Occludin Is Involved in Adhesion, Apoptosis, Differentiation and Ca\(^{2+}\)-Homeostasis of Human Keratinocytes: Implications for Tumorigenesis

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Abstract

Tight junction (TJ) proteins are involved in a number of cellular functions, including paracellular barrier formation, cell polarization, differentiation, and proliferation. Altered expression of TJ proteins was reported in various epithelial tumors. Here, we used tissue samples of human cutaneous squamous cell carcinoma (SCC), its precursor tumors, as well as sun-exposed and non-sun-exposed skin as a model system to investigate TJ protein alteration at various stages of tumorigenesis. We identified that a broader localization of zonula occludens protein (ZO)-1 and claudin-4 (Cldn-4) as well as downregulation of Cldn-1 in deeper epidermal layers is a frequent event in all the tumor entities as well as in sun-exposed skin, suggesting that these changes result from chronic UV irradiation. In contrast, SCC could be distinguished from the precursor tumors and sun-exposed skin by a frequent complete loss of occludin (Ocln). To elucidate the impact of downregulation of Ocln, we performed Ocln siRNA experiments in human keratinocytes and uncovered that Ocln downregulation results in decreased epithelial cell-cell adhesion and reduced susceptibility to apoptosis induction by UVB or TNF-related apoptosis-inducing ligand (TRAIL), cellular characteristics for tumorigenesis. Furthermore, an influence on epithelial differentiation was observed, while there was no change of E-cadherin and vimentin, markers for epithelial-mesenchymal transition. Ocln knock-down altered Ca\(^{2+}\)-homeostasis which may contribute to alterations of cell-cell adhesion and differentiation. As downregulation of Ocln is also seen in SCC derived from other tissues, as well as in other carcinomas, we suggest this as a common principle in tumor pathogenesis, which may be used as a target for therapeutic intervention.

Introduction

Tight Junctions (TJs) are multiprotein complexes formed by transmembrane proteins, e.g. occludin (Ocln), claudins (Cldns), and junctional adhesion molecules (JAMs), which are associated with intracellular plaque proteins, e.g. ZO-1, 2 and 3 and MUPP-1. From simple epithelia it is known that TJs seal neighbouring cells and control the paracellular pathway for solutes, water, and cells (barrier function). In addition, they restrict the diffusion of apical and basolateral membrane components (fence function), coordinate signalling molecules and play a role in cell differentiation and proliferation [1,2,3,4,5,6]. A role of TJs in paracellular barrier function was also shown in the multi-layered epithelium of the skin [7,9], and an involvement of Cldn-1 in proliferation and differentiation of keratinocytes was suggested [9,10,11].

There are frequent changes in TJ protein localization and/or expression in the course of carcinogenesis. For Cldns, up- or downregulation, as well as altered localization were described, dependent on the tumor entity. For instance, in breast cancer Cldn-1, -2, and -7 are downregulated, while Cldn-4 is upregulated and in colorectal- and pancreatic-cancer Cldn-1, -2, and -7 are upregulated [12,13,14,15,16]. For Ocln, mainly a downregulation was observed in various tumors [17,18,19,20]. Downregulation of Ocln is a common feature of epithelial-mesenchymal-transition (EMT) in tumors derived from simple epithelial cells, and regulation of Ocln was described by the oncogenic Ral1 pathway as well as by the transcription factors slug and snail [21,22,23,24,25]. Furthermore, an involvement of Ocln in apoptosis has been suggested, but there is conflicting evidence whether apoptosis is enhanced or suppressed (see also discussion) [26,27,28,29,30]. For ZO-1 down- or upregulation was observed in different tumor entities [17,31,32], and its redistribution from cell-cell-borders to the cytoplasm and nucleus was described in EMT [e.g. 33]. Decreased expression of TJ proteins suggests that
Tissues, Cells, Antibodies, qPCR Primers and siRNAs

Samples of human SCC (n = 46; 31 male, 15 female; age 19–93 y, mean: 73 y), AK (n = 25, 12 male, 13 female; age 61–92 y, mean 72 y) sun-exposed skin (n = 11, 4 male, 7 female; age 16–89 y, mean: 67 y) and non-sun-exposed skin (n = 17, 9 male, 8 female; age 20–78 y; mean 42 y) were obtained from our clinical department. Grading of the SCC was performed according to the world health organisation classification [37]. Human foreskin keratinocytes were isolated and cultured as described [45]. Antibodies used are summarized in Table 1. FAM™ dye-labeled real-time PCR (qRT-PCR) TaqMan® MGB probes for Ocn (Hs00170162_m1), involucrin (Hs00843077_s1), TG1 (Hs01075010_m1), Cldn-2 (Hs00256660_s1), Cldn-12 (Hs00221623_m1) and GAPDH (Hs03929097_g1) were purchased from Applied Biosystems (Carlsbad, CA, USA). Ready-to-use siRNAs for human Ocn (Hs_OCNL_7; SI03225999, Hs_OCNL_9; SI04360034), ZO-1 (SI02655149), and control siRNA (1027280) were purchased from Qiagen (Hilden, Germany). Stealth siRNAs for gene silencing of Ocn in human organotypic skin models [46] were purchased from Life Technologies (Darmstadt, Germany; siRNA1: HSS107401; siRNA3: HSS181629; control: 12935–300). Due to technical reasons different siRNAs had to be used for cell culture and skin model experiments.

