intermediate filament protein. *Cell* 60: 585–95

Matsuda Y, Naito Z, Kawahara K *et al.* (2011) Nestin is a novel target for suppressing pancreatic cancer cell migration, invasion and metastasis. *Cancer Biol Ther* 11:512–23

Pan Y, Jing R, Pitre A *et al.* (2008) Intermediate filament protein synemin contributes to the migratory properties of astrocytoma cells by influencing the dynamics of the actin cytoskeleton. *FASEB J* 22:3196–206

Piras F, Perra MT, Murtas D *et al.* (2010) The stem cell marker nestin predicts poor prognosis in human melanoma. *Oncol Rep* 23:17–24

Reya T, Morrison SJ, Clarke MF *et al.* (2001) Stem cells, cancer, and cancer stem cells. *Nature* 414:105–11

Santini R, Vinci MC, Pandolfi S *et al.* (2012) HEDGEHOG-GLI signaling drives self-renewal and tumorigenicity of human melanoma-initiating cells. *Stem Cells* 30:1808–18

Sellheyer K, Krahl D (2010) Spatiotemporal expression pattern of neuroepithelial stem cell marker nestin suggests a role in dermal homeostasis, neovasculogenesis, and tumor stroma development: a study on embryonic and adult human skin. *J Am Acad Dermatol* 63:93–113

Xue XJ, Yuan XB (2010) Nestin is essential for mitogen-stimulated proliferation of neural progenitor cells. *Mol Cell Neurosci* 45: 26–36

Coculture Model of Sensory Neurites and Keratinocytes to Investigate Functional Interaction: Chemical Stimulation and Atomic Force Microscope–Transmitted Mechanical Stimulation Combined with Live-Cell Imaging

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TO THE EDITOR

There is growing evidence for functional interactions between nociceptive nerve endings and non-neuronal cells modulating sensory function in health and disease. In the skin, non-neuronal cells like keratinocytes actively participate in nociceptor sensitization and thus in encoding of noxious stimuli (Lumpkin and Caterina, 2007; Gold and Gebhart, 2010). *In vivo* approaches for live-cell imaging of the interplay of sensory terminals and surrounding cells are hampered by the small diameter of the endings and the difficult experimental accessibility. Moreover, selectively stimulating one type of cell while recording responses from both is challenging. Our goal was therefore to establish an *in vitro* model that would allow studying potential functional interactions between nociceptive terminals and keratinocytes.

To this end, we developed a coculture model of sensory endings and keratinocytes in a compartmented Campenot chamber (Figure 1a; Campenot *et al.*, 2009; Roggenkamp *et al.*, 2012). Compartmented chambers for coculture (Chateau *et al.*, 2007; Roggenkamp *et al.*, 2012) have the advantage of a spatial segregation of neurites (Ns) from their somata (Figure 1a) compared with a coculture in one compartment (Ulmann *et al.*, 2009; Pereira *et al.*, 2010a,b). Moreover, in the Campenot chamber, fluid isolation between the compartments allows different culture media and factors for neuronal somata in one compartment and for Ns and keratinocytes in the other, mimicking the *in vivo* structural and environmental conditions. In our experiments, isolated somata of porcine dorsal root ganglion neurons (Obreja *et al.*, 2008) were grown in the central compartment; Ns outgrown into the lateral compartments served as a model for sensory endings. These Ns were cocultured with primary isolated porcine keratinocytes (see Supplementary information).

Using this approach, we obtained areas of confluent keratinocytes in spatial contact with sensory Ns (Figure 1b and c). By using atomic force microscopy (AFM), we visualized nanoscaled surface topographies of adjacent Ns and keratinocytes (Figure 1d). In the past, AFM had been established as a tool for the characterization of cell–cell contacts comprising also the desmosomal junctions between the keratinocytes (Fung *et al.*, 2010). In comparison, our images suggest cell–cell contacts not only between keratinocytes but also possibly between Ns and keratinocytes. For functional investigations, we implemented live-cell imaging combined with chemical or mechanical stimulation, allowing selective activation of one cell type while recording responses simultaneously from both types. Responses to these stimuli were visualized using the non-ratiometric calcium dye *Fluo8* acetoxymethyl ester. To activate only Ns, a membrane-depolarizing concentration of KCl was added to the somata containing central compartment. The induced depolarization is transmitted along the Ns into the lateral compartment, indicated by a calcium response (Supplementary Figure S1a online). For specific activation of nociceptive Ns, capsaicin was added to the lateral compartment (Supplementary Figure S1b online; Caterina *et al.*, 2007).

