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Claudin proteins and the regulation of epithelial proliferation and differentiation: a mutual correlation

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Claudin proteins and the regulation of epithelial proliferation and differentiation: a mutual correlation

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of the Ph.D. program in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

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To Mom and Dad

whose eternal love, compassion and support is so pure and priceless

that can never be put down in words.

To my Farshad

whose patient love enabled me to go through the loneliest and hardest

moments of my life.

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And maybe a small thank you to my mice that bore with me for the period of my studies.

Abstract

One mode of cell-cell adhesion between epithelial and endothelial cells is mediated by tight junctions (TJs), complex structures which serve two major roles: 1) they constitute a barrier to the passage of ions and small molecules through the paracellular space and 2) they restrict the movement of lipids and proteins between the apical and basolateral compartments of the plasma membrane. It is now widely recognized that the Claudin (Cldn) family of tetraspan membrane proteins is crucial for TJ assembly and barrier function. Although originally proposed as structural components of TJs, recent evidence implicate the signaling function of Cldns. Notably, a bilateral correlation between Cldns and signal transduction pathways regulating cell proliferation/differentiation is emerging; on one hand Cldns can undergo changes in response to altered proliferation-differentiation, and on the other hand changes in Cldns can result in proliferation and/or differentiation perturbations. Along this line, studies from our laboratory have confirmed that such connection exists. Using the mouse epidermis as a model system, we first reported that overexpression of endogenous Cldn6 in this tissue perturbs both the balance of other Cldn isoforms as well as the normal process of epidermal differentiation. To elucidate the mechanism behind this effect, we looked towards the Cldn6 C-terminal tail; a region that may interact with yet-unidentified molecules involved in transducing signals of epidermal differentiation. Therefore, deletion of the tail domain was assumed to lead us to Cldn6 signaling function; indeed expression of tail-ablated Cldn6 not only disrupted epidermal differentiation but also increased cell proliferation. Loss of epidermal homeostasis was apparently due to the loss of Cldn homeostasis. We were next curious to know whether the correlation of Cldns to proliferation-differentiation is of a reciprocal

nature. Therefore, two well-known models of tissue injury response and chemical carcinogenesis were used, in order to induce proliferation-differentiation aberrancy. Examining various Cldns in both models indicated that in response to perturbed proliferation-differentiation Cldns undergo changes, further raising a two-sided regulatory function between Cldns and intracellular signaling pathways.

Table of Contents

List of Figures

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

List of Tables

Chapter 1:

Chapter 2:

List of Abbreviations

Ca^{2+}	- Calcium ion
CaSR	- Ca^{2+}-sensing receptor
cAMP	- cyclic Adenosine Mono Phosphate
CDK4	- Cyclin dependent kinase 4
CE	- Cornified Envelope
Cldn	- Claudin
CNS	- Central Nervous System
DMBA	- 7, 12-dimethylbenz[a] anthracene
E	- Embryonic age
EBS	- Epidermolysis Bullosa Simplex
EK	- Epidermolytic Hyperkeratosis
EPB	- Epidermal Permeability Barrier
ERK/MAPK	- Extracellular signal-regulated kinase/Mitogen-activated protein kinase
Fiz1	- Flt3 interacting zinc finger 1
G1	- growth phase 1
GFP	- Green Fluorescence Protein
Ha	- Hair acidic keratin
Hb	- Hair basic keratin
HaCaT	- Human keratinocyte cell line
HB	- Hepatoblastoma
IF	- Intermediate Filament
IRS	- Inner Root Sheath

Inv	- Involucrin
JAM	- Junctional Adhesion Molecule
K	- Keratin
Lef	- Lymphoid enhancer factor
MAGUK	- Membrane-associated guanylate kinase
MAGI	- Membrane associated guanylate kinase inverted
MDCK	- Madin-Darby Canine Kidney cells
miRNA	- MicroRNA
miR-29	- MicroRNA 29
mRNA	- messenger RNA
MUPP-1	- Multi-PDZ domain protein 1
Na^+	- Sodium ion
PATJ	- Pals1-associated tight junction protein
PDZ	- Postsynaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), Zonula occludens-1 protein (ZO-1)
PKB/Akt	- Protein kinase B
PKC	- Protein Kinase C
PI3 kinase	- Phosphoinositide 3 kinase
PTEN	- Phosphatase and tensin homolog
Raf-1	- Murine leukemia viral oncogene homolog 1
RNAi	- RNA interference
S	- Synthesis phase
SC	- Stratum Corneum

Scarf	- Skin calmodulin-related factor
SCC	- Squamous cell carcinoma cells
SJ	- Septate Junction
Tcf	- T cell factor
TER	- Transepithelial Resistance
TJ	- Tight Junction
TPA	- 12-O tetradecanoyl phorbol 13-acetate
3'-UTR	- 3' untranslated region of mRNA
UV	- Ultraviolet
WNK4	- With No Lysine (K) kinase 4
ZO	- Zonula Occludens
ZONAB	- ZO-1 associated nucleic acid binding protein

Thesis Format

This thesis is written based on the format of collection of manuscripts. Chapter 1 provides an introduction to the work where the relevant background, supporting data from other researchers' work as well as overview of our past research leading to the current one will be discussed. Chapter 2 is a paper (manuscript #1) entitled "Role of the Cldn6 Cytoplasmic Tail Domain in Membrane Targeting and Epidermal Differentiation In Vivo". This paper has been published in Molecular and Cellular Biology. Aug. 2006, 26 (15): 5876-5887. Chapter 3 consists of a second paper (manuscript #2) entitled as "Claudin expression modulations reflect an injury response in the murine epidermis" and published in Journal of Investigative Dermatology. Jan. 2008, 128 (1): 237-240. Chapter 4 of this thesis presents a third paper (manuscript #3) entitled "Changes in the distribution pattern of Claudin tight junction proteins during the progression of mouse skin tumorigenesis". This paper is published in BMC Cancer. Oct. 2007, 7: 196. The last chapter of this thesis is intended to give an overall discussion of the entire work.

Chapter 1

General Introduction

Skin, the largest organ in human body, serves as a critical interface providing a protective barrier between the environment and the body. Therefore, skin is constantly exposed to a broad spectrum of environmental insults including chemical and physical pollutants (Bicker and Athar, 2006). Only by virtue of its highly organized structure and homeostatic functions, skin defends multicellular organisms against microbial invasion, UV irradiation, temperature fluctuations, dehydration as well as chemical intoxication (Fitzpatrick, 1993). This organ is composed of three major layers: epidermis, dermis and hypodermis or subcutaneous tissue (Fitzpatrick, 1993). It also comprises several specialized appendages including hair follicles, sweat glands and sebaceous glands (Fitzpatrick, 1993). The uppermost layer of the skin i.e. epidermis, which is a stratified squamous epithelial tissue, is composed of three cell types: the major class of epidermal cells is "keratinocytes" and the other two include melanocytes and langerhans cells (Fitzpatrick, 1993). Melanocytes are the pigment-producing cells of the epidermis, which originate from the neural crest and they impart color of the skin and hair as well as some protection against UV irradiation (Fitzpatrick, 1993). Langerhans cells are antigen-presenting cells of the skin arising from the bone marrow, which play immune function in this organ (Holikova et al, 2001). In spite of well-documented roles of both melanocytes and langerhans cells in the skin, they would not be the focus of this thesis. Instead, keratinocytes of the epidermis which confer this tissue both its barrier function (the result of keratinocyte maturation/differentiation) and its homeostatic ability (the result of a fine balance between proliferation and differentiation/apoptosis of keratinocytes) will be

discussed (Fitzpatrick, 1993). Before proceeding to the role of keratinocytes in establishment and maintenance of epidermal homeostasis as well as permeability barrier, it is crucial to become familiar with the process of morphogenesis in this tissue. It is also to be mentioned that from now on the term "epidermis" refers to the murine epidermis which has been used in our investigations as a preferred model system.

1.1) Epidermal morphogenesis

The epidermis is derived from one of the three embryonic germ layers, which in this case would be the ectoderm. During gastrulation in mouse embryo, primitive ectoderm gives rise to surface ectoderm at embryonic age 5-6 (E5-E6). Surface ectoderm will then give rise to several different fates including epidermis and epidermal appendages (Landstrom and Lovtrup, 1979; Wolpert et al., 1998). Under a poorly understood set of signals, at age E8-E12, the mouse epidermis is formed as a single layer of proliferating cells namely stratum germinatium or basal layer, which is overlain by periderm. Subsequently, at E12-E14, stratum germinatium gives rise to an intermediate layer (stratum intermedium) whose further stratification begins at day E15 and results in formation of spinous, granular and cornified layers by days E17-E18 (Bonneville 1968; Hanson 1947; DuBrul 1972, Turksen and Troy 1998). At this stage, establishment of the fully formed epidermis with a completed epidermal permeability barrier (EPB) is accompanied by the loss of the periderm (E18) (Weiss and Zelickson, 1975, I; Bonneville 1968; Hanson 1947; DuBrul 1972, Turksen and Troy 1998; Byrne et al., 1994; Hardman et al., 1998).

1.2) Mature epidermis as an efficient permeability barrier

Although formation of all epidermal layers is completed before birth (Weiss and Zelickson, 1975, I) the epidermis undergoes a complex process of maturation/differentiation postnatally (Weiss and Zelickson, 1975, II), which results in acquisition of a functional permeability barrier (Weiss and Zelickson, 1975, II) (Figure 1). Such differentiation process starts at the basal epidermal layer (stratum basale) which is located on top of the dermis and anchored to the basal lamina via hemidesmosomal junctions. This layer contains basal keratinocytes which are nucleated and relatively undifferentiated cells that actively proliferate and provide a continuous supply of new cells to sustain the homeostasis of the epidermis (Cotsarelis et al., 1989; Lavker et al., 1993). Daughter cells of basal keratinocytes then move upwards away from the basal layer and begin their 2 week journey to the surface of the epidermis, through a number of other layers. The second epidermal layer is the spinous layer (stratum spinosum), getting its name because of the presence of extensive desmosomal connections between cells, which appear as narrow projections. The third layer of the epidermis, granular layer (stratum granulosum), is characterized by the presence of granules. These granules contain products of keratinocyte differentiation which are used for the assembly of cornified envelope (CE) of the stratum corneum (SC). Indeed, the SC is the uppermost layer of the epidermis which accommodates terminally differentiated keratinocytes (known as corneocytes). Corneocytes consist of a stable array of filamentous proteins (namely keratins) contained within a covalently cross-linked protein envelope (Matoltsy and Matoltsy, 1966). These cells are flattened in shape, anucleate, dehydrated and

therefore non-viable which eventually will be removed from the surface of the epidermis by desquamation (Weiss and Zelickson, 1975, II; Fitzpatrick, 1993).

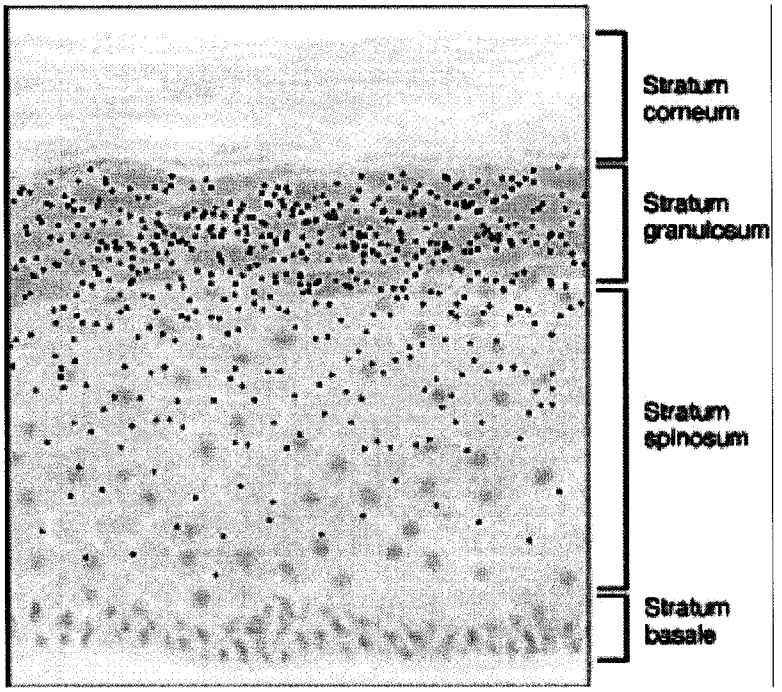

Figure 1. Epidermal differentiation. The diagram illustrates the stages of differentiation in the epidermis. All layers above the basal layer (stratum basale) of the epidermis constitute the suprabasal compartment. (Schematic diagram courtesy of Lauren O'Malley)

The composite of billions of corneocytes is responsible for the protective function of the epidermis (Elias and Friend, 1975; Fitzpatrick, 1993), and what makes these cells play such a role is their unique protein-lipid composition (Kalinin et al., 2002; Hohl 1990) of which "keratins" constitute up to 85% of a fully differentiated keratinocyte (Fuchs, 1995). Keratins are the major proteins synthesized by keratinocytes and are fibrous

proteins that provide structural stability and tensile strength. Structurally, they belong to the superfamily of intermediate filaments (IFs) (Sun and Green, 1978; Fuchs and Green, 1978; Steinert, 1993; Fuchs 1995; Fuchs and Weber 1994). IFs, which were first described by Holtzer and colleagues (Ishikawa et al., 1968), are ubiquitous cytoskeletal proteins in higher metazoans (Erber et al., 1998). They are composed of polypeptide units, which share a tripartite domain structure. A central long α-helical domain is flanked on both ends by non-helical head and tail domains. This α-helical rod is the major driving force triggering their self-assembly, thereby formation of 10 nm filamentous structures (Holtzer et al., 1985; Fuchs and Weber, 1994). The largest group of IF proteins are keratins which are the most abundant proteins in many epithelial cell types. Keratins are obligate heteropolymers between type I or acidic keratins (K9-K20; K23; Ha1-Ha8; IRS1-4) and type II or basic keratins (K1-K8; Hb1-Hb6; K6irs1-4) (Fuchs 1995; Fuchs and Weber 1994; Coulombe et al., 2004). In fact, type I and type II keratins are typically coexpressed as specific pairs and due to their intrinsic ability to self-assemble, they form coiled-coil heterodimers (Fuchs 1995). Approximately, 20000 heterodimers further assemble into an IF which provides major protection against mechanical stress (Fuchs 1995). Although primarily providing structural support to the cell, keratins have recently been attributed additional non-mechanical roles including regulation of key signaling pathways that control cell growth and survival (Kim et al., 2006; Kim and Coulombe 2007). The expression of keratins depends on the cell type, the status of differentiation and the function of the tissue. Moreover, mutations in genes encoding different keratin subtypes have been linked to a number of genetic skin disorders such as epidermolysis bullosa simplex (EBS) and epidermolytic hyperkeratosis

(EH) (Fuchs 1992; Coulombe and Fuchs 1993). The former is caused by point mutations in K14 and the latter by mutations in differentiation-specific keratin, K10 (Cheng et al., 1992). Both EBS and EH are blistering human diseases resulting from perturbation of keratin assembly, thereby their inability to provide mechanical strength to the skin (Cheng et al., 1992; Corden and McLean, 1996).

Among all proteins produced by keratinocytes, keratin proteins are perhaps the most useful and widely used markers for the study of epidermal differentiation, as the differentiation status of each epidermal cell layer is marked by distinct sets of these proteins (Roop et al., 1983). The first keratins expressed in the developing epidermis are K8 and K18 whose expression coincides with the emergence of the single-layered ectoderm during mouse embryogenesis (Franke et al., 1979; Fuchs 1995). In the adult, these two keratins are typically associated with the simple epithelial tissues but not the stratified ones (Fuchs 1995; Eckert et al., 1997). Upon stratification at E15, the expression of K8/K18 will be down-regulated along with the onset of expression of K5 and K14 in the proliferative compartment of the epidermis i.e. basal layer (Fuchs 1995; Byrne et al., 1994). In addition, a minor type I keratin, K15, is also expressed in basal cells (Lloyd et al, 1995). Some other keratins such as K6 and K16 are not normally expressed in the epidermis and their expression will be induced suprabasally during wound healing, in response to retinoic acid treatment, or in hyperproliferative diseases of the skin including skin cancers (Weiss et al., 1984; Paladini et al., 1996; Markey et al., 1992; Fuchs 1995).

Besides keratins, two other major classes of keratinocyte-expressed proteins are CE precursor proteins and transglutaminases whose expression patterns are restricted to

specific layers of the epidermis and therefore mark different stages of epidermal differentiation (Eckert et al., 1997). In the following paragraph, a brief picture of keratinocyte differentiation with respect to the biochemical changes that happen in this process is presented (Table 1). However, as detailed aspects of this process are beyond the scope of this thesis, for a more comprehensive knowledge on this issue the reader is referred to these reviews (Eckert et al., 1997; Eckert et al., 2005; Fuchs, 1995).

Table 1. Keratins and CE proteins during epidermal differentiation

Protein	Layer-specific location	Function
K14/K5	Basal layer	IFs; structural stability of keratinocytes
K1/K10	Spinous and granular layers	IFs; structural stability of keratinocytes
Involucrin	Spinous and granular layers	CE precursor protein; backbone for cross-linkage of all other CE precursor proteins
Filaggrin	Granular layer	CE precursor protein; with ability to bundle keratin filaments
Hornerin	Granular layer	CE precursor protein; with structural and functional similarity to filaggrin
Loricrin	Granular layer	CE precursor protein; major component of CE
SPRRs	Granular layer	CE precursor protein; they function as bridges that link together other precursor proteins
S100 proteins	Granular layer	CE precursor protein; containing calcium-regulated EF hand motifs
Transglutaminases	Spinous and granular layers	Catalyzing formation of Nε-(γ-glutamyl) lysine cross-links between CE precursor proteins

As basal keratinocytes differentiate, they shut down the expression of K5 and K14 and instead they induce the expression of a new set of keratins namely K1 and K10 (Fuchs 1995; Fuchs and Green 1980) which are early markers of keratinocyte differentiation. Moving from the basal layer and approaching the spinous layer, keratinocytes start expressing involucrin (Watt and Green 1981) which is one of the main CE precursor proteins whose expression is induced upon an increase in intracellular Ca^{2+} level. Indeed, calcium ion (Ca^{2+}) is one of the most important regulators of keratinocyte differentiation whose effect is partly exerted through altering gene expression of terminal differentiation proteins such as involucrin, loricrin and filaggrin (Bikle et al., 2004) as well as Ca^{2+}-binding proteins such as Scarf proteins (Hwang and Morasso, 2003; Hwang et al., 2005). Within the epidermis, Ca^{2+} shows a gradual increase in concentration towards the upper strata (Menon et al., 1985; Elias et al., 2002). Following the spinous layer, epidermal cells in the granular layer accumulate lamellar bodies as well as keratohyalin granules (Elias and Friend 1975; Presland et al., 1992). The former develops from the Golgi complex and contains large amounts of barrier lipids (free fatty acids, cholesterol and its esters and ceramides) which will be later deposited into the CE. The latter structure contains two other members of CE precursor protein family: profilaggrin (Dale et al., 1985), a protein which is activated upon cleavage and facilitates aggregation of keratin filaments in the CE; and hornerin (Makino et al., 2001; Makino et al., 2003), a novel protein which has functional and structural similarity to profilaggrin. Ultimately, transition of terminally differentiated keratinocytes from the granular layer to the cornified layer is accompanied by the expression of a new set of CE proteins such as

loricrin (Hohl and Roop 1994), small proline-rich proteins (SPRRs) (Cabral et al., 2001), Ca^{2+}-binding S100 proteins (Robinson et al., 1997) and transglutaminases (Kim et al., 1995). Concomitant with this transition, a number of processes takes place which results in assembly of the CE and the stabilization of keratin network. They include crosslinkage of CE precursors proteins by the function of transglutaminase; aggregation of keratin filaments mainly by the function of filaggrin; extrusion of barrier lipids into the intercellular spaces; destruction of cell organelles; loss of water content and nucleus as well as flattening of keratinocytes which are now called corneocytes. In other words, corneocytes are the final dead cornified cells of the epidermis which are enriched in proteins and embedded in a lipid matrix resembling a "brick and mortar" composition, and provide the vital mechanical and permeability barrier functions essential for the survival of multicellular organisms in the terrestrial environment.

As described above, the process of epidermal differentiation leads to the formation of CE whose unique composition has long been believed to be the only contributor to the establishment of a functional EPB (Kalinin et al., 2002; Hohl 1990). However, recently evidence has emerged that tight junctions (TJs) may also play a crucial role in barrier function of the epidermis (Brandner et al., 2002; Brandner et al., 2006; Shchuter et al., 2004). Indeed, it was due to the advances in electron microscopy that the first observation of TJ structures was made through the pioneering work by Farquhar and Palade (1965). Networks of anastomosing fibrils, characteristic of TJs, were identified by these two scientists in freeze fracture replica prepared from amphibian skin (Farquhar and Palade, 1965). This observation was followed by other works indicating the existence of TJs in

squamous stratified epithelia of several mammals, and in particular in the epidermis (Elias et al., 1977; Morita et al., 1998). TJs were also observed in cultured human keratinocytes (Kitajima et al., 1993) and more recently in human skin (Brandner et al., 2002; Schluter et al., 2004) where typical TJ structures, resembling "kissing points", were observed in the uppermost layer of the human epidermis (stratum granulosum), using ultra-thin section electron microscopy. In addition to direct observation of TJ structures, localization of TJ proteins in the epidermis of mammalian skin with normal barrier function further verifies the presence of a TJ system in the skin (Brandner et al., 2002; Brandner et al., 2003; Langbein et al., 2002; Pummi et al., 2001; Furuse et al., 2002; Morita et al., 1998; Langbein et al., 2003).

