., 2000). To characterize these mice for use in developmental studies, we crossed the K5-tTA mice with reporter mice that express the *lacZ* gene downstream from a tetracycline-regulated promoter (TRE-*lacZ*) (Fig. 1A). In the absence of doxycycline, K5-tTA/TRE-*lacZ* double transgenics express the β-galactosidase enzyme wherever the tTA molecule is expressed. Whole-mount staining with Xgal substrate during the critical time points of development for this study demonstrated that the K5-tTA line follows the endogenous pattern of K5 expression faithfully. At E12.5 the β-galactosidase enzyme (and hence the tTA expression) is observed on the lateral surface with distinct sites of expression in the maxillary region (whisker pad) and a stripe on the lateral surface. At E13.5 the expression has spread along the ventral and lateral surface with near complete expression of the β-galactosidase enzyme on the entire embryo surface observed by E14.5 (Fig. 1B). The developmental pattern observed in the K5-tTA/TRE-*lacZ* double transgenics is essentially identical to the endogenous K5 pattern (Byrne et al., 1994).

A Myc-tagged full-length *Klf4* cDNA was cloned downstream of tetracycline response elements (TRE) and a minimal promoter (Fig. 1C). We tested this construct in vitro and observed a significant increase in the levels of KLF4 protein with induction (data not shown). We generated transgenic founders expressing *Klf4* from this tetracycline-regulated promoter (TRE-*Klf4*). These mice were crossed with the previously characterized tet-OFF transgenic line expressing the tetracycline transactivator tTA from the basal keratin 5 promoter (K5-tTA) (Diamond et al., 2000). In the absence of doxycycline, K5-tTA/TRE-*Klf4* double transgenics (K5-*Klf4* DTs) should express *Klf4* in the basal cells of the epidermis, following the expression pattern of K5. Immunohistochemistry with a KLF4 antibody demonstrates that KLF4 in the K5-*Klf4* DTs is now expressed in the basal and suprabasal cells of the stratified epidermis, whereas control littermates show only the normal pattern of suprabasal expression (Fig. 1D). An antibody against the Myc epitope at the N terminus of the ectopic KLF4 protein stains only the basal cells of the epidermis of the K5-*Klf4* DTs (Fig. 1D). The K5-*Klf4* DTs have a spatial misregulation of the KLF4 protein in the basal cells of the epidermis. To query the temporal misregulation in the K5-*Klf4* DTs, we examined *Klf4* mRNA expression from E10.5 to E14.5. A northern blot shows the endogenous band at 3.5 kb and ectopic *Klf4* at 2.5 kb. The transgenic *Klf4* is expressed as early as E10.5, consistent with the published expression of the

keratin 5 promoter (Byrne et al., 1994). Weak expression of the endogenous *Klf4* transcript is observed at E10.5 and E11.5. Previously published data shows that the endogenous *Klf4* at this stage is in the mesenchymal cells of the first branchial arch, not in the overlying single layered epidermis (Garrett-Sinha et al., 1996). Strong expression of *Klf4* initiates at E12.5 when the transcript can be localized in the developing epidermis. Hence, K5-*Klf4* DTs show both a spatial and a temporal misexpression of *Klf4*.



Analysis of *Klf4* expression in bipartite transgenic mice K5-tTA/TRE-*Klf4*: an allelic series of phenotypic presentation and expression of *Klf4*

Of the ten TRE-*Klf4* founders, we characterized three that had single insertion sites and expressed *Klf4* as double transgenics. The K5-*Klf4* DTs were an allelic series with varying phenotypic severity (Fig. 2A). All of the K5-*Klf4* DT lines exhibited open eyes at E16.5, even though the eyelids normally fuse at E16. Line 2 K5-*Klf4* DT had subtle abnormalities in the shape of the skull in the maxillary region. The whisker pad was narrower and fewer whiskers were observed. Dissection of the oral cavity revealed a cleft palate. Line 6 showed abnormalities that were more severe in line 10. Lines 6 and 10 K5-*Klf4* DTs were runted and had taut skin, lacking the folds or wrinkles visible on normal littermates. Their skulls are abnormally shaped with cleft palates, open mouths and almost complete absence of whisker pads. They also had shorter protruding limbs with normal digits. Examination of skeletal preparations stained with Alcian Blue (to reveal cartilage) and Alizarin Red (an indicator of calcification) showed that the DTs had almost normal sized limb bones of appropriate shape (Fig. 2A). The failure of complete protrusion may be due to abnormal regulation of epidermal proliferation (see below). K5-*Klf4* DT line 10 had an omphalocele (gastrointestinal protrusion into umbilical cord).

To determine if the phenotypic severity correlated with levels and pattern of expression, we characterized the three lines of the K5-*Klf4* DTs. In all three K5-*Klf4* DTs, KLF4 is expressed in both basal and suprabasal cells (representative data shown in Fig. 1D). Although the pattern of expression was the same, the levels of *Klf4* differed between the lines. A northern blot of E16.5 mRNA shows the endogenous band at

