Models of oral and vaginal candidiasis based on *in vitro* reconstituted human epithelia

Martin Schaller[1], Katherina Zakikhany[2], Julian R Naglik[3], Günther Weindl[1]& Bernhard Hube[2,4]

[1]Department of Dermatology, Eberhard Karls University, Liebermeisterstr, 25, D-72076 Tuebingen, Germany. [2]Robert Koch-Institute, FG16, Nordufer 20, D-13353 Berlin, Germany. [3]King's College London, Department of Oral Immunology, King's College London Dental Institute, London SE1 9RT, UK. [4]Friedrich Schiller University Jena and Department of Microbial Pathogenicity, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knoell Institute, 07745 Jena, Beutenbergstrasse 11a, Germany. Correspondence should be addressed to M.S. (martin.schaller@med.uni-tuebingen.de)

Published online 18 January 2007; doi:10.1038/nprot.2006.474

This protocol describes the setup, maintenance and characteristics of models of epithelial *Candida* infections based on well-established three-dimensional organotypic tissues of human oral and vaginal mucosa. Infection experiments are highly reproducible and can be used for the direct analysis of pathogen–epithelial cell interactions. This allows detailed investigations of *Candida albicans* wild type or mutant strain interaction with epithelial tissue or the evaluation of the host immune response using histological, biochemical and molecular methods. As such, the models can be utilized as a tool to investigate cellular interactions or protein and gene expression that are not complicated by non-epithelial factors. To study the impact of innate immunity or the antifungal activity of natural and non-natural compounds, the mucosal infection models can be supplemented with immune cells, antimicrobial agents or probiotic bacteria. The model requires at least 3 days to be established and can be maintained thereafter for 2–4 days.

INTRODUCTION

Investigating human mucosal infections

The mucosal epithelium is of central importance in host defense and immune surveillance, as it is the primary cell layer that initially encounters environmental microbes with pathogenic potential. Other commensal microorganisms are regular inhabitants and harmless colonizers of mucosal surfaces with often important roles for the protection and immune regulation of epithelial tissues. Both types of microbes interact with epithelial cells, leading to either a favorable coexistence (commensal) or a breach of the mucosal barrier and subsequent cell injury (pathogen)[1]. Barrier function alone is usually adequate to restrain commensal microbes, but is often insufficient to protect against microbial pathogens. Accordingly, the oral epithelium is able to secrete a variety of defense effector molecules and to orchestrate an immune inflammatory response to activate myeloid cells in the submucosal layers to clear the invading pathogens[2,3].

Epithelial tissue interaction and penetration is central in understanding pathogenic effects of infectious agents. However, the testing protocols have traditionally involved animals or animal cell cultures as an *in vivo* model of infection. The relevance of these approaches is often limited as human-specific characteristics and end points related to human pathological processes are commonly overlooked. Human cell lines that closely parallel the *in vivo* situation and allow studies of relevant physiological functions and microbial responses are therefore highly desirable. Recent advances in tissue engineering and molecular and cellular biology have elucidated ways to construct tissues using carefully designed scaffolds on which cells can grow and differentiate. These bioengineered tissues can be manipulated to express phenotypic surface markers and to produce immune modulatory molecules.

The complex interactions between microbial virulence attributes of a pathogen and defense mechanisms of the human host are of particular interest in the case of *Candida* species, which can either colonize asymptomatically as commensals or cause direct immunopathology as pathogens. Given that *Candida* is the most common fungal pathogen of man, models of mucosal *Candida* infections (candidiasis) that closely parallel the *in vivo* situation and allow the study of relevant physiological functions are particularly beneficial. A number of epithelial tissue models have been utilized to study *C. albicans* infections[4–6]; however, in this protocol, we will focus on the use of well-established three-dimensional organotypic epithelial models of human oral and vaginal mucosa provided by SkinEthic Laboratories. SkinEthic is a leading tissue production company specialized in the reconstruction of human epidermal and epithelial tissues for *in vitro* test applications for the pharmaceutical, chemical, academic and consumer product industry (http://www.skinethic.com).

The epithelial cells are seeded on inert filter substrates that are lifted to the air–liquid interface in a humidified-air incubator. A fully defined nutrient medium feeds the basal cells through the filter substratum. After 5 days, a stratified epithelium is formed that closely resembles human epithelium *in vivo*. As the oral and vaginal epithelial tissues are reconstituted in a physiologically natural environment and on a chemically defined medium, they express all natural major markers of the epithelial basement membrane and of epithelial differentiation and behave like human *in vivo* epithelium when treated with pharmacologically active but also irritating products. The models also exhibit tissue repair mechanisms that reflect the natural wound healing processes *in vivo*.

