CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Tight Junction Proteins Claudin-1 and Occludin Are Important for Cutaneous Wound Healing

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Accepted for publication
February 7, 2017.

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Tight junction (TJ) proteins are known to be involved in proliferation and differentiation. These processes are essential for normal skin wound healing. Here, we investigated the TJ proteins claudin-1 and occludin in *ex vivo* skin wound healing models and tissue samples of acute and chronic human wounds and observed major differences in localization/expression of these proteins, with chronic wounds often showing a loss of the proteins at the wound margins and/or in the regenerating epidermis. Knockdown experiments in primary human keratinocytes showed that decreased claudin-1 expression resulted in significantly impaired scratch wound healing, with delayed migration and reduced proliferation. Activation of AKT pathway was significantly attenuated after claudin-1 knockdown, and protein levels of extracellular signal-related kinase 1/2 were reduced. For occludin, down-regulation had no impact on wound healing in normal scratch assays, but after subjecting the cells to mechanical stress, which is normally present in wounds, wound healing was impaired. For both proteins we show that most of these actions are independent from the formation of barrier-forming TJ structures, thus demonstrating nonbarrier-related functions of TJ proteins in the skin. However, for claudin-1 effects on scratch wound healing were more pronounced when TJs could form. Together, our findings provide evidence for a role of claudin-1 and occludin in epidermal regeneration with potential clinical importance. (*Am J Pathol* 2017, 187: 1301–1312; <http://dx.doi.org/10.1016/j.ajpath.2017.02.006>)

Cutaneous wound healing (WH) is a complex and well-orchestrated process that proceeds in three different phases: inflammation, regeneration, and remodeling.¹ To rebuild the lost epidermis, keratinocytes from the surrounding tissue need to increase proliferation and mobility to cover the wound bed.¹ It is known that keratinocytes migrate into the wound as an epithelial sheet.² To achieve this uniform movement, adhesion between adjacent cells and the underlying surface has to be carefully regulated.³ Chronic wounds are often stuck in the inflammation phase with, among others, increased proinflammatory cytokine levels^{4–6} and misregulated proliferation and migration of keratinocytes,⁷ resulting in an absent or reduced coverage of the wound.

Tight junctions (TJs) are cell–cell junctions known to be important for the formation of a paracellular barrier in the skin.⁸ In addition, further functions that are important for WH have been linked with TJ proteins in

keratinocytes: claudin-1 has been shown to influence keratinocyte proliferation,⁹ claudin-1 and occludin are involved in epithelial differentiation,^{10–13} and occludin is important for cell–cell adhesion.¹¹ In studies using human skin organ culture models¹⁴ or samples taken from human suction blisters¹⁵ it was shown before that TJ proteins claudin-1, zonula occludens (ZO)-1, and occludin are present early in newly formed regenerating epidermis of normal healing wounds. A link between WH and TJs/TJ proteins has also been described in other organs. In urethral full thickness wounds, occludin was present in the newly formed cells.¹⁶ In rabbit corneal WH, fragments of TJ strands were found in areas behind the leading cells already 2 days after wounding. When the wound was

Supported by the German Ministry of Economics grant KF239060SK1 (J.M.B.).

Disclosures: None declared.

closed, TJ strands were also seen in the central area.¹⁷ In rat cornea, occludin was found at the wound edge and in the regenerating epithelium during all WH phases,¹⁸ whereas in a type 2 diabetic rat model, corneal wound closure rates were decreased and associated with a lower expression of occludin.¹⁹ The same effects were observed in the cornea of vitamin D receptor knockout mice.²⁰ Also consistent with these findings, butyrate was found to promote scratch WH of porcine small intestinal epithelial cells while inducing a significant increase in mRNA for occludin and ZO-1.²¹

Thus, we hypothesized that TJ proteins are involved in skin WH. Therefore, we investigated the expression and localization of claudin-1 and occludin in spontaneous WH *ex vivo* and *in vivo* and compared this with chronic wounds. Because we observed a down-regulation of occludin and claudin-1 in chronic wounds, we further investigated the consequences of this down-regulation for processes important for WH and provide evidence that both proteins play roles in WH.

Materials and Methods

Tissues, Antibodies, siRNAs, Inhibitors

Human skin samples from infant donors (<5 years) used for cell culture were obtained after medical circumcisions. Human adult skin for WH models (WHMs) was obtained from patients undergoing plastic surgery. Tissues from chronic wounds were obtained from the borders of chronic leg ulcers that had existed for at least 1 month without healing. The patients (four men, nine women; aged 35–83 years) had mixed (venous and arterial) of various degrees, $n = 9$) and diabetic ($n = 4$) ulcers. Ulcers were located in the lower leg ($n = 10$) or either the popliteal, ankle, or instep ($n = 1$ each). The samples were used after diagnostic procedures had been completed. Tissue of normally healing wounds ($n = 2$) (mean age of patients, 56 years) was taken by consent from healthy volunteers. Wounds were generated by performing a punch biopsy (diameter, 0.6 cm; depth, 0.5 cm) at the lower back.²² The usage of the different tissues was approved by the ethics committee of the Ärztekammer Hamburg (OB-15/04, WF-28/12, WF-61/12). Porcine ears were obtained from a local slaughterhouse after slaughtering of the animals for human consumption. For antibodies, siRNAs, and inhibitors see [Table 1](#).

