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Permeability barrier dysfunction in transgenic mice overexpressing claudin 6

Kursad Turksen[1,2,3,*] and Tammy-Claire Troy[1]

[1]Ottawa Health Research Institute, [2]Divisions of Dermatology and Endocrinology, Department of Medicine, Ottawa Hospital and [3]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada
*Author for correspondence (e-mail: kturksen@ohri.ca)

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SUMMARY

A defective epidermal permeability barrier (EPB) in premature birth remains a leading cause of neonatal death as a result of its associated complications, which include poor temperature stability, infection by micro-organisms through the skin, and the outflow of water. Despite its importance in survival, the mechanisms involved in the formation and maintenance of the EPB are not well understood. To address the possibility that claudins, a new superfamily of tight junctional molecules, are involved, we engineered transgenic mice with claudin 6 (Cldn6) overexpressed via the involucrin (*Inv*) promoter. Interestingly, the *Inv-Cldn6* transgenic animals die within 2 days of birth, apparently due to the lack of an intact EPB as evidenced by increased water loss and the penetration of X-gal through the skin. Barrier dysfunction was manifested biochemically by the aberrant expression of late epidermal differentiation markers, including K1, filaggrin, loricrin, transglutaminase 3, involucrin, repetin, members of the SPRR family and the transcriptional regulator Klf4. The overall claudin profile of the epidermis was also modified. Our data suggest that repetin and SPRR1A and 2A are downregulated in response to the downregulation of Klf4 in the transgenic animals, which would contribute to decreased protein crossbridging leading to fragile, defective cornified envelopes. These results provide new insights into the role of claudin 6 in epithelial differentiation and EPB formation. In addition, the epidermal phenotype of these transgenic mice, which is very reminiscent of that in pre-term infant skin, suggest that they will be an important and novel model for studies on human premature EPB-related morbidity.

Key words: Permeability barrier, Tight junctions, Claudins, Claudin 6, Mouse, Skin, Epidermis, Keratin, Involucrin, Filaggrin, Small proline rich proteins, Kruppel-like factor

INTRODUCTION

The epidermal permeability barrier (EPB) is crucial for the survival of organisms as it retards dehydration and inhibits the invasion of micro-organisms and noxious materials through the skin (Cartlidge, 2000; Rutter, 2000). One highly pertinent example is the condition seen in premature human infants (babies born before 32 weeks of gestation) in which an aberrant EPB has life threatening consequences (i.e., dehydration, loss of ability to maintain body temperature and toxicity related to resorption of external chemicals) (Harpin and Rutter, 1983; Kalia et al., 1998). In fact, 400 000 premature babies are born each year in the USA, 25% of whom develop life threatening conditions (Griffin and Moorman, 2001). The EPB first forms during development through a complex series of events coinciding with the final stages of epidermal differentiation, and this process occurs throughout life as part of the epidermal program.

The epidermis is a continuously renewing tissue composed of cells at different stages of differentiation overlain by a stratum corneum (Fuchs and Byrne, 1994; Turksen and Troy, 1998). In response to discrete signals, epidermal cells in the basal compartment become irreversibly committed to terminal differentiation. Upon leaving the basal layer, cells downregulate the expression of K5 and K14 and upregulate K1 and K10. As these cells progress further along the differentiation pathway they express proteins involved in both the scaffold function and the eventual formation of the insoluble cornified envelope (CE) (for a review, see Reichert et al., 1993). The CE is constructed through the sequential expression, processing and deposition of several other distinct proteins that are cross linked by disulphide and Nε-(γ-glutaminyl) lysine isodipeptide bonds, the formation of which is catalysed by transglutaminase (Hohl, 1990). The cross-linked CE proteins include involucrin, loricrin, small proline rich proteins (SPRRs), calcium binding S100 proteins, cystatin A (keratolinin), repetin sciellin, NICE-1 and late envelope proteins (LEPs) as well as several others (Presland and Dale, 2000; Marenholz et al., 2001; Marshall et al., 2001). The EPB results from the assembly of these proteins into CEs, which are sealed together via lipids in a bricks and mortar fashion (Nemes and Steinert, 1999; Steinert, 2000). Although numerous CE proteins and several EPB-specific lipid components have been identified as contributing to EPB formation, it seems likely that other, as yet unidentified, components may be involved. Even though their location in the epidermal differentiation locus (EDL) (Hardas et al., 1996; Carver and Stubbs, 1997) has been mapped, there is still very little known about the signaling pathway(s) that are involved in the regulation of both its individual components and their complex assemblies in this locus.

Tight junction (TJ) formation is a prerequisite for the formation of the epidermal permeability barrier and the maintenance of barrier function (e.g. Mitic and Anderson, 1998) in addition to the sealing of proteins into cornified envelopes (CEs). However, the paucity of knowledge of EPB formation is due, in large part, to our lack of understanding of TJs and how they contribute to the EPB. The molecular nature of TJs is becoming better understood with the recent cloning of a super-family of integral membrane proteins called claudins (Morita et al., 1999; Turksen and Troy, 2001). The claudin family consists of at least 20 highly conserved members with great diversity in tissue distribution (Morita et al., 1999; Tsukita et al., 2001). Although their cell and tissue distribution during development (as well as in adult tissue) is not known, it appears that there is a need for more than one claudin to make a tight TJ (Tsukita et al., 2001). Together with the existence of a large number of claudins, it appears that the overall levels and combinations of claudin molecules in a given cell type and tissue must be very precisely regulated to provide the degree of sealing required for EPB formation.

To test the hypothesis that the deregulation of claudin 6 in epidermal cells would disrupt differentiation and lead ultimately to an aberrant EPB, we constructed a transgenic mouse in which claudin 6 was overexpressed in its endogenous location, namely the suprabasal layers of the epidermis. Such mice exhibit an increased trans-epidermal water loss that results in lethality within 24 to 48 hours of life. Barrier dysfunction was proved biochemically with the clear demonstration of abnormalities in the expression of epidermal differentiation markers, including K1, filaggrin, loricrin, transglutaminase 3, involucrin, repetin, SPRRs and the transcription factor Klf4 (Kruppel-like transcription factor 4; also known as GKLF), in the epidermis of *Cldn6* transgenic animals. In addition, overexpression of claudin 6 resulted in decreased expression of other claudins. This study is the first example of a role for claudins in epidermal differentiation and demonstrates that the precise balance of claudin 6 in the suprabasal layer of the epidermis regulates both the structure and function of the epithelium.

MATERIALS AND METHODS

The generation of *Cldn6* transgenic mice

Replacing the *lacZ* insert of the pInv plasmid (H3700-pL2) (Carroll et al., 1993; Carroll et al., 1995) with the mouse claudin 6 coding sequence created the *Inv-Cldn6* expression vector. A 660 bp fragment containing the mouse claudin 6 cDNA was introduced into the *Not*I site of the *Inv* cassette and the resultant construct was designated pInv-Cldn6. The transgene was excised from the plasmid vector by digestion with *Sal*I to release the *Inv* promoter and the downstream *Cldn6*/SV40poly(A) DNA sequence, and was purified using the QIAamp tissue kit (Qiagen, Mississauga, Canada) according to the manufacturer's instructions. Transgenic mice were generated using eggs collected from superovulated FVB female mice mated with FVB males (Jackson Laboratory, Bar Harbor, Maine) at the OHRI Transgenic Mouse Facility as previously described (Turksen et al., 1992; Hogan et al., 1996). Transgenic animals were screened by PCR (Table 1) using genomic DNA from tail clippings.