In coculture of Ns and keratinocytes in the lateral compartment, we observed an interaction between both cell types. In the experiment shown in Figure 2a–d, KCl was first applied to the central compartment. After an immediate

Abbreviations: AFM, atomic force microscopy; N, neurite

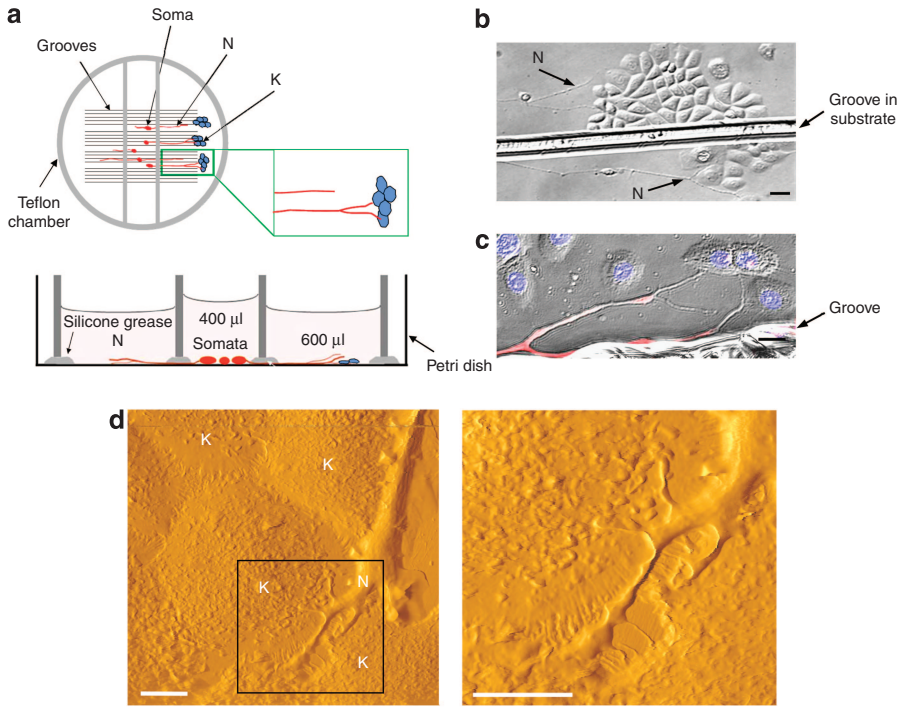

Figure 1. Compartmented culture chamber adapted for functional investigations of neurites and keratinocytes in coculture. (**a**) Layout of the chamber and cultured cells with neuronal somata in the central compartment and outgrown neurites and keratinocytes in the lateral compartment. (**b–d**) Cocultures of neurites and keratinocytes. (**b**) Bright-field microphotograph of two areas of keratinocytes with neurites, divided by a groove in the substrate. Bar = 30 µm. (**c**) Merged microphotographs of bright-field differential interference contrast and immunofluorescence for the pan-axonal neurofilament marker SMI-312R (1:3,000, Covance) in the neurite (red) and 4′,6-diamidino-2-phenylindole in the keratinocytes (blue). Bar = 20 µm. (**d**) Atomic force microscope topography image. Left: topographies showing keratinocytes (K) and ending of a neurite (N). Right: enlarged section showing potential junctions between K and N. Image was taken with the contact mode; applied forces were reduced to a minimum of 10–50 pN. Bars = 10 µm.