Immunoﬂuorescence Microscopy

Immunofluorescence microscopy was performed as previously described [45]. For details see Table 1. An Axioskop-2 microscope (Carl Zeiss; Jena/Oberkochen, Germany), a CCD-camera (Hamamatsu Photonics; Hamamatsu City, Japan), and an Openlab 2.0.9 software (Improvision; Coventry, UK) were utilized to visualize and evaluate the stained sections. ZO-1 and especially Ocn are very sensitive antigens influenced by slight deviations of fixation. Therefore samples were only evaluated when positive staining in the “uninvolved epidermis” (Figure 1) and/or internal positive controls (e.g. sweat glands) confirmed the success of staining procedure and stainings were repeated at least twice. The stainings were analyzed by two independent investigators (SR and JMB). The number of evaluated samples was as follows: SCC (Ocn: n = 35, ZO-1: n = 32, Cldn-1, JAM-A: n = 46; Cldn-4: n = 30, Inv: n = 11, TG1: n = 11, E-cadherin: n = 12, vimentin: n = 10 ), BD (Ocn: n = 19, ZO-1: n = 25, Cldn-1: n = 25, JAM-A: n = 26, Cldn-4: not tested), AK (Ocn: n = 18, ZO-1: n = 23, Cldn-1, JAM-A: n = 24, Cldn-4: not tested), KA (Ocn: n = 19, ZO-1: n = 22, Cldn-1: n = 24; JAM-1: n = 25; Cldn-4: n = 9, sun-exposed skin (Ocn: n = 9, ZO-1: n = 9, Cldn-1: n = 10; JAM-A: n = 9; Cldn-4: n = 11), non-sun-exposed healthy skin (Ocn: n = 11, ZO-1: n = 15; Cldn-1: n = 16; JAM-A: n = 15; Cldn-4: n = 15).

Human Organotypic Skin Models (3D Models)

Pre-confluent human primary keratinocytes were transfected with the respective siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Keratinocytes were then incubated for 24 h before seeding onto a fibroblast collagen gel as described below. Organotypic skin models were prepared as described previously [47]. Briefly, a 2.5 ml suspension of collagen type I (Advanced Biomatrix, San Diego, CA, USA) containing 2.5×10⁶ fibroblasts was poured into cell-culture inserts (3 µm pore size; BD Bioscience, Bedford, MA, USA) and allowed to gel for 2 h at 37°C without CO2. The gels were then equilibrated with KGM medium (Lonza, Basel, Switzerland) at 37°C in a CO2 incubator for 2 h, and 1.5×10⁶ keratinocytes, transfected with the respective siRNAs, were seeded onto each collagen gel. After overnight incubation at 37°C the medium was removed from both the inserts and external wells, and
10 ml serum-free keratinocyte defined medium (SKDM), consisting of KGM without bovine pituitary extract and supplemented with 1.3 mM calcium (Sigma, Vienna, Austria), 10 μg/ml transferrin (Sigma), 50 μg/ml ascorbic acid (Sigma), and 0.1% bovine serum albumin (Sigma), was added to each external well. The organotypic skin models were cultured for 7 days and medium was changed every second day.

### Table 1. Antibodies and nuclear dyes.

| Target          | Clone/lot | Company               | Dilution IF | Dilution Western-blot |
|-----------------|-----------|-----------------------|-------------|------------------------|
| Cldn-1          | 2H10D10   | Zymed Laboratories    | 1:150       |                        |
| Cldn-4          | 3E2C1     | Zymed Laboratories    | 1:50        |                        |
| Zo-1            | 1A12      | Zymed Laboratories    | 1:80        |                        |
| Ocln            | OC-3F10   | Zymed Laboratories    | 1:80        | 1:2000                 |
|                 | 71–1500   |                       |             |                        |
| JAM-A           | Rm-JAM-1  | R&D systems           | 1:30        |                        |
| TG1             | BC1       | Antibody online       | 1:20        | 1:500                  |
| Involucrin      | SY5       | Novo Castra           | 1:200       | 1:2000, pH 8.0         |
| Ki67            | MIB-1     | DAKO                  | 1:50        |                        |
| E-Cadherin      | GP53      | Progen                | 1:1000      | 1:500                  |
| Vimentin        | NCH-38    | DAKO                  | 1:50        | 1:100                  |
| Actin           | AC-15     | Sigma                 | 1:10000     |                        |
| Tubulin         | DM-1A     | Calbiochem            | 1:2000      |                        |
| Nuclei          | DAPI      | Boehringer Mannheim   | 1:5000      |                        |

Abbreviations: Cldn, claudin; JAM, junctional adhesion molecule; IF, immuno-fluorescence; Ocln, occludin; TG1, transglutaminase 1; ZO, zonula occludens.

