

# An *In Vitro* HSV-1 Reactivation Model Containing Quiescently Infected PC12 Cells

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## Abstract

Advances in the understanding of the infection and reactivation process of herpes simplex type 1 (HSV-1) are generally gained by monolayer cultures or extensive and cost-intensive animal models. So far, no reliable *in vitro* skin model exists either to investigate the molecular mechanisms involved in controlling latency and virus reactivation or to test pharmaceuticals. Here we demonstrate the first *in vitro* HSV-1 reactivation model generated by using the human keratinocyte cell line HaCaT grown on a collagen substrate containing primary human fibroblasts. We integrated the unique feature of a quiescently infected neuronal cell line, the rat pheochromocytoma line PC12, within the dermal layer of the three-dimensional skin equivalent. Transmission electron microscopy, a cell-based TCID<sub>50</sub> assay, and polymerase chain reaction analysis were used to verify cell latency. Thereby viral DNA could be detected, whereas extracellular as well as intracellular virus activity could not be found. Further, the infected PC12 cells show no spontaneous reactivation within the *in vitro* skin equivalent. In order to simulate a physiologically comparable HSV-1 infection, we achieved a specific and pointed reactivation of quiescently HSV-1 infected PC12 cells by UVB irradiation at 1000 mJ/cm<sup>2</sup>.

**Key words:** alternative testing; herpes simplex virus type 1; *in vitro* reactivation model; quiescent infection; virus reactivation

## Introduction

OVER THE PAST 15 YEARS, an array of organ-similar structures have been developed in the field of tissue engineering, which are already used as standardized test systems for biomedical research in toxicology, immunology, and pharmacology.<sup>1–4</sup> Organotypic reconstituted skin models represent a suitable alternative for animal testing because they can mimic the three-dimensional (3D) environment of the native skin.<sup>5–7</sup> Different disease models like a tumor model, infection models for pathogenic fungi, and a wound-healing model could be successfully established using this system.<sup>5,6,8</sup>

We extended the applications of this system by developing a reactivation model for herpes simplex type 1 (HSV-1) infections, one of the most common skin diseases. Following a primary infection, HSV establishes a life-long static latency—a characteristic feature of all herpes viruses—within the trigeminal ganglion. Thereby, the virus enters the nerves at the primary infection site and migrates into the cell body of the neuron where the circular viral genome can persist as an episomal molecule in a latent state.<sup>9–11</sup> At this point the viral lytic gene expression is silenced.<sup>10,12–15</sup> The mechanism of latency and the subsequent reactivation are poorly understood.

Currently, infection and reactivation mechanisms are mainly studied using monolayer culture systems and animal models.<sup>13,16–27</sup> The established *in vitro* models in general lack the neuronal component and, therefore, fail to provide an insight into the latency and reactivation mechanisms.<sup>28–30</sup>

In contrast, the novel HSV-1 model presented here shows a significant modification by integrating a quiescently infected neuronal cell line (PC12) within the dermal layer. Additionally, in this report we describe a specific reactivation of the virus. In consideration of the integration of a latently infected neuronal component and the targeted reactivation of the herpes simplex virus, this HSV-1 model ensures a closer approximation to the *in vivo* situation *in vitro*. With this model, it is now possible to investigate the molecular mechanisms involved in virus reactivation process. This model additionally provides the possibility to perform drug screens.

## Material and Methods

### Cell culture

The primary fibroblasts were isolated from foreskin tissues obtained from surgery as described previously.<sup>5</sup> All donors were aged from 4 months to 7 years.

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The spontaneously immortalized human keratinocytes (HaCaT) were obtained from German Cancer Research Center (Heidelberg, Germany). HaCaT cells (passages 38–43) and isolated primary fibroblasts (passages 2–5) were cultured routinely in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, penicillin (50 U/mL; Gibco), and streptomycin (0.05 mg/mL; Gibco). Cells were subcultured by trypsinization using a 0.05% trypsin solution containing 0.53 mM EDTA\*4Na (Gibco) in phosphate-buffered saline (PBS).

Rat pheochromocytoma PC12 cells, obtained from American Type Culture Collection (ATCC; CRL-1721), were cultured on collagen I-coated (50 ng/mL collagen I in 0.1% acetic acid) culture dishes in RPMI 1640 medium containing 10% horse serum (HS) and 5% FCS supplemented with 2 mmol/L glutamine, penicillin (50 U/mL), and streptomycin (0.05 mg/mL; Gibco). The cells were subcultured using Ca<sup>2+</sup>/Mg<sup>2+</sup> free PBS.

