Assessing the *In Vivo* Epidermal Barrier in Mice: Dye Penetration Assays

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Journal of Investigative Dermatology (2014) **135**, e29. doi:10.1038/jid.2014.495

INTRODUCTION

The formation of a properly functioning epidermal barrier is a prerequisite for terrestrial life. The epidermis not only protects from external influences such as pathogens, chemicals, and UV light, but also prevents dehydration. To maintain proper barrier function, the epidermis undergoes a dynamic turnover driven by proliferating keratinocytes in the basal layer that, upon induction of differentiation, will move upward through the stratum spinosum and stratum granulosum while undergoing a terminal differentiation process to ultimately form the stratum corneum (SC). This layer is in direct contact with the outside environment and provides important structural and innate immune barrier properties. Barrier function is initiated when stratum granulosum keratinocytes start secreting and crosslinking specific proteins and lipids into the intercellular space and at the same time transform their membrane into a so-called cornified envelope (e.g., by linking different structural proteins such as loricrin to the inner side of the cell membrane). Consequently, keratinocytes shed their nucleus to become corneocytes forming the SC.

In recent years it has become clear that the SC is not the sole structure providing barrier properties to the epidermis. It is now clear that specialized intercellular junctions, called tight junctions (TJs), are present between cells of the granular layer, where they ensure proper barrier function (Furuse *et al*., 2002; Tunggal *et al*., 2005). TJs have been well characterized in simple epithelia where they form a paracellular size- and ion-specific barrier to separate tissue compartments. The size and ion selectivity of TJs is mainly based on which members of the claudin family of transmembrane proteins are located in the TJs of different tissues. The epidermis expresses several claudins, and loss of claudin-1 in mice results in perinatal lethality attributable to rapid dehydration (Furuse *et al*., 2002). In addition, TJs are crucial for immune surveillance by Langerhans cells (Kubo *et al*., 2009).

The importance of the two barriers is further confirmed by observations that mutations in SC proteins such as filaggrin are linked to diseases characterized by an impaired skin barrier, such as atopic dermatitis and ichthyosis vulgaris (Palmer *et al*., 2006; Smith *et al*., 2006). In addition, human claudin-1 mutations are associated with neonatal sclerosing cholangitis associated with ichthyosis (Hadj-Rabia *et al*., 2004). However, the exact molecular mechanisms of how TJs and the SC contribute to the formation, maintenance, and restoration of the skin barrier are not well understood. In addition, it is not known whether and how TJs and the SC cooperate to form a fully functional skin barrier.

Transgenic mouse models have been helpful tools to investigate how proteins implicated in epidermal barrier function contribute to the formation and maintenance of skin barrier function. Next to assessing whether such mice have a

BENEFITS OF DYE PENETRATION ASSAYS

- Dye penetration assays are a powerful tool to assess potential leakiness of the epidermal barrier *in vivo*.
- A combination of different assays provides an indication about the functionality of both the stratum corneum and the tight junction barrier.
- Application of different molecules can point to the nature of a barrier defect.

LIMITATIONS

- Dye penetration assays do not provide a molecular explanation for a barrier defect.
- Application of a single method may not display the exact origin of a barrier defect.
- Tight junction penetration assays are often difficult to evaluate in adult mice because their stratum granulosum is hardly visible.
- The functional threshold of the assays and the physiological barrier may differ.

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dysfunctional skin barrier as measured by, for example, transepidermal water loss, it would be important to assess their SC and TJ barrier function. To this end, different dye penetration assays have been developed to determine a potential SC or TJ barrier defect in these mouse models. These assays can be subdivided into assays that use dyes to assess either the outside-in or the inside-out penetration to analyze SC and TJ permeability, respectively. Dye diffusion assays provide a rapid and relatively cost-effective tool to assess epidermal barrier function. Note that diffusion of each of the described dyes through a barrier was shown to correlate with a physiological dysfunction of the respective barrier; however, the threshold of a barrier for being permeable for a dye or physiological solutes or water can differ.

INSIDE-OUT TIGHT JUNCTION BARRIER ASSAY: BIOTIN DIFFUSION

The functionality of the TJ barrier can be tested using tracer molecules of different sizes that cannot pass a properly functioning TJ barrier. Sulfo-NHS-esters of biotin will efficiently label proteins by binding primary amine-containing macromolecules on the cell surface. Once injected into the dermis, biotin reagents will diffuse through the intercellular space but will not pass a functional TJ barrier in the granular layer. Thus, diffusion of biotin above the stratum granulosum indicates a disrupted TJ barrier (Figure 1).

