

## Article

# Comparison of Culture Media for *in-vitro* Expansion of Oral Epithelial Keratinocytes: Implications for Testing E-liquid Flavors

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**Abstract: Background:** Expansion of OKF6/TERT-2 oral epithelial cells *in vitro* is important for studying the molecular biology of disease and pathology affecting the oral cavity. Keratinocyte Serum-Free Medium (KSFM) is the medium of choice for this cell line. This study compares three media for OKF6/TERT-2 cultures: KSFM, Dulbecco's Modified Eagle Medium/Nutrient Mixture of Hams F-12 (DMEM/F12) and a composite medium comprised of DMEM/F-12 and KSFM (1:1 v/v), referred as DFK. The toxicological effects of electronic cigarette liquids (E-liquids) on OKF6/TERT-2 cells cultured in these media were also compared. **Methods:** Cells were cultured in KSFM, DMEM/F12 or DFK and cellular morphology, growth, wound healing and gene expression of mucins and tight junctions were evaluated. Additionally, cytotoxicity was determined after E-liquid exposures. **Results:** Switching from KSFM to DMEM/F12 or DFK 24-hours post-seeding leads to typical cellular morphologies, and these cultures reach confluency faster than those in KSFM. Wound-healing recovery occurred fastest in DFK. Except for *claudin-1*, there is no difference in expression of the other genes tested. Additionally, E-liquid cytotoxicity appears to be amplified in DFK cultures. **Conclusions:** DMEM/F12 and DFK are alternative media for OKF6/TERT-2 cell culture to study molecular biology of disease and pathology, provided cells are initially seeded in KSFM.

**Keywords:** oral; mucosa; mucins; tight junctions; wound-healing; E-liquids; cytotoxicity; viability; confluency

## 1. Introduction

The epithelial lining of the oral mucosa is a critical barrier protecting the subepithelial and distal airway tissues from the environment and is one of the first mucosal surfaces that inhaled or ingested substances encounter. The early exposure of cells in the upper respiratory tract and oral cavity makes them a valuable tool for studying biology, to include, but not limited to pathophysiology, host-microbe interactions, innate immunity, toxicology, and pathophysiology. OKF6/TERT-2 cells are oral mucosal epithelial keratinocytes, originally isolated from a human male, that have been immortalized via telomerase 2 retroviral transduction and expression, as well as deletion of the p16INK4a regulatory protein [1]. The OKF6/TERT-2 cell line has shown significant research utility, with publications using these cells to investigate topics such as to carcinogenesis of oropharyngeal malignancy [2–4], infectious disease [5], periodontal disease [6,7], and various tobacco products such as conventional cigarettes [8], shisha [9], and chewable tobacco [10].

Keratinocyte serum-free medium (KSFM) is a culture medium optimized for the growth of human keratinocytes that is widely used to culture epithelial cell lines ranging from hepatocytes [11], to urothelial cells [12], to corneal epithelial cells [13]. This me-

dium, commonly containing 0.09 mM calcium chloride supplemented with 30 µg/mL pituitary bovine extract, 0.2 ng/mL epithelial growth factor (EGF), and 100 U/mL ampicillin/streptomycin, has been the primary medium for culturing OKF6/TERT-2 cells in current literature [5,7,14–16]. Dickson et al. [1] described a protocol in which this cell line was cultured in KSFm, which has since been widely cited and replicated [17–21]. Dulbecco's Modified Eagle Medium/Nutrient Mixture of Hams F-12 (DMEM/F-12) is another standard basal medium commonly containing 1.05 mM calcium chloride [22]. DMEM/F-12, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin is used for the culture of a wider spectrum of cells, including fibroblasts, neurons, muscle cells, and cell lines including HeLa [22]. DMEM/F-12 with 10% FBS has less frequently been used to culture OKF6/TERT-2 cells [23,24]. To our knowledge regarding the OKF6/TERT-2 cell line, no studies use DMEM/F12 without FBS. Most other studies have used the KSFm methodology as described by Dickson et al. [1] which does not include FBS. To mimic this KSFm protocol as close as possible, all cell cultures in this study include media without FBS.

OKF6/TERT-2 cells were originally established in KSFm with its own set of nutrients [1], but DMEM/F12, which consists of a different set of nutrients, has also been used [23,24] and its composition is readily available online [22]. In contrast, the composition of KSFm is proprietary, but after conversations with ThermoFisher technical support (Waltham, MA, USA) it is evident that the composition of KSFm differs substantially from that of DMEM/F12. It is possible that OKF6/TERT-2 access to a combination of both sets of nutrients may increase the rate of cellular growth. The justification for a comparison of the growth of the OKF6/TERT-2 cell line in KSFm, DMEM/F12 and a 1:1 v/v mixture of DMEM/F-12 and KSFm (from this point on referred to as DFK) is two-fold: First, to establish that these three media support the growth of OKF6/TERT-2. Second, to determine potential benefits, such as associated costs and the time to reach confluency, which would ultimately impact the use and versatility of this cell line. We note that other orally-derived cell lines, such as human gingival fibroblasts [25,26], dental pulp stem cells [27], and normal human epidermal keratinocytes [28] are more commonly cultured in DMEM/F12. As such, the DFK combination medium for the growth of OKF6/TERT-2 oral epithelial cells may provide other researchers with increased flexibility for cell culturing.

The putative benefits of the DFK aggregate medium may be a consequence of compositional differences of the two media. There is a near 10-fold higher calcium concentration in DMEM/F12. Extracellular calcium is a highly-investigated media component shown to have variable effects on cell proliferation, depending upon cell-type, with some cells such as human gingival keratinocytes showing increased proliferation at low calcium concentrations [29]. Another study has shown that immortalized human keratinocytes can proliferate at a wide range of calcium concentrations [30]. In the dermal keratinocyte literature, it has been found that low-calcium conditions may stimulate proliferation, but a “calcium-switch” at a threshold of 0.1 mM initiates cellular differentiation [31]. Other differences, such as glutamine (1,020 mg/L in KSFm vs 365 mg/L in DMEM/F-12) and pyridoxine (0.06 mg/L in KSFm vs 2.013 mg/L in DMEM/F-12) concentrations, may play a role in the culturing of OKF6/TERT-2 cells. Based on conversations with ThermoFisher technical support, arginine, asparagine, and d-calcium pantothenate also differ in concentrations between KSFm and DMEM/F12. This raises the question of whether DFK would improve OKF6/TERT-2 cell culture conditions based on accessibility to both sets of nutrients.

In addition to comparing growth characteristics, including time-to-confluency and morphology of OKF6/TERT-2 cells in KSFm, DMEM/F12 and DFK, the functional characteristics of these cells should also be measured to evaluate a viable alternative culture medium. Similar to epithelial cells from other anatomic sites, the barrier function of the oral epithelium is critical in maintaining a well-defined “inside” vs. “outside” environment by demarcating the apical and basolateral domains of adjacent cells in the superficial mucosa [32]. This is accomplished through functional protein complexes known as tight junctions,

which include claudin-1, occludin, and zonula occluden protein 1 (ZO-1). These tight junctions assist in an array of functions, including the regulation of paracellular transport, cell polarity, and importantly, sustaining the functional semipermeable barrier of the oral epithelium [33]. Another barrier function is the ability to close wounds after mechanical injury, which helps prevent microbial and environmental hazards from reaching the connective tissue.