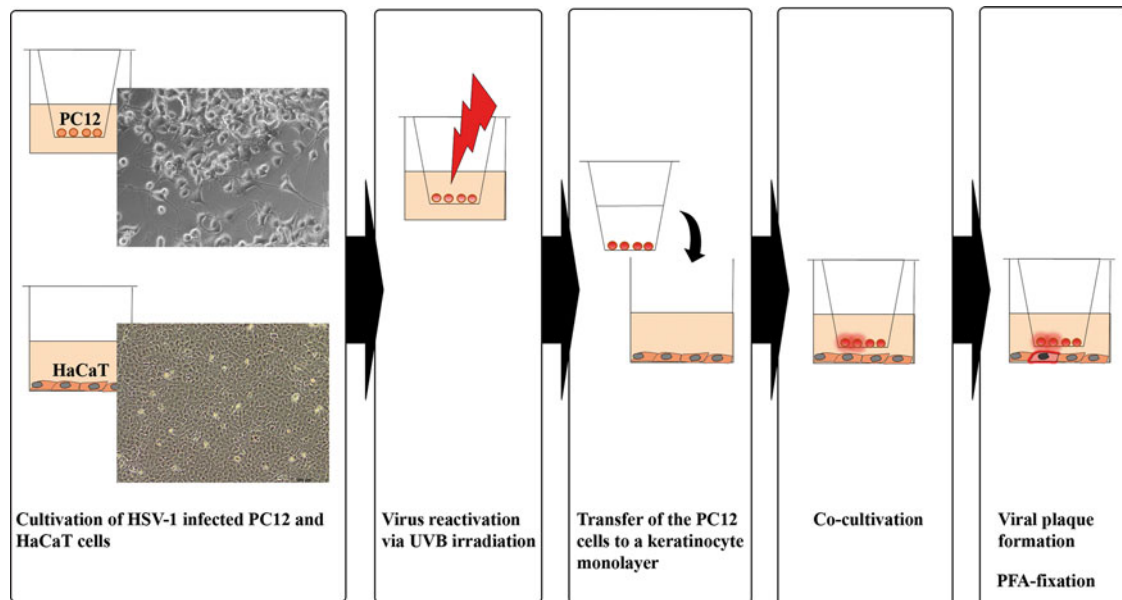
African green monkey kidney cells (Vero B4) were obtained from DMSZ (ACC 33, Braunschweig, Germany). Vero B4 cells were maintained in RPMI 1640 medium containing 10% FCS supplemented as described for DMEM. All cells were cultured at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

For coculture analysis HaCaT cells were seeded on 24-well plates at a density of 2 × 10<sup>5</sup> cells/well. Cells were cultivated in DMEM containing 10% FCS. HSV-1 infected as well as noninfected PC12 cells were seeded in collagen I-coated inserts (1 × 10<sup>5</sup> cells/insert) with a 3-μm anopore membrane (Nunc, Wiesbaden, Germany). Ultraviolet light (UV) radiation was used to achieve HSV-1 reactivation. The quiescently infected PC12 cells were exposed twice within 24 h. Based on the determined value for the radiation intensity of 0.64 mW/cm<sup>2</sup> (Stratalinker UV-Crosslinker 1800) we used UVB exposure

times of 8 min (288 mJ/cm<sup>2</sup>), 13 min (500 mJ/cm<sup>2</sup>), and 26 min (1000 mJ/cm<sup>2</sup>). After the UVB irradiation the PC12 cell-containing inserts were transferred into a 24-well plate onto a HaCaT monolayer. After cocultivation for 24 h, the PC12 cell-containing inserts were transferred again onto a new 24-well plate. The cells of the previous 24-well plate were fixed with paraformaldehyde. The transfer started after the first UVB irradiation and was finalized after 7 days of coculture. A schematic overview of the coculture approach is shown in Figure 1. All 24-well plates were analyzed by microscopy and immunohistochemical staining using a rabbit polyclonal antibody against HSV-1 (BioGenex, Fremont, CA; 1:100) using the ABC substrate development Kit (BioGenex).

*Organotypic cell culture*

The dermal layer was prepared using a fibroblast/PC12-collagen gel. Primary human skin fibroblasts as well as the quiescently HSV-1-infected PC12 cells were suspended in rattail collagen I (6 mg/mL in 0.1% acetic acid solution) and neutralization solution (ratio 2:1) at a density of 1.4 × 10<sup>5</sup> cells/mL (PC12) and 2.5 × 10<sup>5</sup> cells/mL (primary fibroblasts). Six hundred microliters of the cell/collagen suspension was transferred in a 24-well insert (pore size 8 μm; Nunc) and incubated submerged in DMEM at 37°C. After incubation for 24 h ± 2 h, 50 μL of fibronectin (50 μg/mL) was spread onto the gels. The epidermal layer was generated by HaCaT cells that were seeded onto the fibroblast/PC12-collagen gel at a density of 2 × 10<sup>5</sup> cells/well. The organotypic cultures were grown submerged in keratinocyte growth medium 2 (KGM; Basal Medium and SupplementPack; PromoCell, Heidelberg, Germany) with descending FCS concentration (5%, 2%, 0%).



