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Claudin Tight Junction... What's your function ?

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Claudin Tight Junction...What's Your Function?

The Role of Claudin 6 in

Epidermal and Hair Follicle Differentiation

By

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Abstract

Incomplete permeability barrier (PB) is characterized by epidermal defects and remained a leading cause of neonatal death as a result of its associated complications, which include physical and chemical damages, microbial infections, dehydration, and heat loss. This phenotype results from poorly cornified envelope formation that may be a reflection of defects in later stages of differentiation in the epidermis. Despite its importance in survival, the mechanisms involved in the formation and maintenance of the EPB are not well understood. To elucidate the role of Claudin 6, a member of a new super family of TJ molecules, in the skin, we overexpressed it using the promoter for the differentiation marker, involucrin (Carrol et. al. 1993). Homozygous INV-Claudin 6 animals exhibit epidermal differentiation aberrations that result in incomplete formation of EPB and neonatal death (Turksen and Troy 2002). Heterozygote animals have milder EPB defects and survive to adulthood. All heterozygotes have pronounced abnormalities in their coat characterized by fuzzy hairs and a matte texture. Histologically many hair follicles and hair fibers exhibit structural and morphological abnormalities, suggesting that the hair follicle differentiation program has been compromised. In agreement with our hypothesis, the anagen phase of the hair cycle (starting at P14) was shortened, and the catagen-telogen transition was prolonged. Expression of epidermal differentiation markers K14 K5, K15, K1, Loricrine, Involucrin, Filaggrin and transglutaminase3 were perturbed in the transgenic epidermis. Furthermore, K17 revealed patchy

expression in the transgenic epidermis while no expression was seen in wild type. The expression of hair keratins was downregulated in Inv-Claudin 6 hair follicles while the expression of K5, K6, K15 and K17 was maintained. Overall, changes in the differentiation program, abnormalities in hair fibers and hair cycling support the notion that Claudins are crucial members of signaling pathway(s) which modulate cell physiology and epidermal and hair follicle differentiation.

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List of Abbreviations

AP -Alkaline Phosphatase

DP -Dermal Papilla

EPB -Epidermal Permeability Barrier

HF -Hair Follicle

Inv -Involucrin

IRS -Inner Root Sheath

JAM -Junction Adhesion Molecule

K (Number) - Keratin (Number)

ORS -Outer Root Sheath

PB - Permeability Barrier

RAR - Retinoic Acid Receptor

SEM -Standard Error of the Mean

Shh -Sonic Hedgehog

TGF-α - Transformation Growth Factor α

TJ -Tight Junction

Wa-1 - Wave 1

Zo - Zonulla

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Introduction

Skin is the largest organ in the body and is essential for survival. The main role of skin is to protect against mechanical, chemical and microbial invasion and functions as a permeability barrier (PB) by preventing water loss. The epidermis is the outermost layer of the skin and morphologically is a multilayered structure and a continuously regenerating tissue composed of cells at different stages of differentiation (Fuchs and Byrne, 1994; Turksen and Troy, 1998). Therefore it provides an excellent model to follow the differentiation process by morphological and biochemical criteria (Turksen and Troy 2002; Mazallupo et. al. 2003). In this model, proliferation and death are essential cellular processes to keep the functional homeostasis. Proliferation is required to produce as many cells as needed to form the functional structure, and its inhibition allows terminal differentiation. On the other hand, cell death is essential both to eliminate cells within this tissue that have to achieve the right size and form and to eliminate unnecessary cells in the mature structure. Therefore the consequence of conditions that perturb this homeostasis such as wounding, inflammatory stimuli, and UV radiation (as a result of this balance) can lead to diseases such as cancer and psoriasis. In recent years, many signals that control proliferation, differentiation, and cell death have been characterized, several of which are shared, showing the close interactions between these processes (King and Cidlowski 1995; Millar 2002). The maintenance of the correct proportions of proliferating and differentiating cells presumably involves the interplay of intra-

and extra-cellular signals, but the factors that control a cell's decision to multiply or differentiate remain unknown.

Epidermis and hair follicle development and differentiation

After fertilization the zygote of a multicellular organism proceeds by a process called cleavage, a series of mitotic divisions whereby the egg cytoplasm is divided into many smaller, nucleated cells. These cleavage-stage cells are called blastomeres. Gastrulation which takes place over the next few days at around E6 is the process of highly coordinated cell and tissue movements whereby the cells of the blastula are dramatically rearranged. The blastula consists of many cells, the positions of which were established during cleavage. During gastrulation, these cells are given new positions and new neighbors, and the multilayered body plan of the organism is established. These germ layers are: outer ectoderm, inner endoderm, and interstitial mesoderm. The cells that will form the endodermal and mesodermal organs are traveling inside the embryo, while the ectoderm cells that will form the skin and nervous system are covering the outside surface. The covering ectoderm cells at E8.5 start to form the presumptive epidermis. This tissue is originally one cell layer thick but in most vertebrates and at around embryonic age of E13.5 becomes a two-layer structure. In the following days the outer layer gives rise to periderm, a temporary covering tissue that shed once the inner layer forms the true epidermis. The inner layer, the germinative or basal layer, gives rise to the whole cells of epidermis, a self renewing tissue that maintains homeostasis by constant proliferation of basal

layer of rapidly dividing progeny of stem cells (Potten 1974; Potten and Morris 1988; Braun et al 2003).

Hair follicle like all other epithelial appendages such as nail, glands and teeth, also develop from the single-layered ectoderm during embryogenesis (Hogan et al., 1986; Hardy et al 1992; Wolpert et al., 1998; Bard, 1993; Zhao and Hogan 1996; Miller 2002; Tobin et al 2003). To that end, a series of signals sent between dermal cells and the overlying surface epithelial cells eventually result in the differentiation of the hair shaft, root sheaths, and dermal papilla (Hardy, 1992; Worst et al 1982). At embryonic age E8-E12 the progenitor cells residing in ectoderm respond to various signals thereby committing themselves to the hair follicle lineage. The first signal arising in the mesenchyme causes a histologically determinable thickness of epidermis known as a placode. Then signals from the placode cause a dermal condensate which in turn provides signals that regulate the proliferation and movement of epithelial cells down into the dermis to form the hair germ. Further, epithelial cells surrounding the dermal condensate eventually develop into the hair follicle dermal papilla. At E14.5, hair buds appear as local thickenings of epidermis surrounded by condensed mesenchymal cells (Sengel 1976; Hardy, 1992). Once formed, hair buds continuously elongate and penetrate into dermal tissue. By the day E16-E17 four stages of induction, initiation, elongation and differentiation (Holbrook and Minami 1991) of hair follicle are complete and is morphologically recognizable. After morphogenesis, hair follicles enter a phase of structural regression (catagen) and quiescence (telogen). The follicle reactivation follows by the first postnatal anagen, the actual initiation of

hair follicle remodeling. Hair follicle formation occurs but once in the lifetime of an individual, so a mammal is born with a fixed number of follicles, which does not normally increase thereafter (Stenn and Paus 2001).

Thus, development of embryonic mouse skin from E13.5 to E16.5 includes the principal morphogenic events described above. Among the most intriguing questions is how the apparently homogenous epidermal cells generate signals to form hair follicle by a certain subset of epidermal cells, the phenomena which has not been clarified yet.

Mature epidermis and hair follicle

The mature epidermis and hair follicle are regenerating structures formed and maintained by a balance between epithelial cell multiplication and terminal differentiation (Fuchs E. 1990; Turksen and Troy 1998). This is a characteristic that highlighted them as a model for studies of the genetic regulation of morphogenesis (Cotsarelis and Millar 2001; Bull et al 2002; Braun et al 2003). However, the molecular mechanisms that control epidermal differentiation, hair follicle (HF) morphogenesis, and its cyclic transformation in mammals are not yet fully understood. In this regard, the availability of many transgenic, knockouts, and spontaneous mutant mice exhibiting hair growth and skin defects has been essential in the understanding of HF and epidermal biology (Sundberg and King 1996; Yamanishi 1998; Koster 2003).

(A) Mature epidermis: In epidermis, as the basal cells withdraw from the cell cycle, the transiently amplifying cells migrate upward to the outer layer of skin

and commit to terminal differentiation (Byrne and Fuchs 1994; Turksen and Troy 1998). The keratinocytes are the major epidermal cell types and comprise about 90%-95% of epidermal cells. These cells are organized in different layers namely: (1) the stratum corneum or outer layer which is made of flattened epithelial cells in multiple layers, (2) the translucent or transitional layer which is a translucent, thin layer of cells that is sometimes visible in thick skin; however, nuclei and other organelles are not visible, (3) the suprabasal layers which is composed of three to five layers of cells, including spinous and granular layer, in different terminal differentiation stages and eventually (4) the basal or germinative layer (Reichert et al., 1993; Turksen and Troy 1998; 2002).

All keratinocytes contain keratin intermediate filament, a structural protein that provide a fundamental integrity together with the microfilaments and microtubules and constituting up to 85% of a fully differentiated keratinocyte (MacKenzie 1970; Christophers 1971; Allen and Potten 1974; Potten 1974; Byrne and Fuchs 1994; Fuchs 1995). The tightly regulated expression of these keratin genes in complex epithelia, suggest the existence of a direct link between keratin gene expression and epithelial cell function. This ability helps detect the various differentiation markers in epidermis and hair follicle, the characteristic that has been tremendous improvement in evaluation of the differentiation process (Takahashi et. al. 1999).

Vertebrate keratins are subdivided into two groups: acidic type I (keratin 9 -20) and basic type II (K1–K8) proteins that are typically co-expressed as specific pairs (Moll et al., 1982; Domagala 1988; Fuchs and Weber 1994). Keratin 8 and

18 are the first keratins expressed during mouse development, starting as early as E7.5 (Brulet et al. 1980; Jackson et al. 1981; Paulin et al., 1980; Oshima et al., 1983), followed by K19 and K7 (Hesse and Magin 2001). At later stages, they are expressed in the trophectoderm and its derivatives and in the embryonic and extra-embryonic endoderm (Jackson et al. 1981). As it goes, K19 keeps its exhibition in adult epidermis among the basal cells where it expresses in Merkel cells, a minor population (<1%) of mechanosensory cells. Another type I keratin, K15, together with K17 are also early markers of the initiation of epithelial tissue formation and are known to be expressed after birth in epithelial appendages (Lloyd et al 1995; Whitebread and Powell 1998; McGowan and Coulombe 1998). Earlier studies have shown that the K15 mRNA is expressed in all layers of stratified epithelia (Leube et al 1988). The conclusion drawn from these studies was that the K15 expression starts in the basal layer but is independent of the vertical differentiation of migrating keratinocytes. Recent studies, however, have shown that K15 is not expressed in the suprabasal layer but instead is specifically localized in the basal keratinocytes (Lloyd et al 1995). Despite the fact that K15 is regarded as specific for basal keratinocytes, as a consequence of the lack of a suitable antibody, its distribution in normal and epithelial-related diseases has not been investigated in detail. On the other hand during normal skin development in mouse, onset of expression of K17, a member of type I keratin family, occurs in E10.5 ectoderm at sites of epithelial-mesenchymal interactions, such as placodes, and reflects a commitment toward a nonepidermal fate (McGowan and Coulombe 1998). In adult mouse skin, K17

gene is expressed in specific subsets of epithelial cells within all appendages, but is excluded from epidermis (McGowan and Coulombe 1998; Bernot et al. 2002). A member of type II keratin, K6, is first detected at E12.5 in vibrissae and at E14.5 in the periderm, where it coexists with K17, and in a subset of cells within primary hair germs, but not placodes (McGowan and Coulombe 1998). Periderm cells participating in temporary epithelial fusions, which involve epithelial cell migration, express K6 and K17 (Mazzalupo and Coulombe 2001). Similar to keratin 17, K6 gene downregulates its expression in epidermis after birth while this gene is still expressed in specific subsets of epithelial cells such as hair follicle outer root sheath (McGowan and Coulombe 1998). In adult mammalian skin, keratin 6 and keratins 17 are among the genes that are rapidly upregulated after injury and serve as one of the most sensitive indicator of an altered epidermal differentiation program as in hyperproliferation or wound healing (Coulombe 1997; McGowan and Coulombe 1998). In addition to these keratins the basal layer of keratinocytes in contact with the basement membrane is expressing keratin 5 (type I) and keratin 14 (type II) as the predominant keratin pair (Byrne and Fuchs 1994). This expression occurs at E8.5 at the early bi-layered epithelial cells stage and persits after birth as well (Dale et al 1980; Moll et al 1982; Schweitzer and Winter 1982; Banks-Schlegel 1982; Fisher and Holbrook 987; Byrne and Fuchs 1994).

While most of the above mentioned keratins are basal cells specific, K1 (type II) and K10 (type I) are expressed as the predominant keratin pair in cells undergoing terminal differentiation (Fuchs 1995). Expression of K1 and K10 is

detected post K5 and K14 at around E15.5 where transcripts are found in the early suprabasal layer of epidermis (Byrne and Fuchs 1994). In mature epidermis as the basal cells become committed to terminal differentiation they stop dividing and move into the suprabasal compartment. To that end, cells in the spinous layer switch off K14 and K5 and induce K1 and K10, required for the marked mechanical resistance of these cells (Vassar et al 1991; Fuchs et al 1992; Byrne and Fuchs 1994; Fuchs 1995). As these cells differentiate further, the keratinocytes flatten out to form the granular layer, characterized by keratohyalin granules. Finally, the keratinocyte becomes cornified and structural membrane proteins, such as Involucrin, filaggrin and loricrin crosslink with the keratins to form an envelope meanwhile the nucleus breaks down and the cells die forming the cornified layer. In addition the mechanical attributes of the cornified layer are afforded by its rigidity and extraordinary insolubility. These properties are a result of extensive cross-linking of the constituent proteins by the action of transglutaminases (TGases) (Matoltsy 1966; Haydock 1993; Watt 1989; Hohl 1990; Reichert 1993). To date, a few distinct TGase enzymes are known to be present in the epidermis (Holbrook 1993; Watt 1989; Hohl 1990; Reichert1993), of which TGases 1 and 3 seem to be the most important and may have complementary or perhaps overlapping roles in the cross-linking of various cornified layer structural proteins (Candi et al 1995). Now, a growing number of structural proteins and small proline-rich proteins (SPRR) proteins are also known to be components of the epidermal cornified layer (Presland and Dale, 2000; Marenholz et al., 2001; Marshall et al., 2001).

Thus the relative consistency of these findings have provided the developmental biologists with the opportunity to pursue a logical prediction to reconcile morphological studies in other organs such as hair follicle as well as tissue reconstitution experiment.

B) Mature hair follicle: The mature hair follicle is a complex composed of both epithelial and mesenchymal cells. This structure is quite complex as it comprises seven distinct epithelial layers, each the product of a terminal differentiation pathway. These layers are organized in concentric circles around the main axis of the hair. From the inside out, they are the medulla, the cortex, and the cuticle (which form the hair shaft), another cuticle, Huxley's layer and Henle's layer which form the inner root sheath (IRS), and the outer root sheath (ORS), an epithelium that covers around the follicle and is contiguous with the epidermis (Hardy 1992; for review see: Fitzpatrick). The formation of such a complex structure is proposed to involve interlayer communication as well as epithelial-dermal signaling to control and regulate the differentiation process (Millar et al, 1999; Lin et al, 2000; Botchkarev and Kishimoto, 2003). Further, the mature hair follicle has mesenchymal cells (the dermal papilla) at its base which plays a critical role in successful postnatal hair cycle, a hallmark that made hair follicle stands as a unique model for studying regeneration and organ renewal. In this model system, scientists were looking for the location and property of the cells that support this regenerative property (Sperling, 1991; for review see: Fitzpatrick, 1993; Braun et al 2003). Because the bulb of the follicle shows significant cell division, it was implicit in the older literature that the site

for important cell division and new anagen formation was in the bulb (Van Scott 1963). Later studies implicated that this area is a non-cycling region of the follicle in a specialised region of the outer root sheath (Cotsarelis et al. 1990; Lavker et al. 1993; Lyle et al. 1998; Morris and Potten, 1999; Akiyama et al. 2000; Taylor et al. 2000; Fuchs et al. 2001; Oshima et al. 2001; Trempus 2003). This region is defined by the point of insertion of the arrector pili muscle and is known as the bulge (Cotsarelis et al. 1990). Biochemical studies also support the notion that cells in the bulge segment and its surrounding mesenchyme are special. For example the bulge cells are rich in keratin 15 (Lyle et al 1998) and keratin 19 (Lane et al 1991), two markers of early markers of the initiation of epithelial tissue formation.

