ORIGINAL ARTICLE

Modeling dermatophytosis: Guinea pig skin explants represent a highly suitable model to study Trichophyton benhamiae infections

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ABSTRACT

Dermatophyte infections are a growing health concern worldwide with increasing patient numbers, especially in children. However, detailed knowledge about infection mechanisms and virulence factors are scarce. This study aimed to establish an infection model based on guinea pig skin explants mimicking the in vivo situation as closely as possible to survey the pathogenesis of dermatophytoses. A fundamental prerequisite was the detailed description of native guinea pig skin and its morphological changes during tissue culture because comprehensive data on guinea pig skin characteristics were not available. Skin explants were harvested from healthy, adult guinea pigs and transferred to cell culture inserts. One group was inoculated with defined suspensions of colony-forming units of zoonotic Trichophyton benhamiae isolates; others served as controls to assess the tissue viability during the 10-day culture. Samples were taken on days 3, 5, 7 and 10 and processed for histological and immunohistochemical analysis. Standard tissue culture conditions provoked acantholysis and regional orthokeratotic alterations. The reduced desquamation caused hyperkeratosis paralleled by hypogranulosis or regional hyperplasia. During T. benhamiae infection, keratinocyte proliferation came to a complete halt on day 5 whereas the number of terminal deoxynucleotidyl transferase dUTP nick end labeling assay-positive cells increased moderately up to day 7. Hyphae grew massively into the skin explants causing strong keratinolysis and tricholysis. By the end of the culture, complete disintegration of the basement membrane and dermal tissue was observed. A realistic and reliable skin infection model was established to study dermatophytoses in general and cutaneous T. benhamiae infections in particular.

Key words: dermatophytosis model, guinea pig skin explants, tissue culture, Trichophyton benhamiae, zoonoses.

INTRODUCTION

Superficial mycoses, namely fungal infection of the skin, hair and nail, are the fourth most common infectious condition worldwide with a global prevalence of over 626 million cases in 2016.1 Especially young and immunocompromised individuals suffer from long-lasting infections and serious treatment side-effects. Moreover, the increasing number of patients affected by zoophilic dermatophytes, such as Trichophyton benhamiae or Microsporum canis, highlights the great adaptability and resilience of these pathogens and the high risk of zoonotic transmission. Extensive research has been performed on environmental reservoirs and transmission routes, predisposing host conditions and dermatophyte virulence factors that contribute to dermatophytoses.2 Nevertheless, molecular details during manifestation and course of infection remain elusive. This may be attributed to the problem that in vitro culture systems often mimic the in vivo situation insufficiently, namely agar plates do not display the host tissue 3-D structure or imitate the mode of infection appropriately. Accordingly, Staib et al.3 revealed substantial differences in the gene expression profile of T. benhamiae (also known as Arthroderma benhamiae, one of a teleomorphic species of the T. mentagrophytes complex) grown in liquid media versus during animal infection.

To shed light on the pathomechanisms involved in dermatophytoses, a model closely resembling the infection site is of key importance. Skin explants (SE) represent such a suitable...
experimental approach as they offer the 3-D structure and physiological environment of the host tissue which is superior not only to agar plates, but also to primary keratinocyte cultures and commercially available cell lines.\textsuperscript{4,5} Even reconstructed multilayered epidermis models lack important physiological features such as hair follicles (HF) and a continuous basement membrane (BM). Especially the former are crucial for infection studies because HF were postulated as portals of entry already in 1977.\textsuperscript{6}

In addition, SE allow for multiple, continuous sampling while still contributing to the concept of 3R.\textsuperscript{7}

We pursued an SE approach based on guinea pig (GP) skin for several reasons: (i) GP are natural carriers and hosts of different dermatophyte species, ruling out the question of susceptibility; (ii) GP skin bears a high similarity to human skin in terms of cell and molecule composition\textsuperscript{8,9} as well as HF anatomy;\textsuperscript{10} and (iii) they are long-established and widely accepted model animals in the context of skin research concerning wound healing, toxicological testing and radiation therapy.\textsuperscript{11} Nevertheless, a detailed description of native (hairy) GP skin and the assessment of histological changes during long-term tissue culture are lacking.