**FIG. 1.** Schematic overview of the coculture approach. The viral indicator cell line HaCaT was cultivated in 24-well plates and the quiescently infected PC12 cells in collagen I-coated inserts on a membrane with a pore size of 3 μm. The PC12 cells were then exposed separately to ultraviolet (UV)B light, twice within 24 h. Cells were UVB-irradiated for 8 min (288 mJ/cm<sup>2</sup>), 13 min (500 mJ/cm<sup>2</sup>), and 26 min (1000 mJ/cm<sup>2</sup>). After the UVB irradiation, the PC12 cell-containing inserts were transferred onto the HaCaT monolayer. After cocultivation for 24 h the PC12 cell-containing inserts were transferred again onto a new 24-well plate. The cells of the previous 24-well plate were fixed with paraformaldehyde (PFA). The inserts were transferred daily, starting after the first UVB irradiation ended after 7 days. HSV-1, herpes simplex type 1.

After the submersion phase (5–7 days) the organotypic cell cultures were lifted to the air–liquid interface using Airlift media (KGM, without bovine pituitary extract (BPE) and human epidermal growth factor (hEGF), with additional 1.88 mM  $\text{Ca}^{2+}$ ) for 10–12 days. Medium was replaced every 2–3 days.

For HSV-1 reactivation the skin models were exposed to UVB in accordance to the reactivation conditions in coculture. For microscopy and immunohistochemistry the 3D skin models were formalin-fixed and paraffin-embedded 7 days after UVB irradiation.

#### Virus determination and infection

HSV-1 strain HF was obtained from the ATCC (VR-260). Virus titer was determined by a plaque assay on Vero B4 cell monolayer as described by Blaho et al.<sup>31</sup> Virus infectivity in supernatants was determined by TCID<sub>50</sub> (half-maximal tissue culture infective dose) assay as described by LaBarre and Lowy.<sup>32</sup>

To establish a latent infection in differentiated and in undifferentiated PC12 cells, both were infected with HSV-1 strain HF at a multiplicity of infection of 20. Differentiation of PC12 cells was performed as described elsewhere.<sup>25</sup> After an inoculation for 2 h at 37°C cell cultures were treated with sodium citrate buffer (pH 3) as described by Su et al.<sup>25</sup> After low-pH treatment, cultures were incubated at 37°C with fresh medium with or without nerve growth factor (NGF; differentiated or undifferentiated PC12 cells, respectively). To monitor HSV-1 release, the supernatant was collected and the virus load was determined by TCID<sub>50</sub> assay.

HSV-1 was detected by polymerase chain reaction (PCR) in infected PC12 cells. Primers 5'HSV-1\_LAT (5'-GAC AGC AAA AAT CCC CTG AG-3') and 3'HSV-1\_LAT (5'-ACG AGG GAA AAC AAT AAG GG-3') were used to detect HSV-1 latency-associated transcript (LAT). Amplification was performed by a touchdown PCR amplification as follows: (i) one cycle at 95°C for 5 min, (ii) 33 cycles at 95°C for 30 sec, at 68°C for 30 sec ( $\Delta T$ : -0.5°C), and at 72°C for 60 sec, and (iii) last cycle at 72°C for 5 min.

#### Immunohistochemistry

Histologic sections were cut at 3  $\mu\text{m}$  (mikrotom RM 2145, Leica, Solms, Germany) and either used for hematoxylin and eosin staining or for immunohistochemical analysis. Immunohistochemical staining of the PC12 cells was performed by using the DAKO Advance detection system. The primary antibody (Tyrosinhydrolase; Abcam) was optimized to a dilution of 1:400. IgG2a isotype was used as a negative control.

Immunohistochemistry to detect HSV-1 antigens was performed using rabbit polyclonal antibody against HSV-1 (BioGenex) at a dilution of 1:100 with the ABC substrate development Kit (BioGenex).

#### Electron microscopy

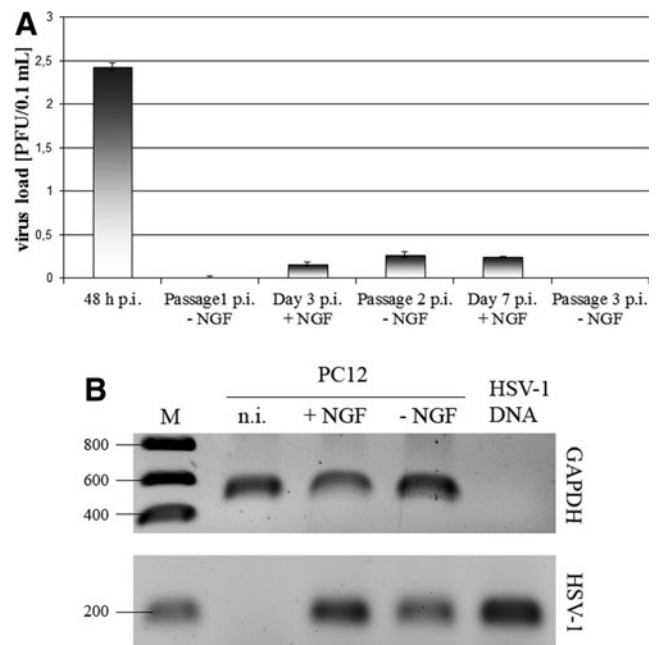
For transmission electron microscopy (TEM) analyses PC12 cells were seeded at a density of  $1 \times 10^5$  cells in a collagen I-coated 24-well insert (pore size 1  $\mu\text{m}$ ; Nunc). Cells were fixed in 2.5% glutaraldehyde in PBS (pH 7.3). Following treatment in 1% osmium tetroxide solution ( $\text{OsO}_4$ ), 2% uranylacetate (UA) and dehydration in acetone series (in ascending

