

Article

HaCaT Keratinocytes: A Differentiation-Competent Platform for Episomal Replication of HPV Type 11

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Abstract

Few differentiation-competent models exist to study early intra-nuclear processes of human papillomavirus (HPV) in keratinocytes. Early HPV DNA replication is usually studied by transfecting transformed or tumor-derived cell lines (C33A, HEK293/HEK293T, CIN612). While these lines support episome replication, their transformed state and oncogene expression can confound interpretation, and they do not undergo the normal keratinocyte differentiation required for the HPV life cycle. We therefore evaluated HaCaT, a spontaneously immortalized, non-transformed keratinocyte line with reversible differentiation, as a model for HPV episomal replication. We optimized culture conditions—particularly extracellular calcium—to toggle HaCaT cells between basal-like proliferation and differentiation, and refined transfection parameters to deliver plasmid vectors required for HPV11 episomal replication. HaCaT cells display differentiation-associated morphological changes and keratin marker expression comparable to primary keratinocytes. In transient luciferase-based origin replicon assays, HPV11 plasmid replicons showed origin-dependent replication in both undifferentiated and differentiated HaCaT cells. Because Ca^{2+} -driven differentiation rewires keratinocyte nuclear organization, this Ca^{2+} -controlled HaCaT system enables evaluation of early viral nuclear processes, including episomal replication and differentiation-associated increases in replication activity, in a nuclear architecture-dependent epithelial context without exogenous viral oncogenes or cellular transformation.

Keywords: HPV; episomal replication; keratinocyte differentiation; nuclear architecture; HaCaT; calcium signaling; DNA tumor viruses; transfection



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1. Introduction

Papillomaviruses (HPVs) and some polyomaviruses (PyVs) replicate in stratified epithelia [1,2], but most in vitro viral episomal replication studies use transformed cell lines. Common models include C33A (an HPV-negative cervical carcinoma line) [3], HEK293-derived cells expressing viral oncogenes [4] or CIN612 cells that carry HPV31 in an episomal state [5]. These cell lines support viral origin-dependent replication, yet either lack epithelial differentiation completely, or, if they do undergo differentiation, the presence of viral oncoproteins leads to questions as to whether results reflect what occurs in non-transformed keratinocytes. Thus, a non-transformed cell culture model that supports viral episome replication with physiological keratinocyte differentiation is needed. HaCaT is a spontaneously immortalized human keratinocyte cell line that, although immortalized, is not transformed and cannot produce tumors in xenograft models; however, its immortalization and ability to undergo keratinocyte differentiation make it a promising potential model

system for HPV [6]. Established from adult skin by Boukamp et al. in 1988 [7], HaCaT achieved immortality through endogenous genetic alterations (without the introduction of SV40 or other sources of exogenous oncogenes). It is a monoclonal line capable of indefinite growth in vitro without feeder layers or supplemental growth factors [8]. Importantly, HaCaT cells retain the capacity for normal epidermal differentiation: they exhibit proper morphogenesis and express key keratinocyte differentiation markers (keratins K5/K14 in the basal state and K1/K10 in differentiated cells, plus late differentiation markers like involucrin and filaggrin) in a well-established sequential epidermal pattern [8]. Notably, they can toggle between a proliferative basal-like state and a differentiated state in response to extracellular calcium (Ca^{2+}) concentration changes [9], retaining essential keratinocyte markers while allowing for genetic manipulation. This ability is a key advantage, enabling experimental control over the differentiation status in one cell line. However, there are some limitations to using HaCaT cells, which include the existence of certain genetic aberrations that promote hyper-proliferation and alter late differentiation kinetics (e.g., TP53 mutations, chromosomal imbalances) [10]. In addition to HaCaT, other cell types have been used to study HPV replication, including normal oral keratinocytes (NOKs), U2OS osteosarcoma cells, and W12 cervical keratinocytes, each providing useful experimental contexts but also presenting limitations related to epithelial differentiation, transformation status, or in modeling differentiation-linked viral replication processes [11–13]. To overcome these deficiencies, near-diploid immortalized lines such as N/TERT 1 and 2G (hTERT-immortalized keratinocytes) [14] and the spontaneously immortalized NIKS line [10] have been used to retain improved barrier formation and more physiological differentiation profiles. However, these newer lines tend to proliferate more slowly and often require more demanding culture conditions compared to robust easily cultured HaCaT cells [15]. Findings in HaCaT should be validated in primary keratinocytes or near-diploid models when possible.

HPV and polyomavirus genomes replicate extra-chromosomally via virus-encoded initiator and helicase proteins (HPV E1/E2 or SV40 large T antigen) that recognize the viral origin, separate the dsDNA helix, and recruit the host's DNA replication machinery [16–20]. A widely used approach to quantify viral DNA replication in cultured cells is to co-transfect an origin-containing luciferase reporter plasmid with viral initiator protein expression vectors and measure replication using a dual-luciferase readout [16,21]. This transient replicon system, first developed for HPV and SV40 [16] and later adapted to the JC virus [21], provides sensitive measurements of viral origin-dependent episome replication in highly transfectable, transformed cell lines (e.g., C33A). Although effective, non-keratinocyte or heavily transformed host cells might distort viral regulatory interactions that normally depend on keratinocyte differentiation. Indeed, productive HPV genome amplification in vivo is tightly linked to the differentiation process of the host keratinocyte [22], molecular cue/s that are absent in standard transformed monolayer cultures. By demonstrating viral DNA replication in HaCaT keratinocytes, this study helps address this gap. The goal of this study is to establish and validate HaCaT cells as a differentiation-competent, transfectable keratinocyte platform for studying early viral episomal replication. Accordingly, the experimental design was optimized for comparative, origin-dependent replication readouts under controlled differentiation states, rather than for defining the molecular determinants of differentiation-dependent amplification.

In this study, we demonstrate that HaCaT keratinocytes support robust replication of HPV11. Notably, HaCaT cells do not possess viral oncoproteins (HPV E6/E7 or SV40 LT) that could artificially enhance replication, so the DNA synthesis we observe is driven only by the introduced viral genome replication factors in a near-normal cellular environment. This appears to be the first reported demonstration of the replication of transfected papillomavirus or polyomavirus DNA in HaCaT cells, although recent work has demon-

strated HPV genome amplification in primary keratinocytes [23]. HaCaT thus emerges as a convenient surrogate for primary keratinocytes, offering a more physiological epithelial context along with the practical advantages of being an immortal cell line. At the same time, monolayer HaCaT cultures provide tight experimental control over differentiation state and reproducible recovery of episomal DNA, while lacking the tissue-level complexity of organotypic raft models. Accordingly, this system is well suited for investigating early, differentiation-associated viral processes, including origin-dependent episomal replication, under controlled conditions, whereas later stages of the viral life cycle require more complex three-dimensional models. Furthermore, this platform enables examination of host–virus interactions and evaluation of antiviral strategies in a keratinocyte setting without the confounding effects of viral transformation or oncogene expression.

2. Materials and Methods

2.1. Cell Culture and Calcium-Induced Differentiation

HaCaT keratinocytes (a gift from S. Sinha, University at Buffalo, Buffalo, NY, USA; originally obtained from Professor Fusenig [7]) were cultured in high-glucose DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 4 mM L-glutamine (Gibco) (standard “full” medium, containing ~1.8 mM Ca^{2+}) or in calcium-free DMEM (high-glucose, no glutamine, no Ca^{2+} ; Gibco) supplemented with 10% calcium-depleted FBS and CaCl_2 to a final Ca^{2+} concentration of either ~0.06 mM (low- Ca^{2+} medium) or 2.8 mM (high- Ca^{2+} medium). Calcium was removed from FBS by batch incubation with Chelex-100 resin (Bio-Rad, Hercules, CA, USA) for 1 h at 4 °C with gentle rocking, followed by sterile filtration through a 0.22 μm filter unit. After use, the resin was regenerated by stripping with 2 bed volumes of 1 N HCl, rinsing with 5 bed volumes of H_2O , treating with 2 bed volumes of 1 N NaOH, and washing with 5 bed volumes of H_2O before reuse.

For the in vitro differentiation model, proliferating HaCaT cells were adapted to low- Ca^{2+} medium [0.06 mM] for ≥ 3 passages to maintain a basal, undifferentiated phenotype [9]. Cells were then shifted to high- Ca^{2+} medium (1.8–2.8 mM) for up to 120 h (5 days) to induce differentiation. Differentiation was monitored by (a) phase-contrast microscopy at 0, 4, 24, 48, 72, 96, and 120 h to document the transition from elongated, spindle-shaped cells to a tightly packed “cobblestone” morphology [24] and (b) Western blotting of whole-cell lysates for keratin 5 (KRT5; basal marker) and keratin 1 (KRT1; early differentiation marker). Rabbit monoclonal antibodies to KRT1 and KRT5 were kindly provided by S. Sinha. As numerous standard housekeeping markers routinely used for the normalization of Western blot studies have been shown to fluctuate significantly in response to keratinocyte differentiation [25,26], Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) staining of cell lysates was instead used to demonstrate comparable loading. Densitometric analysis of immunoblots was performed using ImageJ software (NIH, Bethesda, MD, USA, version 1.53), with band intensities quantified after background subtraction and normalized to total protein levels determined by Ponceau S staining.

2.2. Transfection of HaCaT and HEK293T Cells

HaCaT keratinocytes are relatively resistant to plasmid transfection: standard lipid-based reagents typically yield ~10–30% GFP-positive cells, with >50% efficiencies rarely achieved [27]. Reported contributors include a robust plasma membrane barrier and a propensity for stress-induced growth arrest or differentiation, as well as cytotoxicity and terminal differentiation under harsh transfection conditions [27,28]. To maximize transfection efficiency, we optimized several parameters based on previous studies [9,27,28].