Moreover, the totally defined and serum-free culture environment allows the detection of very small quantities of inflammatory mediators, cytokines or growth factors secreted by the epithelium in response to topical application of test substances to be assessed in a very reproducible way. Biological controls before use include guaranteed absence of HIV-integrated pro-viral DNA, hepatitis C viral DNA, cytomegalovirus DNA, mycoplasma, hepatitis B antigen HBs and bacteria and fungi (http://www.skinethic.com).

Use of the reconstituted human epithelia as a model to study *C. albicans* pathogenicity on mucosal surfaces

The use of the SkinEthic reconstituted human epithelia (RHE) models was pioneered to study *C. albicans* infection and specifically the role of the secreted aspartyl proteinases (SAP) gene family in *C. albicans* pathogenicity[7]. The *SAP* family was shown to be differentially regulated during RHE-*C. albicans* infections. By using a combination of *SAP*-disrupted mutants and proteinase inhibitors, including the HIV-proteinase inhibitors saquinavir or indinavir, it was demonstrated that certain members of the Sap subfamily were responsible for inducing tissue damage (infection) in the oral or vaginal models[8–10].

In recent years, the RHE models have been widely used to analyze the expression patterns of many *C. albicans* genes and to evaluate the consequence of gene disruption on pathogenicity and environmental sensing[11–17]. Recently, we have analyzed the genome-wide expression pattern of *C. albicans* during RHE infection and have compared these data to *in vivo* transcriptional profiles from patient samples (Zakikhany, Naglik, Schmidt-Westhausen, Holland, Schaller & Hube, unpublished data). These *in vivo* transcript profiling data confirmed trends observed during experimental oral RHE infections, thus demonstrating the value of the models as surrogates of *in vivo* infections.

Use of RHE to study the epithelial immune response against *C. albicans*

During *C. albicans* infection of the oral RHE, using real-time RT–PCR, a significant increase in expression of interleukin (IL)-1α, IL-1β, tumor necrosis factor-α, granulocyte macrophage-colony stimulating factor and IL-8 was revealed[18]. Protein studies confirmed the gene expression data and supported the hypothesis that *C. albicans* infection induces an epithelial cytokine pattern that may favor a chemotactic and Th-1-type immune response and an environmental switch from an anti- to a pro-inflammatory milieu. Addition of the aspartyl proteinase inhibitor pepstatin A strongly reduced the cytokine response, and mutants lacking *SAP* genes caused reduced tissue damage and had a significantly reduced potential to stimulate cytokine expression in the vaginal model[19]. These observations support the hypothesis of an active host–fungus interaction at the epithelial surface, which comprises a dynamic adaptation of both the host and *C. albicans* during the transition from commensalism to parasitism.

Supplementation of the *C. albicans* infection models with host cells

A protective anti-*Candida* Th1-type epithelial response is likely to contribute to the recruitment of polymorphonuclear cells (PMNs) and lymphocytes to the site of mucosal infection to deal with the fungus. To test this hypothesis in the oral RHE model, a PMN supplementation assay to study the effect of PMNs during experimental oral candidiasis was established[20]. Infection of RHE with *C. albicans* alone induced IL-1α, IL-1β and tumor necrosis factor-α, with strong upregulation of granulocyte macrophage-colony stimulating factor and IL-8, which was directly correlated with chemoattraction of PMNs to the site of infection. The addition of PMNs enhanced production of the majority of these cytokines. Notably, *C. albicans*-induced tissue damage was significantly reduced when PMNs migrated through a modified perforated basal polycarbonate filter or when PMNs were applied to the apical epithelial surface. Interestingly, this protection of the epithelial tissue was also observed when PMNs were placed on the basal side of non-perforated filters, which prevents cell–cell interactions and cell migration but allows free passage of soluble factors, that is, cytokines[20]. Finally, the addition of saliva to the surface of the oral RHE or the addition of Lactobacilli to the vaginal model in the absence of PMNs also inhibits the *C. albicans* infection phenotype (unpublished results).

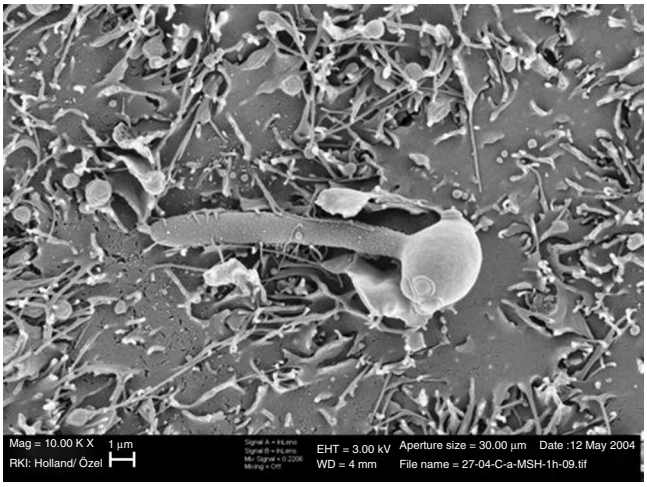

Figure 1 | Scanning electron micrograph of *C. albicans* interacting with the oral RHE. After 1 h of RHE inoculation, fungal hyphal cells were located on the surface of the epithelial tissue. Epithelial cell protrusions grew toward and surrounded the elongated germ tube. Picture by Gudrun Holland and Muhsin Özel, Robert Koch-Institute, Berlin.