1.3) TJs, complex structures contributing to the EPB formation

Tight adherence of epithelial cells results in the formation of epithelial sheets that line organ cavities and serve as barriers between body compartments (Farquahar et al., 1963; Gonzalez-Mariscal et al., 2003). TJs are one type of intercellular junctional complex that impart tightness of epithelial and endothelial sheets. In fact, TJs create intercellular seals, which are selectively permeable to diffusion of water and solutes through the paracellular space. This sealing function is called "gate function" of TJs (Johnson, 2005; Gonzalez-Mariscal et al., 2003). They also make a boundary between the apical and basolateral domains of plasma membranes, thereby preventing intramembranous diffusion of lipids and proteins. This function of TJs is termed the "fence function" (Shin et al., 2006; Gonzalez-Mariscal et al., 2003). TJs have recently been recognized as complex,

multiprotein structures that also have a role in other cellular processes such as proliferation, differentiation, vesicle trafficking, and regulation of cell polarity (Shin et al., 2006; Gonzalez-Mariscal et al., 2003; Lapierre 2000; Miyoshi and Takai 2005). Components of TJs can be classified into two major groups: 1) integral membrane proteins that mediate direct contact between neighboring epithelial cells, and include occludin (Furuse et al., 1993; Tsukita and Furuse 1999; McCarthy et al., 1996; Schulzke et al., 2005), claudins (Cldns) (Furuse et al., 1998 a and b; Furuse et al., 2002; Turksen and Troy 2001; Turksen and Troy 2004; Van Itallie and Anderson 2004; Van Itallie and Anderson 2006) and junctional adhesion molecules (JAMs) (Itoh et al., 2001; Morris et al., 2006; Thomas et al., 2004). 2) cytoplasmic or plaque proteins that are associated with the transmembrane proteins and serve diverse roles. Some of them act as adapters linking transmembrane proteins to the actin cytoskeleton, thereby stabilizing the structure of TJs. Some interact with a variety of signaling proteins, thereby conferring upon TJs the characteristics of a signaling platform. Some of these cytoplasmic constituents of TJs include zonula occludens 1, 2, and 3 (ZO-1, ZO-2, ZO-3) (Anderson et al., 1988; Itoh et al., 1999; Itoh et al., 1997; Inoko et al., 2003; Stevenson et al., 1986; Stevenson et al., 1988; Jesaitis and Goodenough, 1994), Pals1-associated TJ protein (PATJ) (Lemmers et al., 2002, Shin et al., 2005), multi-PDZ domain protein 1 (MUPP-1) (Hamazaki et al., 2002), symplekin (Keon et al., 1996) and cingulin (Citi et al., 1988).

ZO-1 is the first TJ protein which was isolated and identified through the work of Goodenough's group in 1986 (Stevenson et al., 1986). This protein was isolated from a TJ-enriched fraction from mouse liver. As a monomeric 220 kDa protein, it was found to

be localized on the cytoplasmic surface of TJs. ZO-2 and ZO-3 were later identified (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994; Balda et al., 1993) and together with ZO-1 constitute the membrane-associated guanylate kinase (MAGUK) homologues of the TJs (Anderson et al., 1995; Woods and Bryant, 1993).

Occludin was the first integral membrane protein of TJs that was identified in chicken liver (Furuse et al., 1993). It consists of four transmembrane domains, two extracellular loops and two intracellular domains (reviewed in Feldman et al., 2005). Expression of occludin into fibroblasts which are normally devoid of occludin and neither make TJs, resulted in increased intercellular adhesion (Van Itallie and Anderson 1997). However, loss of occludin from the TJs did not abrogate the barrier function (Saitou et al., 1998). In addition, studies of occludin knockout mice revealed that neither the morphology of TJs nor the barrier function was affected (Saitou et al., 2000). These results suggested that there must be another transmembrane protein involved in forming the TJs and a search for such a molecule uncovered Cldn family of proteins, which have similar structure but no sequence homology to occludin (Furuse et al., 1998).

1.4) Cldns; major constituents of TJs, protagonists of the barrier formation scenario

Using a chicken liver fraction, Tsukita and colleagues (Furuse et al., 1998) first identified claudin-1 and -2. Transfection of these two Cldns into fibroblasts resulted in formation of well-developed network of strands as evidenced upon freeze fracture analysis (Furuse et

al., 1998). Subsequent data base searching and genomic cloning led to the identification of more family members, expanding the number of Cldns to 24 in mammals up to now (Furuse and Tsukita, 2006). It is of particular interest that the number of Cldn genes identified in some other species even exceeds those in mammals. For example, analysis of the whole genome of the teleost fish, Fugu rubripes (Fugu fish), revealed presence of 56 Cldn genes of which 35 are orthologs of 17 mammalian Cldn genes (Loh et al., 2004).

An important characteristic of Cldn molecules which is also accounted for differential barrier properties in diverse epithelia is diversity of their expression. In other words, different Cldns show tissue-specific expression, thereby resulting in different barrier properties in various TJ-containing epithelia (reviewed in Tsukita et al., 2001). For example, the proximal tubule of the kidney nephrons has the lowest paracellular permeability of any epithelia, and is responsible for reabsorption of a substantial portion of sodium ions. It has been demonstrated that Cldn2 is selectively expressed in this nephron segment (Reyes et al., 2002; Enck et al., 2001). When overexpressed in MDCK cells, Cldn2 increases the permeability for Na^+ along with a marked reduction in transepithelial resistance (TER) (VanItallie et al., 2003). Therefore, selective expression of this Cldn in the proximal tubule confers both cation selectivity as well as leakiness to this segment of nephrons. In addition, it appears that cell type-specific variations in TJs are also determined by different combinations of Cldn isoforms. For example, high level of Cldn2 expression MDCK type II cells results in low TER and cation selectivity, while MDCK type I cells with high TER express low levels of Cldn2 (Furuse et al., 2001; VanItallie and Anderson, 2006).

Structurally, Cldns possess the same features as occludin, including four transmembrane domains, two extracellular loops, one cytoplasmic loop as well as N- and C-terminal tails (Morita et al., 1999) (Figure 2).

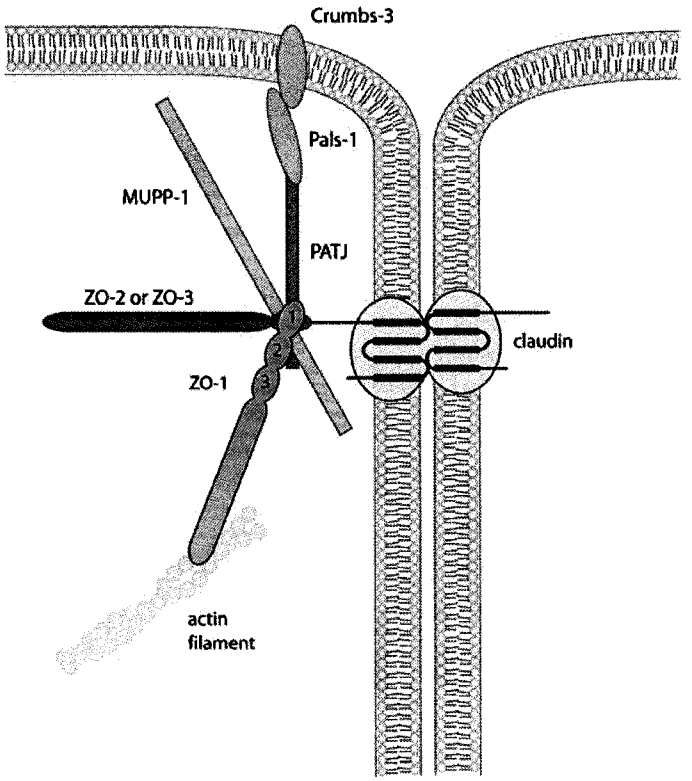

Figure 2. Schematic of Cldns and interacting molecules at the TJs. (Schematic diagram courtesy of Lauren O'Malley)

Through engagement of their extracellular loops, Cldns exhibit two types of interactions: between different TJ strands and within individual strands in a homotypic or heterotypic manner (Furuse et al., 1999; Turksen and Troy 2004). At the paracellular space, interaction of extracellular loops of Cldns from neighboring cells happens in some but

not all combinations. Co-culturing of fibroblasts singly transfected with Cldn1, 2 or 3 showed that Cldn3 strands associated with Cldn1 and Cldn2 of the neighboring cells, but Cldn1 did not interact with Cldn2 strands (Furuse et al., 1999).

The importance of Cldns extracellular loops is reflected in this widely accepted notion that interactions between the loops result in the formation of charged-pores which determine both magnitude of paracellular permeation and its selectivity for ionic charge. Creation of paracellular resistance and charge selectivity by Cldns has been attributed to the presence of charged amino acids on the extracellular loops (VanItallie and Anderson, 2006). In this context, charge-reversing mutagenesis studies have demonstrated the involvement of the first extracellular loop of Cldns in determining paracellular permeation (VanItallie et al., 2003; Colegio et al., 2002).

Although important in determining paracellular permeability properties through engaging their extracellular loops, Cldns have also been broadly studied with respect to their C-terminus, a region which connects these membrane molecules with the intracellular milieu (Figure 2). The C-terminal cytoplasmic tail has a sequence that diverges throughout the Cldn family and except in the case of Cldn12; they end in PDZ-binding motifs. These motifs directly interact with cytoplasmic PDZ-domain-containing proteins including ZO-1, ZO-2, ZO-3, MUPP1 and PATJ (Itoh et al., 1999; Hamazaki et al., 2002; Lemmers et al., 2002; Roh et al., 2002; Tsukita et al., 2001), forming a dense localized network of PDZ interactions under the Cldn strands. In spite of critical role of PDZ motif in PDZ-dependent interactions, some recent studies have suggested the importance of other regions of the cytoplasmic tail. Ruffer and Gerke replaced the entire cytoplasmic domain of Cldn1 with a GFP tag and demonstrated that the tail truncated protein fails to

localize to the plasma membrane and instead accumulates in intracellular vesicles (Ruffer and Gerke, 2004). In addition, the findings from our lab demonstrated that the overexpression of a tail-truncated mutant of Cldn6 mislocalizes Cldn6 and other Cldn proteins to the cytoplasm, emphasizing the importance of the Cldn tail domain in membrane targeting (Arabzadeh et al., 2006). The importance of Cldn tail in controlling paracellular permeability is also evidenced by a study where swapping tails between two different Cldns significantly affects the TJ barrier properties through effects on protein stability (VanItallie et al., 2004). Modifications of C-terminal tail have also been reported to affect Cldn localization as well as barrier function. Two types of post-translational modification recognized for Cldn proteins up to now include lipid modification and phosphorylation. With respect to the former, the presence of conserved cysteines near the cytoplasmic ends of the second and fourth transmembrane domains of all 24 Cldns make them targets of acylation with palmitic acid, a type of modification which has been reported in other integral membrane proteins as well (Stipp et al., 2003; Bijlmakers and Marsh, 2003; Kalinina and Fricker, 2003; VanItallie et al., 2005). Therefore, based on such speculation Anderson's group provided the first demonstration that Cldns are palmitoylated at these two membrane-proximal sites (VanItallie et al., 2005). In addition, in the case of Cldn14, mutation of either or both of these sites abolished palmitoylation of Cldn14 and reduced the TJ localization of this Cldn, suggesting the importance of fatty-acid acylation in efficient localization of Cldns into TJs (VanItallie et al., 2005). Moreover, the presence of potential serine and/or threonine phosphorylation sites in the C-terminal domains of Cldns make them likely targets for this type of modification. In fact, a number of studies have shown phosphorylation of these sites. Phosphorylation of

Cldn5 in response to cAMP was correlated with the enhancement of barrier function in cultured endothelial cells (Ishizaki et al., 2003). On the other hand, Yamauchi and colleagues demonstrated phosphorylation of Cldn1through Cldn4 by the threonine-serine kinase WNK4 where a disease-causing mutant form of WNK4 hyperphosphorylated Cldns and increased the paracellular permeability in the epithelial cells (Yamauchi et al., 2004). Decreased TER and increased paracellular permeability have further been shown in ovarian cancer cells where a constitutive phosphorylated form of Cldn3 was overexpressed (D'Souza et al., 2005). In summary, post-translational modification of the Cldn tails does appear to further influence Cldn localization and integration into TJs as well as the barrier function.

Both animal models and correlation of Cldn mutations with some inherited human diseases have provided enough evidence suggesting that Cldns are crucial for the barrier function of TJs (Table 2). Some lines of evidence are provided here: Cldn1 knockout mice die from dehydration within first day of birth due to severe defects in epidermal barrier function (Furuse et al., 2002). Similarly, Cldn6 overexpression generates a defective epidermal barrier leading to the death of transgenic mice within 48 hours of birth (Turksen and Troy 2002). Cldn11 expression is restricted to the sertoli cells of the testis as well as in the myelinating cells that insulate the axons, thereby allowing the saltatory conduction of the action potential in CNS (Gow et al., 1999). Cldn11-knockout mice exhibit male infertility and neurological defects consistent with loss of TJ fibrils in sertoli cells and CNS myelin cells (Gow et al., 1999). Cldn14-null mice show degeneration of cochlear hair cells and therefore deafness (Ben Yosef et al., 2003).

Consistent with the mouse model, mutations in Cldn14 have been found to cause nonsyndromic deafness in two large Pakistani families (Wilcox et al., 2001). Cldn1 gene mutations are reported in neonatal sclerosing cholangitis with ichthyosis (Hadj-Rabia et al., 2004). In addition, mutations in Cldn16 have been found in patients with hypomagnesemia and hypercalciuria where normal paracellular permeability to calcium and magnesium is impaired (Simon et al., 1999; Hou et al., 2005).

Table 2. Cldn knockout and transgenic mice as well as genetic diseases of Cldn mutations

Gene	Mouse phenotype	Disease	Reference
Cldn1			
knockout	Skin barrier dysfunction		Furuse et al., 2002
mutation		Neonatal sclerosing cholangitis with ichthyosis	Hadj-Rabia et al., 2004
Cldn5 knockout	Size-selective blood-brain barrier defect		Nitta et al., 2003
Cldn6 transgenic	Skin barrier dysfunction		Turksen and Troy, 2002
Cldn11 knockout	Male infertility and CNS myelin defect		Gow et al., 1999
Cldn14			
knockout	Degeneration of cochlear hair cells		Ben Yosef et al., 2003
mutation		Nonsyndromic deafness	Ben Yosef et al., 2003
Cldn16 mutation		HHNC[a]	Simon et al., 1999

[a] Hypomagnesemia hypercalciuria with nephrocalcinosis

In summary, the up-and-coming picture of TJs at the molecular level shows that the complex structure of TJ is the result of dynamic homotypic and heterotypic interactions

among its components which of those, Cldns seem to play a pivotal role. Therefore, Cldn alterations –up or down (as evidenced by overexpression or knockout studies) - have been suggested to ultimately alter proportions of other TJ-associated molecules, thereby disrupting the balance required for the functional integrity of TJ fibrils. This can in turn result in attenuation of permeability barrier function in different epithelia, contributing to the pathology of many diseases where increased tissue permeability is a common feature (Burgel et al., 2002; Kucharzik et al., 2001; Wolburg et al., 2003; Mullin 2004; Morin 2005; Swisshelm et al., 2005).

Although Cldns are well-known for their structural role, another emerging theme of Cldn studies is consistent with the notion that changes in the expression of these molecules may also affect other cellular processes. In other words, the new picture of Cldn proteins visualizes them in the establishment of epithelial barriers by at least two means. First, they control barrier function by limiting paracellular diffusion. This occurs through homo- and heterophilic interactions among themselves as well as with other TJ-associated transmembrane proteins (Wittchen et al., 1999; Gonzalez-Mariscal et al., 2003; Chiba et al., 2008). Second, they may also regulate the barrier formation by modulating cellular events including proliferation and differentiation. This function is supposedly executed through the interactions of these molecules with their yet-unidentified intracellular partners. The following section provides evidence to the effect of Cldns on the processes of cell proliferation and differentiation.

1.5) Role of Cldns in permeability barrier formation seems to extend beyond the structural role

Although the contribution of Cldns to TJ barrier formation has long been known to arise from their structural role, recent studies propose that Cldns are multifunctional players of TJ barrier formation, extending their function beyond the borders of homotypic and heterotypic interactions into the realm of interplay with essential cellular processes such as proliferation and differentiation (Turksen and Troy, 2001; Turksen and Troy, 2002; Troy et al., 2005; Arabzadeh et al., 2006; Troy et al., submitted; Atasoy et al., submitted; Dhawan et al., 2005; Tamura et al., 2008). Whether Cldns are directly or indirectly linked to these cellular events is an issue that remains to be elucidated.

For example, the association of Cldns with cell cycle regulatory pathways was reported recently, where increased expression of Cldn1 in human colon carcinoma cells led to the activation of β-catenin/Tcf-Lef signaling and its target genes (myc, cyclin D1), all of which are well known modulators of cell proliferation, survival and invasion (Dhawan et al., 2005). In another study, ablation of Cldn15 led to the enhanced proliferation of normal crypt cells resulting in megaintestine in Cldn15 deficient mice (Tamura et al., 2008). The association of Cldn-based signaling and cell differentiation has been extensively demonstrated in a series of studies from our own laboratory. First, ectopic expression of Cldn6 in murine epidermis disrupted the epidermal differentiation, as evidenced by abnormalities in the expression of keratins and cornified envelope proteins (Turksen and Troy, 2002; Troy et al., 2005). Furthermore, individual expression of three different cytoplasmic tail-mutated forms of Cldn6 in the epidermis of transgenic mice

resulted in deregulation of epidermal differentiation as well as proliferation to varying degrees (Arabzadeh et al., 2006; Troy et al., submitted; Atasoy et al., submitted).

It is noteworthy that the connection of Cldn proteins with cell proliferation-differentiation has not only been addressed by the in vitro and in vivo genetic manipulations as mentioned above, but it has also been noticed in the context of cancerous cells whose homeostasis (i.e. the balance between cell proliferation and differentiation) is obviously abrogated. For example, altered expression of Cldn5 has been associated with increased proliferation of esophageal tumor cells (Takala et al., 2007), while reduced expression of Cldn4 was shown to correlate with loss of differentiation in gastric adenocarcinoma (Lee et al., 2005). In addition, increased expression of Cldn1 along with its mislocalization from the cell membrane into the nucleus was strongly correlated with changes in the cellular phenotype of human colon carcinoma cells (Dhawan et al., 2005), which are consistent with a lack of differentiation as well as increased growth and aggressiveness of this type of tumor. Results of our own studies have also demonstrated for the first time the striking changes in expression of epidermal Cldns which accompany loss of cell polarity and differentiation during the formation of epidermal tumors (Arabzadeh et al., 2007).

Taken together, these data suggest that Cldn proteins of TJ signaling pathway cross talk with the components of cell proliferation-differentiation pathway; therefore changes imposed on players of either pathway can supposedly be reflected in alteration of the other one. As partly mentioned above, such a concept has been repeatedly asserted through our own investigations. Therefore in the next part, I will first present the

foundation of our research on Cldns and then upon how the mutual relationship between these molecules and cell differentiation and proliferation events was further addressed in our studies.

1.6) Statement of problems and objectives

The research on the process of epidermal differentiation has been going on in our laboratory for over a decade. First, by using an in vitro system (embryonic stem cells differentiating into embryoid bodies) that mimics early events of development in mouse embryo; we attempted to study and identify genes regulating the process of epithelialization (Turksen and Troy, 2001). One of the genes identified in this study belonged to the Cldn tight junction family, namely Cldn6. We next demonstrated that the product of the Cldn6 gene is one of the earliest proteins expressed during the commitment of embryonic stem cells to the epithelial cell fate, as its expression coincides with that of an early epithelial marker, keratin 8 (Turksen and Troy, 2001). This finding raised the question whether Cldn6 may also have a role in the process of differentiation in the stratified epithelium of the skin, namely the epidermis. Therefore, Cldn molecules, and in particular Cldn6, were brought into the picture of our further investigations in the context of epidermal differentiation and barrier function. To address the involvement of Cldn6 in these processes, our lab took a genetic approach and using an epidermis-specific promoter (involucrin, Inv. promoter); overexpression of Cldn6 was targeted to its endogenous location in the skin, namely the suprabasal compartment of the epidermis (Turksen and Troy, 2002). Both homozygous and heterozygous mice overexpressing

Cldn6 displayed epidermal differentiation abnormalities and skin barrier dysfunction at birth, the severity of which was apparently dependent on the level of Cldn6 overexpression. In other words, aberrant differentiation was severe enough to compromise barrier function of the epidermis to the water loss in the former mice, thereby resulting in their neonatal death (Turksen and Troy, 2002). However, the heterozygous counterparts underwent a reparative process of epidermal differentiation after birth which in turn rescued the barrier function of the epidermis and allowed their survival into adulthood (Troy et al., 2005). These findings suggested that a precise balance of Cldn6 regulates the function of the epidermis, clearly by affecting the differentiation process in this tissue. However, the mechanism behind this effect remained to be elucidated. At this point, our attention turned to the C-terminal tail of Cldn6, knowing that C-terminus of Cldns in general is a region that anchors these molecules to the cell cytoskeleton and participates in a number of intracellular interactions (for reviews see; Miyoshi and Takai, 2005; Tsukita et al., 2001; Van Itallie and Anderson, 2004). Although the precise function and extent of these interactions largely remain unknown, they suggest the potential of Cldns for signal transduction in addition to their structural role. Therefore, the C-terminus of Cldn6 was **hypothesized** to engage in a yet-unidentified pathway transducing signals of epidermal differentiation. To this end, one good approach to find out about the function of Cldn6 in the context of epidermal differentiation was the use of different Cldn6 tail deletions. Therefore, three different truncations of the Cldn6 tail (CΔ206, CΔ196, CΔ187) were engineered and expressed under the control of the same Inv. promoter in the epidermis of transgenic mice (Arabzadeh et al., 2006; Troy et al., submitted; Atasoy et al., submitted). Although a

contributor to the studies of transgenic mice expressing two shorter deletions in the cytoplasmic tail of Cldn6 (Troy et al., submitted: study of Inv-Cldn6-cΔ196 mice; Atasoy et al., submitted: study of Inv-Cldn6-cΔ206 mice), my work primarily comprised analysis of mice expressing the tail-ablated Cldn6 (Cldn6-cΔ187) which is lacking nearly the entire C-terminal portion of the molecule (Arabzadeh et al., 2006). As presented in the second chapter of this thesis (***manuscript #1: Arabzadeh et al., 2006***), Inv-Cldn6-cΔ187 mice were neither lethal nor did they exhibit an epidermal differentiation abnormality at birth. However, a detailed analysis of these mice revealed an anomalous program of epidermal proliferation-differentiation starting after the first week of birth. In addition, we demonstrated that disruption of epidermal homeostasis was in fact triggered by the altered localization of several Cldn isoforms as well as transgene itself in the epidermis (Arabzadeh et al., 2006). Along with four other kinds of transgenic mice generated in our lab (homozygous and heterozygous Cldn6 overexpressing mice, Inv-Cldn6-cΔ196 mice and Inv-Cldn6-cΔ206 mice), Inv-Cldn6-cΔ187 mice further supported the presumed signaling that may intertwine Cldns with components of the cellular differentiation-proliferation pathway. Consistent with this notion, it seemed quite likely to us that such interconnection may be of reciprocal nature; meaning that deregulating differentiation-proliferation itself may also lead to altered homeostasis of Cldn molecules. Should this concept be correct, it will have the potential to propose that Cldns are indicators of a differentiation-proliferation anomaly which has been shown to be the common characteristic of many diseases including cancers of epithelial origin. Therefore, our **next hypothesis** was whether altered differentiation-proliferation of the epidermis may also

lead to changes in the Cldn profile. To this end, two types of in vivo approaches were taken to recapitulate this interconnection.