Here, we provide the first comprehensive description of GP skin characteristics and inherent morphological changes during long-term culture. Furthermore, we present data that prove the newly established guinea pig SE (GPSE) model highly suitable for studying adherence to and invasion of skin by the emerging zoonotic dermatophyte \textit{T. benhamiae}.

METHODS

\textbf{\textit{Trichophyton benhamiae} inocula}

\textit{Trichophyton benhamiae} strains from human patients (\(n = 10\); Laboratory for Medical Microbiology, Möltsch, Germany) and infected GP (\(n = 10\); identity of all strains confirmed by internal transcribed spacer [ITS] sequencing [see below] and mass spectrometry [data not shown]) were used for infection experiments. These included strains of both acknowledged colony phenotypes, namely white (\(n = 1\), identical to GenBank accession no. LN874022.1) and yellow (\(n = 19\), identical to GenBank accession no. MF614429.1).\textsuperscript{12,13} To obtain highly concentrated colony-forming unit (CFU) solutions, \textit{T. benhamiae} isolates were incubated for 10 days at 28°C and 10% CO\(_2\) in Nutrient Broth I (Sifin Diagnostics, Berlin, Germany), 20% Kolliphor\textsuperscript{10} P407, 0.5% glucose, 0.6 mg/mL thiamin and antibiotics (50 \(\mu\)g/mL gentamycin, 50 \(\mu\)g/mL chloramphenicol, all Sigma-Aldrich, Munich, Germany). Cultures were centrifuged (4°C, 3350 g, 30 min), the pellet was resuspended in 10 mL phosphate-buffered saline (PBS; pH 7.35), filtered (40-\(\mu\)m cell strainer) and stored at –80°C in freeze medium (50% fetal bovine serum, 30% glycerin and 20% Nutrient Broth I). Aliquots were thawed for standard plate counts and CFU-determination.

\textbf{GPSE culture and infection experiments}

Guinea pig SE were harvested from healthy laboratory GP (\(n = 15\) BFA; \(n = 10\) Dunkin-Hartley, albino; all aged 1–2 years) euthanized all but one for experiments not related to this study (state approval by Landesdirektion Sachsen, animal permit numbers: T4/16, T48/16, T45/17, T07/18). Skin of 20 of these animals was used for pre-experiments on culture conditions and infection doses, and all others for infection experiments.

Routine mycological sampling similar to Mackenzie’s hair brush technique\textsuperscript{12} was conducted to exclude external bacterial and/or fungal contamination. The flank region of each animal was clipped, the skin was disinfected (70% ethanol) and spurious skin patches were excised. After removal of excessive fatty tissue, explants of approximately 2 mm\(^2\) were transferred to cell culture inserts placed in 12-well plates (Greiner BioOne, Frickenhausen, Germany). The lower cavity was supplied with growth medium composed of 3:1 Dulbecco’s modified Eagle’s medium (4.5 g/L \(\nu\)-glucose) and F12 (Gibco by Thermo Fisher Scientific, Schwerte, Germany), 10% GP serum (Biowest by Sigma-Aldrich), 10 ng/mL epidermal growth factor (EGF), 40 ng/mL recombinant human fibroblast growth factor (rhFGF; both Biomol, Hamburg, Germany) and antibiotics (100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin; both Sigma-Aldrich).

For infection experiments, 1 × 10\(^3\) CFU of each \textit{T. benhamiae} strain suspended in 2 \(\mu\)L PBS were applied to the center of the top of GPSE directly after transfer to the well plate (Fig. 1). Inoculated GPSE were maintained for 10 days at 30°C, 5% CO\(_2\) and 95% relative humidity with media change at days 3 and 7. At culture days 3, 5, 7 and 10, one GPSE per tested strain was removed, fixed in 4% neutrally buffered paraformaldehyde and paraffin embedded.

\textbf{Histological and immunohistochemical analysis}

One-micrometer-thin sections were stained with hematoxylin–eosin or a periodic acid–Schiff (PAS) reaction was carried out.
(standard protocols). Specimens were analyzed using an upright Olympus BX 51 microscope (Olympus Deutschland, Hamburg, Germany). Histological features of native GP skin and morphological changes of GPSE during culture with special focus on epidermis, BM and HF were recorded. Epidermal (BM to outer most layer of stratum corneum conjunctum) and dermal thickness (BM to top of fatty tissue) were measured using an inverse Nikon microscope TE2000 and NIS-Elements AR 4.2 software (five random measurements per section; Nikon Metrology, Alzenau, Germany).