Tools to investigate RHE infections

Numerous protocols have been successfully used to observe the pathogenesis of experimental mucosal infection processes. Host–pathogen interactions on the cellular level can be monitored by scanning (**Fig. 1**) and transmission electron microscopy[8,9]. Epithelial damage can be visualized by histological analysis of the embedded RHE[7–9,20–22] and quantified by the extracellular lactate dehydrogenase (LDH) activity in the culture medium released by damaged epithelial cells[9,16,20]. Furthermore, immunoelectron microscopy[8,9,16,21], confocal laser microscopy (unpublished results), fluorescence-activated cell sorting[19] and ELISA[18] can be used to measure and localize protein expression, and RT–PCR[7–9,18–20,23] and microarray-based transcriptional profiling (unpublished results) for gene expression studies.

The overall RHE infection procedure is outlined in a flowchart form (**Fig. 2**).

MATERIALS

REAGENTS

- ▲ CRITICAL All materials must be sterile and maintained under sterile conditions
- Fetal bovine serum, certified, heat-inactivated (Invitrogen Gibco, cat. no. 10082-139)
- Giemsa stain (Carl Roth, cat. no. T862.1): for a working solution dilute 1:20 in distilled water
- HiPerFect transfection reagent (Qiagen, cat. no. 301705)
- Histopaque-1077 (Sigma, cat. no. 11191)
- Histopaque-1119 (Sigma, cat. no. 10771)

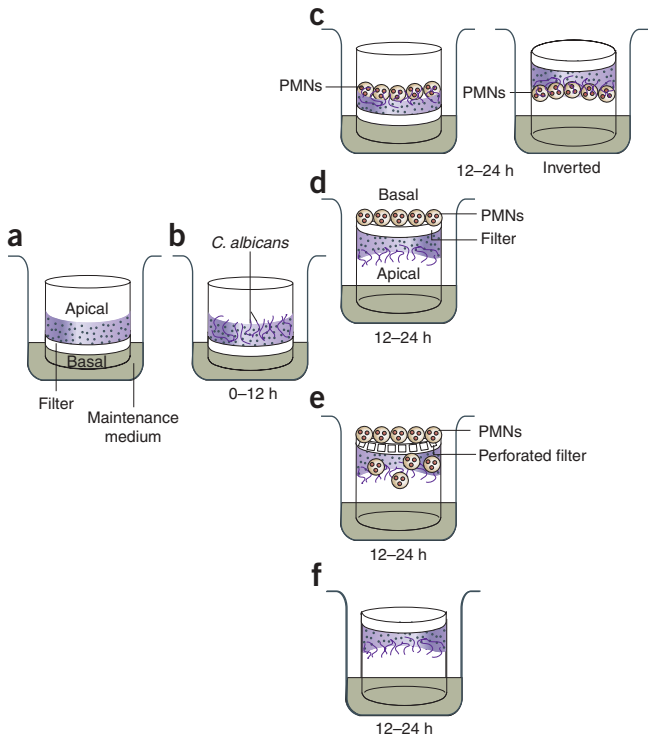

Figure 2 | Schematic diagram of the experimental design. (**a**) RHE on a microporous polycarbonate filter fed by a fully defined nutrient medium through the filter substratum. (**b**) Infection of RHE by *C. albicans*. (**c**–**f**) The model of oral candidiasis was supplemented with PMNs 6 or 12 h after inoculation in three different ways. (**c**) First, PMNs (10^6 cells in 50 µl PBS) were added directly to the apical epithelial layers of the preinfected model. Six hours after PMN supplementation, the samples were inverted to mimic experimental conditions of the following samples. (**d**) Second, PMNs were added to the basal side of the polycarbonate filter. PMNs were not able to migrate through this microporous layer. (**e**) Third, the polycarbonate filters were perforated with a thin needle before addition of the PMNs to enable transepithelial migration. All samples were incubated for further 6 or 12 h after addition of PMNs. Culture medium was applied to the basal side of the filter every 60 min to feed the cells. Histological sections and LDH analysis of the supplemented samples were compared to that of the non-supplemented inverted model of oral candidiasis (**f**). Reprinted with permission from Schaller *et al.*[20]

