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## Authors
Celli, Anna
Zhai, Yongjiao
Jiang, Yan
et al.

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# Tight Junction Properties Change During Epidermis Development

**A Celli**[1,*], **Y Zhai**[1,*], **YJ Jiang**[2], **D Crumrine**[1], **PM Elias**[1], **KR Feingold**[1,2], and **TM Mauro**[1]

[1]Dermatology, University of San Francisco and Veterans Affairs Medical Center, San Francisco, California 94121, USA

[2]Medical Services, University of San Francisco and Veterans Affairs Medical Center, San Francisco, California 94121, USA

## Abstract

In terrestrial animals, the epidermal barrier transitions from covering an organism suspended in a liquid environment in utero, to protecting a terrestrial animal postnatally from air and environmental exposure. Tight junctions (TJ) are essential for establishing the epidermal permeability barrier during embryonic development, and modulate normal epidermal development and barrier functions postnatally. We now report that TJ function, as well as claudin-1 and occludin expression, change in parallel during late epidermal development. Specifically, TJ block the paracellular movement of Lanthanum ($La^{3+}$) early in rat in vivo prenatal epidermal development, at gestational days 18–19, with concurrent upregulation of claudin-1 and occludin. TJ then become more permeable to ions and water as the fetus approaches parturition, concomitant with development of the lipid epidermal permeability barrier, at days 20–21. This sequence is recapitulated in cultured human epidermal equivalents (HEE), as assessed both by ultrastructural studies comparing permeation of large and small molecules, and by the standard electrophysiologic parameter of resistance (R), suggesting further that this pattern of development is intrinsic to mammalian epidermal development. These findings demonstrate that the role of TJ changes during epidermal development, and further suggest that the TJ-based and lipid-based epidermal permeability barriers are interdependent.

### Keywords

Epidermal Development; Tight Junction; Epidermal Permeability Barrier; Transepithelial Resistance

## BACKGROUND

Epidermis must transition from a prenatal epithelium in which regulated water and ion flux may be beneficial, to a postnatal epidermis that must provide an essentially impermeable

Corresponding Author: Theodora Mauro, MD, Dermatology (190) SFVAMC, 4150 Clement St., San Francisco CA 94131, USA, TEL: 001 (415) 750-2091, FAX: 001 (415) 750-2106, maurot@derm.ucsf.edu.
*These authors contributed equally

Celli A.: Performed research, designed experiments, analyzed data, wrote manuscript
Zhai Y.: Performed research, designed experiments, analyzed data
Jiang YJ: Performed research
Crumrine D: Performed research, analyzed data
Elias PM: designed research, analyzed data, wrote manuscript
Feingold KR: designed experiments, provided reagents
Mauro TM: designed experiments, analyzed data, wrote manuscript

barrier to water, ions and toxins or bacteria. Defective epidermal permeability function is devastating, especially for premature infants (<33 wks gestation), whose skin cannot yet protect against water, calorie and electrolyte loss (Hammarlund and Sedin, 1979; Harpin and Rutter, 1983) or sepsis due to microbial invasion (Marcoux et al., 2009)

The relative roles of Tight Junctions (TJ) and the lipid-based barrier in maintaining the epidermal permeability barrier has been the subject of recent intense interest (O'Neill and Garrod, 2011), with some studies supporting a primary role for the lipid based barrier in post-natal epidermis (Behne et al., 2003a; Behne et al., 2003b; Elias and Feingold, 1988; Elias et al., 1978; Elias et al., 1977; Elias et al., 1988; Elias et al., 1998; Fluhr et al., 2004a; Fluhr et al., 2004b; Holleran et al., 1993; Holleran et al., 2006; Proksch et al., 1991), while others show that TJ are essential for perinatal survival and normal epidermal function (Brandner, 2002; Furuse et al., 2002; Morita et al., 1998; Pummi et al., 2001; Troy et al., 2007a; Troy et al., 2007b; Turksen and Troy, 2002; Vockel et al., 2010).

## QUESTIONS ADDRESSED

We hypothesized that TJ form the major water and ion barrier early in development, and that this function changes when the lipid barrier is established. Further, we hypothesized that the barrier function of TJ would change during development, blocking water and ions early, but only larger molecules once the lipid barrier was in place.

