Patient-derived in vitro skin models for investigation of small fiber pathology

Franziska Karl1, Maximiliane Wußmann2, Luisa Kreß1, Tobias Malzacher1, Phillip Fey2, Florian Groeber-Becker2,3 & Nurcan Üçeyler1

1Department of Neurology, University of Würzburg, Josef-Schneider-Str. 11 97080, Würzburg, Germany
2Translational Center Regenerative Therapies, Fraunhofer Institute for Silicate Research, Neunerplatz 2 97082, Würzburg, Germany
3Department of Tissue Engineering & Regenerative Medicine, University Hospital Würzburg, Röntgenring 11 97070, Würzburg, Germany

Correspondence
Nurcan Üçeyler, Department of Neurology, University of Würzburg, Josef-Schneider-Straße 11, 97080 Würzburg, Germany. Tel: +49 931 201 23542; Fax: +49 931 201 623542; E-mail: uceyler_n@ukw.de

Funding Information
The study was supported by the Interdisciplinary Center for Clinical Research (Interdisziplinäres Zentrum für klinische Forschung, IZKF) University of Würzburg (N.U.: NZ53). N.U. was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG: UE171-5/1).

Abstract
Objective: To establish individually expandable primary fibroblast and keratinocyte cultures from 3-mm skin punch biopsies for patient-derived in vitro skin models to investigate of small fiber pathology. Methods: We obtained 6-mm skin punch biopsies from the calf of two patients with small fiber neuropathy (SFN) and two healthy controls. One half (3 mm) was used for diagnostic intraepidermal nerve fiber density (IENFD). From the second half, we isolated and cultured fibroblasts and keratinocytes. Cells were used to generate patient-derived full-thickness three-dimensional (3D) skin models containing a dermal and epidermal component. Cells and skin models were characterized morphologically, immunocyto- and histochemically (vimentin, cytokeratin (CK)-10, CK 14, ki67, collagen1, and procollagen), and by electrical impedance. Results: Distal IENFD was reduced in the SFN patients (2 fibers/mm each), while IENFD was normal in the controls (8 fibers/mm, 7 fibers/mm). Two-dimensional (2D) cultured skin cells showed normal morphology, adequate viability, and proliferation, and expressed cell-specific markers without relevant difference between SFN patient and healthy control. Using 2D cultured fibroblasts and keratinocytes, we obtained subject-derived 3D skin models. Morphology of the 3D model was analogous to the respective skin biopsy specimens. Both, the dermal and the epidermal layer carried cell-specific markers and showed a homogenous expression of extracellular matrix proteins. Interpretation: Our protocol allows the generation of disease-specific 2D and 3D skin models, which can be used to investigate the cross-talk between skin cells and sensory neurons in small fiber pathology.

Introduction
Small fiber neuropathy (SFN) is a subgroup of sensory neuropathies, which selectively impairs thinly myelinated A-delta and unmyelinated C nerve fibers and characteristically leads to acral burning pain, para- and dysesthesias. The peripheral nerve endings of the small fibers terminate in the epidermis as nociceptors, where they are exposed to direct influences from the environment, but also from the surrounding skin cells. It is striking, that intraepidermal innervation is reduced in SFN patients, while pain is a key symptom. Thus, hyperexcitability of the dorsal root ganglion sensory neurons or the remaining peripheral nociceptors is assumed. Indeed, we found an increase in pro-inflammatory cytokine gene expression in skin punch biopsies of SFN patients that may contribute to nociceptor sensitization. Interestingly, this increase was not based on cutaneous inflammatory cell infiltration, which raises the question of a potential role of surrounding skin cells as a source for algesic mediators. Keratinocytes secrete neuroactive molecules capable of modulating sensory neurons such as neurotrophins, adenosine triphosphate, and cytokines, and carry nociception-associated ion channels, growth factor, and cytokine receptors. In a recent study using human two-dimensional (2D) culture models, the interaction of...
keratinocytes and sensory nerve fibers via formation of sensory units was shown.4 Another study applying optogenetics in mice revealed that activation of epidermal keratinocytes elicits action potentials in sensory neurons.5 These studies suggest an active role of keratinocytes in sensory processing and support the novel concept of cutaneous nociception. Furthermore, skin cells release axon guidance cues like netrins and semaphorins, which may direct nerve fiber growth.6,7 Thus, evidence is increasing for a potential pathophysiological role of keratinocytes in peripheral innervation and sensory neuron sensitization, and novel experimental approaches are needed for translational in-depth analysis of the underlying mechanisms, which is hampered by the limited availability of human biomaterial.