- *Lactobacillus acidophilus* cells (ATCC)
- May–Grunwald stain (Carl Roth, cat. no. T863.1)
- Methanol (Carl Roth, cat. no. 7342.1)
- Phosphate-buffered saline, Dulbecco's-PBS (1×), liquid (Invitrogen Gibco, cat. no. 14190-094)
- RPMI 1640 medium (1×), liquid (Invitrogen Gibco, cat. no. 21875-042)
- Sabouraud dextrose agar (Difco, cat. no. 211584)
- SkinEthic Growth Medium (small bottle, 125 ml) (SkinEthic, cat. no. SGM/S) ▲ CRITICAL Can only be purchased together with SkinEthic RHE ▲ CRITICAL Store in the dark
- SkinEthic Maintenance Medium (small bottle, 125 ml) (SkinEthic, cat. no. SMM/S). Note that the pH of the maintenance medium for vaginal RHE is higher than the natural pH of vaginal tissue ▲ CRITICAL Can only be purchased together with SkinEthic RHE ▲ CRITICAL Store in the dark
- SkinEthic Reconstituted Human Oral Epithelium, small, age day 5 (SkinEthic, cat. no. RHO/S/5)
- SkinEthic Reconstituted Human Vaginal Epithelium, small, age day 5 (SkinEthic, cat. no. RHV/S/5)
- Sodium chloride (NaCl) solution, 0.2% (w/v, Sigma or equivalent)
- NaCl solution, 0.9% (w/v, Sigma or equivalent)
- NaCl solution, 1.6% (w/v, Sigma or equivalent)
- Trypan blue stain, 0.4% (w/v, Invitrogen Gibco, cat. no. 15250-061)
- Ultrapure water (e.g., MilliQ; Millipore)
- YPD (yeast, peptone, dextrose) broth (Difco, cat. no. 242820)

EQUIPMENT

- CO_2 incubator
- Counting chamber (Neubauer)
- Incubation shaker, with temperature control
- Laminar-air flow workstation
- Light microscope
- Microscope slides
- Refrigerated microcentrifuge
- Surgical disposable scalpels, sterile (Feather; nos. 11 and 21)
- Table-top centrifuge
- Thin needle, sterile
- Six-well tissue-culture plate, sterile with lid (Greiner Bio-one, cat. no. 657160 or equivalent)
- Tweezers, sterile

PROCEDURE

Semisynchronization of fungal cells ● TIMING start 2 days before planned delivery of the RHE

1| Cultivate yeast cells for 24 h at 37 °C on YPD agar in an incubator.

2| Take a sample of the culture, suspend it in 5 ml 0.9% NaCl solution and in a 15-ml sterile Falcon tube and collect the cells by centrifugation at 500*g* for 10 min at 25 °C (room temperature). Wash cells twice by discarding the supernatant, resuspending the pellet in 5 ml sterile 0.9% NaCl solution and centrifuging at 500*g* for 10 min at 25 °C. Discard the supernatant, resuspend the pellet in 5 ml sterile ultrapure water and use a Neubauer chamber to assess the number of *C. albicans* cells. Suspend a sample of approximately 2×10^5 cells in 10 ml YPD. Culture the suspension for 16 h at 25 °C with orbital shaking (150 r.p.m.).

3| Repeat washing procedure from Step 2. Take a suspension of 4×10^6 cells and incubate with shaking (150 r.p.m.) in 10 ml fresh YPD medium for 24 h at 37 °C. After washing three times with 10 ml PBS for 10 min at 500*g* and 25 °C, adjust the final inoculum to the desired density (4×10^7 cells per ml) with PBS solution.
▲ CRITICAL STEP This procedure is useful to ensure that all *C. albicans* cells are in a similar growth phase when added to the RHE surface. This is important to get reproducible results.

Preparation and preincubation of the oral or vaginal RHE tissue culture ● TIMING start 1 day before inoculation

4| Upon arrival, carefully remove the multiwell plate from the aluminum foil packaging. Open the 24-well plate under a sterile airflow and remove the sterile filter paper.

▲ CRITICAL STEP It is very important to ask SkinEthic that the RHE and all culture media should be prepared without antibiotics and antimycotics.

5| With tweezers, carefully take out each 0.5 cm^2 insert containing the epithelial tissue, rapidly remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on the sterile filter paper, and immediately place in a 35 mm ∅ culture dish (six-well plate) previously filled with 1 ml of SkinEthic Maintenance Medium (at room temperature). Note that if larger amounts of tissue are needed, RHE tissue with 4 cm^2 can be used and cultivated in 2 ml of Maintenance Medium. If smaller amounts of tissue are required, then specialized high-throughput 24-well (0.33 cm^2) or 96-well (0.1 cm^2) RHE tissue plates can be utilized and cultivated in 0.5 or 0.1 ml of Maintenance Medium, respectively.
▲ CRITICAL STEP Make sure that the level of the medium outside of the RHE insert does not extend above the epithelial surface.
▲ CRITICAL STEP Act quickly as the epithelial cultures dry out rapidly when not in contact with medium. Make sure that no air bubbles are formed underneath the insert, as this might prevent the tissues being fed by the medium.