At the onset of phase of hair growth and in response to messages from the dermal papilla, epithelial stem cells residing in the bulge region divide and the daughter transient amplifying cells migrate to the hair germ to initiate a new cycle of hair growth (Lin et. al. 2000; Millar et. al. 1999; Oliver and Jahoda, 1988; Oshima et. al. 2001; Taylor et. al. 2000). Further signals from dermal papilla make the matrix cells travel upwards to form the hair shaft and inner root sheath space. This is a developmental characteristic which occurs over the total lifetime of a mammal and like epidermis regeneration is thought to require many of the cellular signals integral to other morphogenetic and regenerating systems. During hair follicle regeneration that manifests by a cycle, the inferior hair follicle dramatically reforms itself over the cycle but the upper, permanent follicle undergoes substantial remodeling (Chase 1954). Although it is clear that

the amplification and differentiation of progenitor cells progress along a tightly regulated pathway, virtually nothing is known of the individual steps in this process or of the identity of the key regulators of hair follicle morphogenesis. Therefore, the genes and signaling molecules involved in hair follicle stem cells and commitment to the hair follicle lineage remain to be identified.

The hair follicle regeneration process occurs in three main stages: growth (anagen), regression (catagen) and rest (telogen) (Hardy, 1992). During the hair cycle, signals being transmitted from dermal papilla are essential; however the embryonic dermal signal as well as the ectodermal signal (to make dermal papilla) is no longer required (Cotsarelis et. al. 1990).

Anagen

In response to a stimulus of the dermal papilla, the germinative epithelial cells in the bulge region proliferate and form other follicular epidermis (Cotsarelis et. al. 1990; Oliver and Jahoda, 1988; Oshima et. al. 2001; Taylor et. al. 2000; Wilson et. al. 1994). This is the initiation of a new growth phase of the hair follicle, namely, anagen. Under normal circumstances, the first postnatal anagen starts at P2, right after the late embryonic telogen, terminates at around P16-P18 and is comprised of 6 subphases, anagen I-VI (Chase et. al. 1953; Straile et. al. 1961; Paus et al 1997). When anagen is triggered, embryologic events are being repeated briefly and a new hair follicle is formed. With progression of early anagen proliferation, the proliferated cells in the bulge region of hair follicle grow downward and completely engulf the dermal papillae. In this model, a reduction in the expression of integrins or cadherins could encourage the departure of

nonstem daughters from their place (Alonso and Fuchs 2003). Once they reach their destined depth, the cells in the central cylinder reverse their growth direction and now progress distally. At this point, the prominent upward flow of epithelial cells is formed; these originate from the hair matrix, where active proliferation takes place. Proliferation of hair matrix cells results in the formation of the hair shaft and IRS (Oshima et al. 2001; Taylor et al. 2000). The dermal papilla is necessary to both induce and maintain the hair follicle (Arase 1990; Kollar 1970; Weinberg et al 1993).

The most important question in this context is: How does the dermal papilla initiate the proliferation of the cells in bulge region and make them to move upward or downward and execute a particular differentiation program? Although the answer to this question is still unclear, some advances have come from studies on the hair lineage. For instance, onset of the first postnatal anagen fails to occur in mice that progressively lose β-catenin from the epidermis and follicular epithelium, indicating that activation of wnt signaling pathway in the epithelium is necessary for this process (Huelsken et al 2001). In other studies the specific wnts, including wnts3, 3a, 4, 5a, 10a, 10b, and 11, that are expressed within the precortical cells of the developing hair follicle found to be responsible in hair follicle cycling and development certain wnt proteins (Reddy et al 2001). These date suggesting that, as in morphogenesis, wnts play an important part in conveying inductive signals between the follicular epithelium and mesenchyme of postnatal follicles. Sonic hedgehog (Shh) also seems to play its important morphogenetic functions during anagen. While not required for

anagen onset, shh is necessary for subsequent events of anagen, including proliferation of epithelial cells and downgrowth of the follicle into the dermis (Wang et al 1999). Ectopic expression of Shh is capable of inducing resting follicles to enter a growth phase (Sato et al 1999).

Catagen and telogen:

After anagen, cyclic behavior of hair follicle leads it to a regression stage of catagen during which epithelial elements undergo apoptosis-driven diminution (Weedon et al 1981) and the hair bulb shrinks (Kligman et al 1959). The catagen stage of the hair cycle has been divided into eight subphases beginning with late anagen and ending in early telogen (Straile 1961; Paus et al 1997; Muller-Rover 2001). The first sign of regression in the catagen follicle is the withdrawal of papilla cell fibroblast projections from the basement membrane (Botchkarev and Paus, 2003). The papilla shrinks, probably through the loss of extracellular matrix substance. The end of bulbar epithelial cell division coincides with massive epithelial cell apoptosis in well-defined regions of the regressing hair follicle (Lindner et al 1997). The epithelial elements migrate toward the surface, and by late catagen, only secondary epithelial hair germ cells in bulge area, which is located at the level of the arrector pili muscle, are left. A decrease to one-third of the hair follicle's former length occurs during the upward migration (Lever and Schaumburg 1990). Associated with the upward migration, the residual epithelial stem becomes surrounded by a thickened basement membrane zone; the perifollicular connective tissue also becomes wrinkled and folded during late catagen, culminating in development of a club follicle surrounded by a club hair.

The club follicle thus finally matures to leave a club hair during the resting stage or telogen. Telogen then persists until the next anagen phase begins.

During the hair cycle, like hair follicle morphogenesis, the initiation of a new growth phase and the subsequent downgrowth, proliferation, and differentiation of the follicle requires an orchestrated flexibility and a well controlled multiplicity among the cells. The diversity of the morphologic differentiation pathways of mature hair follicle is partly reflected by the respective keratin patterns that are generally suitable indicators for the type of epithelial differentiation (Moll et al 1982; Winter et al 1994; Mitsui et al 1998). Thus the keratin profile of the hair follicle is mainly composed of cytokeratins K5, K6, K14, K16, and K17, which are contributed by the three layered ORS, and the hard keratins of the hair shaft (Kopan and Fuchs 1989). K6 is expressed constitutively in the innermost layer of the ORS of hair follicles (Takahashi et al 1998; Winter et al 1998; Langbein et al 1999; 2001). K17, on the other hand, is expressed early during embryonic development prior to formation of the hair follicle placodes in the single layer ectoderm. It later shows an upregulation in the ORS and bulge but is downregulated in the epidermis (McGowan and Coulombe 1998) and present in the entire ORS of mature hair follicles as well as in sweat and sebaceous glands (Panteleyev 1997; McGowan and Coulombe 1998). Recently another epithelial keratin, K6hf, has been identified as specific constituents of the companion layer , highly differentiated flattened cells, which lie directly adjacent to Henle's layer of the IRS (Ito 1986; 1988). This protein is expresses together with the keratins K6, K16, and K17 (Winter et al 1998; Wojcik et al 2001). In

addition, follicular epithelial cells such as hair cortex were reported by Powell et al in 1991 to express the hair-specific keratins, such as type I keratin Ha3 and Ha2, during the early differentiation stage. Keratin intermediate filament-associated proteins, such as high sulfur protein B2 and ultra high sulfur proteins, during the later stage were found localized in the hair cortex. In contrast, the characterization of the keratins expressed in the IRS, consisting of the Henle and Huxley layers as well as the IRS cuticle, has not been well identified; however previous immunohistochemical and gel electrophoresis studies suggest proteins such as anti tricohyalin AE15 as IRS specific keratins (Manabe et. al. 1996). These two layers of functionally distinct keratinocytes, the IRS and ORS, surround the growing hair shaft for physical and biochemical support. Looking for a hair shaft specific marker, Lynch and his colleagues in 1986 introduced AE13 as a marker of the hair shaft that continues its expression during hair cycle.

While the shaft, the IRS, and the ORS constitute major components of the differentiated middle and upper portions of the hair follicle, the lower bulbous portion contains predominantly undifferentiated and proliferating keratinocytes. Like epidermis, this is the characteristics that made hair follicle an excellent model to follow differentiation and proliferation as well as cell migration. In this model keratinocytes retain the flexibility required for activities such as cell migration and proliferation (mitosis), processes that require the ability to undergo dynamic shape changes on a relatively rapid time scale. In addition, cell adhesion which primarily depends on the cadherin and integrin families of molecules plays crucial roles in segregation, sorting, rearrangements and

migration of cell populations, providing the basis for the dynamic morphogenetic processes of epidermis and hair follicle formation (Steinberg 1987; Gumbiner 1992; 1996; Takeichi 1995; Rubin et al 1996). In contrast, terminally differentiated keratinocytes should display the rigidity and resilience expected of cells taking part in the building of an effective barrier at the skin surface (Nemes and Steinert 1999; Steinert 2000). In this context some fundamental questions can be asked including: What are the cell junctions necessary to achieve the specific characteristic of keratinocytes? How do they participate in the barrier function of epidermis and its appendages such as hair follicle? What are the molecular inputs necessary to achieve this barrier specificity? How similar or dissimilar are the molecular controls of epidermis and hair follicle?

Cellular junctions

Cell junctions can be classified into three functional groups: (1) anchoring junctions, which mechanically attach cells (and their cytoskeletons) to their neighbors or to the extra-cellular matrix (Farquhar and Palade 1963; Koch et al 1994) (2) communicating junctions, which mediate the passage of chemical or electrical signals from one interacting cell to its partner (Kam et al 1986) and (3) occluding junctions, which can seal cells together in an epithelial cell sheet in a way that prevents even small molecules from leaking from one side of the sheet to the other (Farquhar and Palade 1963; Schneeberger and Lynch 1992; Gumbiner 1993; Anderson and van Itallie 1995; Morita et al 1999).

The main types of anchoring junctions in vertebrate tissues are adherens junctions, desmosomes and hemidesmosomes. Adherens junctions are

connecting sites for bundles of actin filaments, whereas desmosomes and hemidesmosomes are connecting sites for intermediate filaments (Stappenbeck 1992; Kouklis et al 1994; Kotch et al 1994). In transition from surface of basal layer to the upper suprabasal layer of epidermis, the differentiating epidermal cells strengthen themselves by adding to their extensive cytoskeleton of keratin filaments linked to tightly adhesive desmosomes (Fuchs and Cleveland 1998; Kowalczyk et al. 1999). As the terminally differentiated epidermal cells reach near the outer surface of the skin, they produce and secrete lipids, which organize into bilayers cornified envelope (for review, see Kalinin et al. 2002). In the 1950s and 1960s, experiments were done showing that solvent extraction of epidermis dramatically increased water permeability, implicating lipids in cutaneous barrier function (Onken and Moyer, 1963; Matoltsy et al, 1968; Barnes et al 1968; Scheuplein and Ross 1974). Then, other studies showed that once the organization of this lipid bilayer is completed, the epidermal cells have performed their barrier function and have no further need for metabolic activity (Wertz and Downing 1983; Lange et al 1985; Madison et al, 1990; Anderson 2001). Now the important question is whether the intercellular junctions play any role in providing this barrier together with the lipid envelope.

In the past several decades the roles of cell junctions and tight junctions in particular, in the barrier function of mammalian stratified epithelia, especially of the epidermis, have been mostly ignored (Squier 1973, Elias 1977). In 1990s, although tight junctions have been occasionally observed in epidermis, and tight junction proteins were expressed in epidermis (Morita et al, 1998 and 1999), their

importance was not appreciated until the recent development of a claudin-1 knockout mouse (Furuse et al, 2002). They addressed whether TJs along with the bilayers lipid structure in the wild type epidermis can create a primary barrier to the diffusion of materials through the paracellular pathway and whether the barrier function of TJs is affected in mice with targeted mutations genes such as claudin1, involved in TJ structure. Interestingly this group obtained evidence for the direct involvement of TJs in the function of the mammalian epidermal barrier as they showed that genetic removal of claudin-1 in mice induces neonatal death, which is associated with rapid appearance of wrinkles in the skin and dysfunction of the epidermal permeability barrier. Later on, other studies supported this notion and showed that tight junctions play a critical part in maintaining the epithelial permeability barrier by: (1) sealing the plasma membranes of adjacent cells together to create a continuous, impermeable, or semipermeable barrier to diffusion across the cell sheet and (2) acting as barriers in the lipid bilayer to restrict the diffusion of membrane transport proteins between the apical and the basolateral domains of the plasma membrane in each epithelial cell (Turksen and Troy 2002; Tsukita and Furuse 2002). In contrast, although it is now believed that during embryonic development, the epidermal keratinocytes give rise to hair follicles and later on, direct continuity to keratinocytes of the ORS (Moll R et al. 1982; Heid et al 1988; Moll I et al. 1988; Holbrook and Minami 1991; Schirren et al. 1997), little is known about the morphological and biological importance of TJs in hair follicles. However, earlier studies on hair follicles have shown them to be

part of the barrier system of mammalian skin (for reviews see: Lauer et al. 1995; Schafer and Lademann 2001).

Tight junctions

Tight junction (TJ) strands are composed of at least three types of membrane-spanning proteins: (A) occludin (Furuse et al 1993); (B) members of the claudin family (Morita et al 1999; Tsukita et al 2000; Tsukita and Furuse 2002); and (C) members of the junction adhesion molecule (JAM) family (Martin-Padura et al 1998). Interactions of these membrane proteins with the actin-based cytoskeleton and with membrane-associated proteins are important for the structural organization of the junctional complex at the plasma membrane and for signal transduction (Turner 2000, Roh 2002). Although the molecular structure of this interaction is still not known, the extracellular domains of rows of occludin and claudin proteins in the plasma membrane of one cell probably form extremely tight links with similar rows of claudin and occludin in the adjacent cell, essentially fusing two adjacent cells and creating an impenetrable seal. In other words, the long C-terminal cytosolic domain of occludin and claudin is bound to that of a group of large cytosolic proteins (ZO-1, ZO-2, and ZO-3) that, in turn, are bound to other cytoskeletal proteins and to actin fibers (Stevenson et al 1986; Jesaitis and Goodenough 1994; Staddon et al 1995; Itoh et al 1997; Morita et al 1999; Itoh et al 1999). These interactions appear to stabilize the linkage between occludin molecules that is essential for integrity of the tight junction.

(A) Occludin was the first and best-known integral membrane protein of TJs (Furuse et al 1994; Hirase et al 1997; Huber et al 2001). This ~65-kDa

membrane protein was identified and characterized biochemically, localized at the ultrastructural level in the TJs of intestinal epithelial and liver cells, and designated as `occludin' (from the Latin word occludere) by Furuse et al. in 1993. Further studies revealed that the amino acid sequences of three mammalian (human, murine, and canine) occludins are very closely (~90% identity) related to each other (Ando-Akatsuku et al 1996), reflecting its developmental importance.

(B) The claudins are a family of more than 23 highly conserved TJ proteins which share the same membrane structure and have various tissue distribution patterns (Morita et al 1999). Many tissues express several different claudins which can interact with each other (Morita et al 1999, Furuse et al 1999). Selectivity and specificity of paired TJ strands are determined by the type of claudins present and their mixing ratios (Tsukita 2000, Furuse 2001, and Van Itallie 2001). Yet, for some claudins it was proposed to take additional roles in cell–cell adhesion and embryonic development (Brizuela 2001; Gregory 2001, Nishiyama 2002).