Three transfection reagents were evaluated in HaCaT cells: (a) Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA; lipid-based), (b) TransIT-X2 (Mirus Bio, Madison, WI, USA), and (c) FuGENE 6 (Promega, Madison, WI, USA; non-liposomal polymeric reagent). Each reagent can form complexes with negatively charged DNA, facilitating cellular uptake. All transfections followed manufacturers' general protocols with in-house optimization as described. HaCaT cells were seeded in 12-well plates at ~ 1.5 or 2.5×10^5 cells per well (30–50% confluency) to assess the effect of cell density. Transfections were performed in undifferentiated [0.06 mM Ca^{2+}] and differentiated [1.8 mM Ca^{2+}] conditions to evaluate the impact of calcium-induced differentiation on transfection efficiency. Each reagent was tested by transfecting $1 \mu\text{g}$ per well of a CMV-GFP reporter plasmid, varying the reagent: DNA ratio according to manufacturer recommendations to identify optimal conditions. AD293 cells (an adherent, highly transfectable HEK293T-derived line; Invitrogen, Carlsbad, CA, USA) were used in parallel as a positive control.

At 72 h post-transfection, live cells were stained with Hoechst 33342 ($0.001 \mu\text{g/mL}$, added directly to warm medium) and imaged without washing. Fluorescence images were acquired in overlay mode on an ECHO Revolve K(2) R4 inverted microscope (Discover Echo Inc., San Diego, CA, USA) with a $10\times$ Olympus (Olympus Corp., Tokyo, Japan) objective and Echo Pro software (version 6.4.2; Discover Echo Inc.), and phase-contrast images were obtained on a Motic AE31 Elite inverted microscope (Motic, Xiamen, China) equipped with a GT5.0 color camera (Motic) and $10\times$ objective. Images were captured in DAPI (DNA) and FITC/GFP channels and overlaid to confirm GFP-positive cells [15]. Transfection efficiency was quantified by counting GFP⁺ cells versus total Hoechst⁺ nuclei in multiple fields per well using ImageJ. The optimal DNA: transfection reagent ratio for each system was defined as the condition yielding the highest percentage of GFP⁺ HaCaT cells with minimal toxicity (based on morphology and nuclei counts). These conditions were used in subsequent experiments.

In preliminary optimization, the following parameters were systematically evaluated for HaCaT growth, maintenance and transfection: (a) *Cell growth*: subconfluent cultures consistently showed higher transfection efficiency than confluent cultures; (b) *Calcium/differentiation*: cells in low- Ca^{2+} (undifferentiated) medium were more efficiently transfected than those in high- Ca^{2+} (differentiated) medium, consistent with reduced susceptibility of differentiated keratinocytes [9]; (c) *DNA to reagent ratio*: plasmid and reagent amounts were titrated to maximize transfection with minimal toxicity [9]; (d) *Serum and antibiotics*: complexes were formed in serum- and antibiotic-free Opti-MEM to avoid interference, and after 4–6 h the medium was replaced with fresh growth medium (low or high Ca^{2+} , without antibiotics); (e) *Promoter strength*: vectors driven by the cytomegalovirus (CMV) immediate-early promoter were used to compensate for modest transfection efficiency; CMV promoters are effective in HaCaT and other difficult-to-transfect cells [16,29]; and (f) *DNA titration*: amounts of each plasmid used in HPV11 and SV40 replication assays were carefully titrated. All plasmids were prepared using endotoxin-free maxi-preps to further enhance transfection performance.

2.3. DNA Replication Assays

Transient viral DNA replication was quantified using a dual-luciferase plasmid replication assay [16,21,30]. Cells were transfected simultaneously with (i) a Firefly luciferase reporter plasmid bearing the viral origin of replication (ori) and (ii) one or more plasmids expressing the cognate viral replication protein(s). Ori-dependent DNA replication increases Firefly luciferase signal relative to an internal Renilla luciferase plasmid lacking a viral ori, which serves as a control for transfection efficiency and cell number [16]. This high-throughput assay provides a sensitive surrogate readout for origin-dependent DNA

replication, enabling relative comparisons across conditions. However, it does not directly measure episomal DNA replication or maintenance, and DNA-based methods such as Southern blotting or DpnI-based qPCR remain required for direct quantification [16].

HaCaT cells were seeded ~24 h before transfection at 2.5×10^4 cells per well in opaque white 96 well plates (Corning Inc., Corning, NY, USA). Two culture conditions were tested: low- Ca^{2+} [0.06 mM] and high- Ca^{2+} [1.8 mM] medium to assess how differentiation status at transfection affects replication. AD293 and C33A cells (ATCC HTB-31; American Type Culture Collection, Manassas, VA, USA), were maintained in standard 1.8 mM Ca^{2+} medium, served as positive controls for robust SV40 and HPV11 ori plasmid replication routinely observed in our laboratory.

Two assay systems were used: SV40 (polyomavirus) and HPV11 (papillomavirus). For both, cells were co-transfected with a Firefly ori^+ reporter, viral replication protein expression plasmid(s), and a control ori^- Renilla plasmid. For SV40, the reporter was pFLORI-40 (Firefly linked to the SV40 ori) and replication was driven by SV40 large T antigen (LT). For HPV11, the reporter was pFLORI-11 (Firefly linked to the HPV11 ori), with E1 and E2 supplied in trans from separate expression vectors [16,31]. DNA amounts were empirically optimized, and very low ori plasmid input was used so that increases in Firefly signal emphasized replicated DNA over initial plasmid uptake [16].

For DNA replication assays in 96-well plates, HaCaT transfections used 0.4 μL TransIT X2 (Mirus) per well, chosen for its combination of efficiency and low toxicity. DNA–reagent complexes were prepared in 100 μL Opti MEM according to the manufacturer’s instructions, incubated for 20 min at room temperature with gentle shaking, and added dropwise to overnight cultures in triplicate. After 4–6 h, the medium was gently replaced. Cells were then incubated at 37 °C, 5% CO_2 for 72 h without drug selection, as replication was driven by transient viral protein expression. To test inhibition by a broad-spectrum DNA polymerase inhibitor, aphidicolin (5, 10, or 50 μM) was added to designated C33A triplicates (SV40 and HPV11 assays) 4–6 h after transfection together with the medium change. Culture conditions (passage number, plating density, plasticware) were kept constant, and all assays were performed in triplicate. Data were averaged to reduce well-to-well variability and are reported as mean % DNA replication $\pm$ SEM. This optimized protocol yielded consistent gene expression in HaCaT cells suitable for downstream functional analyses.

2.4. Dual-Luciferase Assays (HPV11 and SV40): Luciferase Readout and Data Analysis

Cells were lysed in 1 $\times$ lysis buffer (Promega). Firefly and Renilla luciferase activities were measured sequentially using the Dual-Glo Luciferase Assay System (Promega) and a multi-mode microplate reader (Cytation 1; BioTek Instruments, Winooski, VT, USA) controlled by Gen5 software (version 3.11; BioTek Instruments) with a 7-s integration time per well. For each well, Firefly luminescence (reflecting replicated ori plasmid) was divided by Renilla luminescence (reflecting transfection efficiency) to yield a normalized relative replication value [16]. Background from non-replicating controls or samples lacking viral replication proteins was subtracted, and replication efficiency was expressed as a percentage of a positive control (e.g., C33A, AD293, or HaCaT cells transfected with 10 ng LT or E1, defined as 100% for SV40 or HPV11 assays, respectively). The dual luciferase assay was highly sensitive and specific. The co-expression of HPV11 E1 and E2 produced a robust increase in HPV11 ori reporter signal, whereas omission of either protein abolished replication, consistent with the requirement for both factors in origin-dependent HPV11 DNA replication.

3. Results and Discussion

3.1. HaCaT Morphology and Differentiation Markers

In basal conditions [low, 0.06 mM Ca^{2+}], HaCaT keratinocytes maintained a dividing, highly undifferentiated phenotype. Morphologically, low- Ca^{2+} cultures showed elongated, spindle-shaped cells that were loosely adherent and lacked the cobblestone-like contacts typical of differentiated keratinocytes (Figure 1A,B), consistent with previous reports of HaCaT cells grown under low calcium exhibiting basal-like morphology [9,32]. In low Ca^{2+} , cells expressed the basal marker keratin 5 (KRT5), whereas the early differentiation marker keratin 1 (KRT1) remained unchanged up to 120 h (Figure 1C). Densitometric analysis normalized to total protein confirmed a progressive decline in KRT5 expression after 72 h, while KRT1 levels remained low and essentially unchanged throughout the time course (Figure 1D). Upon prolonged growth (72–120 h) in low Ca^{2+} , the cells gradually adopted a more cuboidal, contiguous monolayer appearance (Figure 1B,E) and lost KRT5 expression; KRT1 levels remained low (Figure 1C). These findings indicate that confluency-driven contact inhibition can induce some aspects of keratinocyte differentiation even without the usual differentiation trigger of high Ca^{2+} .