Furuse *et al*. (2002) were the first to successfully use this method to provide evidence for the presence of continuous occludin-based TJs in the granular layer. They found that loss of claudin-1 increased transepidermal water loss, leading to perinatal death in claudin-1-deficient mice accompanied by biotin leakage above the granular layer, highlighting the importance of TJs for epidermal water barrier function (Furuse *et al*., 2002). The assay was also used to provide evidence that epidermal deletion of the adherens junction protein E-cadherin impaired TJs, resulting in a phenotype strikingly similar to that of the claudin-1 mice (Tunggal *et al*., 2005). The assay also demonstrated that TJs function as an intercellular permeability barrier to small molecules (~550 Da) in human epidermis (Yuki *et al*., 2011).

To test the TJ barrier in mice, 10 mg/ml EZ-Link sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) in phosphate-buffered saline containing 1 mM $CaCl_2$ is injected intradermally into the back skin of newborn mice. After 30 minutes of incubation, mice are sacrificed and the skin is isolated and embedded in Tissue-Tek and snap-frozen in liquid nitrogen. Paraformaldehyde-fixed cryosections are stained with an appropriate primary antibody that recognizes a TJ protein to mark these junctions in the granular layer (frequently used targets are zonula occludens-1 or occludin). After being washed with phosphate-buffered saline (PBS), sections are incubated with an appropriate fluorescent-labeled secondary antibody to label TJs, in combination with a different fluorescent-labeled streptavidin to follow penetration of biotin and 4′,6-diamidino-2-phenylindole to label nuclei. After repeated washings with PBS, sec-