Cell Culture

Human keratinocytes were isolated from foreskin and cultured as described.²³ Keratinocytes were maintained in either serum-free Gibco Keratinocyte-SFM (Life Technologies, Carlsbad, CA) or serum-free keratinocyte growth medium-2 (Promocell, Heidelberg, Germany) with defined growth supplement and 100 µg/mL penicillin/streptomycin

under low-calcium conditions. Experiments were performed in passage 2 to 5.

Human and Porcine *ex Vivo* WHMs

Human *ex vivo* WHMs were established and cultured as described in Neub et al,²⁴ porcine models as described in Pollok et al.²³ In short, after washing and disinfecting the pig ears, punch biopsies (diameter, 6 mm) were taken from intact parts of the plicae. Fat, subcutis, and parts of the lowermost dermis were removed, and subsequently wounds were generated by taking a punch biopsy (diameter, 3 mm) from the central part of the 6-mm biopsy, thus removing the epidermis and the upper dermis at this area and generating a WHM ([Supplemental Figure S1A](#)). The *ex vivo* WHMs were then placed on gauze with the dermis side down and put separately into the wells of a 12-well plate, filled with Dulbecco's modified Eagle's medium (Biochrom, Berlin, Germany) supplemented with fetal calf serum, hydrocortisone, and penicillin/streptomycin, to be cultured under air–liquid interface conditions for up to 5 days. For human skin, generation of WHMs was identical except washing and disinfecting had already been done at the operating room.

WH progress was defined as follows: fresh, directly after wounding; early, <25%; intermediate, 25% to 50%; advanced, 51% to 75%; late, 76% to 99%; closed, 100% wound area covered.

siRNA Treatment

Keratinocytes were transfected by using HiPerFect Transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, cells were transfected by a fast forward protocol. Cells (2×10^5 cells/mL) were transfected with a 1:1 mixture of HiPerFect reagent and a 20 µmol/L solution of target or control siRNA, respectively (final dilution of siRNA, 1:200). Efficiency of knockdown (KD) was controlled by Western blot analysis after 2 to 4 days.

Cell Scratch Assays

Keratinocytes were seeded at 2×10^5 or 1×10^5 cells per well in 12- or 24-well plates, respectively, and grew to confluency. Then keratinocyte monolayers were wounded using a 200-µL pipette tip, resulting in a scratch wound of a width of around 670 µm. Fresh medium was added after washing the cells twice with phosphate-buffered saline (PBS). In each of two wells per condition, five areas of the scratch were photographed at t_0 (immediately after the scratch) and every 12 hours for the next 36 hours. Scratch wound area was measured using TScratch software version 1.0 [Koumoutsakos group (CSE Lab), ETH Zürich, Switzerland].²⁵

To inhibit proliferation, mitomycin C was dissolved in fresh medium to a final concentration of 15 µg/mL and added to the cells 3 hours before scratching. After scratches

Table 1 List of Reagents Used in the Study

Application	Name	Company	Dilution
siRNA			
siRNA KD	Hs_CLDN1_5 (claudin-1 siRNA no. 2)	Qiagen (Hilden, Germany)	1:200
siRNA KD	Hs_CLDN1_8 (claudin-1 siRNA no. 1)	Qiagen	1:200
siRNA KD	Hs_OCLN_7 (occludin siRNA no. 2)	Qiagen	1:200
siRNA KD	Hs_OCLN_11 (occludin siRNA no. 1)	Qiagen	1:200
siRNA KD	AllStars Negative Control	Qiagen	1:200
Primary antibodies			
WB	Claudin-1; mouse; mAb	Thermo Scientific (Waltham, MA)	1:2000
IF	Claudin-1; rabbit; pAb	Thermo Scientific	1:150
WB	Occludin; rabbit; pAb	Thermo Scientific	1:3000
IF	Occludin; mouse; mAb	Thermo Scientific	1:100
WB	α -Tubulin; mouse; mAb	Merck (Darmstadt, Germany)	1:2000
WB	β -Actin; mouse; mAb	Merck	1:80,000
WB	ERK1/2; mouse; mAb	Merck	1:500
WB	p-ERK1/2; rabbit; mAb	Merck	1:500
WB	AKT (pan); rabbit; mAb	Cell Signaling (Danvers, MA)	1:600
WB	p-AKT (Ser473); rabbit; pAb	Cell Signaling	1:300
WB	p38; rabbit; pAb	Cell Signaling	1:1000
WB	p-p38 (Thr180/Tyr182); rabbit; pAb	Cell Signaling	1:200
IF	Ki-67; mouse; mAb	Dako (Hamburg, Germany)	1:20
Secondary antibodies			
WB	Goat anti-rabbit, HRP; pAb	Jackson Immuno (West Grove, PA)	1:5000
WB	Goat anti-mouse, HRP; pAb	Jackson Immuno	1:5000
IF	Goat anti-rabbit, F(ab') ₂ Alexa 488	MoBiTec (Göttingen, Germany)	1:600
IF	Goat anti-mouse, F(ab') ₂ Alexa 488	MoBiTec	1:600
IF	Goat anti-rabbit, F(ab') ₂ Alexa 594	MoBiTec	1:1250
Inhibitors			
Inhibition	Mitomycin C (M0503)	Merck	
Inhibition	MK-2206 (M1837)	AbMole BioScience (Kowloon, Hong Kong)	