RNA isolation and RT-PCR

Total RNA was extracted from tissues that were frozen in liquid nitrogen and homogenised in TRIzol® reagent according to the manufacturer's instructions (Life Technologies, Burlington, Canada). The isolated RNA was treated with Rnase-free DNase I (Life Technologies) and the first strand cDNAs were synthesised using random primers and 1 μg of each RNA sample. PCR was then performed using specific primers (Table 1). Products were run on agarose gels, stained with ethidium bromide and relative band intensity was quantified using Bio-Rad Molecular Analyst software Version 1.2 (Bio-Rad Laboratories, Hercules, California).

Immunohistochemistry

Backskin samples from transgenic and non-transgenic littermates were fixed in Bouin's solution (75% saturated picric acid, 20% formaldehyde and 5% glacial acetic acid) at room temperature, dehydrated through a series of ethanol washes, embedded in paraffin and sectioned. One of each sample was stained with Haematoxylin/Phloxine/Safranin and the others were processed for immunofluorescence as described (Turksen and Aubin, 1991), using antibodies to the following: claudin 6 (1:100) (Turksen and Troy, 2001), K5 (1:100), K1 (1:100), K6 (1:200; a gift from Dr Pierre Coulombe), filaggrin (1:100), loricrin (1:100), transglutaminase 3 (1:100; a gift from Dr Len Milstone), claudin 1 (1:100; Zymed Laboratories, San Francisco, CA) and claudin 2 (1:100; Zymed). Secondary antibodies against rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used at a 1:50 dilution. Images were captured with a Zeiss fluorescence microscope (Carl Zeiss, Mississauga, Canada) on Kodak T-Max film.

Skin permeability assays

X-gal staining

To assess the epidermal permeability barrier, we used the skin permeability assay as described previously (Hardman et al., 1993). Briefly, unfixed and freshly isolated embryos were rinsed in PBS then immersed in 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside (X-gal) reaction mix at pH 4.5 (100 mM $NaPO_4$, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ and 1 mg/ml X-gal) and incubated at room temperature overnight. This assay depends on the barrier-dependent access of X-gal to untreated skin. At low pH, skin contains abundant endogenous β-galactosidase activity, which cleaves X-gal to produce a coloured precipitate (Hardman et al., 1993). After staining, embryos were photographed using a 35 mm Nikon digital camera and images were processed with Adobe Photoshop.

DPM measurements

Another method of assessing permeability barrier integrity is through DPM measurements to assess the skin's impedance, thus trans-epidermal water loss. The DPM (dermal phase meter) (Nova Technology Corporation, Portsmouth, New Hampshire) measures the biophysical qualities of the skin, which for our purposes, included hydration measurements (Segre et al., 1999). A defective EPB results in the relative DPM values being higher than under normal conditions, reflecting water loss through the skin. The use of EDWINA software (Nova Technology Corporation) allows for a digital recording of all DPM readings over a given period of time. These results were then interpreted into graphical form using Microsoft Excel.

Cornified envelope extraction

CE from transgenic and wild-type back skin was prepared as described previously (Hohl et al., 1991). Newborn skin was incubated in CE extraction buffer (0.1 M Tris-HCl, pH 8.5, 2% SDS, 20 mM dithiothreitol and 5 mM EDTA) at 95°C for 15 minutes and pelleted through centrifugation. The process was repeated once to give a sample of purified CEs.

Immunoblot analysis

Whole cell protein extracts were collected in Laemmli sample buffer (in the presence of freshly added β-mercaptoethanol and PMSF) by boiling for 5 minutes followed by a brief sonication. 20 μg of protein was separated on a 7.5% SDS-PAGE gel, transferred to nitrocellulose

Table 1. Primer sequences

Gene	Forward Primer	Reverse Primer	Size (bp)
Inv. Pro/Cldn6	5′-GAT CCG GTG GTG GTG CAA ATC-3′ (KT102)	5′-GGA ATT CCT CAC AGC AGC AAA AG-3′ (KT98)	590
Inv. Exon/Cldn6	5′-CTG CCT CAG CCT TAC TGT GAG-3′ (KT323)	5′-ATA GTA GCC CTC CAC CCA GCA-3′ (KT324)	692
Cldn1	5′-ATG GCC AAC GCG GGG CTG-3′	5′-TTC CCA CTA GAA GGT GTT GGC-3′	635
Cldn2	5′-ATG GCC TCC CTT GGC GTT CA-3′	5′-TCA CAC ATA CCC AGT CAG GC-3′	692
Cldn3	5′-ATG TCC ATG GGC CTG GAG AT-3	5′-TCA GAC GTA GTC CTT GCG GT-3′	660
Cldn4	5′-ATG GCG TCT ATG GGA CTA CA-3′	5′-TTA CAC ATA GTT GCT GGC GG-3′	633
Cldn6	5′-ATG GCC TCT ACT GGT CTG CA-3′	5′-TCA CAC ATA ATT CTT GGT GGG A-3′	660
Cldn7	5′-ATG GCC AAC TCG GGC CTG-3′	5′-TCA CAC GTA TTC CTT GGA GG-3′	636
Cldn8	5′-CTG TCA GCT GGG TTG CCA AT-3′	5′-TTC GGC GTG GAA ACT CCG TT-3′	229
Cldn9	5′-ATG GCT TCC ACT GGC CTT GA-3′	5′-TCA CAC ATA GTC CCT CTT ATC C-3′	653
Cldn10	5′-GTC ATC ACC ACA GCC ACT TA-3′	5′-TTC TCC GCC TTG ATA CTT GG-3′	526
Cldn11	5′-ATG GTA GCC ACT TGC CTT CA-3′	5′-GAG TAG CCA AAG CTC ACG AT-3′	470
Cldn14	5′-ACG CTC ATC ACC ACC ATT CT-3′	5′-GAG GAG ATG AAG CCC ATG TA-3′	452
repetin	5′-ATC CTC CCT GGC TCC TGC CT-3′	5′-TGT TGC CTG ATT TCT GAT GCT GAC TTC CAT A-3′	914
SPRR1A	5′-ACA CAG CCC ATT CTG CTC CG-3′	5′-TGC AAA GGA GCG ATT ATG ATT-3′	453
SPRR1B	5′-ATA CAG AGT ATT CCT CTC T-3′	5′-TCT CTT CAG TGA ATT CTG AG-3′	537
SPRR2A	5′-CTG AGA CTC AAG TAC GAT GTC TTA CTA CC-3′	5′-TTT TCT GTG AGG AGC CAT CAT AGG CAC-3′	447
SPRR2B	5′-CTG AGA CTC AAG TAC GAT GTC TTA CTA CC-3′	5′-CTG GGA AAC CCT CAT TTA TTC TTG GGT GGA-3′	323
SPRR2C	5′-GAT CCT GAG GCT CCA GCA CAA TG-3′	5′-GGA TTC TCT TCT CCT GAA TTC TTC TGA AGC-3′	283
SPRR2D	5′-ACC CGA TCC TGA GAA TCC AGC ACT-3′	5′-TTT GTC CTG ATG ACT GCT GAA GAC-3′	307
SPRR2G	5′-ATC CTG AGA CTT CAG AAA GAT G-3′	5′-TTG TTC TGA AGA TTG CTG CCA TGC-3′	273
SPRR3	5′-GTC AAC ATC CTT TGT AAG CAT GAC-3′	5′-CAG CTC CTC AAG GGT CTG GCT GGC-3′	771
KLF4	5′-GCA GTC ACA AGT CCC CTC TC-3′	5′-CTG TGT GAG TTC GCA GGT GT-3′	197
filaggrin	5′-GCT TAA ATG CAT CTC CAG-3′	5′-AGT CAG TCC TAT TGC AGG-3′	330
loricrin	5′-TCA CTC ACC CTT CCT GGT GC-3′	5′-CAC CGC CGC CAG AGG TCT TC-3′	100
transglutaminase 3	5′-CTC AAA CCT AAC GCA TCT TTC GGC GCA AC-3′	5′-GGG CTT CCG CAC ATG AGC CTG TTC CAG-3′	453
involucrin	5′-GGT GTA CAG AAG CTT CCA AGA TGT CC-3′	5′-GGC ATT GTG TAG GAT GTG GAG TTG G-3′	251
GAPDH	5′-CAG TAT GAC TCC ACT CAC GG-3′	5′-GTG AAG ACA CCA GTA GAC TCC-3′	167