6| Place the 35 mm ∅ culture dishes (six-well plates) in the incubator at 37 °C, 5% CO_2 and saturated humidity for 24 h.
▲ CRITICAL STEP SkinEthic Maintenance or Growth Medium should be stored at 4 °C in the dark. Use the media at room temperature, but do not preheat the media.

Infection of oral or vaginal RHE with *C. albicans*

7| For inoculation of the 0.5 cm^2 RHE, add *C. albicans* cells (2×10^6) in 50 µl PBS to each insert. Add 50 µl PBS alone to uninoculated controls.
▲ CRITICAL STEP Make sure that the inocula are equally distributed on the epithelial surface.

8| Incubate the inoculated and uninoculated cultures at 37 °C with 5% CO_2 at 100% humidity for the desired test period (usually a maximum of 48 h).

9| Using SkinEthic Maintenance Medium, change the medium after 24 h if cultures are maintained for up to 48 h only. Using SkinEthic Growth Medium, if cultures are maintained for more than 48 h, change medium daily (1 ml of medium per tissue insert per day).
▲ CRITICAL STEP For coincubation experiments with inhibitors or antimicrobial substances, make sure that both the inoculum and the maintenance medium contain the desired concentration of the agent.

10| The RHE can be further manipulated or analyzed in a number of ways. If comparing epithelial damage by *C. albicans* mutants, follow option A. For supplementation with PMNs and to perform a transepithelial migration assay, follow option B. For supplementation with bacteria, follow option C. For other options not discussed in detail in this protocol, see **Box 1**. If you wish to knockdown RHE gene expression using RNA interference prior to infection (steps 7 to 9), a more detailed description is provided in **Box 2**.

(A) Comparing epithelial damage

(i) Oral or vaginal RHE is widely used for comparing epithelial damage by *C. albicans* mutants with the corresponding wild-type strains. To investigate the consequence of gene disruption of putative virulence factors on tissue damage, use a test period of 12 and 24 h.
▲ CRITICAL STEP A wider range of incubation times can be found in the literature. Tissue destruction appears to progress differentially depending on the protocol used.
▲ CRITICAL STEP Always include uninoculated controls to ensure that the morphology of the RHE was normal before inoculation.

BOX 1 | FURTHER MODIFICATIONS THAT CAN BE MADE TO THE RHE MODEL SYSTEM

Several further modifications to the RHE model system are possible to analyze certain aspects of mucosal infections, host–pathogen interactions or antimicrobial agents. These include:

- using membranes to prevent direct contact of the pathogen to epithelial cells
- supplementation with saliva or components of saliva
- supplementation with antimicrobial substances
- supplementation with antimicrobial peptides
- supplementation of host immune cells (T cells, dendritic cells)
- supplementation of cytokines
- modification of temperature or pH
- supplementation of iron chelators, antifungal, epithelial cell-related inhibitors
- infection with genetically, chemically or otherwise modified microorganisms
- knockdown of RHE gene expression using RNA interference
- preventing specific protein–protein interactions with blocking antibodies

(B) Supplementation of the oral or vaginal RHE with PMNs and transepithelial migration assay

(i) Isolate PMNs from heparinized whole blood using Histopaque-1119 in combination with Histopaque-1077 (Sigma) according to the manufacturer's protocol.

(ii) Wash the PMNs recovered from the interface in 10 ml PBS by centrifugation for 10 min at 200*g* (at room temperature) and aspirate the supernatant. To remove residual erythrocytes, subject cells to hypotonic lysis by resuspending the pellet in 5 ml ice-cold 0.2% NaCl. After exactly 30 s, restore isotonicity by adding 5 ml of 1.6% NaCl and centrifuge. Remove the supernatant, repeat washing step with 10 ml PBS two times and resuspend the PMNs at a concentration of 4×10^7 per ml in RPMI 1640 medium (Sigma) in the presence of 10% (v/v) FCS.
▲ CRITICAL STEP The 30 s limit is essential because a more prolonged period of hypotonicity will result in neutrophil damage.

(iii) Use Giemsa staining and light microscopy to ensure that a pure population of PMNs ($>$ 90% purity) with typical morphology has been isolated. Smear cell preparation on standard microscope slides and air dry. Fix cells with methanol for 1 min and stain with May–Grunwald solution for 4 min. Rinse with water and add Giemsa stain for 4 min. After washing slides with water, air dry the slides and examine stained slides under a light microscope.

(iv) Assess the numbers of vital and non-vital leukocytes per sample using vital staining by the trypan blue dye exclusion method. The viability should be $\geq$95% in all experiments.