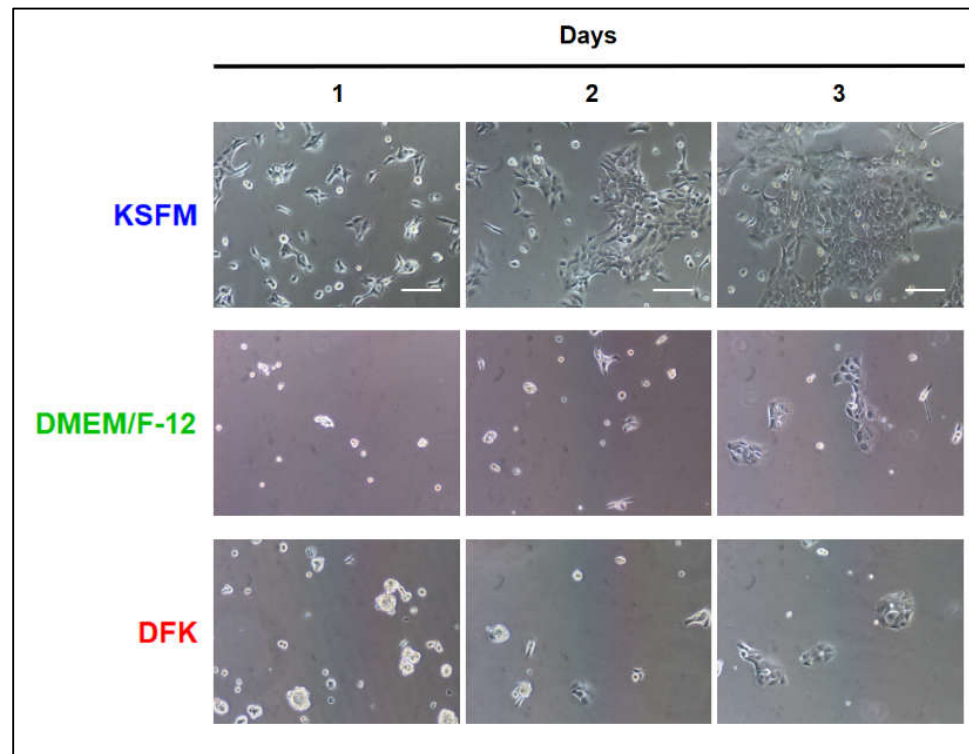
Within the oral cavity, after swallowing, roughly 800  $\mu$ L of saliva remain on oral surfaces [34], providing lubrication and moisture. Of this, 5 to 10% of the dry weight is composed of mucin glycoproteins, a major component of the saliva [35,36], as well as the respiratory [37] and GI tracts [38]. At least 20 mucins have been identified in the saliva that are functional protective substances that play a role in salivary flow and composition and therefore dysregulation may increase susceptibility to pathogens, such as *Candida albicans* [39], and dental decay [40]. Mucin glycoproteins are expressed by a wide range of epithelial cells, typically on the apical membrane and as a gelatinous component or as a lubricant and protective agent in the saliva [41,42]. Mucins play a pivotal role in cellular growth, differentiation, and signaling, as well as homeostasis and innate immunity within the oral cavity [41,42]. Mucin genes such as *muc1* and *muc4* are expressed broadly in epithelial cells of the body, including the upper aero-respiratory tract and oral cavity [43]. Over- and under-expression of mucin genes and other modifications, such as aberrant glycosylation, have been implicated in situations of epithelial dysfunction, including malignant transformation [44,45] and breakdown of the nasal epithelial barrier [46]. For the OKF6/TERT-2 cell model to be representative of *in vivo* physiology, the production of mucins should remain consistent. Therefore, comparable expression of the *muc1* and *muc4* genes, in conjunction with tight junctions, will be used to further assess the functional equivalency of the OKF6/TERT-2 cell line grown in these media.

The OKF6/TERT-2 cell line has conventionally been cultured using a standard KSFM medium. However, the flexibility of this cell line to be cultured in other media, such as DMEM/F12 or DFK, remains to be determined. The ability to diversify the growth and culture requirements of the OKF6/TERT-2 cell line amplifies its research potential. Therefore, the aim of this study is to compare KSFM to DMEM/F12 and DFK as media that can support OKF6/TERT-2 cell cultures, analyzing growth, morphology, tight junctions, and mucin glycoproteins gene expression, as well as tissue repair. Exploring the effects of electronic cigarette liquids (E-liquids)  $\pm$  flavors, which have previously demonstrated a significant impact on the oral microenvironment [47–54], is an important research direction. From a practical point of view, toxicological experiments were also performed using E-liquids  $\pm$  flavors to determine which of these media renders the cell cultures more suitable for such studies.

## 2. Results

### 2.1. Seeding OKF6/TERT-2 cells in KSFM or DMEM/F12 or DFK

After attempting to seed OKF6/TERT-2 cells in KSFM or DMEM/F12 or DFK, cells adhered well to the surface when seeded with the KSFM media, as seen by day 1 in Figure 1. Cells in KSFM present the correct morphology with pseudopodia extensions, forming small microcolonies in the well. By day 1, few cells remain adhered to the surface when seeded with DMEM/F12 or DFK, and these cells appear rounded, with a different phenotype when compared to cells in KSFM. By days 2 and 3, however, cells cultured in DMEM/F12 or DFK begin to present the correct morphology, with scarce microcolonies. On the other hand, by days 2 and 3, cells in KSFM begin to grow confluent, with a much higher cell number per field of view (Fig 1). Our results indicate that when seeding OKF6/TERT-2 cells, KSFM should be the medium used as it yields the correct phenotype and enhanced growth within one day, compared to DMEM/F12 or DFK.

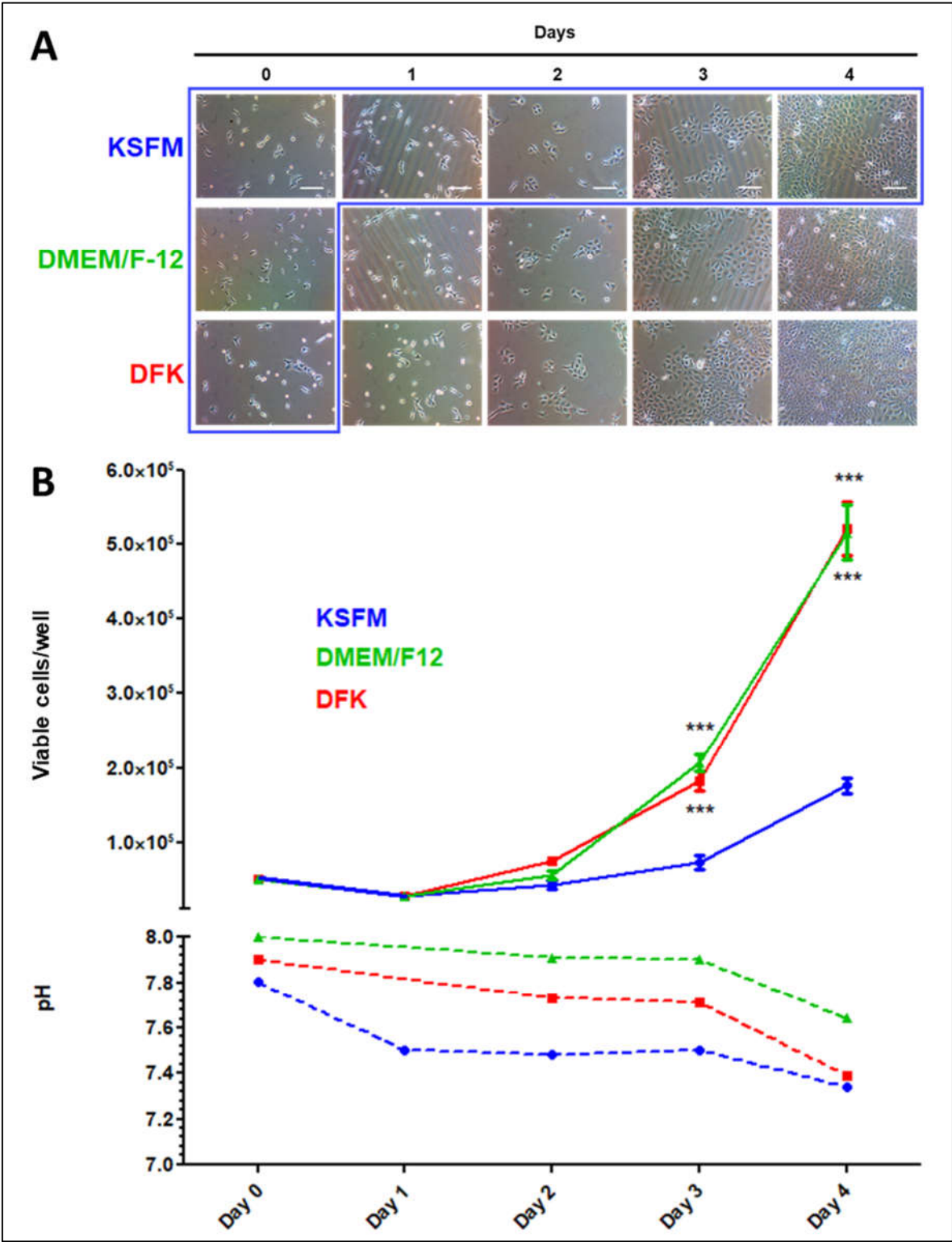


**Figure 1.** Light microscopy displaying cellular morphology and confluency of OKF6/TERT-2 cells seeded in KSFM, DMEM/F12 or DFK media over the course of 3 days. Micrographs are representative images from three independent experiments. Total magnification = 100X and the white bars = 100  $\mu$ m.