IF treatment for TG1: Citrate buffer, 2×10 min microwave, 0.001% trypsin, DAKO Block overnight. IF treatment for all others: TEC buffer, 2×10 min microwave, 0.001% trypsin, DAKO block.

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Figure 1. Schematic drawing of a squamous cell carcinoma denoting the different areas of the tumor.

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siRNA Experiments

Primary human keratinocytes were transfected under low Ca\textsuperscript{2+} conditions by using HiPerFect Transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Briefly, cells were transfected by a fast forward protocol. 100000 cells/ml were transfected with a 1:1 mixture of HiPerFect reagent (1:200) and 100 nM of Ocln-siRNA or control-siRNA, respectively. Efficiency of knock-down was controlled by Western-blot and qRT-PCR analysis. Efficiency of knock-down was assessed after 24 h. After transfection keratinocytes were trypsinized and seeded in 30 min at room temperature. KGM (20 ml) was added and the siRNA solution and 5 ml OPTI-MEM medium (Gibco). After 30 min at room temperature, KGM (20 ml) was added and the solution was poured onto the keratinocytes monolayer (25 ml) for 24 h. After transfection keratinocytes were trypsinized and seeded onto a fibroblast collagen gel. Efficiency of knock-down was controlled by Western-blot and qRT-PCR analysis.

Cell Adhesion Assays

Intercellular adhesion under high Ca\textsuperscript{2+} conditions was tested using a hanging drop assay which was modified from [48]. Briefly, following 48 h of siRNA silencing cell suspensions of 550000 single cells per ml were prepared. 35 μl drops of the suspensions were placed under the lids of 60×15 mm tissue culture dishes. To limit evaporation 2 ml of PBS was added to the bottoms of the dishes. After indicated time points the drops were pipetted and placed in an improved Neubauer hemocytometer chamber. Pictures of 4×12 Neubauer-squares of 0.0625 mm\textsuperscript{2} each were taken using a Leica DM LS microscope with 10× objective magnification and a Leica EC3 digital camera (Leica Biosystems, Nussloch GmbH, Nussloch, Germany). The numbers of particles in the squares were determined automatically by utilizing FIJI software [49] and the particles analyzer tool. The relative cell adhesion activity was evaluated according to [50] by calculating the cell aggregation number as defined previously [52].

Statistical Analysis

Statistical significance of proliferation, adhesion, Ca\textsuperscript{2+}-barrier-function and apoptosis was determined by Student’s t-test. TJ protein expressions were cross-tabulated with tumor grading, differentiation markers, and sun-exposure and the significance of associations were tested using Fisher’s Exact Test. P-values <0.05 were considered as statistically significant (* p<0.05, ** p<0.01, *** p<0.001).
This indicates that complete loss of Ocbl and downregulation of Cldn-1 in the lowermost and JAM-A and Cldn-1 in the uppermost layers is a frequent feature in SCC. This also applies for the broader localization of Cldn-4 and ZO-1.

**Localization Patterns of TJ Proteins in AK, BD, and KA**

We wondered whether the alterations found in SCC may also be seen in in-situ carcinomas (AK, BD) or in tumors of lower malignancy (KA).

Complete loss of Ocbl was found in only 5% of AK, 11% of BD, and 29% of KA, demonstrating that its absence is much more characteristic for SCC (80%). Contrariwise, broader localization of Ocbl was found more frequently in the precursor tumors (42–71%, as compared to 29%, Table 2).

For ZO-1, complete loss was observed in 9% of AK, 12% of BD but not in KA (25% in SCC). Frequency of broader localization was similar between the tumor entities. Furthermore, there was no clear-cut difference between SCC and the other tumors as
Figure 3. Immunolocalization of ZO-1 and OcIn in healthy, non-sun-exposed skin and SCC. Immunolocalization of ZO-1 (A1, A2, B1, B2, C1, C2), and OcIn (D1, D2, E1, E2, F1, F2), in healthy, non-sun-exposed skin (A1, A2, D1, D2) and SCC (B1, B2, C1, C2, E1, E2, F1, F2). (A1, B1, C1, D1, E1, F1: epifluorescence pictures; A2, B2, C2, D2, E2, F2: overlay of epifluorescence and phase contrast pictures). For both molecules an example of broader localization (B1, B2, E1, E2) and loss of the protein (C1, C2, F1, F2) are shown. Note that exposure time in C1, C2, F1, F2 was substantially higher than in the other figures in order to show that also with very high exposure time no OcIn or ZO-1 staining was seen in these samples. Bars: 50 μm.