(v) Supplementation of the preinfected RHE with PMNs can be performed in three ways (**Fig. 2c–e**)[20]. For option 1, add PMNs (2×10^6 cells in 50 µl RPMI 1640 supplemented with 10% FCS) directly to the apical epithelial layers of the model 6 or 12 h after inoculation with *C. albicans*. For option 2, invert the infected model 6 or 12 h after infection with *C. albicans* using two tweezers and add the PMNs to the basal side of the polycarbonate filter. In these samples, the filter prevents cell–cell contact and PMN migration, but allows soluble factors to pass through, that is, cytokines. For option 3, perforate the polycarbonate filter with a thin needle before addition of the host cells to enable transepithelial migration of PMNs.
▲ CRITICAL STEP As a control, add PMNs to an uninfected RHE. Additional controls should include uninfected and infected samples that are inverted and supplemented with RPMI 1640 medium/10% FCS.

(vi) Invert and incubate all samples for a further 6 or 12 h after addition of the immune cells at 37 °C with 5% CO_2 at 100% humidity.
▲ CRITICAL STEP Carefully place inverted samples in the middle of each well and ensure that the sample is not moving toward the edge of the well, which may cause loss of medium. Make sure that the maintenance medium is added to the basal side of the inverted samples every 60 min to feed the keratinocytes/PMNs.

(C) Supplementation with bacteria

(i) Supplementation of the vaginal RHE with probiotic bacteria can be performed in three ways. For the first method, inoculate the vaginal RHE with *C. albicans* (2×10^6) together with *L. acidophilus* (10^7 cells) in 50 µl PBS for 12 and 24 h at 37 °C with 5% CO_2 at 100% humidity. For the second method, add Lactobacilli (10^7 cells) in 50 µl PBS directly to the apical epithelial layers of the model 6 or 12 h after inoculation with *C. albicans*. Incubate for a further 6 or 12 h. For the final method, preincubate the uninfected RHE with *L. acidophilus* (10^7 cells) in 50 µl PBS for 2 h before inoculation with *C. albicans*. Incubate the samples for 12 or 24 h at 37 °C with 5% CO_2 at 100% humidity.

BOX 2 | KNOCKDOWN OF RHE GENE EXPRESSION USING RNA INTERFERENCE

(i) Prepare transfection reagent and siRNA solutions according to the manufacturer's protocol. We have successfully used HiPerfect Transfection Reagent (Qiagen) for a transfection period of 24 h and achieved 60–75% efficiency at siRNA concentrations of 10 nM (unpublished results). For a general protocol on RNA interference please refer to ref. 24.
▲ CRITICAL STEP Using fluorescently labeled control siRNA, we observe that only the uppermost apical epithelial cell layers appear to be successfully transfected whereas the remaining layers of the RHE show no fluorescence. In addition, siRNA molecules do not appear to pass through the polycarbonate filter from the basal side (unpublished observations). However, in the experimental system, there may not be a requirement for a complete knockdown of gene expression throughout the RHE, as only the uppermost cell layers are directly interacting with the fungal cells in the early phase of infection.

(ii) Incubate the RHE with 50 µl of the transfection complexes (diluted in PBS) for an appropriate period of time at 37 °C with 5% CO_2 at 100% humidity.
▲ CRITICAL STEP Make sure that the transfection complexes are equally distributed on the epithelial surface.
▲ CRITICAL STEP The optimal incubation time for gene silencing analysis depends on the RHE model, the gene targeted and the method of analysis. The amount of transfection reagent and siRNA required for optimal performance may vary, depending on the RHE model and gene target. We recommend carrying out empirical testing by performing time-course experiments and vary amounts of transfection reagent and siRNA. Real-time RT–PCR and confocal fluorescence microscopy can be used to assess transfection and knockdown of gene expression.

(iii) Remove the transfection solution and wash apical epithelial layers with 50 µl PBS. Change the maintenance medium and proceed to inoculation with *C. albicans* cells according to the standard procedure.