## 2.2. OKF6/TERT-2 cells seeded in KSFM and switched to DMEM/F12 or DFK

OKF6/TERT-2 cells were seeded in KSFM and 24 hours later, they were either kept on this medium or switched to DMEM/F12 or DFK for the remainder of the experiment. Throughout the entire experiment, the cell morphology appears to be similar regardless of the media used (Fig 2A). However, the cellular confluency progresses slightly faster in the DMEM/F12 and DFK cultures. Cell counts demonstrate a significant difference in cell numbers between the DMEM/F12 or DFK compared to the KSFM cultures on day 3 (Fig 2B, upper Y axis,  $p < 0.001$ ) supporting the visual observation in Figure 2A. On day 4, all cell cultures appear nearly 100% confluent, but the cell counts in both DMEM/F12 and DFK cultures are about three times higher than those in KSFM cultures. These results indicate that, although cultures in KSFM do reach confluency, a protocol of seeding with KSFM (day 0) and switching to DMEM/F12 or DFK by day 1 is more efficient in terms of growth rate. Light microscopy suggests the possibility that cells cultured in DMEM/F12 and DFK, as compared to KSFM, have a smaller cellular size (Fig 2A) which could account for the larger cell number (Fig 2B, upper Y axis). Alternatively, it is possible that cells cultured in DMEM/F12 or DFK have adopted a cuboidal (taller) morphology while maintaining a similar cellular size. For this reason, 3D confocal imaging is presented below. In addition, pH levels were measured throughout the course of the growth experiment (Fig 2B, lower Y axis). Results indicate that there is a drop in pH, starting at 8.0, 7.91 and 7.79 and ending with 7.64, 7.39 and 7.34 for DMEM/F12, DFK and KSFM, respectively. This decrease in pH for all spent media is consistent with cellular growth and metabolic activity. For the remainder of the study, OKF6/TERT-2 cells seeded and maintained in KSFM are compared to cells seeded in KSFM and switched to DMEM/F12 or DFK.





**Figure 2.** Light microscopy displaying cellular morphology and confluency of OKF6/TERT-2 cells seeded in KSFM and either kept in this medium (blue box) or switched to DMEM/F12 or DFK 24 hours later until the end of the experiment. Micrographs are representative images from three separate experiments (A). Quantification of viable OKF6/TERT-2 cells in both media was measured via trypan blue exclusion and presented by solid lines where each point represents the mean  $\pm$  SEM ( $n = 3$  to 6). \*\*\* =  $p < 0.001$ . The media pH levels are presented by dashed lines and represent pooled spent media (B).

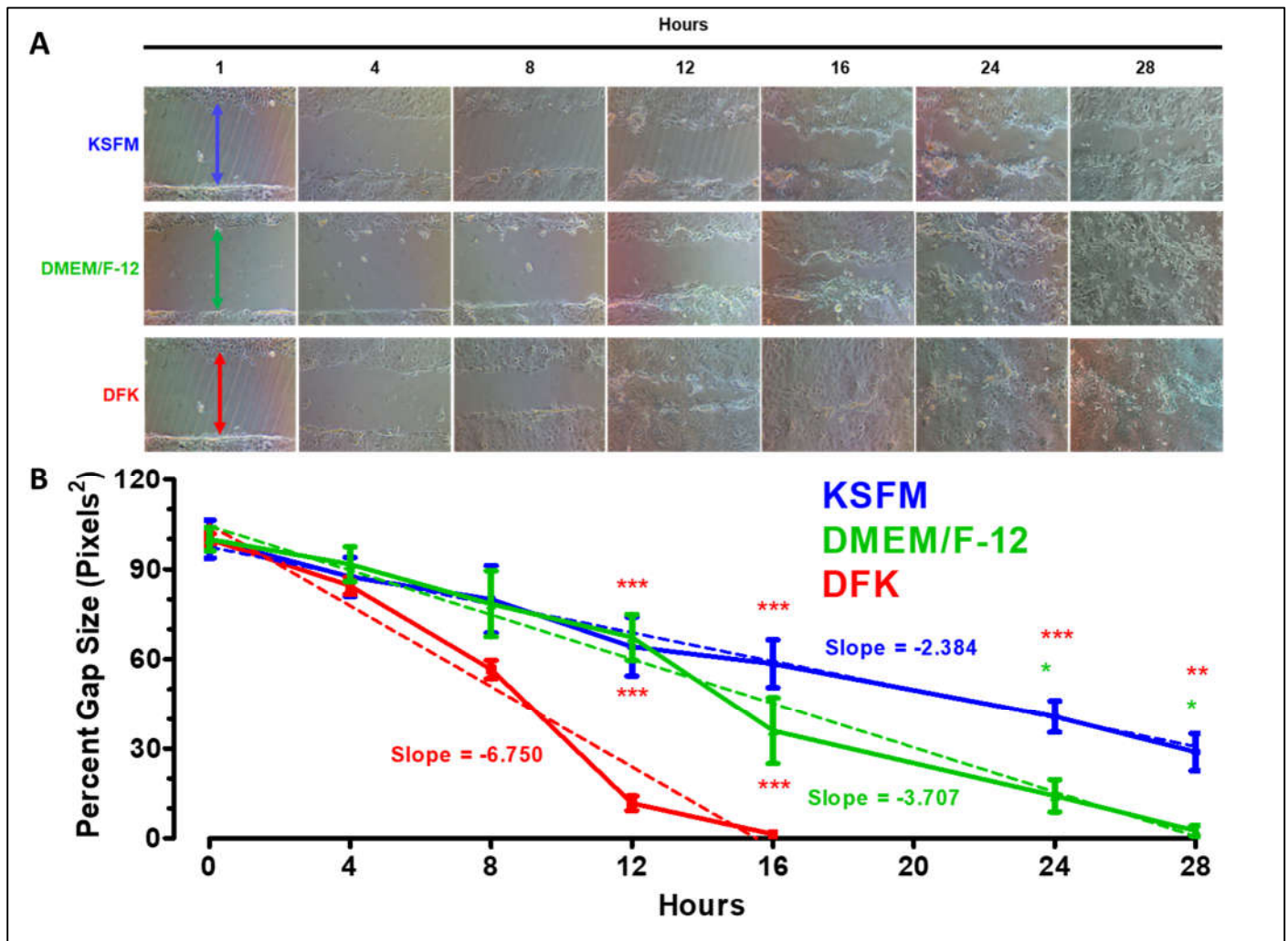
### 2.3. Wound healing assay in KSFM, DMEM/F12 and DFK

An important function of oral epithelial cells is their ability to close wounds after injury, which also helps limit microbes, microbial products, or environmental materials from entering connective tissues or the bloodstream. A wound-healing assay comparing the effects of each media on OKF6/TERT-2 monolayer recoveries from mechanical injury provides insight into the cell culture performance based on the nutrients present in the media. OKF6/TERT-2 epithelial cells demonstrate a significantly greater wound recovery rate in DFK compared to cultures in DMEM/F12 and KSFM (Figure 3A). For all practical purposes, 16 h cultures in DFK exhibit complete closure (Figure 3A). Cultures in DMEM/F12 completely recover by 28 hours and those in KSFM do not fully recover within the timeframe of the experiment (Fig 3A). Quantification of the gap size indicates a significant difference at 12 and 16 hours of wound healing, where cells in DFK close the gap faster ( $p < 0.001$ ) compared to the other two conditions. Linear regression analyses reveal that the correlation coefficients ( $R^2 \geq 0.8$ ) for each culture illustrate a stable progression towards wound healing. Furthermore, the absolute value of the slopes indicate that faster recovery is correlated to greater steepness, where  $DFK > DMEM/F12 > KSFM$  (Fig 3B). Hence, OKF6/TERT-2 cells cultured in DFK recover faster from the injury compared to the other two media tested in the wound-healing assay. Thus, DFK provides a more suitable set of nutrients that supports better monolayer performance after mechanical injury.

The OKF6/TERT-2 cell model was originally established in KSFM (see introduction) and it is required for routine cell culture maintenance and passaging. In addition, KSFM is required for initial seeding of cell cultures for all experiments (Fig 1 and 2). Based on the cellular growth (Fig 2) and wound healing (Fig 3) results, two points are discerned. First, OKF6/TERT-2 cells grow faster in DFK and DMEM/12 media as compared to KSFM. Second, wound healing of OKF6/TERT-2 cells occur faster in DFK as compared to the other two media. For these reasons and given that OKF6/TERT-2 cells grown in KSFM and DFK yield the most polar results, the remainder of the study focuses on comparing cellular and molecular aspects of OKF6/TERT-2 cell cultures in these two media. Nonetheless, if resources are limited, DMEM/F12 remains a viable and accessible option for culturing OKF6/TERT-2 cells after seeding them in KSFM.