In the first one, an injury response was induced in the murine epidermis using a single application of the phorbol ester, 12-*O* tetradecanoyl phorbol 13-acetate (TPA). TPA, a protein kinase C activator and tumor promoter, has long been used as a chemical stimulus in experimental model of the wound/injury response (Marks and Furstenberger, 1993; Kumar and Holmgren, 1999). In this model, the general response of the epidermis consists of a hyperplasia (result of increased proliferation and aberrant differentiation) combined with an inflammatory reaction. Both elements of this response were observed in our study, but more notably perturbation of epidermal homeostasis was accompanied by modulations of Cldns in the epidermis as described in the third chapter of this thesis ***(Manuscript #2; Arabzadeh et al., 2008)***.

In the second approach, we used classical model of murine skin chemical carcinogensis which involves use of a single dose of a carcinogen followed by repetitive applications of TPA. This model is very well-known for progressive changes in epidermal cell morphology, proliferation and differentiation. In fact, epidermal tumors will develop when keratinocyte proliferation and differentiation becomes dysregulated, resulting in sustained epidermal hyperplasia. Using this model, we observed dramatic changes in Cldn distribution and localization which happened in parallel with loss of differentiation and permeability barrier changes as described in the fourth chapter of this thesis ***(Manuscript #3; Arabzadeh et al., 2007)***.

In summary, our studies provide strong evidence that Cldns as principal components of TJ barrier intertwine with components of cell differentiation-proliferation pathway and they may be reciprocally interdependent.

Chapter 2

Role of the Cldn6 cytoplasmic tail domain in membrane targeting and epidermal differentiation in vivo

by

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Abstract

It is widely recognized that the Claudin (Cldn) family of four tetraspan transmembrane proteins is crucial for tight junction (TJ) assembly and permeability barrier function; however the precise role of the tail and loop domains in Cldn function is not understood. We hypothesized that the cytoplasmic tail domain of Cldn6 is crucial for membrane targeting and hence in epidermal permeability barrier (EPB) formation. To test this hypothesis via a structure-function approach, we generated a tail deletion of Cldn6 (CΔ187) and evaluated its role in epidermal differentiation and EPB formation through its forced expression via the Involucrin (Inv) promoter in the suprabasal compartment of the transgenic mouse epidermis. Even though a functional barrier formed, Inv-CΔ187 mice displayed histological and biochemical abnormalities in the epidermal differentiation program, and stimulation of epidermal cell proliferation in both the basal and suprabasal compartments of the interfolliclar epidermis, leading to a thickening of the epidermis after one week of age that persisted throughout life. Although some membrane localization was evident, our studies also revealed a significant amount of not only Cldn6, but also Cldn10, Cldn11 and Cldn18 in the cytoplasm of transgenic epidermal cells, as well as activation of a protein unfolding pathway. These findings demonstrate that overexpression of a tail truncation mutant of Cldn6 mis-localizes Cldn6 and other Cldns to the cytoplasm and triggers a postnatal increase in proliferation and aberrant differentiation of the epidermis, emphasizing the importance of the Cldn tail domain in membrane targeting and function in vivo.

Introduction

Claudins (Cldns) comprise a family of integral membrane proteins involved in the formation of tight junction (TJ) fibrils, which are responsible for the formation and maintenance of the epidermal permeability barrier (EPB) (36, 38, 40, 42). Recent studies indicate that Cldn6 overexpression (34, 40) and Cldn1 deletion mutants (15) are associated with EPB defects in vivo, and that the level of Cldn expression appears to be crucial in EPB integrity and function (34, 38, 40). Knowledge of the overall structure-function of the Cldn family of proteins is limited, however they are known to have three distinct and characteristic functional domains: (i) four transmembrane-spanning regions, (ii) two extracellular loops responsible for permeability barrier formation within the paracellular space and specific ion selectivity residing within the first external loop (8, 43, 44); and (iii) a cytoplasmic C-terminus that functions to anchor to the cytoskeleton apparently through scaffolding molecules such as zonula occludens-1 (ZO-1) (12, 22, 36, 38, 42, 43). It has been demonstrated that PDZ-binding sequences at the C-terminus of the Cldn cytoplasmic tail are responsible for their association with other PDZ domain proteins (16, 19, 22). However, although predicted to be important, the role of the tail domain in the targeting of Cldns to the membrane, as well as the regulation of their function and stability in the stratified epithelium has not been demonstrated.

In this study, we investigated the consequences of expressing a tailless Cldn6, namely CΔ187, in the suprabasal layer of the mouse epidermis. Expression of CΔ187 resulted in accumulation of not only Cldn6 but also Cldn10, Cldn11, and Cldn18 in the cytoplasm and elicited histological and biochemical perturbations in epidermal proliferation and

differentiation evident after one week of age and persisting throughout life. These results confirm the importance of the cytoplasmic tail domain of Cldn molecules in membrane targeting and hence their function in TJs in vivo.

Materials and Methods

Generation of Transgenic Mice

Inv-CΔ187-FLAG transgenic mice were generated following the same strategy as was described for the generation of our full-length Inv-Cldn6 transgenic mice (40). Briefly, the FLAG® epitope tag (Sigma-Alderich) was fused to mutant tailless mouse Cldn6 by PCR and CΔ187-FLAG was subcloned into pCRII vector and sequenced for verification. The lacZ insert of the pInv plasmid (H3700-pL2) (6, 7) was replaced with the CΔ187-FLAG coding sequence to create the Inv-CΔ187-FLAG expression vector. A 585bp fragment containing CΔ187-FLAG cDNA was introduced into the NotI site of the Inv cassette and the resultant construct was designated pInv-CΔ187-FLAG. SalI digestion was used to excise the plasmid vector by releasing the Inv promoter as well as the downstream Cldn6/SV40poly(A) DNA sequence, and purification was done by the QIAamp tissue kit (Qiagen) according to the manufacturer instructions. Ovum collected from superovulated CD1 mice were used to generate transgenic mice at the OHRI Transgenic Mouse Facility as previously described (40). Genomic DNA extracted from ear trimmings was used to screen for the presence of the transgene by PCR analysis using Cldn6 forward and FLAG® reverse primers (**Table 1**).

Table 1: PCR Primers

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Size (bp)
Cldn6 / FLAG	ATGGCCTCTACTGGTCTGCA	TCACTTATCGTCGTCGTCCTTG	585
GAPDH	CTCAACTACATGGTCTACAT	AGTAGACTCCACGACATACT	180
Atf4	GATATCTCTGAAGGAGACAG	GACCTCTTCTATCAGGTCTT	462
Atf6	GAGTATATGCTAGGACTGGA	CTGAGAGACTCTGTTGTGTT	449
Eif2ak4	GATTACCTACACAGCAACTC	GTCTCAATGTAGTCTTGTCC	449
Eif2s1	GATGACAAGTACAAGAGACC	CATCTGTATCTGTGACCACT	433
Ern1	CTAACGCCTACTCTGTATGT	GTGTGTTGTCTGAAGTCTG	406
Hspa5	ATAAGAGAGAGGGAGAGAAG	CTTCAGATCAGAGTCTTCC	422

RNA isolation and RT-PCR

Backskin samples were dissected from the mid-dorsal region of Inv-CΔ187 transgenic mice and their age-matched wild type counterparts. Samples were immediately frozen in liquid nitrogen, and then homogenized in TRIzol® (Invitrogen) reagent for total RNA isolation according to the instructions of the manufacturer. The isolated RNA was subjected to DNase (Invitrogen) treatment and first strand cDNA was synthesized using random hexamers (Applied Biosystems) and 1μg of each RNA sample. PCR analysis was then performed as previously described using specific primers (see **Table 1**). RT-PCR products were visualized on ethidium bromide-containing agarose gels and images were acquired using Bio-Rad Molecular Analyst Software Version 1.2 (Bio-Rad Laboratories).

Immunohistochemistry and Histology

For immunohistochemical and histological analyses both Inv-CΔ187 and wild type backskin samples were collected at the following ages: newborn, one-, two- and three-week as well as one-, three- and five-month old.

a) Sample Collection: At the time points described above, backskin samples (~1cm^2) were dissected from the mid-dorsal region of Inv-CΔ187 transgenic and wild type mice. Frozen sections were required for FLAG®, Ki67 and Occludin immunostaining, whereas all other staining and histology (H&E) was performed on paraffin sections. For frozen sections: skin samples were orientated in HistoPrep™, submerged in isopentane containing dry-ice and 5μm sections were cut as previously described (37). Samples were warmed at room temperature for 3 minutes followed by fixation in methanol at –20°C for 10 minutes and washing in PBS before immunostaining (37). For paraffin sections: skin samples were fixed in Bouin's fixative (75% saturated picric acid, 20% formaldehyde and 5% glacial acetic acid) for 12-16 hours at room temperature and dehydrated through a series of ethanol washes before being embedded and sectioned. Sections (5μm thick) were dewaxed and rehydrated followed by antigen unmasking and washing steps prior to histological analysis and immunostaining (35).

b) Immunohistochemistry: Non-specific antibody binding was blocked (10% goat serum, 0.8% BSA, 1% gelatin in PBS) for 30 minutes at room temperature followed by incubation in wash buffer (0.8% BSA, 1% gelatin in PBS). Primary antibodies were appropriately diluted in incubation buffer (1% goat serum, 0.8% BSA, 1% gelatin in PBS) and sections were incubated for 1-2 hours at room temperature followed by incubation in wash buffer. Antibodies against the following were used: FLAG® (M2 monoclonal) (1:440; Sigma), K15 (1:100) (UC55), K5 (1:100) (5054), K14 (1:100) (199), K1 (1:100) (UC81), Involucrin (1:100; BabCO), Filaggrin (1:100; BabCO), Loricrin (1:100) (UC84), TGase-3 (1:100; a gift from Dr. Len Milstone), K6 (1:100; BabCO),

K17 (1:500; a gift from Dr. Pierre Coulombe), Ki67 (1:25; abcam), Cldn1 (6:100; Zymed Laboratories), Cldn6 (1:50), Cldn10 (1:25) Cldn11 (1:50), Cldn18 (1:50) and Occludin (1:100; Zymed). Secondary antibodies against rabbit, mouse, rat and chicken conjugated to FITC or Texas Red (Jackson ImmunoResearch Laboratories) were used at a 1:50 dilution for 1 hour at room temperature followed by incubations in wash buffer and PBS. Sections were incubated with Hoechst (1:50; Sigma) for 15 minutes before mounting with Mowiol® 4-88 (Calbiochem) containing 2.5% 1,4 diazobicyclo-[2,2,2]-octane (DABCO) (Sigma).

c) Photography: Images were captured with a brightfield/fluorescence capable Zeiss Axioplan 2 microscope (Carl Zeiss Canada Ltd) equipped an AxioCam camera (Carl Zeiss Canada Ltd) and Axio Vision 2.05 software (Carl Zeiss Canada Ltd). Adobe Photoshop version 7.0 (Adobe Systems, Inc.) was used for image processing.

Protein Isolation and Immunoblotting

Proteins were extracted from freshly dissected backskin samples (0.4g) by homogenizing in SDS extraction buffer (62.5mM Tris pH 6.8, 25% Glycerol, 2% SDS, 2% β-mercaptoethanol, with pepstatin A and a complete mini protease inhibitor cocktail [Roche Diagnostics] tablet) followed by high-speed centrifugation at 4°C. The supernatant containing the proteins was collected and assayed for protein concentration. Proteins were incubated at room temperature for 30 minutes in sample reducing buffer (62.5mM Tris pH 6.8, 6M Urea, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 2% β-mercaptoethanol), boiled for 5 minutes and centrifuged at high speed for 10 minutes, then

10μg samples were separated on 12% SDS-PAGE gels, transferred to nitrocellulose and incubated in blocking buffer (5% skim milk in TBS/0.1% Tween-20 [TBS-T]) for 1 hour at room temperature. Primary antibodies were diluted in incubation buffer (1% goat serum, 0.8% BSA, 1% gelatin in TBS-T) and blots were incubated overnight at 4°C with antibodies against FLAG® (polyclonal) (1:500; Sigma) and GAPDH (1: 20 000, abcam). After washing in TBS-T, blots were incubated for 1 hour at room temperature in HRP-conjugated secondary antibodies against rabbit or mouse (1:20 000; Amersham Biosciences) diluted in 5% skim milk/TBS-T. Following washes in TBS-T, blots were incubated in ECL™ Western blotting detection reagents (Amersham Biosciences) and proteins were visualized on Kodak BioMax XAR film (Kodak). Films were digitally scanned and images were processed with Adobe Photoshop version 7.0 (Adobe Systems, Inc.).

Transfection of HaCat cells and Immunofluorescence Analysis

HaCat cells were plated on coverslips 24 hours prior to transfection at a density of 250 000 cells/35mm dish in 2ml DMEM (high glucose without L-glutamine; Invitrogen) supplemented with 10% fetal calf serum (Hyclone), 0.1mM non-essential amino acids (Invitrogen) and 1mM sodium pyruvate (Invitrogen) without antibiotics. For transfection, 0.5μg of Inv-cΔ187-FLAG was added to 250μl unsupplemented DMEM and in a separate tube 10μl Lipofectamine™ 2000 reagent was mixed into 250μl unsupplemented DMEM. Followed by incubation for 5 minutes at room temperature, 250μl DMEM/DNA mixture and 250μl DMEM/Lipofectamine™ 2000 mixture were combined and incubated for 20 minutes at room temperature. The transfection mixture

(500μl) was added dropwise to each 35mm dish and the cells were incubated for 5 hours at 37°C / 5% CO_2. The media was then replaced with DMEM supplemented with 10% fetal calf serum, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen). After 24 hours, cells on coverslips were fixed with methanol for 10 minutes at –20°C and were rinsed with PBS. Cells were incubated in a humidified chamber for 30 minutes with antibodies against FLAG® (M2 monoclonal) (1:440; Sigma) and following a wash in PBS, secondary antibodies against mouse conjugated to FITC (Jackson ImmunoResearch Laboratories) were used at a 1:50 dilution for 30 minutes at room temperature. Following a wash in PBS, cells were incubated with Hoechst (1:100; Sigma) for 10 minutes and mounted onto slides with Mowiol® 4-88 containing 2.5% DABCO. Observation by epifluorescence and photography was as described above.

Animal Photography

Animals were euthanized by isofluorane/CO_2 and images were acquired using a Nikon COOL-PIX950 digital camera (Nikon Canada) and processed using Adobe Photoshop version 7.0.

Results

Generation of Inv-CΔ187 Transgenic Mice

We have previously demonstrated that Cldn6 cDNA overexpressed in differentiating mouse epidermal cells results in disruption of the epidermal differentiation program and a dysfunctional EPB, leading to rapid postnatal death from dehydration (34, 40). To begin

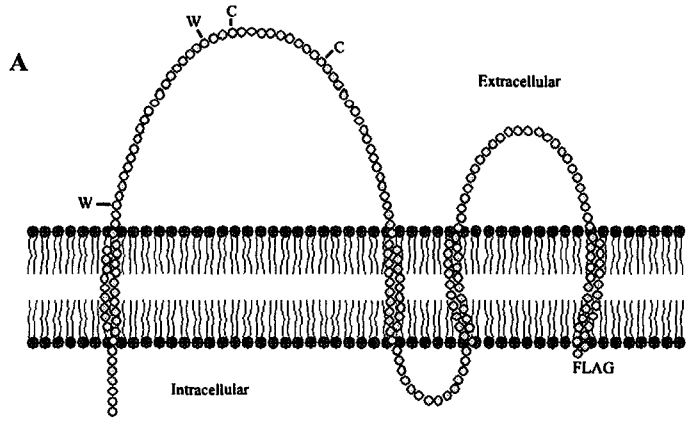
a structure-function analysis of Cldn6 domains in these activities, we created a "tailless Cldn6 mutant" (CΔ187) by deleting the C-terminal cytoplasmic tail domain after amino acid 187, leaving 4 residues next to the fourth transmembrane spanning region (**Figure 1A&1B**). We used transgenic mouse technology to express a FLAG® epitope-tagged CΔ187 cDNA under the control of a 3.7kb 5'-flanking element of the human Involucrin gene (Inv), the same promoter as was used for our full-length Cldn6 model (40), to drive transgene expression to the suprabasal cells of the epidermis where TJs are localized (**Figure 1C**) (6, 7, 40). PCR using Cldn6 forward (from the start codon) and FLAG® reverse (from the 3' end) primers (585bp) revealed that three transgenic founder mice (two female and one male) were generated (**Figure 1D & Table 1**) and lines were established exhibiting indistinguishable phenotypes. To examine the localization of CΔ187 in the transgenic epidermis, we used immunohistochemistry with anti-FLAG® antibodies (**Figure 1E**). As expected, there was no FLAG® protein expression in the wild type epidermis while expression was restricted to the upper spinous and granular layers of the transgenic epidermis. Notably, however, rather than expression primarily localized to cell-cell junctions, FLAG®-tagged CΔ187 was abundant in the cytoplasm, presumably due to inefficient membrane targeting (**Figure 1F**). In addition, immunoblotting using anti-FLAG® antibodies confirmed a ~19.5kD band in the Inv-CΔ187 (TG) and not the wild type (WT) backskin samples (**Figure 1G**). To complement our in vivo observations and to verify the cytoplasmic accumulation of CΔ187-Cldn6, we transfected the same construct into cultured basal-like, undifferentiated, exponentially growing monolayers of HaCat cells, where it has been demonstrated that Cldn6 is not expressed (32, Turksen and Troy, unpublished observations). Again CΔ187 accumulated

in the cytoplasm, not at the cell membrane (**Figure 1H**), confirming a defect in the ability of CΔ187 to target to the membrane in vivo and in vitro.

Next we explored the consequences of the expression of CΔ187 in the overall phenotype of transgenic mice as compared to the phenotype of transgenic mice overexpressing full-length Cldn6 previously described (34, 40). Phenotypically, Inv-CΔ187 transgenic neonates appeared comparable to their wild type counterparts. In fact, newborn transepidermal water-loss (TEWL) measurements (DPM value of 103) and X-Gal staining (no penetration) confirmed that Inv-CΔ187 transgenic mice did not display the barrier dysfunction at birth seen in their full-length Cldn6 counterparts (not shown). In addition, cornified envelopes extracted from newborn CΔ187 epidermis were essentially indistinguishable from wild type extracts (not shown) providing further support that indeed an intact barrier was achieved during development despite the expression of a tailless Cldn6. However, with the emergence of hair fibres, Inv-CΔ187 transgenic mice are easily identifiable by their coat appearance, which is not as sleek looking as their wild type counterparts (**Figure 1I**), a phenotype that persists throughout life. Overall, the hair phenotype of the Inv-CΔ187 transgenic mice is similar to that of the Inv-Cldn6 mice and appears not to be directly related to the other epidermal defects we observe in either case (34); therefore it has not been investigated further here.

Figure 1: Inv-CΔ187 Transgenic Mice. The tailless Cldn6 (CΔ187) mutant was created by deleting the C-terminal cytoplasmic tail domain after amino acid 187, leaving 4 residues next to the fourth transmembrane spanning region (**1A**). The protein sequence of Cldn6 is shown with the transmembrane spanning regions enclosed within a box, the CXXC motifs underlined and the truncation at amino acid 187 indicated (**1B**). Transgenic mice were created using the Inv promoter to drive the expression of CΔ187 to the suprabasal cells of the epidermis where TJs are localized (**1C**) and transgenic mice were identified using Cldn6 forward and FLAG reverse primers (585bp) (**1D**, positions marked with red arrows on **1C**). Transgene localization was restricted to the upper spinous and granular layers of the transgenic epidermis as visualized by immunohistochemistry using anti-FLAG® antibodies (**1E**); however, there was a prominent cytoplasmic accumulation (**1F**), presumably due to inefficient membrane targeting. Immunoblot analysis with anti-FLAG® antibodies confirmed a ~19.5kD band in the Inv-CΔ187 (TG) and not the wild type (WT) backskin samples using anti-GAPDH as a loading control (**1G**). HaCat cells transfected with Inv-CΔ187-FLAG also showed a significant cytoplasmic accumulation, confirming a defect in the ability of CΔ187 to target to the membrane both in vivo and in vitro (**1H**). The CΔ187 transgenic mice are easily identifiable by their coat appearance, which is not sleek as compared to the wild type; a phenotype that persists throughout life (**1I**).