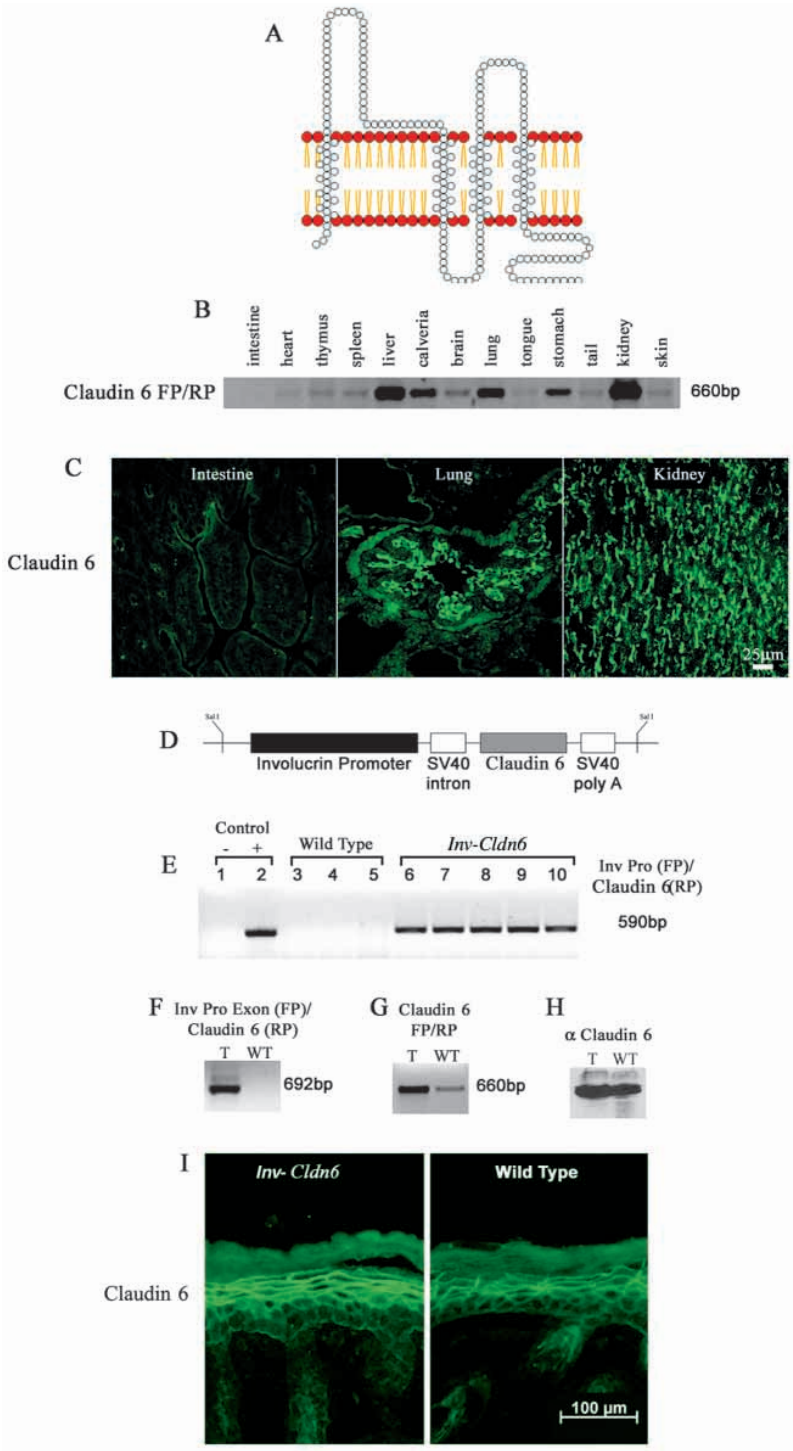
and processed for immunoblot analysis as previously described (Troy and Turksen, 1999). Antibodies against K1 (1:10000), filaggrin (1:5000, Babco, Richmond California), loricrin (1:20000), transglutaminase-3 (1:5000; a gift from Dr Len Milstone) and involucrin (1:10000, Babco, Richmond, CA) were used. For claudin 6 detection, proteins were extracted from backskin epidermis samples in a freshly prepared 9 M Urea/20 mM Tris solution in the presence of β-mercaptoethanol and PMSF. Samples were submerged in extraction buffer for 30 minutes at room temperature followed by sonication. 50 μg of protein were separated on a 15% SDS-PAGE gel and transferred to nitrocelluose for 20 minutes at 70 V. Claudin 6 antibodies were used at a 1:5000 dilution in 3% BSA in PBS. HRP-conjugated secondary antibodies against rabbit (Amersham Pharmacia Biotech, Baie d'Urfe, Canada) were used at a 1:10000 dilution. The ECL+ Plus western blotting detection system (Amersham Pharmacia Biotech) was used for detection on Kodak film (Kodak, Rochester, NY). The approximation of relative band intensities was estimated by scanning the films using the Bio-Rad Molecular Analyst software as previously described.

RESULTS

Generation and expression analysis of *Inv-Cldn6* transgenic mice

We previously identified and characterised *Cldn6* cDNA in differentiating mouse embryonic stem (ES) cells (Turksen and Troy, 2001). A computer prediction indicated that claudin 6 is an integral membrane protein with four transmembrane domains consisting of a long and unique cytoplasmic tail (Fig. 1A). Newborn mouse kidney and liver expressed high levels of *Cldn6* mRNA. Skin, tail, stomach, tongue, lung, brain, calvaria, spleen, thymus and heart expressed low to medium levels and the mRNA was undetectable in intestine (Fig. 1B). Immunohistochemistry on wild-type sections of intestine, lung and kidney supported these RT-PCR findings (Fig. 1C). Interestingly, in preliminary studies, we found that *Cldn6* expression in the newborn mouse epidermis was low and present primarily in the differentiating cells. To test the hypothesis that claudin expression is tightly regulated and that disturbing its homeostasis may alter epidermal differentiation and tissue function, we prepared transgenic mice overexpressing claudin 6 at its endogenous site in the skin.