To determine the epidermal proliferation rate, an anti-Ki-67 staining was performed. Sections were dewaxed, rehydrated and subjected to heat-induced epitope retrieval in citrate buffer (pH 6, 10 X Antigen Retriever, Sigma-Aldrich; 20 min, steam cooker). After permeabilization with Triton X100 (0.25% in PBS; Applichem, Darmstadt, Germany; 10 min, room temperature [RT]), blocking of unspecific binding sites was carried out using 10% normal goat serum (Jackson ImmunoResearch by Dianova, Hamburg, Germany) in PBS (30 min, RT). Afterwards, the fluorescein isothiocyanate (FITC)-coupled anti-Ki-67 antibody (clone SolA, rat monoclonal, no. 11-5698-82; Thermo Fisher Scientific) was applied (1:50 in PBS, 4 h, RT). After three washing steps with PBS, the nuclear counterstain with bisBenzimide H 33342 trihydrochloride (Hoechst; Sigma-Aldrich) followed (20 min, RT). All basal cells were counted, then all Ki-67-positive cells were counted and their ratio was calculated in percentage. HF matrix cells of each section served as an intrinsic positive control due to a high number of proliferating cells.

The terminal deoxynucleoitidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay was conducted according to the manufacturer’s instructions (Biotium TUNEL Assay Apoptosis Detection Kit, no. 30063; Linaris, Dossenheim, Germany) to determine the apoptosis rate. Each staining process included a negative control (TdT omission) and a positive control (pretreatment with Dnase I [500 U/mL] in 50 mmol/L Tris-HCl buffer [pH 7.4] with 1% bovine serum albumin and 5 U/µL MgCl2; Thermo Fisher Scientific). Additionally, GP jejunum sections located in the stratum basale were rarely observed. The stratum spinosum consisted of 3–6 progressively flattening keratinocyte layers. The stratum granulosum was a narrow but prominent cell rim filled with basophilic intracytoplasmic granules. It connected to a clearly distinct eosinophilic band without discernible cell contours and a well-pronounced multilayered stratum corneum conjunctum consisting of (mostly) flat, tightly connected, enucleated cornocytes. A proliferation rate of 8.15 ± 2.99% was assessed while only 1.57% of basal keratinocytes stained TUNEL-positive. The apoptosis rate was determined just as the proliferation rate.

To detect fungal elements in GPSE, an NHS fluorescein-coupled anti-Trichophyton antibody was developed. For detailed production and purification protocols, see Appendix S1. Epitope retrieval for anti-Trichophyton staining was achieved by Proteinase K-incubation (20 min, RT). Afterwards, the anti-Trichophyton antibody was applied (4 h; RT, 2 µg/mL in PBS with 5% rabbit serum). Thorough washing with PBS and the nuclear counterstain (see above) ensued. Sections were screened using a Nikon Eclipse Ni microscope equipped with ProgRes CF cool camera and ProgRes Capture Pro 2.8.8 software (all Jenoptik, Jena, Germany).

Statistics
The morphometric data were analyzed using SigmaStat 2.03 and SigmaPlot 7.0 software (Systat Software, Erkrath, Germany). To test for statistically significant differences between infected and uninfected GPSE and time points, respectively, Student’s t-test and the Mann–Whitney –U test were employed. P-values of less than 0.05 were considered significant (*P < 0.05 and **P < 0.001) and results were presented as mean ± standard deviation.

RESULTS
T. benhamiae inocula and GPSE infection
The combination of a higher CO₂ pressure and a reduced glucose concentration resulted in highly concentrated CFU suspensions (1 × 10⁸ to 2.5 × 10⁸ CFU/mL). Plate counts before and after freeze–thaw cycles showed negligible loss of CFU or viability. Pre-experiments on infection doses found 1 × 10³ CFU/explant suitable because it guaranteed a macroscopically visible fungal growth while GP tissue destruction did not occur until the end of culture. Using CFU suspensions, namely single countable units, proved highly reproducible and superior to mycelia plugs or scrapings from agar plates where the real amount of infectious units can hardly be determined. The centered positioning and the surface tension of the CFU droplet ensured GPSE invasion from the top only.