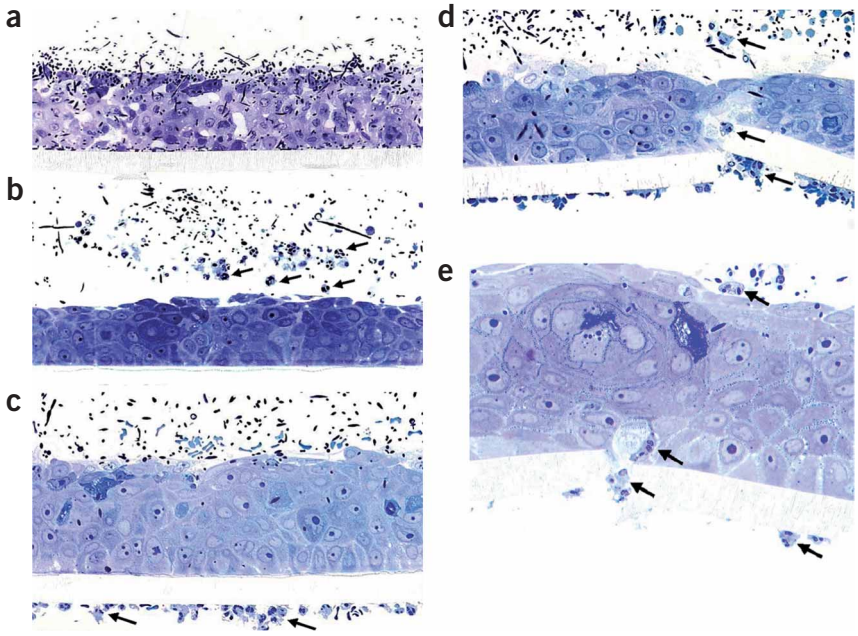

Figure 3 | Light micrographs of oral RHE 24 h after inoculation with *C. albicans* SC5314 in the absence and presence of PMNs. (**a**) *C. albicans* invasion of all epithelial layers by 24 h with extensive edema and vacuolization in the absence of PMNs (see also **Fig. 1b**). (**b**) Strongly reduced virulence phenotype of SC5314 when PMNs (arrows) were added to the apical epithelial layer 12 h after inoculation (see also **Fig. 1c**). (**c**) Firm attachment of PMNs (arrows) added to the basal side of the non-perforated filter 12 h after inoculation of an inverted culture resulting in a protective effect similar to **b** (see also **Fig. 1d**). (**d**) Attenuated virulence phenotype of *C. albicans* was also seen when PMNs (arrows) were added to preinfected and inverted cultures after 12 h to the basal side of a perforated filter enabling transepithelial migration of the immune cells through the infected epithelium (see also **Fig. 1e**). (**e**) Chemoattraction and transepithelial migration of PMNs (arrows) through a pore of the polycarbonate layer of the RHE into epithelial layers and to the surface of the mucosa (see also **Fig. 1f**). Reprinted with permission from Schaller *et al.*[20]

Collection of the culture medium and removal and dissection of the epithelia

11| At selected time points after establishment of the oral or vaginal model, you can remove the culture medium and/or dissect the epithelium.

12| Transfer culture medium to 1.5-ml microcentrifuge tubes and centrifuge at 10,000*g* for 5 min at 4 °C. Aliquot the supernatants into new tubes and store samples for subsequent determination of epithelial cell damage by LDH assays and epithelial cytokine secretion by ELISA.

■ **PAUSE POINT** The media can be stored at 2–8 °C for up to 1 week for analysis of LDH activity and at −70 °C for up to 1 year for ELISA.

13| Carefully take out each 0.5 cm^2 insert, invert and place it on a culture dish.

14| If necessary, gently remove the PMNs, which have been applied to the basal side of the filter, using a scalpel (no. 21) for separate analysis.

15| Cut out the polycarbonate filter with the attached epithelial tissue from the basal side of the plastic insert with a sharp scalpel (no. 11).

16| Place the removed tissue (epithelial side up) on a culture dish and use a disposable scalpel (no. 21) to cut the RHE into several sections for separate analysis (e.g., histology, confocal laser microscopy, electron microscopy) if necessary.

▲ **CRITICAL STEP** Epithelial cell damage by fungal cells can be visualized by histological investigation and analyzed by the release of LDH from epithelial cells into the surrounding medium. The LDH levels in the cell culture medium are representative for the overall epithelial damage of the entire RHE, whereas histological sections represent only a distinct small part of the infected tissue. Therefore, histological changes should be evaluated on the basis of at least 50 sections from five different sites for each RHE sample.

ANTICIPATED RESULTS

Inoculation of the oral or vaginal RHE with reference wild-type strain SC5314[25] leads to hyphal production caused by contact with the epithelial tissue (**Fig. 1**) and should induce signs of tissue damage characterized by edema, vacuolization and detachment of keratinocytes by 24 h (**Fig. 3a**). A temporal progression of epithelial invasion and damage should be clearly evident in time-course experiments by both microscopic analysis of histological sections and increased LDH secretion into the culture medium.

Addition of PMNs to the apical layers of the oral or vaginal epithelium 6 and 12 h after inoculation should significantly reduce *C. albicans* penetration and epithelial damage (**Fig. 3b**). Fungal growth and tissue damage should also be significantly reduced when PMNs are applied to the basal side of the intact polycarbonate filter (**Fig. 3c**) or when PMNs migrate through the filter after perforation with a thin needle (**Fig. 3d,e**).