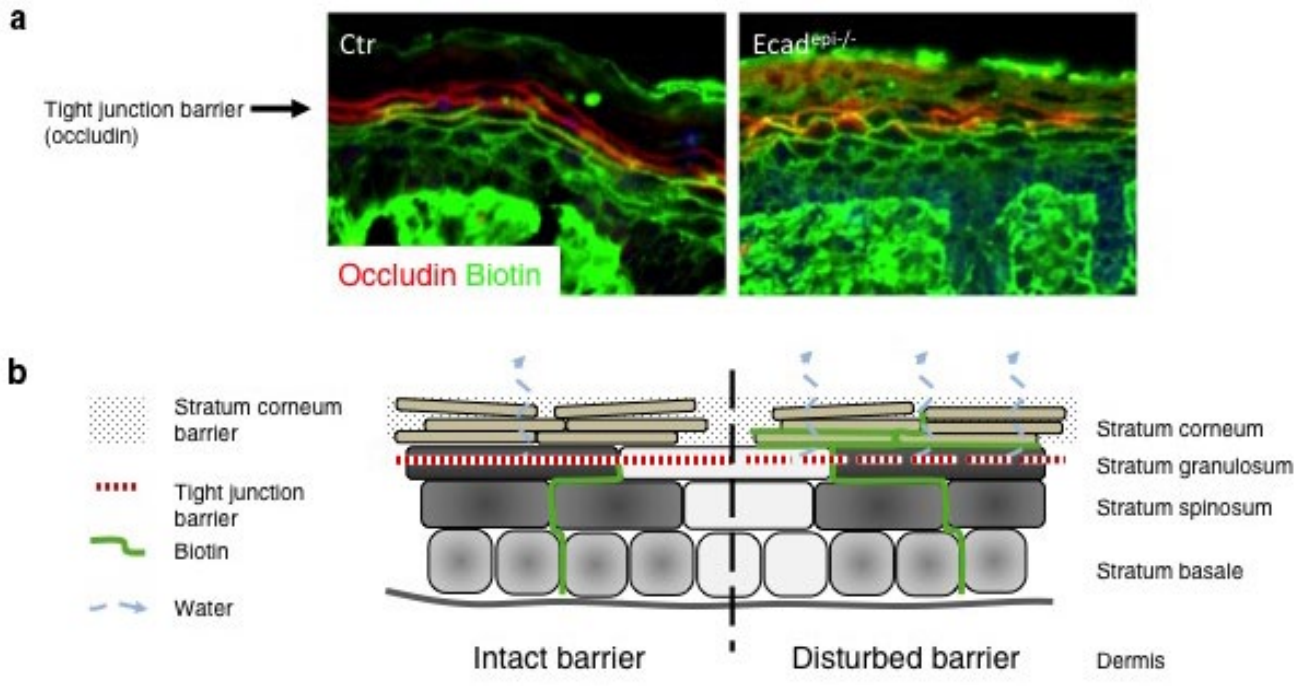

Figure 1. Inside-out barrier: biotin diffusion assay. (**a**) In healthy skin, the diffusion of subcutaneously injected biotin (green) is restricted by the tight junctions of the granular layer (marked by occludin staining in red). Epidermal deletion of, for example, E-cadherin, disturbs epidermal tight junction function as shown by penetration of biotin through the tight junction layer. Reprinted from Tunggal *et al*. (2005); copyright 2005 by John Wiley and Sons. (**b**) Schematic representation of paracellular biotin diffusion through the epidermal barrier in healthy versus disturbed condition.

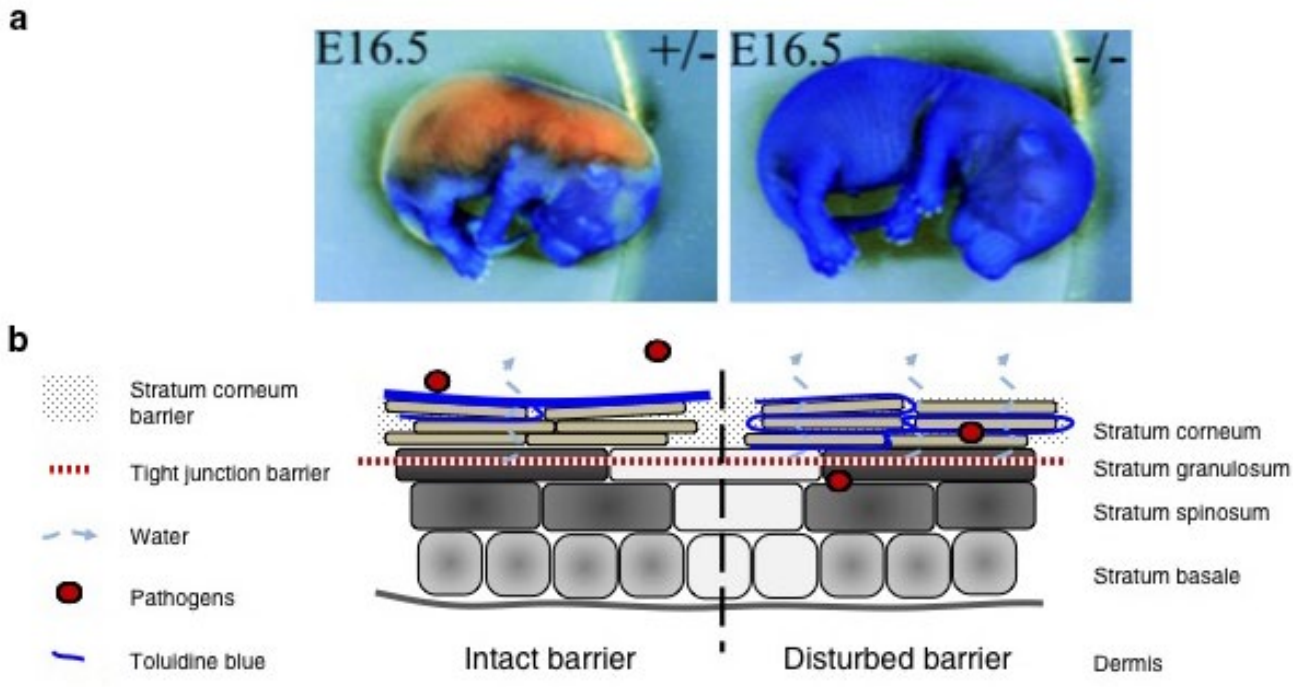

Figure 2. Outside-in barrier: toluidine blue barrier penetration assay. (**a**) Toluidine blue staining of 16.5-day-old embryos shows dye penetration at the ventral but not the dorsal side, where the barrier already has been formed (left). In mice, the first signs of a functional stratum corneum barrier appear at embryonic day 16.5 (E16.5) at the dorsal side of the embryo and progress toward the ventral side, with completion at E18.5. Mouse embryos deficient for loricrin show a clear delay in the formation of the stratum corneum barrier, as shown by whole-body penetration of toluidine blue at E16.5 (right, –/–). (**b**) Schematic representation of toluidine blue penetration through the stratum corneum barrier. Modified from Koch *et al*. (2000).

tions are mounted in mounting medium and analyzed with a fluorescence microscope to determine whether TJs are present at intercellular contacts in the granular layer and whether biotin has penetrated past these TJs.