Table 2. Protein expression and localization of TJ proteins in the various skin tumors.

| Protein | Squamous cell carcinoma | Keratoacanthoma | Bowens Disease | Actinic keratoses |
|---------|-------------------------|-----------------|----------------|-----------------|
|         | Loss | Altered | Loss | Altered | Loss | Altered | Loss | Altered |
| Cldn-1  | 9%   | 60%/81% 2 | 0%   | 68%/80% 2 | 4%   | 55%/66% 2 | 4%   | 83%/83% 2 |
| Cldn-4  | 17%  | 91% 2   | Not tested | Not tested | Not tested | Not tested | 0%   | 91% 2   |
| JAM-A   | 0%   | 85% 1   | 0%   | 88% 1   | 0%   | 78% 1   | 4%   | 73% 1   |
| OcIn    | 80%  | 29% 2   | 29%  | 71% 2   | 11%  | 42% 2   | 5%   | 54% 2   |
| ZO-1    | 25%  | 67% 3   | 0%   | 88% 3   | 12%  | 65% 3   | 9%   | 75% 3   |

Loss: complete loss of expression, altered: altered localization. % in “loss of expression” denotes the percentage of all tumors, % in “altered localization” denotes the percentage of positive tumors.

1downregulation in the uppermost layers.
2downregulation in the lowermost layers.
3broader expression.
Abbreviations see table 1.

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concerning Cldn-1, Cldn-4, and JAM-A (Table 2). Similar to SCC samples, we observed also in the SCC precursor samples spots with lacking expression of TJ proteins.

The data indicate that alterations found for Cldn-1, Cldn-4, ZO-1, and JAM-A are common for UV-induced skin tumors while the frequent loss of Ocln appears as specific for SCC.

Localization Patterns of TJ Proteins in Sun-exposed Versus Non-sun-exposed Skin

Because the alterations for Cldn-1, Cldn-4, and ZO-1 were observed in all UV-promoted skin tumors and a broader localization of Ocln was frequent in all tumor entities except for SCC, we wondered whether these alterations could also be identified in chronically sun-exposed skin. Indeed, a broader localization of Ocln, ZO-1, and Cldn-4 was seen more frequent in the epidermis of sun-exposed as compared to non-sun-exposed skin with high statistical significance (Ocln: \( p = 0.001 \), ZO-1: \( p = 0.001 \), Cldn-4: \( p = 0.001 \)) (Figure 4). We found no significant influence of age and sex on TJ protein localization. However, a complete loss of Ocln, as in SCC, was not observed in sun-exposed skin. Also spots with loss of staining were less frequent than in the tumors (data not shown). For Cldn-1, we observed a downregulation in the lowermost epidermal layers in sun-exposed skin which was less frequent in non-sun-exposed skin (\( p < 0.001 \)) (Figure 4). Downregulation in the uppermost layers - as frequently seen in the tumors - was not observed. From this data we conclude that most TJ protein alterations observed in skin tumors are likely to be induced by UV-irradiation, but loss of Ocln and downregulation of Cldn-1 in the uppermost layers may not be related to UV.

Knock-down of Occludin Alters Epidermal Differentiation Markers but not Markers for EMT

Altered differentiation is a common feature in tumor progression. Accordingly, irregularity of epidermal differentiation markers involucrin and TG1 was described in SCC [38,39,40]. Therefore we investigated whether downregulation of Ocln may influence epidermal differentiation. When using two different siRNAs in a 3D skin model, we could strongly downregulate Ocln protein expression (Figure 5A). This was associated with downregulation of the differentiation marker involucrin and an upregulation of TG1 (Figure 5A). The same effect was seen for involucrin when investigating 2D cell cultures (data not shown). On mRNA-level, a slight and partly significant upregulation for involucrin and TG1 was observed (Involucrin: Ocln siRNA1:1.4±0.4, n.s., Ocln siRNA3:2.2±0.5, \( p < 0.01 \); TG1: Ocln siRNA1:1.4±0.1, \( p < 0.001 \); Ocln siRNA3:1.7±0.6, n.s.; Ocln: Ocln siRNA1:0.4±0.2, \( p < 0.01 \); Ocln siRNA3:0.4±0.2, \( p < 0.01 \); n = 3).