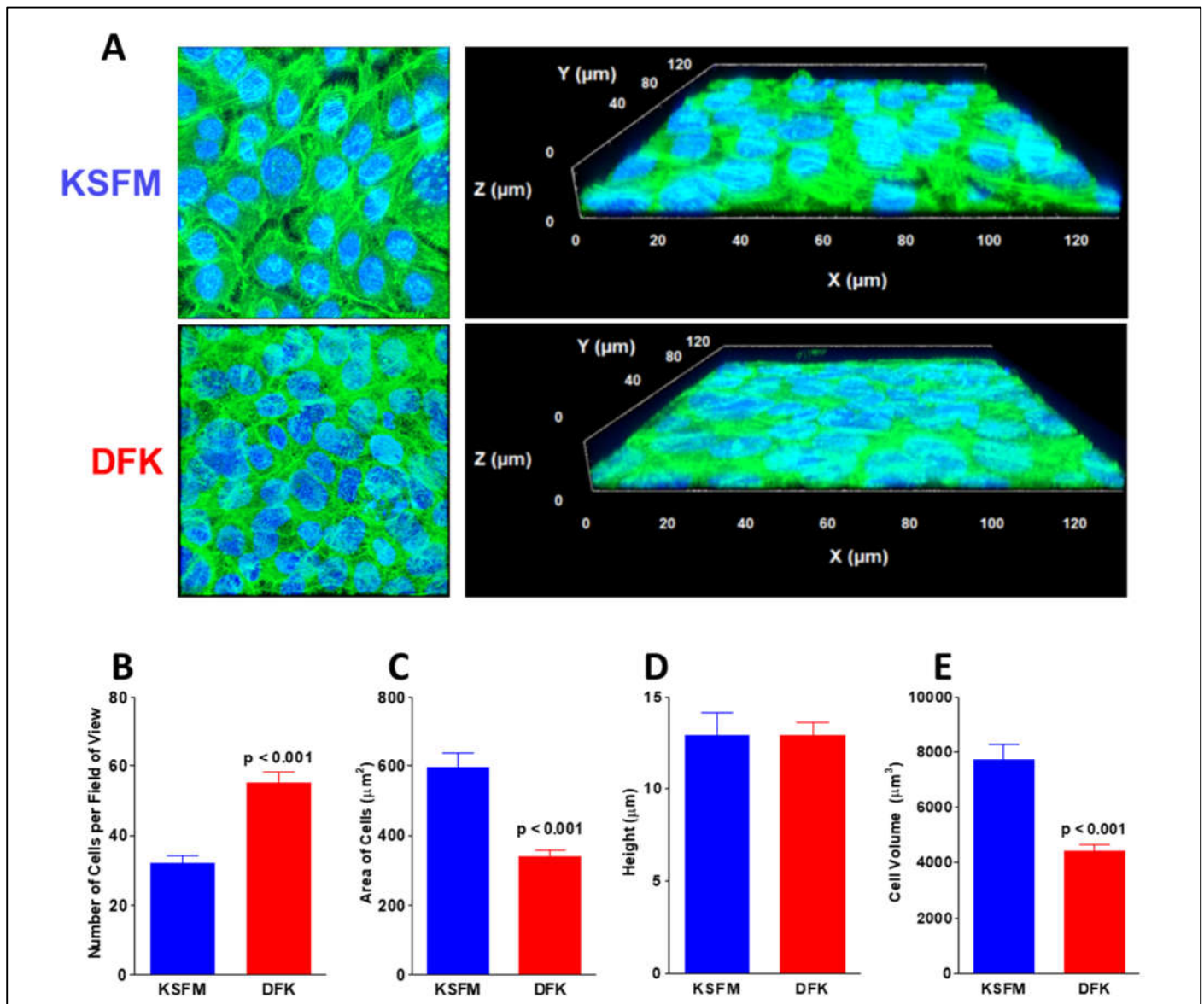
### 2.4. Confocal analysis of OKF6/TERT-2 morphology after growth in KSFM and DFK

Mucosal epithelial cells are mainly of squamous morphology, demonstrating a high width to height ratio [55]. The results in Figure 2 could be explained by a change in DFK-cultured OKF6/TERT-2 cellular morphology, where the cells may elongate in height (i.e., cuboidal) or decrease their overall cellular size. If the cells adopt a more cuboidal phenotype, they will increase in height. Conversely, if the cells remain in a squamous morphology, they simply decrease in overall cellular size. To discern between these two possible outcomes, confluent monolayers were stained and observed under confocal microscopy, yielding a quantification of the z-axis (height). OKF6/TERT-2 cells grown for four days in KSFM and DFK are shown in Figure 4A, where the left side shows two-dimensional images, and the right side shows three-dimensional views of the cultures. No conspicuous difference in morphology is observed. However, as shown in Figure 4B the number of cells per field of view is 1.73 times higher in DFK as compared to KSFM ( $p < 0.001$ ), which agrees with the results in Figure 2. Figure 4C shows that the mean area of OKF6/TERT-2 cells in KSFM is significantly larger than cells in DFK ( $p < 0.001$ ). The height, as indexed by Z-stacks (Fig 4D), reveals that cells reach roughly 13  $\mu\text{m}$  in height with no significant difference observed between media. Furthermore, figure 4E show that cells grown in KSFM have a significantly higher cellular volume ( $p < 0.001$ ).



**Figure 3.** Wound healing assay of OKF6/TERT-2 cells grown in KSFM, DMEM/F12 and DFK. Representative images show the wound-healing process over time from two independent experiments. The colored arrows at 0 hours indicates the gap width of the initial wounds (A). ImageJ was used to measure the gap size over time. Each time-point represents the mean  $\pm$  SEM ( $n = 4$ ) of the gap size as a percentage of the initial size. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ . Dashed lines indicate linear regressions (B).

Consequently, our results favor the idea of a decrease in cell size. Overall, the results indicate the morphology of the cells remains squamous and does not change based on the media but the cells in DFK are smaller in size than those in KSFM. A potential explanation is that cells grown in DFK undergo mitosis faster and do not have enough time to accumulate cellular content, hence they are smaller and more numerous per surface area.



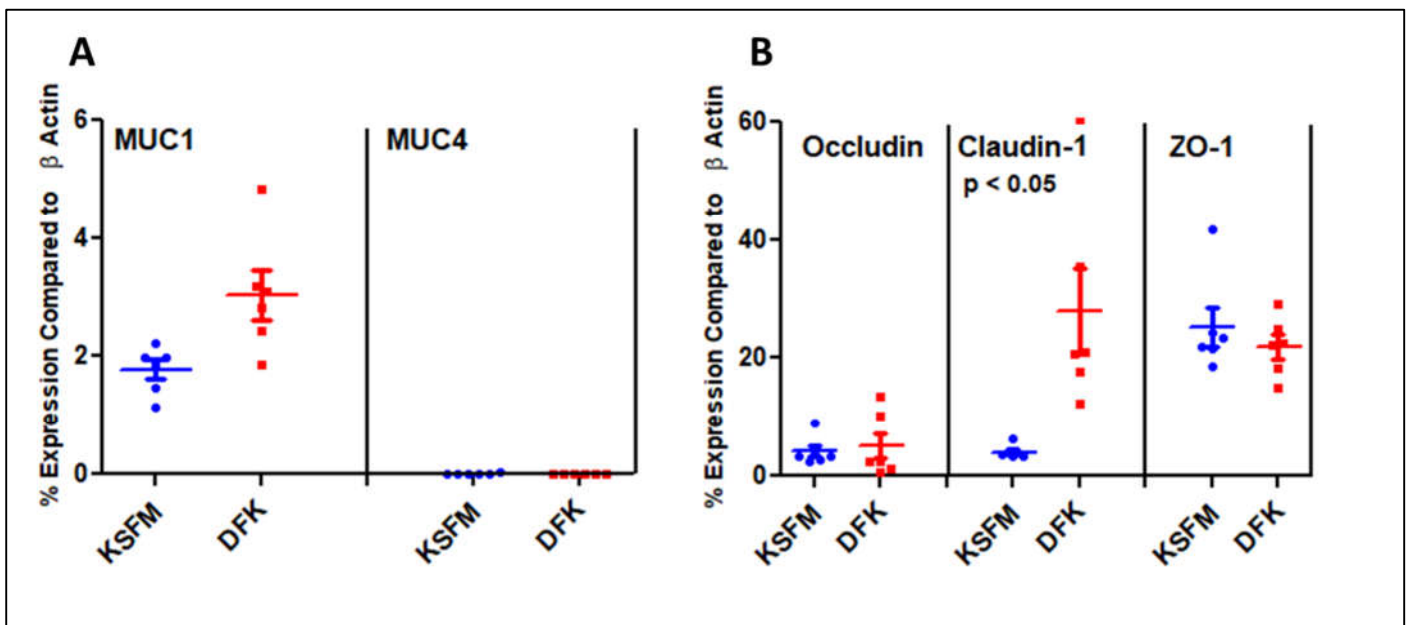
**Figure 4.** Confocal imaging and cellular size of confluent OKF6/TERT-2 cells grown in KSFM and DFK. Representative confocal (2D) images of cell cultures grown in KSFM and DFK by day 4 from two independent experiments. Phalloidin green labels actin filaments and DAPI blue labels cellular nuclei. Each frame is 135  $\mu\text{m}$  per side, total magnification = 630X. Z-stacks were tilted to render 3D images (A). Mean number of cells per field of view  $\pm$  SE in both media (B). Average area of cells  $\pm$  SE ( $n = 8$ ) was calculated by dividing the total area of the field of view by the mean number of cells (C). Average height  $\pm$  SE ( $n = 17$  to 18) of cells after growth in each media was indexed by the Z-stacks (D). Average cell volume  $\pm$  SE was calculated by multiplying area ( $n = 8$ ) times the average height of cells (E).

