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Growth Factor Cocktail to Facilitate Epithelial Differentiation of Exfoliated Deciduous Teeth Stem Cells

(Koktel Faktor Pertumbuhan untuk Memudahkan Pembezaan Epitelium bagi Sel Stem Gigi Susu yang Terkelupas)

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ABSTRACT

Epithelial cells are important in the regeneration of oral mucosal tissue. The cell source is commonly derived from tissue biopsy, which is obtained through surgery. Stem cells from human exfoliated deciduous teeth (SHED) were demonstrated to differentiate into multiple cell types. As it can be readily available from exfoliated deciduous teeth, it can be induced and become a potential source of epithelial cell for oral tissue study. This study aims to examine a mixture of growth factors in the differentiation of epithelial-like cells obtained from human exfoliated deciduous teeth (SHED) stem cells. This growth factor cocktail constitutes hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), insulin-like growth factor 2 (IGF-II), and epidermal growth factor (EGF). After introducing the cocktail, the treated SHED were assessed for epithelial characteristics and markers using cell proliferation test, morphological transformation, protein and gene expression by immunofluorescence staining and cytometry assessment, respectively. The proliferation rate was analysed statistically using Analysis of Variance (ANOVA) with Repeated Measures (p<0.05). SHED cells demonstrated morphological changes on the 7th day after using the cocktail. These transformations are in alignment with the identification of genes associated with epithelial cells and positive stain outcomes for pan-cytokeratin, E-cadherin, and p63. The cell proliferation test indicated that proliferation of cells and growth factor introduction were significantly correlated. The growth factor cocktail used for this research facilitated SHED differentiation for epithelial-like cells. The outcomes validate the production of epithelial cells using SHED; tissue production studies focus on these aspects immensely.

Keywords: Epithelial characterization; epithelial growth factors; epithelial markers; SHED differentiation

ABSTRAK

Sel epitelium adalah sel utama untuk penjanaan semula tisu mukosa mulut. Sel ini selalunya diperoleh daripada biopsi tisu melalui pembedahan. Sel stem gigi susu manusia terkelupas didapati boleh diterbitkan kepada beberapa jenis sel. Oleh kerana ia boleh diperoleh dengan mudah di dalam gigi susu yang terkelupas, maka ia boleh diinduksi dan berpotensi menjadi sumber sel epitelium untuk kajian tisu mulut. Kajian ini bertujuan untuk mengkaji campuran faktor pertumbuhan di dalam pembezaan sel epitelium yang diperoleh daripada sel stem gigi susu manusia terkelupas (SHED). Koktel faktor pertumbuhan ini dirumus daripada faktor pertumbuhan hepatosit (HGF), faktor pertumbuhan keratinosit (KGF), faktor pertumbuhan seperti insulin -2 (IGF-II) dan faktor pertumbuhan epidermis (EGF). Selepas dirawat dengan koktel faktor pertumbuhan, sel SHED dinilai untuk ciri dan penanda epitelium menggunakan kelajuan percambahan sel, transformasi morfologi dan pengekspresan protein dan gen menggunakan pewarnaan imunofluoresensi dan penilaian sitometri. Kadar percambahan dianalisis secara statistik menggunakan ANOVA sehala dengan pengukuran berulang (p<0.05). Sel SHED menunjukkan perubahan morfologi pada hari ke-7 selepas menggunakan koktel. Transformasi ini adalah sejajar dengan pengenalpastian gen yang dikaitkan dengan sel epitelium dan hasil pewarnaan positif untuk pan-sitokeratin, E-cadherin dan p63 menggunakan pewarnaan imunofluoresensi dan penilaian sitometri. Analisis statistik ujian menunjukkan bahawa percambahan sel dan pengenalan faktor pertumbuhan berkorelasi dengan ketara. Koktel faktor pertumbuhan yang digunakan untuk kajian ini didapati menggalakkan pembezaan SHED kepada sel seperti epitelium. Hasil kajian mengesahkan penerbitan sel epitelium daripada sel SHED setelah koktel digunakan; kajian penghasilan tisu meletakkan nilai yang tinggi pada aspek ini.

Kata kunci: Faktor pertumbuhan epitelium; pembezaan SHED; penanda epitelium; pencirian epitelium

Introduction

Epithelial cells are tightly arranged on the surface lining of various organs and tissues like skin, oesophagus, lungs, cornea, and oral mucosa. From an embryological perspective, epithelial cells are produced in the ectodermal germ layer (Kolltveit et al. 2010; Queiroz et al. 2010). These cells are critical because they constitute the defence system by creating a cell sheet that lines the human body surface externally and internally. This layer constitutes mechanical and chemical isolation for the body and its surroundings (Le Bras & Le Borgne 2014; Liu et al. 2011; Roignot et al. 2013).