Because Ocln was also described to be involved in epithelial-mesenchymal transition [21,22,25], we investigated E-cadherin as an epithelial marker as well as vimentin as a mesenchymal marker for EMT in the Ocln knock-down models. However, we did not find any changes of these markers (Figure 5A).

Further, we looked for correlations between involucrin, and TG1, on one hand and Ocln expression on the other hand in the SCC samples as well as for correlations of Ocln with the SCC grade. We found that Ocln-positive tumors were to a higher percentage differentiated tumors (Grade I; 57.1%) than Ocln-negative tumors (25.9%) (Table 3). Characteristically, all Ocln-positive tumors were also positive for involucrin (Table 4). However, also 84% of Ocln-negative tumors were still positive for involucrin (see Table 3, 4). For TG1, 71% of the Ocln-negative tumors showed an upregulation in the epidermal parts, however, this was also the case in 100% of Ocln-positive tumors (Table 4).

Figure 4. Distribution patterns of TJ proteins in normal skin from sun-exposed and non-sun-exposed areas. Percentages denote distribution patterns of the various TJ proteins in sun-exposed (sun; e.g. face, lower arms) and non-sun-exposed areas (no-sun; e.g. abdomen, bottom). ***, \( p < 0.001 \) between sun-exposed and non-sun-exposed skin.

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Figure 5. Influence of Ocln on epidermal differentiation and EMT markers, cell-cell adhesion and paracellular Ca\(^{2+}\) permeability. (A) (left side) Western Blot analysis of 3D skin models after silencing of Ocln with 2 different siRNAs shows downregulation of Ocln and of the epidermal differentiation marker involucrin and an upregulation of TG1. There is no alteration for the EMT markers E-cadherin and vimentin. Same amounts of protein were loaded and actin or tubulin were used as gel loading controls. A representative experiment is shown (n = 3). (right side) Semiquantitative analysis of Ocln, involucrin, TG1, E-cadherin and vimentin. Band intensities were normalized to actin (Inv, TG1) or tubulin (E-cad, vim). Subsequently, the values were normalized to control siRNA treated cells (n = 3; mean ± SEM; *p < 0.05, ***p < 0.001 compared to control siRNA). (B) Calcium induced cell-cell adhesion was investigated with a hanging drop assay at the indicated time points. Significantly greater numbers of particles (indicating less cell-cell adhesion) were found in the suspensions of Ocln knock-down cells compared to the controls (*: p < 0.05 n = 3). (C) Electrophysiological studies of paracellular Ca\(^{2+}\)-permeability in Ocln siRNA-treated cultured keratinocytes revealed an increase in paracellular permeability for Ca\(^{2+}\) in Ocln knock-down cells compared to cells treated with control siRNA (n = 6, mean ± SEM). (D) Example for the knock-down of Ocln in siRNA treated submerged cells. Mean knock-down of Ocln in the cells used for experiments in Figure 5 B, C and 6 was 76% ±/− 9%.

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Knock-down of Occludin Reduces Cell-cell Adhesion

We asked whether the alteration of Occludin might also influence keratinocyte adhesion, because loss of adhesion is also a prerequisite for tumor invasion and metastasis. Indeed, significantly reduced keratinocyte-keratinocyte adhesion was seen in response to Occludin knock-down, as shown by a cell adhesion assay in a time kinetic analysis (Figure 5B, D).

Knock-down of Occludin Increases Paracellular Permeability for Ca\(^{2+}\)

Cell-cell adhesion as well as cell-differentiation is dependent on Ca\(^{2+}\)-homeostasis. Therefore, we investigated whether decreased Occludin levels may also alter Ca\(^{2+}\) permeability of keratinocyte-sheets. Indeed, increased paracellular Ca\(^{2+}\) permeability (126% ± 5%) was observed in Occludin knock-down cells as compared to control-siRNA-treated cells (Figure 5C, D). Consequently, knock-down of Occludin also reduced transepithelial resistance (data not shown). Because Cldn-2 and Cldn-12 are known to be key-players in Ca\(^{2+}\)-permeability in intestinal cells [53] we investigated the influence of Occludin knock-down on mRNA expression of these claudins. While there was no influence on Cldn-12, we observed a significant downregulation of Cldn-2 to 0.56 ± 0.05 of the level of control cells (n = 3).

Knock-down of Occludin Reduces the Susceptibility of Keratinocytes for Induction of Apoptosis

A tumor-promoting effect may also result from inhibition of apoptosis. We therefore addressed the question whether downregulation of Occludin may also result in a reduced sensitivity of keratinocytes for proapoptotic stimuli. We tested here TRAIL, which plays a particular role in keratinocyte regulation of apoptosis [54], and UVB-irradiation, a typical apoptosis inducing stimulus in the skin.

