Supply Chain Disruptions During COVID-19 Pandemic Uncover Differences in Keratinocyte Culture Media

Mary C. Moran[1,2,3], Eleanor M. Pope[1,3], Matthew G. Brewer[1] and Lisa A. Beck[1]

Various culture media are used to propagate keratinocytes (KCs) in vitro. The COVID-19 pandemic resulted in supply chain shortages necessitating substitutions to standard laboratory protocols, which resulted in many laboratories having to use culture media different from those they typically use. We screened available media on the KC line N/TERT2G and found that biological responses varied considerably across three culture media: KC serum-free media, KC growth medium 2, and defined media. We observed qualitative and quantitative differences in proliferation; KCs cultured in defined media had significantly lower proliferative capacity. KC differentiation was assessed by western blot for CLDN1, occludin, cytokeratin-10, and loricrin. Elevated expression of differentiation markers was observed in cells cultured in either KC growth medium 2 or defined media compared with those in cells cultured in KC serum-free media. KC barrier function was measured by transepithelial electrical resistance. KCs cultured in KC growth medium 2 and defined media developed significantly higher transepithelial electrical resistance than those cultured in KC serum-free media, and when treated with IL-4 and IL-13 or IL-17A, we observed variable responses. H&E staining on day 5 -post-differentiation showed greater epithelial thickness in KCs cultured in defined media and KC growth medium 2 than in those cultured in KC serum-free media. These findings show that the choice of culture media impacts the biological response of KCs in a manner that persists through differentiation in the same media.

JID Innovations (2022);**2:**100151 doi:10.1016/j.xjidi.2022.100151

INTRODUCTION

Within the field of epidermal biology, various culture media are used to propagate keratinocytes (KCs). Although the same media is commonly used within a laboratory, supply chain disruptions that occurred throughout the COVID-19 pandemic required our laboratory and laboratories around the globe to identify substitutions for many standard protocols. We found that the use of alternative KC culture media gave us highly variable results in the immortalized KC line, N/TERT2G (Dickson et al., 2000; Moran et al., 2021).

Differences in cell behavior based on culture media have been observed by other groups. Broadbent et al. (2020) found that the type of media used for differentiation and maintenance of airway epithelial cells impacts experimental results, including morphology, epithelial integrity, and response to viral infection. Zorn-Kruppa et al. (2016) also commented on KC characteristics altered by the propagation media. Key differences to note in these studies include that (i) primary KCs were used, (ii) comparison was performed with propagation media different from the one we studied (e.g., KC growth medium 2 [KGM2], DermaLife, and EpiLife; some of these media and supplements were back ordered so we were unable to include them in our study), and (iii) they used a protocol for KC differentiation (high calcium added to the culture media rather than differentiation media), which is not commonly used.

We sought to assess relevant biological responses (i.e., proliferation, protein expression, barrier function, etc.) of KCs cultured in different media. Even though researchers may be aware that cell behavior may vary with media composition, we hoped to share the significant differences that can be observed among culture media and further highlight that media is not interchangeable.

In this study, we assessed the question of whether the choice of culture media affects critical aspects of KC biology. We measured several characteristics of the KC cell line N/TERT2G, which were propagated in three routinely used culture media: KC serum-free media (KSFM) (number 17005042, Gibco, Waltham, MA), KGM2 (number C-20211, PromoCell, Heidelberg, Germany), and defined media (number 10744019, Gibco) (Table 1). We focused on four KC assays: proliferation, differentiation marker expression, barrier function, and susceptibility to viral infection (Figure 1).

[1]*Department of Dermatology, University of Rochester Medical Center, Rochester, New York, USA; and* [2]*Department of Microbiology & Immunology, University of Rochester Medical Center, Rochester, New York, USA*

[3]*These authors contributed equally to this work.*

Correspondence: Mary C. Moran, Department of Dermatology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, New York 14642, USA. E-mail: mary_moran@urmc.rochester.edu

Abbreviations: KC, keratinocyte; KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; OCLN, occludin; TEER, transepithelial electrical resistance

Received 10 March 2022; revised 18 July 2022; accepted 20 July 2022; accepted manuscript published online XXX; corrected proof published online XXX

Cite this article as: JID Innovations 2022;2:100151

Table 1. Media Composition

Media Type	Vendor	Catalog Number	Known Supplements Added by Vendor (Concentration)	Supplements Added by Our Laboratory (Concentration)	Calcium Concentration (mM)
Keratinocyte Serum-Free Medium (KSFM)	Gibco	17005042	BPE (0.2% v/v) EGF (5 ng/ml)	Penicillin (10 units/ml) Streptomycin (10 μg/ml) Amphotericin B (500 ng/ml)	0.09
KGM2	PromoCell	C20111	BPE (0.4% v/v) EGF (0.125 ng/ml) Insulin (5 μg/ml) Hydrocortisone (0.33 μg/ml) Epinephrine (0.39 μg/ml) Transferrin (10 μg/ml) $CaCl_2$ (0.06 mM)	Penicillin (10 units/ml) Streptomycin (10 μg/ml) Amphotericin B (500 ng/ml)	0.06
Defined media	Gibco	10744019	No information was provided on supplements	Penicillin (10 units/ml) Streptomycin (10 μg/ml) Amphotericin B (500 ng/ml)	0.09
EpiLife	Cascade Biologics	MEPI500CA	BPE (0.2% v/v) EGF (0.2 ng/ml) Insulin (5μg/ml) Hydrocortisone (0.18 μg/ml) Transferrin (5 μg/ml) $CaCl_2$ (0.06 mM)	Penicillin (10 units/ml) Streptomycin (10 μg/ml) Amphotericin B (500 ng/ml)	0.06
DermaLife	LifeLine Cell Technology		BPE (0.4%) Insulin (5 μg/ml) Hydrocortisone (0.08 μg/ml) Epinephrine (0.33 μg/ml) Transferrin (5 μg/ml) $CaCl_2$ (0.05 mM) TGFα (0.5 ng/ml) L-glutamine (6 mM) Penicillin/streptomycin (100 μg/ml)		