Figure 1



B

mastglqilg ivltllgwvn alvscalpmw kvtafignsi vvaqmvwegl

wmscvvqstg qmqckvydsl lalpqdlqaa ralcvvtlli vllgllvyla

gakcttcved rnsksrlvli sgiifvisgv ltlipvcwta hsiiqdfynp

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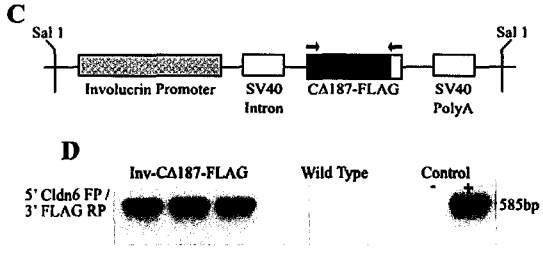
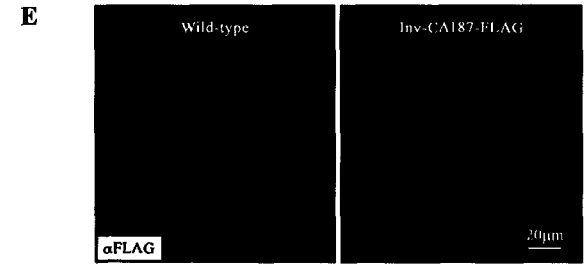
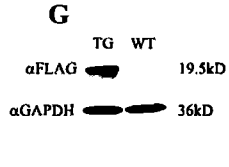
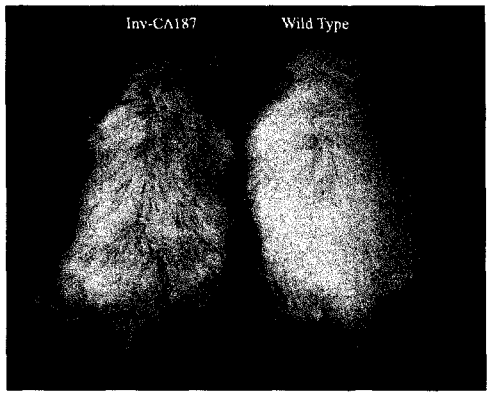
stsvphsrgp seyptknyv

CΔ187







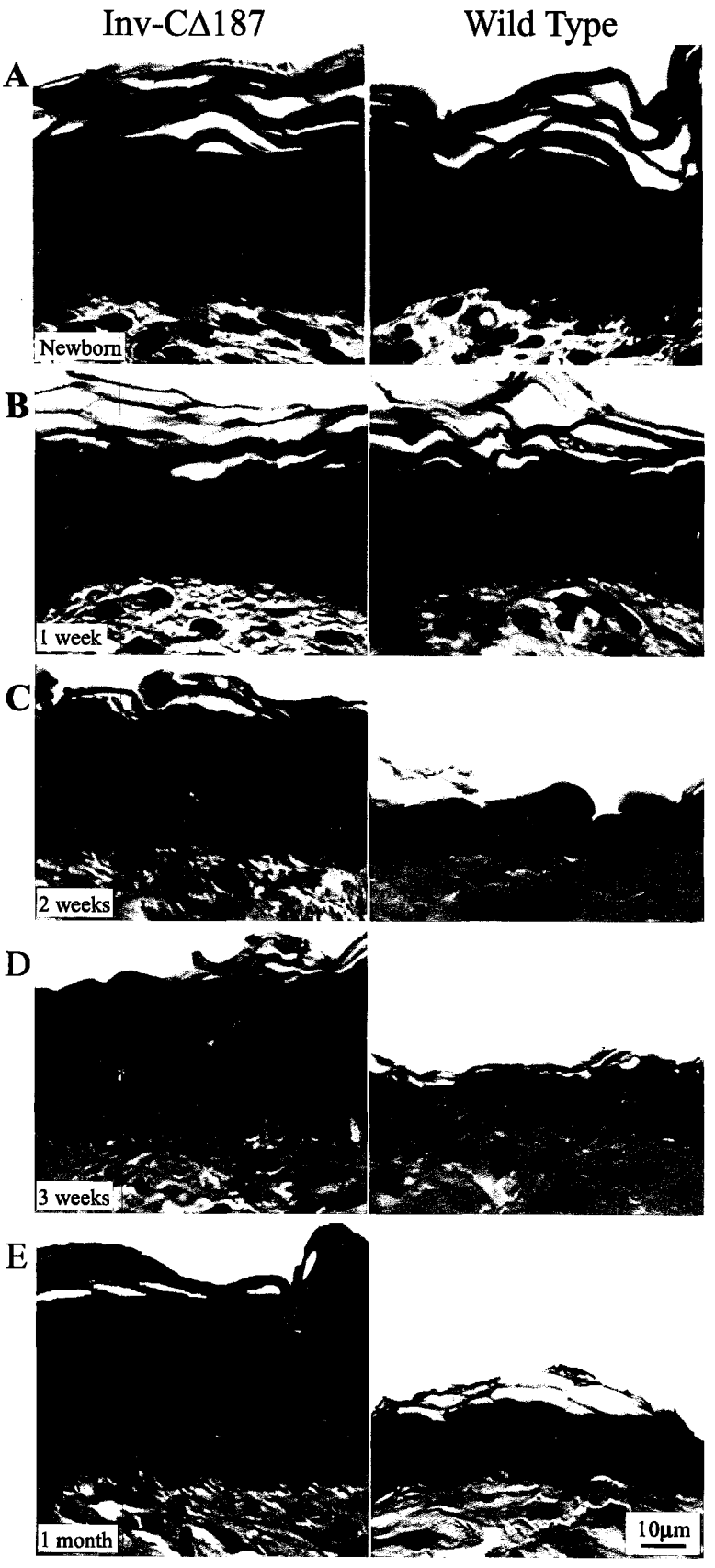


Expression of Inv-CΔ187 Elicits Morphological Changes in the Epidermis

A histological analysis of newborn, one-, two- and three-week old as well as one-, three- and five-month old Inv-CΔ187 backskin samples as compared to their age-matched wild type counterparts was done to examine the morphology of the transgenic epidermis. As anticipated from the observations described above no obvious differences were seen in epidermal morphology in the Inv-CΔ187 (left panel) versus wild type (right panel) newborn or one week old backskin samples (**Figure 2A & 2B**). However, after one week of age, a progressive thickening of the transgenic epidermis versus the normal thinning pattern of the wild type epidermis was seen (**Figure 2C-D**). After three weeks of age and persisting throughout life, a thick epidermis was present with striking morphological abnormalities in architecture and differentiation (see below). The transgenic backskin was characterized by an increase in the number of spinous layers, wherein cells exhibited some degree of disorganization, abnormalities in the upper differentiating layers including parakeratosis with the prevalent appearance of nuclei as well as an obvious but improperly packed granular layer, and a thicker stratum corneum (**Figure 2E**).

Figure 2: Histological Abnormalities in the Inv-CΔ187 Epidermis. A histological analysis of Inv-CΔ187 backskin samples as compared to their age-matched wild type counterparts revealed no obvious differences in the epidermal morphology of the Inv-CΔ187 (left panel) versus wild type (right panel) at newborn (**2A**) or one week of age (**2B**). However, after one week of age, a progressive thickening of the transgenic epidermis versus the normal thinning pattern of the wild type epidermis was seen (**2C-D**). After three weeks of age and persisting throughout life, a thick epidermis was present in the transgenic samples characterized by an increase in the number of spinous layers, wherein cells exhibited some degree of disorganization, abnormalities in the upper differentiating layers including parakeratosis with the prevalent appearance of nuclei as well as an obvious but improperly packed granular layer, and a thicker stratum corneum (**2E**).

Figure 2


Inv-CΔ187
Wild Type
A
Newborn
B
1 week
C
2 weeks
D
3 weeks
E
1 month
10μm

Inv-CΔ187 Transgenic Mice Exhibit Abnormalities in Epidermal Differentiation

It has been demonstrated that alterations in the normal distribution of keratin markers, as well as scaffold and cornified envelope markers, is an excellent means of determining the integrity of the epidermal differentiation program (9, 13, 14, 25, 28, 39). Therefore, a immunohistochemistry analysis was done of early, later and terminal differentiation markers in backskin samples from Inv-CΔ187 transgenic mice versus their age-matched wild type littermates. We will report on the analysis of newborn (**Figures 3&4, left panel**) and one-month old (**Figures 3&4, right panel**) backskin samples; samples from animals from three weeks to five months of age gave similar and consistent results to those at one month. Consistent with the histological results summarized above, expression of differentiation markers was indistinguishable between newborn (**Figures 3&4, left panel**) and one-week old (not shown) transgenic versus wild type animals. However, there was a progressive alteration in the expression of epidermal differentiation markers from two to four weeks of age that remained consistent throughout life. For example K15 expression, while limited to the basal layer, became sporadic in transgenic samples rather than uniform as in wild type (**Figure 3A, right panel**). Expression of K5 and K14 expression extended beyond the usual/wild type basal layer through all the suprabasal layers of the transgenic epidermis (**Figure 3B & 3C, right panel**). Expression of. K1 also showed a broadened expression pattern, overlapping with the suprabasal expression compartment of K5/K14, in the Inv-CΔ187 backskin (**Figure 4A, right panel**). A similarly expanded expression compartment in the transgenic epidermis was observed for various structural proteins including Involucrin (**Figure 4B, right**

panel), Filaggrin (**Figure 4C, right panel**), Loricrin (**Figure 4D, right panel**) and TGase-3 (**Figure 4E, right panel**).

Figure 3: Perturbation of Early Markers of Epidermal Differentiation. Immunohistochemical analysis of early differentiation markers was evaluated by immunofluorescence in backskin samples from newborn and one-month old Inv-CΔ187 transgenic mice as compared to their age-matched wild type. Consistent with our histological observations, in the newborn transgenic mouse epidermis (**left panel**) there was no deviation from the wild type expression of K15 (**3A**), K5 (**3B**) or K14 (**3C**), where expression was restricted to the basal layer. However, in the 1-month samples (**right panel**), K15 expression, while limited to the basal layer, became sporadic in transgenic samples rather than uniform as in wild type (**3A**). Expression of K5 (**3B**) and K14 (**3C**) extended beyond the usual/wild type basal layer through all the suprabasal layers of the transgenic epidermis.

Figure 3

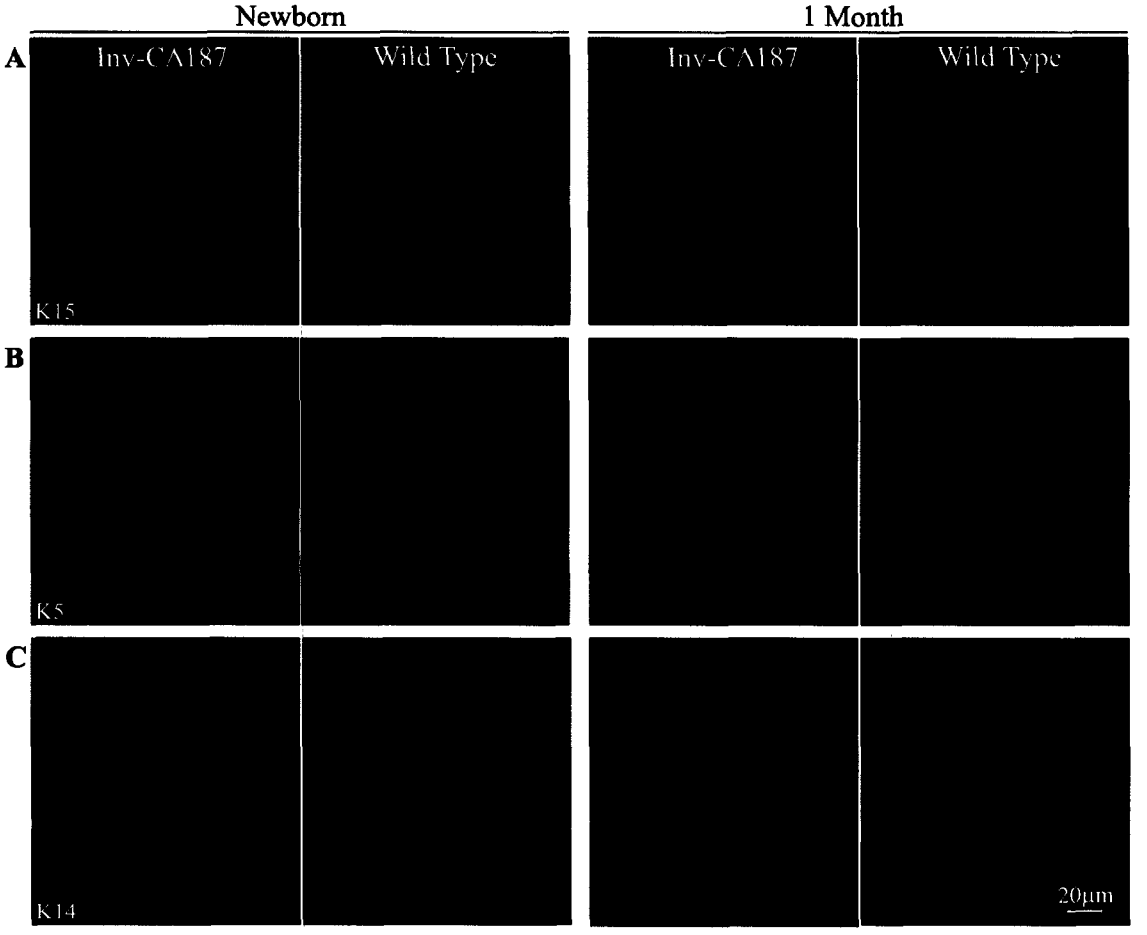
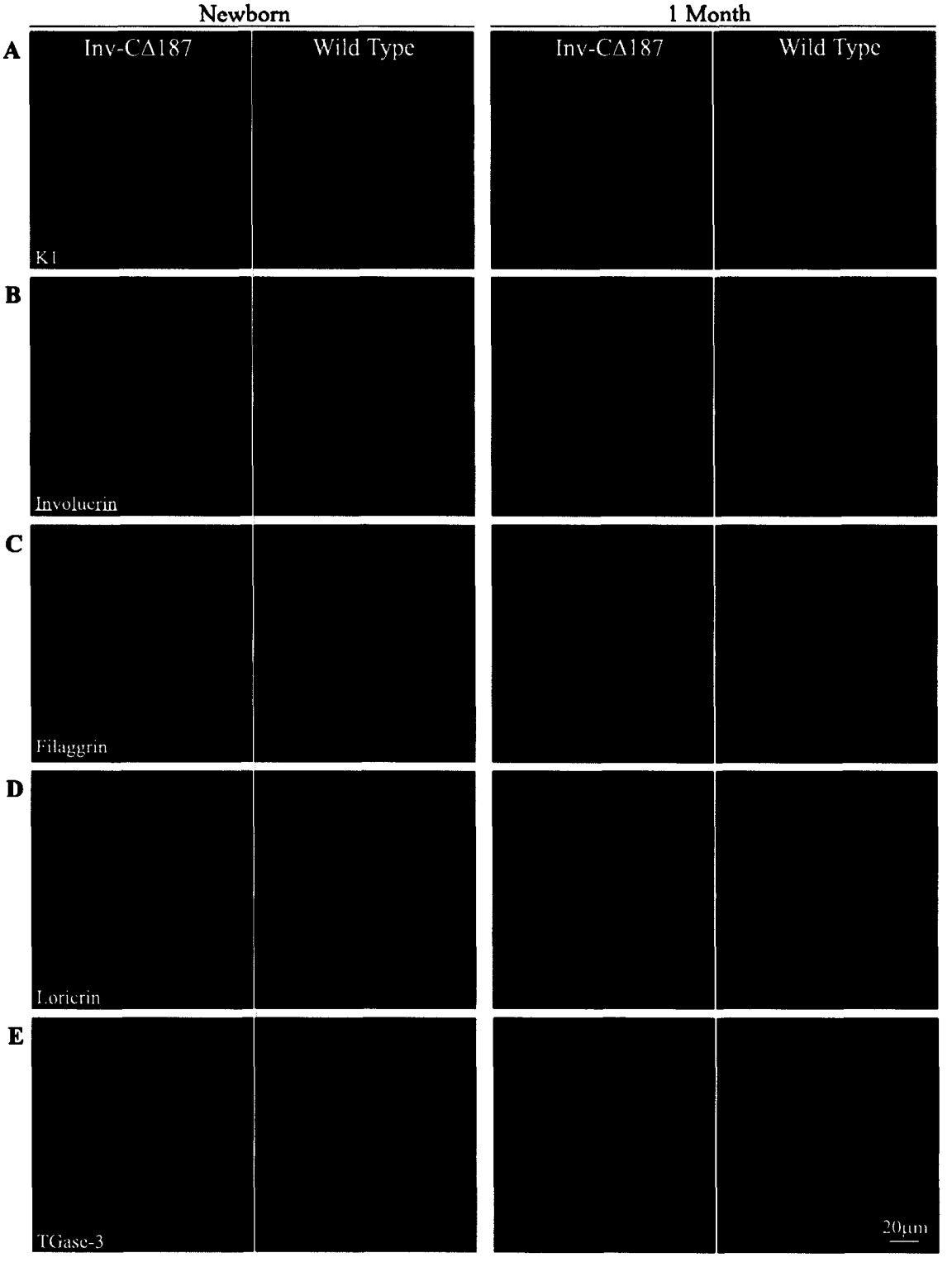

Newborn
1 Month
A
Inv-CA187
Wild Type
Inv-CA187
Wild Type
K15
B
K5
C
K14
20μm

Figure 4: Aberrance of Later and Terminal Differentiation. Immunohistochemical analysis of markers of later and terminal epidermal differentiation was also evaluated in age-matched wild type and Inv-CΔ187 backskin samples. Concurrent with the expression of early epidermal differentiation markers, there was no apparent modification in the expression of K1 (**4A**), Involucrin (**4B**), Filaggrin (**4C**), Loricrin (**4D**) or TGase-3 (**4E**) in the newborn transgenic epidermis as compared to the wild type (**left panel**). However in the one-month-old samples (**right panel**), K1 showed a broadened expression pattern (**4A**) and a similarly expanded expression compartment in the transgenic epidermis was observed for various structural proteins including Involucrin (**4B**), Filaggrin (**4C**), Loricrin (**4D**) and TGase-3 (**4E**).

Figure 4


Newborn
1 Month
A
Inv-CΔ187
Wild Type
Inv-CΔ187
Wild Type
K1
B
Involucrin
C
Filaggrin
D
Loricrin
E
TGase-3
20μm

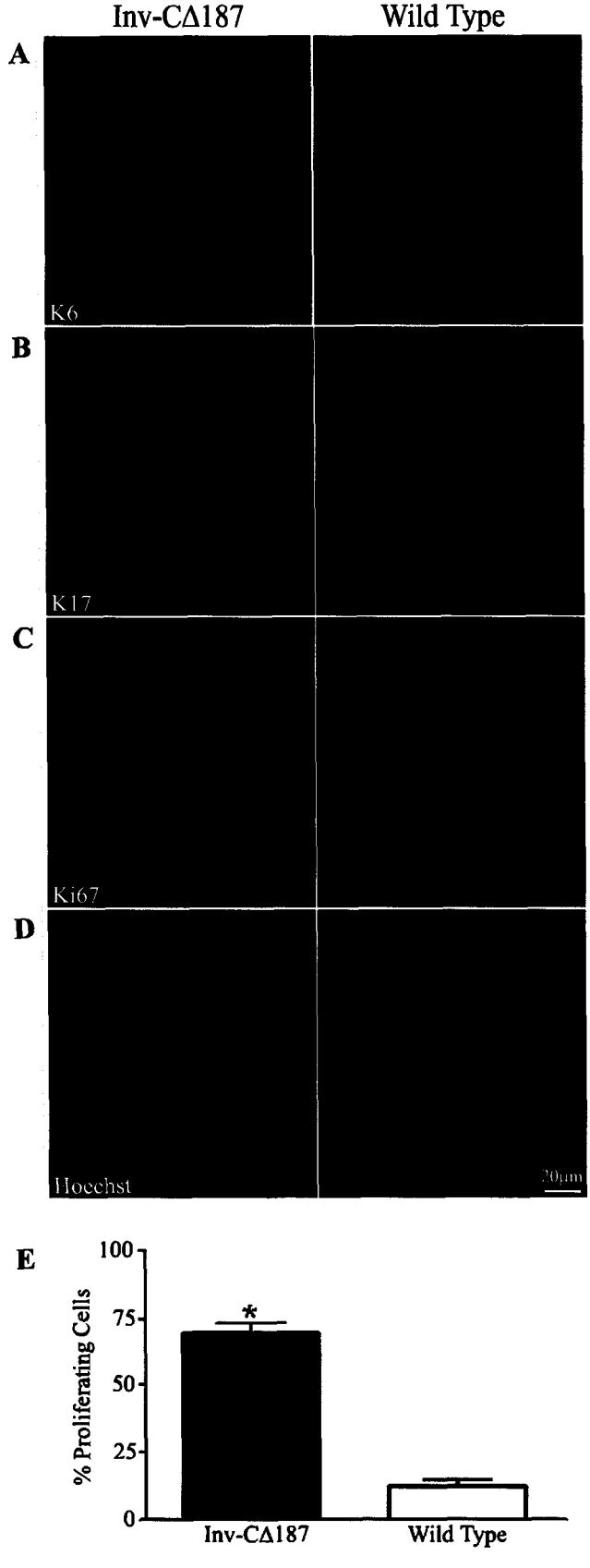
CΔ187 Increases Basal Cell Proliferation and Induces Suprabasal Cell Proliferation

Histological observations of a thickened epidermis and the apparent perturbations in the epidermal differentiation program were suggestive of an increased proliferation rate in the Inv-CΔ187 transgenic epidermis. Therefore, immunostaining was done for K6 and K17, differentiation markers that are generally not present in the normal adult interfollicular epidermis except under conditions reflective of abnormal cell proliferation and differentiation (20, 23, 24, 41). Results indicated that there was no abnormal proliferation in the newborn or one-week old transgenic backskin samples (not shown). However, with the thickening of the transgenic epidermis, there was an obvious persistent increase in the expression of these proliferation-associated markers (one-month-old backskin samples are shown; **Figure 5A & 5B**). We also assayed for Ki67, a cell cycle related nuclear protein expressed by proliferating cells in the G1, S, G2 and M phases of the cell cycle. In the wild type epidermis, Ki67-positive and Hoechst-stained cells were confined to the basal cell layer as expected (2, 29) (**Figure 5C & 5D, right panel**). However in the Inv-CΔ187 transgenic epidermis, consistent with the other analyses reported above, there were Hoechst-stained nuclei in the upper layers (**Figure 5D, left panel**) and not only was the number of Ki67-positive basal cells increased by ~4-fold ($p<0.001$; using the student's t-test) (**Figure 5E**), but a significant proportion of Ki67-positive cells were also found in the suprabasal cell layers (**Figure 5C, left panel**). In addition, immunohistochemical staining for macrophages (F4/80 antigen) and T-cells (CD3 molecular complex) in sections of Inv-CΔ187 transgenic epidermis compared to the wild type did not reveal any accumulation or immune infiltration (not shown),

indicating that the observed changes in proliferation rate in the epidermis of the transgenic mice is likely not due to the expression of immune cell-derived cytokines (17).

Figure 5: Proliferation in the Inv-CΔ187 Epidermis. To address the potential proliferation defect observed in the Inv-CΔ187 transgenic epidermis, immunostaining for proliferation-associated markers including K6, K17 and Ki67, was performed. Backskin samples from one-month-old Inv-CΔ187 transgenic mice as compared to their age-matched wild type revealed that K6 (**5A**) and K17 (**5B**) expression was precociously localized to the basal to suprabasal layers of the transgenic epidermis (**left panel**), while there was no interfollicular expression in the wild type epidermis (**right panel**). In addition, the Inv-CΔ187 transgenic epidermis was characterized with Hoechst stained nuclei in the upper layers (**5D**) and not only was the number of Ki67-positive basal cells increased by ~4-fold ($p<0.001$; using the student's t-test) (**Figure 5E**), but a significant proportion of Ki67-positive cells were also found in the suprabasal cell layers (**5C**).