Claudin 6 is a novel tight junction molecule with four transmembrane domains that is critical for the development of epidermis in mice (Turksen and Troy, 2002). Using mass immunoblot analysis of 406 markers in early hair follicle progenitor cells, it was demonstrated that Claudin 6 is involved in hair follicle differentiation during development. Therefore these data suggest that the newly identified member of the Claudin family of tight junctional molecules likely constitutes part of the cross-talk between tight junctions and some signaling pathways to function in the regulation of the initiation and cycling of hair follicles.

To investigate the role of Claudin 6 and its function in the development of the hair follicle, we established transgenic mice lines that express Claudin 6 under the control of the involucrin promoter (Inv) (Carrol et al., 1993). The involucrin was chosen since, in the skin, involucrin is restricted to post-mitotic epithelial cells (the upper spinous and granular layers) and serves as a marker of the epidermal and follicular terminal differentiation. Thus the Inv-Claudin 6 transgene extends the expression of Claudin 6 to the suprabasal layers of the epidermis, the hair follicles, the urothelium and all other involucrin-expressing stratified epithelia (Turksen and Troy 2002). While homozygous Inv-Claudin 6 transgenic mice die within 2-3 day of birth due to severe dehydration as a result of poor epidermal permeability barrier formation (Turksen and Troy, 2002), the heterozygous mice have problems in epidermis differentiation as well as hair follicle differentiation and cycle disruption, but would survive and reach adulthood.

The phenotype observed in our heterozygous transgenic mouse models provided us the opportunity to address the following questions concerning hair follicle morphogenesis and cycling: What are the differences in histological analysis of skin samples in comparison to the wild type? What are the overall proportions of hair fibre composition in these animals versus the wild type? What happens during the differentiation of hair follicles that causes their formation to be disorganised? What are the defects in the hair cycle? Which of the differentiating steps is missing/modified causing the phenotypes observed in our transgenic animals?

Having the hypothesis that Claudin 6 modulates the balance between growth and differentiation in self renewing epithelia and contributes to hair follicle regulation, the main goal of my thesis was directed towards providing a detailed description of Claudin 6 function and its role in the development of the hair follicle using a targeted overexpression strategy in transgenic mice.

MATERIALS AND METHODS

Transgenic Mice

The transgenic construct utilizes mouse Claudin-6 cDNA (Turksen and Troy 2001) containing the complete coding sequence (nucleotides 1-660). The Claudin-6 cDNA was ligated into the Not I site of the expression vector H3700-pL2 (Carroll et al., 1993), which carries the human involucrin promoter and other regulatory elements. The INV–Claudin-6 transgene was excised from the parent plasmid by digestion with Sal-I, and was isolated and purified using the Qiagen Gel Extraction Kit (Qiagen, Mississauga, Canada) The construct was injected into fertilized oocytes of FVB mice at the Loeb Transgenic Facility of the Ottawa Heath Research Institute. Transgenic animals were identified by PCR analysis of genomic DNA isolated from tail samples.

Tissue harvesting

A) Paraffin Sectioning:

Skin samples were harvested from backskin of Inv-Claudin 6 heterozygous as well as wild type. They were collected at various time points to get representative samples of cycling hair: 4, 8, 12, 14, 16, 18, 22 and 23 days after birth. Samples were fixed in Bouin's fixative (75% saturated picric acid, 20% formaldehyde, 5% glacial acetic acid) or paraformaldehyde (4% Paraformaldehyde-PBS (8 g NaCl, 0.2 g KCl, 1.15g Na_2HPO_4, 0.2 g KH_2PO_4, in 1 liter H_20) (100 ml), 4 g paraformaldehyde, 60 μl 0.1N NaOH) overnight and dehydrated by placing two times in 30%, 50% and 70% ethanol, 30 minutes each for paraffin sectioning.

After formalin/bouine fixation and prior to paraffin embedding, tissues were dehydrated and infiltrated with paraffin. This was achieved on an automated tissue processor. Cassettes were loaded into a plastic basket which was mounted to a rotating disk on the processor. The disk submerged the cassettes successively for 1 hour each in graded ethanol (usually 70%, 95%, 100% 2X each), then xylene (3X). Finally, tissues were infiltrated with molten paraffin and were eventually embedded in paraffin and sectioned on a programmed microtome (Thermo Shandon) for 4 μm cut.

B) Frozen Sectioning:

In those cases that paraffin sections were not suitable for certain staining, frozen sectioning was performed as following:

Tissues were oriented in cryomatrix medium (Thermo Shandon) and were dived into the dry-ice chilled iso-penthane for 6 sec. The frozen blocks were kept in –80 °C for long term and -20 for short term use. Then they were ready to be sectioned on a programmed cryostat microtome (Thermo Shandon) for 4 μm cut.

Hair Fibre Analysis

Hair fibres were plucked from the coats of Inv-Claudin 6 as well as wild type mice using forceps at different ages in a hair cycle (8, 12, 14, 16, 18, 20, 24 days after birth) and were mounted on glass slides for visualisation. The four main types of hair fibres (zigzag, awl, auchene and monotrich) were easily discernible using stereomicroscopy (Leica) an 8x objective. To perform statistical analysis, using

the same dissecting microscope, we computed the composition of the hair fibres by five times count for each fibre, repeated three times, and the differences were judged as significant if the P value was ≤ 0.05

Statistical analysis

Counted hair fibre samples were tabulated using Microsoft Excel 2002. Means of all counts and SEM (standard error of the mean) and t test were calculated using Excel 2002 software. Differences were judged as significant if the P value was ≤ 0.05 as determined by the Mann-Whitney U/Wilcoxon rank tests.

The Wilcoxon signed rank test is an alternative to the paired t test. Each paired sample is dependent, and the data are continuous. The assumptions needed to use the Wilcoxon signed rank test are less stringent than the assumptions needed for the paired t test. It requires only that the paired population be distributed symmetrically about its median.

Skin permeability assays, X-gal staining

To assess the epidermal permeability barrier, the skin permeability assay was performed (Hardman et al., 1998). Unfixed and freshly isolated mice at 5 serial time points of 0, 2, 4, 8, 12 days after birth of both Inv-Claudin 6 and wild type were rinsed in PBS then immersed in 5-bromo-4-chloro-3-indolyl-ßD-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. The mice were rinsed in many changes of 1X PBS.

Residual fixative could inhibit the enzyme. After staining, mice were photographed using a 35 mm Nikon digital camera and images were processed with Adobe Photoshop 7.

Histological Analysis

Paraffin sectioned samples were stained with Hematoxylin and Eosin (H&E) for morphological and histological analyses and were also processed for Picrosirius red staining to stain collagen as following:

Samples were deparaffined by baking at 50°C for 15 minutes and incubating 3 times in toluene, 5 minutes each. They rehydrated by placing three times in 100% ethanol, 5 minutes each, then in 95%, 70% ethanol, 5 minutes each and water 2 times, 5 minutes each.

A) Sirius red staining:

After rehydration slides were stained in saturated picric acid (Sigma) with 0.1% Sirius red F3BA (Aldrich Chemicals) for an hour room temperature, washed in 0.01 N hydrochloric acid for 2 minutes, and rapidly dehydrated through graded alcohols starting at 70%, then to toluene, and finally cover slipped (22x50 mm Fischer scientific) in Permount (Fischer scientific).

B) H&E:

After rehydration slides were stained with Hematoxylin (Harris modified, Fischer scientific) for two minutes. Harris modified Hemotoxylin working solution was 800 ml of concentrate and 100 ml of dH_2O. Then sections were washed in water for 10 minutes and Eosin (Fischer scientific) (200 ml stock Eosin (10 g Eosin, 100 ml of dH_2O, 800 ml of 95% alcohol) , 600 ml 80% alcohol and 4 ml glacial acetic acid) for three minutes before dehydration as explained for Sirius red staining.

Immunofluorescence analysis

Paraffin sections were rehydrated as were described for histological evaluation and processed for immunohistochemical analysis as followings:

Slides were plated in a plastic beaker filled with 1X Citrate buffer (Sodium Citrate dihydrate 2.41 g, Citric acid monohydrate 0.378 g and ddH_2O 900 ml) and heat in a microwave oven 2 times, 5 minutes each. Citrate buffer was removed off the slides using distilled water, and slides were allowed to slowly cool down in hot water for 5 minutes. Slides were rinsed in PBS 3 times, 5 minutes each. Then the slides were blocked with blocking solution (5% Heat inactivated goat serum in washing buffer (0.8% BSA, 0.1% Gelatin in 1XPBS). They were incubated for 30 minutes at room temperature in a humidified chamber. Samples were then washed in washing buffer for 5 minutes. Then the slides were incubated in a humidified chamber for 1 hour with primary antibody solution (primary antibody in incubation buffer (1% Heat inactivated goat serum in washing buffer). They were washed three times with washing buffer, 10 minutes each.

The following first antibodies were performed (diluted in incubation buffer):

K5 (1:100), K14 (1:100), K1 (1:100), K6 (1:100) (Babco), K17 (1:500) ; (a gift from Dr Pierre Coulombe), Filaggrin (1:100), Loricrin (1:100), Transglutaminase 3 (1:100; a gift from Dr Len Milstone), Involucrin (1:100), K15 (1:100), H3 (1:100), AE13 (1:2) and AE15 (1:2) (a gift from Dr. Tung-Tien Sun) . In case of hair-keratin staining frozen sections were performed.

After the blocking and the first antibody incubation, the slides were incubated in a humidified chamber with secondary antibody solution (secondary antibody in incubation buffer) according to manufacturer's protocols (Secondary antibodies Anti-Rabbit and Mouse IgG (F(ab2)), FITC Labeled (Jackson Immuno Research Laboratories) were used at a 1:50 dilution). They were washed 3 times with washing buffer 15 minutes each time followed by 3 times, 5 minutes each rinsed in PBS.

After the last stage and prior to microscopy observation, about 100 μl of Mowiol (2.4 g Mowiol, 6 g Glycerol, 6 ml dH_2O, 12 ml 0.2M Tris pH 8.5) was applied on the section and slides were mounted using coverslip (22x50 mm Fischer scientific).

Alkaline Phosphatase (AP) histochemistry

To perform AP staining the following procedure was obtained on frozen sections: Sections were fixed 15 minutes in ice cold formalin (1:10 formaldehyde in 1X PBS) at room temperature and rinsed with distilled water. They were incubated in distilled water for 15 minutes, followed by a 45 minutes incubation in substrate

solution (0.0025 g Naphtol AS MX-PO_4 , 100 µl N,N-Dimethylformamide, 12.5 ml 0.2M Tris-HCl (pH 8.3), 12.5 ml distilled H_2O, 0.015 g Red violet LB salt, in 25 ml). Slides were rinsed with H_2O and were ready for observation.

Photography

In all histological, immunohistochemical and AP analysis, images were captured with a Zeiss fluorescence/bright field microscope (Carl Zeiss) equipped with a Zeiss digital AxioCam camera using Axio Vision 2.05 software. Images were then processed with Adobe Photoshop version 7.

Results

Generation and expression analysis of Inv-Cldn6 transgenic mice

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). 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. 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. 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. 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. 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, with transgenic mice

exhibiting appreciably higher levels in relative terms in agreement with the PCR results.

Analysis of morphology and barrier acquisition in Inv-Claudin 6 mice

The defective epidermal permeability barrier (EPB) usually manifests itself in the loss of body heat, weight and dehydration, events that remain a leading cause of neonatal death as a result of its associated complications (Harpin and Rutter, 1983; Kalia et al., 1998; Turksen and Troy 2002). Together, all these symptoms were counted for the weakness in EPB. Homozygous Inv-Claudin 6 mice were unable to prevent the penetration of dyes through their skin and suffered an early postnatal death as a result of rapid water loss across the skin surface (Turksen and Troy 2002); however the heterozygous counterparts are healthy and fertile and beside their distinct appearance, they reach to adulthood. To assess if heterozygous counterparts are also unable to regulate the processes necessary to establish the epidermal permeability barrier, we analyzed several states of postnatal growth of both heterozygous Inv-Claudin 6 and wild type pups with a whole-mount skin permeability assay using X-gal staining. This assay measures the permeability of the epidermis to a dye solution which produces a blue colored reaction in the skin. At low pH, skin contains abundant endogenous ß-galactosidase activity, which cleaves X-gal to produce a colored precipitate. 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). However the heterozygous Inv-Claudin 6 mice acquire the epidermal barrier through the

same pattern after birth. At P0, while the wild type mice have already established a barrier and didn't show a blue appearance due to X-gal staining, the Inv-Claudin 6 animals are completely blue, introducing an obvious defect in their EPB formation. At P2, P4 and P6 the transgenics still present a blue skin appearance, however at P8 they are at the beginning of barrier acquisition process, presenting a white dorsal skin, imitating dorsal-ventral pattern of EBP completion (figure 1) (Hardman et al 1998). By P12 the Inv-Claudin 6 littermates have established the barrier and they appear similar to wild type.

Heterozygous INV–Claudin 6 Transgenic Mice Exhibit Abnormalities of the Skin

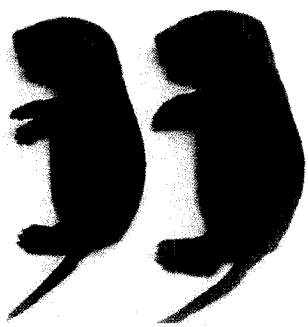
The appearance and texture at gross level of morphology in heterozygous Inv-Claudin 6 animals does not look like the homozygous counterparts that was previously reported (Turksen and Troy 2002), indicating that these animals may not have drastically disturbed epidermis. However, unlike their homozygous counterparts, the heterozygous pups did not possess a severely aberrant skin.

Similar to what we see in homozygous transgenics, the heterozygous animals were readily identifiable solely on the basis of their curly whiskers (Figure 2a). They have a smaller body size, and only with the emergence of coat hairs did the heterozygous pups acquire their most evidently distinguishable characteristic: **wavy** hair.

Overall appearance of the coat in Inv-Claudin 6 heterozygous is delayed compared to wild type (about two days) but while this was most apparent once the coat was well formed (day 12 onward), it was possible to identify Inv-Claudin

Figure 1: Inv-Cldn6 transgenic mice acquire a delayed EPB. ß-gal assays were performed to assess the integrity of the EPB of transgenic animals as compared to the wild type. At P2 the wild-type presents a white appearance a characteristic for a normal EPB while the transgenic pops possessed totally blue skin and a defective EPB (a). At P8 there was no penetration of X-gal through the EPB of dorsal part of Inv-Claudin 6 animals while the rest of the bosy skin remained blue (b).

Inv-Claudin 6



Wild type

2 day-old

Inv-Claudin 6

8 day-old

Figure 1

6 mice from day 9 or 10 on the basis of the waviness of the initial pelage hair. Both the wavy coat and curled whisker phenotype persisted throughout the life of the animals (figure 2c). In addition, heterozygous Inv-Claudin 6 mice open their eyes later than their wild type littermates. The eyes of wild type mice opened at 15 days of age while those of transgenic mice opened anywhere from 18 to 22 days of age (figure 2b).

In general these animals did not express any other gross abnormalities either in the epidermis or hair coat beside what we described.