order: 30%, 50%, 70%, 90%, 100%; 15 min each), the samples were embedded in Spurr's resin and polymerized 48 h at 60°C. Ultrathin sections were obtained with a diamond knife on an ultramicrotome and picked up on pioloform-coated grids (150 mesh). Sections on grids were post-stained for 2 min with 1% UA and 6 min with 1% lead citrate by floating them on single drops of the staining solution at room temperature, then rinsed in deionized water and dried. Sections were observed by EM 10C/CR electron microscope (Zeiss, Jena, Germany) at 60 kV.

## Results

### Generation of a HSV-1 quiescently infected PC12 cell line and characterization of the latency state

Previous studies indicated that differentiated PC12 cells that are infected by HSV-1 permit inducible virus production.<sup>17,25</sup> In order to test whether differentiation using NGF is required to establish a quiescently infection in PC12 cells,



**FIG. 2.** (A) TCID<sub>50</sub> assay of the supernatant of HSV-1-infected PC12 cells. Supernatants were used either differentiated with NGF (100 ng/mL) or nondifferentiated. The virus titer in nondifferentiated PC12 cells show decreased viral titer already after the first passage post infection (p.i.). The supernatant of differentiated PC12 cells also decreased viral activity already after a cultivation period of 3 days p.i. (B) Semiquantitative PCR analysis to confirm the presence of the HSV-1 genome within the infected PC12 cells. Cells were used either undifferentiated or NGF treated. Thereby, no significant differences are observed between differentiated (21 days p.i.) and undifferentiated PC12 (passage 9 p.i.) cells on DNA level. HSV-1 genome isolated from the virus stock solution was used as a positive control. Mock-infected PC12 cells served as a negative control. GAPDH was used as a loading control. Both the differentiated and undifferentiated positive control cells as well as the PC12 cells in passage 9 p.i. showed the predicted fragment size of 195 bp ( $n=3$ ). TCID<sub>50</sub>, tissue culture infective dose<sub>50</sub>; NGF, nerve growth factor; PCR, polymerase chain reaction; PFU, plaque-forming units.

undifferentiated as well as differentiated PC12 cells were used for infection with HSV-1.

Interestingly, the virus titer showed no significant differences in differentiated or undifferentiated PC12 cells after infection. Furthermore, no cytopathic effect (CPE) was observed in either cell population at any time post infection (p.i.; data not shown).

The supernatant of both HSV-1-infected PC12 populations was assayed via TCID<sub>50</sub>. Again, no difference was observed between differentiated and undifferentiated PC12 cells with regard to the TCID<sub>50</sub>. Maximum virus activity was detected in the supernatant of PC12 cells 48 h p.i. Already after the first passage the virus load converged to zero (Fig. 2A), indicating entry into a latent infection stage.

Since no differences between the cell populations were detected, all further experiments were performed with the undifferentiated PC12 population. To ensure a successful infection of the PC12 cells, the presence of the viral genome within the cells was analyzed and identified by PCR up to passage 9 (Fig. 2B).

To further characterize the status of the HSV-1 infection, we analyzed the cells at different stages of infection using TEM analysis. If HSV-1 persists in a latent state as a stable episomal element within the cell nucleus, no production of infective viral particles should be visible. The ultrastructure of the HSV-1-infected PC12 cells is shown in Figure 3. As expected, 48 h p.i. PC12 cells showed fully enveloped intracellular as well as extracellular virions (Fig. 3A-i, ii). In addition, there were a number of assembled but nonenveloped viral capsids within the nucleus (arrows in Fig. 3A-ii).