Figure 5


Inv-CΔ187
Wild Type
A
K6
B
K17
C
Ki67
D
Hoechst
20μm
E
100
75
50
25
0
% Proliferating Cells
*
Inv-CΔ187
Wild Type

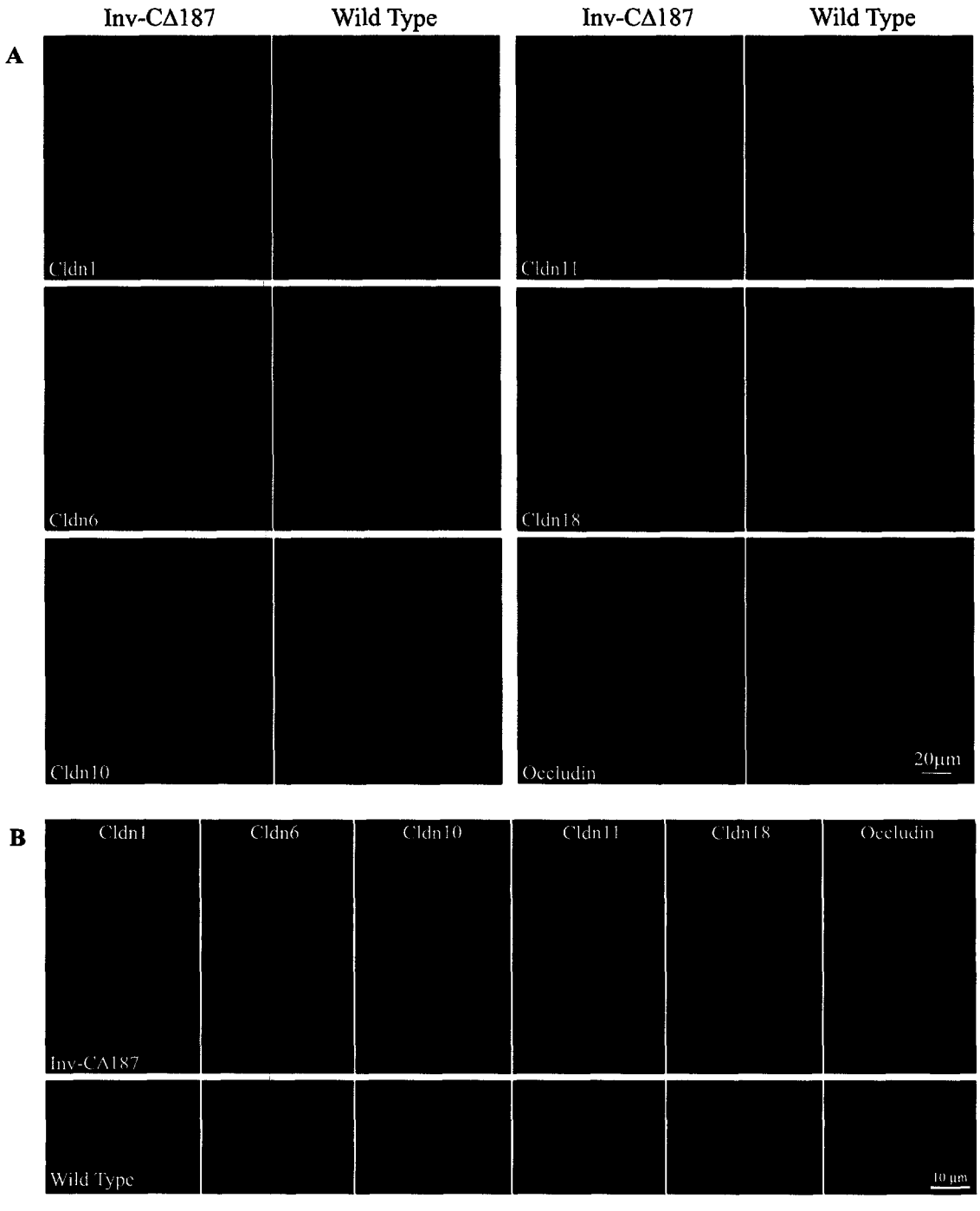
Perturbations in Cldn Expression in Inv-CΔ187 Transgenic Mice

Different epithelial cells exhibit a complex profile of Cldn TJ molecules, the composition of which has recently been attributed to the formation of a diverse array of selective permeability barriers for different epithelia (1, 10, 18, 34, 38, 42). Concomitantly, it is thought that a precise Cldn expression profile characterizes epidermal differentiation and that, in response to injury or disease, modifications in the epidermal differentiation program are reflected in changes in the Cldn profile and vice versa (15, 27, 34, 40). Cldn2, Cldn3 and Cldn5, which are normally not expressed in the epidermis, were not observed in either wild type or transgenic skin at any age sampled (not shown). As was observed with the expression of epidermal keratins and other differentiation markers, newborn and one-week old transgenic backskin samples also exhibited no differences in the expression or localization of any of the typical epidermal Cldns assayed (not shown). However, in parallel with the thickening of the epidermis, there was a marked difference in the transgenic expression profile of epidermal Cldns (**Figure 6**). Cldn6, Cldn10, Cldn11 and Cldn18, which are normally restricted to the differentiating compartment of the mature epidermis starting with the upper spinous layers (15, 34, 40), were expressed in a clearly expanded zone (one-month old samples; **Figure 6A & 6B**). On the other hand, whereas Cldn1 is expressed in the basal to suprabasal layers of the wild type epidermis, the basal layer was essentially devoid of Cldn1 expression in the transgenics (**Figure 6A & 6B**). Notably, and reminiscent of the expression of the transgene product (**Figure 1F**), Cldn6, Cldn10, Cldn11 and Cldn18 localization shifted to varying degrees from the membrane of wild type to the cytoplasm of transgenic mice (**Figure 6B**, higher magnification). Altered localization of Cldn1 expression to the cytoplasm was less

pronounced as compared to the other Cldns, and the localization of Occludin (another TJ-associated integral membrane protein that is normally associated with the granular layer in epidermal cells) (26, 27, 47) remained membranous (**Figure 6B**).

Figure 6: Cytoplasmic Accumulation of Cldns in the Inv-CΔ187 Epidermis. Given the complex profile of Cldn TJ molecules in epithelial cells we systematically analysed the expression of Cldn1, Cldn6, Cldn10, Cldn11 and Cldn18 in backskin samples from one-month old Inv-CΔ187 transgenic mice as compared to their age-matched wild type. In the transgenic epidermis, Cldn6, Cldn10, Cldn11 and Cldn18 were expressed in a clearly expanded zone (**6A & 6B**-higher magnification). Cldn1 is expressed in the basal to suprabasal layers of the wild type epidermis, while the basal layer of the transgenic epidermis was essentially devoid of Cldn1 expression. Reminiscent of the expression of the transgene product (**Figure 1F**), Cldn6, Cldn10, Cldn11 and Cldn18 localization shifted to varying degrees from the membrane of wild type to the cytoplasm of transgenic mice (**6B**). Altered localization of Cldn1 expression to the cytoplasm was less pronounced as compared to the other Cldns, and the localization of Occludin remained membranous.

Figure 6

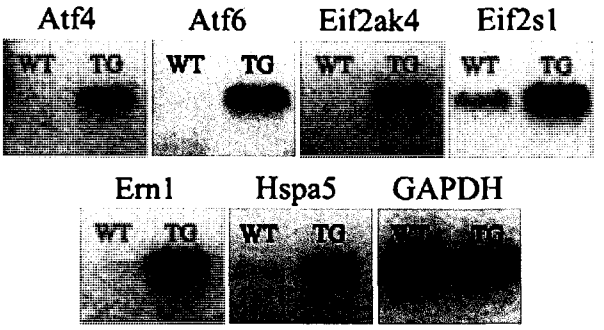

A
Inv-CΔ187
Wild Type
Inv-CΔ187
Wild Type
Cldn1
Cldn11
Cldn6
Cldn18
Cldn10
Occludin
20μm
B
Cldn1
Cldn6
Cldn10
Cldn11
Cldn18
Occludin
Inv-CA187
Wild Type
10 μm

Activation of an Unfolded Protein Signaling Pathway in the Inv-CΔ187 Epidermis

The cytoplasmic accumulation of Cldns observed in the Inv-CΔ187 transgenic mouse epidermis is reminiscent of certain other proteins where unusual cytoplasmic accumulation has been attributed to signaling activation through an unfolded protein pathway (30, 31). RT-PCR analysis with RNA extracted from the backskin of Inv-CΔ187 transgenic (TG) and wild type (WT) mice (**Figure 7**) indicated that the unfolded protein signaling pathway components activating transcription factor 4 (Atf4), activating transcription factor 6 (Atf6), eukaryotic translation initiation factor 2αkinase 4 (Eif2ak4), eukaryotic translation initiation factor 2, subunit 1α (Eif2s1), endoplasmic reticulum to nucleus signaling 1 (Ern1) and heat shock 70kD protein 5 (Hspa5) are activated in our transgenic model.

Figure 7: Activation of an Unfolded Protein Response Pathway in the CΔ187 Epidermis. In an attempt to explain the cytoplasmic accumulation of Cldns observed in the Inv-CΔ187 transgenic epidermis, the potential activation of a pathway involving protein folding was investigated by RT-PCR of RNA extracted from the backskin of Inv-CΔ187 transgenic (TG) mice as compared to the wild type (WT). There was an upregulated expression of various genes involved in protein folding in the Inv-CΔ187 epidermis as compared to the wild type; including Atf4, Atf6, Eif2ak4, Eif2s1, Ern1 and Hspa5.

Figure 7



Discussion

In this study, we took a structure-function approach to elucidate whether the cytoplasmic tail of Cldn6 plays a crucial role in epidermal differentiation and the formation of a physiologically intact EPB. Using transgenic mouse technology, we expressed a complete tail truncation of Cldn6 at its endogenous site of expression, the TJ compartment of the epidermis, via the Inv promoter (Inv-CΔ187). While Inv-CΔ187 animals displayed an apparently normal prenatal epidermal development and a functioning EPB, histological and immunohistochemical characterization revealed abnormalities in the epidermal differentiation program with progressive thickening of the epidermis and aberrant epidermal marker expression that was evident after one week of age and persisted throughout life. This was accompanied by increased basal cell proliferation and induced suprabasal and interfollicular cell proliferation. Although some membrane association was evident, a significant amount of not only Cldn6, but also Cldn10, Cldn11 and Cldn18 mis-localized to the cytoplasm of transgenic epidermal cells, an observation correlating with activation of the unfolded protein signaling pathway in the Inv-CΔ187 epidermis. These findings demonstrate the importance of the tail domain in targeting Cldn6 to the membrane for normal proliferation and differentiation in the mouse epidermis.

Loss of the Cytoplasmic Tail Domain of Cldn6 Results in Epidermal Hyperproliferation and Differentiation Defects

A normal wild type epidermis starts out as a multilayer structure at birth and undergoes thinning until the mature two to three cell layer thick epidermis is achieved within two

weeks after birth (46). The abnormal thickening of the Inv-CΔ187 epidermis reflects a disrupted epidermal proliferation and differentiation program presumably as a result of inefficient membrane targeting and mis-localization of Cldn6 to the cytoplasm (see also below). The fact that other Cldns are also both mis-localized and aberrantly expressed points towards activation of not only coordinate regulatory pathways but also potential compensatory pathways that rescue EPB function but do not rescue the tightly coupled epidermal proliferation-differentiation program. Whether Cldns are linked directly to signaling for epidermal cell proliferation or the observed hyperproliferation results from differentiation anomalies is not yet known. However, one potential explanation for the proliferation and differentiation changes observed is activation of an unfolding protein pathway (30, 31). The cytoplasmic accumulation of CΔ187 is reminiscent of that observed for numerous molecules where a folding defect has inhibited their normal functioning and is consistent with our data showing up-regulation of Atf4, Atf6, Eif2ak4, Eif2s1, Ern1 and Hspa5, all of which are expected if the unfolded protein response signaling pathway is activated. Notably, it is becoming apparent that the prolonged presence of unfolded proteins as occurs in the Inv-CΔ187 mice may result in certain disease states, including hyperproliferation situations such as tumorigenesis (for review see 21, 48).

Epidermal specific keratins and epidermal terminal differentiation markers such as Involucrin, Filaggrin, Loricrin and TGase-3, proteins involved in scaffold function as well as the eventual formation of the cornified envelopes of the stratum corneum (5), are excellent indicators of the orderly progression of epidermal differentiation (4, 9, 14, 25),

which is clearly disrupted in the Inv-CΔ187 transgenic mice. In this regard, expression of basal-specific K5 and K14 was deregulated and extended throughout the epidermis of the transgenic mice, indicating that the thick epidermis maintains basal-like characteristics throughout, supporting the hyperproliferative state. In addition, the observed broad and overlapping expression compartments of the early differentiation marker K1 as well as some of the late differentiation markers (i.e., Involucrin, Loricrin, Filaggrin and TGase-3) suggest that the terminal differentiation program and processing of late epidermal differentiation markers are also dysregulated. The anomalous expression of K6, which is normally not associated with the interfollicular epidermis except under specific skin conditions such as wound healing and psoriasis as well as during the development of epidermal tumours (11, 41), also supports this view. Similarly K17 is normally expressed in the basal layer of the epidermis during development and within the first few days after birth before its expression is turned off (24). Finally, amongst markers with abnormal expression domains, the hyperproliferation state may also be related to the fact that Cldn1 expression is lost in the basal layer of the transgenic epidermis (see also below), which parallels Cldn1 ablation in cancerous cells (33). Taken together, these observations suggest that the disrupted proliferation-differentiation program in the Inv-CΔ187 mice may render them susceptible to epidermal tumors, a possibility being pursued further in these animals.

The Cytoplasmic Tail Domain of Cldn6 is Necessary for Cldn Homeostasis and Membrane Targeting in the Epidermis

As already mentioned, a precise Cldn expression profile characterizes epidermal differentiation and modifications to the Cldn expression profile are reflected in changes to the epidermal differentiation program (15, 27, 34, 40). Thus, for example, in our two different transgenic models in which native (Inv-Cldn6) or tail-truncated (Inv-CΔ187) Cldn6 is overexpressed, a common feature is loss of the usual Cldn expression profile. Our Inv-CΔ187 transgenic mouse model underscores the intimate link between Cldn expression, heterophyllic interactions and normal differentiation in the epidermis. In addition to the accumulation of CΔ187 in the cytoplasm of the transgenic epidermis, there was also increased accumulation of Cldn6, Cldn10, Cldn11 and Cldn18 to varying degrees; while the subcellular Cldn1 localization was not as drastically modified, its expression domain was. Since details of potential Cldn-Cldn interactions in the cytoplasm and/or TJ fibrils have not yet been elucidated, our observations support two potential mechanisms whereby CΔ187 acts as a dominant negative to affect other Cldns. In the first, heterophilic Cldn-Cldn assembly would occur in the cytoplasm before membrane targeting; in this model, mutant mis-localized Cldn6 would bind to and inhibit (or delay) membrane targeting of other Cldns, causing their accumulation in the cytoplasm. In the second, heterophilic Cldn-Cldn assembly would occur in the membrane; in this model, a small amount of mutant membrane-localized Cldn6 may destabilize not only its own but binding of other Cldns in TJ strands, leading to fibril dissolution and Cldn accumulation in the cytoplasm. It is also possible that both

mechanisms are operative. In any case, the resultant changes in Cldn homeostasis have profound consequences on epidermal proliferation and differentiation.

Although the C-terminal tail domain is diverse amongst different Cldns, the CXXC (where C is cysteine and X is any amino acid) (see underlined sequences of **Figure 1B**) tetrapeptide sequence motif which is conserved in the cytoplasmic tail domain of several Cldns, signals for palmitoylation, a posttranslational modification in a number of integral membrane molecules essential for their targeting to and stabilization in the membrane (3). The importance of palmitoylation in the posttranslational modification and membrane targeting of Cldn14 has also recently been demonstrated; where its mutation resulted in cytoplasmic accumulation (45). Although the poor membrane localization and cytoplasmic accumulation of CΔ187 in the transgenic mice is reminiscent of Cldn mis-localization when the CXXC is mutated, the tail domain CXXC motif remains intact in our mutant; indeed, there are two additional amino acids in the transgene sequence before the FLAG® tag was introduced, suggesting that palmitoylation defects cannot account for mis-localized CΔ187 and its consequences in the epidermis. It also seems unlikely that the second conserved CXXC motif in the Cldn internal loop, well-removed from our truncation site, contributes to the mis-localization although its role in palmitoylation and membrane targeting remains to be investigated. Nevertheless, we cannot rule out the possibility that the tail and/or the internal loop CXXC motif has become dysfunctional due to conformational changes in the CΔ187 mutant or that introduction of the FLAG® epitope tag results in high turnover of membrane molecules thereby contributing to excessive cytoplasmic accumulation, issues we are currently exploring. However, our

data support the notion that there exist other crucial determinants for stable TJ incorporation in the cytoplasmic C-terminal sequence of Cldns that up to now have not been implicated in specific protein-protein interactions.

Most Cldns, including Cldn6, have a conserved PDZ-binding YV sequence at the C-terminal end of the tail domain that serves as an interaction site for Cldn binding with other PDZ domain proteins (16, 19, 22). Such interactions may be responsible for the linking of Cldns to the cytoskeleton and/or to Cldn signaling pathways. However, to date the only PDZ protein shown to act potentially as a link between the actin cytoskeleton and Cldns is ZO-1 (12). Nevertheless, it seems likely that other TJ-related PDZ domain-containing proteins that interact with Cldns will be identified.

The Effect of CΔ187 on the Barrier Function of Epidermal Cells

While disruption of Cldn homeostasis is a common feature of overexpression of both native and tail-truncated Cldn6, neither the severe defects in developmental EPB formation nor the measurable dehydration across the skin with neonatal lethality that characterize Inv-Cldn6 mice are seen in the Inv-CΔ187 neonates. Furthermore, and again in contrast to Inv-Cldn6 mice, the epidermis of Inv-CΔ187 neonates was quite comparable to that of the wild type during development and at birth. Strikingly, even after the postnatal differentiation and skin thickness defects are expressed in the Inv-CΔ187 transgenic epidermis, there is again no measurable barrier defect observed. The reason for this is not obvious, but must reflect functionally adequate, even if immunohistochemically abnormal, epidermal TJs. Whether this is due to the fact that

there is sufficient residual Cldn6 and other Cldns properly targeted to the membrane to participate in TJs with adequate barrier function, if not normal function for the postnatal epidermal proliferation-differentiation program, is not known. However, it is also important to emphasize that the complete Cldn composition of the epidermis is not yet known, and it is therefore possible that the Inv-CΔ187 epidermis may express other Cldns that can compensate and rescue barrier function but not the entire proliferation-differentiation sequence in the transgenic mice.

In summary, through the generation of transgenic mice that express tailless Cldn6 in the suprabasal layer of the epidermis we provide evidence for the importance of the cytoplasmic tail domain of Cldns in membrane targeting and epidermal proliferation-differentiation in vivo. It is likely that the cytoplasmic accumulation of CΔ187 may play an important role in the aberrant proliferation observed in the transgenic mouse epidermis, possibly through "gain of function" activity, its precocious interaction with membrane or cytoplasmic molecules and/or activation of a protein folding defect pathway. In addition, we speculate that the Cldn6 cytoplasmic tail domain contains additional motifs to those already known that may play a role in targeting of CΔ187 to TJ fibrils. The Inv-CΔ187 mice provide an excellent animal model system to further investigate the role of Cldn in all these processes.

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Chapter 3

Claudin expression modulations reflect an injury response in the murine epidermis

by

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TO THE EDITOR

The mature epidermis is maintained throughout life in response to discrete signals whereby epidermal cells in the proliferative basal compartment become irreversibly committed to terminal differentiation and move upwards away from the basal layer to maintain a constant thickness and homeostasis (Mack et al., 2005; Turksen and Troy, 1998). However in response to injury or in experimentally induced hyperplasia/tissue remodeling following the topical application of phorbol esters (Molloy and Laskin, 1987), epidermal homeostasis is perturbed, resulting in changes in epidermal terminal differentiation and barrier selectivity. In vivo studies have demonstrated that the epidermis is characterized by a defined differentiation-dependent expression of Claudins (Cldns) (Arabzadeh et al., 2006; Troy et al., 2007; Troy et al., 2005; Turksen and Troy, 2002), a family of tetraspan membrane proteins that comprise a major component of tight junction (TJ) fibrils essential to the structure and function of TJs (Furuse and Tsukita, 2006; Turksen and Troy, 2004; Van Itallie and Anderson, 2006). Recent studies have also shown that changes in Cldns may contribute to changes observed in cell permeability and barrier selectivity (Furuse et al., 2002; Troy et al., 2005; Turksen and Troy, 2002). However, no systematic analysis of Cldns during epidermal repair is yet available.

In this study we examined the expression of Cldns in the mouse epidermis during the course of TPA-induced injury, transient hyperproliferation and repair. Our data indicate that Cldns are modulated in parallel with epidermal cell morphology and differentiation changes in a fashion indicative of cell polarity and barrier selectivity changes. Specifically, the differentiation compartment-specific Cldns occupied a broader zone of

expression, with a loss of membranous localization. In addition to becoming cytoplasmic, Cldn1 was downregulated in the epidermal basal compartment and was observed to shuttle between the cytoplasm and the nucleus, thus suggesting a potential molecular basis for the changes observed in TJ modulation in response to injury.

The dorsal backskin of several one-month-old CD1 mice was treated with a single dose of TPA (25 μg/ml in acetone); all animal studies were conducted according to the regulations of the Canadian Council on Animal Care. Samples (~1cm^2) were dissected from the mid-dorsal region of treated mice as well as their untreated and vehicle-treated counterparts at 4, 6, 12, 24, 48, 72 and 96 hours post application and processed for immunofluorescence as described (Arabzadeh et al., 2006; Troy et al., 2007). Histological analysis of skin samples from TPA-treated CD-1 mice revealed expected modifications in the morphology of the TPA-treated epidermis (**Figure 1, column 1**). From 12 to 72 hours, the epidermis underwent a gradual thickening with characteristic changes in cellular morphology, including some degree of suprabasal keratinocyte disorganization as well as an expanded stratum corneum. The thickening pattern and histological abnormalities of the treated epidermis were not sustained and underwent normalization commencing after 96 hours indicative of the repair mechanism of the epidermis.