To determine if the phenotypic severity correlated with levels and pattern of expression, we characterized the histology of the Inv-Claudin 6 heterozygous as well as wild type at different time points. At P2, histological analyses did not yield obvious differences in the thickness of wild type and transgenic skin; however our results showed that the thickening of the subcutaneous fat layer began at this age in wild type (Figure 3a and b). To analyze whether the growth in skin thickness that happens through the normal processing of skin occurs in our transgenes, we checked the morphological changes in other time points. Not surprisingly, at P4, the wild type subcutaneous fat layer had grown and was much thicker than the transgenic, but there were no detectable differences in the thickness of the epidermis (figure 3c and d). However dysmaturation (lack of progressive flattening in the suprabasal layers), prolonged the retained of nuclei in the upper strata and disorganization in natural cuboidal shape of basal cells in transgenes, which reflects the improper packing of these cells, were observed in some areas (figure 3c2 vs. c3). The significantly thinner subcutaneous fat layer in

Figure 2: Heterozygous transgenic mice display curly whiskers at birth **(a)**. Wild type and Inv-Claudin-6 littermates are shown at 15 days **(b)**. Delayed hair growth and eye opening, a runted appearance and the frequent smaller size are characteristic of transgenic mice at this age. Adult transgene mice show a pronounced fuzzy hair coat and curly whiskers **(c)**.

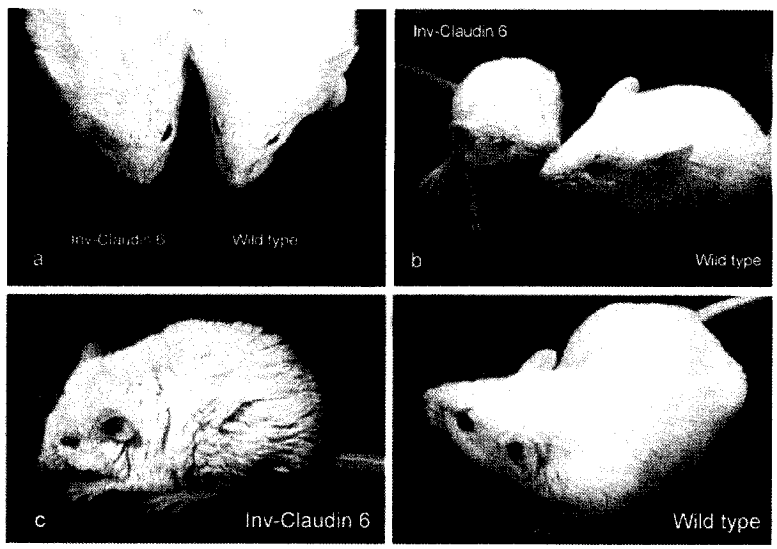


Figure 2

Figure 3: Histological sections of 2 and 4 day old wild type and Inv-Claudin 6 mice backskin. Histological analyses did not show obvious differences in the thickness of wild type and transgenic skin at P2 **(a, b)**. At P4 there were no detectable differences in the thickness of the epidermis **(c1 vs. c2)**. Retained nuclei in the upper strata and disorganization in natural cuboidal shape of basal cells in transgenics were observed in some areas **(c3)**.Some structural defects of the Inv-Claudin-6 hair follicle include a crooked shape (arrowheads d). In addition there is also a transgene-associated skin subcutaneous fat reduction at day 4 **(d)**.

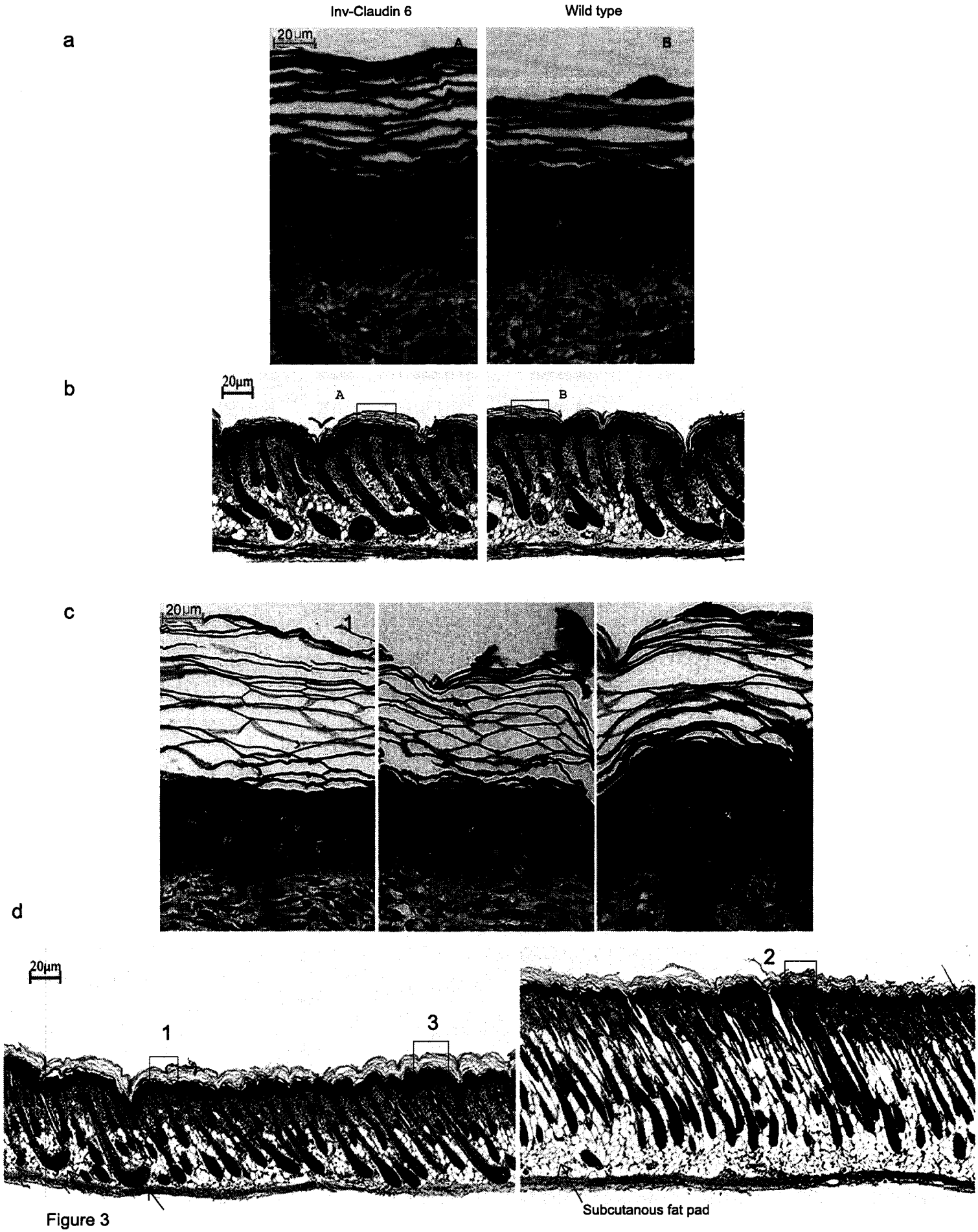

Inv-Claudin 6
Wild type
a
20µm
A
B
b
20µm
A
B
c
20µm
1
d
20µm
1
3
2
Subcutanous fat pad

Figure 3

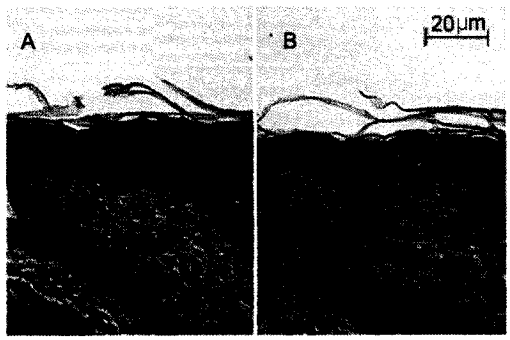
Inv-Claudin 6 mice reflects the EPB problems that caused postnatal dehydration. As the EPB of transgenic animals shows a delayed completion compared to the wild type, their subcutaneous fat layer gradually became thicker and was of an thickness equal to wild type at P12 (figure 4a). However, also at P12, the heterozygous transgenic animals began to exhibit a slightly thicker epidermis, but otherwise had no major histological abnormalities (figure 4b). Gradually, the transgenic epidermis thinned and after 4 days, at P16, it was comparable with wild type (figure 4c). This indicates that even though there appears to be a delay in the developmental timeline in which the transgenic skin lags the wild type, but they will eventually be comparable with the wild type morphology.

In addition to differences in the epidermis and some of the hair follicles, the inertial matrix in the dermis of the transgenic mice appeared to be less dense (figure 5a). This was substantiated by Sirius Red staining which is known to stain the inertial collagen matrix. Overall this staining supported our observation on H&E stained sections that indeed there were differences in dermal matrix. The defect in collagen density in the dermis, confirms the developmental problems that transgenic animals suffer. In addition we see some very strong/increased staining in the stratum corneum, which can reflect an increase in overall negative charge due to an upsurge in sulphonic acid group, the basic groups that the picrosirius stain reacts with, however the detailed model for such an increase remains to be identified (figure 5b).

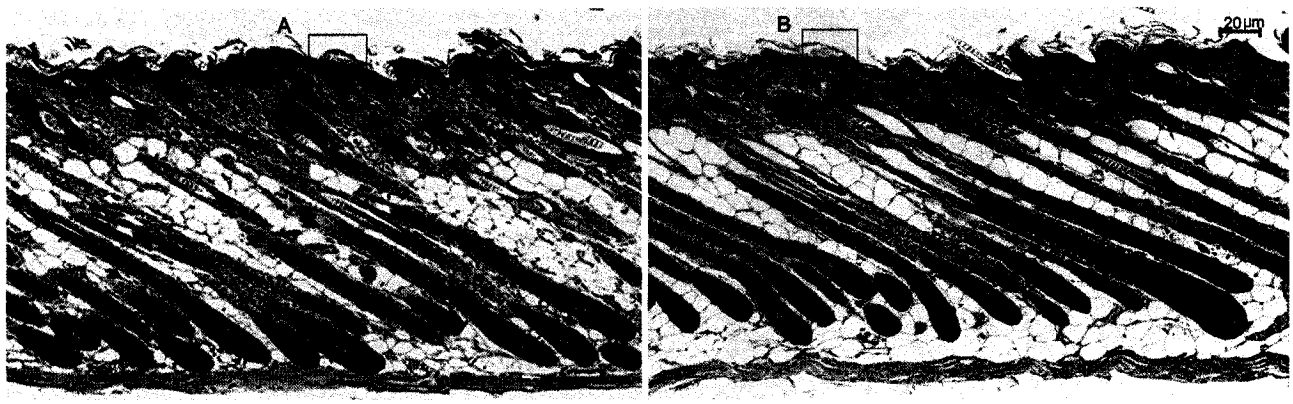
In addition to the indicated differences in epidermis, disorganization in some of the hair follicle architecture including a crooked shape was also evident

Figure 4: Inv-Claudin 6 backskins subcutaneous fat layer was of an equal thickness to wild type at P12 **(b)**. However the transgenic animals exhibit a slightly thicker epidermis, but no major histological abnormalities **(a)**. Gradually, the transgenic epidermis thinned and after 4 days, at P16, it was comparable with wild type **(c)**.

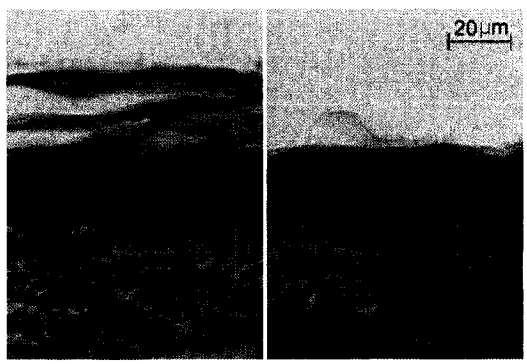
a



b



c



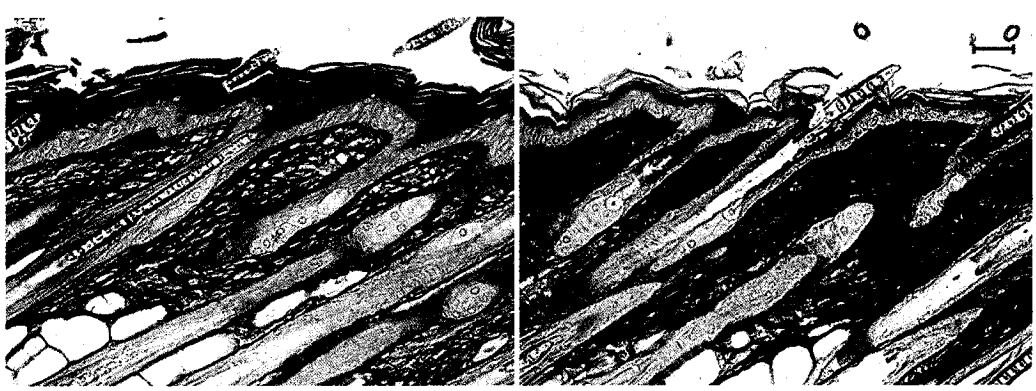
Inv-Claudin 6 Wild type

Figure 4

Figure 5: The density of the inertial matrix in the dermis (collagen) of the transgenic mice appeared to be defective **(a)**. The strong/increased staining in the stratum corneum may reflect an increase in overall negative charge due to an upsurge in sulphonic acid group, the basic groups that the picrosirius stain reacts **(b)**. Bar 20 μm

a

b



Inv-Claudin 6 Wild type

Figure 5

(figure 3b arrowheads). As were abnormalities previously associated with the emergence of waved hair (Luetteke et al., 1993, 1994; Mann et al., 1993) but overall the orientation of the hair follicles was not drastically different in wild type comparing to transgenic.

Transgenic Mice Display Abnormal Proportion of Hair Fibre Composition, Defective hair shaft differentiation and Abnormal Hair Cycle.

The fully developed coat of heterozygous Inv-Claudin 6 pups had a matte texture and the distinct appearance of sheep, alterations that were observed in both male and female transgenic mice. The lack of a sleek look normally associated with wild type suggests probable differences in the composition of hair fibers (Sundberg et al 1999).

By the time the full coat is formed, four distinct morphological recognizable fibers can be characterized on the body of mice: monotrich, awl, auchene, and zigzag hairs (Sundberg and King 2001). In wild type mice the guard hairs (monotrich, awl and auchene) make up 56% of the fibres and zigzag fibres only 44% providing for the smooth coat normally observed (Sundberg et al., 2001). In many studies, the differences in overall portional of hair fibre distribution in several transgenic mice with coat abnormalities show differences in this proportion (Dong et al. 2002; Vielkind et al., 1996).

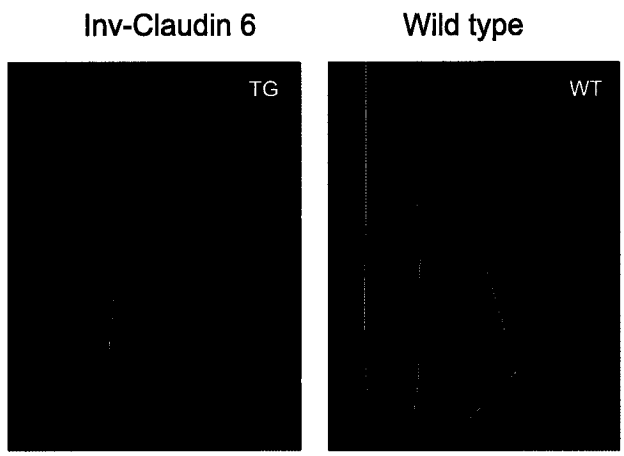
To identify if there is any differences in hair fibre composition, analysis was performed to find out the overall proportion of its composition in the wavy transgenic phenotype as compared to the straight hairs of the wild type mice. To

do so, we randomly plucked fibers from backskin of both wild type and transgenic using forceps and computed the composition carefully using the dissecting microscope (five times count for each fibre, three times repetition for each experiment and the differences were judged as significant if the P value was ≤ 0.05). Removal of the hairs in the transgenic was as easy as wild type and club hairs appeared to be morphologically similar. Interestingly, although all major hair types, auchene, awl, zigzag and monotrich, were present in the transgenic animals, the proportion of the hair types was distinctly different in transgenic mice (figure 6a). Wavy, zigzag fibres accounted for about 55 percent of hairs in the transgenic coat compared to 40 percent in wild type and resulted in a significant decrease in proportions of the other three hair types (figure 6b). At the microscopic level, many of the hair fibres were misshapen disorganized and their medullae contained disorganized septates (figure 6c). Collectively these data suggest structural defects in hair fibres, however fibre fragility or loss of club hair was not observed in these animals.