## EXPERIMENTAL DESIGN

Rat fetuses were harvested from day 17 to day 22 of gestation. Cell culture, immunoblotting, electron microscopy, light and confocal microscopy were performed using standard methods (see Supporting Information).

## RESULTS

### TJ Expression and Function Change During Rat Embryonic Development

Mirroring mice and humans (Cartlidge, 2000), the rat epidermal lipid-based barrier consistently develops late in rat gestation, around gestational day 20–21 (rats are born gestational day 22) (Aszterbaum et al., 1992; Hanley et al., 1996). Relative Claudin-1 and occludin protein expression levels peaked at day 18/19, then decreased at days 20–21 (Fig 1A), the period during which the lipid barrier is established (Hanley et al., 1996). $La^{3+}$, an electron dense element with a hydrated radius (0.4 nm) similar to that of $Na^{+}$ (0.3 nm), was blocked at sites of TJ in the SG at day 18 (Fig 1B and Supplemental information (SI) figures 1A and B) but permeated through TJ sites in the SG and was blocked instead at the location of the epidermal lipid barrier, at the base of the SC, after the lipid-based permeability barrier was formed postnatally (Fig 1C and SI figure 1D). Secreted lipid processed into bilayers was noted in postnatal epidermis (Hanley et al. 1996), denoting a functional lipid barrier in this epidermis. These experiments demonstrate that TJ were able to block ion and water flux through the epidermis transiently in utero, but lost this ability late in gestation. Conversely, a lipid-based barrier was not formed early in gestation, but developed late in gestation and was able to block ion and water flux postnatally.

### TJ Changes Are Recapitulated in a Human Epidermal Equivalent Model (HEE)

HEE are useful models of epidermal differentiation, as they reproduce both the epidermal differentiation and the lipid barrier seen in skin, and can be used for electrical measurements, because they do not contain hair follicles, eccrine glands or dermis.

Morphology and TJ protein expression was similar in HEE and rat fetal epidermis, with development of a functional lipid-based barrier by 11–12 days (SI Figure 2). EM micrographs revealed structures typical of tight junctional complexes in cultures at days 5–6 (SI figure 3). $La^{3+}$ perfusion was blocked at TJ sites in the SG at days 5–6 (SI figure 2C and SI figure 3), when relative claudin-1 and occludin expression was high (SI Fig 2 B), corresponding with days 18–19 in rat skin. Likewise, $La^{3+}$ permeated through these sites and was instead blocked at the SG/SC interface by the lipid barrier at day 11 (SI Fig 2 F and SI figure 4), as seen in postnatal rat skin (compare to Fig 1C). Because $La^{3+}$ permeation cannot measure the global permeability barrier function of the epidermis, we additionally measured electrical parameters (De Benedetto et al., 2010). Transepithelial resistance (TER) peaked at day 7 (Fig 2), when $La^{3+}$ permeation was blocked at TJ sites (SI Fig 2 C). TER dropped precipitously until day 9, corresponding to decreases in occludin expression. However, TER then peaked again at day 10–11 (Fig 2), correlating with the development of a SC, secreted and processed lipid, and a competent lipid-based barrier that blocked $La^{3+}$ permeation at the SC/SG interface (SI Fig 2G).

### TJ Block Paracellular Movement of Macromolecules Later in Development

TJ have been noted to block larger molecules, such as biotin, in postnatal epidermis (Kirschner et al., 2010). HEE impeded passage of biotin at TJ sites, even as they no longer blocked $La^{3+}$ flux (SI Fig 5). These experiments suggest that TJ function changes as the epidermis matures. The evolution of TJ permeability likely corresponds to different physiologic requirements for TJ's at various stages of epidermal development.

## CONCLUSIONS

TJ are essential for establishing the epidermal permeability barrier during embryonic development, and modulate normal epidermal development and barrier functions postnatally. TJ block the paracellular movement of Lanthanum ($La^{3+}$) early in rat in vivo prenatal epidermal development and early in HEE differentiation, concurrent with upregulation of claudin and occludin. TJ then become more permeable to ions and water as the lipid epidermal permeability barrier develops. However, TJ continue to block paracellular access by large molecules, even though they become permeable to ions, suggesting an important role for these structures in postnatal epidermis. These findings demonstrate that the role of TJ changes during epidermal development, and further suggest that the TJ-based and lipid-based epidermal permeability barriers are interdependent.