Fig. 1. Characterization of the K5-tTA and TRE-*Klf4* transgenic mice. (A) Diagram of the K5-tTA and TRE-*lacZ* transgenes. (B) The patterned expression of the driver K5-tTA transgene revealed by crossing with the tester TRE-*lacZ* line is essentially identical to the endogenous K5 expression with lateral expression at E12.5, spreading ventrally and then dorsally to cover almost the entire surface of the embryo by E14.5. (C) Diagram of the TRE-*Klf4* construct used for in vitro studies and microinjection. A unique *Bam*HI and a Myc tag were inserted into the *Klf4* cDNA by PCR. This fragment was inserted into a vector containing TRE, CMV minimal promoter, and a β-globin poly(A)+ sequence. (D) Anti-KLF4 and anti-Myc immunohistochemistry of frozen sections of wild-type (wt) and K5-*Klf4* DT (DT) epidermis. The endogenous KLF4 is expressed in suprabasal cells and the Myc-tagged ectopic protein is expressed in basal cells. The dotted line indicates the basement membrane; the bracket, basal layer. Representative data from line 2 K5-*Klf4* DT is shown. (E) Northern blot analysis of mRNAs isolated from pools of skins of K5-*Klf4* DTs at E10.5, E11.5, E12.5, E13.5 and E14.5. Embryos hybridized with a *Klf4* cDNA probe, revealing that the ectopic *Klf4* transcript is expressed as early as E10.5. The endogenous *Klf4* is 3.5 kb and the transgenic *Klf4* is 2.5 kb in the K5-*Klf4* DTs. G3PDH probe is a control. Data is from line 10 K5-*Klf4* DT.

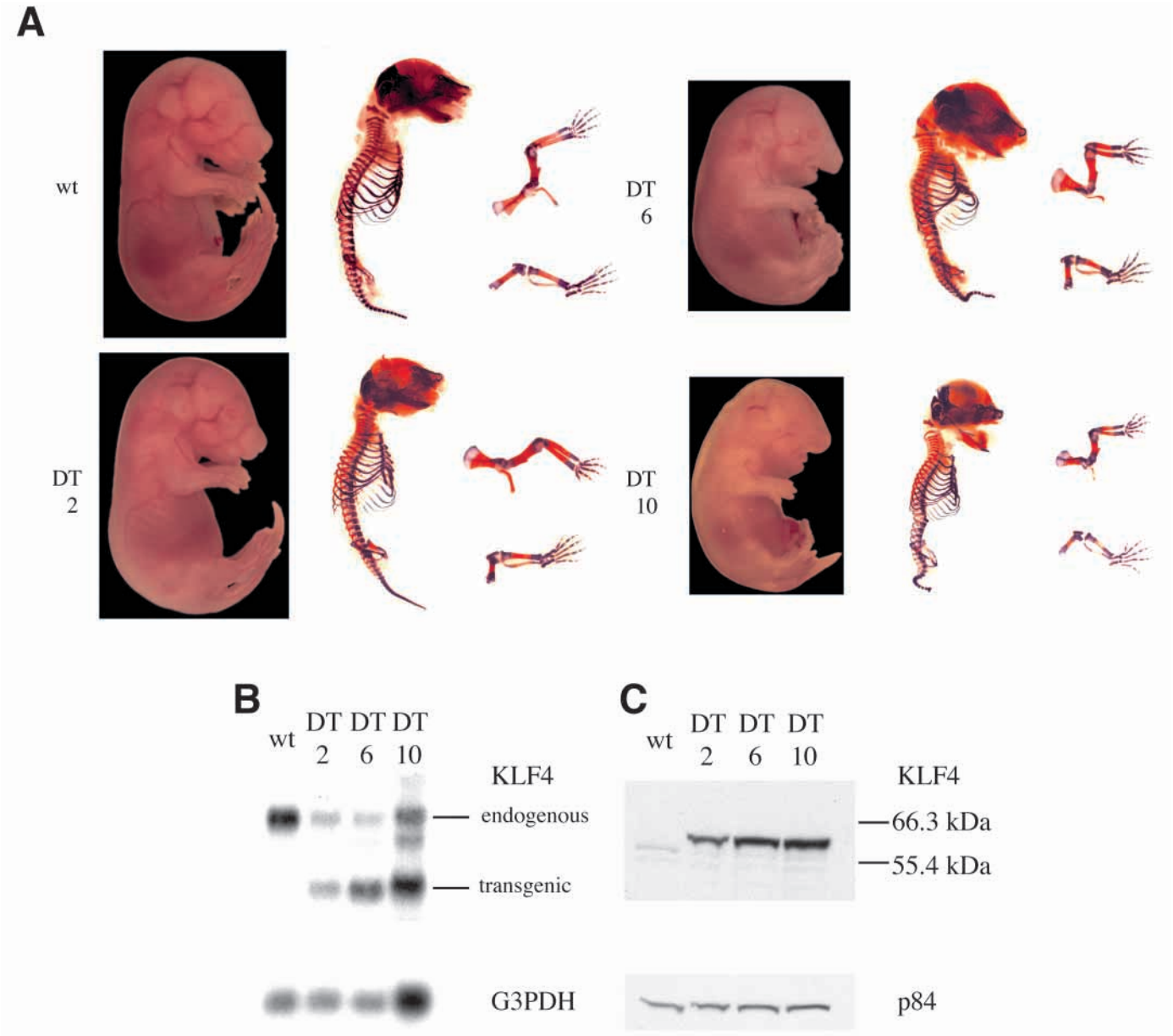


Fig. 2. Allelic series of morphology and expression levels in three lines of TRE-*Klf4* mice that were crossed with K5-tTA mice. (A) Gross morphology and skeletal preparations of wild-type and the three lines of K5-*Klf4* DTs. (B) Northern blot analysis of mRNAs isolated from E16.5 embryonic skin from the line indicated and hybridized with a *Klf4* cDNA probe. The endogenous *Klf4* is 3.5 kb and the ectopic *Klf4* is at 2.5 kb in the K5-*Klf4* DTs. Lines 6 and 10 show a faint band at 3 kb that may be an alternatively spliced form of the endogenous *Klf4* gene. G3PDH probe is a control and signals are quantified using Phosphorimager. (C) Western blot analysis of proteins isolated from E16.5 embryonic skin from the line indicated and hybridized with anti-KLF4 polyclonal antibody. The endogenous KLF4 protein is 60 kDa and the ectopic KLF4 protein has a slightly higher molecular mass because of the N-terminal tag. Hybridization with p84 antibody, that recognizes an 84 kDa nuclear matrix protein, is a control.