Abbreviations: BPE, bovine pituitary extract; $CaCl_2$, calcium chloride; KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media.

RESULTS AND DISCUSSION

When propagating N/TERT2G in KSFM, KGM2, and defined media, we observed similar cell morphology but differences in how quickly culture flasks reached confluency (Figure 2a). To quantify changes in proliferation across different media, we used the Click-iT EdU Proliferation Assay. N/TERT2G cells were plated in 96-well plates (28,000 cells/well), and after 24 hours, 10 μM 5-ethynyl-2′-deoxyuridine was added to each

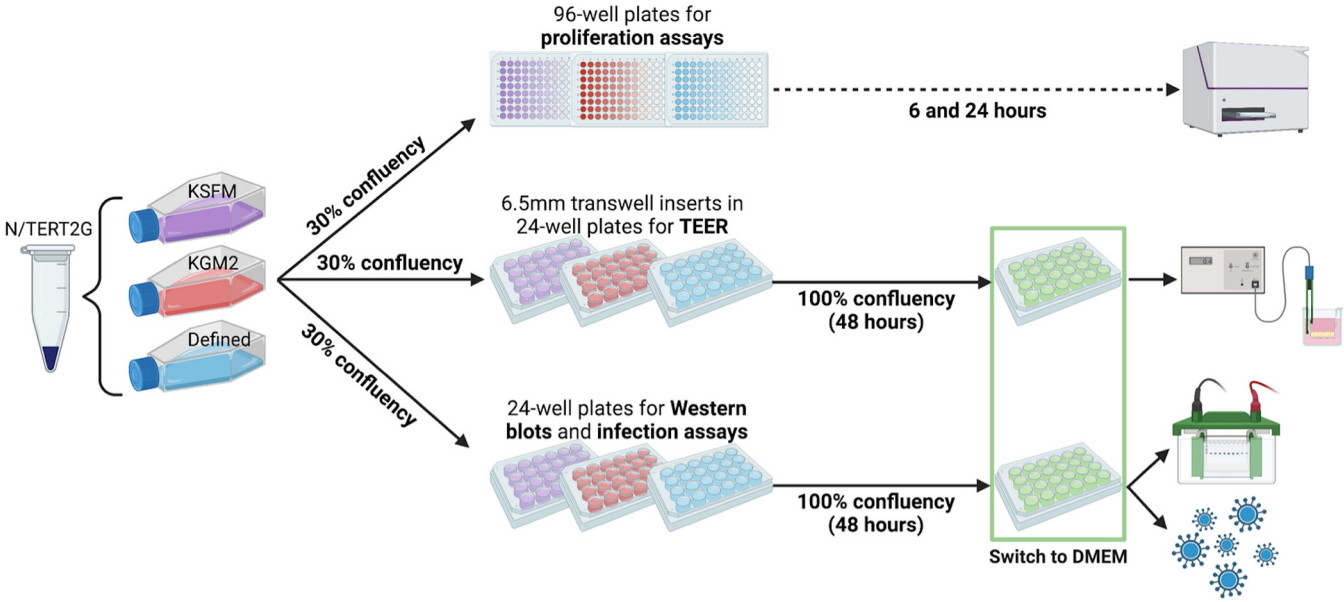

Figure 1. Schematic of cell culturing and experimental methods. N/TERT2G cells were cultured in different media, including KSFM, KGM2, and defined media. Once they reached 30% confluency, cells were plated at equal numbers in 96-well plates for proliferation assays, 6.5 mm transwell inserts in 24-well plates transwells for TEER assays, or 24-well plates for western and infection assays. In all assays, cells reached confluency 48 hours after plating (24-well pates/transwells) and were switched to DMEM media containing calcium (1.8 mM) to initiate differentiation. The Schematic was created using BioRender.com. KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; TEER, transepithelial electrical resistance.