Transgenic animals were generated by placing mouse *Cldn6* cDNA under the control of a 3.7 kb 5′-flanking element of the human involucrin gene (*Inv*) (Fig. 1D). The Inv promoter was logical since it has previously been shown to be sufficient to drive the expression of a transgene in the suprabasal cells of the epidermis (Carroll et al., 1993; Caroll et al., 1995) where involucrin is normally expressed (Rice and Green, 1977). Seventeen transgenic mice were generated. Among them seven were mosaic and survived while the remaining founders died within 48 hours. Lines were established from the mosaic founders exhibiting similar phenotypes and transgenic mice were identified using PCR (Fig. 1E; Table 1). To ensure amplification of mRNA of transgenic, but not non-transgenic skin, RT-PCR was conducted with primers spanning the junction of the *Inv* exon and *Cldn6* sequences (Fig. 1F). Conversely, with *Cldn6* forward and reverse primers, we performed RT-PCR and a 660 bp band diagnostic of mouse *Cldn6* was amplified both endogenously and exogenously (Fig. 1G). It was estimated that expression of the transgene was approx. 8-fold higher than endogenous *Cldn6* expression. Protein analysis also revealed a significant increase in the expression of claudin 6 in the transgenic epidermis (Fig. 1H). Overexpression of claudin 6 was further confirmed by indirect immunofluorescence on frozen sections of transgenic and wild-type backskin using polyclonal antibodies specific for claudin 6. As expected, the claudin 6 protein in wild-type and transgenic epidermis was restricted to the upper spinous and granular layers, where it localizes to cell-cell junctions (Fig. 1I), with transgenic mice exhibiting appreciably higher levels in relative terms in agreement with the PCR results.



The claudin profile of *Inv-Cldn6* transgenic epidermis is disrupted

We next examined whether, as predicted, the perturbation of one claudin would disrupt the expression and homeostasis of the other claudins. Using primers specific for known mouse claudins (Table 1), we first did pilot PCR runs at 25, 30 and 35 cycles to find the linear range of signal detection (Fig. 2A) and we then quantified the band intensities (Fig. 2B) in RNA samples from transgenic and wild-type mouse epidermis. *Cldn6* expression is clearly increased in transgenic mice indicating the substantial level of overexpression and ease of detection of the transgene. However, *Cldn1* expression is only slightly decreased in the transgenic epidermis: ~2-fold at 30 cycles and marginally at 35 cycles. This is supported by the immunohistochemical analysis of *Cldn1* expression in backskin samples indicating no obvious differences between the normal and transgenic samples (Fig. 2C).

RT-PCR results further indicated that other claudins were decreased to varying degrees in response to the overexpression of *Cldn6*, i.e., decreases were seen in *Cldn3* (~2.6-fold at 35 cycles), *Cldn4* (~2.4-fold at 30 cycles), *Cldn7* (~1.7-fold at 35 cycles), *Cldn8* (~2.5-fold at 30 cycles), *Cldn10* (~2.6-fold at 35 cycles), *Cldn11* (~2-fold at 30 cycles) and *Cldn14* (~2-fold at 35 cycles). *Cldn9* remained unchanged as no detectable signal was evident in either wild-type or transgenic samples (not shown). There was no *Cldn2* expression detectable by RT-PCR or immunohistochemistry analysis (Fig. 2C). As antibodies for other specific claudins become available, they will also be assessed and compared to our RT-PCR results. A control with no reverse transcriptase shows that there was no DNA contamination in the RNA samples used and a control with primers for GAPDH indicated that signal differences observed are truly due to claudin expression rather than sample inconsistencies.

Fig. 1. Claudin 6 structure, distribution and transgenic phenotype. (A) Molecular structure analysis of claudin 6 predicts it to be a 4 trans-membrane domain integral membrane protein that has varying tissue distribution in newborn mice. (B) Expression of claudin 6 was detected strongly in liver and kidney while there were varying degrees of expression in heart, thymus, spleen, calveria, brain, lung, tongue, stomach, tail and skin. There was no detectable expression in intestine. (C) RT-PCR results were supported by immunohistochemical staining of wild-type sections of intestine, lung and kidney tissues. (D) Mouse *claudin 6* was cloned into the *Not*I site of an expression vector containing the human involucrin promoter/enhancer and 17 transgenic animals were generated. (E) PCR analysis was used to detect expression of the *claudin 6* transgene using primers spanning the involucrin promoter to the *claudin 6* coding sequence (Table 1). A 590 bp band was detected in lane 2 (plasmid DNA control) as well as in lanes 6-10 (positive transgenic mice). There was no band detectable in lane 1 (PCR control) and lanes 3-5 (non-transgenic mice). (F) RT-PCR was also performed using primers spanning the exon sequences present in the involucrin promoter to the *claudin 6* coding sequence in order to identify only the transgene expression (692 bp). (G) *claudin 6* forward and reverse primers also identified the endogenous expression (660 bp), which is approximately 8-fold less than that of the transgenic expression. Western blotting of proteins extracted from the epidermis of transgenic and wild-type mice with claudin 6 antibodies revealed a 2.5-fold increase in the transgenic samples over the wild-type expression (H). The increased transgene expression was also demonstrated through indirect immunofluorescence with a marked increase of claudin 6 protein detected in the upper spinous and granular layers of transgenic backskin samples as compared to their wild-type counterparts (I).

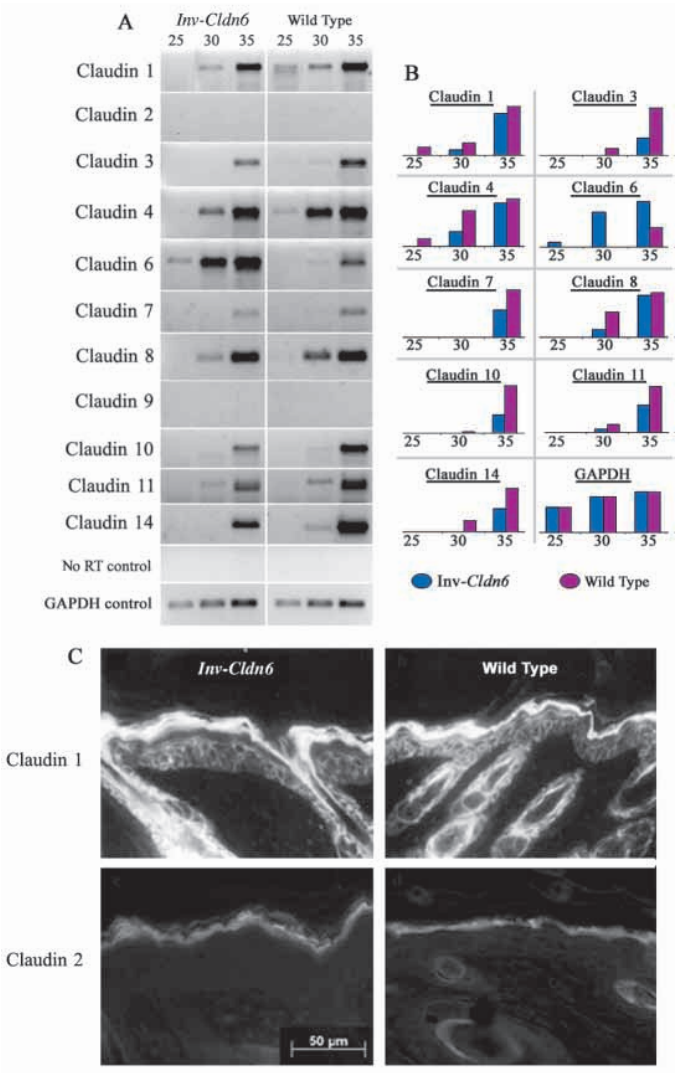


Fig. 2. *claudin 6* overexpression disturbs the overall claudin distribution. (A,B) RT-PCR analysis of various claudins using RNA extracted from newborn epidermis revealed that the overexpression of *claudin 6* resulted in modification of the expression profiles of other claudins in transgenic epidermis. Semi-quantitative RT-PCR analysis at 25, 30 and 35 cycles shows the range of signal detection between the epidermis of wild-type and *Inv-Cldn6* transgenic mice. *claudin 2* and *9* were undetectable in both samples.
(C) Immunofluorescence analysis of transgenic and wild-type backskin samples supports the RT-PCR results, showing virtually no difference in claudin 1 staining and no claudin 2 detection. Labelling in the SC is due to non-specific binding of the secondary antibody.