ACKNOWLEDGMENTS We thank all the previous undergraduate and graduate students who have completed a project or a thesis in the laboratory and helped to build the foundations of these protocols. We thank Birgit Fehrenbacher, Renate Nordin, Helga Möller and Hannelore Bischof, University of Tuebingen, for excellent technical assistance and Gudrun Holland and Muhsin Özel, Robert Koch-Institute, for providing **Figure 3**. M.S. and G.W. were supported by the Deutsche Forschungsgemeinschaft (Sch 897/1; Sch 897/3), B.H. by the Robert Koch-Institute, the Deutsche Forschungsgemeinschaft (Hu 528/8; Hu 528/10) and the European Commission Union (QLK2-2000-00795; "Galar Fungail consortium") and M.S. and J.N. by NIH grant R01 DE017514-01 and J.N. by a personal Wellcome Trust Value in People (VIP) award.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Godaly, G. *et al.* Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection. *J. Leukoc. Biol.* **69**, 899–906 (2001).
2. Backhed, F. & Hornef, M. Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? *Microbes Infect.* **5**, 951–959 (2003).
3. Tjabringa, G.S. *et al.* Host defense effector molecules in mucosal secretions. *FEMS Immunol. Med. Microbiol.* **45**, 151–158 (2005).
4. Dieterich, C. *et al. In vitro* reconstructed human epithelia reveal contributions of *Candida albicans* EFG1 and CPH1 to adhesion and invasion. *Microbiology* **148**, 497–506 (2002).
5. Dongari-Bagtzoglou, A. & Kashleva, H. Development of a novel three-dimensional *in vitro* model of oral *Candida* infection. *Microb. Pathog.* **40**, 271–278 (2006).
6. Okeke, C.N. *et al.* Reverse transcription–3′ rapid amplification of cDNA ends-nested PCR of ACT1 and SAP2 mRNA as a means of detecting viable *Candida albicans* in an *in vitro* cutaneous candidiasis model. *J. Invest. Dermatol.* **114**, 95–100 (2000).
7. Schaller, M., Schafer, W., Korting, H.C. & Hube, B. Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol. Microbiol.* **29**, 605–615 (1998).
8. Schaller, M. *et al.* Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol. Microbiol.* **34**, 169–180 (1999).
9. Schaller, M. *et al.* The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an *in vitro* model of vaginal candidiasis based on reconstituted human vaginal epithelium. *Infect. Immun.* **71**, 3227–3234 (2003).
10. Korting, H.C. *et al.* Effects of the human immunodeficiency virus (HIV) proteinase inhibitors saquinavir and indinavir on *in vitro* activities of secreted aspartyl proteinases of *Candida albicans* isolates from HIV-infected patients. *Antimicrob. Agents Chemother.* **43**, 2038–2042 (1999).
11. Heymann, P. *et al.* The siderophore iron transporter of *Candida albicans* (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. *Infect. Immun.* **70**, 5246–5255 (2002).
12. Zhao, X. *et al.* ALS3 and ALS8 represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. *Microbiology* **150**, 2415–2428 (2004).
13. Zhao, X., Oh, S.H., Yeater, K.M. & Hoyer, L.L. Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151**, 1619–1630 (2005).
14. Green, C.B. *et al.* RT-PCR detection of *Candida albicans* ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. *Microbiology* **150**, 267–275 (2004).
15. Li, D., Bernhardt, J. & Calderone, R. Temporal expression of the *Candida albicans* genes CHK1 and CSSK1, adherence, and morphogenesis in a model of reconstituted human esophageal epithelial candidiasis. *Infect. Immun.* **70**, 1558–1565 (2002).
16. Albrecht, A. *et al.* Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. *J. Biol. Chem.* **281**, 688–694 (2006).
17. Klengel, T. *et al.* Fungal adenylyl cyclase integrates CO_2sensing with cAMP signaling and virulence. *Curr. Biol.* **15**, 2021–2026 (2005).
18. Schaller, M. *et al.* Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J. Invest. Dermatol.* **118**, 652–657 (2002).
19. Schaller, M., Korting, H.C., Borelli, C., Hamm, G. & Hube, B. *Candida albicans*-secreted aspartic proteinases modify the epithelial cytokine response in an *in vitro* model of vaginal candidiasis. *Infect. Immun.* **73**, 2758–2765 (2005).
20. Schaller, M. *et al.* Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an *in vitro* model of oral candidosis. *Microbiology* **150**, 2807–2813 (2004).
21. Korting, H.C. *et al.* Reduced expression of the hyphal-independent *Candida albicans* proteinase genes SAP1 and SAP3 in the efg1 mutant is associated with attenuated virulence during infection of oral epithelium. *J. Med. Microbiol.* **52**, 623–632 (2003).
22. Rouabhia, M. *et al.* Virulence of the fungal pathogen *Candida albicans* requires the five isoforms of protein mannosyltransferases. *Infect. Immun.* **73**, 4571–4580 (2005).
23. Schaller, M., Mailhammer, R. & Korting, H.C. Cytokine expression induced by *Candida albicans* in a model of cutaneous candidosis based on reconstituted human epidermis. *J. Med. Microbiol.* **51**, 672–676 (2002).
24. Amarzguioui, M. *et al.* Rational design and *in vitro* and *in vivo* delivery of Dicer substrate siRNA. *Nature Protocols* **1**, 508–561 (2006).
25. Gillum, A.M., Tsay, E.Y. & Kirsch, D.R. Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae* ura3 and *E. coli* pyrF mutations. *Mol. Gen. Genet.* **198**, 179–182 (1984).