A promising tool to study interactions between peripheral nociceptors and skin cells is three-dimensional (3D) full-thickness skin model (FTSM).8 FTSM enables the investigation of human cell–cell and cell–matrix interactions which is particularly useful when suitable animal models are lacking. However, published protocols require large biopsies of several cm² area,9 highly proliferative juvenile cells,10 or a combination of both, and are therefore not applicable when only few millimeter skin samples of adult patients are available as obtained for routine diagnostics. Our aim was to establish individually expandable primary keratinocyte and fibroblast 2D and 3D cell culture models from 3-mm skin punch biopsies and to generate patient-derived FTSM as a new in vitro tool for the investigation of the pathophysiological impact of skin cells on small fiber pathology.

**Methods**

**Patient recruitment**

Patients with SFN were prospectively recruited at the Department of Neurology, University of Würzburg, Germany, according to current criteria11 and after exclusion of large fiber polyneuropathy using clinical examination and nerve conduction studies. Additionally, age- and sex-matched healthy controls were recruited. For the establishment of subject-derived skin models, skin punch biopsies were obtained from two patients with SFN and two healthy controls (Table 1).

**Isolation of adult skin cells**

**Skin punch biopsy**

Figure 1 depicts our experimental setup. A 6-mm skin punch biopsy (apparatus by Stiefel GmbH, Offenbach, Germany) was taken in local anesthesia from the calf 10 cm above the lateral malleolus of two patients with idiopathic SFN and two healthy controls following a standard procedure. Each skin sample was divided in two 3-mm parts: one part was used for immunohistochemical determination of the intraepidermal nerve fiber density as practiced in clinical routine; the second part was processed for the isolation of adult human dermal fibroblasts (hDF) and epidermal keratinocytes (hEK). The epidermis was manually separated from the dermis using a scalpel (B. Braun, Melsungen, Germany). Both halves were further processed for primary cell cultures of hDF and hEK immediately after partitioning.

**Table 1. Patient characteristics.**

|            | SFN-1   | SFN-2   | Ctr-1   | Ctr-2   |
|------------|---------|---------|---------|---------|
| Sex        | Male    | Male    | Male    | Male    |
| Age        | 54      | 44      | 69      | 29      |
| SFN etiology | Idiopathic | Idiopathic | N/A    | N/A    |
| Current pain intensity (on numeric rating scale 0–10) | 8/10 | 4/10 | N/A | N/A |
| Pain localization and character | Acral burning | Acral burning | N/A | N/A |
| Pain duration | 6 years | 6 years | N/A    | N/A    |
| IENFD at calf (fibers/mm) | 2       | 2       | 7       | 8       |

Abbreviations: Ctr, control; IENFD, intraepidermal nerve fiber density; N/A, not applicable; SFN, small fiber neuropathy.

**hDF culture**

To obtain hDF, the dermis was cut into three small pieces placed in a T25 flask which were covered with 10 µL of Dulbecco’s Modified Eagle Medium, F-12 Nutrient Mixture (DMEM/F-12) with 1% penicillin/streptomycin, and with 10% fetal calf serum (fibroblast medium, Thermo Fischer Scientific, Carlsbad, USA), and incubated for 1h at 37°C, 5% CO₂ to ensure cell attachment. Thereafter, 1.5-mL fibroblast medium was carefully added. Medium was changed every 2–3 days until cell outgrowth was observed. Subsequently, cells were expanded and further processed at passage four.