3.5 kb for all of the mice and ectopic *Klf4* expression at 2.5 kb in the K5-*Klf4* DTs (Fig. 2B). Line 2 showed equal intensity of the endogenous and transgenic *Klf4*. K5-*Klf4* DT lines 6 and 10 have a higher level of expression of the transgenic *Klf4* transcript and a concomitant decrease in the endogenous *Klf4* transcript. This supports the biochemical analysis that KLF4 is able to bind to sites within its own promoter and autoregulate its own transcription (Mahatan et al., 1999).

To determine if these changes in *Klf4* transcript level manifest as changes at the protein level, we examined the expression for the three K5-*Klf4* DT lines by western blot. The endogenous KLF4 protein is barely detectable in the skin of wild-type E16.5 embryos (at 60 kDa) whereas the transgenic KLF4 protein is detected in all K5-*Klf4* DT lines (Fig. 2C). Correlating with the mRNA results, expression was less in line 2 than lines 6 and 10, which were equivalent. The relative intensities of the transgenic to endogenous protein are more significant than the level of RNA. There are three possible reasons for this, (i) the KLF4 antibody has an increased affinity for the ectopic protein, (ii) this protein has increased stability

or (iii) the transgenic *Klf4* transcript is preferentially translated. The levels of *Klf4* mRNA and protein correlate with the severity of the phenotypes observed: K5-*Klf4* DT line 2 has an almost normal appearance and lower levels of transgenic *Klf4* are expressed than in lines 6 and 10.

Analysis of morphology and barrier acquisition in K5-*Klf4* DTs

Since *Klf4* deficiency results in a failure of epidermal barrier formation, we analyzed whether the ectopic expression of *Klf4* in the DTs conversely accelerated this process. We analyzed the state of differentiation with a whole-mount skin permeability assay. This assay measures the permeability of the epidermis to a dye solution which produces a blue colored reaction in the skin. In wild-type animals, skin permeability changes dramatically from E16 to E17 as the barrier is acquired in a dorsal to ventral pattern (Hardman et al., 1998).

The K5-*Klf4* DTs acquire the epidermal barrier one day earlier in development than the control littermates (Fig. 3). At E15.5, the K5-*Klf4* DTs have already established a 15-60% barrier, depending on the line, while the control embryos are completely blue. At E16.5, K5-*Klf4* DTs are almost completely white while the control embryos are only beginning the process of barrier acquisition. By E17.5 even the control littermates have established the barrier and they appear similar to the K5-*Klf4* DTs. Regressing, at E14.5 neither the controls nor K5-*Klf4* DTs have initiated the process of barrier acquisition (data not shown). Although all three K5-*Klf4* DT lines had accelerated barrier acquisition, the initiation and degree of coverage was more pronounced in lines 6 and 10 than in 2, correlating with the levels of *Klf4* mRNA and protein expressed.

K5-*Klf4* DTs exhibit accelerated epidermal differentiation with proper restriction of protein expression, but decreased proliferation

To determine whether the acceleration in dye exclusion of the K5-*Klf4* DTs was occurring through the normal process of epidermal differentiation, we analyzed the underlying morphological changes. We examined the histology of the K5-*Klf4* DT epidermis during embryogenesis as the epidermal barrier is acquired. In wild-type mice, at E15.5 the epidermis has stratified and differentiated with a basal (K5/K14 positive) and one or two layers of suprabasal (K1/K10 positive) cells. The E15.5 mouse epidermis lacks a granular layer or a stratum corneum and is still covered by periderm. During the next 2 days, the upper layer of cells thickens, flattens and forms the stratum corneum (SC). Impermeability to the dye solution precedes the development of the typical SC but is associated with a dark flattened precursor SC (Hardman et al., 1998).

The K5-*Klf4* DT epidermis at E15.5 is already more organized with increased stratified layers, granular cells and a precursor SC than that of the wild type. The K5-*Klf4* DT epidermis at E15.5 resembles the E16.5 wild-type sample. By E16.5, the K5-*Klf4* DT epidermis has already matured considerably with multiple granular layers of flattened enucleated keratohyalin containing cells, and multiple layers of SC. By E17.5, the K5-*Klf4* DT and wild-type control epidermis appear identical with multiple layers of granular and SC. At the level of gross morphology, the K5-*Klf4* DT epidermis appears normal, but advanced in development by at least 1 day (Fig. 4). Examination of E16.5 epidermis at the ultrastructural level gave similar results, documenting the presence of mature granular layers and SC in the K5-*Klf4* DTs (data not shown).