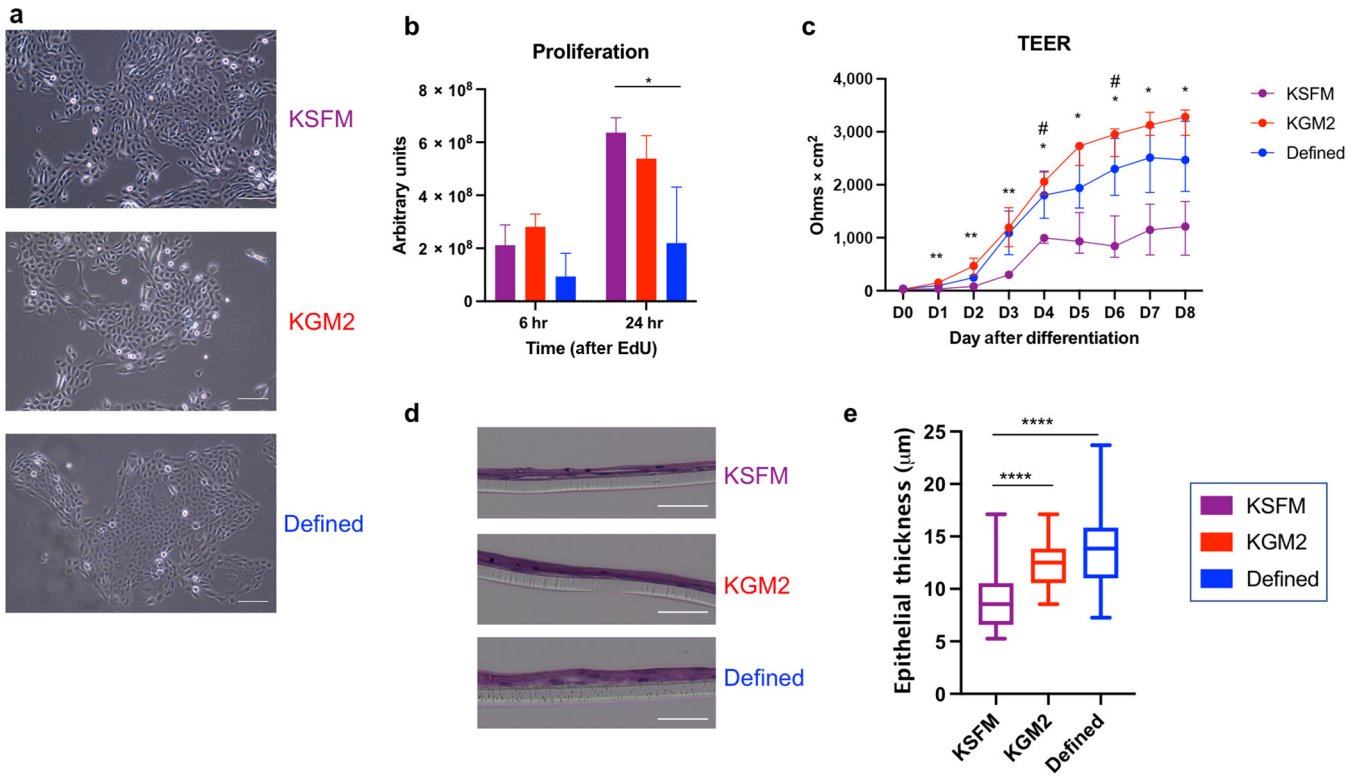

Figure 2. Propagation of KCs in KGM2 and defined media leads to greater barrier integrity and epithelial thickness than in KSFM. (**a**) Cells were cultured in KSFM, KGM2, or defined media. Cell morphology was similar across all media. Bar = 200 μm. (**b**) Proliferation was measured in undifferentiated cells cultured in KSFM, KGM2, or defined media using the Click-iT EdU assay 6 and 24 hours after addition of EdU (n = 3 experiments). Data were analyzed using Friedman test with Dunn's posthoc test (pairwise). Cells were cultured in KSFM, KGM2, or defined media, grown to confluency, and switched to DMEM media containing calcium (1.8 mM) to initiate differentiation. (**c**) TEER was measured for 8 days after differentiation (n = 5 experiments). Data were analyzed using Friedman test with Dunn's posthoc test (pairwise) comparing KSFM versus KGM2 (*) or KSFM versus defined media (#). (**d**) Membranes were removed from transwells on D5, formalin fixed, processed, embedded, sectioned, and stained for H&E. Bar = 50 μm. (**e**) Epithelial thickness was measured across five representative images per media type; data were analyzed with Kruskal–Wallis test with Dunn's posthoc test (unpaired). Significance: *#$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are presented as median (Q1, Q3). D, day; EdU, 5-ethynyl-2′-deoxyuridine; hr, hour; KC, keratinocyte; KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; Q, quartile; TEER, transepithelial electrical resistance.

well and incubated for 6–24 hours (Figure 1). Before adding the 5-ethynyl-2′-deoxyuridine, we observed decreased confluency of cells cultured in defined media, whereas confluency was comparable for cells cultured in KSFM and KGM2 (data not shown). This finding was confirmed by the 5-ethynyl-2′-deoxyuridine fluorescent readout, which showed a significant decrease in the proliferative capacity of N/TERT2G cells grown in defined media compared with those grown in KSFM ($P < 0.05$) (Figure 2b). In all media, we observed increased proliferation from 6 to 24 hours; however, the degree of this was lower in cells cultured in defined media (Figure 2b).