Indeed, significantly decreased apoptosis was evident after 3 h (data not shown) and 20 h (Figure 6A, B) of TRAIL-treatment in Occludin knock-down keratinocytes compared to control siRNA-treated cells as seen by using two different siRNAs (Figure 6A, B). In addition, a clear decrease of UVB-induced apoptosis was seen in Occludin knock-down cells (Figure 6C).

Thus, Occludin knock-down selectively prevented apoptosis induction by TRAIL and UVB, which may suggest a role of Occludin loss in tumor progression of SCC, based on apoptosis resistance to different stimuli.

Of note, the effects of Occludin knock-down were not seen in all keratinocyte cultures. The effects on TRAIL-induced apoptosis were seen in 50% of the primary keratinocyte cultures (n = 14) that originated from different donors, and the suppressive effects on UVB-induced apoptosis were seen in 40% of cultures (n = 10). This was independent from the level of Occludin knock-down. All primary cultures that showed an effect of Occludin knock-down on UVB-induced apoptosis showed a similar effect when using TRAIL.

No primary cytotoxicity (necrotic cell death) was seen in response to TRAIL at early time points (3 h). It was only enhanced in the TRAIL-treated keratinocyte cultures after 20 h, interestingly both in the Occludin-siRNA treated cells and in control cells (Figure 6D), arguing for a secondary cytotoxic effect due to absence of removal of apoptotic cells from cell culture.

Knock-down of Occludin Shows Only Minor Effects on Keratinocyte Proliferation

Increased proliferation represents a characteristic feature of in-situ and malignant tumors. Therefore, we asked whether Occludin

Table 3. Correlation of presence of TJ proteins and tumor grading of SCC.

| Tumor grading | Ocln Pos | Ocln Neg | ZO-1 Pos | ZO-1 Neg | Cldn-1 Pos | Cldn-1 Neg | Cldn-4 Pos | Cldn-4 Neg |
|---------------|----------|----------|----------|----------|------------|------------|------------|------------|
| I             | 57.1%    | 25.9%    | 27.3%    | 25.0%    | 32.5%      | 50%        | 20.8%      | 40%        |
| II            | 28.6%    | 40.7%    | 45.4%    | 37.5%    | 42.5%      | 25%        | 50%        | 20%        |
| III           | 14.3%    | 33.4%    | 27.3%    | 37.5%    | 25%        | 25%        | 29.2%      | 40%        |

Ocln, positive; Neg, negative. Further abbreviations see table 1.

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Table 4. Correlation of the presence of TJ proteins and differentiation markers involucrin and TG1 as well as EMT markers E-cadherin and vimentin in SCC.

| Involucrin | TG1 | E-Cadherin | Vimentin |
|------------|-----|------------|----------|
| Pos        | Neg | Normal     | Up-regulated | Normal or slightly down-regulated | Strongly down-regulated | Pos | Neg |
| Ocln pos   | 100% 7/7 | 0% | 0% | 100% 4/4 | 67% 4/6 | 23% 2/6 | 20% 1/5 | 80% 4/5 |
| Ocln neg   | 84% 21/25 | 16% 4/25 | 29% 2/7 | 71% 5/7 | 86% 6/7 | 14% 1/7 | 0% 0/5 | 100% 5/5 |

Abbreviations see table 1 and 3. n/m denotes the positive/negative or normal/upregulated cases compared to the total amount of cases of a specific category.
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might also contribute to the regulation of keratinocyte proliferation. We did, however, not observe increased proliferation of human keratinocytes in 2D cultures at low Ca\(^{2+}\) conditions when treated with Ocln-siRNA, whereas at high Ca\(^{2+}\) conditions cell proliferation was slightly reduced. Also in 3D skin models no significant influence of Ocln knock-down on keratinocyte proliferation was evident (data not shown).

**Discussion**

Here, we demonstrate that downregulation of Ocln in keratinocytes results in decreased cell-cell adhesion, reduced susceptibility to induction of apoptosis, altered epidermal differentiation, and altered Ca\(^{2+}\) homeostasis. This hints for a significance of the Ocln loss proven here in a large panel of cutaneous SCC as compared to SCC precursors and to normal skin. In addition, we demonstrate that changes of Cldn-1, Cldn-4, JAM-A, and ZO-1 can not only be found in SCC but also in precursor lesions and sun-exposed skin.

We identified a frequent loss of Ocln in cutaneous SCC which was not found in its precursors or sun-exposed and non-sun-exposed skin. In line with our results loss or downregulation of Ocln has also been described in most samples of lingual and bronchial SCC [55,56] suggesting Ocln downregulation as a common characteristic of SCC, irrespective from its origin. In addition, Ocln downregulation was also observed in other cancer entities, e.g. gastric cancer, hepatocellular carcinoma, and breast cancer [17,18,19], hinting for a general role in tumorigenesis. In breast cancer cell lines it was shown that overexpression of Ocln can decrease invasiveness and cell motility in vitro and inhibits tumor development and metastasis in mouse experiments in vivo [30]. On the other hand, knock-down of Ocln in breast cancer cell lines resulted in increased invasiveness [18].