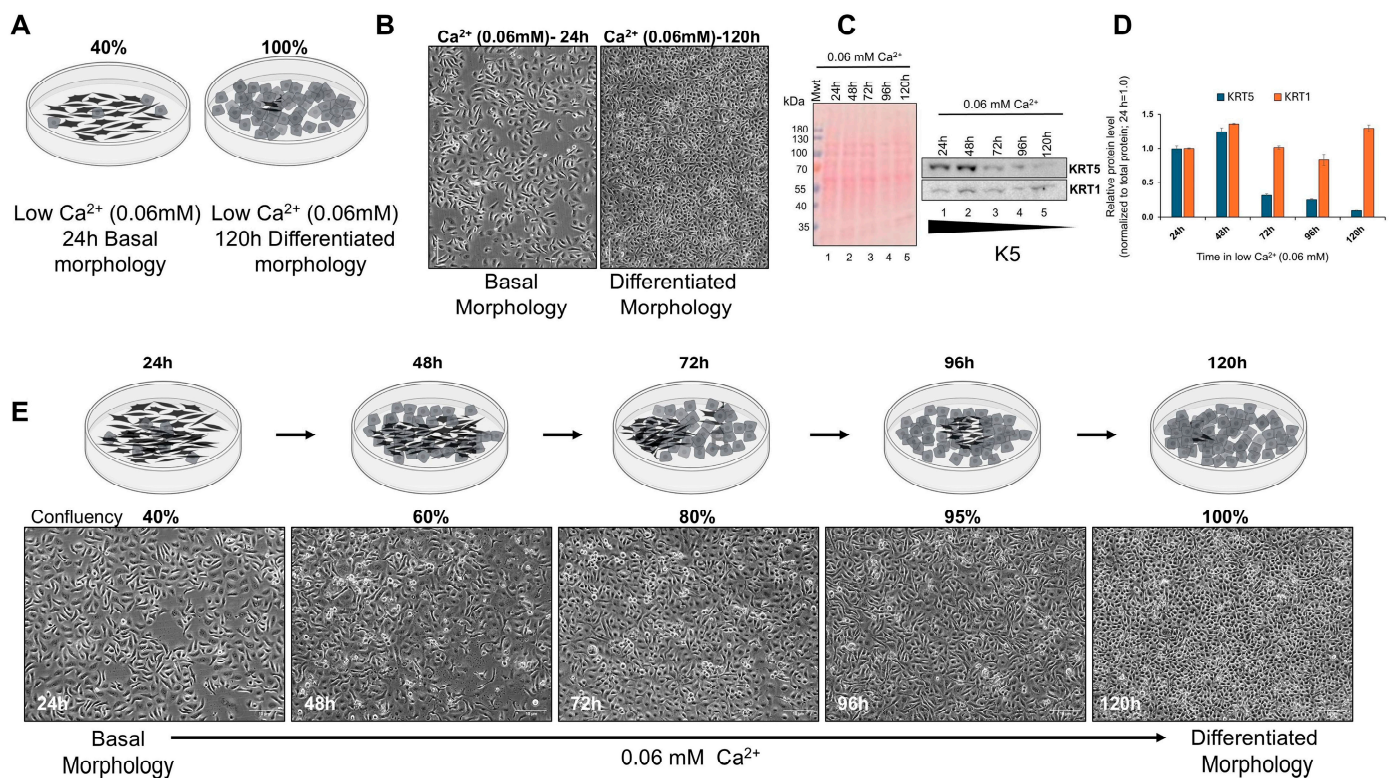


Figure 1. Morphological and molecular characteristics of HaCaT cells under low- and high-density growth conditions. HaCaT cells maintained for at least three passages in Dulbecco's Modified Eagle Medium (DMEM) containing low calcium [0.06 mM Ca^{2+}] were used in this experiment. (A) The schematic illustrates HaCaT cells cultured in low-calcium medium [0.06 mM Ca^{2+}], with a spindle-shaped basal morphology at 24 h (~40% confluency) that progressively transitions to a cobblestone morphology characteristic of differentiated cells (100% confluency) by 120 h. (B) Phase-contrast micrographs of HaCaT cells in low-calcium medium at 24 h and 120 h closely correspond to the schematic representation. (C) Western blot analysis shows that the cells express keratin 5 (KRT5), which

is abundant in the undifferentiated basal keratinocyte phenotype at 24–48 h in low-calcium [0.06 mM] medium (**upper** panel). KRT5 expression begins to decline after 72 h and is barely detectable by 120 h. In contrast, keratin 1 (KRT1) expression remains consistently low throughout the 24–120 h period (**lower** panel). Thus, KRT5 serves as a reliable marker of undifferentiated HaCaT keratinocytes. Ponceau S staining of total protein is shown as a loading control, as housekeeping proteins varied with differentiation. (**D**) Densitometric quantification of KRT5 and KRT1 protein levels from the Western blot shown in (**C**). Ponceau S staining of total protein is shown as a loading control, as housekeeping proteins varied with differentiation. Band intensities were quantified using ImageJ, normalized to total protein per lane by Ponceau S staining, and expressed relative to the 24 h time point (set to 1.0). Data represent mean $\pm$ SEM from three independent experiments. (**E**) HaCaT cells cultured in low Ca^{2+} [≈ 0.06 mM] were examined microscopically at 24 h intervals up to 120 h. The white scale bars in the lower-right corners = 10 μm . At 24 h, the cells exhibited a small, elongated, fibroblast-like spindle morphology and were loosely attached, indicating the absence of tight junctions. The cells also lacked desmosomes, characteristic of differentiated keratinocytes. Over time, cell morphology transitioned progressively from predominantly spindle-shaped basal cells at 48 h to a firmly attached, almost fully contiguous cobblestone pattern by 120 h.

Cell density and cell–cell contact are themselves established drivers of early keratinocyte differentiation, indicating that confluency-associated morphology and differentiation are mechanistically linked rather than independent phenomena [33]. In contrast, shifting HaCaT cells to high- Ca^{2+} medium (1.8–2.8 mM) rapidly induced a differentiated morphology, even without high-density culturing. Within 4 h at 2.8 mM Ca^{2+} , cells became more cuboidal and tightly packed, forming a junction-rich “cobblestone” monolayer of differentiated keratinocytes (Figure 2A), consistent with prior observations [9]. KRT1 expression increased steadily between ~ 72 to 120 h after the shift to high Ca^{2+} (Figure 2B) in contrast to what was observed in low- Ca^{2+} confluent cultures, which suppressed KRT5 expression without KRT1 induction (Figure 1). Densitometric analysis normalized to total protein confirmed a progressive and time-dependent increase in KRT1 expression following calcium addition, with minimal induction at early time points and maximal accumulation observed between 96 and 120 h (Figure 2C).

These observations are consistent with established models of Ca^{2+} -dependent keratinocyte differentiation. Low- Ca^{2+} cultures remain in a basal-like state (KRT5-high) but as the cells divide and the dish becomes more confluent with the characteristic differentiated morphology, KRT5 expression decreases while KRT1 remains constantly low. In contrast, sustained growth in high Ca^{2+} promotes a switch toward a differentiated, high-KRT1 state. This behavior mirrors the epidermal pattern in vivo, where basal keratinocytes co-express K5/K14 and suprabasal cells replace these with K1/K10 (and in subsequent layers, involucrin) as differentiation proceeds [9,24].

To evaluate the context of our findings, we surveyed the literature on HaCaT cells for (a) Ca^{2+} -responsive markers (Table S1) [7,8,24,32,34–38]; (b) lipid-based transient transfection strategies (Table S2) [9,27,39–41]; and (c) use in HPV replication studies (Table S3) [12,42–46]. Prior work showed that HaCaT cells support the replication of both low- and high-risk HPV types, but with important differences: low-risk HPV11 genomes can be maintained as stable episomes for extended periods (weeks to months) in culture, enabling long-term drug and mechanistic studies, as demonstrated by the establishment of basal-like HaCaT cells harboring episomal HPV11 genomes [43]. In contrast, high-risk HPV16/18 genomes are more difficult to sustain episomally in HaCaT cells and instead favor short-term episome models [12,42,47,48]. Such transient systems sacrifice long-term maintenance readouts but yield consistent reproducible results for episomal functions (e.g., E1/E2-dependent origin firing) under tightly controlled conditions. These studies are particularly useful for comparing multiple HPV types under identical, short-term conditions. Hence, HaCaT cells occupy a functional middle ground between highly transfectable tumor

cell lines (e.g., HeLa, HEK293, C33A) and refractory primary keratinocytes: as HaCaT cells are genetically stable, and capable of executing key stages of epidermal differentiation, they are suitable for molecular dissection of the HPV life cycle, including origin-specific plasmid replication by both high-risk (HPV16, 18) and low-risk (HPV6, 11) types [42].

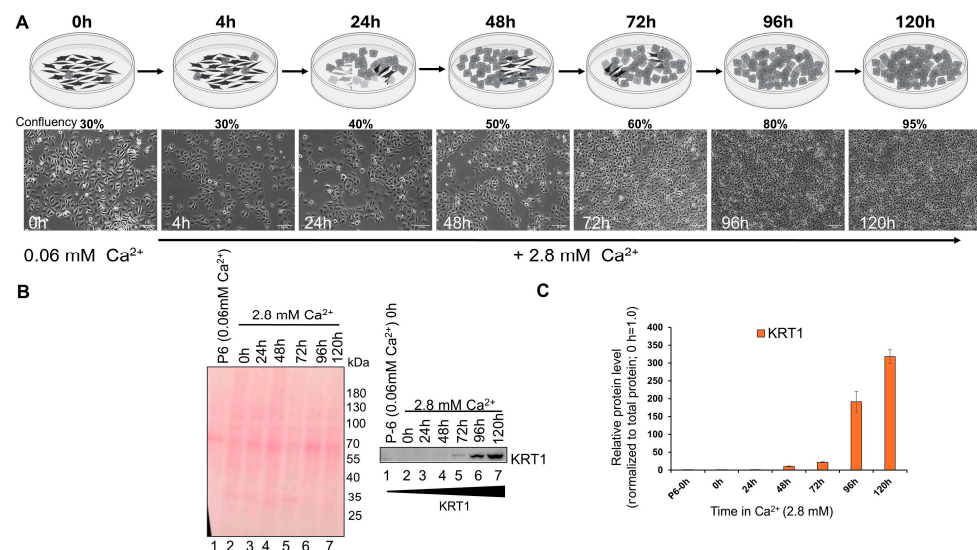


Figure 2. Calcium-induced morphological and molecular differentiation of HaCaT keratinocytes. HaCaT cells that were maintained for three passages in Dulbecco's Modified Eagle Medium (DMEM) containing low calcium [0.06 mM Ca²⁺] were used in this experiment. Overnight cultures in low Ca²⁺ were switched to DMEM supplemented with high calcium (2.8 mM Ca²⁺) and monitored for calcium-induced morphological and molecular changes. Protein lysates were collected at 0 h (prior to calcium addition), 4 h after supplementation, and subsequently at 24 h intervals up to 120 h for Western blot analysis. (A) Distinct morphological changes were observed as early as 4 h following calcium addition, with cells transitioning from an elongated, spindle-shaped morphology to a more cuboidal form. The white scale bars in the lower-right corners = 10 μ m. Morphological differentiation progressed over time, culminating at 120 h in a firmly attached, confluent monolayer displaying the characteristic cobblestone appearance of mature keratinocytes. (B) Western blot analysis shows that keratin 1 (KRT1) expression increased progressively with time in elevated calcium, reaching maximal levels at 120 h in 2.8 mM Ca²⁺. Ponceau S staining of total protein is shown as a loading control, as housekeeping proteins varied with differentiation. The cultures began to reach confluence at approximately 72 h, consistent with the onset of terminal differentiation. (C) Densitometric quantification of KRT1 protein levels from panel B. Band intensities were quantified using ImageJ, normalized to total protein (Ponceau S staining), and expressed relative to the 0 h time point (set to 1.0). Data represent mean $\pm$ SEM from independent experiments ($n = 3$).