## MATERIALS AND METHODS

### Animals

Timed-pregnant Sprague–Dawley rats (plug date = day 0) were obtained from Simonsen Laboratories (Gilroy, CA). Rat fetuses were harvested from day 17 to day 22 of gestation, after anesthetizing and sacrificing maternal animals, according to an SFVAMC IACUC-approved protocol. Separate maternal animals were used for each day. Postnatal rat pups (day 1–4) were used as controls.

### Electron Microscopy

Electron microscopy and lanthanum permeation were performed using the methods outlined in (Schmuth et al., 2001). Briefly, rat skin samples or human lifted culture samples to be used for electron microscopy were fixed in modified Karnovsky's solution. The basal (dermal) side of half of these samples were exposed to 4% lanthanum nitrate in 0.05 M Tris buffer (Sigma Aldrich, Saint Luis, MO) containing 2% glutaraldehyde, 1% paraformaldehyde, pH 7.4, for 1 h at room temperature, to assess lanthanum permeation.

The other half of the paired samples were prefixed in half-strength Karnovsky's fixative, followed by postfixation in 1% $OsO_4$, to assess morphology. After postfixation, all samples were dehydrated in a graded ethanol/propylene oxide series, and embedded in an Epon-epoxy mixture. Ultrathin sections were collected and assessed either unstained or after further lead citrate contrasting in a Zeiss 10 A (Carl Zeiss Inc., NY) electron microscope operated at 60 kV.

### Immunoblotting

Cell culture and heat separated epidermis samples were homogenized, and proteins were isolated using RIPA buffer (Sigma-Aldrich, Saint Luis, MO) containing protease inhibitors (Roche Applied Science, Indianapolis, IN). Protein quantification for equal loading was made with Thermo Scientific Pierce's BCA assay kit. SDS-PAGE and transfer was performed using NuPAGE Novex 4–12% sodium dodecyl sulfate-polyacrylamide gels and nitrocellulose membranes according to Invitrogen's NuPAGE protocol. Membranes were blocked in PBS with 5% non-fat dry milk and 0.05% Tween-20 and incubated with the following primary antibodies and dilutions: Claudin 1, polyclonal antibody (Invitrogen by Life Thecnologies, Carlsbad, CA) used at 1:1000 dilution with an anti-rabbit secondary antibody used at 1:2000; Occludin, monoclonal HRP-lined antibody (Invitrogen by Life Thecnologies, Carlsbad, CA), used at a dilution of 1:1000. Chemiluminescent detection was performed with ECL Plus detection reagent (Thermo Scientific Pierce, Walthman, MA) using the Fujifilm LAS-3000 imaging system. β-Actin was used as a control.

### Epidermal Skin Equivalents

Epidermal skin equivalents (HEE) were prepared by seeding 500 μL of a $10^6$human keratinocytes/ml in CNT-07 (CELLnTEC, Bern, Switzerland) on 12 well Millicell hanging 0.4 μm PET inserts (Millipore, Billerica, MA). Inserts were precoated with CellStart (Invitrogen by Life Thecnologies, Carlsbad, CA) in a 50X DPBS dilution. 1ml CNT 07 (CELLnTEC, Bern, Switzerland) was added to the well. 72 hours after seeding (day 3) the media was switched to the differentiation media CNT 02 3D (CELLnTEC, Bern, Switzerland) both on the inside and outside of the insert. Cultures were submerged in differentiation media for 16 hours and then lifted to the air media interface by removing the excess media from inside the Millicel insert and lowering the volume of the differentiation media on the outside to 500 μl. Cultures were fed every day with 500 μl of differentiation media until harvested. The Declaration of Helsinki protocols were followed, and the protocol was approved by the UCSF-SFVAMC Committee on Human Research.

### Light Microscopy

Samples of lifted cultures were harvested and halved for light and electron microscopy (see below). Samples for light microscopy were fixed in 5% formaldehyde. Epon-embedded, and 5 μm sections were stained by hematoxylin/eosin.