![Graphs](https://example.com/graphs.png)
Previous reports of cutaneous SCC could not identify such an Ocln loss though the protein was partly found to be restricted to cells with keratinization such as cancer pearls [43,44]. As we also observed Ocln staining in some of our SCC, this discrepancy might be due to the limited number of cases investigated before. Any staining procedure problems could be largely excluded because we exclusively evaluated tumors with internal positive controls (see materials and methods).

Addressing the question of consequences of reduced Ocln levels in keratinocytes, we investigated epidermal differentiation, markers for EMT, cell-cell adhesion, Ca\(^{2+}\)-permeability, apoptosis, and proliferation.

We demonstrate that downregulation of Ocln results in downregulation of involucrin and increase of TG1 protein levels in 3D skin models, indicating an influence of Ocln on epidermal differentiation. Several studies described a downregulation of involucrin in poorly differentiated SCC, while it was strongly expressed in highly differentiated SCC [38,39,40,44]. For TG1 increased staining intensity was described in the epidermal part of cutaneous SCC, [41], a frequent (60%) upregulation was also observed in SCC of the lung [57]. However, we do not see a clear correlation of downregulation of Ocln and changes of involucrin and TG1 in our SCC tissues. This reflects that tumor progression is influenced by various internal and external factors that, depending on the tumor-microenvironment, might overrule the effect of downregulation of Ocln and which we cannot mimic in our cell culture system. In addition, we cannot rule out that in SCC alteration of Ocln might not only be a cause but also a consequence of altered differentiation, because the chicken-egg question ‘‘TJs – differentiation’’ has not been solved yet [58]. Therefore the final contribution of Ocln downregulation to altered epidermal differentiation in SCC still has to be clarified.

As a putative underlying mechanism of altered epidermal differentiation we identified an increased paracellular Ca\(^{2+}\)-permeability in keratinocytes and therefore altered Ca\(^{2+}\)-homeostasis. Increased permeability might result in an altered tissue Ca\(^{2+}\)-gradient which is known to influence keratinocyte differentiation [59,60,61]. Altered Ca\(^{2+}\)-homeostasis is also likely to be involved in reduced cell-cell adhesion observed in our Ocln knock-down keratinocytes, because Ca\(^{2+}\) is known to be essential for the formation of functional adherence junctions and desmosomes [62,63,64]. Reduced cell-cell interaction is a typical hallmark in tumor progression. Involvement of Ocln in cell-cell adhesion was described before for fibroblasts ectopically overexpressing Ocln [50]. Because increased Ca\(^{2+}\)-permeability in Ocln knock-down cells could also be a secondary effect due to the alteration of other TJ proteins, namely Cldn-2 and 12 which are critical for Ca\(^{2+}\)-permeability in the intestine [65] we also investigated the mRNA levels of these TJ proteins. There was no change of Cldn-12, but there was a significant decrease of Cldn-2. However, because Cldn-2 normally mediates Ca\(^{2+}\)-permeability, its downregulation counteracts the increased permeability and might be a compensation mechanism.

Downregulation of Ocln has been described to be involved in EMT. Especially in the context of TGF\(\beta\) induced EMT recent studies in simple epithelial cells indicate high significance of Ocln as a key regulatory component mediating complex formation of PAR6 together with type I TGF\(\beta\) receptors. Upon exposure to TGF\(\beta\), PAR6 is phosphorylated and binds to Smurf1, an E3 ubiquitin ligase, which in turn mediates ubiquitination of RhoA. Loss of Rho is important for the dissolution of TJs and for EMT [66,67,68]. However, we did not observe an influence of Ocln knock-down on E-cadherin and vimentin, typical markers for EMT, in our 3D cultures. We also did not observe a correlation of presence/absence of Ocln and the downregulation of E-cadherin or the upregulation of vimentin in our SCC (Table 4). Therefore it is unlikely that Ocln plays a major role in EMT in cutaneous SCC. This might reflect a difference between tumors derived from simple and multilayered epithelia.