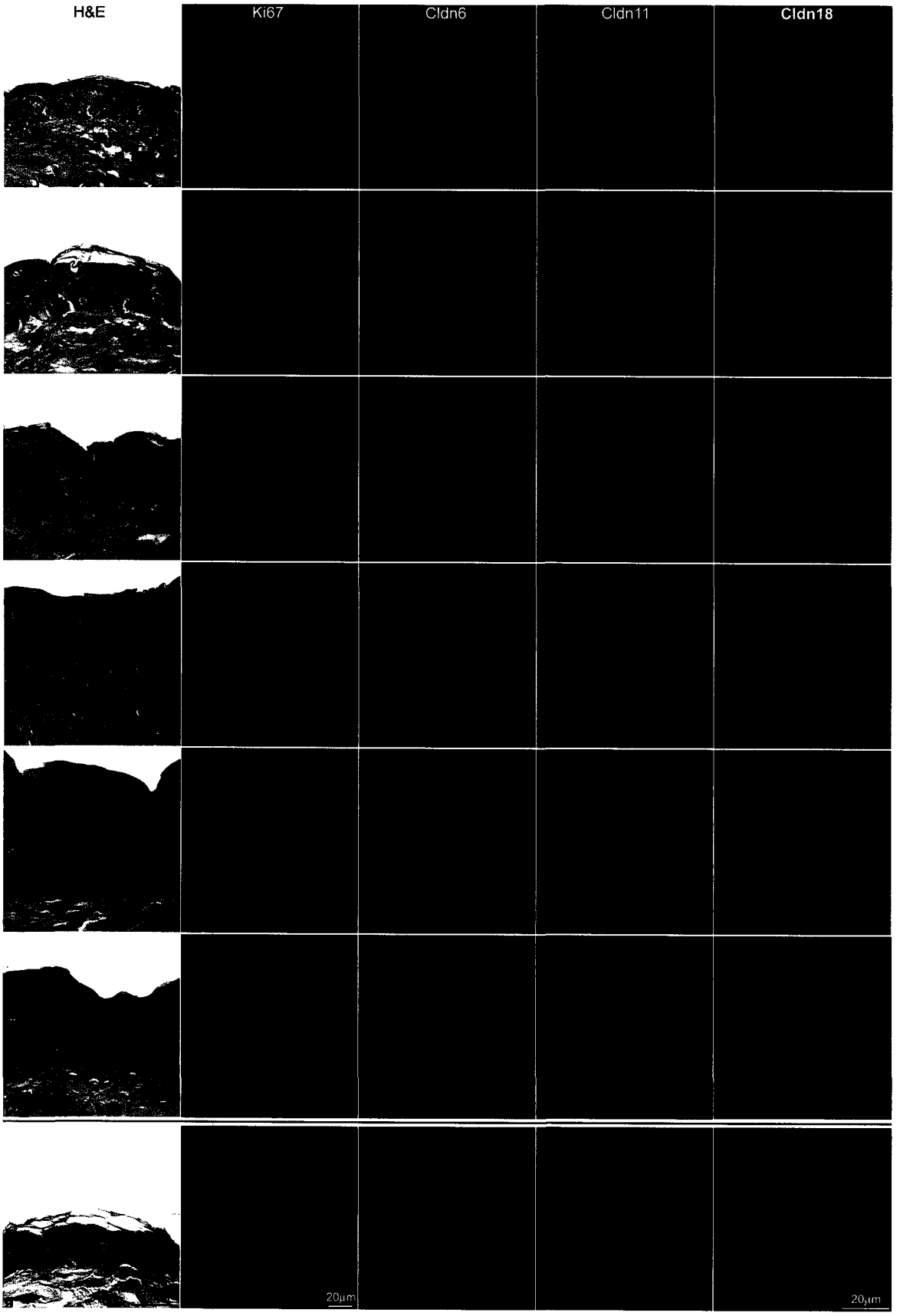
To complement our histological observations, immunohistochemical analyses were performed to evaluate the expression of classical markers of the epidermis. Beginning 12 hours after TPA treatment, immunofluorescence analyses revealed that K14 was

localized in all the basal to suprabasal layers, and the expression compartment of K1 was significantly expanded (not shown). In addition, loricrin expression occupied a thicker zone of expression beginning 48 hours after TPA treatment (not shown). After 96 hours as the epidermis underwent normalization, so too did the expression compartments of these markers. K6 expression, which is generally absent in the adult wild-type interfollicular epidermis except under hyperproliferative conditions, was evident 12 hours after TPA treatment where its expression was maintained throughout the experimental protocol, with an indication of downregulation evident after 96 hours (not shown). Immunolocalization with anti-Ki67 antibodies, a nuclear protein expressed by proliferating cells during all phases of the cell cycle, confirmed the proliferation response of the TPA-treated epidermis (**Figure 1, column 2**). In addition, immunostaining for the T-cell receptor-associated CD3 complex indicated that from 24 to 72 hours there was a modest increase in the dermal infiltration of CD3-positive T cells that was reduced in response to tissue repair at 96 hours (not shown).

We next analyzed by immunofluorescence the expression of those Cldns we have previously demonstrated to be important in epidermal differentiation and the formation of the epidermal permeability barrier (Arabzadeh et al., 2006; Troy et al., 2007; Troy et al., 2005; Turksen and Troy, 2002). Commencing 12 hours after TPA application, Cldn6 (**Figure 1-column 3**), Cldn11 (**Figure 1-column 4**) and Cldn18 (**Figure 1-column 5**) (the suprabasal cell-specific Cldns) were expressed in an expanded zone, where there was also a shift in their subcellular localization from the membrane to the cytoplasm.

Figure 1: Morphological changes, epidermal hyperproliferation and changes in the epidermal expression of suprabasal-associated Cldns in response to a single exposure to TPA. Backskin samples from CD-1 mice at 4 (**row a**), 12 (**row b**), 24 (**row c**), 48 (**row d**), 72 (**row e**) and 96 (**row f**) hours post TPA-application were compared to their acetone-treated counterparts (**row g**) for histological changes (H&E) and epidermal proliferation was confirmed through the increased localization of Ki67-positive cells by immunofluorescence. Immunolocalization of Cldn6, Cldn11 and Cldn18 revealed expanded epidermal expression compartments after a single exposure to TPA. In addition, there was a shift in Cldn localization from the membrane to the cytoplasm from 24 to 72 hours. In the repairing epidermis 96 hours after TPA treatment, membrane localization of the suprabasal-specific Cldns was evident with an overall reduction in the expression compartment.

Figure 1



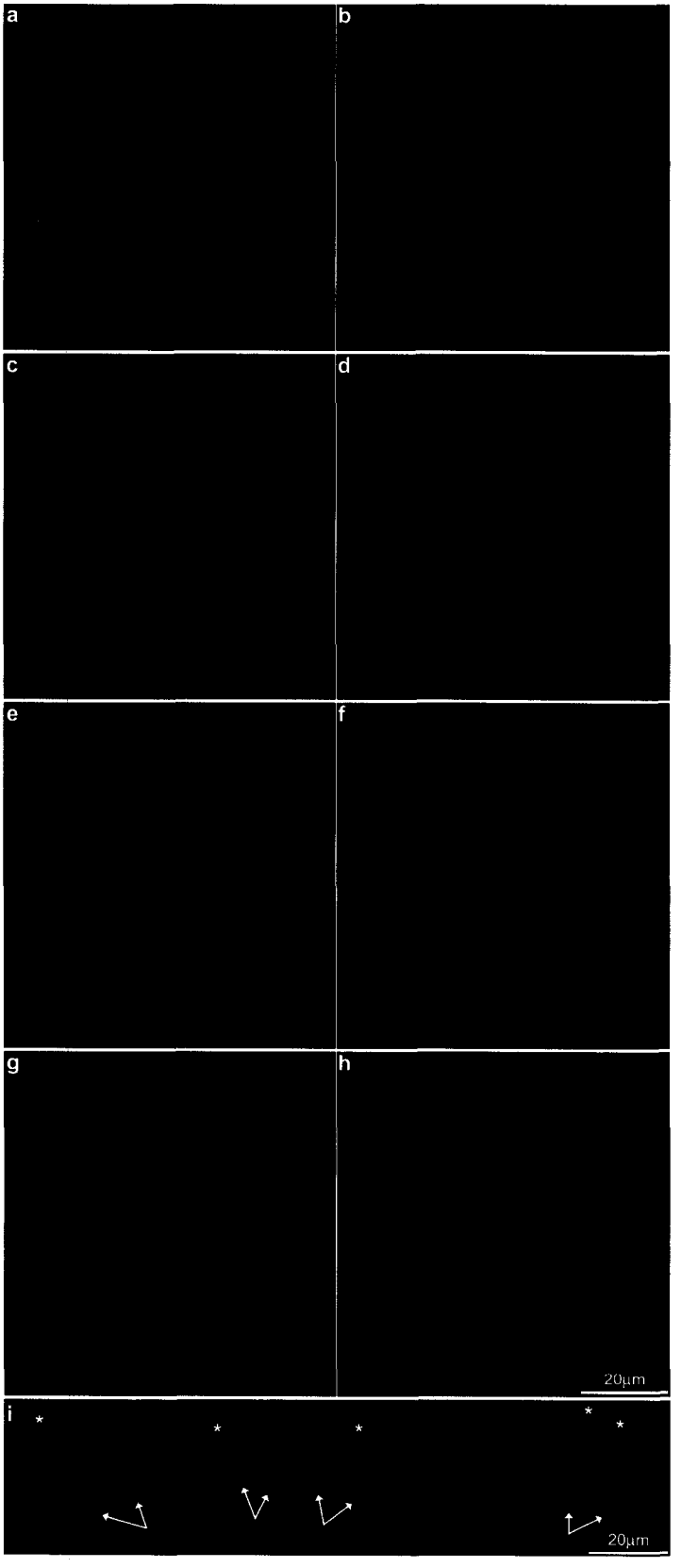
Transient changes in epidermal differentiation and proliferation resulted in reversible Cldn expression and localization modifications, indicating that the TJ-based permeability barrier was perturbed from 12 to 72 hours after a single epidermal exposure to TPA, and that it was in repair-mode by 96 hours. Although the molecular mechanism is not known, the role of TPA in the activation of protein kinase C (PKC) has been demonstrated (Hennings et al., 1992) and therefore PKC may be acting indirectly by changing nuclear receptors (e.g. RAR, PPAR) and/or growth factor signaling (e.g. EGF, IGF) in the epidermis (Hatoum et al., 2001; Kumar et al., 1994; Stewart and O'Brian, 2005) resulting in downstream changes affecting Cldn localization. Or since the cytoplasmic tail domain of several Cldns contain putative PKC sites, a direct PKC-dependent phosphorylation of Cldns may be involved in the relocalization of Cldns from the membrane to the cytoplasm. However either a direct or indirect PKC-dependent modulation of Cldn localization in the epidermis in response to TPA is yet to be demonstrated.

Beyond the cytoplasmic relocalization of the suprabasal-specific Cldns, the distribution of Cldn1 (Zymed Laboratories Inc.; cat. # 71-7800) was also perturbed, albeit in a different manner. In the normal mature epidermis, Cldn1 occupies both the basal and suprabasal compartments as observed in the vehicle-treated epidermis (**Figure 2a**). In response to TPA exposure, Cldn1 expression adopted a progressively more cytoplasmic localization as observed from 4 to 72 hours after treatment, especially in the lower strata of the epidermis (**Figure 2b-g**). In addition, some nuclear association was evident from 12 to 72 hours commencing in the basal layer and moving upwards into the lower suprabasal layers. By 96 hours post-treatment the membranous basal to suprabasal

localization of Cldn1 was mostly restored, with little cytoplasmic and nuclear association detected indicative of the normalization of the epidermis (**Figure 2h**). Co-immunolocalization using anti-Lamin B and anti-Cldn1 antibodies clearly demarcates the nuclear lamina in the cells of the epidermis, and supports the notion of the nuclear association of Cldn1 (**Figure 2i**).

Figure 2: Cldn1 relocalization in response to a single application of TPA. Immunofluorescence analyses revealed that in the acetone-treated epidermis (**a**), the mature localization of Cldn1 was maintained in the basal and suprabasal layers. Cldn1 localization was modulated as observed after 4 (**b**), 6 (**c**), 12 (**d**), 24 (**e**), 48 (**f**), 72 (**g**) and 96 (**h**) hours in response to a single application of TPA. Cldn1 became increasingly cytoplasmic, especially in the lower strata of the epidermis where some nuclear association was observed from 12 to 72 hours after treatment; nuclei are demarcated in (**i**) by co-immunolocalization with Lamin B. By 96 hours, membrane association and localization in all strata was indicative of epidermal normalization. Co-immunolocalization demonstrates that while Cldn1 nuclear association is evident in the basal and immediate suprabasal layers (arrows), the nuclei in the upper strata are void (star). Please note that in **a-h** Cldn1 antigen is detected using FITC-conjugated goat anti-rabbit antibodies (green); in **i** Cldn1 antigen is detected using Alexa Fluor 594-conjugated donkey anti-rabbit antibodies (red) and Lamin B antigen is detected using Alexa Fluor 488-conjugated chicken anti-goat antibodies (green).

Figure 2



Although the molecular mechanism is not understood, it is widely recognized that TPA induces affects on the TJ and its components (Mullin and O'Brien, 1986; Ojakian, 1981). Specifically, a TPA-induced dislocation of ZO-1, a TJ scaffolding molecule, has been observed (Weiler et al., 2005). Since ZO-1 has been noted to shuttle between the cytoplasm and the nucleus under some circumstances (Gonzalez-Mariscal et al., 1999; Islas et al., 2002; Riesen et al., 2002), it is interesting to speculate that indeed a similar mechanism may be acting on Cldn1. Conversely however, in the case of the epidermis, ZO-1 is only localized in the terminal differentiation compartment (Arabzadeh et al., 2006; Morita et al., 1998), and not in the basal and lower suprabasal layers where we report the TPA-depended shuttling of Cldn1; therefore this observed phenomenon is likely not due to a Cldn1/ZO-1 interaction. Whether Cldn1 has nuclear export and import sequences remains to be demonstrated, in addition to whether or not Cldn1 interacts with other proteins as part of its shuttling.

Given that nuclear translocation of various membrane-associated proteins has been demonstrated in the regulation of cell proliferation and differentiation, it is likely that the nuclear association of Cldn1 has a role in the observed epidermal response to injury; however the mechanism remains to be elucidated. Collectively, our studies provide persuasive evidence that mechanisms involving posttranslational and gene expression modifications are important in the dynamic changes occurring in Cldn expression in the disruption and normalization of epidermal homeostasis.

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Chapter 4

Changes in the distribution pattern of Claudin tight junction proteins during the progression of mouse skin tumorigenesis

by

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Abstract

Background

Despite the fact that morphological and physiological observations suggest that the tight junction (TJ)-based permeability barrier is modified/disrupted in tumorigenesis, the role of members of the Claudin (Cldn) family of TJ proteins is not well-understood. Using a well-established two-stage chemical carcinogenesis model, we investigated the temporal and spatial changes in expression of those Cldns that we have previously demonstrated to be important in epidermal differentiation and the formation of the epidermal permeability barrier, i.e., Cldn1, Cldn6, Cldn11, Cldn12 and Cldn18.

Methods

The lower dorsal backskin of mice was treated topically with 7,12-dimethylbenz(*a*)anthracene (DMBA; 0.25 mg/ml in acetone) and following a 10-day incubation period, 12-O-tetradecanoyl-phorbol-13-acetate (TPA; 25 μg/ml in acetone) was applied three times a week to the same area. Backskin samples were dissected 2, 4, 6, 8 and 12 weeks after the initiation of the experimental protocol and immunohistochemistry was performed on sections using antibodies against the following: Cldn1, Cldn6, Cldn11, Cldn12, Cldn18, Ki67 and CD3.

Results

Our data indicate that along with the changes in epidermal cell morphology and differentiation that occur during tumor formation, there is a dramatic change in Cldn distribution consistent with cell polarity and barrier selectivity changes. Specifically, in

the early stages of DMBA/TPA treatment, the suprabasal-specific Cldns occupy an expanded zone of expression corresponding to an increased number of suprabasal epidermal cell layers. As tumorigenesis progressed, the number of suprabasal epidermal layers positive for Cldn6, Cldn11, Cldn12 and Cldn18 was reduced, especially in the lower strata of the expanded suprabasal zone. In addition, a variably reduced cell membrane association of those differentiation-specific Cldns was observed, especially within the infiltrating epidermal structures. In contrast, Cldn1 (which is normally expressed in all the living layers of the epidermis) remained restricted to the cell membrane throughout the tumorigenesis protocol. However commencing 2 weeks after treatment there was a marked decrease in the number of Cldn1-positive basal cells, and the zone of Cldn1-null epidermal cells was expanded up into the lower stratified epidermis throughout the progression of DMBA/TPA treatment. In addition, there was no Cldn1 localization in the infiltrating epidermal structures of the tumorigenic epidermis.

Conclusions

This is the first demonstration of the changes in Cldn expression in the progression of DMBA/TPA-induced skin tumors; however further investigation into the molecular mechanisms regulating the observed changes in barrier selectivity during tumorigenesis is required.

Background

Disruption of epithelial cell polarity and cell-cell junctions with concomitant changes in the expression of junctional proteins during primary tumor formation is considered to be a hallmark of cancer cell invasion and metastasis [1]. Amongst the junctional complexes, the role in tumor formation of specific tight junction (TJ) proteins essential for cell polarity and the formation and maintenance of heterogeneous permeability barriers is not well understood. In vivo studies have demonstrated that the epidermis is characterized by a defined differentiation-dependent expression of Claudins (Cldns), a family of tetraspan membrane proteins that comprise a major component of TJ fibrils essential to the structure and function of TJs [2-6]. Recent studies have also shown that changes in the distribution pattern of diverse Cldns may contribute to changes observed in cell permeability [7-10]. However, no systematic analysis of the expression and/or localization of various Cldns during skin tumorigenesis is yet available.

Understanding the molecular basis of skin tumor development has been greatly facilitated by the use of animal model systems in which tumor development can be carefully controlled [11]. For example, the classical mouse two-stage carcinogenesis model provides an excellent system in which to study the stages and molecular mechanisms involved in squamous cell carcinoma [12]. The chemical carcinogenesis process can be divided into three distinct phases: initiation, promotion and progression. Initiation results from exposure to a mutagenic carcinogen, followed by the application of a promoter to alter gene expression and increase cell proliferation to ultimately result in tumor

formation. Therefore, we used this model to elucidate the changes occurring in Cldn expression during the progression of epithelial tumors.

Methods

DMBA/TPA treatment: The coat on the dorsal side of one-month-old CD1 wild type mice was shaved one day prior to the initiation of the experimental protocol and mice were shaved once a week as required until the coat failed to re-grow. The lower dorsal backskin of mice was treated topically with 7,12-dimethylbenz(*a*)anthracene (DMBA; 0.25 mg/ml in acetone) and following a 10-day incubation period, 12-O-tetradecanoyl-phorbol-13-acetate (TPA; 25 μg/ml in acetone) was applied three times a week to the same area. Experimental results were highly reproducible in three independent assays, each comprising three mice per time point (treated vs. control) and a minimum of 2-3 biopsies per mouse. All animal studies were conducted according to the regulations of the Canadian Council on Animal Care.

Sample Collection: Backskin samples (~1 cm^2) were dissected from the lesion and/or tumor regions of the mid-dorsal backskin from DMBA/TPA-treated mice as well as their vehicle-treated controls; sampling was done 2, 4, 6, 8 and 12 weeks after the initiation of the experimental protocol. Since the fixation method routinely used in our laboratory is rather stringent (see below) and not appropriate for all antibodies, frozen sections were required for Cldn1, Ki67 and CD3 immunostaining [2]. All other immunolocalization and histology (Hematoxylin & Eosin; H&E) were performed on paraffin sections. For frozen sections: skin samples were embedded in HistoPrep™ and solidified in dry ice-

chilled isopentane. Sections (5 μm) on slides were warmed at room temperature for 3 minutes, and then fixed for 10 minutes in methanol at –20°C, followed by washing in PBS prior to immunostaining. For paraffin sections: skin samples were fixed for 12-16 hours in Bouin's fixative (75% saturated picric acid, 20% formaldehyde and 5% glacial acetic acid) at room temperature, followed by ethanol dehydration (30%, 50%, 70%, 95%, 100%) , paraffin embedding and sectioning (5μm). Prior to H&E and immunostaining, sections were dewaxed and rehydrated followed by antigen unmasking and washing steps [5].

Immunohistochemistry: Paraffin and frozen sections were blocked for non-specific antibody binding by a 30-minute room temperature incubation (10% goat serum, 0.8% BSA, 1% gelatin in PBS) followed by several washes in wash buffer (0.8% BSA, 1% gelatin in PBS). Primary antibodies appropriately diluted in incubation buffer (1% goat serum, 0.8% BSA, 1% gelatin in PBS) were applied for 1-2 hours at room temperature; antibodies against the following were used: Ki67 (1:25) (cat. # ab833; abcam, Cambridge, MA), CD3 (1:100) (cat. # 555273; BD Biosciences, Franklin Lakes, NJ), Cldn6 (1:50) (custom antibody generated from hen #3677 against mouse Cldn6 sequence-CYSTSVPHSRGPSEYPTKNYV, Aves Labs, Inc., San Diego, CA), Cldn11 (1:50) (custom antibody generated from hen #3680 against mouse Cldn11 sequence-CRKMDELGSK, Aves Labs, Inc.), Cldn12 (1:50) (custom antibody generated from hen #5186 against mouse Cldn12 sequence-CZRKLRLITFNRNEKNLTIYT, Aves Labs, Inc.), Cldn18 (1:50) (custom antibody generated from rabbit #A9953 against mouse Cldn18 sequence-CRTEDDEQSHPTKYDYV, Open Biosystems, Huntsville AL) and

Cldn1 (6:100) (cat. #71-7800; Invitrogen, Burlington, Canada). Following incubation in wash buffer, secondary antibodies against rabbit, rat and chicken conjugated to FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a 1:50 dilution in incubation buffer for 1 hour at room temperature. Images were acquired using a Zeiss Axioplan 2 brightfield/fluorescence capable microscope outfitted with an AxioCam camera and Axio Vision 2.05 software (Carl Zeiss Canada Ltd, Toronto, Canada) before processing with Adobe Photoshop version 7.0 (Adobe Systems, Inc., San Jose, CA).