Native GP skin
The histological examination of native GP skin revealed the usual mammalian stratification of epidermal layers, dermis and parts of the underlying subcutis, which is illustrated in Figure 2. Native GP epidermis displayed a thickness of 57.39 ± 16.27 µm. Basal keratinocytes were of cuboidal to cylindrical shape with oval nuclei resting on a continuous, slightly undulating BM. In pigmented animals, melanocytes located in the stratum basale were rarely observed. The stratum spinosum consisted of 3–6 progressively flattening keratinocyte layers. The stratum granulosum was a narrow but prominent cell rim filled with basophilic intracytoplasmic granules. It connected to a clearly distinct eosinophilic band without discernible cell contours and a well-pronounced multilayered stratum corneum conjunctum consisting of (mostly) flat, tightly connected, enucleated cornocytes. A proliferation rate of 8.15 ± 2.99% was assessed while only 1.04 ± 1.57% of basal keratinocytes stained TUNEL-positive. The epidermis was supported by a well-developed demnis (1524.0 ± 184.18 µm). A classical “epidermal trias” was not present due to physiologically absent apocrine sweat glands; HF accompanied by sebaceous glands and a few Musculi arrectores pilorum were dispersed throughout the dermis (no spatial pattern). Compound HF were rare; mostly individual HF with separate isthmus and outer root sheath (ORS) were seen. As expected in hairy skin, a pronounced papillary body of the stratum papillare interdigitating with the epidermis was not observed.
The epidermal thickness increased during culture to 92.55 ± 13.05 µm (day 10); the number of stratum corneum layers increased and individual corneocytes enlarged, while underlying epidermal layers flattened. On day 3, morphology was not markedly altered, the BM was still intact and only a few keratinocytes showed a brightened appearance and beginning vacuolization. The sebaceous glands appeared increasingly empty. Scattered pyknotic nuclei in ORS were observed. From day 5 onwards, basal keratinocytes became prolate and dyskeratotic; proceeding acantholysis, swelling of degenerating cells and orthokeratotic hyperkeratosis were seen. In the dermis, thinning ORS were noticed. On day 10, the BM showed a discontinuous appearance. All keratinocytes flattened, the stratum granulosum disappeared and the staining intensity of the eosinophilic band below the stratum corneum was reduced. Numerous pyknotic nuclei were observed in the epidermal layers as well as in the ORS.

During culture, the proliferation of basal keratinocytes decreased to 2.86 ± 3.60% (day 10). TUNEL-positive cells were most numerous on day 7 (day 7, 21.51 ± 26.82%; day 10, 19.96 ± 22.59%), displaying a high variability throughout the culture. Immunofluorescent images are shown in Figure 3.

Infection experiments
The histological findings in infected GPSE—illustrated in Figures 4 and 5—revealed a significant influence of *T. benhamiae* strains on the epidermal proliferative capacity. Already on day 3, the epidermis displayed a significantly higher thickness of 105.19 ± 20.14 µm (*P* ≤ 0.05) with increasing values towards the end of culture (day 5, 109.85 ± 12.36 µm, *P* < 0.001; day 7, 131.88 ± 30.2 µm, *P* < 0.05; day 10, 125.97 ± 23.77 µm, *P* < 0.05). On day 3, hyphae were found mostly in the stratum corneum disjunctum accompanied by strong keratinolysis. Otherwise, normal skin morphology was not altered. On day 3, the germinative strata of the epidermis were invaded as well. Occasionally, hyphae were also found at HF isthmi and dermal papillae. Dermatophyte keratinolysis of the stratum corneum and tricholysis continued, and accelerated acantholysis and orthokeratotic hyperkeratosis (+) due to reduced desquamation is observed. On (d) d7 and (e) d10, acantholysis proceeds (small black arrows) and the BM shows a partly loosened appearance. Basal and differentiating keratinocytes become prolate; the stratum granulosum and the eosinophilic band above it become indistinguishable (PAS reaction; bars 100 µm).
Pyknotic nuclei were observed. By no later than day 7, the BM was penetrated, dermis, HF and sebaceous glands were invaded and in some parts severely destroyed. Sixteen of the 20 *T. benhamiae* strains sporulated again: numerous globose, sessile microconidia throughout the whole tissue were found. On day 10, GPSE were completely disintegrated and overgrown; hard and soft keratins were equally digested by all applied *Trichophyton* strains.