#### 2.5. Expression of Mucins and Tight Junction genes after growth in KSFM and DFK

Oral epithelial cells have several functions, including the production of mucins and tight junctions. Both of these cellular factors aim to prevent foreign materials, such as microbial products or environmental hazards, from interacting directly with oral tissues. In the case of mucins, *muc1* and *muc4* are among the most abundant in oral epithelial tissues. Tight junction genes *occludin*, *claudin-1* and *ZO-1* yield the most important proteins of these structures. These genes were tested via qPCR and the results, presented in figure 5, are compared to the levels of  $\beta$ -actin expression in terms of percentages. OKF6/TERT-2 cells grown in DFK express a slightly higher level of *muc1* (3%) compared to their KSFM counterparts (1.8%), but this was not significant ( $p < 0.08$ ). A much lower, but detectable



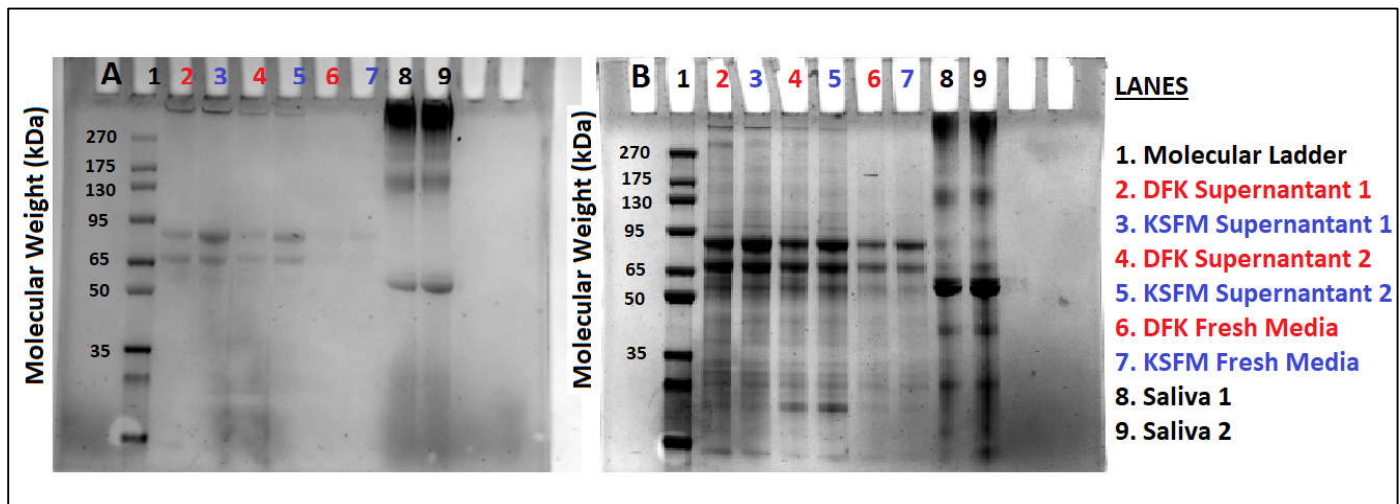
level of *muc4* was found in these cells (0.01% in both cultures), once again, with no significant difference among them (Fig 5A). For the tight junction genes, *ZO-1* was highly expressed in both cell cultures, with amounts of roughly 22 and 25% in KSFM and DFK, respectively (compared to  $\beta$ -actin). Interestingly, *claudin-1* was upregulated in cells grown in DFK to 28%, which is significantly different than the 4% found in KSFM ( $p < 0.05$ ). Cells grown in both DFK and KSFM expressed about 4% of *occludin* with no significant difference among the different cultures (Fig 5B). Taken together, OKF6/TERT-2 cells grown in DFK or KSFM express similar levels of *muc1*, *muc4*, *occludin* and *ZO-1*. However, the growth of OKF6/TERT-2 in DFK yields seven times higher expression of *claudin-1* compared to that in KSFM, suggesting a more robust formation of tight junctions in the former medium.



**Figure 5.** QPCR: Expression levels of mucins and tight junction genes compared to  $\beta$ -actin as a percentage. Horizontal lines represent the mean  $\pm$  SE of six data points from three independent experiments.

## 2.6. Release of Mucins after growth in KSFM and DFK

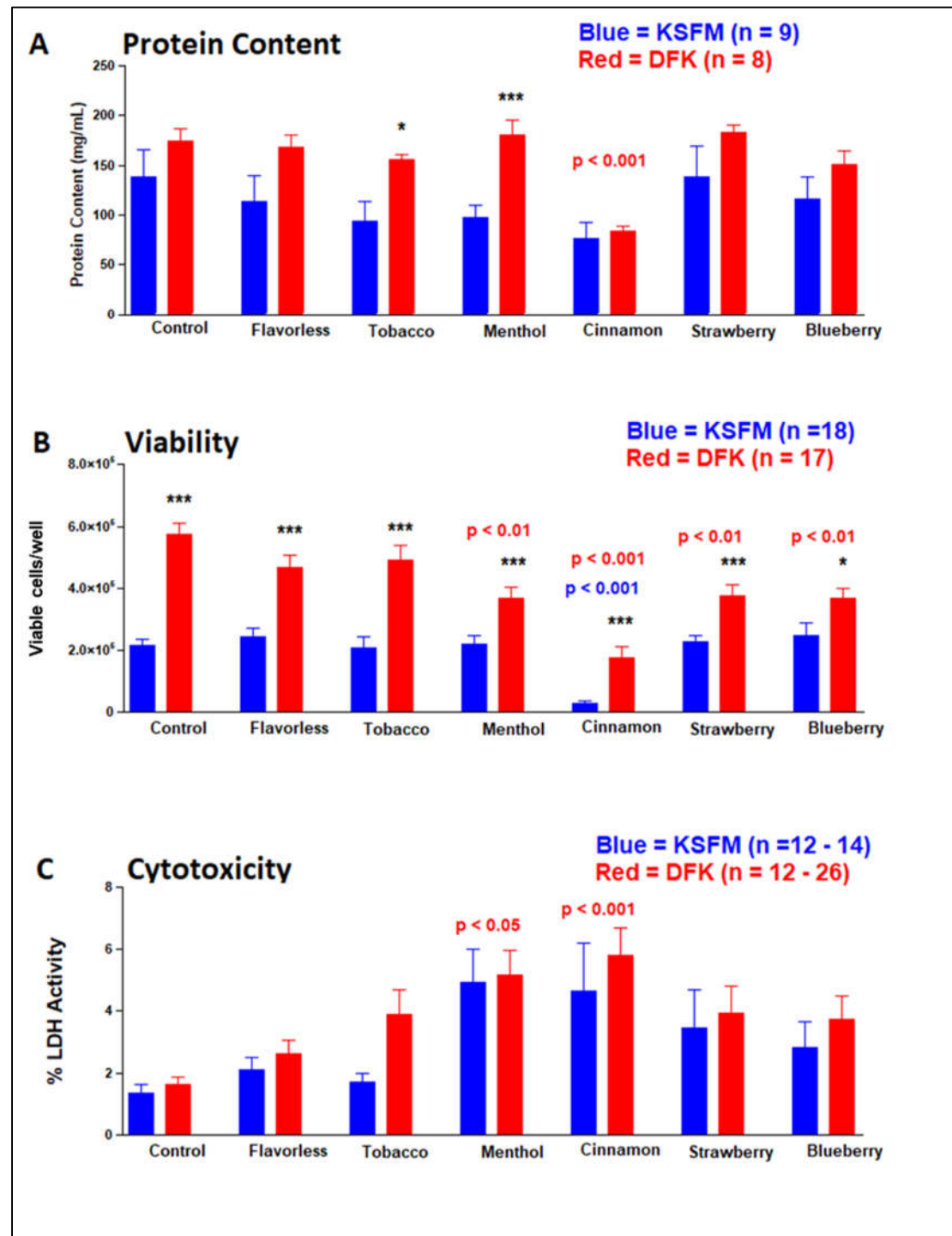
After quantifying roughly 1 to 4% expression of *muc1* compared to  $\beta$ -actin, the possibility that OKF6/TERT-2 cells could be releasing *muc1* glycoproteins into solution, as is the case with *in vivo* oral epithelial cells, was tested using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Because of the high levels of mucin glycosylation, MUC1 travels very little in SDS-PAGE and would appear as a high molecular-weight (MW) band. To test whether mucins are released by OKF6/TERT-2 cells *in vitro*, supernatants from DFK and KSFM cultures were concentrated, separated by SDS-PAGE and all glycoproteins were stained using the periodic-acid-Schiff (PAS) protocol, followed by Coomassie staining. Figure 6A shows high MW bands above 270 kDa in the supernatants of both DFK and KSFM cultures (lanes 2 – 5), which are not observed in concentrates of fresh DFK or KSFM media (lanes 6 and 7), indicating that such high MW glycoproteins were produced and secreted by OKF6/TERT-2 cells during culture. These high MW materials are of similar size as those in human saliva (lanes 8 – 9) albeit, much less abundant. In contrast, when the gel is further stained in Coomassie (Fig 6B), several bands in all samples (lanes 2 – 5), control media (lanes 6 and 7) and saliva (lanes 8 and 9), are revealed indicating the presence of several proteins. Our results indicate that our cell model, whether cultured in KSFM or DFK, mimics the behavior of *in vivo* oral epithelial tissues in terms of glycoprotein production and secretion.