### TER

TER measurements were compared in HEE from day 3 to day 11 after the cells were plated. Cultures were lifted on day 4. Transepithelial electrical resistance (TER) of the epidermal equivalent was measured using an ohmmeter (EVOM; World Precision Instruments, Sarasota, FL). Measurements were performed taking care of having the same volume of culture medium in the transwell and in the outside well everytime. 1 ml culture media was placed in the outside well and 500 ul culture medium was added to the transwel prior to each measurement.

### Biotin permeation

EZ-Link Sulfo-NHS-LC-Biotin No Weigh Format Mw:556.59 g/mole (Thermo Scientific, Rockford, Il) was added to DPBS in a final concentration of 1mg/ml. 3D skin equivalents (SE) were cultured for 11 days, excised from insert and placed lifted side up on a 50 ml drop of biotin solution for 30 minutes. SEs then were submerged in OCT medium and snap frozen in liquid nitrogen. 5um frozen sections were cut on a cryostat (Leika, Germany), and counterstained with streptavidin-DyLite 633 (Thermo Fisher, Rockford, IL) in a 1:1000 dilution. Confocal images were acquired on a Zeiss 510 meta microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) using a 20X air objective and an optical slice of 2μm.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **TJ** | Tight Junctions |
| **ENaC** | Epithelial Sodium Channel |
| **SC** | Stratum Corneum |
| **TER** | Transepithelial Resistance |
| **SG** | Stratum Granulosum |
| **HEE** | Human Epidermal Equivalents |
| **TEWL** | Transepidermal Water Loss |

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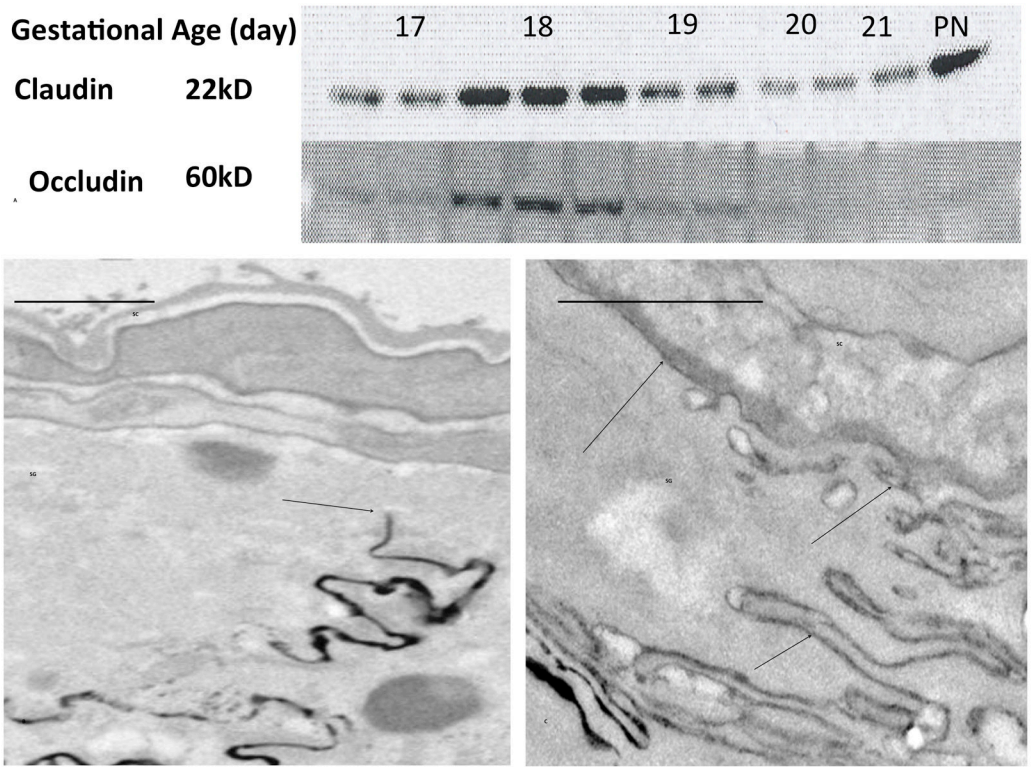