We observed a role of Ocln in apoptosis sensitivity of keratinocytes. Thus, reduced apoptotic responses of keratinocytes were seen in response to TRAIL and UVB after Ocln knock-down. TRAIL activates the extrinsic apoptotic pathway, while UVB may induce both, extrinsic and intrinsic apoptotic pathways [69,70]. In line with our results, a relation of Ocln with extrinsic apoptotic pathways has also been shown in mammary gland cells. Following disruption of TJs by an Ocln-specific peptide, Ocln became associated with the death-inducing signalling complexes (DISC) of death receptors and the extrinsic apoptotic pathway was activated. In mammary gland cells from Ocln knock-out mice this peptide did not induce apoptosis [27]. However, because TRAIL is less effective in differentiated keratinocytes [71,72], we investigated its effect under low Ca\(^{2+}\) conditions. Under these conditions only a low proportion of Ocln is present at the cell-cell borders [73,74]. Therefore the observed effect of Ocln knock-down in keratinocytes is likely to include an Ocln cell membrane localization-independent effect, while data presented for mammary gland cells suggest that Ocln moves through the plasma membrane to activate the death receptor [27]. In general, the role of Ocln in apoptosis is ambiguous and might depend on the cell type. In HeLa cells over-expression of Ocln enhanced the sensitivity to H\(_2\)O\(_2\)-induced cell death [30], suggesting, in line with our results and the results in mammary gland cells [27], a supportive role of Ocln for the induction of apoptosis. In contrast, in primary hepatocytes from Ocln-deficient mice increased numbers of apoptotic cells were observed and in immortalized cells from this origin apoptosis induced by serum-free conditions was more pronounced than in wild-type cells [20]. Yu et al [29] observed an increase of apoptotic cells in MDCKII cultures after siRNA-mediated Ocln knock-down but this increase was primarily due to the retention of apoptotic cells in the monolayers while overall apoptosis ratio was not affected compared to control cells. We do not observe a significant increase in the number of apoptotic cells in Ocln knock-down cells without treatment with UV or TRAIL compared to control cells. Therefore we conclude that the mechanism in MDCK II cells is different to keratinocytes. We also excluded by our experimental design that the reduced amount of apoptosis observed here in Ocln knock-down cells after induction of apoptosis with TRAIL and UV was due to decreased retention of the apoptotic cells in the monolayers. Interestingly, in our experiments only 40–50% of primary keratinocyte cultures from different donors revealed decreased response to proapoptotic stimuli after Ocln knock-down. Further clarification of the role of Ocln in keratinocyte apoptosis regulation will be a challenging task for future projects.

For Cldn-1, we found downregulation in uppermost and lowermost layers of cutaneous SCC and predominantly absence in the invasive parts, but a complete loss was only found in 9%. This is in agreement with Ouban et al [75] who found Cldn-1 in 91.7% of SCC from different origins. Concerning the downregulation in the uppermost and lowermost layers there was no difference for Cldn-1 between SCC and its precursors, hinting for an early event in skin hyperplasia. This is supported by findings in neoplasia induced by 7,12-dimethylbenz(a)anthracene and 12-O-tetradecanoyl-phorbol-13-acetate treatment of mouse models, which also exhibit downregulation of Cldn-1 in the basal cell layer [76].
Looking for explanations for the common alterations of Claudin-1, Claudin-4 and ZO-1 in the various skin tumors, we hypothesized that chronic UV exposure might induce some of these changes. Therefore, we investigated sun-exposed versus non-sun-exposed skin. Indeed, we observed a significant downregulation of Claudin-1 in lower layers and broader localization of Occludin, ZO-1, and Claudin-4 in a high proportion of chronically sun-exposed skin. Our data fit nicely to results reported in an UBV-irradiated murine skin model [77] describing a broader ZO-1 localization throughout the upper epidermis. A broader localization of ZO-1 was also shown in irradiated human skin xenografts and of Occludin in irradiated skin equivalents [78]. Downregulation of Claudin-1 in upper layers which was found in SCC and its precursors, was not observed in sun-exposed skin. Also, opposed to skin tumors, spots of loss for the various proteins were rarely seen in sun-exposed skin. Therefore these events seem to be independent from chronic UV-irradiation.

Conclusion

In conclusion, we demonstrate a frequent loss of Occludin in cutaneous SCC but not in its precursors. We confirm our hypothesis that Occludin knock-down in keratinocytes is involved in the promotion of tumorigenic features and show reduced susceptibility to TRAIL- and UV-induced apoptosis as well as reduced cell-cell adhesion. This may play a role in SCC tumorigenesis, as well as in other carcinomas, as loss of Occludin is a common feature in tumors. Other changes of TJ proteins were identified in cutaneous SCC as well as in precursor lesions and sun-exposed skin, and may therefore characterize initial steps in tumorigenesis induced by UV-irradiation.

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Author Contributions

Conceived and designed the experiments: SR MZK UO JE JMB. Performed the experiments: SR MZK UO NK SV MM RR CB. Analyzed the data: SR MZK UO NK PV DDD EV RR IM JMB. Wrote the paper: SR MZK UO NK SV PV DDD JE MM EV RR IM JMB.

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