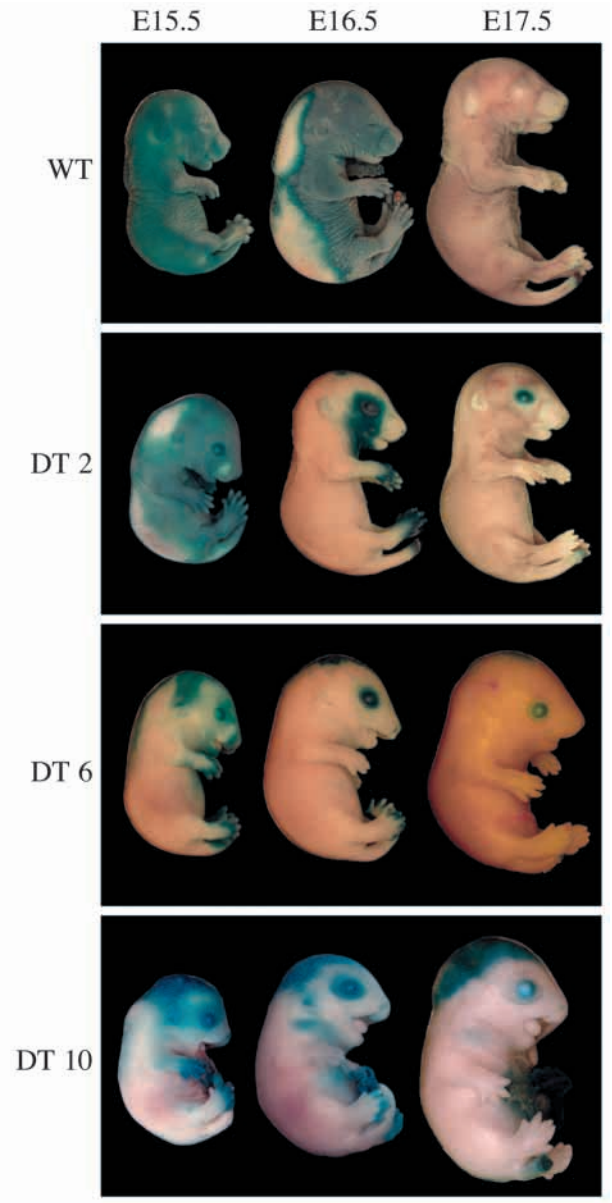


Fig. 3. Skin barrier acquisition is accelerated in the K5-*Klf4* DTs lines. Barrier-dependent dye exclusion assays on three lines of K5-*Klf4* DTs at the ages indicated.

To further analyze the hypothesis that ectopic *Klf4* expression can accelerate epidermal terminal differentiation, we assessed the developmental expression of specific markers: keratin 14 (basal layer), keratin 1 (spinous layers), filaggrin (granular layers) and loricrin (precursor of cornified envelopes). We observed no difference in K14 and K1 expression, as both were detected in all embryonic stages in the proper cell layer. By contrast, loricrin and filaggrin are expressed in the proper cell layer but earlier in development in the K5-*Klf4* DTs. Filaggrin staining is absent in wild-type at E16.5. By contrast, the pattern and intensity of filaggrin in

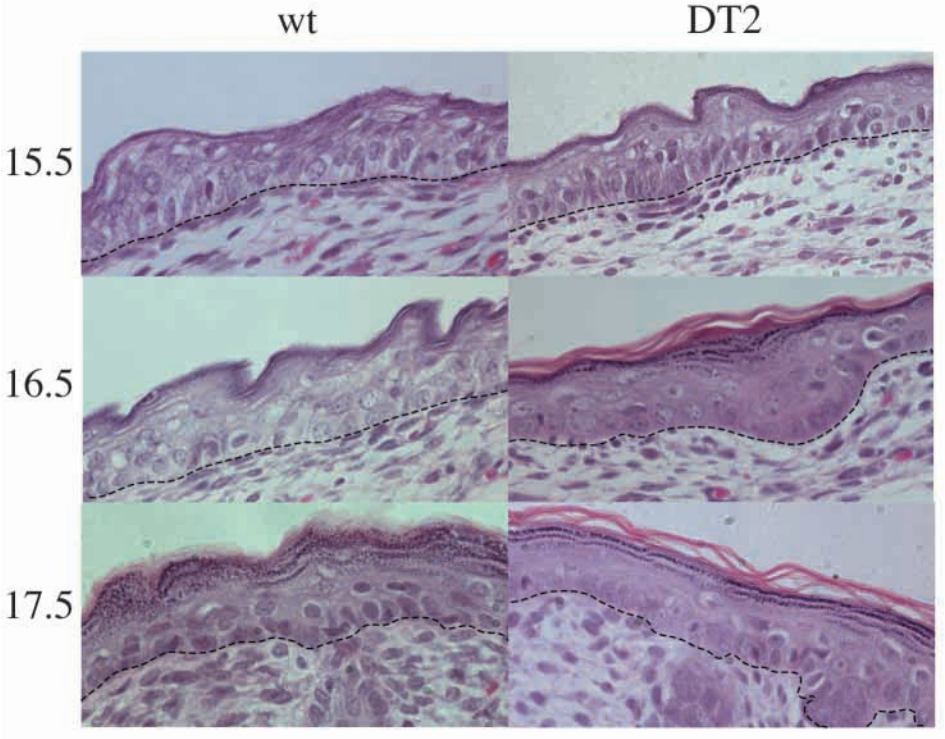


Fig. 4. Histology of line 2 K5-*Klf4* DT epidermis during development. Control and line 2 K5-*Klf4* DT epidermis at the ages indicated were fixed in paraformaldehyde, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin. The K5-*Klf4* DT epidermis has accelerated differentiation with normal histology. Similar results were obtained for all three lines except that lines 6 and 10 had more advanced granular and SC layers at E15.5 than line 2. The dotted line indicates the basement membrane.

E16.5 K5-*Klf4* DTs is similar to wild-type E17.5. Patchy filaggrin staining is observed as early as E15.5 for lines 6 and 10. Very similar results are obtained with loricrin with no wild-type expression at E16.5 but expression in the granular cells of K5-*Klf4* DT epidermis at E16.5 with patchy expression as early as E15.5 in lines 6 and 10 (Fig. 5). The K5-*Klf4* DTs exhibit a normal restriction of expression 1 day earlier in development than control littermates. Since proliferation and differentiation are tightly coupled in the epidermis, we next sought to determine if proliferation was affected in the K5-*Klf4* DTs.