In contrast to the early phase of infection (48 h p.i.) infective viral particles could not be observed in passage 9 of quiescently infected PC12 cells (Fig. 3B-i, ii). The only particles that could be found were dense-core secretory granules. PC12 cells are known to contain a large number of secretory

granules for storage of small molecules, processing enzymes, neuropeptides, and peptide hormones.<sup>33-35</sup>

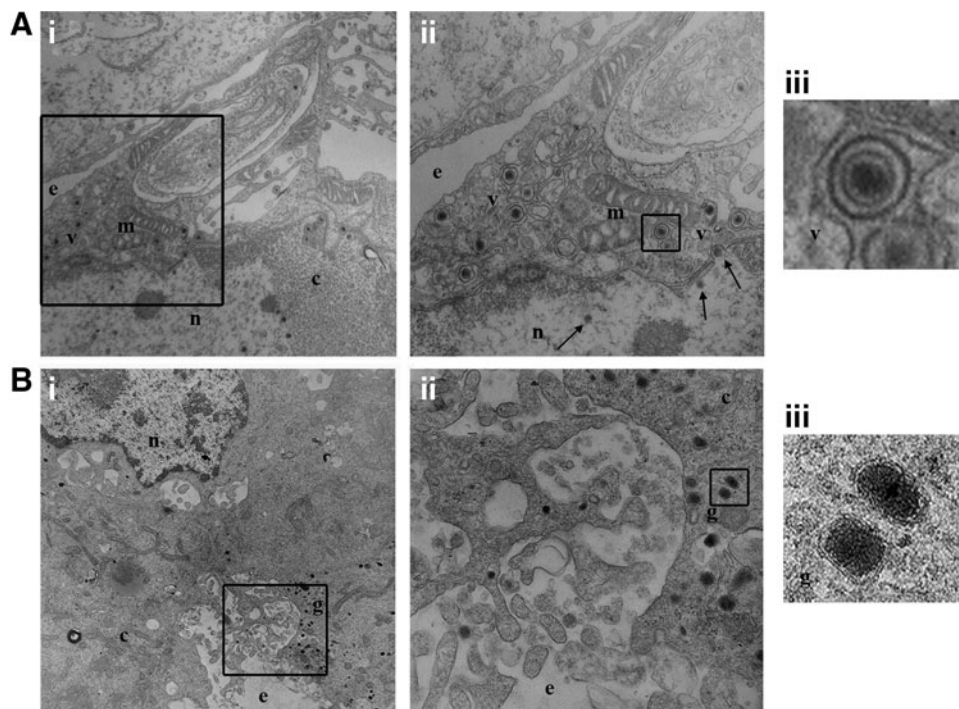
Thus, viral DNA could be detected, whereas extracellular and intracellular virus activity could not be shown in the infected PC12 cell culture. These observations correlate with the phenotype of latently infected cells.

*In vitro reactivation of HSV-1-infected PC12 cells in coculture*

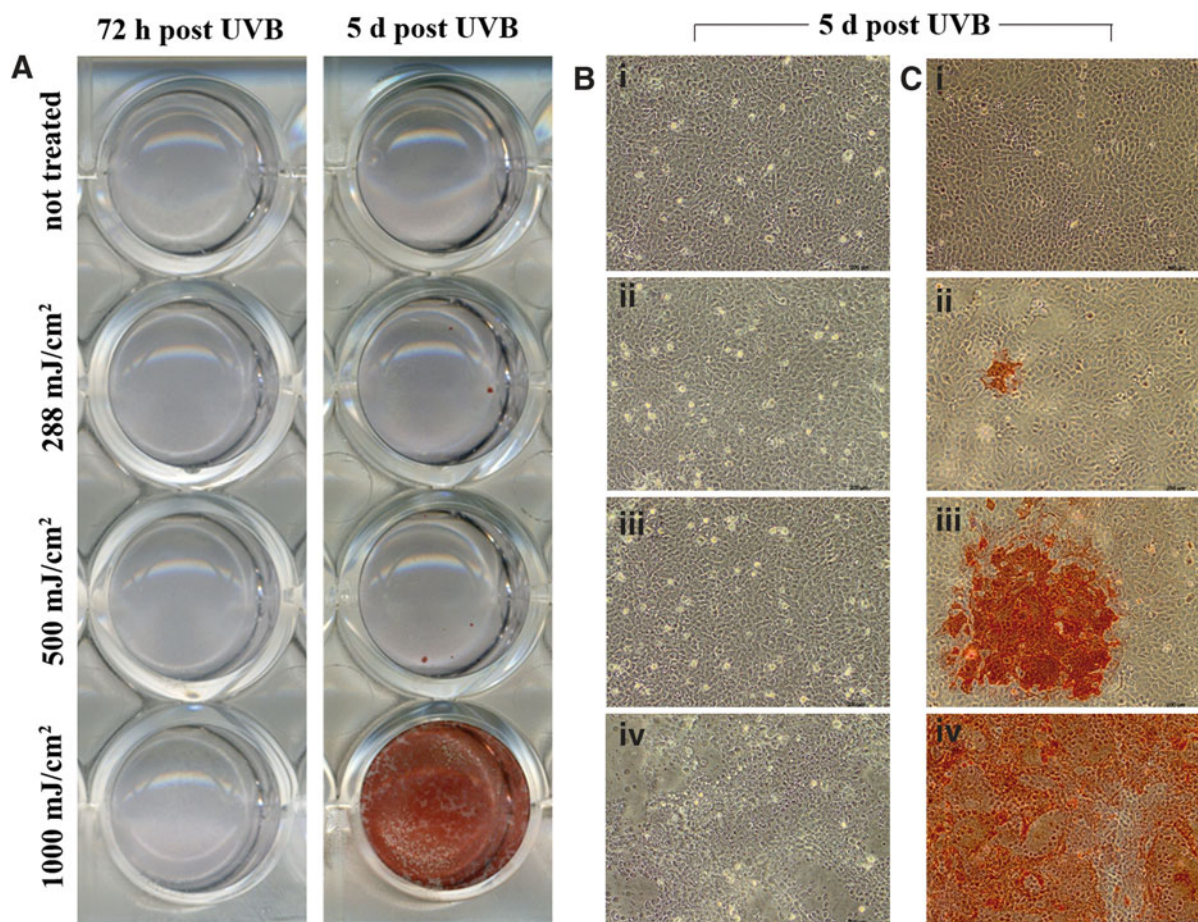
UV light is known to induce reactivation of herpes simplex virus *in vivo* as well as *in vitro*.<sup>36-39</sup> This leads to a CPE and the formation of viral plaques on monolayer cell cultures. To test whether reactivation of HSV-1 is possible in principle, quiescently infected PC12 cells were UVB-irradiated and cocultured on HaCaT cells. Thereby, the HaCaT cell line represented an indicator cell line for viral plaque formation in case of a successful reactivation. Immunohistochemical staining for HSV-1 antigens is shown in Fig. 4C-i-iv. The HaCaT cells showed lytic infection at different levels proportional to the UVB dose (Fig. 4B-ii-iv). Infected cells displayed well-defined red-stained plaques, whereas noninfected cells remained blue. The highest virus reactivation was detected on HaCaT monolayer cells growing in coculture with PC12 cells that were irradiated at the highest UVB dose of 1000 mJ/cm<sup>2</sup> (Fig. 4B-iv, C-iv). Nonirradiated PC12 cells showed no virus reactivation at all time points tested (Fig. 4B-i, C-i).