All 20 *T. benhamiae* isolates caused similar histopathological alterations in the GPSE as described above and did not display any temporal or spatial invasion pattern. No differences between colony phenotypes (yellow vs white) or strains of different origin (human vs GP) were seen.

The proliferation rate of infected GPSE decreased significantly with time: on day 3, 3.58 ± 2.33% of basal keratinocytes showed positive Ki-67 staining. On days 5, 7 and 10, no positive signal was observed (days 5 and 7 were significantly different compared with uninfected control, *P* < 0.05). For days 3 and 5, elevated TUNEL values compared with uninfected controls were noticed (day 3, 6.73 ± 16.50%; d5, 33.03 ± 17.61%). As reported previously, TUNEL values for later time points could not be determined due to a severe cellular dissolution leaving individual basal cells indistinguishable.

### DISCUSSION

**Native GP skin**

Guinea pig skin comprises the typical mammalian skin structure. Slightly differing descriptions of, for example, stratum corneum architecture and HF arrangement are found. However, in our samples a well-developed stratum corneum conjunctum consisting of numerous layers of flat, neatly stacked corneocytes was observed. For hairless GP, compound HF with 3–13 individual follicular structures stretching halfway into the dermis are mentioned. In the present samples, neither an overall spatial pattern nor predominantly compound HF were observed; only in a few animals HF were arranged in parallel lines, as described previously.

The most striking peculiarity seen in our samples of GP skin was a thin eosinophilic layer without discernible cell boundaries between the strata granulosum and corneum. This distinct layer of unknown composition is also seen after Ladewig staining and PAS reaction (standard protocols, Fig. S1) but was not mentioned by many authors even though it is as obvious in their figures as it is in ours. Only Spearman and Riley speculate that it may correspond to the compressed basal part of the “horny layer” and state that it must not be confused with the stratum lucidum. Although there is no official term for this...
Figure 4. *Trichophyton benhamiae* strains invade and destroy guinea pig skin explants (GPSE) during culture. (a–d) Periodic acid–Schiff (PAS)-stained sections of infected GPSE during the course of culture illustrate massive growth of fungal tissue. (a) On day (d) 3, very few hyphae are found in the stratum corneum (large black arrows). (b) On d5, the germinative strata of the epidermis are invaded as well. Keratinolysis of the stratum corneum disjunctum continues. Note hyphae at the hair follicles’ isthmus (*). (c) Ongoing dermatophyte keratinolysis of epidermal strata is seen, the basement membrane (BM) and deeper structures are not only invaded but in some parts severely destroyed by no later than d7. Microconidia (small black arrows) as well as hyphae inside hair and outer root sheath (large black arrows) are observed. (d) On culture d10, all epidermal structures, the BM and the dermis are completely disintegrated and overgrown by fungal elements. Note hyphae inside hair (tricholysis; large black arrow) and numerous microconidia (small black arrows) dispersed throughout the whole tissue (PAS reaction; bars = 100 µm). (e–h) Representative immunofluorescence stainings of infected GPSE with anti-*Trichophyton* fluorescein isothiocyanate (depicted in green) illustrate the same process of invasion and destruction during culture as described above. Note the massive increase of mycelium towards the end of culture. (g) Globose, sessile microconidia (small white arrows) are found in clusters from d7 onward (nuclear counterstain with bisBenzimide 33342 [Hoechst] is depicted in blue; bars = 100 µm). [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 5. Quantitative evaluation of guinea pig skin explants (GPSE) over the course of culture reveals a significant influence of *Trichophyton benhamiae* on epidermal thickness and proliferative ability. (a) Comparison of epidermal thickness during culture of uninfected (black) versus infected (gray) GPSE. The epidermis of uninfected GPSE increases from 57.39 ± 16.27 µm (native) to 92.55 ± 13.05 µm (day [d]10). The epidermis of infected GPSE is significantly thicker compared with uninfected controls already on d3 (d3: 105.19 ± 20.14 µm; *P* ≤ 0.05; **P** ≤ 0.001). (b) The percentage of Ki-67-positive cells in uninfected GPSE decreases during culture from 8.15 ± 2.99% (native) to 2.86 ± 3.60% (d10). In infected GPSE, basal keratinocytes halted proliferation completely on d5 (infected d5/d7/d10: 0%; uninfected control d5: 3.09 ± 2.32%; *P* ≤ 0.05). (c) The percentage of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in uninfected GPSE increases from 1.04 ± 1.58% (native) to 19.97 ± 22.6% (d10) with its maximum on d7 of culture (21.52 ± 26.82%) and is highly variable. Due to severe cellular dissolution leaving basal cells indistinguishable, TUNEL-positive cells could not be determined after d5. PAS, periodic acid–Schiff.
the authors describe an increasing number of sub-stratum corneum layers with increasing hyperkeratosis as seen in our observation. The penetration of epidermal tissue by Trichophyton equinum and a subsequent hyperkeratosis of human stratum corneum was described comparable with our observation. The invasion of T. mentagrophytes hyphae into human full-thickness SE is characterized as undirected and meandering as it is seen in our model. Dermatophyte colonization of the hair shaft and cortex after animal infection were reported but no tricholysis as seen in our GPSE. Certainly, the extent of tissue destruction in the later phase of culture is not as severe in immunocompetent patients where host immune defense mechanisms are still intact. However, this model depicts deep dermal or disseminated dermatophytooses which are reported in immunocompromised or -suppressed patients and underline its validity. Interestingly, three of the 20 fungal isolates (one strain of human origin and two isolated from GP) changed their physical appearance from yellow to white or vice versa once applied to the medium, we would like to draw attention to it because it was seen in all our samples after all histological staining methods. Moreover, it tends to bind antibodies unspecifically (Fig. S1) and, hence, forces attention during immunohistochemical analysis.