As KLF4 has been suggested to be a negative regulator of cell proliferation in vitro, we investigated the role of KLF4 in cell proliferation in vivo by analyzing the incorporation of bromodeoxyuridine (BrdU) into replicating DNA. BrdU injected into the peritoneum of pregnant females reaches the skin of their embryos rapidly. Staining the embryonic skin at E16.5 and E17.5 with a BrdU antibody 1.5 hours after injection showed that the number of labeled cells in the skin of K5-*Klf4* DTs was lower than in wild type. At E16.5 approximately 38.4±1.2% (duplicate counting of 200 nuclei of four independent animals produced 73.5, 78.5, 76.5 and 78.5 positive nuclei) and 23.0±3.1% (42.0, 39.5, 50.5, and 46.0 of 200 nuclei) of the cells were BrdU positive in wild-type and K5-*Klf4* DTs, respectively. (Representative data shown in Fig. 6.) At E17.5 approximately 38.1±2.1% (78.5, 76.5, 73.5, and 76.0 of 200 nuclei) and 23.7±3.1% (45.0, 50.5, 44.5, and 49.5) of the nuclei of the 200 cells in the basal layer were BrdU positive in the wild-type and K5-*Klf4* DTs, respectively (data not shown). No spatial difference in BrdU staining was observed as both wild-type and K5-*Klf4* DTs stained exclusively in the basal cells. Proliferation is decreased 40% in the K5-*Klf4* DTs at both E16.5 and E17.5. It is difficult to determine if this is a direct result of *Klf4* overexpression or a secondary effect of accelerated differentiation.

The dye permeability assay measures the transition toward barrier acquisition. However, after the embryo becomes impermeable to the dye, there is a continued maturation, characterized by release of profilaggrin from the keratohyalin granules and processing from the phosphorylated 500 kDa precursor to 26 kDa dephosphorylated filaggrin. Although increased profilaggrin abundance accompanies the change to impermeability, the fully processed, active form of filaggrin is only detected with the full gain in barrier function at E17.5. The skin of K5-*Klf4* DTs already expresses fully processed filaggrin at E16.5 (Fig. 7A). Abundance of the suprabasal protein involucrin does not change significantly with the change in skin permeability or when the K5-*Klf4* DT lines 2 or 6 are compared with controls (Fig. 7B). In K5-*Klf4* DT line 10, the levels of involucrin and processed filaggrin are less than in lines 2 and 6, which may be due to the more severe developmental defects observed for this line. Loricrin is detectable in all of the samples at slightly decreased levels in the K5-*Klf4* DTs (Fig. 7C). Note that from the same sample, filaggrin and loricrin can be detected by western blot, but not by immunohistochemistry because the antibody may only have access to the epitopes of these proteins in restricted cellular locales. Filaggrin and loricrin initially localize to keratohyalin granules, but are then released to the matrix and intracellular side of the cornified envelope. The levels of involucrin, loricrin and filaggrin protein detected in the K5-*Klf4* DTs as compared to controls mirrors the changes observed for their respective transcripts at E16.5 with a large increase in filaggrin, slight decrease in loricrin and no change in the levels of involucrin (data not shown).

Cornified envelopes (CEs) are assembled directly underneath the plasma membrane by sequential incorporation of precursor proteins that are cross-linked by the transglutaminase enzyme. Boiling skin for 10 minutes in the