*Integration of infected PC12 cells within the 3D skin equivalent*

In order to be able to simulate a HSV-1 infection more closely *in vitro*, we established a 3D skin model, composed of three cell types: primary fibroblasts as well as quiescently infected PC12 cells embedded in a collagen matrix that



**FIG. 3.** Electron micrograph of different passages of PC12 cells p.i. with HSV-1. **(A)** Morphology of PC12 cells 48 h p.i. show fully enveloped virions intracellular as well as extracellular (marked as v) **(i, ii)**. High magnification electron micrograph **(ii)** additionally shows the envelopment process within the nucleus (arrows). The enveloped viral particles displayed an average size of ~130–200 nm in diameter. **(B)** In PC12 cells passage 9 p.i. dense-core secretory granules (marked as g) are seen in the cytoplasmic region, whereas no infective viral particles are visible **(i, ii)**. Cells were fixed in 2.5% glutaraldehyde and postfixed in 1% lead citrate and 1% uranylacetate (UA). Nuclear (n), cytoplasmic (c), and extracellular (e) regions are marked; *n*=3.



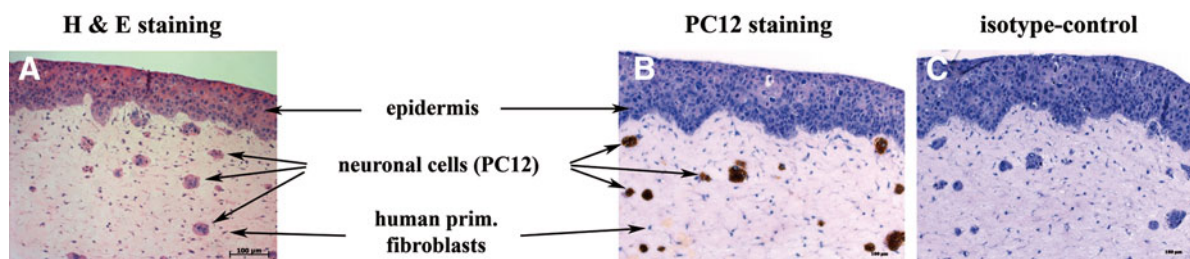
**FIG. 4.** Cytopathic effect (CPE) of HSV-1-infected PC12 cells after UV irradiation. HaCaT cells growing in coculture (**A**) with HSV-1-infected PC12 cells nonirradiated (**B-i**, **C-i**) and post-UVB irradiation at a UVB dose of 288 mJ/cm<sup>2</sup> (**B-ii**, **C-ii**), 500 mJ/cm<sup>2</sup> (**B-iii**, **C-iii**), and 1000 mJ/cm<sup>2</sup> (**B-iv**, **C-iv**). CPEs were visualized by microscopy (**B**) and the viral plaques (red) by immunohistochemistry (**C**) using a highly specific anti-HSV-1 antibody (1:100). UVB-induced reactivation could be observed at all UVB intensities. The highest HSV-1 reactivation could be achieved at 1000 mJ/cm<sup>2</sup> (**B-iv**, **C-iv**). Nonirradiated PC12 cells showed no virus reactivation at any time (**B-i**, **C-i**);  $n=3$ .

represented the dermal layer and a human keratinocyte cell line (HaCaT), which formed the epidermal layer.