Transgenic mice overexpressing *Cldn6* have skin abnormalities

Transgenic newborn mice were immediately identifiable by their smaller size (~30% by weight) as well as the very distinct appearance of their skin, which was red, shiny and sticky to the touch. These neonates lost sufficient body moisture to cause the skin to dry up and crack (Fig. 3A), and the dehydration resulted in death within 24 to 48 hours, suggesting an impairment of the skin's barrier function (see below). Histopathology showed the transgenic epidermis to be thicker and disorganised, with improper packing of cells and with basal cells lacking their usual uniformly cuboidal shape (Fig. 3B-G). In addition, there were areas of the epidermis that had few or no visible keratohyalin granular cells (Fig. 3B compare with C) and the stratum corneum (SC) was moderately thicker and frequently fragmented (Fig. 3D compare with E). Collectively, these findings suggested that certain steps of epidermal terminal differentiation might be retarded or disrupted in the transgenic mice. Strikingly, there was a marked decrease in subcutaneous fat pads (Fig. 2F compare with G) such that the skin of transgenic animals was notably thinner than that of control animals in spite of the thicker epidermis.

Inv-Cldn6 transgenic mice exhibit abnormal epidermal barrier function

To determine whether the observed dehydration was due to defective epidermal barrier function, we compared permeability barrier formation using a β-gal assay (Hardman et al., 1993) on transgenic and wild-type animals at embryonic day (E) 16.5 and E18.5 as well as newborns (Fig. 4A). Typically, the mouse EPB forms 2-3 days prior to birth corresponding to the first detection of the SC in the mouse at E17.5 (Hardman et al., 1993; Aszterbaum et al., 1992; Williams et al., 1998; Elias and Feingold, 2001). Since an intact EPB blocks the penetration of X-gal through the skin, this assay is a reliable means to assess EPB formation. As expected, X-gal penetrated the skin of both transgenic and wild-type animals at E16.5. By E18.5, however, there was no penetration of the dye in wild-type mice whereas transgenic embryos still did not possess an EPB, a situation that continued to exist even after birth.

To compliment the β-gal assay, we also measured the amount of water that was lost through the skin's surface at birth (**t**rans-**e**pidermal **w**ater **l**oss: TEWL) using a dermal phase meter (DPM). In representative experiments, significantly higher DPM values (reproducibly in the range of 440-470) were obtained from transgenic samples than from wild-type samples (reproducibly in the range of 118-122), indicating a ~3-fold increase in water loss through the skin in transgenic newborns (Fig. 4B). To assess further whether the water loss was sufficient to cause the neonatal lethality observed, the weight of newborn transgenic and wild-type mice was tabulated for a period of 6 hours after birth. Transgenic mice lost up to 5% of their birth weight as a result of fluid evaporation attributed to a compromised EPB (not shown). It has been noticed that such a rapid rate of dehydration exists in premature babies, which results in hypovolaemic shock leading to death as observed in *Inv-Cldn6* transgenic mice. These results confirm that the EPB of transgenic animals was poorly formed, indicating that the aberrant expression of claudin 6 affects barrier formation and causes neonatal lethality.

Defective cornified envelopes (CEs) in *Inv-Cldn6* epidermis

We next investigated the CEs of *Inv-Cldn6* and wild-type skin to determine if there was a disruption in their formation and morphology. CEs were isolated by boiling the epidermis in the presence of an ionic detergent and a reducing agent. The CEs of wild-type mice were abundant, uniformly rigid and polygonal (Fig. 4D,F), but within the transgenic samples there were fewer CEs and they were generally more fragile in appearance (Fig. 4C,E). In addition, transgenic CEs were

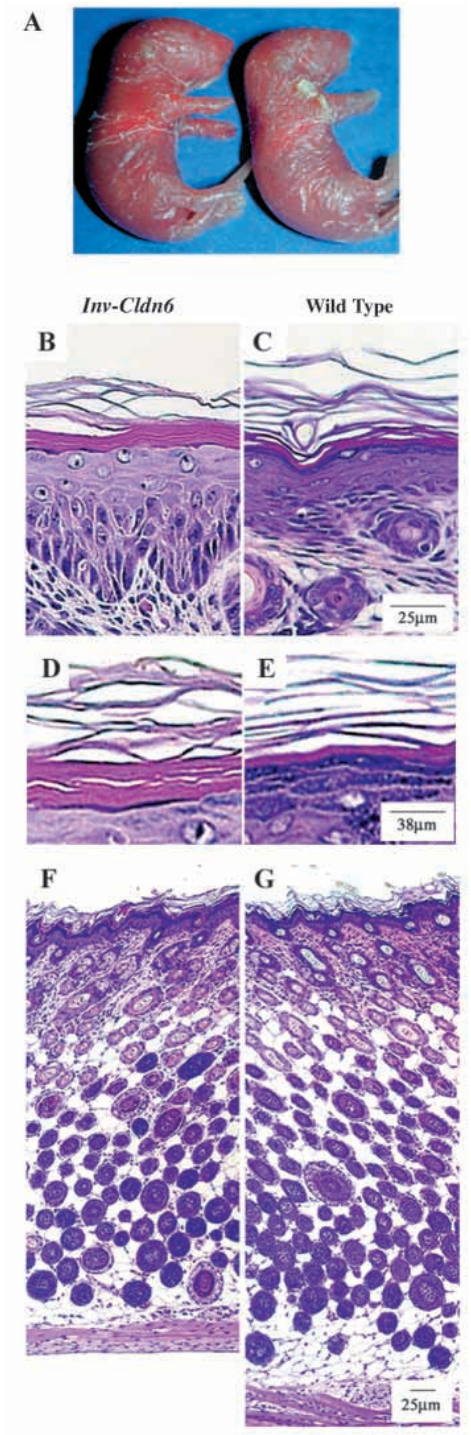


Fig. 3. *Inv-Cldn6* skin. (A) As a consequence of the transgene, the skin of *Inv-Cldn6* neonates is red and shiny in appearance and becomes dehydrated and cracked within a few hours of life. (B,C) Newborn backskin samples from transgenic and wild-type mice were fixed with Bouin's solution, processed and embedded in paraffin and sections were stained with Haematoxylin/Phloxine/Safranin. The transgenic epidermis is highly disorganised with gaps apparent throughout in addition to non-uniform basal cells. (D,E) It is also apparent that there is a dramatic decrease in keratohyalin granular cells and that the stratum corneum is thicker and often fragmented in the transgenic samples. (F,G) There is also an obvious reduction in the occurrence of subcutaneous fat pads resulting in the overall reduction in the thickness of transgenic skin.

morphologically less uniform and mostly rounded in shape reflecting their weakened nature (Fig. 4E). The defect in the shape of the CEs, with many rounded rather than polygonal in appearance, may also explain the thicker less compact nature of the overall SC observed histologically (Fig. 3D).