As KCs differentiate, their proliferative capacity decreases, and they form tight junctions, which are critical for establishing an epithelial barrier. Transepithelial electrical resistance (TEER) is a widely accepted and quantitative measure of tight junction function, where higher TEER values indicate greater barrier function. N/TERT2G cells (75,000 cells/well) were plated in transwells (6.5 mm insert, 0.4 μm polyester membrane, Costar, Corning, NY) and grown to confluency (Figure 1). TEER was measured before inducing differentiation (day 0) and every day after (days 1–8), with media replacements performed every 2 days. Notably, continued growth in KSFM, KGM2, and defined media (without switching to DMEM) did not result in increased TEER (data not shown). After inducing differentiation with DMEM media, we observed that KCs cultured in KGM2 ($P < 0.01$) or defined media ($P < 0.05$) rapidly developed significantly higher TEER than cells initially cultured in KSFM (Figure 2c). This indicates that the initial propagation of KCs in defined media or KGM2 promotes greater barrier function of differentiated cells. H&E staining showed greater stratification and epithelial thickness ($P < 0.001$) in cells that had been cultured in defined media or KGM2 than in those cultured in KSFM, which also may explain the increased level of TEER observed with defined media and KGM2 (Figure 2d and e). We could not delineate from the specimens whether the thicker epidermis was a consequence of taller cells or simply more layers of cells.

To further understand the characteristics of N/TERT2G cells grown in these different media, we analyzed the expression of differentiation markers by western blot analysis (Goleva et al., 2019). On reaching confluency, KCs were switched to high calcium DMEM media (1.8 mM) to induce differentiation (Bikle et al., 2012). In undifferentiated KCs, we observed surprising differences in the expression of the

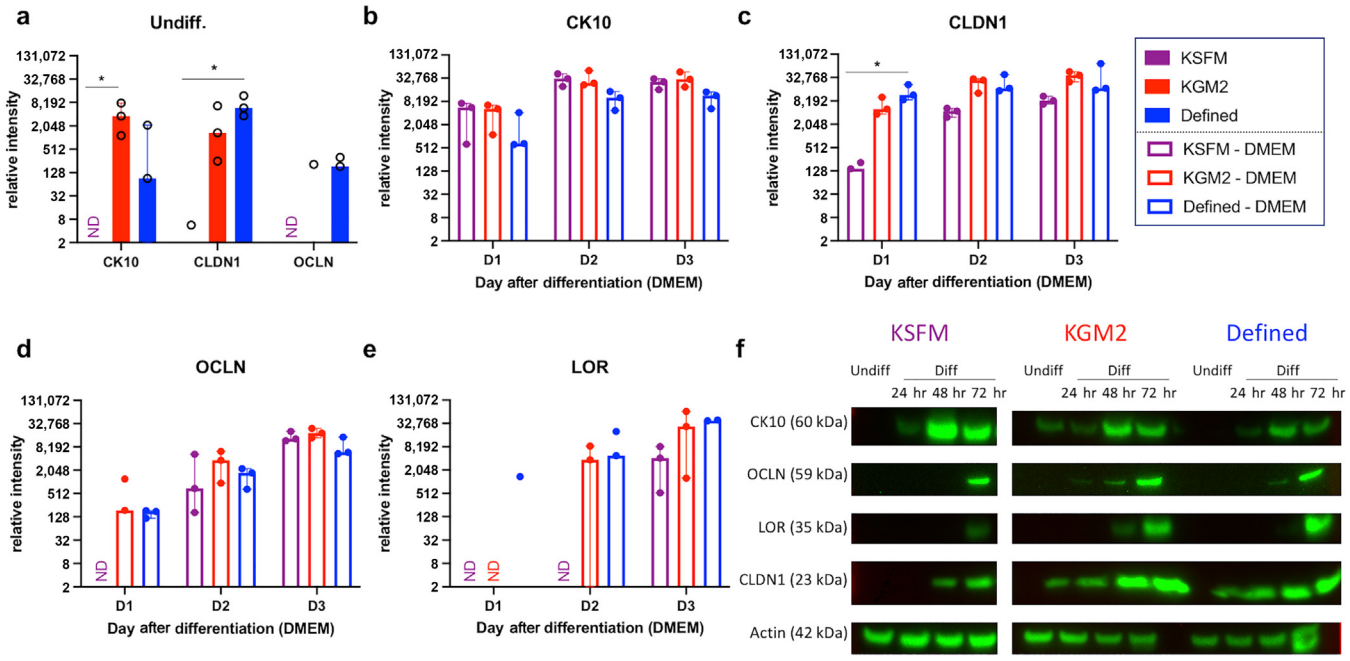

Figure 3. Markers of KC differentiation are significantly impacted by propagation media. Cells were cultured in KSFM, KGM2, or defined media; grown to confluency; and switched to DMEM media containing calcium (1.8 mM) to initiate differentiation. Cell lysates were (**a**) collected from Undiff KCs 24 hours after reaching confluency and (**b–e**) collected from Diff KCs on D1, D2, and D3 after differentiation. Differentiation markers CK10 and LOR and tight junction proteins CLDN1 and OCLN were detected by western blot analysis. (**f**) A representative blot for each protein is provided. Expression was quantified by densitometry with normalization to β-actin expression. n = 3 experiments. Graphs with <3 points indicate that protein was ND in experiments. Solid bars indicate Undiff KCs in respective media; open bars indicate KCs that have been switched from respective media to DMEM. Friedman test with Dunn's posthoc test (pairwise) of KSFM versus KGM2 or KSFM versus defined media. Significance: *$P < 0.05$. Data are presented as median (Q1, Q3). CK10, cytokeratin 10; D, day; Diff, differentiated; hr, hour; KC, keratinocyte; KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; LOR, loricrin; ND, not detected; OCLN, occludin; Q, quartile; Undiff, undifferentiated.