3.2. Calcium-Induced Differentiation and Proliferation

Our time-course experiments indicate that the Ca²⁺-driven differentiation switch in HaCaT cells is progressive: morphological events (enhanced cell–cell adhesion and compaction) are exhibited early, followed by detection of proteins representative of differentiation. Notably, unlike primary keratinocytes, HaCaT cells do not exit the cell cycle upon calcium-induced differentiation. Micallef et al. reported that NHKs respond to elevated Ca²⁺ by differentiating and slowing proliferation, whereas HaCaT cells in high Ca²⁺ exhibit increased proliferation and accumulation in S/G2–M phases [32]. We made a similar observation: in high-Ca²⁺, HaCaTs became more compact and differentiated in shape, but with no obvious growth arrest. This behavior is likely due to the immortalized, aneuploid nature of HaCaT cells [32,49]. Consistent with this phenotype, HaCaT keratinocytes lack activating RAS oncogene mutations and require experimental introduction of oncogenic HRAS for malignant conversion. Nevertheless, they harbor endogenous TP53 mutations

and display altered stress- and proliferation-associated signaling, including NF- κ B activity and inducible expression of differentiation- and stress-responsive factors such as GADD153 (CHOP) and C/EBP β (NF-IL6) under defined conditions [49–52]. Thus, while Ca²⁺ treatment recapitulates many differentiation-associated changes in HaCaT cells (morphology and marker expression), they retain proliferative capacity, unlike normal keratinocytes [32]. This unique property may be informative for modeling dysregulated differentiation in disease. Nevertheless, the ability to reversibly toggle HaCaT cells between basal and differentiated states by modulating Ca²⁺ levels is highly advantageous for studying aspects of early keratinocyte differentiation events and signaling pathways. Increased Ca²⁺ drives a shift from basal to differentiated phenotypes over time, particularly as cultures approach confluence [32]. Conversely, maintaining low Ca²⁺ and subconfluent densities preserves a basal, highly proliferative state and high transfection competence (see below). These insights guided our strategy of using low-Ca²⁺, subconfluent HaCaT cultures for DNA transfection in HPV replication assays, while exploiting high-Ca²⁺ conditions to probe differentiation-associated changes in viral replication.

3.3. Transfection Efficiency in HaCaT Cells

Consistent with previous reports and our preliminary data, HaCaT cells displayed relatively low plasmid transfection efficiencies, typically 20–30% and rarely exceeding ~50% GFP-positive cells even under optimized conditions [9].

Multiple factors contribute to this difficulty, including the cells' differentiation state and sensitivity to transfection reagents. Indeed, prior studies have noted that many chemical transfection methods can induce keratinocyte differentiation or cell death [28]. In our initial trials, standard liposome-based kits used according to manufacturer instructions yielded poor DNA uptake in HaCaT cells. Hence, we systematically explored various conditions to optimize transfection (see Section 2 for details). Critical measures included (i) maintaining cells in an undifferentiated basal state at the time of transfection (in low-Ca²⁺ medium at a proliferative density, ~30–50% confluency); (ii) forming lipid–DNA complexes in serum-free Opti-MEM and replacing with complete medium (either low or high Ca²⁺) after 4–6 h; and (iii) empirically refining reagent-to-DNA ratios.

To attempt to maximize transfection efficiency and minimize HaCaT cell toxicity, we compared three commercial transfection reagents (Lipofectamine-3000, TransIT-X2, FuGENE 6) across two seeding densities (150,000 vs. 250,000 cells per well) and two Ca²⁺ concentrations [0.06 vs. 1.8 mM]. The outcomes, summarized in Figure 3A–L, showed clear advantages to transfecting HaCaT in low-Ca²⁺ conditions.

Morphologically, cells transfected in low-Ca²⁺ medium remained flatter and less differentiated in appearance post-transfection, with fewer dead or detached cells (Figure S1A,B,E,F,I,J). In contrast, parallel cultures transfected in high-Ca²⁺ [1.8 mM] exhibited more rounded, refractile morphologies and substantial cell loss at higher densities, particularly when using Lipofectamine 3000 and TransIT-X2 (Figure S1C,D,G,H). FuGENE 6 was comparatively gentler and preserved cell integrity even in high Ca²⁺ (Figure S1I–L). Across all reagents and densities, low-Ca²⁺ cultures yielded healthier cells and higher transfection rates, consistent with prior observations that basal-state HaCaTs show superior transgene uptake [28].

Quantitatively, each reagent performed best in low-Ca²⁺ cultures seeded at 30–50% confluency. In low Ca²⁺, Lipofectamine 3000 (Figure 3A,B) and TransIT-X2 (Figure 3E,F) gave comparable efficiencies, which dropped sharply when cells were pre-differentiated in high Ca²⁺ (Figure 3C,D,G,H). FuGENE 6 achieved a modest peak (~16% GFP-positive) in 150K low-Ca²⁺ cultures (Figure 3I) but was poor in other conditions (Figure 3J–L). TransIT-X2 yielded the highest efficiency overall, reaching ~28% GFP-positive cells in 150 K

low- Ca^{2+} cultures in 24-well plates (Figure 3E), and was therefore chosen for subsequent DNA replication assays. FuGENE 6, while less efficient, caused the least overt cell stress and may be suitable where maximal viability is paramount.