transient increase in calcium concentration in the N in the lateral compartment, two of the adjacent keratinocytes (K1 and K4) also clearly responded (Figure 2d). There was a delay of 10 and 34 seconds between peak of the neuronal response and response onset in the keratinocytes. Differential interference contrast and fluorescence microphotographs show the arrangement of the keratinocytes and the Ns (Figure 2b and c). As the keratinocytes were not exposed to KCl, their calcium transients can be attributed specifically to activation of the Ns and consecutive release of mediators such as ATP or neuropeptides. It is, however, conceivable that the second responding keratinocyte is activated by local diffusion of compound(s) released from the first one. To test whether the phenotype of the N is nociceptive, a subsequent capsaicin application to the lateral compartment was administered; it induced a transient calcium increase in the N, supporting its nociceptive nature. There was no response in keratinocytes, possibly because of depleted stores in the N. In another experiment, the time course of the responses of the keratinocytes was similar; however, one keratinocyte showed oscillation and possibly a small response after the capsaicin application (Supplementary Figure S2a–c online). When capsaicin was applied to the coculture as first stimulus, there was an immediate response in the N and delayed responses in the nearby keratinocytes (Supplementary Figure S2d–f online). For normal human epidermal keratinocytes, expression of functional TRPV1 has been reported (Jain *et al.*, 2011), but only about 3% of cells responded with a calcium influx within the first 30 seconds after application of capsaicin (Inoue *et al.*, 2002). Similarly, in our cultured porcine keratinocytes, the proportion of cells responding to capsaicin (1 µM) within the first 10 seconds was 2.6% ($n = 1441$ cells; one animal; Supplementary Figure S3 online). Thus, because responses in keratinocytes in the coculture experiments occurred in the vicinity of the Ns, it is likely that they were due to neuronal activation.

Selective activation of keratinocytes was achieved by focal mechanical stimulation. To this end, we implemented the AFM for locally precise and force-controlled stimuli to single keratinocytes. Commonly, it is used as an imaging device for the nanoscaled three-dimensional visualization of surface topographies (Figure 1d) or as a tool to measure intermolecular and intramolecular forces (Hinterdorfer and Dufrêne, 2006; Niland *et al.*, 2011). In this study, the atomic force microscope was combined with live-cell calcium imaging to allow mechanical stimulation of one keratinocyte and simultaneous detection of changes in the calcium concentration in adjacent Ns and keratinocytes (Figure 2e–h). In these experiments, we used a single lumen chamber as the somata-containing compartment (Figure 2e). The mechanical stimulus was applied to one preselected keratinocyte through the tip of the atomic force microscope cantilever, oscillating with a frequency of 1 Hz and a loading force of up to 500 pN for 60 seconds. As the cantilever interferes with the view on the stimulation site, the local response cannot be assessed. In the experiment shown, 6 seconds after starting the mechanical stimulation of a keratinocyte (K stimulation; Figure 2f), a transient increase in calcium concentration in a nearby N was detected, likely due to released endogenous compound(s) from the stimulated keratinocyte. With a delay of 10 and 31 seconds, respectively, adjacent keratinocytes (K3, K4, and K5) responded as well (Figure 2h).

In summary, our study demonstrates, using AFM, structurally, nanoscaled close contact between keratinocytes and N terminals *in vitro*. Functionally, we show a direct cross talk between Ns and keratinocytes in coculture using a compartmented chamber and live-cell imaging. Chemical activation of neurons resulted in calcium responses in keratinocytes and mechanically stimulated