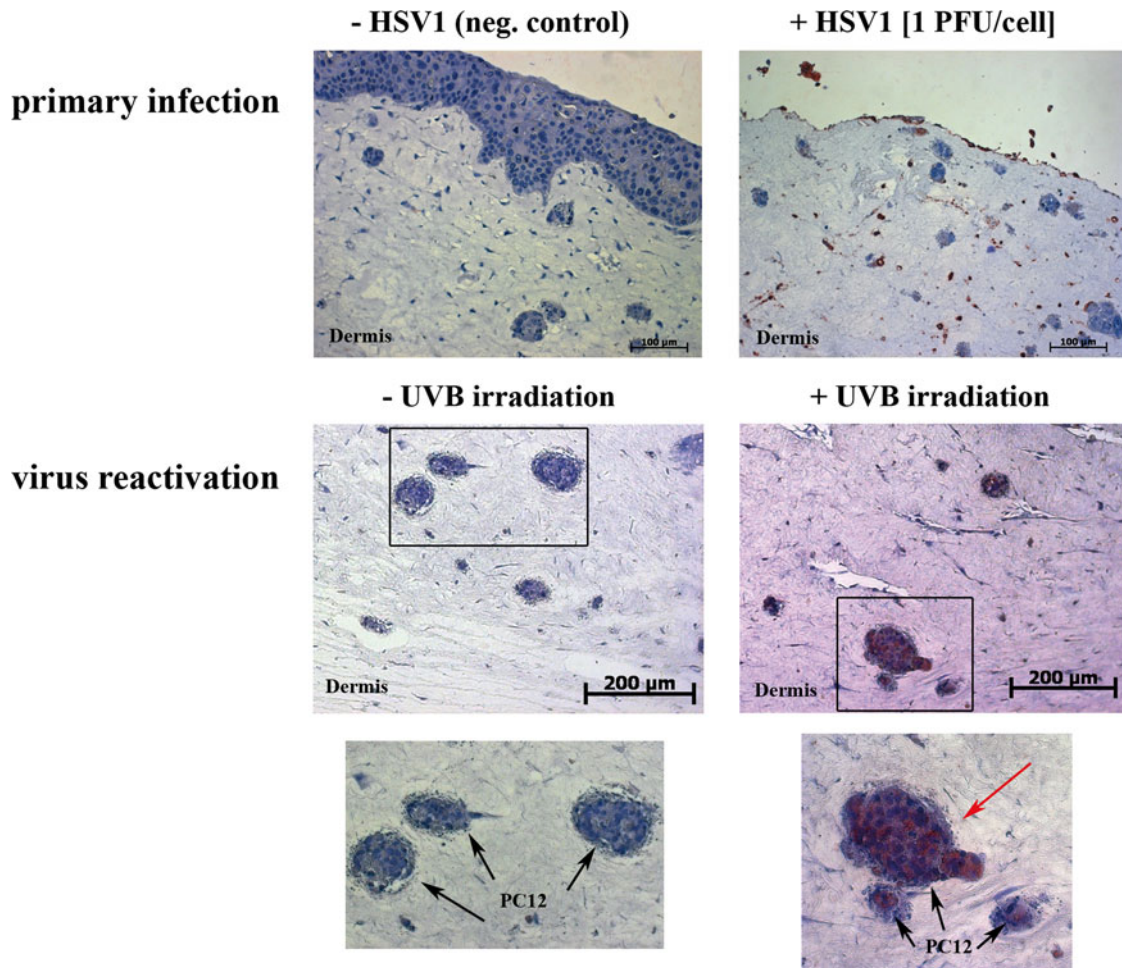
The cross sections of the 3D skin equivalent revealed equally distributed PC12 clusters in the dermal layer and the HaCaT cell line formed a multilayered epidermis (Fig. 5). No CPEs were observed within the skin equivalent. Thus, the infected PC12 cells stayed dormant within the 3D reactivation model and no spontaneous reactivation occurred.

#### *In vitro reactivation of HSV-1-infected PC12 cells in the 3D skin model*

To achieve virus reactivation under defined conditions, we irradiated the skin model with UVB light according to the reactivation conditions of the coculture experiments. The cross sections of the 3D reactivation model are shown in Figure 6. A UVB irradiation twice at 1000 mJ/cm<sup>2</sup> resulted in a



**FIG. 5.** Cross sections of the HSV-1 infection model with quiescently infected PC12 cells integrated into the dermal layer of the skin equivalent. (**A**) Hematoxylin and eosin (H&E) staining of cross sections shows uniformly distributed PC12 cells within the dermal layer of the *in vitro* epidermal model. (**B**) Immunohistochemical staining using a monoclonal antibody raised against a specific neuronal marker (tyrosine hydroxylase) shows PC12 cells in brown. (**C**) Isotype control (IgG2 $\alpha$ ).  $n=3$ .