**Figure 6.** Concentrated materials from OKF6/TERT-2 cell culture supernatants in both KSFM and DFK (lanes 2 – 5), uncultured KSFM or DFK media (lanes 6 and 7) or human saliva (lanes 8 and 9) were separated by SDS-PAGE. PAS staining, specific for glycoproteins, reveals high molecular weight materials in saliva lanes and supernatants (A). Coomassie stain shows all proteins found in the same gel (B). Lanes 2 – 5 represent two independent experiments comparing DFK vs. KSFM culture supernatants. Each lane was loaded with 30  $\mu$ g of protein.

#### 2.7. Effects of E-liquids $\pm$ flavors on OKF6/TERT cells after KSFM and DFK culturing

Protein content and cellular viability were assessed on cell cultures in both media after exposure to 1% E-liquids  $\pm$  flavors (v/v) for 24 hours. Figure 7A shows that the addition of 1% E-liquids  $\pm$  flavors do not alter the total protein content in KSFM cultures with respect to those of untreated controls. Similarly, the addition of 1% E-liquids  $\pm$  flavors do not alter the total protein content in DFK cultures with respect to those of untreated controls, except for the cinnamon flavored E-liquid, which is significantly lower than the control ( $p < 0.001$ ). In contrast, tobacco and menthol conditions between DFK and KSFM show a statistical difference in protein content ( $p < 0.05$  and  $p < 0.001$  for tobacco and menthol, respectively), where in both cases protein levels in DFK are higher than in KSFM (Fig 7A). Figure 7B shows the total number of viable cells in DFK and KSFM cultures in the presence of 1% E-liquids  $\pm$  flavors for 24 hours. In untreated control cultures there is a significant disparity of viable cell numbers between DFK and KSFM cultures ( $p < 0.001$ ), confirming the results in Figure 2. Cinnamon flavor significantly reduces the number of viable cells in both DFK and KSFM cultures ( $p < 0.001$ ). Lastly, menthol, strawberry and blueberry flavors also significantly reduce the total number of viable cells in DFK cultures ( $p < 0.01$ ), but not in KSFM cultures. In general, these results indicate that the detrimental effects of menthol, cinnamon, strawberry, and blueberry flavored E-liquids on OKF6/TERT-2 cells cultured in DFK are amplified in comparison to KSFM. Fig 7C shows the total levels of lactate dehydrogenase (LDH) activity in the culture-supernatants as a percentage of completely lysed cells. Unexpectedly, only a modest amount (roughly between 2 and 6%) of LDH activity is detected in all condition and controls. Although modest, only treatments with menthol and cinnamon, in both KSFM and DFK, yield LDH activity that is roughly 6% and significantly different ( $p < 0.05$ ) compared to their respective controls (Fig 7C). Interestingly, there is no significant difference in LDH activity between KSFM and DFK cultures within the same treatments.



**Figure 7.** Effects of E-liquids ± flavors on OKF6/TERT-2 cells growing in KSFM or DFK. Total protein content (A), cellular viability (B), and cytotoxicity (C) were measured, and each bar indicates their means ± SE. \* =  $p < 0.05$  and \*\*\* =  $p < 0.001$  when comparing KSFM vs DFK within each treatment in three separate experiments. The p values in blue and red signify statistical significance between control and treatment groups for either KSFM or DFK, respectively.

### 3. Discussion

The present study provides evidence that the OKF6/TERT-2 cell line grown in DFK, a novel medium composed of a 1:1 mixture of KSFM and DMEM/F-12, confers comparable morphology, mucin production, as well as *ZO-1* and *occludin* gene expression. However,

monolayer growth, wound healing performance and *claudin-1* expression occur at elevated levels when cells are cultured in DFK. In addition, E-liquids ± flavors seem to be more toxic to OKF6/TERT-2 cells cultured in DFK.

Seeding of OKF6/TERT-2 with KSFM, DMEM/F12 and DFK results in different cellular morphologies and growth rates (Fig 1), indicating that KSFM is required to initiate cell cultures as established by Dickson et al. [1]. However, once the cultures are established, the growth rate can be accelerated by switching to DMEM/F12 or DFK (Fig 2). Both alternatives yield similar growth rates and should be considered in OKF6/TERT-2 studies as these can improve culturing time and spare resources. While OKF6/TERT-2 cultured in DMEM/F12 or DFK yield comparable growth rates, cells cultured in DFK demonstrate quicker monolayer healing rates compared to the other two media. On the other hand, KSFM is required to maintain and passage OKF6/TERT-2 cells routinely grown in the laboratory, as previously established. Consequently, it appears that the combined nutrients from both KSFM and DMEM/F12 augment OKF6/TERT-2 cell growth and wound-healing capacity. Hence, KSFM and DFK were the two media compared for the remainder of this study.

Confocal microscopy with 3D imaging of OKF6/TERT-2 monolayers showed similar morphology of monolayers on both media. However, cells in the DFK medium yielded rapid proliferation without significant alterations to actin filament arrangement (Fig 3A). Both media led to the formation of small cell aggregates within 2 days, however the coalescence of these aggregates occurred 24 hours sooner when cells were grown on DFK (Fig 2A). Regarding other oral epithelial cell lines, investigators have found that telomerase immortalized gingival keratinocytes (TIGKs) demonstrate a typical ‘cobblestone’ appearance 2-3 days after seeding [56], which is similarly observed in OKF6/TERT-2 cells (Fig 1 and Fig 2A) and other oral epithelial cell lines grown in KSFM [57], as well as *ex vivo* oral mucosal epithelial cells grown in culture [58]. Our confocal imaging results indicate this cellular morphology remains unchanged in DFK and KSFM media and is consistent with the squamous morphology seen in the human oral cavity [59–61].

The increased cell density in DFK is the result of increased propensity for rapid proliferation and smaller cell size (Fig 3). Unlike TIGK cells, which rarely reach confluence in KSFM containing 0.04 mM calcium [56], the present study suggests that OKF6/TERT-2 cells grow more rapidly in the DFK composite medium containing 0.4 mM calcium (Fig 2). Higher cytosolic calcium levels correlate with increased cellular differentiation progressing from the proliferative basal layer to the superficial non-proliferative stratum corneum *in vivo* [62–65]. However, the biochemistry behind this process has been complicated by more recent reports of basal layer cells actually containing relatively lower calcium content [66]. Possibly, the higher calcium in DFK, as a result of mixing DMEM/F12 with KSFM, compared to KSFM alone could promote a proliferative phenotype in OKF6/TERT-2 cells, displaying gene expression and function (Fig 5 and 6). However, when cells reach confluence and contact inhibition, they show a more non-proliferative phenotype. As expected, DFK enhances the transition from proliferative to non-proliferative states by reaching contact inhibition faster.

The expression and function of tight junction proteins are critical for epithelial cells from a range of anatomic sites, including the oral epithelium, to maintain a clear barrier between the outside environment and the sub-epithelial tissues. For example, there is a positive correlation between the expression of *claudin-1* and *occludin* with proliferation and migration to close wounds [67]. Since *claudin-1* is overexpressed in OKF6/TERT-2 cells cultured in DFK (Fig 5B) this could potentially support the faster recovery of the wound in this medium (Fig 3).

To remain an appropriate model for research on the oral epithelia, OKF6/TERT-2 cells should demonstrate a comparable expression of tight junctions in any media selected, which was observed in our experiments with both KSFM and DFK. Our results indicate that *occludin* and *ZO-1* are similarly expressed (Fig 5B). This correlates with other studies where tight junction genes are well expressed, and even overexpressed when challenged.



For example, exposure to the commensal organism, *Streptococcus gordonii*, leads to elevated expression of the tight junctions ZO-1, ZO-2 and JAM-A, increasing the paracellular barrier function [68]. On the other hand, oral pathogens, such as *Porphyromonas gingivalis*, alter the expression levels of tight junctions [69–71], which ultimately leads to disease. This alteration in barrier integrity is correlated to susceptibility to severe allergic reactions [72], and permeability of surfaces in many other anatomic sites, notably the intestinal mucosa [73]. Further studies should focus on the role of tight junction genes in the presence of invasive oral bacteria.

MUC1 and MUC4 were found to be similarly expressed when cells were cultured in KSFM and DFK (Fig 5A), indicating that both media are effective in maintaining this phenotype in OKF6/TERT-2 cells. MUC1 is broadly expressed in mucosal tissues [74]. Similarly, mucins are released by OKF6/TERT-2 cells during culture and later found in the supernatant (Fig 6). This is consistent with other findings indicating that membrane-associated mucins are released into solution [75–77], which in the case of the oral cavity, become part of saliva. Mucins also function as decoys for the clearance of microbial infections. For example, MUC1 binds to adenovirus, reducing infection into host cells [78], where the virus binds to O-linked carbohydrates on the mucin [79]. In addition, MUC1 also binds to bacteria, including *Pseudomonas aeruginosa* [80], and *Helicobacter pylori* [81]. Furthermore, mucins could serve as a source of carbohydrates for commensal species, such as *Streptococcus gordonii* catabolism [82] or as decoys for clearance of cariogenic *Streptococcus mutans* [83,84], indicating a role in the maintenance and homeostasis of the oral microenvironment. Since both DFK and KSFM support the expression and release of mucins in OKF6/TERT-2 cells, similar to *in vivo* oral epithelial cells, either media could be used for further studies in mucin expression and function.