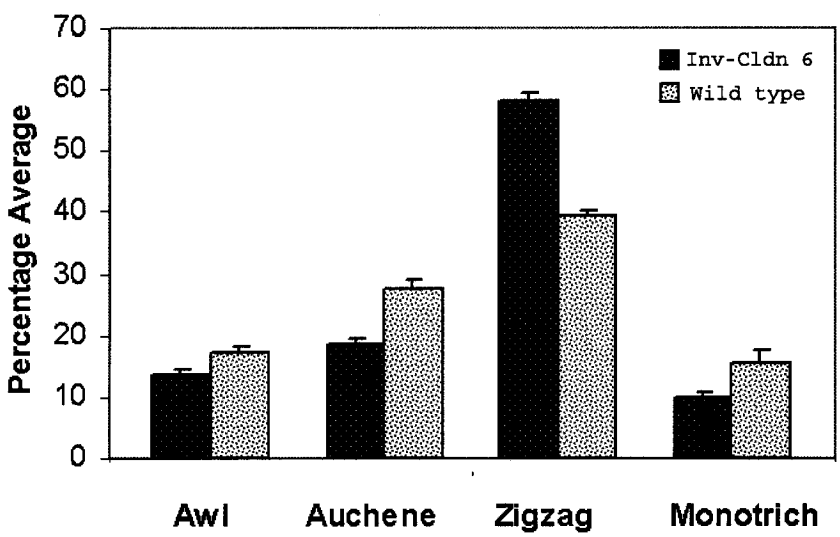
Beside the reduced number of guard hair and defects in hair fibre structure, heterozygous Inv-Claudin 6 mice also show shorter monotrich and zigzag hair fibers, the abnormality that usually associates with hair cycle defects (figure 6d) (Moore et al 1983; Mak et al 2003; Ma et al 2003). During anagen differentiating into the hair fibre that emerges from the hair shaft causes the elongation of the fibre, thus any defects that influences this period duration would explain the abnormalities in hair length. To obtain evidence for the existence of such a relationship, we investigated the cycling of hair follicles by examining skin

Figure 6: The four major hair types (from left to right: monotrich, awl, auchene and zigzag) are evident for both the transgenic and wild type mice **(a)**. However, there are differences in the overall portion of hair fibre distribution in the transgenic samples. There is evident an obvious increase in the number of zigzag fibres in Inv-Claudin-6 mice in addition to a decrease proportion of all the other hair types (TG: Zigzag: ~55%, Awl: ~12%, Auchine: ~23%, Monoritch: ~10% and WT: Zigzag: ~38, Awl: ~17, Auchene: ~29%, Monoritch: ~16%) **(b)**. Changes of hair fibre differentiation in the Inv-Claudin-6 mice show an affected medulla patterning by the irregular septation in the hair **(c)**. Heterozygous Inv-Claudin 6 mice show shorter hair fibers, the abnormality that usually associates with hair cycle defects **(d)**.

a



b



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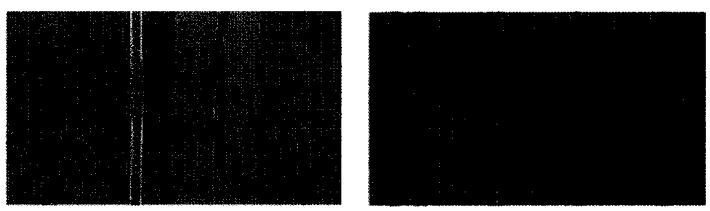


d

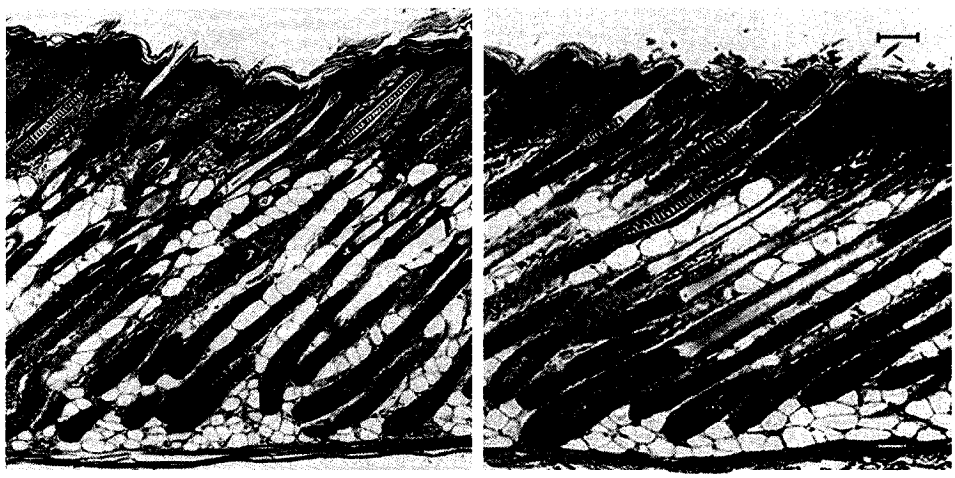
Figure 6

histology. Considering that the first postnatal hair cycle follows is synchronized (Paus et al 1998), the characteristic that provides us with the opportunity to evaluate each hair cycle phases more precisely and to compare wild type and transgenic more accurately, we examined backskin histology of heterozygous Inv-Claudin 6 as well as wild type counterparts at 6 serial time between P4 and P24. First postnatal anagen in wild type mouse starts at around P2. It terminates at around P18 when catagen starts and enter telogen at around P20 while initiates second anagen at around P23 (Paus et al 1998; Muller-Rover et al 2001). During the anagen phase, the bulb of the hair follicle and dermal papillae (DP) still reside in the deep subcutis. As anagen progresses, the bulb is enlarged and the DP is narrowed, and eventually the diameter of the DP becomes smaller than one-third of the bulb diameter (Paus et al 1998). This is the characteristic that serves to distinguish different stages of anagen from the previous and consecutive stages. At P14, wild-type hair follicles were in anagen whereas Inv-Claudin 6 hair follicles were in anagen-catagen transition, characterized by the thinner hair follicles (figure 7a). At P16 the transgenic hair follicles were already in catagen, while the wild type counter parts still show an anagen morphology (figure 7b).

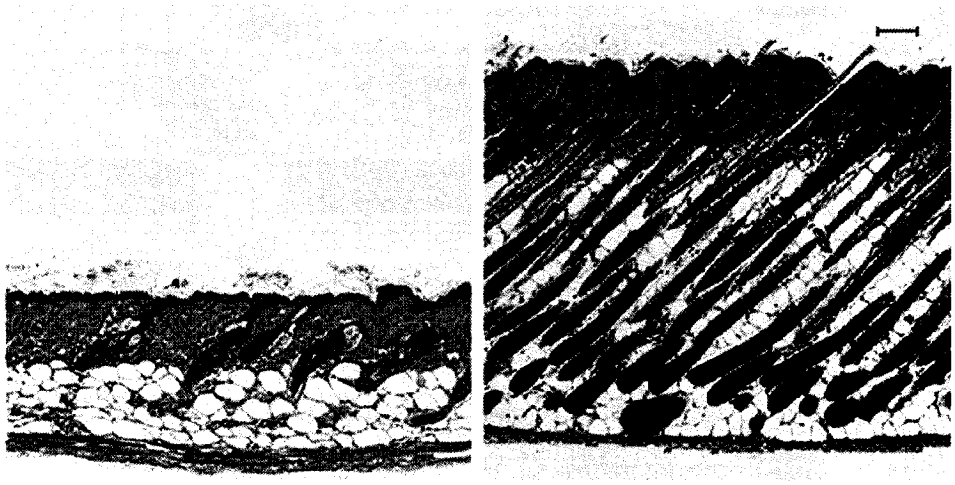
To provide further support for the shorter anagen hair cycle of transgenics, we next analyzed the morphology of DP in both Inv-Claudin 6 and wild type hair follicles and the reason was based on the earlier analysis (Paus et al 1998) that showed the more stretched and narrowed DP is, the more advanced anagen stages are. Alkaline phosphatase (AP) activity and the localization of AP active

Figure 7: Postnatal 14 days (P14) hair follicles show an anagen termination in Inv-Claudin-6 mice whereas wild type littermates are still in mid-anagen (Morphologically late anagen hair follicles look skinny) **(a)**. P16 hair follicles show late anagen in wild type whereas transgene hair follicles are in mid catagen **(b)**. Bar 20 μm

a



b



Inv-Claudin 6 Wild type

Figure 7

cells in hair follicles were well detailed by Handjiski et al in 1994. Using AP immunohistology, they showed DP as a region which displays strong AP activity during the entire hair cycle, according to the form and the position of DP. To obtain evidence whether distinctive hair cycle-dependant changes in morphology of DP in wild type is different with transgenic, we examined alkaline phosphatase staining on frozen sections at P4 (early anagen in both wild type and transgenic hair follicles), P8 and P12 (late anagen stage in Inv-Claudin 6 animals hair follicles). Compared to the relatively short anagen I-III (P0-P4) and IV-V (P5-P10), anagen VI is the longest phase of the mouse hair cycle and this encompasses massive morphologic changes in the follicle, including DP, which follow an easily distinguishable choreography of follicle transformation events (Paus et al 1998). Due to the quickness of anagen I to V transition, comparing different anagen stages in Inv-Claudin 6 hair follicles versus wild type at these time points was not possible and the DP showed an approximately similar shape, volume, and appearance in P4 and P8 in both wild type and Inv-Claudin 6 mice. In contrast and as mentioned before because of the long anagen VI, this comparison was easier at this stage and, not surprisingly, transgenic DP showed a more stretched morphology at P12, a characteristic of a more developed anagen based on DP morphology. These data support our previous finding on a shorter anagen in transgenic mice (figure 8).

Telogen hair follicles are easy to distinguish from catagen ones as they are fully surrounded by interfollicular dermal fibroblasts. Careful histological analysis showed that heterozygous Inv-Claudin 6 hair follicles shift to telogen at

Figure 8: DP showed an approximately similar shape, volume, and appearance in P4 **(a)** and P8 **(b)** in both wild type and Inv-Claudin 6 mice due to alkaline phosphatase staining. In contrast transgenic DP showed a more stretched morphology at P12 **(c)**, a characteristic of a more developed anagen based on DP morphology. Bar 20 μm

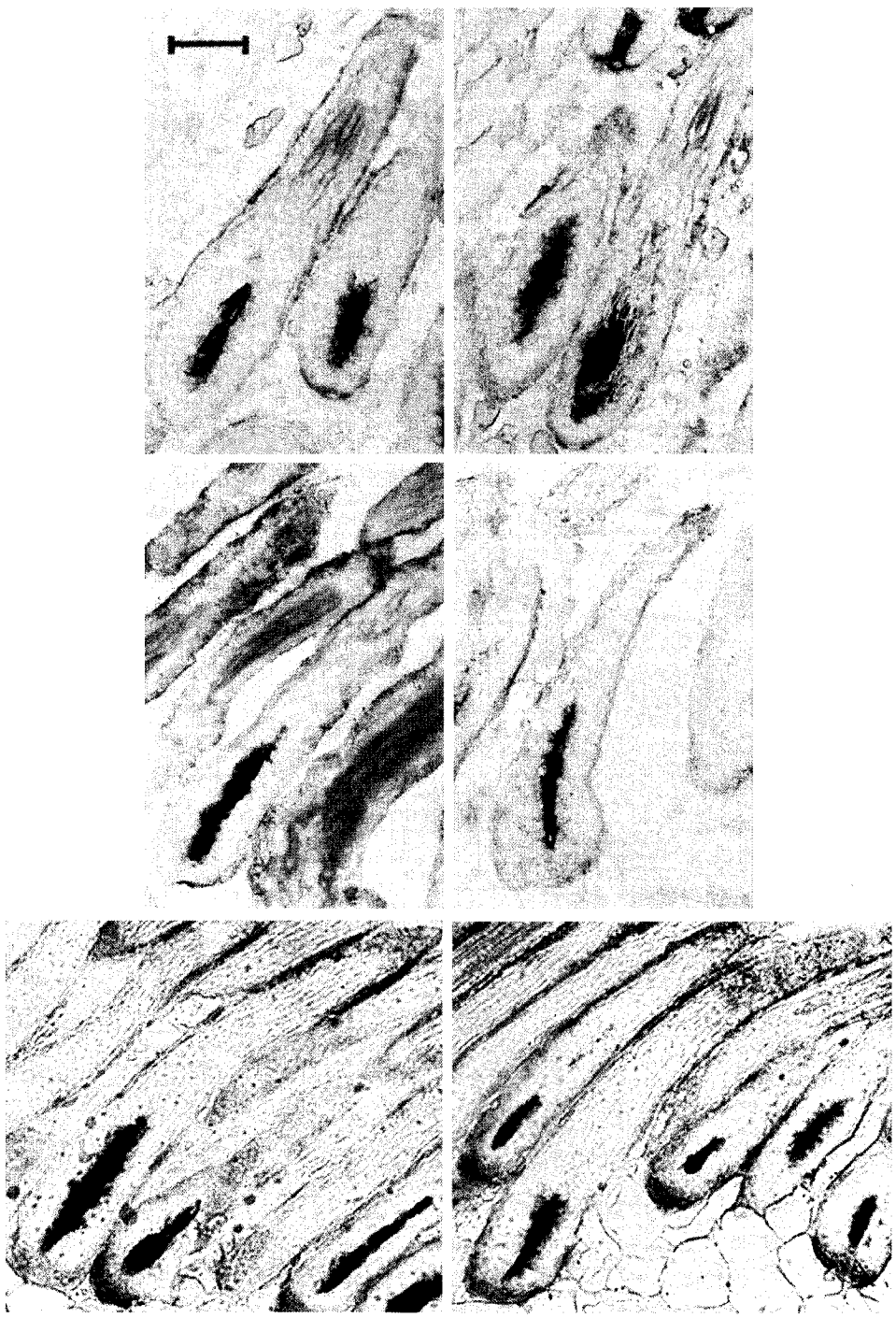


Figure 8

P19 (5 days residency in catagen), while wild type counterparts enter telogen at P20 (3 days residency in catagen) (figure 9a). At P23, wild-type hair follicles were making the transition between telogen and anagen, while hair follicles in Inv-Claudin 6 mice had already entered anagen at P22 (figure 9b).

Taken together, the shorter anagen and the prolonged catagen-telogen confirmed the structural problems in the INV-Claudin 6 mice, as observed through histological examinations.