ERK, extracellular signal-related kinase; HRP, horseradish peroxidase; IF, immunofluorescence staining; KD, knockdown; mAb, monoclonal antibody; p-, phospho-; pAb, polyclonal antibody; WB, Western blot analysis.

were made, cells were washed twice with PBS to remove mitomycin C before adding new medium.

For cell scratch assays under high-calcium conditions to induce or strengthen the formation of TJs, desmosomes, and adherens junctions, confluent cells were incubated for an additional 24 hours with fresh medium with a calcium concentration increased from 0.06 to 1.8 mmol/L, which was maintained for the whole experiment.

Number of donors for the individual experiments is mentioned in the figure legends.

Inhibition of AKT

MK-2206 was used in a concentration of 2 or 5 μ mol/L to inhibit phosphorylation of AKT.²⁶ MK-2206 was added 3 hours before scratching and was resupplemented during regular medium changes throughout the experiment. Efficiency of MK-2206 was checked by Western blot analysis for phospho- (p-)AKT.

Stressed Cell Scratch Assay

To investigate the impact of impaired adhesion during cell migration we performed additional scratch assays in which

the 24-well plates were put on a vortex mixer for 30 seconds every 60 minutes for the first 6 hours after the scratch. Thus, cells were exposed to mechanical stress. Images of the scratch wounds were taken 6 hours after scratching. In every experiment, a second well plate that was not subjected to mechanical stress but otherwise was treated identically was also monitored to serve as a control. Different donors ($n = 4$) were used for occludin siRNA no. 1 and no. 2.

Cell Proliferation Assay

Cells were grown on coverslips to confluent monolayers, scratched, and then fixed 24 hours after wounding. After immunostaining for Ki-67, proliferation was determined by comparing counts for Ki-67-positive cells with the total cell number at the scratch margins evaluated by nuclear DAPI staining. Three visual fields per sample were counted for evaluation.

Cell-Matrix Adhesion Assay

Twenty-five thousand transfected or nontransfected keratinocytes were seeded in 100 μ L of medium per well in

96-well plates in triplicates for each condition. After indicated intervals supernatant fluids were transferred to another plate, and remaining adherent cells were washed twice with PBS, followed by the addition of fresh keratinocyte growth medium. Then MTT assay was performed. For estimation of the total number of viable cells, wells were subjected to MTT assay without removing the supernatant fluid first.

MTT Assay

MTT assay was performed slightly modified after Mosmann.²⁷ Briefly, 10 μ L of MTT stock solution (5 mg/mL) was added to each well and incubated for 3 hours at 37°C. Thereafter, 100 μ L of 2-propanol was supplemented. The plate was incubated overnight at room temperature in the dark. Absorbance was measured at 570 nm. After subtraction of blank values relative amount of adhered, viable cells was determined for each treatment using the following formula: % adhered cells = [OD (cells adhered at different time points)/OD (total amount of viable cells)] $\times$ 100%.

Immunofluorescence Staining

Immunostaining was performed as described previously.^{11,28} For antibody dilutions see Table 1.

Western Blot Analysis

Cultured cells were washed with PBS and subsequently lysed in RIPA buffer supplemented with Phosphatase Inhibitor Cocktail (100 $\times$) (Cell Signaling, Danvers, MA). Equal amounts of total protein determined by Bradford assay were separated by SDS-PAGE and transferred to nitrocellulose. After blocking for 1 hour with 5% w/v dry milk powder in tris-buffered saline with Tween 20 buffer, membranes were probed overnight with the antibodies in dilutions described in Table 1. α -Tubulin and β -actin were used as loading controls.

Statistical Analysis

Data are presented as means + SEM. For statistical tests, *t*-tests or *U*-tests were calculated using Excel 2010 (Microsoft, Redman, WA), random intercept models were analyzed using SPSS version 21 (SPSS Inc., Chicago, IL). For random intercept models, cell donor and technical replicate as well as image position were set as random effects, and time after scratch, the initial wound size, and siRNA treatment were included as fixed effect. Further, we also included the interaction of treatment and time as a fixed effect. Bonferroni correction was used to adjust the calculated *P* values for the multiple comparisons of differences between estimated means. The respective tests are mentioned in the figure legends.