barrier proteins CLDN1 and occludin (OCLN) and a commonly used marker of differentiation, cytokeratin-10. These proteins were below the limit of detection in nearly all the samples cultured in KSFM, whereas expression was detected in samples cultured in either KGM2 or defined media (Figure 3a and f). Undifferentiated cells are reminiscent of cells within the stratum basale of the epidermis, where OCLN and cytokeratin-10 are not typically detected at the protein level; therefore, it is surprising that we could detect these proteins in undifferentiated KCs (Fuchs and Green, 1980; Kirschner et al., 2010). This suggests that KCs cultured in KSFM have protein expression similar to in vivo observations, whereas culturing in KGM2 or defined media may prime cells for differentiation. On differentiation, we observed earlier and/or greater expression of the differentiation markers and barrier proteins CLDN1, OCLN, and loricrin in cells that had been cultured in KGM2 or defined media than in KCs cultured in KSFM (Figure 3b–f). The expression of these proteins, although significantly lower in undifferentiated KCs (KSFM) ($P < 0.05$), became comparable in differentiated cells. These findings reveal that culturing KCs in different media fundamentally alters the cells in ways that impact protein expression in both undifferentiated and differentiated KCs, even after switching to the same media.

The significantly increased barrier function observed in KCs propagated in defined media or KGM2 (Figure 2b) may be explained by our western blot findings, in which two important tight junction proteins, CLDN1 and OCLN, were already detected in undifferentiated KCs propagated in defined media or KGM2 (Figure 3a). Furthermore, the relative intensity of CLDN1 and OCLN strongly correlated with TEER values of matched samples (Table 2).

Our laboratory studies atopic dermatitis and frequently employs the in vitro model of KCs conditioned with type 2 cytokines IL-4 and IL-13 (IL-4/13) to study epidermal biology, with a focus on barrier development (Figure 4a). We observed a range of responses to IL-4/13 treatment, with KSFM-propagated KCs showing an initial drop in TEER (days 2–3), followed by a significant increase in TEER at later timepoints (days 5–8) ($P < 0.05$). Cells cultured in defined media showed no significant TEER changes after IL-4/13 treatment. Finally, we observed a modest but significant decrease in TEER on IL-4/13 treatment in cells that had been cultured in KGM2 (day 6) ($P < 0.05$). To test whether this difference in response to cytokine stimulation was observed with other cytokines, we treated KCs with the type 3 cytokine commonly observed in psoriasis, IL-17A. Similar to IL-4/13 stimulation, we saw a range of responses depending on which media KCs had been propagated in (Figure 4b). KCs propagated in KSFM showed a transient enhancement in TEER (days 2–3), followed by a significant decrease in TEER (days 4–5) ($P < 0.05$). KCs propagated in KGM2 also resulted in a significant decrease in TEER from day 4 to day 8 ($P < 0.01–0.05$). Similar to treatment with IL-4/13, treatment with IL-17A had no significant impact on TEER in KCs propagated in defined media, although a modest decrease was observed.

Table 2. Barrier Function Correlates with the Expression of Tight Junction Proteins

Media	Tight Junction Protein	Pearson r	95% Confidence Interval	*P*-Value
KSFM	CLDN1	0.921	0.435–0.992	0.009
KSFM	OCLN	0.951	0.613–0.995	0.004
KGM2	CLDN1	0.948	0.591–0.994	0.004
KGM2	OCLN	0.984	0.855–0.998	0.0004
Defined media	CLDN1	0.636	−0.363 to 0.955	0.175
Defined media	OCLN	0.985	0.865–0.998	0.0003

Abbreviations: KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; OCLN, occludin; TEER, transepithelial electrical resistance.

TEER and relative intensity of tight junction proteins CLDN1 and OCLN, as measured by western blot quantification, were analyzed for correlation using the Pearson correlation test over the course of 3 days of differentiation. n = 2 matched sample experiments.

This highlights that the culture media used may change the KC inflammatory response. It is possible that culturing KCs in these different media results in differential expression of cytokine receptors, which may contribute to the variable effects of IL-4/13 and IL-17A on KC barrier function.

We have previously shown the state of KC differentiation impacts susceptibility to infection with vaccinia virus (Moran et al., 2021). Because western blot and TEER findings suggest differences in the kinetics of differentiation in cells propagated in defined media or KGM2 from those propagated in KSFM media, we evaluated whether susceptibility to viral infection differed. N/TERT2G cells were grown to confluence, and cells were infected with a low multiplicity of infection of vaccinia virus 1 or 2 days after differentiation. Undifferentiated KCs were similarly infected. We observed comparable infection kinetics over the course of differentiation across the three media. Undifferentiated KCs were most resistant to infection, day 1 differentiated KCs were more susceptible, and day 2 differentiated KCs became less susceptible to infection. Infection was significantly greater in undifferentiated cells (KGM2) than in KSFM ($P < 0.05$), which may be partially due to the more differentiated-like nature of KCs cultured in KGM2, which is supported by the western blot analysis showing increased levels of differentiation markers in KCs grown in this media (Figure 3a). There were no significant differences in infection among the day 1– or day 2–infected cells across the media, indicating that the overall kinetics of susceptibility is similar among the different media types (Figure 5).