Alternative molecules, such as fluorescent-labeled dextrans of different sizes, may be used in this assay to test whether the size-dependent diffusion barrier properties of the TJs are changed in the transgenic mouse of interest. A potential pitfall is the occasional nonspecific binding of the fluorescent-labeled streptavidin to SC structures, which are generally prone to unspecific binding in fluorescence analysis, resulting in a potential false interpretation that suggests leakage of TJs. Therefore, to avoid misinterpretation of the assay, a sufficient number of sections and number of mice must be analyzed.

OUTSIDE-IN STRATUM CORNEUM BARRIER ASSAY: TOLUIDINE BLUE

The SC barrier can be assessed by applying different dyes from the outside and measuring how far these dyes penetrate into the epidermis. One of the most commonly used dyes is toluidine blue, which is a thiazine metachromatic dye exhibiting high affinity for acidic tissue components and, therefore, nuclear material. It was first discovered in 1856 by William Henry Perkin and used in the dye industry. Since then it has been used in a number of medical applications, including its initial use as a diagnostic tool in oral cancer. Hardman *et al*. (1998) first described the use of toluidine blue in investigation of skin barrier dysfunction. It was shown that toluidine blue penetrance into the epidermis is inhibited as the SC epidermal barrier starts to form between days 16 and 17 of gestation. Using this assay at different time points during development, they demonstrated that barrier function spreads dorsally to ventrally and is complete at embryonic day 18.5 (Hardman *et al*., 1998).

To perform the toluidine blue assay, mouse embryos are sacrificed and rinsed in PBS followed by immersion in 25, 50, 75, 100, 75, 50, and 25% methanol to allow toluidine blue penetration. Although the mechanism of methanol treatment is not fully understood, it likely involves extraction of polar lipids. Subsequently, embryos are rehydrated in PBS and immersed in 0.1% toluidine blue for 10 minutes and washed again in PBS. The SC barrier function is evaluated based on the degree of dye penetration. Embryos with SC barrier defects will be stained blue, whereas a functional barrier will prevent staining (Figure 2).

Alternative dyes used for penetration assays to analyze the outside-in barrier are X-gal and Lucifer yellow, which are both applied to untreated skin. These dyes can only be visualized by staining and thus require processing of the tissue. The advantage of using toluidine blue is the immediate readout without any further processing. However, it does not offer the possibility of being combined with immunofluorescence analysis of markers of the barrier.

The use of these penetration assays is magnified in the case of a significantly disrupted barrier (Sevilla *et al.*, 2013; Sugawara *et al.*, 2013).

CONFLICT OF INTEREST

The authors state no conflicts of interest.

ACKNOWLEDGMENT

This article was written as part of the EADV/ESDR summer course Mouse Models in Skin Research, held 22–26 July 2013 at the University of Cologne.

CME ACCREDITATION

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of the Duke University School of Medicine and Society for Investigative Dermatology. The Duke University School of Medicine is accredited by the ACCME to provide continuing medical education for physicians. To participate in the CME activity, follow the link provided. Physicians should only claim credit commensurate with the extent of their participation in the activity.

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SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2014.495.

QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

For each question, more than one answer may be correct.

1. Which are the principal structures participating in the epidermal barrier?

A. Tight junctions.
B. Stratum corneum.
C. Basement membrane.
D. Stratum spinosum.

2. Which are the essential components of the stratum corneum barrier?

A. Protein–lipid crosslinks.
B. Collagen.
C. Adherens junctions.

3. Dye penetration assays show which of the following?

A. The origin of the barrier leakage.
B. Leakage of the tested barrier.
C. Severity of barrier defects.

4. What are the limitations of dye penetration assays?

A. They cannot show leakiness of a specific barrier.
B. Dye penetration may not reflect physiological leakiness.
C. Single assays are not sufficient to determine the origin of the barrier defect.

5. What is the function of the tight junction barrier?

A. Mechanical rigidity.
B. Paracellular diffusion barrier.
C. Separating tissues from outside environment.
D. Protection from UV light.

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