Results

Localization of TJ Proteins in Acute and Chronic Wounds

For the investigation of acute wounds we used a human *ex vivo* WHM¹⁴ and *in vivo* wounds.²² For the comparison with chronic wounds, in which normally no or only small areas of regenerating epidermis can be seen, WHM/*in vivo* wounds at early stages of WH were used, which means with no or restricted regeneration (wound coverage <25%).

Claudin-1 is localized in all viable cell layers in unwounded healthy epidermis *in vivo* (Figure 1A) and in acute human WHMs (6 of 6; data not shown). In addition, at and behind the wound margins of acute wounds of the human WHM claudin-1 could be found in all layers (6 of 6), and the according regenerating epidermis proved to be positive for this TJ protein (4 of 4; *n* = 4 because no regenerating epidermis in two models) (Figure 1, B and D; Supplemental Figure S1A). In some cases (2 of 4), the very first cell(s) were negative (Figure 1B). In contrast, in chronic wounds, claudin-1 was lost in one or several layers in the areas behind the wound margins in all cases (13 of 13) (Figure 1D) and at the wound margins in most cases (11 of 13) (Figure 1, C and D). The majority of cases were negative for a substantial part of the regenerating epidermis (9 of 11, no regenerating epidermis in two cases) (Figure 1, C and D). Of note, when investigating the dynamics of claudin-1 alteration during WH that was performed in porcine *ex vivo* models because of the larger availability of tissue (*n* = 12 to 17, dependent on WH stage), we observed also a loss of claudin-1 in the regenerating epidermis but mainly at later stages (advanced and late WH as well as closed wounds) of WH and to a lower extent. There was no loss of claudin-1 at and behind the wound margins at any stage (Supplemental Figure S1B). Thus, there seems to be a more pronounced and untimely down-regulation of claudin-1 in chronic wounds.

Occludin is restricted to the stratum granulosum in unwounded epidermis *in vivo* (Figure 2A) and in acute wounds of the human WHM (6 of 6; data not shown). We found the same expression pattern behind the wound margins (6 of 6) (Figure 2D) and for 5 of 6 WHMs at the wound margins (Figure 2D). The same was true in multi-layered parts of the regenerating epidermis in the human WHM (3 of 4; *n* = 4 because of no regenerating epidermis in two models) (Figure 2D). When the regenerating epithelial sheet consisted of only one to two layers (leading edge), occludin could be found in the basal layer (2 of 4) (Figure 2B). Expression of occludin was absent in the very first cells of the leading edge (3 of 4) (Figure 2B) but in one case also from the whole regenerating epidermis (1 of 4) (Figure 2D). Strikingly, in chronic wounds, the protein was lost in a substantial part of the regenerating epidermis in all cases (11 of 11) and at the wound margins of most samples (10 of 13) (Figure 2, C and D). In the areas behind the

Figure 1 Localization of Cldn-1 in healthy human skin and acute and chronic human wounds. **A–C:** Immunolocalization of Cldn-1 in healthy skin (**A**), acute wound healing model (wound margin and regenerating epidermis) (**B**), and a chronic wound (wound margin and regenerating epidermis) (**C**). Overlay of Cldn-1 immunofluorescence staining (green) and phase contrast images or DAPI nuclear staining (**B**, inset). White lines mark the outline of the vital part of the epidermis. **D:** Graph showing the proportion of samples of healthy skin, acute, and chronic wounds with Cldn-1 in all layers, SG and SS, SG and upper SS, SG only, or without Cldn-1 at all behind the wound margins, at the wound margins as well as in the regenerating epidermis. $n = 6$ samples of healthy skin; $n = 6$ samples of acute wounds; $n = 4$ samples of acute wounds in regenerating epidermis; $n = 13$ samples of chronic wounds; $n = 11$ samples of chronic wounds in regenerating epidermis (**D**). Scale bar = 20 μm (**A–C**). Cldn-1, claudin-1; Occludin, occludin; SG, stratum granulosum; SS, stratum spinosum.

wound margins of chronic wounds a broader localization was observed with occludin being found in the stratum granulosum as well as the stratum spinosum in 62% of the analyzed samples (8 of 13) (Figure 2D). Investigating TJ protein dynamics in the porcine WHM ($n = 14$ to 22, depending on WH stage), we never observed a complete loss of protein in the regenerating epidermis at any stage of regeneration (Supplemental Figure S1C). Similar to human skin, the very first cells at the leading edge were often negative (data not shown).

To exclude that the differences seen between acute and chronic wounds are due to the *ex vivo* situation of our acute wounds we also investigated two human *in vivo* acute wounds. In these *in vivo* wounds we observed the same localization of TJ proteins as in most human *ex vivo* wounds (data not shown).