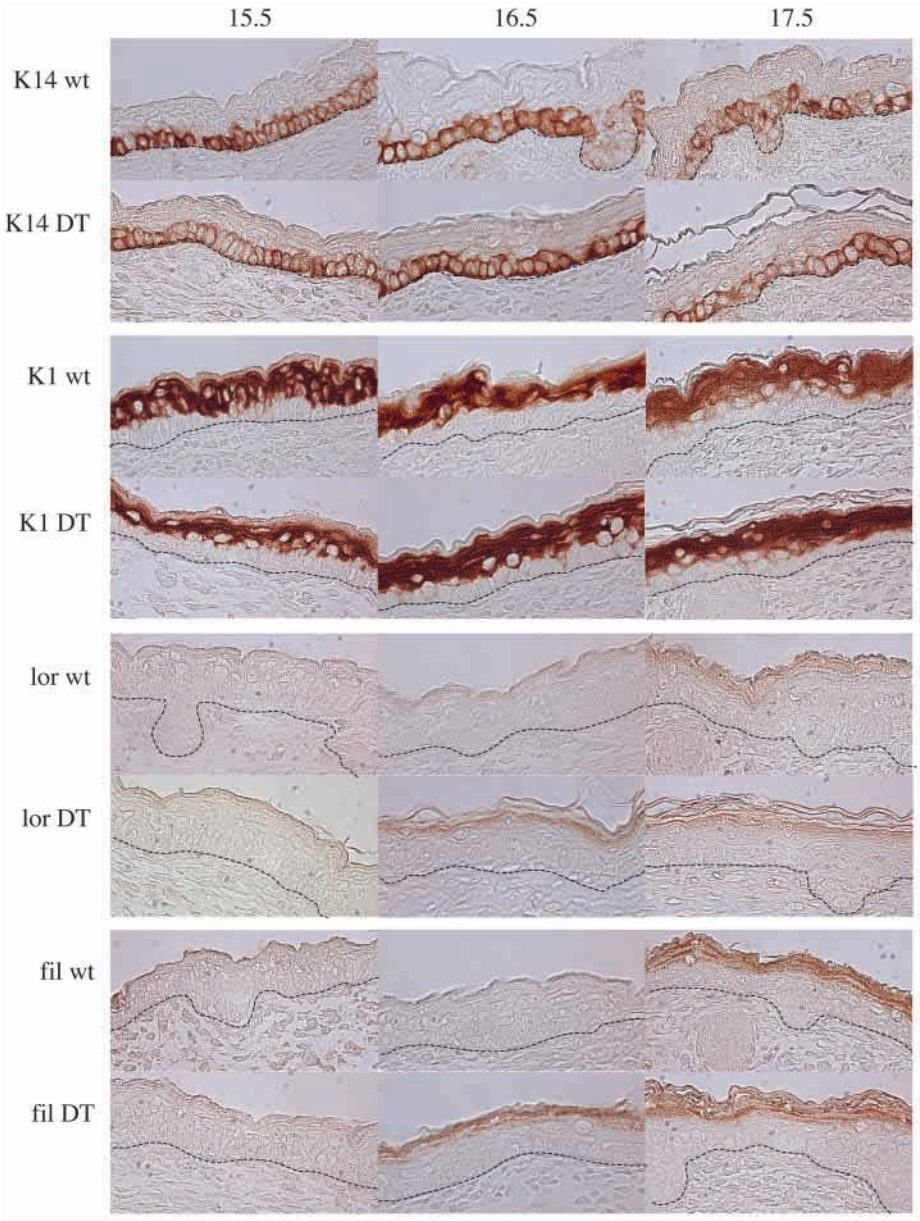


Fig. 5. Epidermal differentiation markers are expressed earlier in development with normal patterns in K5-*Klf4* DTs. Control wild-type and line 2 K5-*Klf4* DT epidermis at the age indicated were fixed in paraformaldehyde, embedded in paraffin and sections were stained with epidermal differentiation markers: keratin 14 (K14; basal layer), keratin 1 (K1; spinous layers), loricrin (lor) and filaggrin (fil; granular layers). All of the markers show the normal restriction of expression, but the K5-*Klf4* DTs express the terminal differentiation markers loricrin and filaggrin earlier in development. The dotted line indicates the basement membrane.

presence of ionic detergent and a reducing agent leaves behind the tough impermeable CEs (Hohl et al., 1991). During the barrier acquisition transition, initially 'fragile' crumpled envelopes are formed from the inner stratum corneum near the granular layer. In post-barrier (including adult) skin, smooth, polygonal, 'rigid' envelopes derived from the outer layers of the SC replace the fragile CEs (Hardman et al., 1998). At E18.5, mature CEs were present in both controls and K5-*Klf4* DT embryos, and in fact CEs can be isolated from E15.5 K5-*Klf4* DT embryos (Fig. 8). These CE exhibit the characteristics of fully mature CEs. In contrast, CEs cannot be isolated from wild-type controls until E16.5 and these are immature fragile CEs.

DISCUSSION

We have shown that ectopic expression of the transcription factor *Klf4* accelerates epidermal terminal differentiation, culminating in proper establishment of the barrier. *Klf4*, normally expressed in the suprabasal cells, was ectopically expressed in the basal cells in K5-tTA/TRE-*Klf4* double transgenic mice. The acceleration in barrier acquisition is manifest in the K5-*Klf4* DTs by the patterned exclusion of a dye solution at E15.5, one day earlier in development than controls. Underlying this dye impermeability at E16.5 are morphological changes, including an increased number of granular and stratum corneum layers, expression of the terminal differentiation markers filaggrin and loricrin, processing of profilaggrin to filaggrin, and assembly of cornified envelopes. By all criteria, *Klf4* ectopic expression accelerates the normal process of terminal differentiation.

During normal development, barrier acquisition first appears at specific canonical locations on the dorsal side and then spreads to the ventral surface (Hardman et al., 1998). The pattern of barrier acquisition in the K5-*Klf4* DT line 2 recapitulates this pattern. In these studies, the expression of *Klf4* is driven from the keratin 5 promoter, which has a pattern of expression distinct from barrier acquisition. Specifically, K5 is expressed on the lateral and ventral surfaces at E13.5 and over the entire embryo, except the neural tube, at E14.5 (Fig. 1A) (Byrne et al., 1994). Since the pattern of barrier acquisition in the K5-*Klf4* DT line 2 animals follows the normal pattern and not that of the K5 promoter, this supports the concept that there are fields of competence in the epidermis ready to respond to a signal(s). K5-*Klf4* DT lines 6 and 10 initiate barrier acquisition on the dorsal surface, but have perturbed cranial patterns, including an area on the top of the head that remains permeable to the dye even at E17.5 when the non-transgenic mice have established full impermeability, reflecting the developmental abnormalities associated with lines 6 and 10. In the K5-*Klf4* DT mice, the ectopic *Klf4* is detected at E10.5 although the barrier acquisition is not observed until E15.5. We hypothesize that this intervening time

is necessary to induce the factors necessary for terminal differentiation.