**hEK culture**

For the extraction of hEK, the epidermis was cut into nine small pieces and placed into three T25 cell culture bottles (three pieces per bottle) with the basal layer facing down toward the cell culture surface. To ensure cell attachment, samples were covered with 10 µL of fibroblast medium and incubated for 1 h at 37°C, 5% CO₂.
Thereafter, 1.5-mL fibroblast medium was carefully added. Cells were inspected daily until keratinocyte outgrowth was observed. Then, medium was changed to keratinocyte medium (EpiLife Medium supplemented with 1% Epilife defined growth supplement, and 1% penicillin/streptomycin, Thermo Fischer Scientific, Carlsbad, USA). After passage two, magnetic-activated cell sorting (MACS) with human Anti-Fibroblast MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used according to the manufacturer’s protocol for purification of the hEK primary cell culture. Medium was changed every 2–3 days. Cells were further processed at passage four.

**Cellular impedance**

Measurements of cellular real-time impedance for hEK and hDF were performed with xCELLigence® RTCA DP (Roche, Basel, Switzerland). About 3000 cells/well \((n = 3/\text{cell type})\) were seeded in 200 µL of the respective medium into an E-plate 96 (ACEA Biosciences Inc., San Diego; USA). Cell-based impedance (as reflected by the cell index) was measured hourly in a time interval of 50–100 h after cell seeding. Cellular doubling time (i.e., period of time that is needed for doubling a cell population) was calculated from the obtained data by fitting the curve to an exponential equation. Each cell line was measured in triplicate and RTCA Data Analysis Software 1.0 (Roche, Basel, Switzerland) was used for analysis. Data are illustrated as line charts or bar graphs (GraphPad Prism 7 software, San Diego, USA), representing the mean and standard deviation.

**Generation of FTSE**

Full-thickness skin equivalents (FTSE) were generated using a Corning® Costar® well plate. First, hDF \((4.5 \times 10^4 \text{ cells})\) were embedded in collagen I hydrogel isolated in-house from rat tails to build the dermal layer of the FTSE. Care was taken to avoid the formation of air bubbles in the highly viscous collagen I hydrogel. The dermal layer was cultivated in Epilife® medium supplemented with 1X human keratinocyte growth supplement.
were fixed with Roti-temperature. FTSE and native skin for immunohistochemistry ethanol, 50% acetone mixture for 10 min at room temperature. Immunocytochemistry and immunohistochemistry SFN-1, Ctr-2; Fig. 2).

IENFD at the lower leg was normal in healthy controls. Both patients with SFN had reduced IENFD, while subjects showed no symptoms or signs of neuropathy. The control subjects showed no symptoms or signs of neuropathy. Both patients with SFN had reduced IENFD, while IENFD at the lower leg was normal in healthy controls (SFN-1, Ctr-2; Fig. 2).

Primary fibroblasts and keratinocyte 2D cell culture

We succeeded in isolating HDF and hEK cells from the 3-mm skin specimens of the SFN patient (SFN-1) and the healthy control (Ctr-2), and established stable HDF (Fig. 3A and B) and hEK (Fig. 3C and D) cell cultures for subsequent application in FTSM. Morphological assessment showed a typical spindle-shape appearance of the HDF, whereas hEK displayed a cobblestone morphology. For cellular characterization, immunoreactions with cell-specific markers were performed. HDF of the SFN patient and the healthy control showed homogeneous expression of the intermediate filament of mesenchymal cells vimentin (Fig. 4A and E). hEK isolated from the SFN patient and the healthy control expressed the basal epidermal marker CK-14 in 2D cell culture but were negative for the intermediate filament of differentiated keratinocytes CK-10 (Fig. 4B, C, F, and G). The nuclear proliferation marker ki67 was equally expressed in hEK of the SFN patient and the healthy control (Fig. 4D and H). We did not detect morphological or immunocytochemical differences between skin cells obtained from the SFN patient and the healthy control.

Adequate cellular viability and constant proliferation of the SFN patient and the healthy control

To assess the viability and proliferation of skin cells in 2D culture, real-time impedance was determined in hEK and HDF of the patient (SFN-1) and the healthy control (Ctr-2). The cell index of both cell types constantly increased indicating adequate viability and constant proliferation without...
Figure 2. Representative photomicrographs of skin biopsy sections immunoreacted with antibodies against protein gene product-9.5 for the detection of intraepidermal nerve fibers (IENF). IENF (indicated by white arrows) were reduced in the skin sample obtained from the lateral calf of a patient with small fiber neuropathy (SFN-1, A) compared to a healthy control (Ctr-2, B). Scale bar represents 100 μm.