Because of the striking down-regulation of claudin-1 and occludin in chronic wounds we investigated the effect of

their down-regulation by siRNA treatment on WH in cell culture. Many experiments were performed under low-calcium conditions to mimic the non- and low-differentiated cells mainly contributing to the leading edge. However, some experiments were also performed with high-calcium cells to take the formation or strengthening of cell–cell junctions into account.

KD of Claudin-1 Results in Impaired Scratch Wound Closure due to Decreased Proliferation and Impaired Migration under Low-Calcium Condition

In general, we observed a significant up-regulation of claudin-1 mRNA in keratinocytes 6 hours (2.3 ± 0.4 -fold; $P < 0.01$) and 24 hours (1.7 ± 0.3 -fold compared with unwounded; $P < 0.05$) after scratch wounding. After KD of claudin-1 by claudin-1 siRNA no. 1 (KD efficiency: $93 \pm 10\%$, which means reduction of claudin-1 protein

Figure 2 Localization of Occludin (Ocln) in healthy human skin and acute and chronic human wounds. **A–C:** Immunolocalization of Ocln in healthy skin (**A**), acute wound healing model (regenerating epidermis) (**B**), and a chronic wound (wound margin and regenerating epidermis) (**C**). Overlay of Ocln immunofluorescence staining (green) and phase contrast images. **White lines** mark the outline of the vital part of the epidermis. **D:** Graph showing the proportion of samples of healthy skin, acute, and chronic wounds with Ocln in all layers, in SG and SS, SG and upper SS, SG only, or without Ocln at all behind the wound margins, at the wound margins, and in the regenerating epidermis. $n = 6$ samples of healthy skin; $n = 6$ samples of acute wounds; $n = 4$ samples of acute wounds in regenerating epidermis; $n = 13$ samples of chronic wounds; $n = 11$ samples of chronic wounds in regenerating epidermis (**D**). Scale bar = $20 \mu\text{m}$ (**A–C**). Ocln, occludin; SG, stratum granulosum; SS, stratum spinosum.

level to $7\% \pm 10\%$), a strong and significant down-regulation of scratch WH was found (Figure 3A). A similar, even though less pronounced, effect for KD efficiency ($83\% \pm 16\%$; which means protein level reduced to $17\% \pm 16\%$) and WH impairment was also observed with claudin-1-specific siRNA no. 2 (Supplemental Figure S2A). KD of claudin-1 with both siRNAs had only minor effects on the protein levels of TJ proteins occludin (no. 1: reduced to $71\% \pm 36\%$; no. 2: $88\% \pm 39\%$; $n = 3$) and ZO-1 (no. 1: reduced to $96\% \pm 15\%$; no. 2: $75\% \pm 11\%$; $n = 3$).

Because scratch WH is mediated by proliferation and migration, we investigated these two cell biological features separately. When investigating migration by performing scratch assays with a pretreatment of mitomycin C to inhibit proliferation, we still observed significantly slower migration of claudin-1 KD cells than controls (Figure 3B). By staining for Ki-67 in non-mitomycin C-treated cells we observed that proliferation at the wound edges of the scratch

wounds was slightly but significantly ($P < 0.05$) reduced to $74\% \pm 7\%$ by claudin-1 KD (Figure 3C). Thus, claudin-1 down-regulation impairs both migration and proliferation. Because migration in part depends on cell–matrix adhesion, we tested whether claudin-1 KD influenced cellular adhesion to the cell culture plastics. However, we did not find a homogeneous effect of claudin-1 KD on cell–matrix adhesion (Supplemental Figure S2B).

KD of Claudin-1 Attenuates AKT Activation and ERK1/2 Protein Levels

Proliferation and migration are mediated, among others, by the AKT and extracellular signal-related kinase (ERK) pathways. Therefore, we investigated the influence of claudin-1 KD on these pathways (Figure 3D).

For AKT, we observed 10 minutes and 6 hours after scratch a significant ($P < 0.05$) decrease of total protein

Figure 3 Effect of Cldn-1 KD and AKT inhibition on wound healing under low-calcium conditions. **A** and **B**: Effect of Cldn-1 KD on cell scratch wound healing without (**A**) and with (**B**) impairment of proliferation by mitomycin C. **C**: Effect of Cldn-1 KD on proliferation. **D**: Effect of Cldn-1 KD on protein levels of AKT/p-AKT, ERK1/2/p-ERK1/2 [left side: Western blot analyses shown exemplarily for one experiment, α -tubulin was used as loading control; right side: densitometric analyses of Western blot analyses with cells from 5 different donors (target protein normalized to α -tubulin; 100%: protein level of siRNA control cells at 0 hours)]. **E** and **F**: Effect of AKT inhibition using MK-2206 on protein levels of AKT/p-AKT [**E**; left side: Western blot analyses shown exemplarily for one experiment, β -actin was used as loading control; right side: densitometric analyses of Western blot analyses with cells from 3 different donors (target protein normalized to β -actin; 100%: protein level of Ds)] and cell scratch wound healing (**F**). All experiments in **A–D** shown here were performed with Cldn-1 siRNA no. 1. Data are expressed as means $\pm$ SEM. $n = 11$ (**A**); $n = 4$ (**B** and **C**); $n = 5$ (0 hours, 10 minutes) or 4 (6 hours) (**D**); $n = 3$ (**E** and **F**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ comparison between treatments; † $P < 0.05$ versus 0 hour of respective treatment, evaluated using a random intercept model (**A** and **B**), t -test (**C–F**), or U -test (**D**). Cldn-1, claudin-1; Ctrl, control; D, dimethyl sulfoxide control; ERK, extracellular signal–related kinase; KD, knockdown; p-, phospho-; U, untreated cells; 2, 2 μ mol/L MK-2206; 5, 5 μ mol/L MK-2206.