**Figure 1. Tight Junction Formation in in Vivo Rat Fetal Development**

A) Tight Junction protein expression in epidermis during the perinatal period. Western blots demonstrate that both claudin-1 and occludin expression peak at gestational day 18/19 and diminish as the fetal rat barrier is formed (day 20) and approaches parturition (day 22). Claudin-1 then peaks postnatally (PN), while occludin remains low. B) $La^{3+}$ permeation at fetal day 18. $La^{3+}$ permeates the viable epidermis until its diffusion is blocked by TJ between the lateral borders of the SG cells (arrow). C) $La^{3+}$ permeation at postnatal day 3. In contrast to panel B, $La^{3+}$ permeates through the lateral borders of the SG (arrows) and is blocked not at the SG, but instead is blocked at the SG/SC interface. SC= Stratum Corneum. SG= Stratum Granulosum. N=2–3 pups. Scale bar = 1 μm

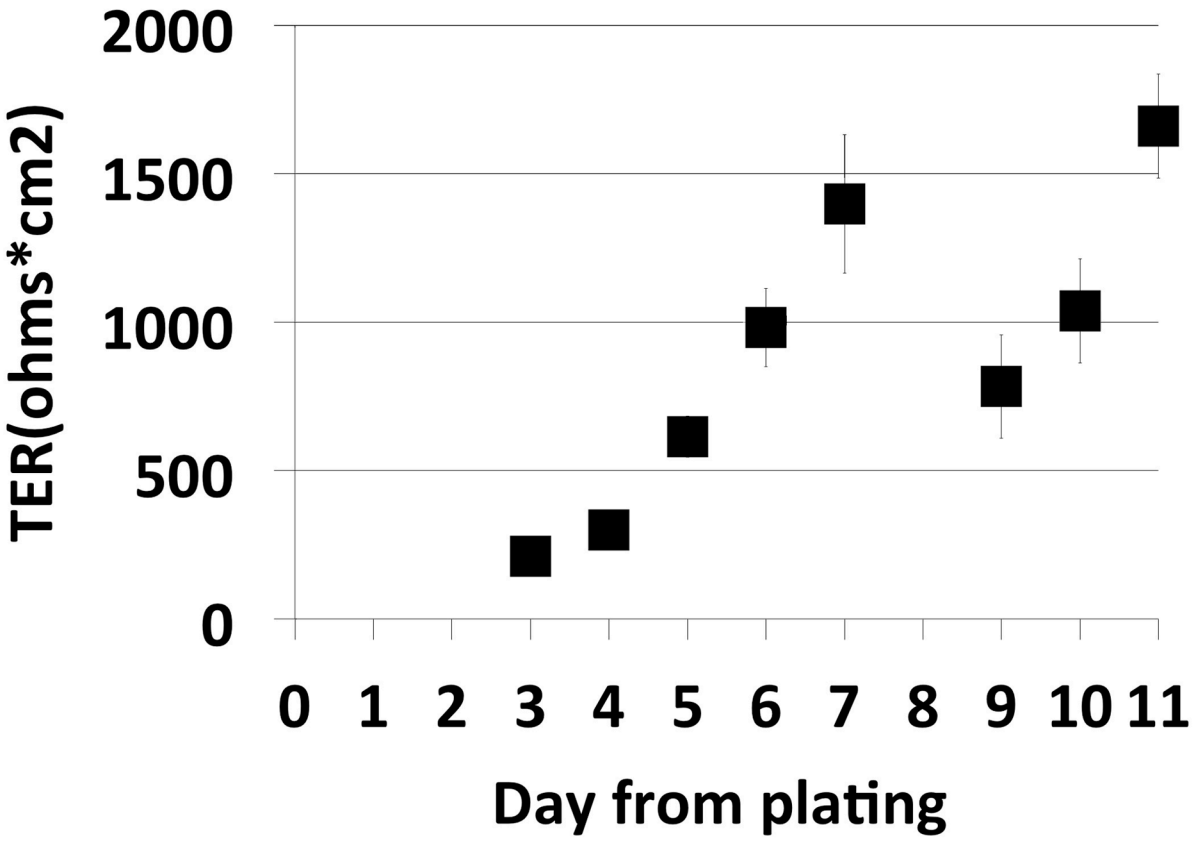


**Figure 2. Transepithelial Resistence measurements in Developing HEE**

TER was measured in HEE cultured with those analyzed in SI Figure 1. An initial maximum is recorded at day 7. TER then drops until day 9, and increases again until day 11. $n = 9–36$ for each time point. Data are presented as the mean +/− SD.