Figure 3. 2D culture of primary human dermal fibroblasts (hDF) and epidermal keratinocytes (hEK). Phase contrast images of hDF culture of a patient (SFN-1) with small fiber neuropathy (SFN, A), a healthy control (Ctr-2, B), and hEK culture of a patient with SFN (C) and a healthy control (D). The morphology was normal in the hDF and hEK of the SFN patient and the healthy control. Scale bar represents 100 μm.
relevant difference between the SFN patient and the healthy control (Fig. 5A and B). In contrast, the doubling time of hDF and hEK obtained from the SFN patient was lower (Fig. 5C, hDF: 217.47 h; hEK: 96.27 h) compared to those from the healthy control (hDF: 89.11 h; hEK: 57.65 h).

Characterization of the FTSM

After 3 weeks, a FTSM was generated in skin cells obtained from two SFN patients (Fig. 6A, B) and two healthy controls (Fig. 6C, D). We first used HE

Figure 4. Expression of cell-specific markers in fibroblasts and keratinocytes. Skin cells of a patient (SFN-1) with small fiber neuropathy (SFN, A–D) and a healthy control (Ctr-2, E–H) were immunoreacted with specific antibodies against vimentin (A, E), cytokeratin (CK)-14, CK-10, and ki67. Human dermal fibroblasts (hDF) expressed vimentin (A, E), whereas human epidermal keratinocytes (hEK) expressed the epithelial marker CK-14 (B, F), but not CK-10 (C, G). hEK also expressed the nuclear proliferation marker ki67 (D, H). Scale bar represents 50 μm.

Figure 5. Real-time impedance measurements using xCELLigence® instrument. Line charts display the cell index within the time frame of 50–100 h after seeding human dermal fibroblasts (hDF, A) and human epidermal keratinocytes (hEK, B) of the patient with small fiber neuropathy (SFN-1) and the healthy control (Ctr-2). Both cell types showed increase in the cell index, without differences between the cells of the SFN patient and the healthy control. Bar graphs show the doubling time (C) of hDF and hEK isolated from the patient with SFN and the healthy control. The doubling time of both cell types from the patient with SFN was lower compared to the healthy control.
Figure 6. Morphological structure of the full-thickness skin model (FTSM). Macroscopic images of the FTSM of two patients with small fiber neuropathy (SFN, A, B) and two healthy controls (Ctr, C, D). Hematoxylin and eosin (HE) stainings of the FTSM (A’-D’), the respective skin punch biopsy (A”-D”) of the patients with SFN, and the healthy control. Morphology was normal in the FTSM of the SFN patients and the healthy controls, and was similar to that of native skin. Scale bar represents 100 μm.
staining to assess the morphological structure of the FTSM. The overall morphology of the FTSM was analogous to the respective HE stains performed on skin punch biopsy sections both in the SFN patients (Fig. 6A–E) and the healthy controls (Fig. 6F–J) consisting of a dermis and a multilayered epidermis with stratum corneum. To further investigate the composition of the FTSM of one patient with SFN (SFN-1) and one healthy control (Ctr-2), we performed immunoreactions with antibodies against selected tissue-specific markers. Both models expressed intermediate filament markers, CK-10 and CK-14, in the epidermal layer. Differentiation between the basal and suprabasal strata of the epidermis with stratum corneum was not possible in both models due to the low number of epidermal layers. The dermal layer showed a homogenous distribution of vimentin, collagen 1, and procollagen (Fig. 7). We did not detect morphological or immunohistochemical differences between the FTSM of the SFN patient and the healthy control. We also performed immunoreactions on native skin from a healthy control subject embedded in paraffin (Ctr-2; Fig. 7). Expression of the markers CK-10, CK-14, vimentin, collagen 1, and procollagen was similar to the FTSM.

**Discussion**

We set out to individually isolate and expand hDF and hEK cell cultures and provide a protocol to isolate both cell types from a single 3-mm skin punch biopsy obtained from adult subjects. We have succeeded in generating 2D cell cultures and a 3D in vitro skin model from individual subject-derived cells which can now be used for in-depth pathophysiological analysis of small fiber damage. We showed that both cell types have the typical morphology, express cell-specific markers, and display constant proliferation as determined by impedance measurement.