levels in claudin-1 KD cells (reduced to $81\% \pm 15\%$ and $75\% \pm 18\%$) compared with unwounded cells, which was not found in control cells (Figure 3D). For p-AKT (Ser473) we observed in claudin-1 KD cells a significant decrease compared with controls 10 minutes and 6 hours [reduced to $57\% \pm 27\%$ ($P < 0.05$) and $49\% \pm 17\%$ ($P < 0.01$), respectively] after scratching (Figure 3D). The relative

amount of p-AKT (p-AKT/AKT) was significantly lower in claudin-1 KD cells than in controls at 0 hours, and the trend continued at later time points (data not shown).

To investigate whether indeed decreased p-AKT activity can result in impaired scratch WH under our low-calcium conditions we inhibited AKT phosphorylation by using MK-2206 (Figure 3E). This resulted in significantly

impaired scratch WH in a dose-dependent manner (Figure 3F).

For ERK1/2 we found in claudin-1 KD cells a trend for down-regulation compared with unwounded cells 10 minutes (to $69\% \pm 25\%$) after scratching that became significant at 6 hours (to $56\% \pm 26\%$; $P < 0.05$). This time-dependent down-regulation was not seen in controls (Figure 3D). Compared with controls at the different time points, we observed a significant down-regulation of ERK1/2 at 10 minutes (reduced to $61\% \pm 22\%$; $P < 0.05$) and a trend at 6 hours (reduced to $54\% \pm 37\%$) (Figure 3D) in claudin-1 KD cells. Levels of phosphorylated ERK1/2 did not differ significantly compared with controls.

Pronounced Effect of Claudin-1 KD on Scratch WH under High-Calcium Condition

The above-mentioned experiments were performed under low-calcium conditions to specifically address conditions in which TJ structures do not form and, therefore, TJ-independent functions of claudin-1 could be investigated. In addition, these undifferentiated conditions are important for the cells at the leading edges of WH and in the basal cell layers of regenerating and nonregenerating skin.²⁹ However, because the formation of cell–cell junctions plays an important role for migrating cellular sheets,^{30–32} we also investigated the influence of claudin-1 under high-calcium conditions, allowing the formation or strengthening of TJs, desmosomes, and adherens junctions. In general, wound closure was slower under high-calcium conditions than for low-calcium conditions (Figure 4 compared with Figure 3A), but also under high-calcium conditions claudin-1 KD resulted in decreased scratch WH (Figure 4). Of note, this decrease was more pronounced than under low-calcium conditions (healed wound area of claudin-1 KD cells in relation to siRNA control: 35%, 24%, and 9% at 12, 24, or 36 hours in high-calcium cells compared with 55%, 53%, and 61% in low-calcium cells) (Figure 4 compared with Figure 3A).

KD of Occludin Results in Decreased Wound Closure in a Stress-Scratch WH Assay under Low- but Not under High-Calcium Conditions

During scratch WH, occludin mRNA was significantly up-regulated 6 hours (1.4 ± 0.2 -fold; $P < 0.05$) and 24 hours (1.4 ± 0.2 -fold compared with unwounded; $P < 0.01$) after scratch wounding. However, down-regulation of occludin expression (no. 1: KD efficiency: $88\% \pm 14\%$, ie, protein level of occludin reduced to $12\% \pm 14\%$; no. 2: KD efficiency: $79\% \pm 26\%$, ie, protein level reduced to $21\% \pm 26\%$) neither resulted in significant changes of WH rates in scratch wound assays under low- or high-calcium conditions (compared with control-siRNA-treated cells; Figures 4 and 5A; Supplemental Figure S2C) nor proliferation (Figure 5B). There was also no substantial influence

Figure 4 Effect of Cldn-1 and Ocldn KD on wound healing under high-calcium conditions. Data are expressed as means + SEM. $n = 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, evaluated using a random intercept model. Cldn-1, claudin-1; Ctrl, control; KD, knockdown; Ocldn, occludin.

on the protein level of TJ protein ZO-1 (no. 1: reduced to $93\% \pm 10\%$, NS; no. 2: $81\% \pm 5\%$, $P < 0.05$; $n = 3$) and a trend for down-regulation of claudin-1 protein level (no. 1: reduced to $59\% \pm 37\%$; no. 2: $41\% \pm 29\%$; $n = 3$).