Overexpression of claudin 6 in the suprabasal layer of the epidermis affects the epidermal differentiation program

In general, keratin and intermediate filament associated protein expression are very reliable biochemical indicators of whether the program of epidermal differentiation is altered (Fuchs and Byrne, 1994; Turksen and Troy, 1998). The expression of epidermal keratin and terminal differentiation markers in the skin of transgenic and normal animals was evaluated by immunofluorescence (Fig. 5). Basal cell-specific keratins K5/K14 were present at similar levels in transgenic and normal skin, indicating that the basal cells continue to express the major structural proteins characteristic of this layer despite their abnormal morphology and arrangement. However, the expression of the suprabasal differentiation marker K1, which is generally restricted to the spinous and granular layers of the epidermis, was higher in the transgenic skin.

Skin samples were further assayed for the expression of K6/K16, keratins normally associated with the outer root sheath of hair follicles and not seen in the interfollicular epidermis unless there is hyperproliferation of the stratified epithelia such as under wound healing conditions (McGowan and Coulombe, 1998a). Patchy K6/K16 expression was seen in the lower strata primarily in the suprabasal layers of the epidermis of *Cldn6* transgenic mice. However, K17 [a marker of early epithelial differentiation expressed normally in some basal cells of newborn epidermis and upregulated during hyperproliferation (McGowan and Coulombe, 1998b)] was similar in wild-type and transgenic samples (not shown). Immunohistochemistry with antibodies against histone 3, a proliferation marker, showed no obvious differences between wild-type and transgenic samples, suggesting that there is not an increase in proliferation rate in transgenic epidermis (not shown).

The expression of the structural proteins filaggrin, loricrin, transglutaminase 3 and involucrin were evaluated by immunofluorescence (Fig. 5) and western blotting (Fig. 6A). All four of these markers were abnormally expressed with a noticeably less compact distribution in transgenic as compared to wild-type epidermis, again indicating a dysfunction in differentiation. Most interestingly, filaggrin expression was seen to extend into the suprabasal layers of the epidermis of transgenic mice. The markedly increased filaggrin expression suggests a dysfunction in the processing of profilaggrin. Western blotting showed that the processing of profilaggrin to filaggrin was enhanced as evidenced by the increased proportion of smaller (processed filaggrin) versus larger sized (processing profilaggrin) bands in transgenic than in wild-type samples. In fact, fully processed filaggrin was shown to be increased approx. 13-fold in the transgenic samples (Fig. 6A). Alterations in filaggrin processing are also supported by our histological observations indicating the discontinuous and disrupted pattern of keratohyalin granules in the transgenic epidermis. Since filaggrin expression changes in accordance with the enhancement or disappearances of granular cells, the abnormal distribution of granular cells supports our hypothesis

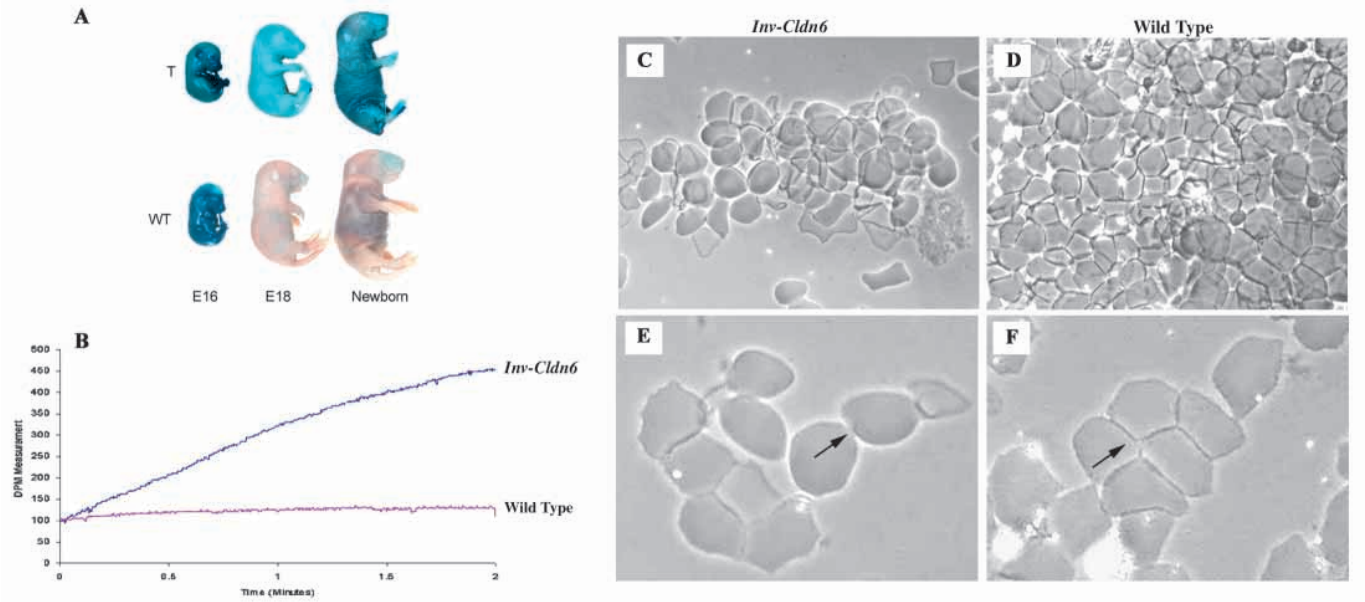


Fig. 4. *Inv-Cldn6* transgenic mice have a defective EPB. (A) β-gal assays were performed to assess the integrity of the EPB of transgenic animals, as compared to the wild type, at different stages of development. Under normal conditions, an intact EPB exists at E17.5 days thereby preventing the penetration of substances such as X-gal. When X-gal enters the epidermis a striking blue staining results. At E16.5 neither the wild-type nor the transgenic embryos possessed an intact EPB, yet at E18.5 there was no penetration of X-gal through the EPB of the wild-type embryos while transgenic embryos remained blue. Even after birth, transgenic neonates did not possess an EPB. (B) In addition to the β-gal assays, DPM measurements were performed to assess the trans-epidermal water loss of transgenic and wild-type neonates. In representative experiments, there was greater than a 3-fold increase in the dehydration of transgenic neonates as compared to the wild type further supporting the notion that the defective EPB causes the massive dehydration and death of the transgenic animals. (C-F) Isolated CE preparations of newborn transgenic and wild-type epidermis showed that the rigid, polygonal structure of the wild-type CEs (D and F) disappeared in the transgenic samples. Transgenic CEs were mostly rounded in shape (E) and overall less abundant (C) leading to the fragile, less compact nature of the stratum corneum that is observed.

that the terminal epidermal differentiation program was not progressing normally in the transgenic animals.

In addition to filaggrin, the expression of loricrin, transglutaminase-3 and involucrin was increased, though more modestly, in the transgenic compared to wild-type samples (~1.5-fold; Fig. 6A). RT-PCR analysis revealed no major detectable alterations in the expression of these markers.