The use of different culture media fundamentally alters KCs in a manner that persists even after switching cells to the same media (DMEM) (Table 3). These studies show that culture conditions are able to induce lasting changes in KCs that impact numerous biological functions. Although understanding which components of each media are responsible for such changes is critically important, the formulations of these media are in part proprietary, and therefore we are unable to test individual components. Our results highlight specific differences between commonly used and commercially available media, so that laboratories may be more aware of what results to expect depending on their media of choice. These findings may also support why specific experimental results may not be repeatable across laboratories if different culture media are being used. This observation should motivate the field to be precise in our experimental methodology reporting, particularly regarding which media and supplements are used and from which vendor.

MATERIALS AND METHODS

Cell cultures

N/TERT2G cells were provided by Ellen H. van den Bogaard and grown to 30% confluency as previously described (Dickson et al., 2000; Moran et al., 2021; Smits et al., 2017). Cells were cultured in one of the three media: KSFM (number 17005042, Gibco), KGM2 (number C-20211, PromoCell), and defined media (number 10744019, Gibco). N/TERT2G cells were switched to DMEM media supplemented with 4 mM glutamine and 1.8 mM calcium ion to induce differentiation. Days after differentiation refers to the number of days since exposure to DMEM. Representative images were taken

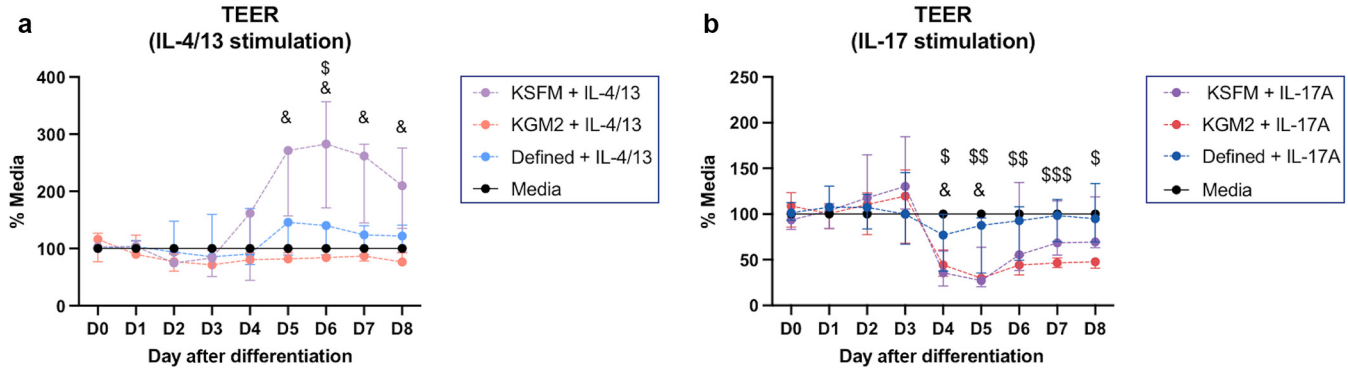

Figure 4. KC propagation media significantly impact barrier responses to cytokine stimulation. Cells were cultured in KSFM, KGM2, or defined media; grown to confluency; and switched to DMEM media containing calcium (1.8 mM) to initiate differentiation. Cells were treated with (**a**) 50 ng/ml of IL-4/13 (n = 3 experiments) or (**b**) 50 ng/ml of IL-17A (n = 4 experiments) at the time of differentiation, and TEER was measured for 8 days after differentiation. Paired *t*-test comparing KSFM with KSFM + cytokines (&) or KGM2 with KGM2 + cytokines ($); black points indicate matched untreated media controls. Significance: $,&$P < 0.05$, $$$P < 0.01$, and $$$$P < 0.001$. Data are presented as median (Q1, Q3). D, day; KC, keratinocyte; KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; Q, quartile; TEER, transepithelial electrical resistance.

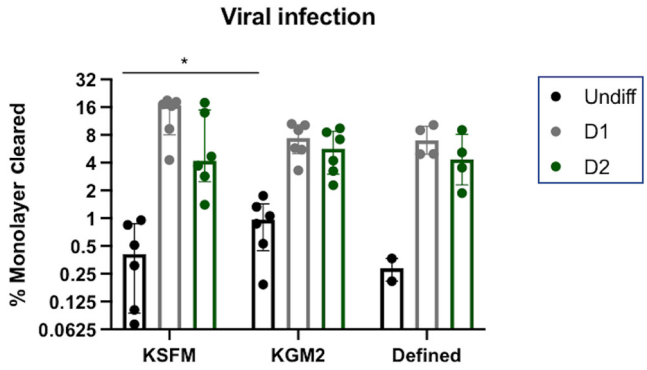

Figure 5. Viral infection kinetics are similar across KSFM, KGM2, and defined media culture media. Cells were cultured in KSFM, KGM2, or defined media; grown to confluency; and switched to DMEM media containing calcium (1.8 mM) to initiate differentiation. Cells were infected with a low MOI (0.0001) of vaccinia virus while Undiff on D1 after differentiation or D2 after differentiation. Plates were stained with crystal violet 48 hours after infection to visualize plaque formation. ImageJ was used to calculate the percentage of the monolayer within each well that was cleared by plaques (n = 4–6 experiments). Wilcoxon matched-pairs signed rank test for Undiff KSFM versus KGM2. Significance: $*P < 0.05$. Data are presented as median (Q1, Q3). D, day; KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; MOI, multiplicity of infection; Q, quartile; Undiff, undifferentiated.

at ×140 magnification using the EVOS XL Core Imaging System (Thermo Fisher Scientific, Waltham, MA).