SFN is a subgroup of sensory neuropathies, which is characterized by burning pain and dysesthesias in hands and feet, while skin punch biopsies typically reveal a reduction in IENFD. The traditional view, that neurodegenerative processes in DRG sensory neurons may underlie SFN pain and peripheral denervation, is increasingly challenged by studies providing evidence for an active role of skin cells in sensory processing and cutaneous nociception. As the main skin cell population, keratinocytes and fibroblasts are in close and active contact with sensory nerve fiber endings, and human and experimental data are pointing toward an intensive interaction between skin...
cells and nociceptors. This novel concept of skin cell involvement in sensory signaling is supported by the expression of nociception-associated receptors and the release of pain-related mediators by fibroblasts and keratinocytes. In addition, keratinocytes release axon guidance cues like netrins and semaphorins, which may act as local pathfinders.

Several studies have shown the interplay between skin cells and sensory neurons. Overexpression of neurotrophins like nerve growth factor or neurotrophin 3 by keratinocytes prolonged neuronal survival. Axonal outgrowth was strongly stimulated when cocultivating neurons with keratinocytes, but was absent in pure neuronal cultures indicating the influence of a potential soluble factor secreted by keratinocytes. Using mice expressing channelrhodopsin in keratinocytes, a recent study revealed that mere activation of the epidermis already evokes action potentials in sensory neurons. In transgenic mice, selective transient receptor potential vanilloid (TRPV) 3 overexpression and heat activation of keratinocytes induced prostaglandin E2 release causing nocifensive behavior. After conditioned expression of the capsaicin receptor TRPV1 in keratinocytes of TRPV1-knockout mice, exclusive stimulation of keratinocytes was sufficient to evoke pain behavior.

While data from experimental studies are encouraging, the limited availability of patient biomaterial remains the main translational roadblock to transfer knowledge from bench to bedside. To overcome this hurdle, 3D skin models have been established. One group developed a hybrid model of human skin with axonal growth of rat DRG neurites into the dermis. In another study, the authors developed a skin model using neonatal foreskin fibroblasts and keratinocytes, coated with human-induced neural stem cells. Recently, a fully human-derived and innervated skin model using fibroblasts and keratinocytes from breast reductive surgeries was developed. The major drawbacks for the methodological transfer into clinical studies are the need of large skin samples, juvenile and highly proliferative cells as prerequisites, or hybrid models obtained from different species, which are either not practicable in clinical routine or do not reflect real-life conditions. Moreover, these tools do not model disease conditions since skin cells and neurons derived from healthy donors were included. Here, we established a protocol, which enables the expansion of patient-derived skin cells under 2D and 3D conditions using only few millimeter of skin from adult subjects obtained under conditions used in clinical practice.

Our study has some limitations. Although keratinocytes and fibroblasts are the most abundant cell types in the skin, various other cells (e.g. immune cells and Schwann cells) were not included in the current model. Also, cutaneous structures, such as blood vessels and nerve fibers, are missing. Future developments improving and extending 2D and 3D cell culture protocols will help refining our model.

Our protocol provides a promising in vitro method for generating disease-specific 2D cultures and 3D skin models from a single 3-mm skin punch biopsy. 2D cultures enable gene expression analysis of distinct skin cells rather than using the entire skin samples. Likewise, our patient-derived 3D skin models facilitate individualized in-depth analysis of cutaneous cell–cell interactions as a potential contributor to small fiber pathology. Our method also provides a solid basis for future developments toward innervated 3D-skin models serving as novel tools in personalized medicine.

**Acknowledgments**

We thank Alina Hohmuth (Department of Neurology, University of Würzburg) for technical help during the early phases of our 2D skin cell protocol establishment.

**Conflict of Interest**

The authors declare that there is no conflict of interest.

**Author Contributions**

NÜ, FGB, and FK contributed to the conception and design of the study. FK, MW, LK, TM, and PF were involved in acquisition and analysis of data. FK and NÜ drafted the manuscript and figures. NÜ, FGB, and MW revised the manuscript critically for important intellectual content.

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