Based on linear regression, results of wound-healing assays demonstrate that OKF6/TERT-2 cells recover within 16 hours in DFK, 28 hours in DMEM/F12 and extrapolated to 41 hours in KSFM (Fig 3). Other wound-healing studies with the same cell line used media containing FBS. For example, OKF6/TERT-2 cells cultured with KSFM + 1% FBS recover nearly 100% by 18 hours [85]. In addition, the same cell line, cultured in Roswell Park Medical Institute (RPMI) medium + 10% FBS recovered by 24 hours after onset of the scratch in the wound-healing assay [86]. In a study by Shaikh et al., using DMEM/F12 + 10% FBS, full recovery of OKF6/TERT-2 cells took over three days [87]. In our hands, cells in cultured in complete DMEM/F12 without FBS (see materials and methods section 4.1), achieved full recovery within two days (Fig 3). In contrast, DFK (a mixture of DMEM/F12 and KSFM) results in a set of nutrients that yield faster wound-healing recovery compared to DMEM/F12 alone (Fig 3). Shaikh and coworkers [87] also tested the effects of E-liquids on OKF6/TERT-2 cells and reported a decrease in viability after treatments, which agree with our results in figure 7B. Alanazi et al. [88] show that the pathogenesis of yeast *Candida albicans* on a human gingival epithelial carcinoma cell line (grown in RPMI + 10% FBS) is increased after the microbe is exposed to tobacco flavored aerosol. While many studies make use of media + FBS, few studies used serum-free media. For example, Catalá-Valentín and coworkers [89] grew OKF6/TERT-2 cells in KSFM, using a protocol like ours, and treated cariogenic *S. mutans* with electronic cigarette aerosol containing menthol and nicotine. Subsequently, researchers found that *S. mutans* adherence to OKF6/TERT-2 cells increases after aerosol treatments. Furthermore, a parallel study by Catalá-Valentín et al. [90] shows OKF6/TERT-2 immunosuppression, where cytokine expression of the cells, challenged with *Staphylococcus aureus*, is significantly decreased after exposure to flavorless electronic cigarette aerosol.

The cytotoxicity assay employed in Fig 7C measures release and activity of LDH in the supernatant suggesting compromised cell membranes but does not specify how the cells are dying (necrosis vs. apoptosis). Although the viability data (Fig 7B) and the LDH activity data (Fig 7C) appear to be counterintuitive, a potential explanation is that E-liquid treatments induce apoptosis where cytoplasmic contents, including LDH, are packaged within apoptotic bodies and are not released into the supernatant. On the other hand, necrosis is a result of cell membrane rupture where cytoplasmic materials are spilled into

the extracellular space (i.e., supernatant). Necrosis could range from a subtle to a more abrupt event, whereas apoptosis is a gradual and organized process resulting in apoptotic bodies. Because Fig 7A shows that the overall protein content is still comparable in most samples, this supports the idea that apoptotic bodies were pelleted with intact cells, lysed and detected in the protein assay.

Future studies from our group aim to dissect the effects of E-liquids  $\pm$  flavors on the oral mucosa, specifically on (1) the cellular and molecular biology, including changes in gene expression and wound healing; (2) the physiological stress response, including glutathione and cytokine alterations; (3) the potential apoptotic events following exposure to E-liquids and; (4) the host-bacteria interactions using both commensal and pathogenic oral species. These future investigations rest on the cell culture protocols and molecular foundations established in the present study.

The oral cavity is frequently the first site of exposure to external insults. Therefore, a biologically representative model of the oral environment, such as the one described in this study, is essential considering the vast array of pathophysiological conditions that may occur in the mouth. For example, the oral epithelium was recently identified to contain angiotensin converting enzyme-2 (ACE2) [95], which works as a receptor for the spike protein on severe acute respiratory syndrome coronavirus-2 (SARS CoV2). A recent study reports the use of chewing gum containing ACE2 decoy proteins protecting the host from microbial infection [96]. Consequently, DMEM/F12 or DFK may facilitate research in such host-pathogen studies. Since our research interests focus on the use of electronic cigarettes and the effects of these on oral mucosa, this study compared the applicability of DFK or KSFM on OKF6/TERT-2 cultures exposed to E-liquids  $\pm$  flavors. An oral epithelial cell line, amenable to multiple culture conditions, without compromising the phenotype of the model, will not only facilitate further research in these areas using monolayer culturing techniques but also provide a basis for further improved organotypic 3D cultures, a more realistic representation of *in vivo* tissues [91–94].

In the present study, a monolayer culturing technique was used to compare the effects of DMEM/F12 and a novel culture medium DFK to KSFM on the OKF6/TERT-2 cell line illustrating that both DMEM/F12 and DFK are viable alternative media and more importantly, showing that these cells retain appropriate characteristics to study various aspects oral physiology. This becomes crucial in studies where OKF6/TERT-2 cells are compared to other cell lines and therefore, using the same media is critical when interpreting the results. While our results are novel and contribute to the field of *in vitro* oral biology, they are not without limitations. For example, organotypic 3-dimensional cultures with multiple cell layers reflect the oral environment more accurately and therefore increase the pertinence of *in vitro* experimentation [91–94]. However, the present study was conducted on the premise that a novel culture medium should be evaluated on a monolayer before extension to more complex 3D models. Additionally, using a single cell line for the evaluation of DFK is another limitation. Future studies should explore additional oral cell lines, such as gingival epithelial cells, in DFK or other media to further expand *in vitro* oral epithelial models as well as host-bacteria models. In addition, expression levels of only five genes were measured. These genes were chosen because they are involved in the maintaining integrity of the oral epithelia. In prospective studies, other genes and gene products, such as toll-like receptors, cytokines, adhesion proteins, etc. should be analyzed. Furthermore, in E-liquid experiments the LDH activity assay was employed, although other protocols to evaluate cytotoxicity are also available. While we are aware of these numerous limitations, it would be impractical and nearly impossible to address them all in a single manuscript.

Based on the results of these experiments, KSFM is essential to seed the OKF6/TERT-2 cell line. However, continued growth of the cultures could be achieved by either keeping the cells in KSFM or switching to DMEM/F12 or DFK. Both DMEM/F12 and DFK yielded faster growth and more dense cultures, which also enhanced the wound healing process. OKF6/TERT-2 cultured in DFK resulted in enhanced expression of *claudin-1*. In addition, the effects of E-liquids  $\pm$  flavors were amplified in DFK cultures. In our study, switching

cultures from KSFM to DMEM/F12 or DFK is a more favorable protocol because of the benefit of decreased culturing time, thus expediting research efforts.

#### 4. Materials and Methods

##### 4.1. Culture media

All culture media reagents and supplies were purchased from ThermoFisher Scientific (Waltham, MA, USA) unless otherwise indicated. KSFM (catalog # 17005042, lot # 2401396) purchase includes the base medium, bovine pituitary extract (BPE) and epithelial growth factor (EGF). BPE and EGF supplements are always provided in excess, at least twice more than needed to prepare KSFM. Complete KSFM was prepared by adding 30 µg/mL BPE, 1 ng/mL EGF, 1 mM glutamine, 0.3 mM calcium chloride and 100 U/mL penicillin/streptomycin as previously described [7]. Complete DMEM/F12 (catalog # 11320033, lot # 2522615) was prepared by adding the remainder EGF and BPE from the KSFM purchase along with glutamine, calcium chloride and penicillin/streptomycin at the same concentrations as complete KSFM. DFK was prepared by mixing complete KSFM and complete DMEM/F12 at a 1:1 (v/v). No filtration is necessary as all above reagents are purchased sterile. All media were stored at 4 °C.

##### 4.2. Cell culture, morphology and growth

OKF6/TERT-2 cells were kindly provided by Dr. Gill Diamond at Louisville University School of Dentistry, but were originally established in the study by Dickson et al. [1]. Cells were routinely cultured and maintained with KSFM in T75 flasks between passages 10 and 20. For all experiments, cells were cultured at 37 °C 5% CO<sub>2</sub>, feeding the cells with fresh media at 24 hours and then every 1 or 2 days until confluent. For seeding experiments to evaluate initial cell morphology and growth (Fig 1), cells were seeded at 50,000 cells/well with KSFM or DMEM/F12 or DFK in 24-well plates. In latter experiments, to evaluate cell morphology and growth, cells were seeded in KSFM and cultured for 24 hours as above. Spent media were removed and replaced with equal volumes of either fresh KSFM or DMEM/F12 or DFK (Fig 2). Cells were imaged at 100× magnification using a Nikon Eclipse TE2000-U inverted microscope equipped with a Nikon Digital Sight DS-Fi1 camera and NIS Elements Imagine Software (Nikon Instruments Inc, Melvin, NY, USA). For each type of media, three to six wells were trypsinized for 30 minutes at 37 °C 5% CO<sub>2</sub> and trypsin was quenched with 1% bovine serum in PBS for one minute. Cells were counted using the trypan blue exclusion assay with the hemocytometer and light microscopy every 24 hours. Spent media were collected every 24 hours of cell growth, pooled according to media type, and stored at 4 °C until pH was measured. For confocal microscopy experiments, cells were seeded in chamber slides using KSFM for the first 24 hours at 37 °C 5% CO<sub>2</sub>. Then, media was changed to fresh KSFM or switched to DFK and grown for four days as above.