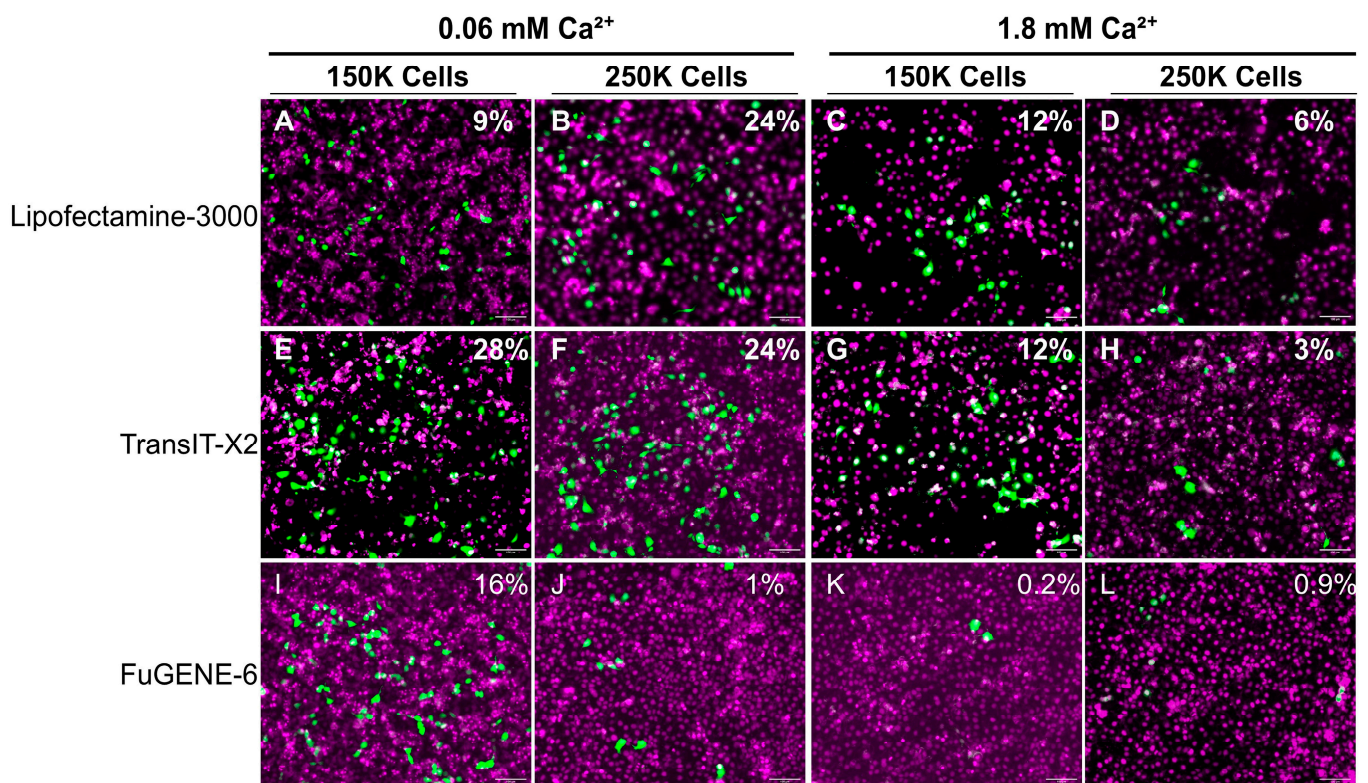


Figure 3. Optimization of transfection efficiency in HaCaT cells under varying calcium concentrations and cell densities. HaCaT cells maintained for ≥ 3 passages in DMEM containing either low calcium [0.06 mM Ca^{2+}] or high calcium [1.8 mM Ca^{2+}] (see Section 2) were used to assess the effects of calcium concentration and cell density on DNA transfection efficiency. Fluorescence microscopy was performed using DAPI (magenta) and FITC (green) filters and the representative images shown are DAPI-FITC overlays. Scale bar = 100 μm . Three commercial reagents were tested: (A–D) Lipofectamine-3000, (E–H) TransIT-X2, and (I–L) FuGENE 6. Cells were seeded in 12-well plates at $\sim 1.5 \times 10^5$ cells per well ((A,E,I) = grown and transfected in low Ca^{2+} and (C,G,K) = grown and transfected in high Ca^{2+}) or at $\sim 2.5 \times 10^5$ cells per well ((B,F,J): low Ca^{2+} ; (D,H,L): high Ca^{2+}), corresponding to ~ 30 – 50% confluence. Each well received 1 μg of a CMV-GFP reporter plasmid complexed with each reagent at the manufacturer’s recommended reagent: DNA ratio. (A–D) Lipofectamine 3000. The highest transfection efficiency ($\sim 24\%$ GFP $^{+}$) was obtained at 2.5×10^5 cells in low Ca^{2+} (panel (B)). Cells in low Ca^{2+} (A,B) showed higher viability than those in high Ca^{2+} (C,D) (Supplementary Figure S1A–D). (E–H) TransIT-X2. Transfection efficiency peaked at ~ 24 – 28% in low- Ca^{2+} conditions (panels (E,F)) and decreased under high Ca^{2+} (G,H). Cell morphology and survival were superior in low- Ca^{2+} cultures (Supplementary Figure S1E–H). (I–L). FuGENE 6. The best efficiency ($\sim 16\%$ GFP $^{+}$) was observed at 1.5×10^5 cells in low Ca^{2+} (panel (I)), with reduced efficiency at higher density or in high Ca^{2+} (J–L). FuGENE 6 produced the least overt cytotoxicity, and cells remained largely intact in both calcium conditions (Supplementary Figure S1I–L), although overall transfection efficiencies were lower than those obtained with Lipofectamine 3000 and TransIT-X2. White bars in the lower right corners represent 100 μm .

To further improve performance, we optimized reagent–DNA ratios using a CMV-GFP reporter (1 μg DNA per well) in HaCaT cells seeded at 150K per 12-well in low- Ca^{2+} medium (Figure 4). Optimal ratios were ~ 3 :1 for Lipofectamine 3000 (3 μL Lipofectamine + 3 μL P3000 per 1 μg DNA; Figure 4A–C), 4:1 for TransIT-X2 (4 μL per 1 μg DNA; Figure 4D–F), and 4–5:1 for FuGENE 6 (4–5 μL per 1 μg DNA; Figure 4G–I).

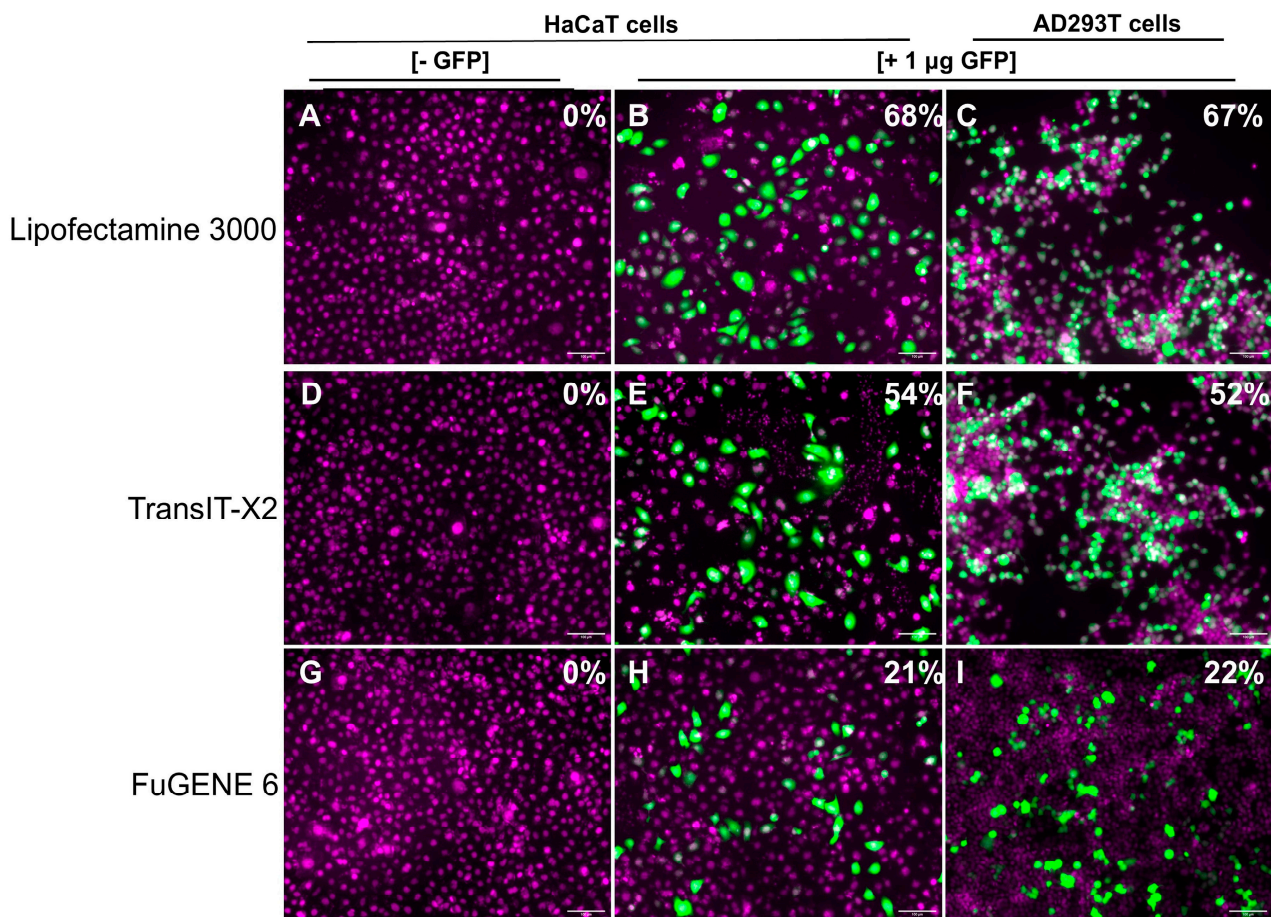


Figure 4. Optimized transfection efficiency in HaCaT and AD293 cells using three transfection reagents. HaCaT cells were cultured for at least three passages in Dulbecco's Modified Eagle Medium (DMEM) containing low calcium [0.06 mM Ca^{2+}] before initiating these experiments. For transfection experiments, HaCaT (A,B,D,E,G,H) and AD293 (C,F,I) cells were seeded at 1.5×10^5 cells per well in 12-well plates and incubated overnight at 37 °C in 5% CO_2 . HaCaT cells were maintained in low-calcium [0.06 mM Ca^{2+}] medium, whereas AD293 cells were maintained in complete DMEM [1.8 mM Ca^{2+}]. Three commercial transfection reagents were evaluated: (A–C) Lipofectamine 3000 (LF-3000), (D–F) TransIT-X2, and (G–I) FuGENE 6. For each reagent, cells were transfected in the absence or presence of plasmid DNA encoding GFP as follows: LF-3000: (A) 0 µg; (B,C) 1 µg with 3 µL-P3000 reagent and 3 µL LF3000; TransIT-X2: (D) 0 µg; (E,F) 1 µg with 4 µL-TransIT-X2; FuGENE 6: (G) 0 µg; (H,I) 1 µg with 4 µL-FuGENE 6. Lipid–DNA complexes were prepared in 100 µL Opti-MEM and added dropwise to each well with gentle agitation. Cells were maintained in 2 mL growth medium (low- or high- Ca^{2+} as appropriate). After 6 h, the medium was replaced with fresh low- or high- Ca^{2+} medium, and incubation continued for 72 h post-transfection. Fluorescence microscopy was performed using DAPI (magenta) and FITC (green) filters, and representative images shown are DAPI-FITC overlays. White scale bars in lower-right corners = 100 µm. Transfection efficiency was quantified using ImageJ. These optimized transfection conditions for all three reagents were empirically determined (see Supplementary Figures S2–S4).

These conditions consistently produced the highest GFP-positive fractions. More extensive titration data are provided in Figures S2–S4. By refining growth conditions and reagent ratios, we developed a reproducible protocol achieving $\geq 50\%$ transfection efficiency in HaCaT while maintaining viability, which is critical for plasmid-based molecular studies. Pseudovirus- and quasivirus-mediated delivery can achieve near-uniform infection of HaCaT cells and efficient infection of primary keratinocytes, providing a highly physiological means of genome delivery; however, these systems have not yet been adapted for scalable, quantitative, origin-dependent replication assays analogous to transient replicon

format [53]. Although electroporation or viral vectors could further improve efficiency [28], our aim was to devise a simple, scalable, plate-based protocol compatible with 96-well formats used for high-throughput HPV and PyV replication assays [54].