The discontinuous keratohyalin granules, fragile CEs and abnormal histological observations led us to ask whether the regulators of the formation of the CEs were defective. We therefore studied the expression levels of repetin and several SPRRs (namely SPRR1A, 1B, 2A, 2B, 2C, 2D, 2G and 3), molecules known to be involved in the cross-linking of CE molecules (Fig. 6C). SPRR2B, 2C and 3 (SPRRs known not to be expressed in newborn epidermis) as well as SPRR1B were not detected, suggesting that the overexpression of claudin 6 did not cause an alteration in the expression of these SPRRs. In contrast, repetin and SPRR1A and 2A expression was decreased while the expression of SPRR2D and 2G was increased in the transgenic epidermis. We next questioned whether there was any disruption in the known regulators of repetin, SPRR1A and 2A that would give us clues to the mechanism of deregulation of these cross-linking proteins. Since a KLF4 binding site exists on the SPRR2A promoter (Sark et al., 1998; Fischer et al., 1996), KLF4 was a potential candidate. Analysis of KLF4 expression in transgenic epidermis by RT-PCR indicated that its levels were decreased approx. 4-fold as compared to the wild type (Fig. 6C). The downregulation of Klf4, given its transactivator role in SPRR expression, may be responsible for the cascade of changes that lead to disruption of the CE cross-linking process and hence the perturbation of epidermal differentiation as well as the processing of late markers that contribute to EPB formation.

DISCUSSION

In this paper we report a novel mouse model in which the overexpression of the TJ molecule, claudin 6, at its endogenous site in the upper strata of differentiating epidermal cells, initiated a cascade of events that resulted in a defective EPB with associated striking phenotypic consequences. The defective EPB manifested itself in the loss of body heat and weight, dehydration and the penetration of external dyes, events that resulted in the demise of the newborn transgenic animals within 24-48 hours of birth. The phenotype of these mice is very reminiscent of that seen in premature infants born before 32 weeks of gestation, with low birth weight, poor temperature stability and high susceptibility to toxicity (Rutter, 2000; Kalia et al., 1998; Lund et al., 1999). While it is known that these infants lack a properly functioning EPB, our transgenic mice provide a powerful animal model system for studying EPB formation as well as for potential therapeutic interventions.

EPB formation occurs during terminal differentiation, as keratinocytes move upwards to the skin surface and undergo both morphological and biochemical changes. Concurrent with their drastic shape and adhesion changes, the keratin component of their cytoskeleton becomes incorporated into the forming CE, a process requiring keratin filament bundling by

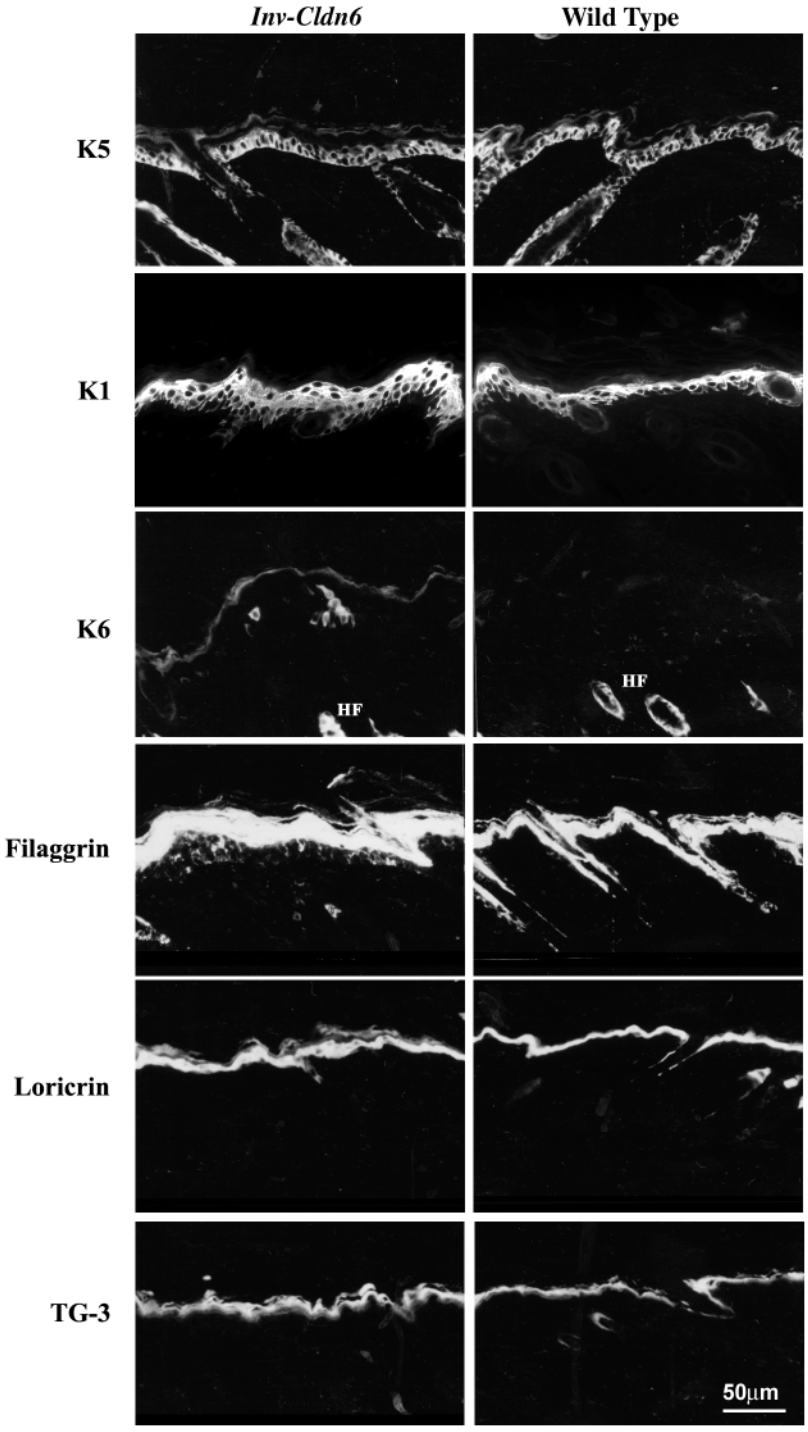


Fig. 5. Transgenic epidermal differentiation markers are perturbed. Newborn transgenic and wild-type epidermis was processed for immunofluorescence to evaluate epidermal differentiation markers. K5 expression was not altered in the transgenic epidermis, however, K1 expression was increased indicating a perturbation in the differentiation program of the transgenic epidermis. In addition, K6, which is not normally expressed in the epidermis, shows some patchy expression in the transgenic epidermis; while normal hair follicle (HF) distribution was not altered. In addition, filaggrin, loricrin and transglutaminase-3 were also assessed. There was aberrance in the expression of these markers in the transgenic epidermis in that they were much more diffuse and expressed in a much broader zone, especially in the case of filaggrin.

filaggrin. The processing of profilaggrin to filaggrin normally occurs in the keratohyalin granules (KGs) of the granular layer under the action of various processing enzymes. Interestingly, western blotting and immunofluorescence analysis revealed a approx. 13-fold increase in processed filaggrin expression in the transgenic epidermis with expression extending into the suprabasal layers of the epidermis; yet the histological appearance of KGs was drastically altered in addition to being aberrantly distributed in the granular layer. The abnormal nature of the KGs suggests an accelerated processing of profilaggrin into filaggrin before the profilaggrin is able to collect and form KGs in the granular layer. Enhanced processing of profilaggrin was supported by western blot analysis of filaggrin expression. These observations are consistent with a previous report in which the overexpression of filaggrin alone was found not to be sufficient for granule formation but required linkers between the filaggrin molecules of profilaggrin (Kuechle et al., 1999). Since the increased levels of filaggrin together with its abnormal expression in the lower strata of the epidermis may contribute to the premature bundling of keratin filaments, the abnormal processing of profilaggrin could account for the observed disturbances in the epidermal differentiation program of the transgenic epidermis. Whether the processing of profilaggrin is stimulated by an increased activation of one of its processing enzymes [e.g. the convertase furin (Pearton et al., 2001)] is not yet known. It is interesting to speculate, however, that reducing furin activity via the topical application of available specific furin inhibitors may be one clinically feasible option for the treatment of EPB dysfunction.