Proliferation assay

N/TERT2G cells were plated in 96-well plates at 28,000 cells/well. After 24 hours, 10 μM 5-ethynyl-2′-deoxyuridine was added to each well and incubated for 6 or 24 hours. Proliferative capacity was assessed by the Click-iT EdU Proliferation Assay for Microplates (C10499, Invitrogen, Waltham, MA), and the protocol was followed as written in the product information sheet.

Western blot analysis

N/TERT2G cells were plated in 24-well plates at 75,000 cells/well. Cell lysates (RIPA buffer containing protease and phosphatase inhibitors with 0.2% SDS) were collected from undifferentiated cells and from cells on days 1, 2, and 3 after differentiation. Samples were run on Invitrogen NuPAGE 4–12% Bis-Tris gels and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with the antibodies provided in Table 4. Antibodies (Table 4) were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). Relative protein expression was determined by densitometry calculated using ImageJ software (National Institutes of Health, Bethesda, MD). Samples were normalized to β-actin expression and protein content (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific).

Table 3. Summary of Key Findings

Assay	KSFM	KGM2	Defined	Figure Number
Proliferation	=	=	↓	2b
TJ barrier function (TEER)	↑	↑↑↑	↑↑	2c
Epithelial thickness	↑	↑↑	↑↑	2d and e
Undifferentiated condition (CLDN1, OCLN, CK10)	↓	↑↑	↑↑	3a
Differentiated condition (D1) (CLDN1)	↓	↑	↑↑	3e and f
Differentiated condition (D2, D3)	=	=	=	3e and f
Response to type 2 cytokines	↑	=	=	4a
Response to type 3 cytokines	↓↓	↓↓	↓	4b
Pattern of viral infection	=	=	=	4

Abbreviations: D, day; CK10, cytokeratin 10; KGM2, keratinocyte growth medium 2; KSFM, keratinocyte serum-free media; OCLN, occludin; TEER, transepithelial electrical resistance; TJ, tight junction.

Table 4. Western Blot Antibodies

Antibodies	Company	Dilution
Anti-CLDN1 (519000)	Invitrogen	1:1,000
Anti–β-actin (C4) HRP	Santa Cruz	1:5,000
Anti–keratin 10 (Poly 19054)	BioLegend	1:1,000
Anti-loricrin (Poly 19051)	BioLegend	1:1,000
Anti-occludin (OC3F10)	Invitrogen	1:500
Anti-mouse IgG HRP (NA931V)	Sigma-Aldrich	1:5,000
Anti-rabbit IgG HRP (NA934V)	Sigma-Aldrich	1:5,000

Abbreviation: HRP, horseradish peroxidase.

TEER/epithelial thickness

TEER measurements were done as previously published (De Benedetto et al., 2011). Measurements of TEER were taken for up to 8 days after the initiation of differentiation and exposure to type 2 cytokines IL-4 and IL-13 at 50 ng/ml of each (number 574004 and 571104, BioLegend, San Diego, CA) or type 3 cytokine IL-17A (317ILB, R&D Systems, Minneapolis, MN). To measure epithelial thickness, membranes were cut from transwells on day 5 after differentiation and fixed in formalin. University of Rochester Medical Center Pathology processed, embedded, sectioned, and stained the membranes for H&E. Representative images were taken using the EVOS XL Core Imaging System (Thermo Fisher Scientific) at ×560. Epithelial thickness was quantified at six equally distributed locations across five representative images per media type using ImageJ software. The straight-line tool was used to span the thickness of the layers and was then measured using the Analyze -> Measure function.

Vaccinia virus infection assay

N/TERT2G cells were plated at a density of 150,000 cells/well in a 24-well plate. Cells were infected at a low multiplicity of infection with the western reserve strain of vaccinia virus (multiplicity of infection of 0.0001) while undifferentiated 1 day after differentiation (day 1) or 2 days after differentiation (day 2). Crystal violet was added to the cells 48 hours after infection. ImageJ software was used to calculate the percentage of the monolayer within each well that was cleared by plaques. To do this, each well was selected with the region of interest tool (circle), and the image was duplicated (right click, duplicate). The total area of the circle was determined using Analyze -> Measure. Next, the outside of the circle was cleared using the Edit -> Clear Outside command, then the Threshold function was applied to the image so that the cleared monolayer (plaques) was white: Image -> Adjust -> Threshold -> Apply. Finally, all areas considered to be plaques were selected using the Edit -> Selection -> Create Selection, and the selection was inverted using the Make Inverse function. The area covered by plaques was measured with the Analyze -> Measure, and the area covered in

plaques was divided by the total area to get a percentage of monolayer cleared.