3.4. DNA Replication Assay Validation

To quantify viral DNA replication, we employed a dual-luciferase reporter assay in which Firefly luciferase (FFluc) is encoded in cis with a viral origin of replication, with a Renilla luciferase (RLuc) plasmid lacking an ori co-transfected as a control [16]. In this system, viral replication (driven by the viral replication proteins, HPV E1/E2 or polyomavirus large T antigen) is measured by FFluc signal relative to RLuc. The increase in FFluc signal is dependent on the replication of the ori-linked reporter plasmid, resulting in a greater number of FFluc-encoding plasmids thereby amplifying the FFluc signal.

We validated the assay in C33A cervical epithelial cells. Co-transfection with SV40 or HPV plasmids produced robust replication signals when 2.5 or 10 ng of wild-type LT or E1 were expressed, with the 10 ng condition defined as 100% replication (Figure 5A). Treatment with the DNA polymerase inhibitor aphidicolin (5–50 μ M) reduced the FFluc/RLuc ratio by >50%, consistent with the known requirement of DNA polymerase-dependent episomal replication. These results are consistent with previous results using these luciferase-based replication assays [16,21,54].

Applying the optimization of transfection for HaCaT cells, we showed that under low- Ca^{2+} , subconfluent conditions, HPV11 origin-dependent replication produced increases in FFluc/RLuc (the lower absolute induction levels were lesser than in C33A cells, likely reflecting the differences in transfection efficiency). Episome replication in HaCaT was dose-responsive, increasing with escalating E1 expression (2.5, 5, and 10 ng E1) and was highly reproducible across experiments, consistent with published studies on this system in other cell types [16,55,56]. Thus, HaCaT maintained in the basal proliferative state can support transient papillomavirus origin-and E1-dependent DNA replication, consistent with previous reports using these dual-luciferase systems [16,56–58]. Viral DNA replication in HaCaT cells was compared under low- and high- Ca^{2+} levels. Using 25K cells per well, we performed replication assays in cells grown and transfected in either low [0.06 mM] or high [1.8 mM] Ca^{2+} medium (Figure 5B). Remarkably, HaCaT cells cultured and transfected under high Ca^{2+} showed an ~1.8-fold increase in HPV11 origin-dependent replication activity compared with low- Ca^{2+} cells, despite lower transfection efficiency in high Ca^{2+} . This suggests that elevated extracellular Ca^{2+} , which promotes keratinocyte differentiation, facilitates increased viral DNA replication per transfected genome. The magnitude of this enhancement is smaller than the ~3–5-fold differentiation-dependent “amplification” reported in HPV stable episome systems [59–61]. This difference likely reflects the fact that stable episome systems are uniformly HPV-positive, whereas transient transfection assays interrogate replication in only a subset of cells, thereby constraining the apparent dynamic range. Whether this modest increase reflects early components of differentiation-associated amplification observed in stable episome systems remains to be determined, as such effects have not previously been detected in transient transfection assays. Nevertheless, these initial findings indicate that Ca^{2+} -induced differentiation can modulate replication in a short-term transfection of HaCaT cells and raise the possibility that Ca^{2+} -induced differentiation can modulate early episomal replication in HaCaT cells, while later amplification stages are expected to require stratified three-dimensional models. A transfectable system that recapitulates HPV genome amplification would be a useful tool for studying requirements for amplification.

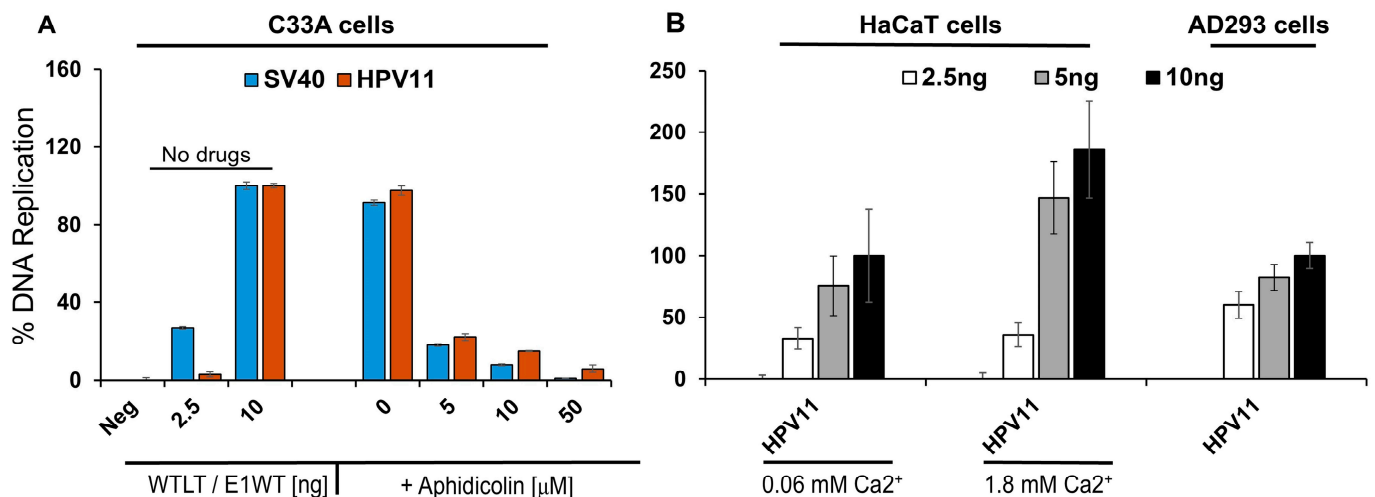


Figure 5. DNA replication efficiency in C33A, HaCaT, and AD293 cells at different calcium levels. HaCaT keratinocytes were maintained for ≥ 3 passages in DMEM containing either low [0.06 mM] or high [1.8 mM] Ca^{2+} and used for transient replication assays. C33A and AD293 cells were cultured in standard DMEM [1.8 mM Ca^{2+}] and seeded at 25,000 cells per well in 96-well plates. A dual-luciferase plasmid replication system was used to quantify replication from SV40 (blue) and HPV11 (orange) origins. **(A)** Basic replication assay in C33A cells. Cells were transfected with 2.5 or 10 ng of the SV40 large T antigen expression plasmid (pLT) or HPV11 E1 expression plasmid (p11E1), together with 2.5 ng of the corresponding origin-containing vector (pFLORI40 or pFLORI11), 7.5 ng of HPV11 E2 expression plasmid (p11E2, for HPV11 assays), and 1 ng of Renilla luciferase plasmid (RLuc) as an internal control. pUC19 carrier DNA was added to bring the total DNA to 100 ng per well. Maximum replication was observed at 10 ng LT or E1 in both C33A and AD293 cells, and relative replication efficiency was expressed as the percentage of the signal at this condition. To assess dependence on host DNA polymerases, aphidicolin was added immediately after the 4–6 h transfection period at 0, 5, 10, or 50 μM in parallel assays. **(B)** Replication assays in HaCaT and AD293 cells. Conditions were optimized to achieve $\geq 50\%$ transfection efficiency in HaCaT, comparable to AD293. Assays were performed in triplicate using HaCaT cells grown in low or high amounts of Ca^{2+} and AD293 cells in standard DMEM. Cells were transfected with 2.5 (white), 5 (grey), or 10 ng (black) p11E1, plus p11E2, pFLORI11, and RLuc at optimized ratios. DNA replication was both ori- and LT/E1-dependent in all three cell lines (C33A, AD293 and HaCaT). At 72 h post-transfection, firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System. Relative replication efficiency in HaCaT cells was normalized to the 10 ng E1 condition in low- Ca^{2+} HaCaT, defined as 100%.

Collectively, our data demonstrates that HaCaT cells provide a physiologically relevant *in vitro* model for transient HPV DNA replication assays. When maintained in a low- Ca^{2+} , subconfluent state, they support robust origin-dependent replication, and when shifted to high Ca^{2+} they reveal differentiation-linked enhancements in replication efficiency. This positions HaCaT as a useful platform for investigating both calcium- and differentiation-dependent regulation of HPV DNA replication in keratinocytes.

4. Conclusions

Overall, our findings show that modulation of extracellular Ca^{2+} enables a controlled comparison of basal and differentiation-associated states in HaCaT-based replication assays. Low Ca^{2+} preserves a basal, proliferative phenotype that is more amenable to plasmid uptake, whereas high Ca^{2+} induces differentiation, alters morphology, and shifts keratin expression ($\uparrow\text{KRT1}$, $\downarrow\text{KRT5}$) without enforcing cell-cycle exit in this immortalized line [32]. Our optimized protocols (subconfluent seeding, basal medium, reagent titration) yielded transfection efficiencies near the upper end of reported values ($\sim 50\%$ with Lipofectamine

3000 and TransIT-X2), including in HaCaT cells maintained under high- Ca^{2+} conditions. These parameters enabled robust HPV11 and SV40 DNA replication assays using dual-luciferase readouts, with kinetics and quantitative behavior comparable to established systems [16].

We also emphasize an important limitation: unlike normal keratinocytes, HaCaT differentiation is not tightly coupled to cell-cycle withdrawal [32]. Accordingly, while this model is well suited for studying episomal replication and early viral processes, it is less appropriate for late events requiring full stratification and barrier formation. Under defined culture conditions, HaCaT cells therefore provide a flexible experimental platform for probing early stages of the HPV life cycle. Finally, the ~1.8-fold increase in HPV11 replication observed under high- Ca^{2+} conditions highlights the utility of this system for dissecting differentiation-associated regulation of early episomal replication.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/v18020230/s1>, Table S1: Previously studied calcium-induced marker expression in HaCaT cells [7,8,24,32,34–38]; Table S2: Previous lipid based transient transfection studies in HaCaT cells [9,27,39–41]; Table S3: Use of HaCaT cells in previous HPV Replication Studies [12,42–48]; Figure S1: (A–L) Effect of calcium concentration, cell density, and transfection reagent on DNA delivery efficiency in HaCaT cells; Figure S2: (A–F) Optimization of Lipofectamine 3000 (LF3000)–mediated transfection in HaCaT cells under low-calcium conditions; Figure S3: (A–F) Optimization of TransIT-X2–mediated transfection in HaCaT cells under low-calcium conditions; Figure S4: (A–H) Optimization of FuGENE 6–mediated transfection in HaCaT cells under low-calcium conditions.