The three lines of TRE-*Klf4* produce an allelic series with levels of *Klf4* mRNA and protein expression correlating with acceleration of barrier acquisition and associated phenotypic severity. Line 2 K5-*Klf4* DTs appear phenotypically normal except for the failure of the eyelids to fuse at E16 and the cleft palate observed at E17.5. At E15.5 dye is excluded from approximately 15% of the surface area. At this same developmental time point, lines 6 and 10 K5-*Klf4* DTs exclude dye from greater than 60% of the surface area and they express higher levels of *Klf4* mRNA and protein. However, in addition to the failure of the eyelids to fuse, there are other more severe defects. Lines 6 and 10 K5-*Klf4* DTs are runted with taut skin and craniofacial abnormalities including defects in the whisker pads, nasal pits and head shape. Line 10 K5-*Klf4* DT had an omphalocele. Although the line 2 K5-*Klf4* DTs appear normal at birth, except for the open eyes, there is a failure to nurse, perhaps due to the cleft palate. They appear dehydrated and die within 24 hours. Further exploration is required to determine the primary cause of death. We are currently investigating, by administering doxycycline during specific developmental

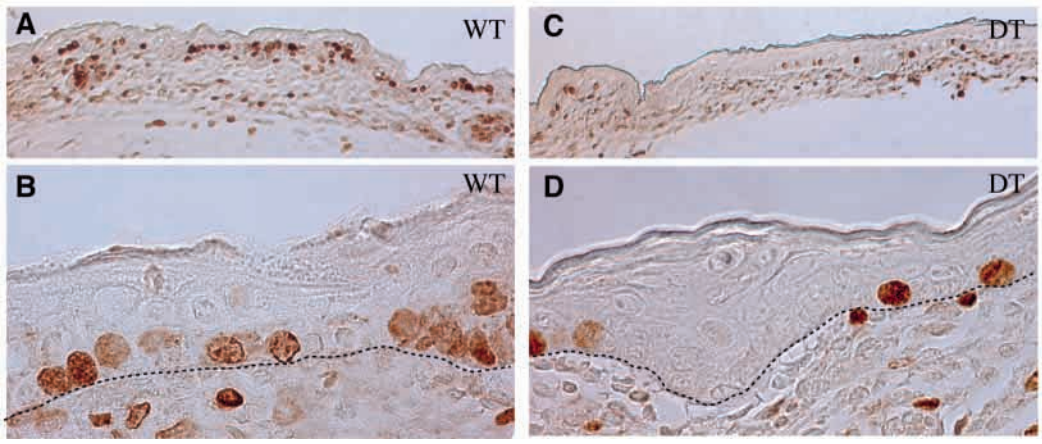


Fig. 6. Immunohistochemistry for BrdU to visualize the number of mitotically active cells in the epidermis. Samples at low magnification (A,C) and high magnification (B,D) of E16.5 wild-type (A,B) and K5-*Klf4* DTs (C,D) reveal a decrease in proliferation in the transgenic mice. The dotted line indicates the basement membrane.

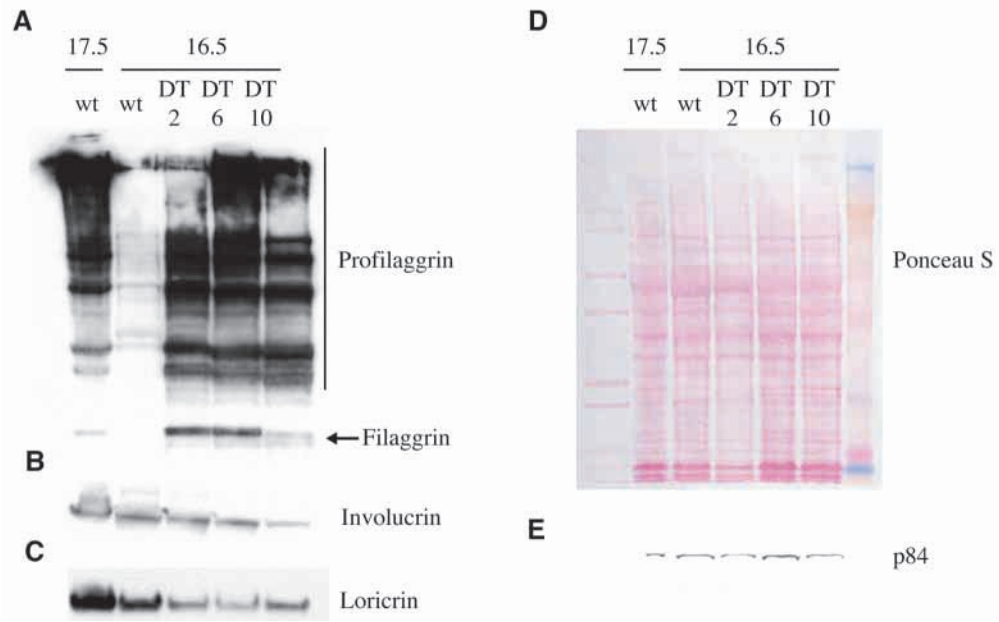


Fig. 7. Structural elements necessary for full barrier acquisition in K5-*Klf4* DTs. Profilaggrin, filaggrin, involucrin and loricrin expression in E16.5 epidermis of wild-type and K5-*Klf4* DTs. (A) Profilaggrin processed to filaggrin is not detected in wild-type epidermis until after the permeability change (E17.5), but is already processed in all of the E16.5 K5-*Klf4* DTs. (B) Involucrin levels do not change during barrier acquisition and there is no difference between the wild-type and K5-*Klf4* DTs. (C) Loricrin levels are slightly decreased in K5-*Klf4* DT lines 2 and 6. (D,E) Ponceau S stainings; p84 expression was used as a control .

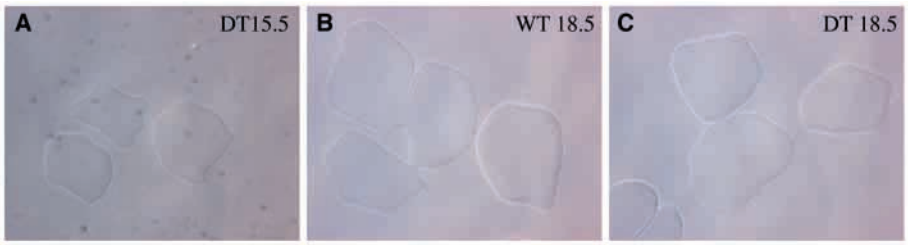


Fig. 8. Cornified envelopes at the stage and genotype specified. Fully mature cornified envelopes are present in E15.5 K5-*Klf4* DTs.

windows, whether we can achieve the acceleration of barrier acquisition without the other abnormalities. In addition, we are utilizing the inducible tetracycline system to investigate the effects of ectopic *Klf4* postnatally.