**FIG. 6.** Immunohistochemical detection of HSV-1 in cross sections of the either nonirradiated or at 1000 mJ/cm<sup>2</sup> UVB-irradiated HSV-1 infection models. Polyclonal rabbit anti-HSV-1 (1:100) was used for immunohistochemical staining. High magnification micrograph shows no virus reactivation within the nonirradiated skin models. Successful reactivation of HSV-1 (red) was achieved after UVB-irradiation at 1000 mJ/cm<sup>2</sup>. HSV-1 is visualized within the PC12 cell clusters (red arrow). *n* = 3.

successful reactivation of the herpes simplex virus within the PC12 cell clusters, as detected by the specific HSV-1 antibody recognizing only fully enveloped viral particles (red-stained spots; Fig. 6). Irradiation at lower intensities did not show visible reactivation (data not shown).

**Discussion**

A characteristic feature of herpes viruses is their ability to establish a life-long episomal latency in neural tissue. During the dormant state, the HSV-1 genome persists as a circular molecule within the nucleus.<sup>40,41</sup> The virus maintains the potential to reactivate and cause recurrent disease.<sup>41,42</sup> Periodic reactivation occurs whereby HSV is set free from the neurons and undergoes further rounds of infection.<sup>10,41,43</sup> The molecular and cellular mechanisms involved in establishing, maintaining, and mediating reactivation from latency are not known completely.

In this report we describe the establishment of a 3D skin model system to study the mechanism of HSV-1 reactivation. The rat pheochromocytoma (PC12) cell line was infected by HSV-1 and showed neither spontaneous reactivation nor

virus replication; however, the virus could be specifically reactivated via UVB. The PC12 cell line has been reported as an *in vitro* HSV-1 infection model resembling latency.<sup>21,25,26</sup> Our results partly confirmed these previous findings and additionally showed that NGF is not necessary to keep the infected cells in a nonproductive state.<sup>17,25</sup> There was no detectable difference between differentiated or undifferentiated PC12 cells after infection with regard to HSV-1 status. HSV-1 DNA was detected in infected undifferentiated PC12 cell culture up to passage 9, whereas extracellular and intracellular virus activity could not be found as determined by a cell-based TCID<sub>50</sub> assay, PCR analysis, and TEM. Only in the early infection phase did the neuronal cells still show viral replication resulting in the production of infective virus particles without lysis, which is in accordance with findings of other studies.<sup>10,42</sup> In contrast to previous studies, spontaneous reactivation from the quiescent state was not observed.<sup>17,25</sup> This suggests that the infected PC12 cell culture produced has unique properties compared with other *in vitro* infection models reported, requiring HSV-1 inhibitors (e.g., acycloguanosine) to maintain latency or showing spontaneous reactivation by an overlay of corneal

fibroblasts.<sup>25,44,45</sup> In our studies virus reactivation only occurred under UVB irradiation conditions. Virus reactivation was shown in the coculture experiments leading to cell lysis of the indicator cell line (HaCaT) and formation of viral plaques, on day 5 post-UVB irradiation.

In order to establish a HSV-1 model, more related to the *in vivo* situation, we integrated these quiescently infected PC12 cells into a 3D-skin model. This integration was accomplished without any evidence of spontaneous virus reactivation. The PC12 cell clusters were equally distributed within the dermal layer and stayed dormant within the 3D skin equivalents. Thereby, the epidermal layer was generated by using the HaCaT cell line. As a remarkable result we achieved a successful reactivation of the dormant virus by exposing the skin model to ultraviolet radiation (UVB) at 1000 mJ/cm<sup>2</sup>, after which virus could be detected within the infected PC12 clusters.

However, the infection and reactivation process is still partially unstable. Different levels of reactivation might be explained by the fact that viral production occurred in a minority of the quiescently infected cell population. Studies based on animal experiments suggest that only a low percentage (4–35%) of HSV-1-infected neurons contain latent HSV-1 DNA.<sup>46,47</sup> Thus, the infection conditions have to be further refined in order to achieve a reproducible reactivation within the 3D HSV-1 model.

Our objective is to improve the current model, more related to human skin, by integrating human neuronal cells. Additionally, existing deficiencies in the formation of a normal stratum corneum requires further improvement of culture conditions or the use of primary keratinocytes. Nevertheless, this novel HSV-1 reactivation model may help to further unravel the molecular and cellular mechanisms of the reactivation process. In the pharmaceutical industry, new antivirals can be tested or identified with the help of the *in vitro* HSV-1 test system, and in addition this skin equivalent can be seen as an alternative to animal tests.

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### Author Disclosure Statement

No competing financial interests exist.

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