Author Contributions: Individual contributions to this work are as follows: Conceptualization, R.D.-R. and T.M.; methodology, R.D.-R.; investigation, R.D.-R.; visualization, R.D.-R.; writing—original draft preparation, R.D.-R.; writing—review and editing, R.D.-R. and T.M.; funding acquisition, T.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

HPV	Human papillomavirus
PyV	Polyomavirus
ORI/ori	Origin of DNA replication
GFP	Green fluorescence protein
KRT	Keratin
C33A	human cervical squamous cell carcinoma cell line (-HPV)
HEK293	Human embryonic kidney (transformed with adenovirus DNA)
CIN612	human cervical epithelial cell line infected with Human Papillomavirus type 31 (HPV-31).

References

- Longworth, M.S.; Laimins, L.A. Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol. Mol. Biol. Rev.* **2004**, *68*, 362–372. [\[CrossRef\]](#) [\[PubMed\]](#)
- Kazem, S.; van der Meijden, E.; Kooijman, S.; Rosenberg, A.S.; Hughey, L.C.; Browning, J.C.; Sadler, G.; Busam, K.; Pope, E.; Benoit, T.; et al. Trichodysplasia spinulosa is characterized by active polyomavirus infection. *J. Clin. Virol.* **2012**, *53*, 225–230. [\[CrossRef\]](#)
- Skok, K.; Gradisnik, L.; Maver, U.; Kozar, N.; Sobocan, M.; Takac, I.; Arko, D.; Kavalari, R. Gynaecological cancers and their cell lines. *J. Cell. Mol. Med.* **2021**, *25*, 3680–3698. [\[CrossRef\]](#) [\[PubMed\]](#)
- Li, J.X.; Zhang, J.; Li, C.H.; Li, Y.F.; Chen, H.M.; Li, T.; Zhang, Q.; Kong, B.H.; Wang, P.H. Human papillomavirus E1 proteins inhibit RIG-I/MDA5-MAVS, TLR3-TRIF, cGAS-STING, and JAK-STAT signaling pathways to evade innate antiviral immunity. *Front. Immunol.* **2025**, *16*, 1549766. [\[CrossRef\]](#)
- Hummel, M.; Hudson, J.B.; Laimins, L.A. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *J. Virol.* **1992**, *66*, 6070–6080. [\[CrossRef\]](#)
- Pyeon, D.; Pearce, S.M.; Lank, S.M.; Ahlquist, P.; Lambert, P.F. Establishment of human papillomavirus infection requires cell cycle progression. *PLoS Pathog.* **2009**, *5*, e1000318. [\[CrossRef\]](#)
- Boukamp, P.; Petrussevska, R.T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, N.E. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **1988**, *106*, 761–771. [\[CrossRef\]](#) [\[PubMed\]](#)
- Colombo, I.; Sangiovanni, E.; Maggio, R.; Mattozzi, C.; Zava, S.; Corbett, Y.; Fumagalli, M.; Carlino, C.; Corsetto, P.A.; Scaccabarozzi, D.; et al. HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes. *Mediat. Inflamm.* **2017**, *2017*, 7435621. [\[CrossRef\]](#)
- Deyrieux, A.F.; Wilson, V.G. In vitro culture conditions to study keratinocyte differentiation using the HaCaT cell line. *Cytotechnology* **2007**, *54*, 77–83. [\[CrossRef\]](#)
- Pavez Lorie, E.; Stricker, N.; Plitta-Michalak, B.; Chen, I.P.; Volkmer, B.; Greinert, R.; Jauch, A.; Boukamp, P.; Rapp, A. Characterisation of the novel spontaneously immortalized and invasively growing human skin keratinocyte line HaSKpw. *Sci. Rep.* **2020**, *10*, 15196. [\[CrossRef\]](#)
- Evans, M.R.; James, C.D.; Loughran, O.; Nulton, T.J.; Wang, X.; Bristol, M.L.; Windle, B.; Morgan, I.M. An oral keratinocyte life cycle model identifies novel host genome regulation by human papillomavirus 16 relevant to HPV positive head and neck cancer. *Oncotarget* **2017**, *8*, 81892–81909. [\[CrossRef\]](#)
- Orav, M.; Geimanen, J.; Sepp, E.M.; Henno, L.; Ustav, E.; Ustav, M. Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. *Sci. Rep.* **2015**, *5*, 15952. [\[CrossRef\]](#)
- Stanley, M.A.; Browne, H.M.; Appleby, M.; Minson, A.C. Properties of a non-tumorigenic human cervical keratinocyte cell line. *Int. J. Cancer* **1989**, *43*, 672–676. [\[CrossRef\]](#)
- Smits, J.P.H.; Niehues, H.; Rikken, G.; van Vlijmen-Willems, I.; van de Zande, G.; Zeeuwen, P.; Schalkwijk, J.; van den Bogaard, E.H. Immortalized N/TERT keratinocytes as an alternative cell source in 3D human epidermal models. *Sci. Rep.* **2017**, *7*, 11838. [\[CrossRef\]](#)
- Moran, M.C.; Pandya, R.P.; Leffler, K.A.; Yoshida, T.; Beck, L.A.; Brewer, M.G. Characterization of Human Keratinocyte Cell Lines for Barrier Studies. *JID Innov.* **2021**, *1*, 100018. [\[CrossRef\]](#)
- Fradet-Turcotte, A.; Morin, G.; Lehoux, M.; Bullock, P.A.; Archambault, J. Development of quantitative and high-throughput assays of polyomavirus and papillomavirus DNA replication. *Virology* **2010**, *399*, 65–76. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ustav, M.; Ustav, E.; Szymanski, P.; Stenlund, A. Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. *EMBO J.* **1991**, *10*, 4321–4329. [\[CrossRef\]](#)
- Bergvall, M.; Melendy, T.; Archambault, J. The E1 proteins. *Virology* **2013**, *445*, 35–56. [\[CrossRef\]](#) [\[PubMed\]](#)