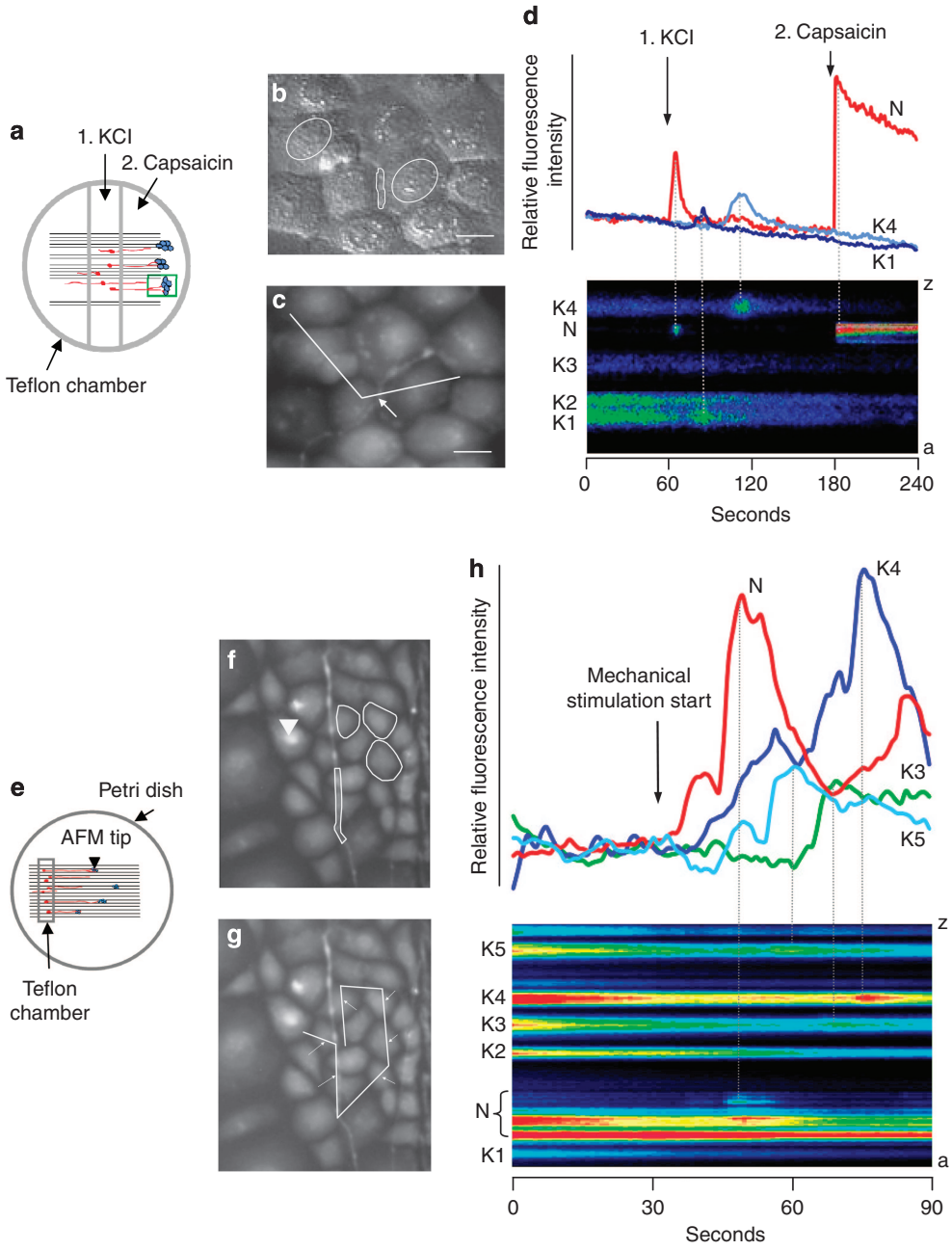

Figure 2. Calcium-imaging recordings from neurites (N) and keratinocytes. (**a**–**d**) Chemical and (**e**–**h**) mechanical stimulation revealing cross talk between both cell types. (**a**) Schema of experimental layout for chemical stimulation of N. Cross-compartment stimulation by KCl (25 mM) application to the somata containing central compartment and subsequent capsaicin (1 μM) application to the lateral compartment. Boxed area indicates recording site. (**b**) Differential interference contrast image with regions of interest for keratinocytes (K1, K4) and a N. Regions of interest apply to the fluorescence intensity plot in **d** (top), start of stimulation with KCl and capsaicin is indicated by arrows. (**c**) Fluorescence image of the same region as shown in **b**; white line represents the scan line (a to z) through K1, K2, K3, N, and K4 for the time-lapse plot in **d** (bottom). K1 to K4 indicate keratinocytes. Culture medium: central compartment, F12 with 50 ng ml^{-1} GDNF; lateral compartment, keratinocyte growth medium (KGM) without growth factors; time in culture: 5 days. (**e**) Schema of experimental layout for selective mechanical stimulation of one keratinocyte. (**f**) Fluorescence image showing a keratinocyte stimulated by the atomic force microscope tip (white triangle, K stimulation), a N, and keratinocytes (K3, K4, and K5). Regions of interest apply to the fluorescence intensity plot in **h** (top) with start of mechanical stimulation indicated by arrow. Mechanical stimulus with an oscillating force of 500 pN maximum and a frequency of 1 Hz for 60 seconds. (**g**) Fluorescence image of the same region as shown in **f**; white line represents the scan line (a to z) through K1, N, K2, K3, K4, and K5 for the time-lapse plot in **h** (bottom). Culture medium: central compartment, F12 with GDNF, 50 ng ml^{-1}; lateral compartment, KGM with GDNF, 50 ng ml^{-1}; time in culture: 7 days.