Because attachment of keratinocytes to each other and to the matrix is important for proper WH,^{3,30} and because KD of occludin results in reduced cell–cell adhesion,¹¹ we subsequently investigated the impact of occludin KD under mechanical stress conditions that challenge cell adhesion. To this end we shook the cell plates of our scratch WH assays at defined time points to induce multidirectional mechanical stress, mimicking movement that normally also takes place *in vivo* and puts a strain on intercellular as well as cell–matrix adhesion. Indeed, in this setting, KD of occludin showed a clear reduction in WH under low-calcium condition (Figure 5C; Supplemental Figure S2D); however, this was not the case under the high-calcium condition (Figure 5D). To elucidate the origin of the influence seen by occludin KD under low-calcium conditions on stressed scratch wound assays we tested whether occludin KD influences cell–matrix adhesion to plastic and fibronectin. However, we did not find consistently decreased cell–matrix adhesion in occludin KD cells (Figure 5, E and F). In addition, because it was shown before that occludin is involved in susceptibility of keratinocytes to apoptosis, we investigated whether our mechanical stress scratch assay induces cell death under low-calcium conditions. However, no significant decrease of cell viability was observed ($89\% \pm 12\%$; $n = 3$).

Discussion

WH is a complex process that involves proliferation, migration, and differentiation of keratinocytes. Because it is known that TJ proteins are involved in some of these processes,^{33,34} we were interested in the role of TJ proteins in

Figure 5 Effect of Occludin KD on wound healing. **A–F:** Effect of Occludin KD on cell scratch wound healing (**A**), proliferation (**B**), mechanically stressed scratch wound assays 6 hours after wounding (change in healed area was determined by subtracting healed areas of nonstressed from stressed cells within the same treatment; results were then subtracted from the values of untreated controls for normalization) under low (**C**) and high (**D**) calcium conditions, and cell–matrix adhesion using fibronectin (**E**) and plastic-coated (**F**) wells (amount of adhered cells is shown as percentage of total viable cell count). All experiments shown here were performed with Occludin siRNA no. 1 unless noted otherwise. All experiments except for those in **panel D** were performed under low-calcium conditions. Data are expressed as means + SEM. $n = 7$ (**A**); $n = 4$ (**B** and **C**); $n = 3$ (**D**); $n = 4$ to 5 depending on the time point (**E** and **F**). * $P < 0.05$ evaluated using a random intercept model (**A**) or t -test (**B–F**). Ctrl, control; KD, knockdown; Occludin, occludin.

WH. We found that there is a striking difference for TJ proteins claudin-1 and occludin between normal and chronic wounds with decreased levels of both proteins in chronic wounds. KD of the TJ proteins revealed that this indeed results in the alteration of cell biological features that contribute to WH. Of interest, the two investigated proteins influence different events of WH.

Claudin-1

Claudin-1 was extensively lost in almost all investigated chronic wounds in the regenerating epidermis and/or at the wound margins, whereas it was present in acute wounds at early WH, except for the very first cells.

Investigating the effect of KD of claudin-1 in cultured keratinocytes under low-calcium conditions, we observed significantly decreased scratch WH. Both impaired proliferation and migration contribute to this decrease. A negative impact of claudin-1 KD on cell proliferation was also shown in endometrial carcinoma cells KLE,³⁵ in airway smooth

muscle cells,³⁶ and in gastric cancer cells.³⁷ However, one has to take into account that aberrant cell signaling such as overactive Ras/ERK pathways is often present in carcinoma cells and may contribute to the result.³⁸ Of note, it was shown before that the KD of claudin-1 in keratinocytes can also result in an increase of proliferation.⁹ Also in atopic dermatitis mouse eczema, reduced claudin-1 expression is correlated with increased proliferation.¹³ The discrepancy between these results and what is described here can be explained because we investigated proliferation in scratch-wounded cells, whereas De Benedetto et al⁹ and Gruber et al¹³ investigated the effect in nonwounded cells. In addition, in the atopic dermatitis mouse model proliferation could also be influenced by inflammatory cytokines. This indicates that claudin-1 can influence proliferation positively and negatively, dependent on the cellular context. Concerning cell migration, a negative impact of claudin-1 KD was also shown in lung carcinoma cells A549,³⁹ hepatocellular carcinoma cells HepG2, and endometrial carcinoma cells KLE.³⁵

To clarify the mechanism of how claudin-1 KD influences proliferation and migration we investigated signaling pathways involved in these processes. We observed a reduction of total protein levels of AKT and ERK1/2 6 hours after wounding in claudin-1 KD cells compared with unwounded cells, which was not seen in controls. In addition, p-AKT (Ser473) level was reduced 6 hours after wounding by claudin-1 KD compared with controls. Taken together, these changes resulted in a decrease in the relative amount of p-AKT.