Guinea pig epidermis thickness varies from 20 to 100 μm; the figure found in our study lies within this range but its comparability is immensely hampered by different means of measuring and a high intra- and interindividual variability. To our knowledge, the proliferation marker Ki-67 is not described for GP skin. Few studies assessed skin viability with other markers, namely proliferating cell nuclear antigen (PCNA) and phosphohistone H3 (pHH3). We tested these markers in immunofluorescent stainings (PCNA: MCA1558, mouse monoclonal; Bio-Rad Laboratories, Munich, Germany; pHH3: S10, no. A301-844A-T, rabbit polyclonal; Bethyl Laboratories by Bio-Rad Laboratories, Munich, Germany; pHH3: S10, no. A301-844A-T, rabbit polyclonal; Linaris) but neither yielded conclusive results. The number of PCNA-positive cells varied significantly depending on the method of specimen fixation and antigen retrieval as reported in other studies. Agreeing with others, we detected an implausibly high amount of pHH3-positive epidermal cells. Hence, these markers were abolished and we analyzed the Ki-67 antigen using three different antibodies. The first one (PAK8956, rabbit polyclonal; Linaris) did not yield clear intranuclear signals and the second (clone Ki-67-P, no. DIA-670-P05, mouse monoclonal; Dianova) was not cross-reactive at all. Data obtained with the anti-Ki-67 FITC antibody from Thermo Fisher Scientific were in good accordance with the labeling index of GP epidermis previously determined (6.13 ± 0.51%). In our study, no TUNEL signals were found in native GP skin which is supported by data for hairless GP skin and human skin, respectively.

### Explant culture (uninfected)

As reported before, our pre-experiments proved autologous serum most potent in maintaining GPSE during tissue culture. The morphological evaluation of GPSE cultured at 30°C confirmed previous findings concerning good SE viability at lower culture temperatures.