Results and discussion

Characteristics of skin tumor progression

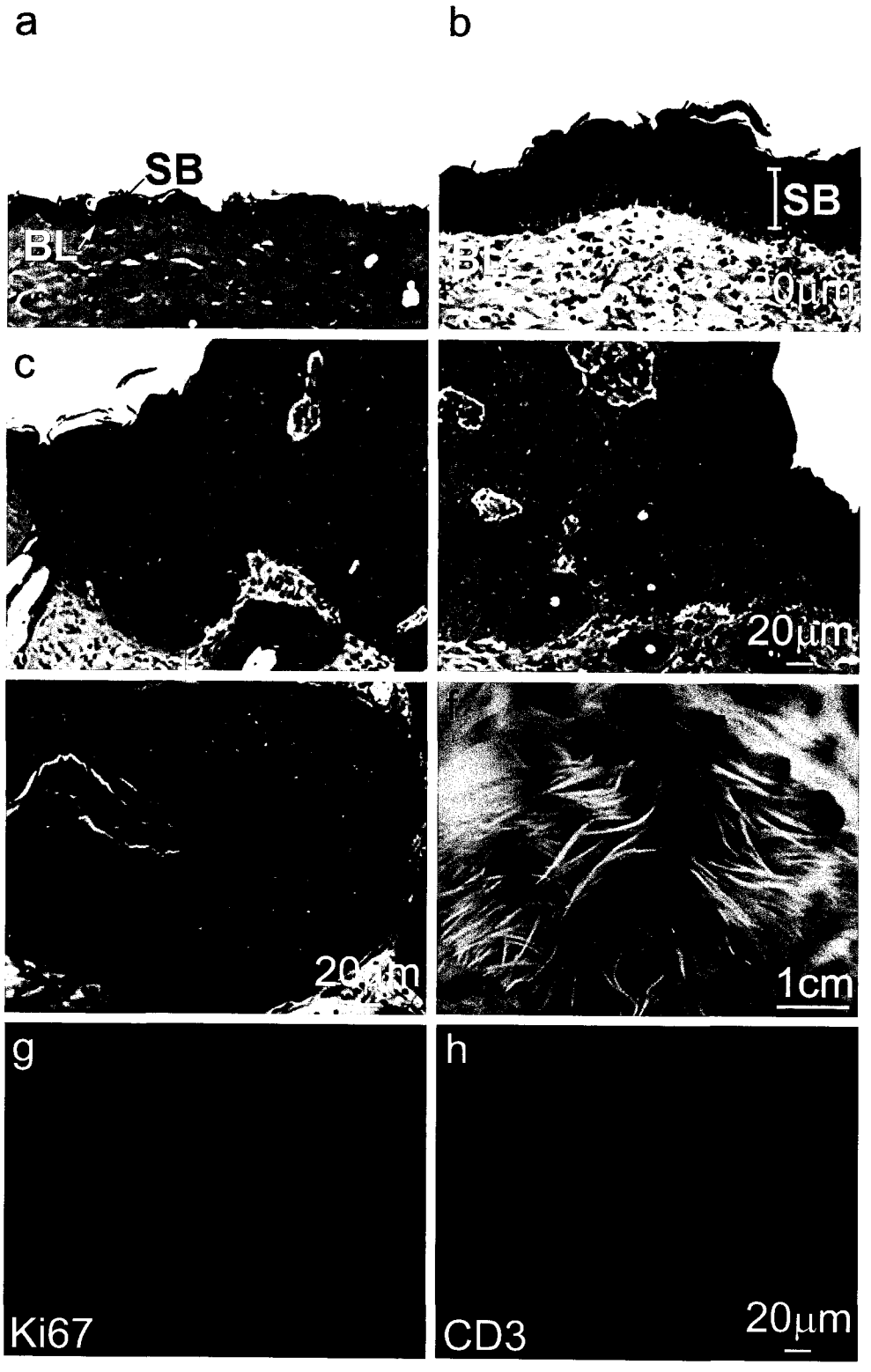
When treated with vehicle (acetone) only for 12 weeks, the epidermis (**Figure 1a**) was morphologically reminiscent of the normal, untreated epidermis in age-matched samples (not shown). Initiation with DMBA followed by 2 to 12 weeks of TPA treatment caused the epidermis progressively to display abnormalities in morphological architecture consistent with tumor formation, including a much thicker suprabasal compartment, an expanded stratum corneum and a more dispersed granular layer (**Figure 1b-e**). In addition, characteristic invaginations of epidermal structures into the dermis were evident (**Figure 1c-e**), as were pseudohorn cysts (**Figure 1c-d**, marked with stars); a photograph depicting the dorsal side of the DMBA/TPA-treated mouse after 12 weeks is shown (**Figure 1f**). Throughout the experimental protocol, immunofluorescence confirmed the expression of Ki67 (**Figure 1g**), a nuclear protein expressed by proliferating cells during all phases of the cell cycle, and the T-cell receptor-associated CD3 complex (**Figure 1h**), indicative of the proliferative and immune infiltrative states of the treated epidermis. The

expression and localization of keratins and epidermal terminal differentiation markers were also progressively disrupted as has been previously described (not shown) [13].

Figure 1: Histological characteristics of skin tumor formation.

H&E staining of the vehicle-treated epidermis after 12 weeks is shown (**a**). The epidermis after 4 (**b**), 6 (**c**), 8 (**d**) and 12 (**e**) weeks of the classical two-stage chemical carcinogenesis protocol revealed striking abnormalities in epidermal architecture consistent with tumor formation, including the appearance of pseudohorn cysts (marked with stars). The dorsal skin of the mouse after 12 weeks of DMBA/TPA treatment is shown (**f**). Both Ki67-positive cells (**g**) and CD3-positive infiltrates (**h**) were evident throughout the experimental protocol (12 weeks is shown). BL: basal layer; SB: suprabasal compartment.

Figure 1



Expression of the suprabasal Cldns in skin tumorigenesis

We next analyzed the expression of those Cldns we have previously demonstrated to be important in epidermal differentiation and the formation of the epidermal permeability barrier [2,4,5,14]. The distribution of Cldn6, Cldn11, Cldn12 and Cldn18 was indistinguishable in the normal (not shown) and vehicle-treated (**Figure 2m-p**) epidermis after 12 weeks, occupying the entire suprabasal compartment (in **Figure 2** the basal layer is marked by a dotted line and the suprabasal compartment is indicated with a bracket). After 2 weeks of DMBA/TPA treatment (**Figure 2a-d**), on the other hand, the suprabasal compartment was expanded, with a corresponding expanded zone of Cldn expression in all the suprabasal layers. However, after 6 (**Figure 2e-h**), 8 (**Figure 3a-d**) and 12 (**Figure 2i-l**) weeks, the treated epidermis displayed a marked reduction in the number of suprabasal cell layers staining positively for Cldn6, Cldn11, Cldn12 and Cldn18; the loss of staining was evident in the lower suprabasal zone (**Figure 3;** representative areas of Cldn-negative suprabasal cells are marked with stars). In addition there was an obvious but somewhat variable shift in the subcellular localization of all four of these Cldns away from the cell membrane that was especially evident in the lower suprabasal layers of the DMBA/TPA-treated epidermis after 8 weeks (**Figure 3a-d**, arrowheads illustrate representative areas where Cldn association is not restricted to cell membranes). Although all of the suprabasal-specific Cldns assayed showed some degree of non-membranous labeling after 8 weeks of treatment, this was especially true for Cldn12 and Cldn18 where the subcellular shift extended higher into the suprabasal zone than for Cldn6 and Cldn11.

In the characteristic epidermal structures infiltrating the dermis of tumors after 8 weeks of treatment, the subcellular translocalization of the suprabasal Cldns away from the cell membrane was even more apparent, with a nearly complete loss of membrane labeling (**Figure 3e-h**; the suprabasal compartment is marked with a double-ended arrow to demonstrate that the view encompasses only suprabasal cells and arrowheads point to representative areas of non-membranous Cldn labeling). In addition, there were many Cldn6-, Cldn11-, Cldn12- and Cldn18-negative suprabasal cell layers evident (note that in **Figure 3,** the stars highlight areas of Cldn-negative epidermal cells).

Figure 2: Suprabasal-specific Cldns in epidermal tumor progression.

Cldn6, Cldn11, Cldn12 and Cldn18 expression changes during epidermal tumorigenesis to occupy a gradually expanding zone of expression as observed after 2 (**a-d**), 6 (**e-h**) and 12 (**i-l**) weeks of the carcinogenesis protocol. While the localization of these suprabasal-specific Cldns shifted to varying degrees away from the cell membrane, there was a correspondingly varied reduction in the number of Cldn-positive epidermal cell layers in the lower strata of the epidermis after 6 and 12 weeks. The acetone-treated epidermis (after 12 weeks) is shown (m-p). A dotted line marks the base of the epidermal basal layer and the suprabasal zone is indicated with a bracket.

Figure 2

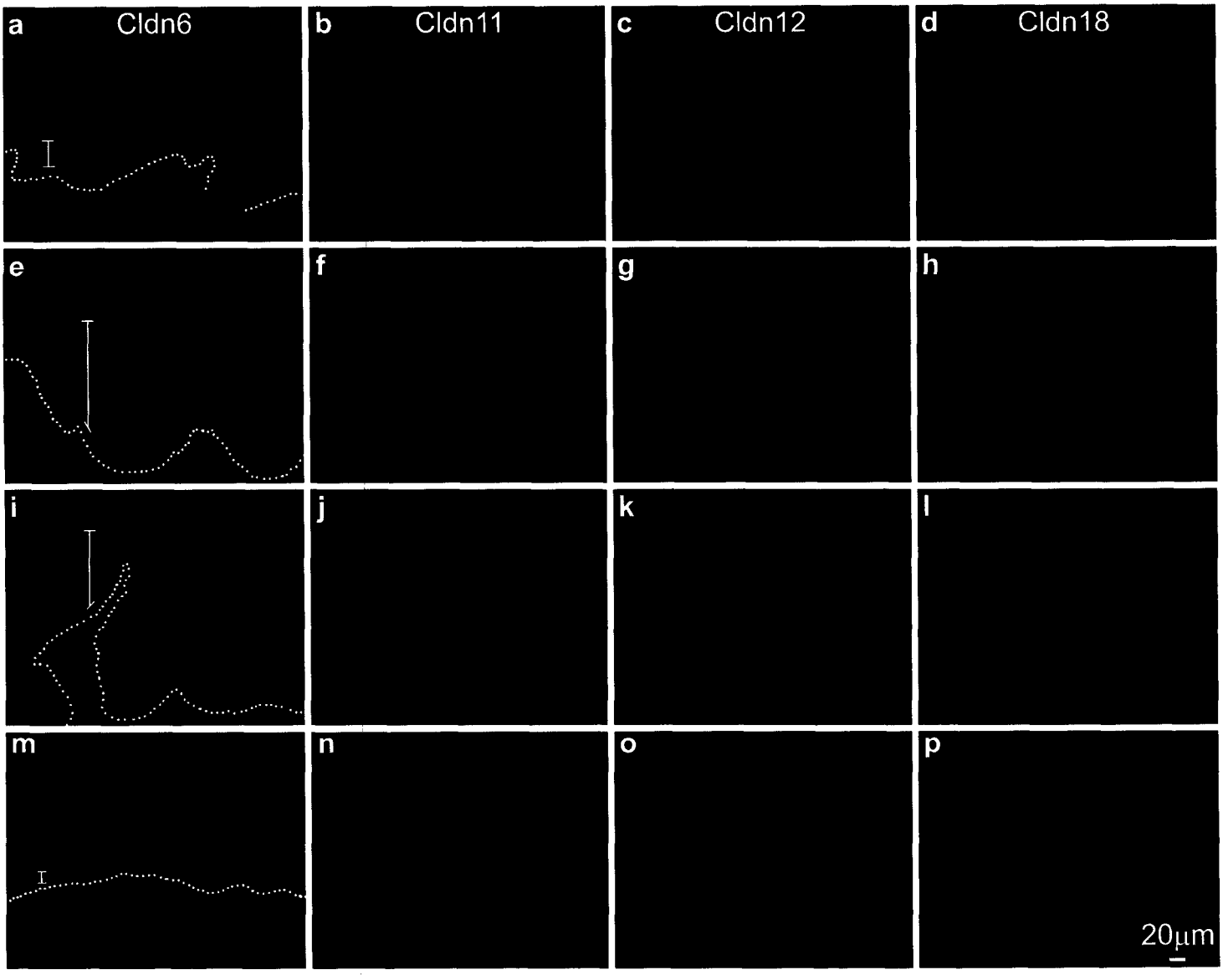
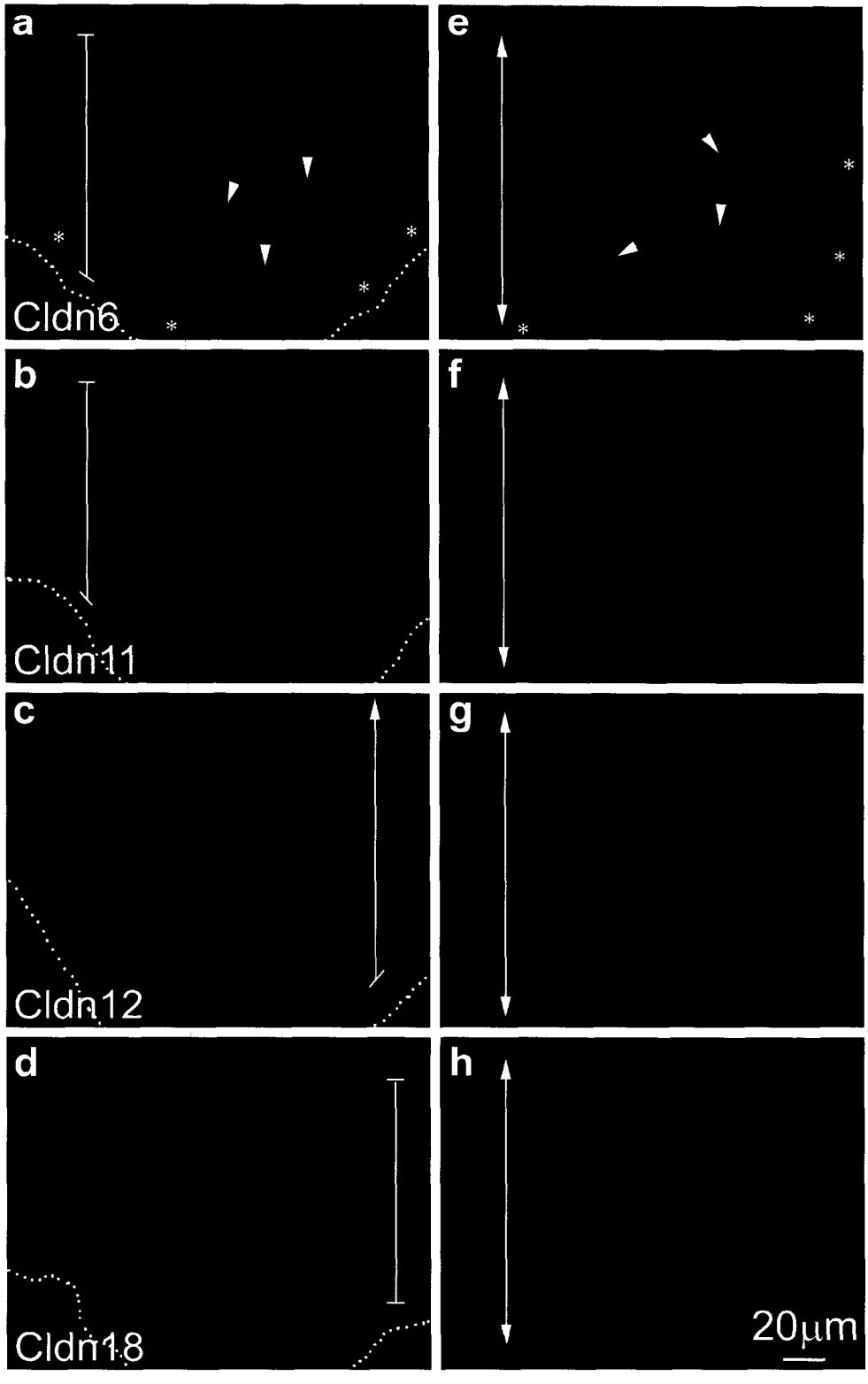


Figure 3: Differential Cldn localization in the tumorigenic epidermis.

Higher magnification immunolocalization images of the epidermis (**a-d**) as compared to the characteristic epidermal structures infiltrating the dermis (**e-h**) better demonstrate the differences in Cldn localization 8 weeks after the initiation of the DMBA/TPA protocol. Membranous Cldn association was more prominent for each Cldn, albeit to varying degrees, in the upper strata of the epidermis; whereas in the lower strata and the epidermal invaginations Cldn localization was less membranous in nature and Cldn-null epidermal cells were more frequently observed. A dotted line marks the basal layer, and a bracket encompasses the epidermal suprabasal compartment in a-d. A double-ended arrow marks the suprabasal compartment in e-h to demonstrate that the entire panel represents suprabasal cells; arrowheads point to representative areas of non-membranous Cldn localization, and stars designate areas of Cldn-negative epidermal cells.

Figure 3



The expanded suprabasal Cldn expression compartment in early DMBA/TPA treatment, followed by the loss of Cldn6, Cldn11, Cldn12 and Cldn18 in the lower strata of the suprabasal compartment as well as the reduced cell membrane association of those Cldns primarily associated with the stratifying/differentiating layers of the normal epidermis raises the question of whether such changes are obligatory for the promotional stage of skin tumor formation. The change in Cldn expression and localization may also impart new permeability properties to the affected and surrounding epidermal cells resulting in their hyperplastic conversion. Our results are consistent with the hypothesis of Daugherty et al. [15] that undifferentiated cells with poor barrier function exhibit a significant intracellular Cldn pool. In addition to transcriptional and translational regulation of expression levels, barrier function may be influenced by Cldn subcellular localization. In this context the observed changes in Cldn localization described in this study may be a result of multiple mechanisms including changes in Cldn phosphorylation. Although a number of Cldns, including the ones assessed in this study, have computer-predicted phosphorylation sites in the cytoplasmic tail domain, the functional significance of such sites has been demonstrated for only a few Cldns; e.g. Cldn3 where post-translational phosphorylation at threonine 192 has been shown to regulate TJ barrier function in ovarian cancer cells [16]. Another possibility is that there is increased endocytosis and/or an inhibition of a post-translational insertion into the ER resulting in Cldn retention in cytoplasmic vesicles. However, further investigation into the molecular mechanism(s) governing Cldn expression and localization in skin tumorigenesis is required.

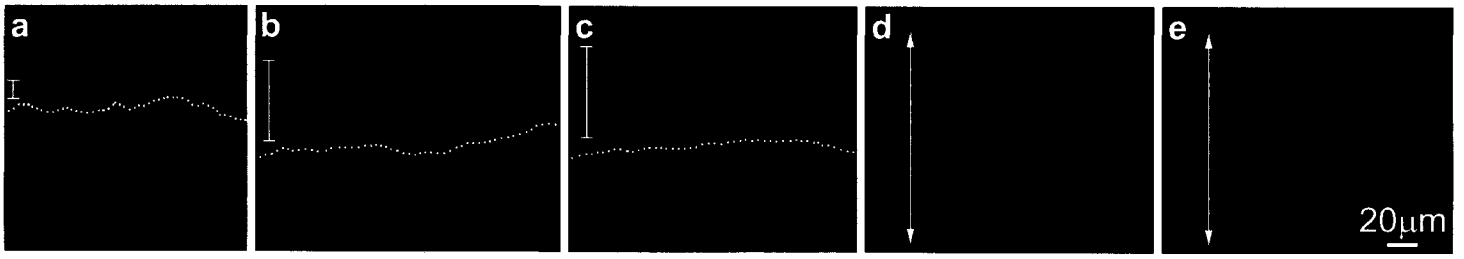
Changes in Cldn1 expression through epidermal tumor progression

Amongst the Cldns in the epidermis, Cldn1 expression is unique and undergoes a maturation switch parallel to the acquisition of epidermal barrier function. In the developing epidermis, Cldn1 expression is first restricted to the stratified layers at E15.5, and by E17.5 it occupies both the basal and suprabasal compartments [4]. This mature expression pattern is maintained throughout life under normal conditions and was not modified when the epidermis was exposed to vehicle over the duration of tumorigenesis experiments (**Figure 4a**, the acetone-treated epidermis after 12 weeks is shown). However in response to DMBA/TPA treatment, Cldn1 lost its normal distribution pattern and adopted an expression like the immature pattern (**Figure 4**). Thus, 2 weeks after initiation of the carcinogenesis protocol, Cldn1-positive cells were decreased in the basal layer of the epidermis, while the entire suprabasal compartment maintained cell membrane-associated Cldn1 expression (**Figure 4b**); by 4 weeks of treatment, the basal layer was essentially devoid of Cldn1 (**Figure 4c**). After 8 weeks of treatment, the zone of Cldn1-negative cells expanded upwards into the lower strata of the suprabasal compartment, with only sporadic presence of Cldn1 protein remaining (**Figure 4d**), a phenomena more exaggerated after 12 weeks (**Figure 4e**). However, and in contrast to the normal suprabasal-specific Cldns, the membranous localization of Cldn1 was preserved throughout the 12 weeks of sampling in the upper strata of the suprabasal epidermis. Reminiscent of the reduced number of Cldn6, Cldn11, Cldn12 and Cldn18-positive epithelial cell layers in the characteristic epidermal structures infiltrating the dermis, these areas were completely devoid of Cldn1 expression (not shown).

Figure 4: Changes in Cldn1 expression in skin tumorigenesis.

In the normal (not shown) and vehicle-treated (**a**, after 12 weeks) epidermis, Cldn1 is localized in the basal and suprabasal layers; however in response to the two-stage chemical carcinogenesis protocol, the number of Cldn1-positive epithelial cells was progressively reduced starting from the basal layer and moving upwards at 2 (**b**), 4 (**c**), 8 (**d**) and 12 (**e**) weeks. Although a distinctly membranous Cldn1 association was maintained in the upper layers of the treated epidermis, as the number of Cldn1-negative epidermal cells in the lower epidermal layers increased, only sporadic Cldn1 localization was evident (**c-e**). The epidermal basal layer is indicated by a dotted line, and the suprabasal compartment is marked with a bracket (**a-c**); note that the basal layer is out of view in panels **d** and **e**; the entire view is therefore the suprabasal compartment and is marked with a double-ended arrow.

Figure 4



Although the molecular mechanisms responsible for the reduced number of Cldn1-positive cells in epithelial tumorigenesis have not been defined, one possibility involves suppression by the Snail family of genes, which are involved in numerous tumors both in vivo and in vitro [17]. In support of this notion, functional Snail-binding E-box motifs have been demonstrated to have a suppressive role on Cldn1 expression in vitro [18,19]. Furthermore, a role for promoter methylation in the silencing of Cldn3, Cldn4 and Cldn7 has been observed in some tumors [20,21], suggesting that methylation could also play a role. Further studies are required to identify the mechanisms responsible for the changes observed in Cldn expression through epidermal tumor progression. However, our studies indicate that in this model the expression and distribution of Cldns change drastically and in a manner consistent with the loss of cell polarity and altered barrier selectivity concomitant with epidermal tumor formation.

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Chapter 5

General Discussion

5.1) Crosstalk between Cldn signaling pathway and differentiation-proliferation processes; a two-way street.