##### 4.3. Wound/Healing assay

OKF6/TERT-2 cells were seeded and grown to confluency in 24-well plates using KSFM. Once confluent, media were removed, and using a sterile 1 mL pipet tip, a scratch (a straight line across the diameter of the well) was made in all cultures. Monolayers were washed twice with PBS to remove excess cellular debris and 1 mL of either KSFM or DMEM/F12 or DFK media were added to all cultures. Scratched monolayers were immediately imaged at 100× magnification (0 hours) using a Nikon Eclipse TE2000-U inverted microscope as indicated above and cultures were incubated at 37 °C 5% CO<sub>2</sub>. To assess wound recovery, cultures were imaged at 4, 8, 12, 16, 24, and 28 hours after scratching.

To quantify and compare the rate of OKF6/TERT-2 wound-heal recovery across all media, the computer image processing program ImageJ with the open source *Wound Healing Size Tool* (WHST) plugin optimized for *in-vitro* wound-heal assay analysis was utilized [97,98]. The WHST supports accurate discrimination between cell monolayer and open

wound area by fixing a line dividing the two regions, driven both by its independent algorithmic analysis as well as user-defined input of variance filter radius values and manual modification of saturation percentage in contrast enhancement. Open wound area was defined by pixel area (pixels<sup>2</sup>) and was quantified using WHST analysis of the imaged monolayers over time.

#### 4.4. Confocal Microscopy

Confluent monolayers in chamber slides were washed in PBS and fixed in 4% para-formaldehyde for 20 minutes, followed by PBS washes and aldehyde quenching using 0.1% glycine. Permeabilization was performed with 0.1% Triton X-100 in PBS for 15 minutes followed by washes and blocking with 1% bovine serum albumin in PBS. Phalloidin-FITC conjugate at 5 µg/mL in PBS was added for 30 minutes to stain F-actin (green). Samples were then washed 3 times with PBS and mounting media containing DAPI were added to stain the cell nucleus (blue). Cell samples were observed under a Carl Zeiss LSM880 laser scanning confocal microscope (Carl Zeiss Inc. White Plains, NY) at 630× magnification with oil immersion using an excitation wavelength of 405 nm and 488 nm for DAPI and FITC, respectively. Z-stacks (height) were acquired at slow speed and high resolution with an optical slicing of 1 µm. The ZEN 3.5 software (Carl Zeiss Inc. White Plains, NY, USA) was used to obtain 3D images. The confocal microscope and software were accessed in the Biological Sciences Department at the College of Arts and Sciences, Lehigh University (Bethlehem, PA, USA). The average number of cells per field view was achieved by counting the number of nuclei (blue) in both KSFM and DFK (n = 8). To calculate the average area of cells, the total area of the field of view (135 µm x 135 µm) was divided by the average number of cells. The average height of the cultures in both media was calculated by averaging the number of slices (each slice = 1 µm) in all Z-stacks (n = 17 for KSFM and n = 18 for DFK). The cell volume was calculated by multiplying the cell area by the average height.

#### 4.5. Expression of mucins and tight junction genes

OKF6/TERT-2 cells were seeded and grown to confluency in 6-well plates using either KSFM or DFK. Once confluent, media were collected and stored. Monolayers were washed twice with PBS to remove excess cellular debris and RNA was collected with the mirVana miRNA isolation kit, 100% ethanol and phenol:chloroform, following the manufacturer's instructions. The RNA concentration was determined with the nanodrop, and the VILO reverse transcription kit was used to obtain cDNA. *β-actin*, *claudin-1*, *occludin*, *zonula occluden* (ZO-1), *muc1* and *muc4* were amplified with TaqMan primers. Cycle threshold (Ct) values were obtained using the QuantStudio 3 qPCR cyclers (Applied Biosystems, Waltham, MA, USA). The initial denaturation was at 95 °C for 20 seconds followed by a total of 40 cycles, where each cycle was 1 second at 95 °C (denaturing) and 20 seconds at 60 °C (annealing and extension). Once Ct values were obtained,  $2^{-\Delta\Delta C_t}$  values were calculated using *β-actin* as control. Data are presented as percentages of *β-actin* expression levels.

#### 4.6. SDS-PAGE for released glycoproteins

Human saliva was collected from five healthy individuals under IRB approval code Cuadra\_S19\_18. Saliva samples were pooled and sterilized following a previously established protocol [47]. Approximately 12 mL of OKF6/TERT-2 cell culture supernatants in KSFM and DFK from two separate experiments were filtered through Amicon Ultra 30K centrifugal filters. The concentrates were resuspended in sterile water to dilute the salts and re-filtered. In addition, fresh KSFM and DFK media, as well as sterile human saliva, were also filter-concentrated. After Amicon filtration, all samples contain macromolecules above 30 kiloDaltons (kD), and protein concentrations were determined with the Micro BCA Protein Assay Kit, following manufacturer's instructions. Samples were adjusted to equal protein concentrations and 30 µg of proteins were separated by SDS-PAGE. Then,



all heavily glycosylated glycoproteins were stained using the PAS protocol [83,99]. After obtaining the image of all glycoproteins present in the gel, Coomassie blue was used to stain the rest of the proteins in the samples and the gel was imaged again.

#### 4.7. Effects of E-liquid treatments: protein concentration, viability and cytotoxicity

E-liquids were prepared as previously described [47,48]. Briefly, the flavorless E-liquid mixture was prepared by mixing equal volumes of propylene glycol and vegetable glycerine and supplemented with 20 mg/mL nicotine. In addition, flavors including tobacco, menthol, cinnamon, strawberry, and blueberry, were added to a final volume of 5% (v/v) to flavorless E-liquid. All E-liquids  $\pm$  flavors and their components were stored at 4°C or at room temperature, respectively.

To test the effects of E-liquids on OKF6/TERT-2 cells, confluent cultures in DFK or KSFM were exposed 1% E-liquids (v/v) dissolved in either media. Confluent monolayers were exposed to these E-liquid treatments at 37°C, 5% CO<sub>2</sub> for 24 hours. Supernatants were removed and stored at -20 °C for LDH cytotoxicity assays. Cells were trypsinized, diluted to a final volume of 1 mL and from the resulting cell suspension, only 10  $\mu$ L were used for cell viability via trypan blue exclusion. The remaining cells were pelleted and stored at -20 °C. Cell pellets were lysed in a final volume of 1 mL 0.2% Triton-X 100 solution, syringe-filtered (0.22  $\mu$ m), and assayed for total protein, as indicated above (section 4.6). The LDH cytotoxicity assay kit was used to perform cytotoxicity assays according to manufacturer's instructions. Briefly, untreated cell monolayers were lysed with lysis buffer (provided in the kit) and used as reference for 100% LDH activity. Supernatants were thawed and 50  $\mu$ L were added to 50  $\mu$ L of the reaction mixture and allowed to incubate for 30 minutes in the dark at room temperature. Finally, 50  $\mu$ L of stopping solution was added to all reactions and absorbance was read at 595 nm.

#### 4.8. Statistical analysis

Means and standard errors of the mean (SEM) were calculated and analyzed for all quantitative experiments. Two-way ANOVA followed by Bonferroni post-hoc analysis was used to compare the effects of KSFM vs DMEM/F12 vs DFK on the growth and wound healing ability of OKF6/TERT-2 cells over time. Additionally, linear regression analyses and correlation coefficients for wound-healing assays were performed over time and extrapolated to the x-intercept. Student's t-test was used to compare the effects of DFK vs KSFM on the height of OKF6/TERT-2 cells (based Z-stacks where each optical slice is 1  $\mu$ m) and the expression of mucin and tight junction genes. For comparison of protein content and viability of OKF6/TERT-2 cells grown in DFK or KSFM in the presence of E-liquid  $\pm$  flavors, a one-way ANOVA followed by Bonferroni post-hoc analysis was used to determine statistical significance between treatment groups within the same media and a student's t-test was used to determine statistical significance between media for each treatment group. All statistical tests were performed using GraphPad Prism® version 5.02 (GraphPad Software, San Diego, CA, USA). For all tests,  $p < 0.05$  was considered statistically significant.

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**Data Availability Statement:** The contributions presented in the study are included in the publication or can be located through the section entitled Supplementary Material. Any further questions or inquiries can be directed to the corresponding author.

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