Once the filaggrin-mediated bundling of keratin intermediate filaments occurs, the development of the CE scaffold continues via the activity of molecules such as involucrin and loricrin in addition to cross-linkers such as SPRRs which contribute to CE rigidity and resistance to mechanical stress (Steinert et al., 1998). Thus, the reduced expression of repetin as well as SPRR1A and 2A in the claudin 6 overexpressing mice almost certainly contributes to the fragile and poorly formed CEs. These and other CE-associated genes (at least 27 known genes, including involucrin, loricrin, transglutaminase, SPRRs, Ca^{2+} binding S100 proteins, late envelope proteins and other less characterised proteins such as NICE-1 and ZIRTL) (Lioumi et al., 1999; Marenholz et al., 2001) constitute a 2 Mb epidermal differentiation locus (EDL) involved in CE formation (Marenholz et al., 2001; Marshall et al., 2001). This locus, as well as the order of the individual genes within, has been mapped to chromosome 1q21 in human (Hardas et al., 1996) and chromosome 3 of mouse (Carver and Stubbs, 1997). However, it is not yet known how these molecules are regulated in this locus or whether their positional order is important. Our data

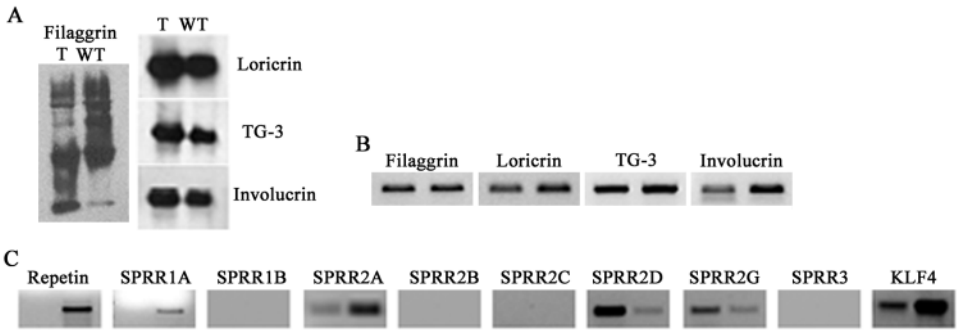


Fig. 6. The overexpression of claudin 6 causes an overall disturbance in the expression of other markers. (A) Western blot analysis revealed the enhanced processing of profilaggrin. The transgenic samples revealed a 'bottom heavy' expression of the smaller sized processed filaggrin while wild-type samples were 'top heavy' indicating a greater abundance of unprocessed profilaggrin in the wild-type newborn epidermis. In fact, processed filaggrin was increased ~13-fold. In addition, the expression of loricrin, transglutaminase-3 and involucrin was increased ~1.5 fold in the transgenic samples. (B) RT-PCR analysis indicated no significant differences with filaggrin, loricrin, transglutaminase-3 and involucrin in the transgenic epidermis versus wild-type epidermis. (C) RT-PCR analysis further indicated that repetin, members of the SPRR family and Klf4 (genes involved in CE formation) were altered in transgenic epidermis. Repetin, SPRR1A, 2A and Klf4 were downregulated in the transgenic samples while the expression of SPRR2D and 2G was increased. SPRR1B, 2B, 2C and 3 remained unchanged.

indicating the downregulation of some members of a SPRR cluster within the locus (SPRR1A and 2A, thought to be crucial for barrier formation) concomitant with upregulation of others (SPRR2D and 2G) are consistent with other evidence that regulation of SPRR family members appears to be very complex (Cabral et al., 2001; Marshall et al., 2000; Song et al., 1999). The results also suggest that SPRR2D and 2G may be upregulated as part of a compensatory response to the downregulation of SPRR1A and 2A in an attempt to maintain barrier function. Interestingly however, the upregulation seen in these mice is either not sufficient for EPB formation or the SPRR family members are not interchangeable with respect to their activity in barrier function.

The changes observed in SPRR1A and 2A expression are consistent with the downregulated expression of Klf4 in our transgenic mice. Klf4 was originally cloned from gut and since then it has been shown to be expressed at higher levels in skin where its localisation is associated with the differentiation compartment of the epidermis (Dang et al., 2000; Bieker, 2001). Supporting its role in differentiation, Klf4 has been shown to be involved in the regulation of at least SPRR2A and it is known that the SPRR2A promoter has a Klf4 binding site. In addition, there is no Klf4 binding site on SPRR2D or 2G indicating that their increased level of expression is independent of Klf4 activity. The differences in binding sites for the transcriptional regulators in the promoter regions of various SPRRs indicate a complex evolution of the members of this family. In addition, it suggests that there are several regulatory mechanisms that may contribute to the tissue-specific flexibility required for the different cross-linking capabilities needed in the diverse epithelia of the organism. Recently, Segre et al. (Segre et al., 1999) generated *Klf4* null mice, using homologous recombination. Interestingly, the transgenic mice die within a couple of days of birth because of a loss of skin barrier function. The loss of barrier function was shown to result in the penetration of external dyes as well as dehydration (measured by the loss of body fluids). However, the *Klf4* null animals did not exhibit the morphological or biochemical alterations in the structural features of the epidermis that we observed in the claudin 6 overexpressing animals, although the *Klf4*$^{-/-}$ epidermis did have perturbations in late differentiation structures such as the cornified envelope. Thus, together, the two studies emphasise the importance of a Klf4 signaling pathway but point towards the need to identify additional important players in the formation of the EPB.

The molecular mechanism by which the deregulated expression of one claudin contributes to changes in the expression of other claudins is unknown but is consistent with our hypothesis that their expression is tightly temporo-spatially regulated in normal epidermal development. The role that Klf plays, if any, in claudin regulation is not yet known. However, it is intriguing that Klfs were initially identified as lineage-specific trans-activators and regulators in hemopoiesis. For example, they regulate specific globin genes in the globin locus that consists of a cluster of several genes not unlike that in the EDL. Our observation that claudins lie upstream of Klf4, which regulates some of the SPRRs, sheds some light on at least one tissue-specific differentiation pathway. It is not unlikely that claudins function as receptors through their specific cytoplasmic domains, which may interact with various adapter molecules in a claudin-containing TJ-dependent signalling pathway that leads to the regulation of tissue-specific gene expression.

In summary, we provide the first clear evidence that claudins are involved in and are crucial for the formation and maintenance of a bona fide EPB. Our results also support the notion that there is tightly controlled expression of the claudin profile in a cell- and tissue-specific manner, that the alteration of one claudin has regulatory repercussions on other claudins, and that a claudin-containing TJ-based signalling pathway involving the regulation of Klf4 is important in epidermal differentiation and EPB formation. Identification of the upstream regulators of Klf4 and the other players in this pathway is currently under investigation. Finally, these mice provide an important opportunity to design new non-invasive therapeutic treatments to enhance EPB formation of premature infants in order to increase their survival.

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