Cultured GPSE maintained their general morphological integrity up to day 5. Epidermal thickening was also observed in human abdominal skin cultures. In contrast to our findings, the authors describe an increasing number of sub-stratum corneum layers up to day 12. Nevertheless, a continuous thinning of the sub-stratum corneum layers with increasing hyperkeratosis as seen in our GPSE is described for human scalp skin cultures. The observed hyperkeratosis cannot be attributed to hyperproliferation given the results from the Ki-67 immunostaining. However, reduced desquamation and altered keratinocyte differentiation as suggested elsewhere probably contribute to this phenomenon. Proliferation rates of less than 1% on day 5 and 7% on day 12 are found for human SE. This is in good accordance with our findings of approximately 2.8% of proliferating cells (day 10). The number of TUNEL-positive cells increased moderately during culture. Indeed, the TUNEL assay, as every method, is subject to inherent technical limitations; for example, the discrimination between different types of cell death, the labeling of fragmented DNA unrelated to cell death and the dependency on pretreatment have been discussed controversially ever since its first description. However, it is a well-established and verifiable assay if: (i) the necessary controls are included; (ii) a thorough histological evaluation of the examined tissue is carried out; and (iii) a second technique is simultaneously performed to critically compare the obtained results. For the latter, we chose the immunohistochemical detection of activated caspase 3 (Cas3; no. ABIN462092, rabbit polyclonal; antibodies-online, Aachen, Germany). Coinciding positive signals for TUNEL and Cas3 in cultured GPSE sections underlining the validity of the conducted TUNEL assay were found.

### Infection studies

Inoculation with T. benhamiae strains led to a successful invasion of all GPSE. Moreover, the relatively low number of 10² CFU was sufficient to induce infection. Reports of different in vivo experiments where more than 10⁴ spores were needed to achieve infection of all experimental animals are found. Indeed, in an animal study in 1976, infection was induced using 10³ and 10⁷ spores in 87.5% and 75% of test animals, respectively, but only when the animals’ skin was thoroughly shaved and the infection site was protected with several layers of gauze, Teflon, foam rubber, Elastoplast and paper tape. In fact, it is hypothesized that infection does not occur in healthy, intact skin, justifying this elaborate infection implementation. However, the easily achieved and mild compromising effect on the skin barrier of disinfecting alcohol used in our study seems sufficient to initialize dermatophyte infection in the GPSE model. The penetration of epidermal tissue by Trichophyton equinum and a subsequent hyperkeratosis of human stratum corneum was described comparable with our observation. The invasion of T. mentagrophytes hyphae into human full-thickness SE is characterized as undirected and meandering as it is seen in our model. Dermatophyte colonization of the hair shaft and cortex after animal infection were reported but no tricholysis as seen in our GPSE. Certainly, the extent of tissue destruction in the later phase of culture is not as severe in immunocompetent patients where host immune defense mechanisms are still intact. However, this model depicts deep dermal or disseminated dermatophytooses which are reported in immunocompromised or -suppressed patients and underline its validity.
GPSE. The association to a colony phenotype is based on specific DNA sequences in the ITS region.12 Because the difference between these phenotypes depends on multiple substituted nucleotides, we would assume spontaneous mutation. Differences between these phenotypes depend on multiple subtypes.12,41,42 However, because conventional identification of dermatophytes in routine diagnostic laboratories still relies on clinical features and culture morphology, we would like to raise awareness of this widely observed pleomorphism.12,41,42

In conclusion, the presented data provides reliable information for intra- and interspecies comparisons concerning skin morphology and long-term skin culture. The newly established model represents a reliable and close-to-reality experimental system for dermatophytosis research, not only with Trichophyton spp. but also with other anthropophilic, zoophilic and geophilic dermatophytes. Moreover, the multi-well approach enabling broad scale use may also be useful in the search for new antifungal therapeutics.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** In native guinea pig epidermis, a continuous eosinophilic layer without discernible cell boundaries is recognized between the strata granulosum and corneum.

**Figure S2.** Double staining with anti-caspase 3 (Cas3) and terminal deoxynucleotidyl transferase dUTP nick end labeling assay reveals co-localization of apoptotic signals in cultured guinea pig skin explants (GPSE).

**Appendix S1.** Production and purification of fluorescein-conjugated antibodies against *Trichophyton benhamiae*