Statistical analysis

Statistics were run using GraphPad Prism (GraphPad Software, San Diego, CA). The Friedman test with Dunn's posthoc test for multiple pairwise comparisons was used to compare proliferation among the media (KSFM vs. KGM2, KSFM vs. defined media, and KGM2 vs. defined media) at both 6 and 24 hours, respectively. The Friedman test with Dunn's posthoc test (pairwise) using KSFM as the control group (KSFM vs. KGM2, KSFM vs. defined media) was run on each day of differentiation (day 0–8) in which TEER was measured. The Kruskal–Wallis test with Dunn's posthoc test for multiple comparisons (unpaired) was used to compare epithelial thickness among the three media (KSFM vs. KGM2, KSFM vs. defined media, KGM2 vs. defined media). For each protein and at each day of differentiation (undifferentiated, day 1, day 2, day 3), the Friedman test with Dunn's posthoc test (pairwise) using KSFM as the control group (KSFM vs. KGM2, KSFM vs. defined media) was used to test for differences in protein expression. Correlation between TEER and tight junction protein expression (CLDN1 and OCLN) across 3 days of differentiation was assessed using the Pearson correlation test. On each day of differentiation (day 0–8), a paired *t*-test was run comparing media alone (KSFM, KGM2, or defined media) with media with cytokines (either IL-4/13 or IL-17) to test whether treatment with cytokines significantly changed TEER within each media. The Wilcoxon matched-pairs signed rank test was used to compare infection in undifferentiated KC cultured in KSFM or KGM2. No statistics were run on defined media of undifferentiated KCs because there was only $n = 2$. Kruskal–Wallis tests with Dunn's posthoc test (unpaired) comparing KSFM with KGM2, KSFM with defined media, and KGM2 with defined media were run on days 1 and 2 of differentiation and showed no significant differences in infection.

Data availability statement

No large datasets were generated or analyzed during this study.

ORCIDS

Mary C. Moran: http://orcid.org/0000-0002-6456-082X
Eleanor M. Pope: https://orcid.org/0000-0002-9577-672X
Matthew G. Brewer: http://orcid.org/0000-0001-7631-5234
Lisa A. Beck: http://orcid.org/0000-0002-8452-667X

AUTHOR CONTRIBUTIONS

Conceptualization: MCM, EMP, MGB, LAB; Formal Analysis: MCM, EMP; Funding Acquisition: LAB, MGB; Investigation: MCM, EMP; Project Administration: MGB, LAB; Resources: MGB, LAB; Supervision: MGB, LAB; Visualization: MCM, EMP; Writing - Original Draft Preparation: MCM, EMP; Writing - Review and Editing: MGB, LAB

CONFLICT OF INTEREST

LAB is a consultant for Abbvie, Allakos, Arena Pharmaceuticals, AstraZeneca, Benevolent AIBio, DermTech, Galderma, Incyte, Janssen, LEO Pharma, Lilly, Novartis, Numab Therapeutics, Pfizer, Principia Biopharma, Rapt Therapeutics, Regeneron, Ribon Therapeutics, Sanofi/Genzyme, Sanofi-Aventis, Stealth BioTherapeutics, and Union Therapeutics and an investigator for Abbvie, Astra-Zeneca, DermTech, Kiniksa, LEO Pharma, Pfizer, Regeneron, and Sanofi.

ACKNOWLEDGMENTS

The authors acknowledge Ellen H. van den Bogaard for providing the N/TERT2G cells. The authors acknowledge Anna De Benedetto for assistance with the proliferation assays, Julie Ryan Wolf for assistance with statistics, and Diana Scott for assistance with sectioning and H&E staining. LAB is supported by the National Institute of Allergy and Infectious Diseases (U01AI152011 and U19AI117673). MCM is supported by the National Institute of Allergy and Infectious Diseases (T32 AI007285). EMP is supported by the National Center for Advancing Translational Sciences (TL1TR002000).

REFERENCES

Bikle DD, Xie Z, Tu CL. Calcium regulation of keratinocyte differentiation. Expert Rev Endocrinol Metab 2012;7:461–72.

Broadbent L, Manzoor S, Zarcone MC, Barabas J, Shields MD, Saglani S, et al. Comparative primary paediatric nasal epithelial cell culture differentiation and RSV-induced cytopathogenesis following culture in two commercial media. PLoS One 2020;15:e0228229.

De Benedetto A, Slifka MK, Rafaels NM, Kuo IH, Georas SN, Boguniewicz M, et al. Reductions in claudin-1 may enhance susceptibility to herpes simplex virus 1 infections in atopic dermatitis [published correction appears in J Allergy Clin Immunol 2011;128;903] J Allergy Clin Immunol 2011;128:242–6.e5.

Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, et al. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 2000;20:1436–47.

Fuchs E, Green H. Changes in keratin gene expression during terminal differentiation of the keratinocyte. Cell 1980;19:1033–42.

Goleva E, Berdyshev E, Leung DY. Epithelial barrier repair and prevention of allergy. J Clin Invest 2019;129:1463–74.

Kirschner N, Houdek P, Fromm M, Moll I, Brandner JM. Tight junctions form a barrier in human epidermis. Eur J Cell Biol 2010;89:839–42.

Moran MC, Pandya RP, Leffler KA, Yoshida T, Beck LA, Brewer MG. Characterization of human keratinocyte cell lines for barrier studies. JID Innov 2021;1:100018.

Smits JPH, Niehues H, Rikken G, van Vlijmen-Willems IMJJ, van de Zande GWHJF, Zeeuwen PLJM, et al. Immortalized N/TERT keratinocytes as an alternative cell source in 3D human epidermal models. Sci Rep 2017;7:11838.

Zorn-Kruppa M, Volksdorf T, Ueck C, Zöller E, Reinshagen K, Ridderbusch I, et al. Major cell biological parameters of keratinocytes are predetermined by culture medium and donor source. Exp Dermatol 2016;25:242–4.