As shown in the literature, Cldn mutant animal models have been extremely valuable for understanding the importance of particular Cldns in barrier function of specific tissues. For example, Cldn1 is crucial for maintaining the epidermal barrier function where its ablation resulted in water loss and neonatal death (Furuse et al., 2002). Similarly, Cldn6 overexpression generated defective skin barrier in mice leading to their postnatal lethality as well (Turksen and Troy, 2002). Cldn5 expression is largely restricted to the vasculature (Morita et al., 1999) including that of retina (Barber and Antonetti, 2003) and brain (Wen et al., 2004). Deletion of its gene resulted in increased permeability of blood-brain barrier and neonatal lethality (Nitta et al., 2003). Cldn11-knockout mice display neurological defects and male infertility consistent with the critical role of this Cldn in TJ formation in axons as well as between sertoli cells of the testis (Gow et al., 1999). Finally, Cldn14 deficient mice are deaf due to cochlear hair cell degeneration (Ben Yosef et al., 2003). These studies collectively suggest that an imbalance in the expression levels of individual Cldns in a given epithelial tissue can be detrimental to the barrier function of that tissue. Whether altered expression of one Cldn also perturbs expression and/or localization of other Cldn molecules (i.e. Cldn homeostasis) is an issue which has been demonstrated in at least some studies (Turksen and Troy, 2002; Troy et al., 2005; Tamura et al., 2008). Therefore, it is perceived that indeed homeostasis of Cldn

molecules in a tissue is tightly linked to the maintenance of barrier functionality in that tissue. In spite of the well-documented role of Cldn changes in permeability barrier function of epithelial tissues, a new concept is emerging and gaining popularity which extends the role of these molecules beyond the boundaries of structural function. In other words, it is believed that Cldn changes may not only alter epithelial barrier properties through compromising the architecture of TJs but they may also affect the cellular pathways controlling events such as cell proliferation and differentiation (Tamura et al., 2008; Turksen and Troy 2002; Troy et al., 2005; Arabzadeh et al., 2006; Troy et al., submitted; Atasoy et al., submitted). Vice versa, it has also been documented that under conditions where cellular homeostasis (i.e. the balance between proliferation and differentiation) is disrupted, Cldn alteration appears to be one of the prominent consequences (Takala et al., 2007; Lee et al., 2005; Dhawan et al., 2005; Arabzadeh et al., 2007). Therefore, it seems that a two-way street bridges Cldn proteins to the components of differentiation-proliferation pathway. Therefore, in the next two parts those supporting evidence to such concept will be discussed. However, before heading to talk about the effect of Cldn molecules on intracellular signaling, I will first try to provide a bigger picture of the emerging function of TJs in regulation of cellular proliferation and differentiation.

5.1.1) Disruption of Cldn homeostasis; triggering aberrant proliferation-differentiation

Although function of TJs as tight intracellular seals has been recognized for over four decades (Farquhar and Palade, 1963), only recently has the role of these junctional complexes in the control of cellular proliferation and differentiation been appreciated (for review see; Matter et al., 2005). Henceforth, two different mechanisms have been known to be responsible for the effect of TJs on cell proliferation and differentiation: regulation of intracellular signaling cascades, and sequestration of cell cycle regulatory transcription factors. With respect to the former mechanism, for example, occludin has been shown to suppress Raf-1 signaling and therefore cell cycle entry via ERK/MAP kinase activation (Li and Mrsny, 2000). Tumor suppressor PTEN binds to TJ adaptor proteins MAGI-2 and -3 (Wu X et al., 2000; Wu Y et al., 2000). This interaction recruits PTEN to the junctions, resulting in dephosphorylation of products of PI3 kinase and thereby inhibition of signaling by PKB/Akt, which is a key regulator of cell proliferation and survival (Kotelevets et al., 2005; Balada and Matter, 2003). In addition, up-regulation of TJ protein cingulin correlates with cell cycle arrest and differentiation, and this is at least partly due to the inhibition of RhoA signaling and cell cycle progression (Aijaz et al., 2005; Guillemot et al., 2004; Bordin et al., 2004). With respect to the second mechanism, ZO-1 has been shown to interact with the Y-box transcription factor ZONAB, a protein required for normal proliferation (Balda et al., 2003; Balda and Matter, 2000). Through this interaction, ZONAB-CDK4 complex will be sequestered in the cytoplasm, thereby reducing nuclear CDK4 and inhibiting progression of G1/S transition (Balda et al., 2003).

Taken collectively, these observations suggest that TJs regulate formation of the barriers not only by restricting paracellular diffusion but also by modulating cell proliferation and differentiation. In such scenario, role of Cldns as crucial components of TJ barrier function cannot be disregarded. Indeed, recent research has also connected Cldn proteins to the downstream pathways regulating proliferation and differentiation of the cell. In this context, genetic manipulations of Cldns have been particularly informative. For example, Tsukita's group demonstrated that loss of Cldn15 expression in intestinal crypt cells resulted in increased rate of proliferation, producing an enlarged intestinal phenotype (megaintestine) without diseased states such as polyps or cancer in Cldn15-knockout mice (Tamura et al., 2008). Notably, the deficiency of Cldn15 changed spatio-temporal distribution of other intestinal Cldns, although expression levels remained undisturbed. Such Cldn changes in turn decreased the number of TJ strands as well as paracellular permeability to ions, which suggestedly through perturbing the ion conditions of crypt microenvironment influenced the cell proliferation (Tamura et al., 2008). In addition to megaintestine in Cldn15 deficient mice, occurrence of megatrachea (enlarged trachea) in *Drosophila* has also been attributed to a mutation in the Mega protein (a transmembrane protein homologous to Cldns), which acts in septate junctions (SJs: invertebrate equivalents of TJs) (Behr et al., 2003). Of particular interest, expression of the mutated Mega protein also resulted in mislocalization of other SJ components as well as reduced transepithelial barrier of SJs (Behr et al., 2003). In our studies, overexpression of Cldn6 in the epidermis of transgenic mice altered the background expression and localization of other types of Cldns, thereby inducing abnormality in the process of epidermal cell differentiation. The severity of perturbed differentiation was dependent on the level of

Cldn6 overexpression and determined degree of epidermal barrier dysfunction (Turksen and Troy, 2002; Troy et al., 2005). Given that lack of Cldn homeostasis was apparently associated with loss of normal differentiation of the epidermal cells in Cldn6 overexpressing mice, we took the next step and sought the mechanism behind this correlation. As cytoplasmic C-terminus of Cldns enables these molecules to make a connection with the intracellular milieu, we presumed this region to be a very good candidate responsible for the crosstalk of Cldns with downstream signaling pathways regulating differentiation and proliferation. Therefore, transgenic mice expressing different truncations of Cldn6 were generated. Intriguingly, ectopic expression of three different tail mutations produced different phenotypes where common denominator was also aberrant keratinocyte differentiation (Arabzadeh et al., 2006; Troy et al., submitted; Atasoy et al., submitted). Although discussing two other shorter truncations is beyond the scope of this thesis, it suffices to say that both transgenic mice developed a reparative process to normalize the epidermal differentiation before adulthood (Troy et al., submitted; Atasoy et al., submitted). However, in the case of deletion of nearly the entire portion of Cldn6 tail (Inv-Cldn6-CΔ187 mice); a postnatal and lifelong state of hyperplasia (epidermal thickening) appeared which itself arose from aberrant differentiation and increased proliferation of epidermal cells (Arabzadeh et al., 2006). Loss of epidermal homeostasis in these mice is believed to be due to inefficient membrane targeting of tailless Cldn6 which also perturbed normal localization of other Cldn isoforms. This observation is indeed consistent with mislocalization of SJ components in *Drosophila* Mega mutants (Behr et al., 2003) and that of intestinal Cldns

in Cldn15 knockout mice (Tamura et al., 2008); where in both cases such mislocalization also triggered a proliferation defect though spared differentiation.

Taken together, along with other researchers' findings, our data strengthen the notion that functions of Cldns are not confined to their structural roles, but they also take part in the regulation of epithelial cell proliferation and/or differentiation. To exert this novel function, each Cldn likely acts in concert with other members of the family, meaning that the overall balance in Cldn repertoire of a given tissue can determine the homeostatic balance between cell proliferation-differentiation in that tissue. In addition, as Cldns are integral membrane proteins, it is believed that they influence the intracellular signaling in an indirect manner, perhaps through Cldn tail-interacting partners. A few of these interacting partners including ZO proteins (Itoh et al., 1999), MUPP1 (Hamazaki et al., 2002) and PATJ had been identified (Lemmers et al., 2002). However recently, our laboratory succeeded to identify other tail interacting proteins among which Fiz1 (a zinc finger-containing transcription factor) and Filamin (an actin crosslinking protein) were found in a yeast two-hybrid screen using Cldn6 cytoplasmic tail as bait. Currently, our lab has undertaken separate projects using these two tail partners, in order to elucidate the molecular mechanism by which Cldn6 regulates epidermal differentiation-proliferation.

5.1.2) proliferation-differentiation anomaly elicits Cldn changes

Although the abovementioned documentation pictures Cldn isoforms or, in a more accurate sense, Cldn homeostasis in an upstream position that can influence downstream

intracellular events, but it would be a matter of question whether these proteins may also be influenced by changes in the same cellular pathways. In other words, do Cldns respond to altered proliferation/differentiation, thereby serving as indicators of anomaly in such essential cellular events?

We tried to answer such question by simulating an in vivo situation where disruption of epithelial cell proliferation and differentiation is well-known. Then, under these conditions we examined Cldn modulations. To this end, we took two different approaches. In the first one, a tissue injury response was elicited in the murine epidermis by topical application of the phorbol ester, TPA.

Both a variety of chemicals (e.g. acetone, detergents and phorbol esters) and mechanical stress (e.g. tape stripping) have been widely used to reproduce tissue injury or experimentally induced hyperproliferative condition in mouse epidermis (Molloy and Laskin, 1987; Jensen and Lavker, 1996; Yang et al., 1995; Malminen et al., 2003). In all cases, disruption of epidermal permeability barrier triggers a homeostatic response in the viable epidermis which is characterized by increased rate of basal cell proliferation along with alteration of normal differentiation (Molloy and Laskin, 1987). Such response is induced to rapidly restore those epidermal functions that have been compromised. As per this injury model, single acute exposure to TPA has been frequently used by our lab to evaluate modulated expression of diverse keratinocyte proteins including keratins, Cldns and Ca^{+2}-sensing receptor (CaSR) (Arabzadeh et al., 2008; Troy et al., submitted; Arabzadeh et al., submitted; Arabzadeh et al., submitted). It is to be mentioned that in the case of CaSR and one particular keratin (K15), our results are separately under

publication and have constituted my two other recent papers (Arabzadeh et al., submitted; Arabzadeh et al., submitted).

With respect to the question posed at the beginning, our data indicated that epidermal Cldns were modulated in parallel with changes in epidermal proliferation and differentiation induced by TPA application (Arabzadeh et al., 2008; described in the third chapter). The most notable Cldn alteration among all was loss of cell membrane association and nuclear localization of Cldn1 which coincided with the peak of proliferation-differentiation perturbation (48-72 hrs post TPA application). This effect was primarily associated with the proliferative compartment of the epidermis (i.e. basal layer). Of particular interest, in a recent study mislocalization of Cldn1 from the cell membrane into the nucleus was reported in human colon carcinoma cells and was further associated with increased activity of β-catenin signaling (Dhawan et al., 2005). β-catenin/Lef-Tcf signaling pathway is one of the best known pathways regulating cell proliferation, where its dysregualtion can result in a range of hyperproliferative disorders including cancer (Eaton and Cohen, 1996; Wong et al., 1998; Sellin et al., 2001; Gat et al., 1998; Chan et al., 1999). In association with Lef/Tcf transcription factors, β-catenin regulates the expression of a number of genes among which Cldn1 has been described as one of its targets (Miwa et al., 2001). As activation of β-catenin signal transduction pathway as well as up-regulation and nuclear localization of Cldn1 are frequent events in colorectal cancer (Dhawan et al., 2005; Miwa et al., 2001; Kinugasa et al., 2007), β-catenin signaling was suggested to be responsible for the altered expression of Cldn1 (Miwa et al., 2001). Whether TPA may work through β-catenin signaling pathway to change Cldn1 localization in epidermal cells is not yet known. However, in a separate

study it was demonstrated that activation of PKC signaling in response to scratching-induced injury of bronchial epithelial cells induces β-catenin activation and cell proliferation (Zhu et al., 2007). Knowing that TPA is a PKC activator (Hennings et al., 1992), TPA-induced injury of epidermal cells may also result in activation of β-catenin signaling, thereby altering localization of Cldn1. Determining the involvement of such potential mechanism would be a matter of further investigations in the laboratory.

Another pathophysiological condition under which loss of homeostatic balance between cell proliferation and differentiation is notoriously known is cancer (Hanahan and Weinberg, 2000). Therefore, in a second approach we embarked on inducing epidermal tumors using the very well known model of two-step chemical (DMBA/TPA) carcinogenesis (Arabzadeh et al., 2007; described in the fourth chapter), in order to evaluate Cldn alterations. In this study, the prominent observation was progressive loss of Cldn1; indeed it started very early in response to DMBA/TPA treatments in the basal layer and expanded upwards into the stratifying layers upon progression of epidermal tumors. Reduced number of Cldn-positive epithelial cell layers upon progression of tumors was not restricted to the Cldn1 but was also evident in the case of differentiation-specific Cldns6, 11, 12 and 18 (Arabzadeh et al., 2007). Our observations from chemical carcinogenesis are consistent with the reports of decreased Cldn expression in several types of cancer (Halasz et al., 2006; Vare et al., 2008; Lee et al., 2005; Rendon-Huerta, 2003; Liebner et al., 2000; Kramer, 2000; Al Moustafa et al., 2002; Kominsky et al., 2003; Quan et al., 2003). Some examples include absence of Cldn1 in glioblastoma multiforme (Liebner et al., 2000), loss of Cldn6 in head and neck squamous carcinoma

(Al Moustafa et al., 2002) and Cldn7 in invasive ductal carcinoma of breast (Kominsky et al., 2003; Quan et al., 2003). Among the studies pointing to the reduced expression of Cldns, a few have drawn a clear correlation between loss of Cldn expression and degree of tumor differentiation/proliferation. Halasz and colleagues demonstrated that the expression of Cldn1 and Cldn2 was significantly lower in embryonal hepatoblastomas (HBs) than in fetal HBs (Halasz et al., 2006). In fact, in embryonal type of HB, hepatocytes display very less differentiated phenotype with high proliferation index, suggesting an inverse correlation between perturbation of proliferation-differentiation and expression of these two Cldns (Halasz et al., 2006). In another study, a strong association was found between Cldn1 and 5 expression and Gleason score in prostate adenocarcinoma (Vare et al., 2008). Lowered expression of both Cldns was correlated with a high Gleason score, indicative of poorly differentiated tumors (Vare et al., 2008). In addition, reduced expression of Cldn4 was also associated with loss of cell differentiation in gastric adenocarcinoma (Lee et al., 2005). Finally, progressive reduction in the degree of epidermal tumor differentiation upon chemical carcinogenesis has long been known (Yuspa et al., 1980; Winter et al., 1980; Nelson and Slaga, 1982; Klein-Szanto et al., 1983), and we found it in parallel with progressive increase in number of Cldn-negative epidermal cell layers (Arabzadeh et al., 2007). Therefore, in light of recent evidence including our findings, it seems that lack of differentiation and increased proliferation (two universal features of a cancerous tissue) apparently affect Cldn proteins and in many, but not all, cases result in reduction/loss of these molecules in a given tumor.

Taken collectively, research on Cldns has recently unveiled interesting but unexplored aspects of these molecules, suggesting a bilateral correlation between Cldn proteins and signal transduction pathways regulating cell proliferation and differentiation. Therefore, changes in Cldn profile of a certain epithelial tissue can affect status of proliferation/differentiation in that tissue, and on the other hand, perturbation of tissue homeostasis can alter Cldn expression and/or localization. A fascinating theme of future studies would be to understand the regulatory mechanisms working on such two-way street, an issue that is a goal of our own further investigations as well. Although the field is nascent, we have taken steps towards this goal and in the last section of this discussion I present an overview of some of our future directions in the area of Cldn regulation.

5.2) Future directions; regulation of Cldns in tumorigenesis

As per Cldn alterations, there are numerous studies reporting changes of these molecules under a variety of pathopysiological conditions including in cancerous tissues (Burgel et al., 2002; Kucharzik et al., 2001; Wolburg et al., 2003; Mullin 2004; Morin 2005; Swisshelm et al., 2005). Although a descriptive analysis of Cldns in diverse tumors suggests that expression levels and/or localization of these molecules are modulated, the regulatory mechanisms governing Cldn modulations are poorly understood. Nevertheless, recent studies have started to unravel some of these mechanisms including modifications at the protein and/or expression level. For example, both suppression of Cldn expression in some types of cancer and overexpression in other types have been attributed to

epigenetic modification of promoter region of Cldns, where degree of DNA methylation (either hypermethylation or hypomethylation) was shown to determine the extent of Cldn expression (Boireau et al., 2007; Litkouhi et al., 2007; Osanai et al., 2007; Honda et al., 2007; Kominsky et al., 2003). In addition, inhibition of Cldn gene transcription by some transcription factors has been recognized as another mechanism responsible for altered Cldn expression in certain tumors (Ikenouchi et al., 2003; Ohkubo and Ozawa, 2004). In the context of changes in Cldn localization, phosphorylation (D'Souza et al., 2005) as well as endocytosis (Ivanov et al., 2004; Utech et al., 2005; Muller at al., 2006) have been shown to decrease surface expression of Cldns, thereby providing one mechanism for the disruption of TJs in cancerous epithelial tissue.

Therefore given the importance of Cldn regulation, our data indicating significant loss of Cldn1 in epidermal tumors urged us to delineate the regulation of this Cldn in normal versus tumorigenic keratinocytes. To this end and based on the current knowledge, our laboratory has focused on two possible mechanisms for this effect: regulation of Cldn1 expression by Snail transcription factor and by microRNA (miRNA). The former one is known to function as an estrogen-regulated suppressor (Fujita et al., 2003; Hemavathy et al., 2000) and its up-regulation in various types of cancer has been correlated with loss of expression of other cell-cell junction molecules such as E-cadherin (Batlle et al., 2000; Poser et al., 2001). The latter one belongs to the family of non-coding small RNAs that function as negative regulators of gene expression via translational suppression and/or mRNA degradation (Ambros, 2004; Bartel, 2004). Notably, the role of miRNAs in cancer development partly arises from their ability to repress the expression of several known tumor suppressor genes (Esquela-Kerscher and Slack, 2006). Therefore, exploring a

potential regulatory link between miRNAs and Cldns remains a very novel and un-investigated area that we wish to pursue it as well.

With respect to the first mechanism, a few recent studies have provided evidence of the suppression of Cldn1 expression through Snail transcription factor (Martinez-Estrada et al., 2006; Ikenouchi et al., 2003; Ohkubo and Ozawa, 2004). Multiple Snail binding sites (E-box motifs) have been found in the promoter regions of Cldn1, Cldn3, Cldn4 and Cldn7, where Snail was shown to directly bind to these regions (Ikenouchi et al., 2003). In addition, overexpression of Snail in mouse epithelial cells repressed Cldn1 expression (Ikenouchi et al., 2003; Ohkubo and Ozawa, 2004). Therefore, using RNAi and overexpression strategies in vitro, the lab is currently investigating the regulatory role of Snail on Cldn1. Our hypothesis is that up-regulation of Snail represses Cldn1 expression resulting in loss of cell polarity and barrier function; both are characteristics of tumor progression. For this purpose, two types of cell lines are being used in these experiments: SCC-13 cell line (a tumorigenic cell line isolated from human facial epidermis) (Rheinwald and Beckett, 1981) versus HaCaT cell line (normal epithelial cells isolated from normal human back skin epidermis) (Boukamp et al., 1988). RNAi knockdown of Snail in SCC cells (SCC-13$^{\downarrow Snail}$ cells) will take place using a retrovirus-mediated delivery system; while normal epithelial cells overexpressing Snail (HaCaT$^{\uparrow Snail}$ cells) will be generated using the pcDNA-DEST53 expression vector in which Snail has been cloned. SCC-13$^{\downarrow Snail}$ cells are expected to display increased levels of Cldn1 (as measured by RT-PCR and immunoblotting) and therefore improved cell polarity and barrier function. On the other hand, we anticipate that HaCaT$^{\uparrow Snail}$ cells show reduction of Cldn1

expression, thereby a decrease in cellular organization/polarity and barrier function. Cellular polarity will be evaluated using an apical-specific fluorescent lipid marker (van IJzendoorn et al., 1997) and barrier function will be examined by transepithelial resistance and paracellular flux measurements, which are invaluable ways of determining the integrity/functionality of epithelial TJs.

With respect to the second potential mechanism (i.e. miRNA suppression of Cldn1), in a preliminary analysis of the miRNA signature of human squamous carcinoma cell (SCC) tumors, we found a number of down-regulated as well as up-regulated miRNA species. Among those showing down-regulation, several are putative regulators of Snail, further supporting the important role of Snail in Cldn1 regulation. Of those identified to be up-regulated, miR-29 is a candidate as its sequence shows complementarity to the predicted miRNA binding site on the 3'-UTR of Cldn1 mRNA. It is worth noting that miR-29 binding site is the only predicted miRNA binding site on Cldn1 which is conserved in mouse and human. Therefore, the possibility of regulatory role of miR-29 in Cldn1 expression will be addressed by a number of in vivo and in vitro assays. First, using real-time qPCR on clinical samples of different stages of human SCC, we will confirm that the levels of Cldn1 and miR-29 are inversely correlated in the progression of human SCC in vivo. We will next demonstrate in vitro that miR-29 regulates the expression of Cldn1. For this purpose, we will use pMIR-REPORT miRNA expression reporter vector system, which contains a firefly luciferase reported gene under the control of a CMV promoter. The 3'-UTR of the luciferase gene has a multiple cloning site for insertion of predicted miRNA targets (in this case Cldn1 3'-UTR). By co-transfecting pMIR-REPORT-Cldn1

(3'-UTR) with pMIR-REPORT-miR-29 into HEK 293 cell (known to be devoid of native TJs as well as endogenous miR-29 activity); we anticipate seeing a reduction in luciferase activity in HEK 293 cells, providing evidence that miR-29 functionally binds the 3'-UTR of Cldn1. Finally, using the same epithelial cell contexts, we also aim for suppression as well as overexpression of miR-29 in SCC-13 and HaCaT cells correspondingly. If miR-29 is involved in Cldn1 regulation, the overexpression of pMIR-REPORT-miR-29 in normal epithelial cells (HaCaT cells) will reduce Cldn1 expression level and affect the phenotype of transfected cells to adopt a disorganized phenotype concomitant with reduced barrier function. On the other hand, suppression of endogenous miR-29 in SCC tumorigenic cells (SCC-13 cells) using commercially available anti-miR-29 inhibitors is expected to increase the level of Cldn1 in SCC cells along with normalization of their cellular phenotype with respect to polarity and barrier function, which will be measured as described above.

Whether either or both of these two potential mechanisms are involved in regulation of Cldn1 in epidermal cells is currently under investigation.

Overall, the studies going on in our laboratory during the last decade have provided invaluable evidence to various aspects of Cldn family of recently identified transmembrane proteins. Some of our scientific attempts as described in this thesis have constituted my work in the lab within the last four years. In addition, the results of this work have now paved the way for the next strides through which we wish to answer more challenging questions and add to the knowledge of Cldns and their role in both physiology and pathophysiology of epithelial cells.

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