In vitro, biochemical studies in CHO cells have suggested that KLF4 binds to and activates its own promoter (Mahatan et al., 1999). Since KLF4 has both activation and repression domains, it may function differently in various cell types (Yet et al., 1998). The decreased expression of the endogenous *Klf4* transcript observed in the epidermis of the K5-*Klf4* DTs counters the hypothesis that KLF4 activates its own promoter, but rather suggests that in vivo KLF4 represses its own transcription.

Various studies have implicated *Klf4* in various growth-related and proliferation pathways of the intestine (Chen et al., 2001; Dang et al., 2000; Dang et al., 2001). In vivo, expression of *Klf4* is restricted to the differentiated cells in the epidermis, thymic epithelium and intestine (Garrett-Sinha et al., 1996; Panigada et al., 1999; Shields et al., 1996). Analysis of the *Klf4*$^{-/-}$ mice does not show a change in proliferation status in the epidermis or intestine (Katz et al., 2002; Segre et al., 1999). At E16.5 and E17.5, K5-*Klf4* DTs exhibit approximately a 40% decrease in proliferation. As the rates of proliferation from E16.5 to E17.5 do not appear to change substantially in either the transgenics or wild type, this appears to be specific for the *Klf4* expression. The observed change in *Klf4* expression in cancer cell lines and intestinal and colonic adenomas in mice and humans is consistent with *Klf4* regulating the balance between proliferation and differentiation. Analysis of *Klf6* in primary human prostate tumors identified loss of heterozygosity at the locus and mutations in the coding sequence, suggesting that *Klf6* is a tumor suppressor gene in this tissue (Narla et al., 2001).

Prenatal maternal injections of pharmacological levels of glucocorticoids are also known to accelerate barrier acquisition (Horbar et al., 2002; Outcomes, 1995). This regime accelerates intestinal, lung and epidermal maturation prior to an anticipated premature delivery and has been shown to reduce morbidity. Physicians do not treat a premature infant with corticosteroids owing to the deleterious side effects, including arrest of lung branching morphogenesis and immune suppression. Developing an understanding of the mechanisms by which glucocorticoids accelerate barrier function development should lead to more specific compounds to achieve this end. With the ectopic expression of *Klf4* we observe patches of hypoplastic skin or underdeveloped dysplastic hair follicles. These defects may be a direct result of the expression of KLF4 or a result of the accelerated differentiation and concomitant decreased proliferation. Mice with ectopic expression of the glucocorticoid receptor from the K5 promoter demonstrate a range of developmental defects ranging from epidermal hypoplasia with underdeveloped dysplastic hair follicles to skin lesions in the cranial and umbilical regions (Perez et al., 2001). The milder phenotype of the K5-*Klf4* DTs suggests that it may be beneficial to enhance the levels of *Klf4* to achieve normal epidermal differentiation in premature infants. However, the highest expression of KLF4 in the K5-*Klf4* DT line 10 with the defects in the cranial regions and the omphalocele are highly reminiscent of the defects seen in the K5-glucocorticoid receptor transgenic mice.

Since the *Klf4*$^{-/-}$ mutants and the K5-*Klf4* DTs have complementary phenotypes, the overlap of genes misexpressed in *Klf4*$^{-/-}$ mutant and K5-*Klf4* DTs is a powerful way to identify genes that may be direct targets of KLF4 in regulating the establishment of epidermal barrier. We examined the mRNA expression of the epidermal genes previously published as misregulated in newborn *Klf4*$^{-/-}$ mutants, *Sprr2a*, repetin (*Rptn*) and *Planh2*/*Serpinb2* at E16.5 in *Klf4*$^{-/-}$, K5-*Klf4* DT and their respective controls (Segre et al., 1999). *Rptn* mRNA is not expressed at detectable levels at E16.5 in any of the four samples. Levels of *Sprr2a* and *Planh2* mRNA show no significant changes at E16.5 between the *Klf4*$^{-/-}$, K5-*Klf4* DT and their respective controls. However, other targets have been identified on microarrays and subtractive hybridization that are misregulated in opposite directions between the *Klf4*$^{-/-}$ mutants and the K5-*Klf4* DTs (data not shown). In the future, this strategy should prove fruitful to identify and examine primary targets of KLF4 in establishing the epidermal barrier.

Targeted ablations of epidermal proteins can perturb early events in epidermal differentiation, resulting in a subsequent defect in terminal differentiation. There are at least three essential components of epidermal permeability barrier: proteins cross-linked to form the cornified envelope, extruded lipids, and tight junctions. Ablations in the transglutaminase 1 enzyme, matriptase, and claudin 1 loci have all produced mice with red, shiny, wrinkled skin and subsequent perinatal lethality due to water loss (Furuse et al., 2002; List et al., 2002; Matsuki et al., 1998). The *Klf4*$^{-/-}$ mice exhibited similar epidermal permeability defects but the skin had an outwardly normal appearance. Although there are several genetic ways to perturb the barrier, the K5-*Klf4* DT mouse is the first to accelerate the acquisition of the barrier. As such, these mice have tremendous potential as animal models to understand how to accelerate this process for a premature infant ex utero.

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