19. An, P.; Saenz Robles, M.T.; Pipas, J.M. Large T antigens of polyomaviruses: Amazing molecular machines. *Annu. Rev. Microbiol.* **2012**, *66*, 213–236. [[CrossRef](#)] [[PubMed](#)]
20. Fisk, J.C.; Chojnacki, M.D.; Melendy, T. Replicative Helicases as the Central Organizing Motor Proteins in the Molecular Machines of the Elongating Eukaryotic Replication Fork. In *The Mechanisms of DNA Replication*; Stuart, D., Ed.; InTechOpen: Hamilton, NJ, USA, 2013; Volume 1, pp. 29–57.
21. Shin, J.; Phelan, P.J.; Chhum, P.; Bashkenova, N.; Yim, S.; Parker, R.; Gagnon, D.; Gjoerup, O.; Archambault, J.; Bullock, P.A. Analysis of JC virus DNA replication using a quantitative and high-throughput assay. *Virology* **2014**, *468–470*, 113–125. [[CrossRef](#)]
22. Graham, S.V. Keratinocyte Differentiation-Dependent Human Papillomavirus Gene Regulation. *Viruses* **2017**, *9*, 245. [[CrossRef](#)]
23. Zwolinska, K.; Bienkowska-Haba, M.; Scott, R.S.; Keiffer, T.; Sapp, M. Experimental Support for Human Papillomavirus Genome Amplification Early after Infectious Delivery. *J. Virol.* **2023**, *97*, e0021423. [[CrossRef](#)] [[PubMed](#)]
24. Borowiec, A.S.; Delcourt, P.; Dewailly, E.; Bidaux, G. Optimal differentiation of in vitro keratinocytes requires multifactorial external control. *PLoS ONE* **2013**, *8*, e77507. [[CrossRef](#)] [[PubMed](#)]
25. Steele, B.K.; Meyers, C.; Ozbun, M.A. Variable expression of some “housekeeping” genes during human keratinocyte differentiation. *Anal. Biochem.* **2002**, *307*, 341–347. [[CrossRef](#)]
26. Ruan, W.; Lai, M. Actin, a reliable marker of internal control? *Clin. Chim. Acta* **2007**, *385*, 1–5. [[CrossRef](#)]
27. Yong, W.; Peng, D.; Wang, L.; Dong, Z.; He, B. Screening of HaCaT clones for CCL20 gene knockout and preliminary exploration of gene-targeting vector transfection approaches in this cell line. *Med. Sci. Monit. Basic Res.* **2015**, *21*, 21–28. [[CrossRef](#)]
28. Distler, J.H.; Jungel, A.; Kurowska-Stolarska, M.; Michel, B.A.; Gay, R.E.; Gay, S.; Distler, O. Nucleofection: A new, highly efficient transfection method for primary human keratinocytes. *Exp. Dermatol.* **2005**, *14*, 315–320. [[CrossRef](#)]
29. Vij, R.; Lin, Z.; Schneider, K.; Seshasayee, D.; Koerber, J.T. Analysis of the effect of promoter type and skin pretreatment on antigen expression and antibody response after gene gun-based immunization. *PLoS ONE* **2018**, *13*, e0197962. [[CrossRef](#)] [[PubMed](#)]
30. Gagnon, D.; Fradet-Turcotte, A.; Archambault, J. A quantitative and high-throughput assay of human papillomavirus DNA replication. *Methods Mol. Biol.* **2015**, *1249*, 305–316. [[CrossRef](#)]
31. Gagnon, D.; Senechal, H.; D’Abramo, C.M.; Alvarez, J.; McBride, A.A.; Archambault, J. Genetic analysis of the E2 transactivation domain dimerization interface from bovine papillomavirus type 1. *Virology* **2013**, *439*, 132–139. [[CrossRef](#)]
32. Micallef, L.; Belaubre, F.; Pinon, A.; Jayat-Vignoles, C.; Delage, C.; Charveron, M.; Simon, A. Effects of extracellular calcium on the growth-differentiation switch in immortalized keratinocyte HaCaT cells compared with normal human keratinocytes. *Exp. Dermatol.* **2009**, *18*, 143–151. [[CrossRef](#)]
33. Poumay, Y.; Pittelkow, M.R. Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. *J. Investig. Dermatol.* **1995**, *104*, 271–276. [[CrossRef](#)]
34. Hohl, D.; Lichti, U.; Breitkreutz, D.; Steinert, P.M.; Roop, D.R. Transcription of the Human Loricrin Gene In Vitro Is Induced by Calcium and Cell Density and Suppressed by Retinoic Acid. *J. Investig. Dermatol.* **1991**, *96*, 414–418. [[CrossRef](#)] [[PubMed](#)]
35. Pleguezuelos, O.; Kapas, S. Differentiation of the HaCaT keratinocyte cell line: Modulation by adrenomedullin. *Br. J. Dermatol.* **2006**, *154*, 602–608. [[CrossRef](#)] [[PubMed](#)]
36. Sark, M.W.J.; Fischer, D.F.; de Meijer, E.; van de Putte, P.; Backendorf, C. AP-1 and Ets Transcription Factors Regulate the Expression of the Human SPRR1A Keratinocyte Terminal Differentiation Marker. *J. Biol. Chem.* **1998**, *273*, 24683–24692. [[CrossRef](#)] [[PubMed](#)]
37. Denning, M.F.; Guy, S.G.; Ellerbroek, S.M.; Norvell, S.M.; Kowalczyk, A.P.; Green, K.J. The Expression of Desmoglein Isoforms in Cultured Human Keratinocytes Is Regulated by Calcium, Serum, and Protein Kinase C. *Exp. Cell Res.* **1998**, *239*, 50–59. [[CrossRef](#)]
38. Du, G.-G.; Luo, X.-D.; Qiu, J.-H.; Zhang, L.-L. Effect of calcium on the activity and expression of integrin beta1 promoter in HaCaT cells and cell migration. *Chin. J. Burn.* **2010**, *26*, 207–211.
39. Steintraesser, L.; Hirsch, T.; Beller, J.; Mittler, D.; Sorkin, M.; Pazdierny, G.; Jacobsen, F.; Eriksson, E.; Steinau, H.U. Transient non-viral cutaneous gene delivery in burn wounds. *J. Gene Med.* **2007**, *9*, 949–955. [[CrossRef](#)]
40. Hung, C.-F.; Hwang, T.-L.; Chang, C.-C.; Fang, J.-Y. Physicochemical characterization and gene transfection efficiency of lipid emulsions with various co-emulsifiers. *Int. J. Pharm.* **2004**, *289*, 197–208. [[CrossRef](#)]
41. Jacobsen, F.; Mertens-Rill, J.; Beller, J.; Hirsch, T.; Daigeler, A.; Langer, S.; Lehnhardt, M.; Steinau, H.-U.; Steintraesser, L. Nucleofection: A New Method for Cutaneous Gene Transfer? *BioMed Res. Int.* **2006**, *2006*, 26060. [[CrossRef](#)]
42. Geimanen, J.; Isok-Paas, H.; Pipitch, R.; Salk, K.; Laos, T.; Orav, M.; Reinson, T.; Ustav, M.; Ustav, E. Development of a Cellular Assay System To Study the Genome Replication of High- and Low-Risk Mucosal and Cutaneous Human Papillomaviruses. *J. Virol.* **2011**, *85*, 3315–3329. [[CrossRef](#)] [[PubMed](#)]
43. Wang, Y.; Li, X.; Song, S.; Wu, J. Development of Basal-Like HaCaT Keratinocytes Containing the Genome of Human Papillomavirus (HPV) Type 11 for Screening of Anti-HPV Effects. *SLAS Discov. Adv. Sci. Drug Discov.* **2014**, *19*, 1154–1163. [[CrossRef](#)]
44. Wang, Y.; Li, X.; Song, S.; Sun, Y.; Zhang, J.; Yu, C.; Chen, W. HPV11 E6 mutation by overexpression of APOBEC3A and effects of interferon- ω on APOBEC3s and HPV11 E6 expression in HPV11.HaCaT cells. *Virol. J.* **2017**, *14*, 211. [[CrossRef](#)]

45. Kaczkowski, B.; Rossing, M.; Andersen, D.K.; Dreher, A.; Morevati, M.; Visser, M.A.; Winther, O.; Nielsen, F.C.; Norrild, B. Integrative analyses reveal novel strategies in HPV11,-16 and -45 early infection. *Sci. Rep.* **2012**, *2*, 515. [\[CrossRef\]](#)
46. Bojilova, E.D.; Weyn, C.; Antoine, M.-H.; Fontaine, V. Extrachromosomal HPV-16 LCR transcriptional activation by HDACi opposed by cellular differentiation and DNA integration. *Oncotarget* **2016**, *7*, 75526–75538. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Hochmann, J.; Millan, M.; Hernandez, P.; Lafon-Hughes, L.; Aiuto, N.; Silva, A.; Llaguno, J.; Alonso, J.; Fernandez, A.; Pereira-Prado, V.; et al. Contributions of viral oncogenes of HPV-18 and hypoxia to oxidative stress and genetic damage in human keratinocytes. *Sci. Rep.* **2023**, *13*, 17734. [\[CrossRef\]](#)
48. Griffin, L.M.; Cicchini, L.; Xu, T.; Pyeon, D. Human keratinocyte cultures in the investigation of early steps of human papillomavirus infection. *Methods Mol. Biol.* **2014**, *1195*, 219–238. [\[CrossRef\]](#)
49. Boukamp, P.; Popp, S.; Altmeyer, S.; Hulsen, A.; Fasching, C.; Cremer, T.; Fusenig, N.E. Sustained nontumorigenic phenotype correlates with a largely stable chromosome content during long-term culture of the human keratinocyte line HaCaT. *Genes Chromosomes Cancer* **1997**, *19*, 201–214. [\[CrossRef\]](#)
50. Lehman, T.A.; Modali, R.; Boukamp, P.; Stanek, J.; Bennett, W.P.; Welsh, J.A.; Metcalf, R.A.; Stampfer, M.R.; Fusenig, N.; Rogan, E.M.; et al. p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis* **1993**, *14*, 833–839. [\[CrossRef\]](#)
51. Lewis, D.A.; Hengeltraub, S.F.; Gao, F.C.; Leivant, M.A.; Spandau, D.F. Aberrant NF-kappaB activity in HaCaT cells alters their response to UVB signaling. *J. Investig. Dermatol.* **2006**, *126*, 1885–1892. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Maytin, E.V.; Habener, J.F. Transcription factors C/EBP alpha, C/EBP beta, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. *J. Investih. Dermatol.* **1998**, *110*, 238–246. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Gilson, T.D.; Gibson, R.T.; Androphy, E.J. Optimization of human papillomavirus-based pseudovirus techniques for efficient gene transfer. *Sci. Rep.* **2020**, *10*, 15517. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Bergvall, M.; Gagnon, D.; Titolo, S.; Lehoux, M.; D'Abramo, C.M.; Melendy, T.; Archambault, J. Requirement for the E1 Helicase C-Terminal Domain in Papillomavirus DNA Replication In Vivo. *J. Virol.* **2016**, *90*, 3198–3211. [\[CrossRef\]](#)
55. Xie, F.; DeSmet, M.; Kanginakudru, S.; Jose, L.; Culleton, S.P.; Gilson, T.; Li, C.; Androphy, E.J. Kinase Activity of Fibroblast Growth Factor Receptor 3 Regulates Activity of the Papillomavirus E2 Protein. *J. Virol.* **2017**, *91*, e01066-17. [\[CrossRef\]](#)
56. DeSmet, M.; Kanginakudru, S.; Rietz, A.; Wu, W.H.; Roden, R.; Androphy, E.J. The Replicative Consequences of Papillomavirus E2 Protein Binding to the Origin Replication Factor ORC2. *PLoS Pathog.* **2016**, *12*, e1005934. [\[CrossRef\]](#)
57. Kanginakudru, S.; DeSmet, M.; Thomas, Y.; Morgan, I.M.; Androphy, E.J. Levels of the E2 interacting protein TopBP1 modulate papillomavirus maintenance stage replication. *Virology* **2015**, *478*, 129–135. [\[CrossRef\]](#)
58. Lehoux, M.; Fradet-Turcotte, A.; Lussier-Price, M.; Omichinski, J.G.; Archambault, J. Inhibition of human papillomavirus DNA replication by an E1-derived p80/UAF1-binding peptide. *J. Virol.* **2012**, *86*, 3486–3500. [\[CrossRef\]](#)
59. Moody, C.A.; Laimins, L.A. Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. *PLoS Pathog.* **2009**, *5*, e1000605. [\[CrossRef\]](#)
60. Hong, S.; Mehta, K.P.; Laimins, L.A. Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. *J. Virol.* **2011**, *85*, 9486–9494. [\[CrossRef\]](#) [\[PubMed\]](#)
61. Kajitani, N.; Satsuka, A.; Kawate, A.; Sakai, H. Productive Lifecycle of Human Papillomaviruses that Depends Upon Squamous Epithelial Differentiation. *Front. Microbiol.* **2012**, *3*, 152. [\[CrossRef\]](#)

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