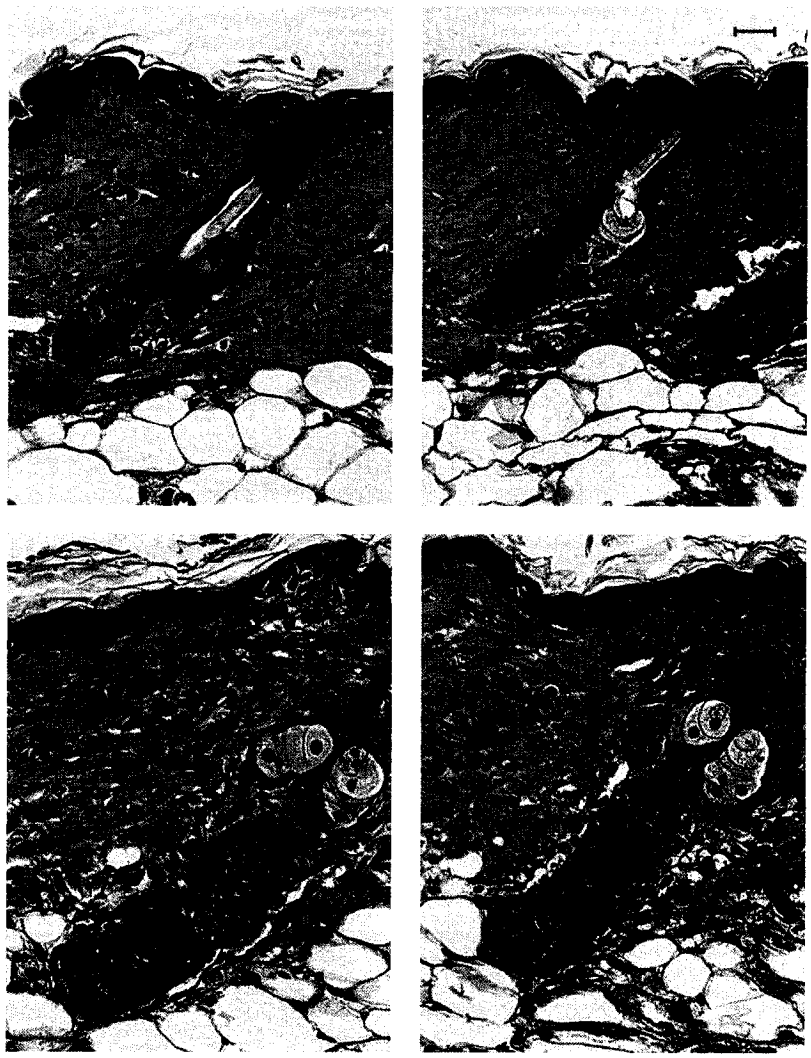
Claudin 6 Overexpression in Suprabasal Layer of Epidermis Affects Keratinocyte Differentiation and Proliferation

The tightly regulated expression of the keratin genes in complex epithelia, suggest the existence of a direct link between keratin gene expression and epithelial cell function (Moll et al 1982; Vassar et al 1989; Fuchs and Byrne 1994; Turksen and Troy 1998; Segre et al 1999). In the other words, beyond the huge number of keratin sequences and their fairly non-descript classification, there is an apparent logic in the regulation of keratin genes that "facilitates" the study of differentiation in epithelial cells and tissues. In all complex epithelia, for instance, progenitor cells transcribe a common set of keratin genes that consists of the type II K5 and the type I K14 genes (along with variable amounts of K15 or K19, two other type I keratins). Post-mitotic, suprabasal cells in these epithelia transcribe other pairs of keratin genes, the identity of which depends on the

Figure 9: In Inv-Claudin-6 mice, catagen and telogen progression is delayed. Inv-Claudin 6 hair follicles shift to telogen at P19 (5 days residency in catagen), while wild type counterparts enter telogen at P20 (3 days residency in catagen) **(a)**. At P22 transgenic mice enter anagen whereas wild type controls enter second postnatal anagen at P23 **(b)**. Bar 20 μm

a

b



Inv-Claudin 6 Wild type

Figure 9

program of terminal differentiation being executed. Thus the K1 and K10 pair is characteristic of the post-mitotic compartment of cornifying epithelia. In epidermis as an important member of the complex epithelia, epidermal specific keratins and markers of terminal differentiation are reliable tools of assessing the differentiation program of epidermis and hair follicle (Fuchs and Byrne, 1994, for review see Fuchs 1995, Turksen and Troy, 1998, 2002). Earlier studies showed usual association between altered morphological appearance and changes in pattern of differentiation (Mann et al 1993; Porter et al., 1996; Reichelt et al., 1997; Reichelt et al 2001; Turksen and Troy 2002; Turksen and Troy 2003). Based on what we observed in epidermis and hair follicle morphology as well as hair cycle, we were wondering whether these alterations in our transgenic animals are consistent with the preliminary data. To that end skin sections from P2, P4, P8 and P12 transgenic and wild type mice were stained with differentiation markers, and processed for immunofluorescence.

K5 and K14 show an age-dependant increase of expression in suprabasal layer of epidermis:

Using rabbit polyclonal keratin 14 and 5 antibody we found that like K14, K5 was expressed in the basal layer of epidermis. Comparing to the wild type, Inv-Claudin 6 animals showed an increased level of immunoreactive cells for both K14 and K5. The increase in suprabasal layer expression of this couple seemed to be age-dependant, as even though the K14/5-positive suprabasal

Figure 10: K5 and K14 show increase of expression in suprabasal layer of Inv-Claudin 6 mice epidermis. The increase in suprabasal layer expression of this couple seemed to be age dependant, as even though the K14/5-positive suprabasal cells were found at P2 **(a, b)**, at P12 the expression was detected all over the suprabasal compartment **(g, h).** In case of K14, the expression was throughout the suprabasal layers up to the uppermost granular layer of epidermis **(b, d, f and h)**. Bar 20 μm

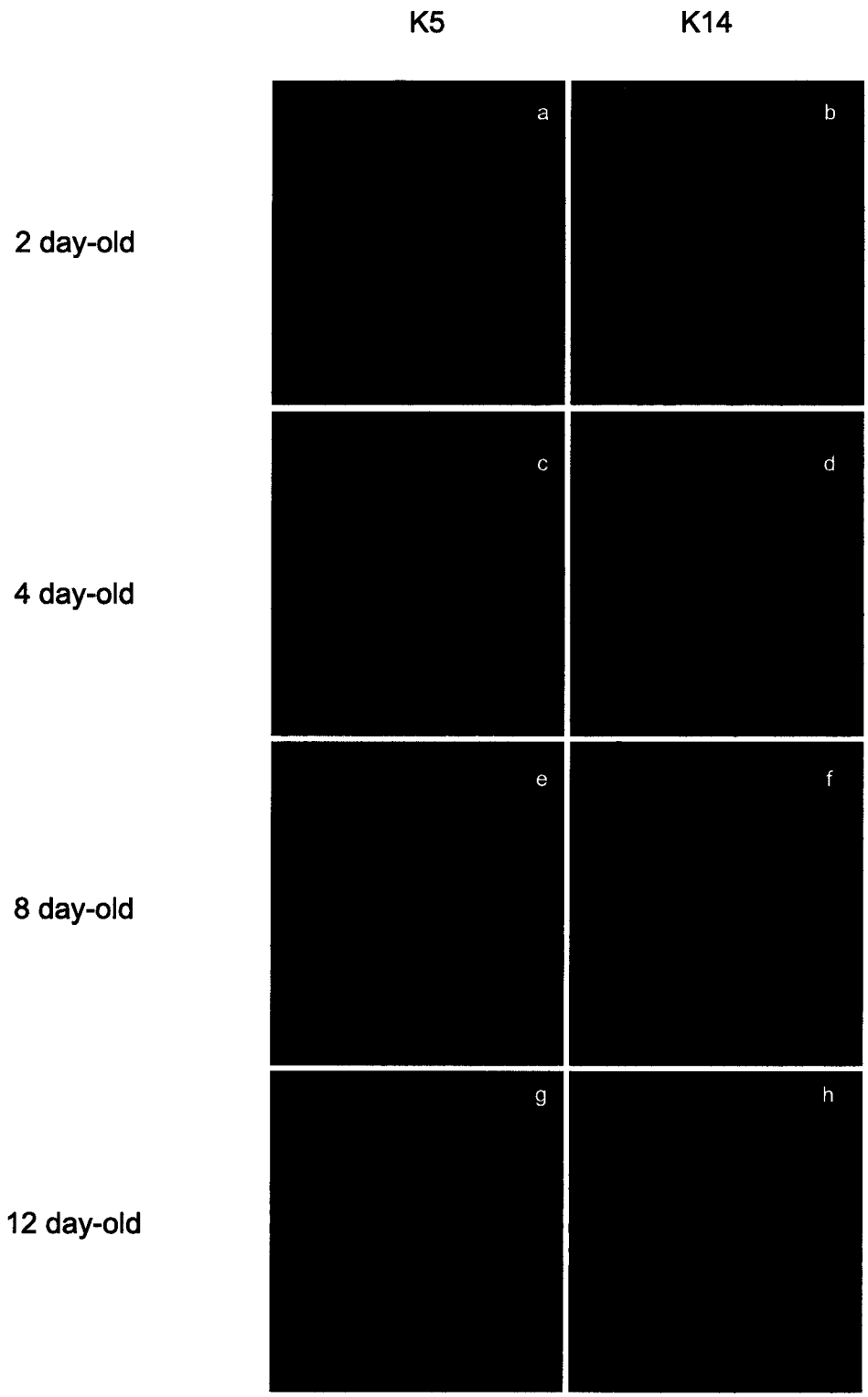

K5
K14
2 day-old
4 day-old
8 day-old
12 day-old
a
b
c
d
e
f
g
h
inv-Claudin 6

Figure 10

Figure 11: K14 and K5 are natural partners and known to be expressed in basal layers of epidermis in wild type. No suprabasal layer expression of either K14 or K5 was observed in wild type. Bar 20 μm

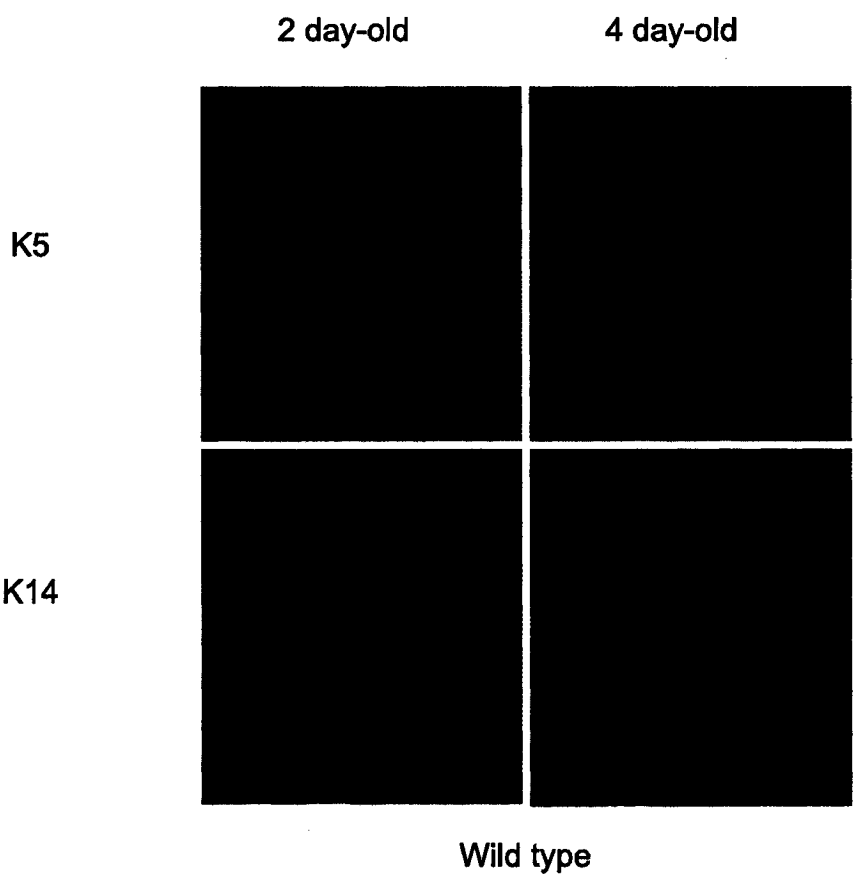


Figure 11

cells were found at P2, at P12 the number of immunoreactive suprabasal layer cell increased and expression was detected all over the suprabasal compartment. Especially in case of K14, the expression was throughout the suprabasal layers up to the uppermost granular layer of epidermis (figure 10, 11). This data suggest that although K14 and K5 are natural partners and known to be expressed in basal layers of epidermis under very tight control, their suprabasal layer expression seem to be uncoupled.

The expression of K15 and K17 in heterozygous Inv-Claudin 6 mice is perturbed:

K15 and K17 are early markers of the initiation of epithelial tissue formation and are known to be expressed after birth in epithelial appendages (Lloyd et al 1995; Whitebread and Powell 1998; McGowan and Coulombe 1998; Waseem et al 1999). McGowan and Coulombe in 1998 showed that in human skin, K17 occur in all types of appendages, but not in interfolliclar epidermis. Interestingly, Waseem et al in 1999 showed that an increase in K14 mRNA and protein expression in epidermis and hair follicles causes a downregulation of the K15 gene, a characteristic that implies the highly related regulation of different keratin genes even if they are impaired.

To elucidate if there is any alteration in expression of either K15 or K17, immunohistochemistry was performed on P2, P4, P8 and P12 backskin sections of both heterozygous Inv-Claudin 6 and wild type mice. The results were the same in all ages for both K15 and K17. In wild type, K15 associates with the basal layer primarily in agreement with Lloyd et al 1995; however in transgenic epidermis its expression decreased to a patchy expression. These

Figure 12: The expression of K15 and K17 in heterozygous Inv-Claudin 6 mice is perturbed. In wild type K15 associates with basal layer; however in transgenic epidermis its expression decreased to a patchy expression. K17 was not found in the epidermis of wild type mice while in transgenic counterparts shows patchy expression in the epidermis. Bar 20 μm

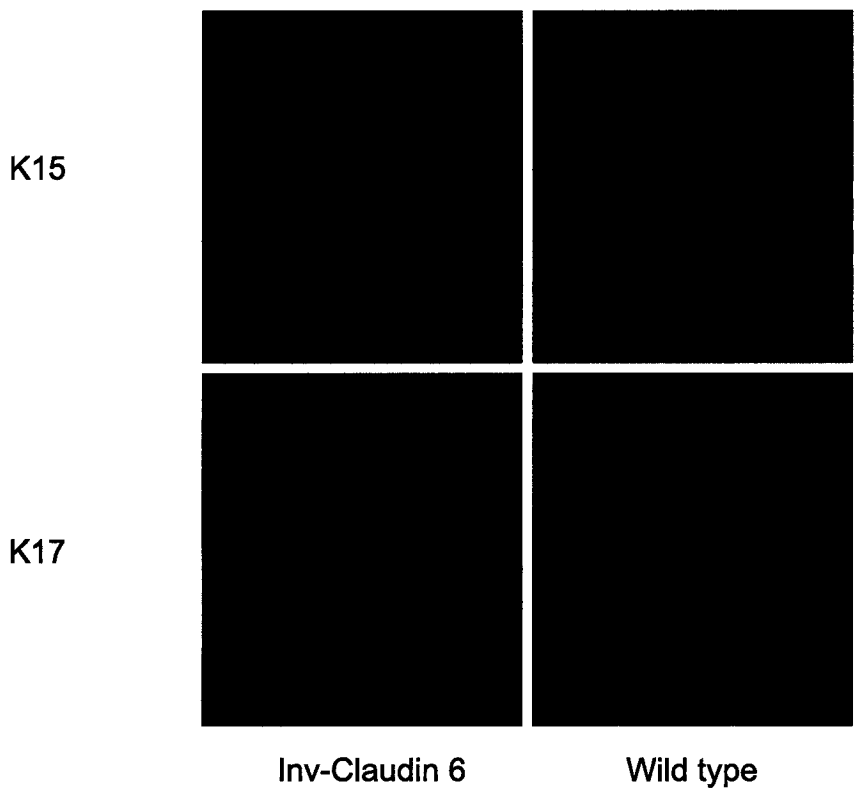


Figure 12

findings are in agreement with the previous data that upregulated K14 causes a downregulation in K15 gene expression (Waseem et al 1999). K17, which is developmentally regulated, was not found in the epidermis of wild type mice while in transgenic counterparts shows patchy expression in the epidermis (figure 12).