keratinocytes activated sensory Ns. *In vivo*, modulation of nonsynaptic cross talk between the cell types has major implications for tissue homeostasis and sensory function in health and diseases such as dermatitis or neuropathy, and the described *in vitro* coculture system permits the detailed study of neuron–keratinocyte interaction.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Andreas Klusch[1,5], Leslie Ponce[2,5], Christian Gorzelanny[3], Ina Schäfer[2], Stefan W. Schneider[3], Matthias Ringkamp[4], Andreas Holloschi[2], Martin Schmelz[1], Mathias Hafner[2] and Marlen Petersen[1]

[1]Department of Anesthesiology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany; [2]Institute of Molecular Cell Biology, University of Applied Sciences Mannheim, Mannheim, Germany; [3]Department of Dermatology, Experimental Dermatology, Venerology and Allergology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany and [4]Department of Neurosurgery, Johns Hopkins University, Baltimore, Maryland, USA
E-mail: marlen.petersen@medma.uni-heidelberg.de
[5]These authors contributed equally to the work.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Campenot RB, Lund K, Mok SA (2009) Production of compartmented cultures of rat sympathetic neurons. *Nat Protoc* 4:1869–87

Caterina MJ, Schumacher MA, Tominaga M *et al.* (2007) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816–24

Chateau Y, Dorange G, Clement JF *et al.* (2007) *In vitro* reconstruction of neuro-epidermal connections. *J Invest Dermatol* 127:979–81

Fung CK, Seiffert-Sinha K, Lai KW *et al.* (2010) Investigation of human keratinocyte cell adhesion using atomic force microscopy. *Nanomedicine* 6:191–200

Gold MS, Gebhart GF (2010) Nociceptor sensitization in pain pathogenesis. *Nat Med* 16:1248–57

Hinterdorfer P, Dufrêne YF (2006) Detection and localization of single molecular recognition events using atomic force microscopy. *Nature Methods Rev* 3:347–55

Inoue K, Koizumi S, Fuziwara S *et al.* (2002) Functional vanilloid receptors in cultured normal human epidermal keratinocytes. *Biochem Biophys Res Commun* 291:124–9

Jain A, Brönneke S, Kolbe L *et al.* (2011) TRP-channel-specific cutaneous eicosanoid release patterns. *Pain* 152:2765–72

Lumpkin EA, Caterina MJ (2007) Mechanisms of sensory transduction in the skin. *Nature* 445:858–65

Niland S, Westerhausen C, Schneider SW *et al.* (2011) Biofunctionalization of a generic collagenous triple helix with the α2β1 integrin binding site allows molecular force measurements. *Int J Biochem Cell Biol* 43:721–31

Obreja O, Klusch A, Ponelis N *et al.* (2008) A subpopulation of capsaicin-sensitive porcine dorsal root ganglion neurons is lacking hyperpolarization-activated cyclic nucleotide-gated channels. *Eur J Pain* 12:775–89

Pereira U, Boulais N, Lebonvallet N *et al.* (2010a) Mechanisms of the sensory effects of tacrolimus on the skin. *Brit J Dermatol* 163:70–7

Pereira U, Boulais N, Lebonvallet N *et al.* (2010b) Development of an *in vitro* coculture of primary sensitive pig neurons and keratinocytes for the study of cutaneous neurogenic inflammation. *Exp Dermatol* 19:931–5

Roggenkamp D, Falkner S, Stäb F *et al.* (2012) Atopic keratinocytes induce increased neurite outgrowth in a coculture model of porcine dorsal root ganglia neurons and human skin cells. *J Invest Dermatol* 132:1892–900

Ulmann L, Rodeau JL, Danoux L *et al.* (2009) Dehydroepiandrosterone and neurotrophins favor axonal growth in a sensory neuron-keratinocyte coculture model. *Neuroscience* 159:514–25