p-AKT was shown to have a positive impact on lamellipodia formation and in turn on migration as well as proliferation and thus to increase scratch healing.^{40–46} Nonetheless, there were also data showing no^{47–49} or inhibiting⁵⁰ effects of AKT kinase activity. To investigate whether the decrease in p-AKT seen in our experiments because of claudin-1 KD can result in decreased scratch WH under our experimental conditions, we inhibited p-AKT formation by MK-2206. This inhibition indeed resulted in significantly impaired scratch WH. Thus, decrease in p-AKT likely contributes to impaired scratch WH observed in our claudin-1 KD cells.

Most of our experiments in primary cultured keratinocytes were performed under low-calcium conditions, reflecting the non- and less-differentiated cells in the lower layers of the epidermis and at the leading edge which mainly contribute to migration and proliferation during WH. Investigation of scratch WH under low-calcium conditions has been used to investigate several cellular processes, including the effect of gap junctions on keratinocyte migration.^{51–54} Under low-calcium conditions no functional TJ structures form,⁵⁵ even though TJ proteins are present.²⁸ Thus, the effects described here for claudin-1 KD elucidate TJ structure independent functions of this protein in keratinocytes. Of note, investigation of the effect of claudin-1 KD on scratch WH under high-calcium conditions revealed that then the effect was even more pronounced.

It was shown before that decrease/loss of claudin-1 under high-calcium conditions/in differentiated epidermal cells impairs TJ as well as skin barrier function.^{12,13,28,56,57} This might support or facilitate bacterial colonization of the wound, further interfering with the WH progress. Thus, it is likely that TJ-dependent and TJ-independent functions of claudin-1 play a role in normal WH and are disturbed in chronic wounds.

Occludin

In addition, occludin was shown to be absent in many chronic wounds, suggesting an important role of occludin in WH. This is supported by data from Safferling et al⁵⁸ who demonstrated an important role of occludin in *in vitro* WH when using three-dimensional reconstructed human skin. Mimetic peptides that recognize and inhibit occludin strongly accumulated at the leading edge of the extending epidermal tongue of the reconstructed human

skin and reduced shield extension accompanied by extrusion of cells. Of note, a heavily increased loss of cells from the regenerating epidermis because of impaired cellular adhesion as a factor contributing to delayed WH was also proposed by Adair.⁵⁹ Indeed, we could show that even though knocking down occludin did not influence scratch wound closure or proliferation under normal scratch wound conditions it resulted in reduced WH in scratch wounds subjected to mechanical stress. However, this was only the case when cells were cultured under low-calcium conditions that mimicked basal cell layers/leading edge cells. Under high-calcium conditions this effect was abolished. This argues for a special role of occludin in adhesion of the basal cell layers/leading edge cells of the regenerating epidermis, whereas in upper layers desmosomes and enforced adherens junctions may play a more important role.

Because cell–matrix adhesion plays an important role in cell migration, we also investigated the effect of occludin KD on adhesion to fibronectin and plastic-coated wells. However, no reproducible alteration of adherence was observed; thus, this can be excluded as an explanation of the reduced WH in our stressed scratch wound assays.

One could also imagine that increased cell death might contribute to the impaired WH under low-calcium conditions because occludin has been shown to be involved in regulation of apoptosis.¹¹ However, on one hand, we could show that our experimental setting did not induce cell death; on the other hand, the suggested correlation between occludin and apoptosis is that overexpression of occludin enhances apoptosis, whereas gene silencing inhibits sensitization.^{11,60,61} Thus, the impaired scratch WH seen in occludin KD cells subjected to mechanical stress is unlikely to be mediated by enhanced apoptosis.

In three-dimensional skin models, we showed before that occludin KD impairs keratinocyte differentiation as seen by reduced expression of differentiation marker involucrin and increased expression of transglutaminase 1.¹¹ Because it is known that proper differentiation is important for WH,^{62,63} this could also contribute to the formation of chronic wounds.

Taking together the effects caused by claudin-1 and occludin KD in human keratinocytes, we conclude that the changes seen in localization/expression of claudin-1 and occludin in chronic human wounds compared with normal healing wounds might well be an important part of the pathogenesis of nonhealing wounds. Decreased levels of claudin-1 result in impaired proliferation, migration, and skin barrier function. Occludin KD impaired cell–cell adhesion at the leading edges. In addition, altered differentiation might play a role. Because claudin-1 and occludin are involved in different effects, and many of these effects are found in cells cultured under low-calcium conditions in which no barrier-forming TJs are present (see above), we conclude that these effects are partly TJ structure independent.

Acknowledgments

We thank Ina Ridderbusch and Konrad Reinshagen (University Hospital Hamburg-Eppendorf, Hamburg, Germany) for providing foreskin samples.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.02.006>.

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