Heterozygous Inv-Claudin 6 mice show an increase in epidermal differentiation layers, but not a hyperproliferation:

As was mentioned before, the basal keratinocytes in epidermis continuously proliferate to regenerate and restore cells lost to the environment. As the daughter cells migrate up through the epidermal layers, they undergo terminal differentiation and growth arrest, meanwhile they downregulate the expression of certain genes such as keratin 14/5 and upregulate the expression of other genes such as keratin 1/10 (for review see Fuchs 1995). This is a characteristic of keratin 1 and 10 that facilitates the study of terminal differentiation in epidermis. On the other hand, keratin 6, K6 gene is synthesized in the periderm, where it coexists with K17, and in a subset of cells within primary hair germs, but not in the placode (McGowan and Coulombe 1998).

To elucidate whether differentiating layers in the transgenic epidermis are altered, we used K1 antibody and the results showed an upregulation, indicating an increase in these layers (figure 13a).

We next studied the expression levels of K6 as a marker of hyper proliferation in the epidermis (McGowan and Coulombe 1998). No K6 staining

Figure 13: Heterozygous Inv-Claudin 6 mice show an increase in epidermal differentiation layers, but not a hyperproliferation. K1 antibody showed an upregulation in transgenic sections, indicating an increased in differentiating layers. No K6 staining was observed in epidermis layer, an indication for nonexistence of hyperproliferation in epidermis. Bar 20 μm

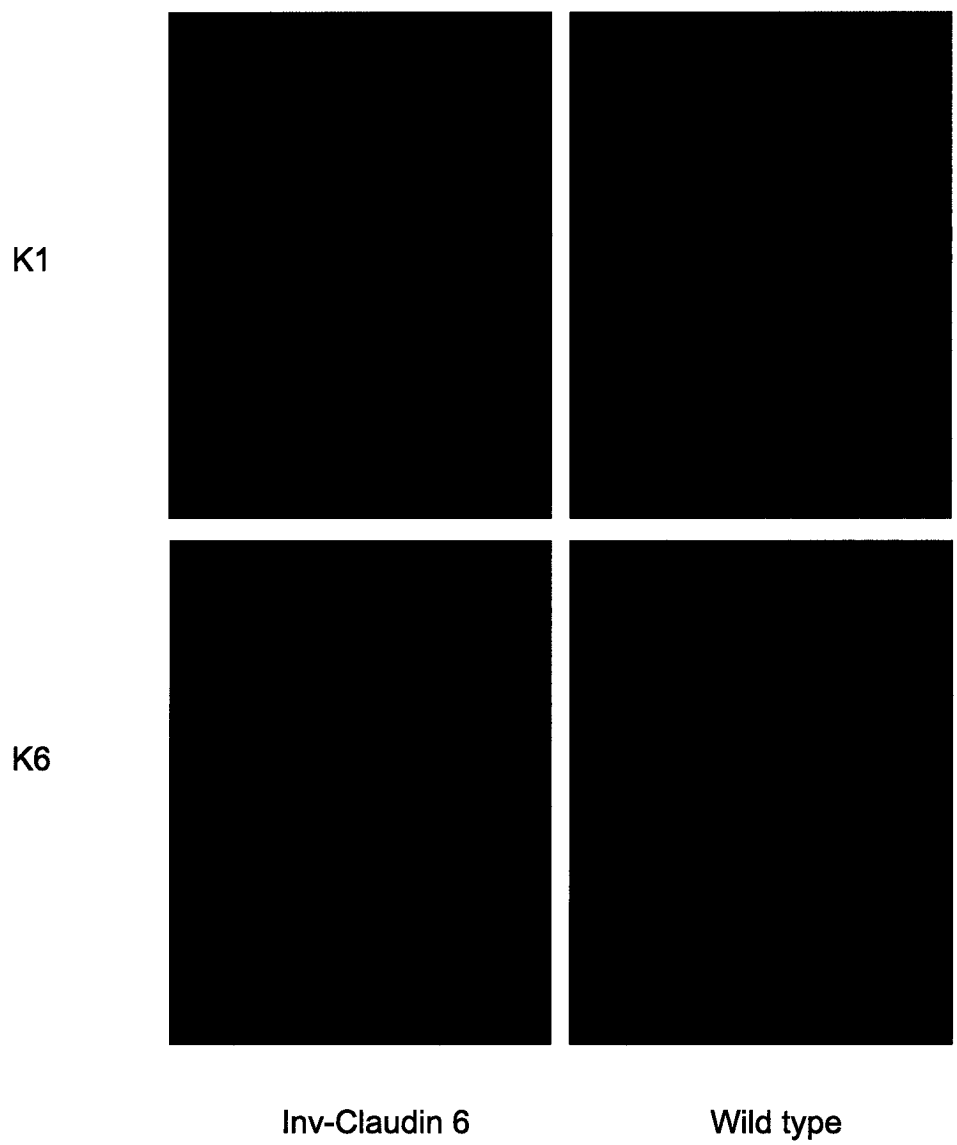


Figure 13

was observed in epidermis layer, an indication for abscence of hyperproliferation in epidermis (figure 13b).

Epidermal terminal differentiation markers show a different expression pattern in heterozygous Inv-Claudin 6 mice:

In the next layer after the spinous layer of the epidermis (the first differentiated layer in the suprabasal layer), the granular layer, late markers of keratinocyte differentiation, including filaggrin, loricrin, involucrin and other structural proteins, are expressed. In addition, compared to lower layers of suprabasal layer of epidermis, the activity of transglutaminase, the enzyme that cross-links the structural proteins into the cornified envelope, is increased (Steinert et al 1997).

To elucidate whether the expression of these late differentiation markers in epidermis are altered, using antibodies for each protein, immunohistochemistry was performed. Involucrin, loricrin, filaggrin and transglutaminase 3 expression in the transgenic epidermis was more diffuse in the lower layer of the epidermis. Filaggrin expression was extended into the suprabasal layers of epidermis, and the markedly increased filaggrin expression suggests a dysfunction in the processing of profilaggrin (Reichelt 2001,Turksen and Troy 2002) (figure 14).

Figure 14: Transgenic and wild-type epidermis was processed for immunofluorescence to evaluate epidermal terminal differentiation markers. Filaggrin, loricrin, Involucrin and transglutaminase 3 expression was altered. 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. Bar 20 μm

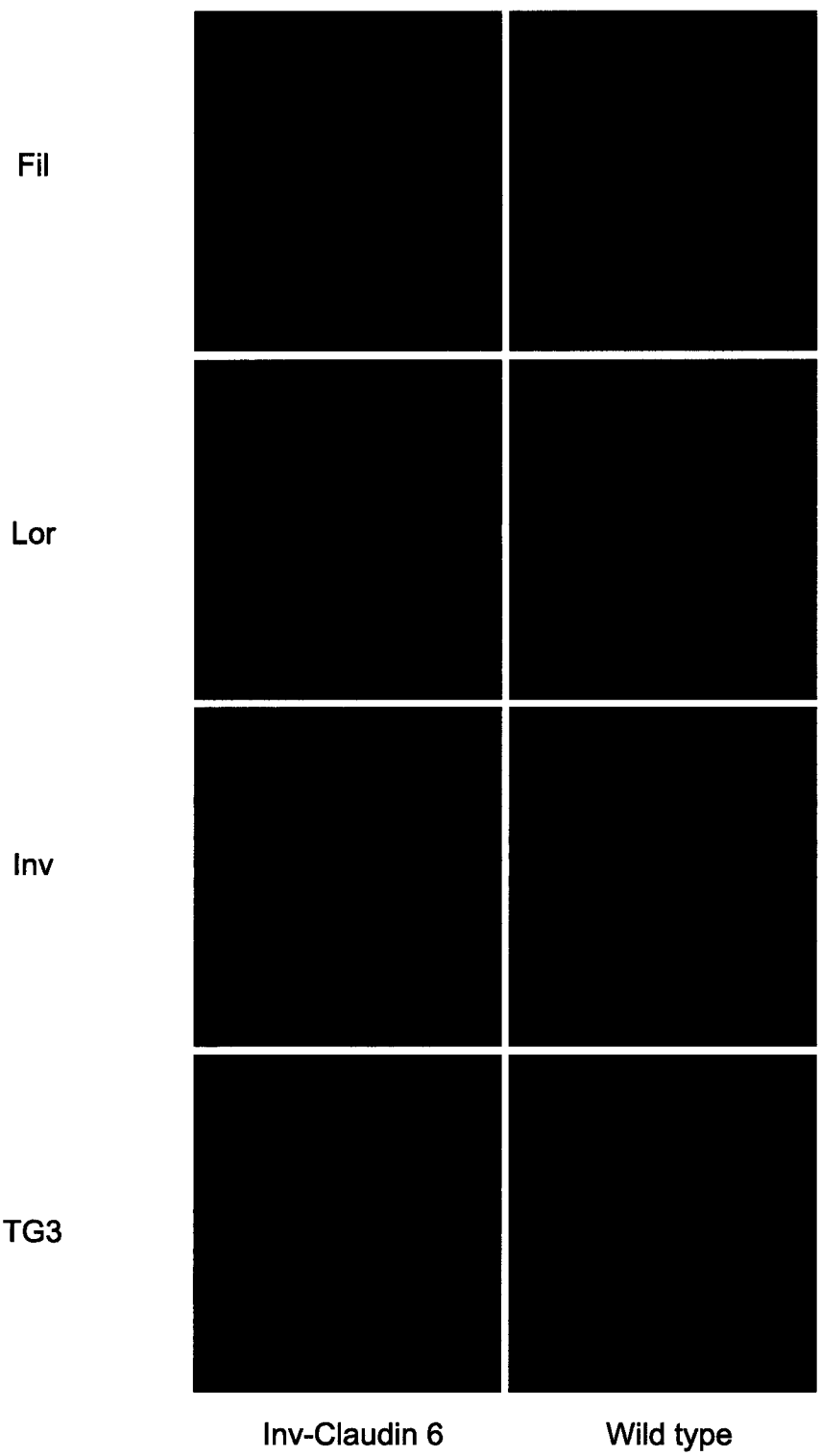


Figure 14

Claudin 6 overexpression results an altered proliferation and differentiation program in hair follicles

To verify if heterozygous Inv-Claudin 6 mice show an aberrant hair follicle proliferation and terminal differentiation, we focused on keratin expression pattern in this organ. In these animals' hair follicle, K5, K14, K15, and K17 expression persisted throughout the hair cycle as observed in wild type. Similarly, K6, which is normally restricted to the inner layer of ORS, was present in both wild type and transgenic hair follicle (figure 15).

Considering the observed changes in transgenic hair structure such as shortened hair fibers and defects in hair fibre architecture including disorganized septates in medullae, we were wondering if these alterations could result from either defective differentiation of the hair matrix cells and its appendages such as hair shaft and IRS. To assay differentiation, we used immunohistochemistry using hair keratins AE13, which reacts with low sulfur hair keratins, and AE15, which reacts with tricohyalin granules, on frozen sections of P4, P8, and P12 Inv-Claudin 6 mice and wild type counterparts. Our results revealed that both AE13 and AE15 were expressed in transgenic hair follicle, but with reduced expression relative to wild type, suggesting the differentiation problems were present in Inv-Claudin 6 mice hair follicles. In addition AE15 pattern of expression was different in transgenic samples, where the constitutive expression of this protein see in wild type IRS was changed to a dotty pattern of expression in transgenic hair follicle (figure 16).

Taken together, the proliferation abnormalities and aberrant hair specific keratin expression in Inv-Claudin 6 hair follicle suggest strong reasons for causing disruptions in their differentiation program, the defects which is in agreement with phenotypic appearance of these animals.

Figure 15: In heterozygous Inv-Claudin 6 mice hair follicles, K5, K17 and K15 expression persisted throughout the hair cycle as observed in wild type. Similarly, K6, which is normally restricted to the inner layer of ORS, was present in both wild type and transgenic hair follicle. Bar 20 μm

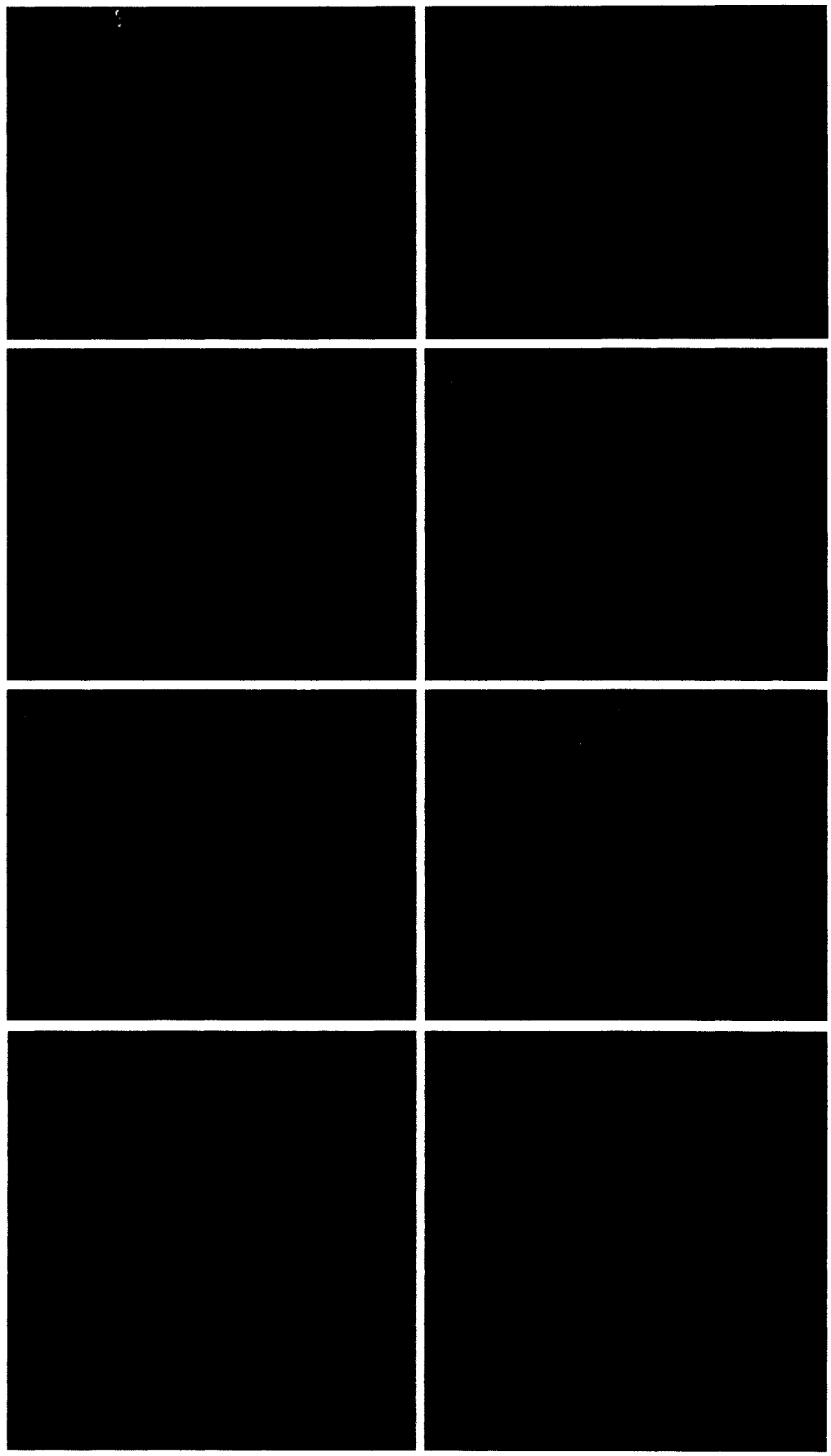


figure 15

Figure 16: Terminal differentiation is perturbed in Inv-Claudin 6 mouse hair follicles. Both AE13 and AE15 were expressed in transgenic hair follicle, but with reduced expression relative to wild type. In addition AE15 pattern of expression was different in transgenic samples, where the constitutive expression of this protein in wild type IRS was changed to a dotty pattern of expression in transgenic hair follicle. Bar 20 μm

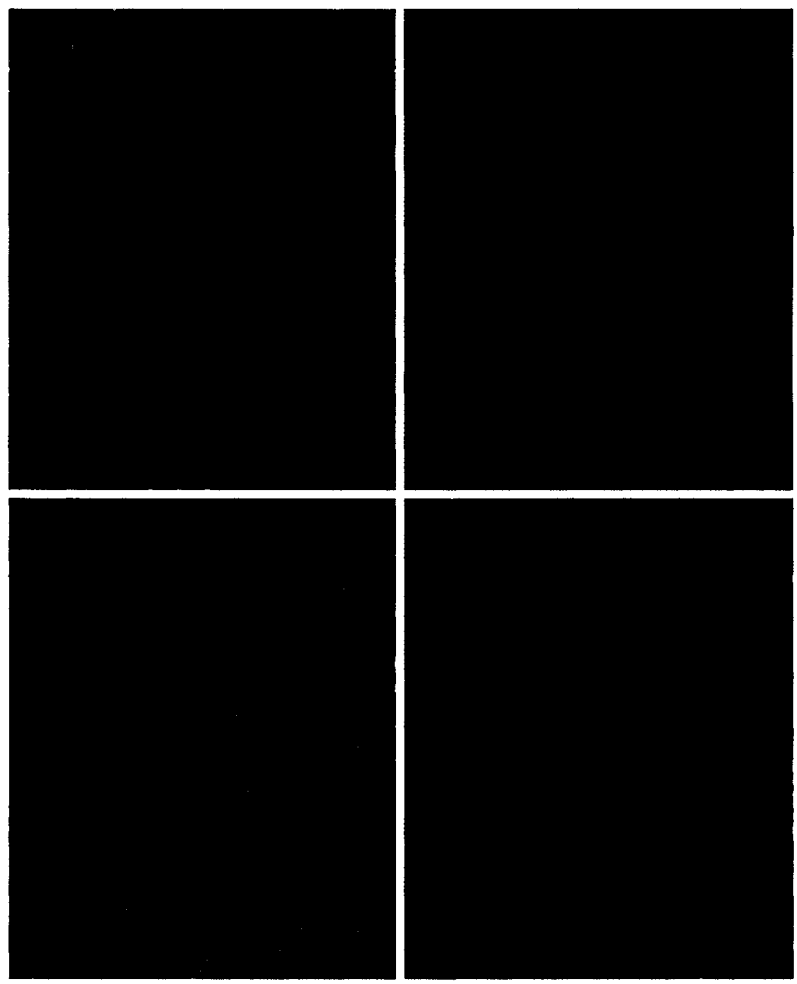


Figure 16

Discussion

Since our in vitro studies on epithelial differentiation showed that Claudin 6 is one of the earliest molecules to be expressed in embryonic stem cells committed to epithelial fate, we examined the consequences of Claudin6 overexpression in transgenic mice. Using the involucrin promoter, Claudin 6 expression was targeted to epithelial cells that have lost the ability to proliferate and is under-going terminal differentiation. In this project, we analyzed the morphological and molecular defects in heterozygous Inv-Claudin 6 mice epidermis and hair follicles. Our findings establish that heterozygous Inv-Claudin 6 mice present two major differences compare to wild type counterparts during the postnatal maturation of epidermis and hair follicles: that of the failure of epidermal barrier to water permeation and that of structural defects in the hair fibres. Therefore, these mice represent a novel transgenic animal model system for studying epidermal permeability barrier formation, potential therapeutic interventions and addressing several important questions about epidermis and hair follicle differentiation.

Earlier studies showed that two copies of Inv-Claudin 6 in the skin of homozygous mice, caused rapid postnatal lethality, due to a defective epidermal barrier (Turksen and Troy 2002). Since heterozygous animal survive to adulthood, the disruptions in the skin barrier may not be as severe as in homozygous pups. Not surprisingly, using whole mount staining with x-gal substrate, we detected defects in the epidermal barrier after birth; however, over the next few days, the heterozygous mice eventually formed functional barriers.

On the other hand, compared to wild type counterparts, histological analysis of heterozygous Inv-Claudin 6 mice revealed a dramatic thinner skin after birth which gradually disappeared in next few days. This probably could be a consequence of skin dehydration followed by changes in permeability barrier. However, by completion of barrier acquisition in next few days the skin dehydration disappears and the skin thickness returns normal.

Although many of the structural proteins and lipids involved in epidermal barrier have been identified, little is known about how barrier function is established or about the regulatory proteins that govern this late-stage process. It is a late stage process because the formation of the cornified envelope is the final step in epidermal differentiation, after which the cells are sloughed off. This is now well accepted that it is the lipid-protein composition of stratum corneum that serves a water-holding function through the formation of lamellar structures within the stratum corneum (Imokawa et al. 1991; Rawlings et al. 1994) to preserve a functional skin barrier. During cornified envelope formation and as a consequence of epidermal terminal differentiation, keratinocytes move upward while undergoing both morphological and biochemical changes. To that end, the keratin component of their cytoskeleton becomes incorporated with specific proteins, such as loricrin and filaggrin in the envelope cells through the crosslinks that affords the suitable dynamics necessary for an efficient barrier acquisition. Past experiments have shown that, loricrin crosslinks to the cornified envelope, whereas filaggrin participates in keratin filament assembly and is required for corneocyte hydration (Dale et al. 1980; Haydock & Dale 1990; Fuchs 1990;

Steven et al. 1990; Ishida-Yamamoto et al 1994).This crosslinking of the constituent proteins is provided by the action of transglutaminases (Melino et al 2000; Kalinin et al 2002). Therefore any dramatic changes in expression of these protein or sudden relocalization of their crosslinks would cause skin permeability status changes. As a support to this hypothesis, several studies have shown that the pattern of keratinocyte differentiation is abnormal in skin with aberrant permeability barrier. In some of these cases the mRNA and protein levels for several genes such as filaggrin, and loricrin are perturbed (Watanabe et al. 1991; Bernerd et al. 1992). Moreover, the gene expression pattern of transglutaminases (Nonomura et al. 1993) and involucrin (Ishida-Yamamoto & Izuka, 1995) is also disturbed in other cases, resulting in abnormal cornified envelope formation (Ishida-Yamamoto & Izuka, 1995) followed by defects in epidermis barrier function. In our transgenic animals, performing immunohistochemical analysis, we have documented the abnormal increased expression levels of loricrin, filaggrin, and transglutaminase 3 as the consequence of the epidermal differentiation defects. The disruption in homeostasis of these structural proteins' gene expression would probably perturb the epidermal permeability barrier is a support for previous data which showed the correlation between different stages in barrier formation and the expression of these structural proteins (Hardman et al 1998; Elias and Feingold 2001). Increased immunoreactive cells of filaggrin have also been found for K10 dominant negative mice (Fuchs et al. 1992) and for epidermolytic hyperkeratosis

(EHK) (Ishida-Yamamoto et al. 1994), though skin hydration and its barrier efficiency is reduced in both cases.

Once the loricrin- and filaggrin-mediated bundling of keratin intermediate occurs, the development of cornified envelope continues via the molecules such as involucrin to increase the rigidity and insolubility of the structure (Yuspa et al 1989; DiColandrea et al 2000; Presland and Dale 2000). Thus, any disruption in their gene expression such as an increase in the expression of these proteins as we observed in the case of involucrin almost certainly contributes to the cornified envelope-related permeability barrier problems. The genes coding for these proteins with other cornified envelope-associated genes such as SPRRs and S100 proteins have been mapped on chromosome 3 of the 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, but our results on the upregulated level of loricrin, filaggrin, involucrin and transglutaminase 3 gene expressions in Claudin 6 overexpressing mice may suggest a modulatory role for Claudin 6 in affecting the expression of these genes.

In addition, the alterations observed in these proteins expression drove our attention to check if the other markers of terminal differentiation, such as K1, are perturbed in these animals. Therefore, we checked their expression during epidermal differentiation.

During terminal differentiation in epidermis, the proliferated basal compartments leave the basal layer, downregulate the expression of K5 and K14 and upregulate K1 and K10 (Fuchs 1990). 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, indicating an increase in the number of differentiating layers. This was a support for previous reports that changes in epidermal differentiation markers including a perturbed expression in K1 in dry skin of healthy persons were observed (Engelke et al. 1997). Also, a reduced skin hydration (and a reduced barrier function) accompanied by disturbed expression of K1 has been reported in psoriasis lesional skin (Hagemann & Proksch, 1996). On the other hand our data also revealed an age related ability of suprabasal layer to keep their K5/K14 expression. This increase was observed in both K14 and K5 case; however compare to K5 the expression of K14 was more diffused throughout the suprabasal layers up to the uppermost granular layer of epidermis. The persistent suprabasal expression of the K5/K14 protein might suggest (1) the half-life of K5/K14 is increased in Inv-Claudin 6 animals (2) suprabasal expression of K5/K14 couple seems to be independent.

The phenotype of affected epidermal permeability barrier in heterozygous Inv–Claudin 6 newborns is consistent with other mutations in mice that lead to barrier defects: the production of a dominant negative retinoic acid receptor (RAR) (Imakado et al., 1995). The RAR mutant animals also exhibit red, shiny skin, possess an abnormal stratum corneum, and frequently die within hours of birth, but do not display epidermal hyperplasia. This was in agreement with what we observe in our transgenics using K6 antibody. Compared to the wild type, these animals did not exhibit any differences in the level of expression of K6

suggesting absence of hyperproliferation of the epidermis. Thus, barrier defects in mice are not necessarily associated with keratinocyte hyperproliferation.

Overall our data suggested that the pattern revealed by barrier assay is initiated when cells starts terminal differentiation. In the other words, epidermal barrier forms in a moving fashion starting from the initiation site as they leave the cell cycle in basal layer and commit terminal differentiation and terminates at the level of cornified envelope. Thus, any alteration in "pre-planned" program of barrier acquisition would ultimately cause defects in maturation of barrier function. The role of a probable cross-talk between the epidermal cells and the underlying mesenchymally-derived dermis is the subject of further experiments.

The other defect the Inv–Claudin6 transgenic mice exhibit, as pointed before, is the abnormalities of the hair coat and whiskers. At the cellular level, transgene expression has two major consequences on hair follicles. One is some hair follicles showing the disruption in their normal architecture, including crookedness, and shorter anagen, thus terminating the hair growth earlier, while exhibiting a delay in regression and resting phase. The second consequence of transgene expression in hair follicle is the disruption of terminal differentiation.

In heterozygous transgenic mice, hair fibres form but have abnormal structure and length. This was consistent with what other groups reported on TGF-α deficient (Mann et al 1993) and wa-1 mutant mice (Crew 1993). In the original paper by Crew, the author describes the wa-1 mouse as having "developed coats which looked exactly as though the animals had been to the hair dresser and had a permanent wave treatment." This description could be applied equally to

Claudin 6 overexpressed heterozygous mice. As hair fibre production and its growth is a consequence of terminal differentiation, we were wondering if like epidermis, Claudin 6 plays a possible modulatory role in terminal differentiation of hair follicle. Interestingly our data supported this probability as markers of hair follicle terminal differentiation, such as AE13 (marker of the hair shaft) and AE15 (marker of the IRS), exhibited an altered pattern of expression in transgenics. However, ORS markers such as K14, K5, K6, K17 and K15 did not show a difference. This was not surprising as Claudin 6 expression was targeted to the epithelial cells that have lost the ability to proliferate and are under-going terminal differentiation but ORS, as an epithelium that covers around the follicle, is contiguous with the basal layer of the epidermis (Hardy 1992) should not present a transgenic associate disruption of protein expression be observed. However, the existence of a probable cross-talk between proliferating and differentiating layers that regulates the developmental process during the hair follicle initiation and cycle is still need to be identified.

Both development of the hair follicle and the subsequent hair growth involve signaling between dermal papilla and matrix cells. To respond to dermal papilla signals, matrix cells proliferate during anagen and soon start to differentiate to several hair cell types of hair shaft and IRS. The fate of these cells is intimately related to cell-cell interaction (Kopan and Weintraub 1993) and is determined as they leave the cell cycle. As our transgens present defects in hair fibre, hair shaft layer and IRS, we consider the role of Claudin 6 in affecting the differentiation of transient amplifying cells of hair matrix.

In addition to differences in hair coat appearance in heterozygous Inv-Claudin 6 mice show hair cycle disruption as well as shorter hair fibre. Recently, both signaling molecules and transcription factors that exhibit altered expression during different cycle phases have been identified (Paus et al 1999; Stenn and Paus 2001). Classical experiments suggest the existence of a built-in control of the hair cycle that resides in the hair follicle itself, and that can be modulated by outside factors (reviewed by Stenn and Paus, 2001). In mouse hair follicles, it has been proposed that such an intrinsic biological clock, termed the `hair cycle clock', controls the length of each phase of the hair cycle (Paus et al., 1999; Stenn and Paus, 2001). Although the molecular nature of this clock is unknown, it may involve hormones or diffusible factors whose expression oscillates in each hair cycle. This view implies that the hair cycle is controlled by sets of molecules that coordinately regulate each phase transitions. According to this model, sets of regulatory factors act at each checkpoint either to promote or suppress the transitions between the phases of the hair growth cycle. The observed length of each phase thus reflects the balanced strength of the promoting and suppressing factors impinging on each transition point. At day 14 Inv-Claudin 6 hair follicle pose an anagen-catagen histological sign while those of wild type remain in anagen. In addition Inv-Claudin 6 hair cycle proceeded to the catagen/telogen in 5 days while wild type pups reside in catagen for only 3 days. Taken together, these Claudin 6 would be predicted to encourage the anagen-catagen transition while playing a suppressive role in the transition to telogen. These mice also have shorter hair fibre, a phenotype which reflects the shortened anagen.

A complex signaling network in hair follicle regulates the specification and differentiation of the hair shaft, the IRS as well as hair cycle. From the analyses we performed, Claudin 6 is likely to be involved in this network (1) in constituting a regulator in the differentiation of transient amplifying cells into hair shaft cells and (2) in hair cycle progression. The successful formation of a hair depends on the progression of hair progenitor cells through several major determination and morphogenetic events. Our results show that Claudin 6 is involved in the switches between transient amplifying cells and pre-cortical cell and in the transitions between the different phases of the hair cycle, however the molecular mechanisms underlying these regulations and the exact role for Claudin 6 are not yet known.

In summary, we provide the first clear evidence that claudins are involved in and are crucial for the formation and maintenance of epidermal permeability barrier as well as the postnatal maturation of hair follicles.

Future perspective:

In this project we report a novel mouse model in which the overexpression of the claudin 6 at its endogenous site in the upper strata of differentiating epidermal cells initiated a cascade of events that resulted in a defective skin permeability barrier with associated striking phenotypic consequences including defective hair fiber production. Therefore, defining developmental regulatory mechanisms in terms of molecular pathways regulating the differentiation of epidermis and hair follicle would be the future research path of what we established in these animals. Understanding these signaling pathways that regulate epidermis and

hair follicle morphogenesis and the identification of those molecules involved in the initiation and cycling of the hair follicle would make our transgenic animals a useful model for studying the causes of various diseases such as some basal cell carcinomas and pilomatricoma (a tumor of hair follicle matrix cells) which result from the inappropriate activation of the signaling pathway (Chan et al., 1999; Chiang et al., 1999; Gat et al., 1998; Oro et al., 1997; St-Jacques et al., 1998). A better understanding of these molecular mechanisms regulating the epidermis and hair follicle may therefore lead to the development of novel therapies for hair loss and various skin disorders.

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