Several areas from the oral cavity like the hard palate, buccal mucosa, and gingiva are appropriate for gathering epithelial cells to study oral tissue regeneration (Gartner et al. 1994). Moreover, external cells can be extracted using adipose induction, skin biopsy, bone marrow cell extraction, and embryonic and umbilical cord cell extraction. Recently, pluripotent stem cell has also been induced (Liu et al. 2011). Typically, gathering such epithelial cells requires minor surgical procedures that might be uncomfortable and invasive (Sen et al. 2011). Nevertheless, it is ideal to distinguish between stem cells and epithelial cells from an oral tissue standpoint. The primary reason is that neural crest cells can also be used for obtaining dental stem cells (Volponi et al. 2010), which might have relatively low phenotypic differences when employed for regenerating oral cells.

In this research, we employed human exfoliated deciduous teeth (SHED) stem cells; these are highly potent cells first identified in 2003 from live pulp remains of exfoliated deciduous teeth (Miura et al. 2003). SHED access does not require invasive or complex processes and the cells have an extraordinary proliferation potential, which is the primary reason for use (Gronthos et al. 2002; Miura et al. 2003). Hence, SHED can offer numerous cells for clinical and experimental requirements. Moreover, these cells can differentiate better than stem cells from other dental areas (Miura et al. 2003). Such properties suggest that SHED is likely to be less mature than postnatal stromal stem cells (Annibali et al. 2014; Miura et al. 2003; Saez et al. 2016; Suchánek et al. 2010). SHED cells can differentiate into several other types like neural cells, osteoblasts, and adipocyte-like cells (Egusa et al. 2012; Miura et al. 2003). Previous research provides information concerning several SHED differentiation forms using different culture media and specific growth factors (Hebert et al. 2009; Wang et al. 2010). SHED cells have exceptional differentiation ability concerning cell variety; hence, they have been emphasised for regenerating the dental tissue. However, there is limited work concerning techniques for differentiation into epithelial-like cells. SHED cells have been a primary source for several experiments; keratinocyte basal medium (KBM) and serum-free keratinocyte growth medium (KGM) were used to create epithelial-like cells (Nam et al. 2009).

However, using TGF-β as the only growth factor was inadequate to trigger SHED epithelial differentiation (Azmi 2017). Our efforts to repeat epithelial differentiation for commercial SHED were not fruitful when performed with one growth factor (data not indicated). Hence, this work attempts to study SHED differentiation using a commercial origin and a mix of several growth factors, namely, hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), insulin-like growth factor 2 (IGF-II), and epidermal growth factor (EGF).

Materials and Methods

Culture of SHED

The commercial SHED (AllCells, USA) was cultured and expanded according to Gronthos et al. (2000) with modifications. SHED between passage 6 and 9 with 70-80% confluence was used for further investigation in this study.

Differentiation of SHED into epithelial-like cells

To induce epithelial differentiation, SHED was cultured in the induction media comprising of minimum essential medium - α modification (α-MEM), 10% foetal bovine serum (FBS), 1% penicillin (100 units/mL)/streptomycin (100 µg/mL). The media was added with a cocktail of selected growth factors: (12.5 ng/mL keratinocyte growth factor (KGF) (Millipore, USA), 25 ng/mL epidermal growth factor (EGF) (Millipore, USA), 12.5 ng/mL hepatocyte growth factor (HGF) (Gibco, USA), and 75 ng/mL human insulin-like growth factor-2 (IGF-II) (Gibco, USA) as per Păunescu et al. (2007) with slight modifications. SHED cultured in growth medium was used as the control. Both cells were kept in 37 ºC incubator with 95% relative humidity and 5% CO_2, and the media were changed every 2 to 3 days. The controls and differentiating cells were viewed and captured every day using an inverted microscope image analyzer Axiovert 25 (Ziess, Germany).

SELECTIVE DIGESTION METHOD FOR EPITHELIAL DIFFERENTIATION

Some cells were observed to begin transforming their shape from spindle to cobblestone-shaped appearance at day 7 post-induction. Therefore, selective digestion based on Farea et al. (2016) was carried out by isolating these cobblestone-shaped cells from the heterogonous cell populations consisting of both cobblestone and spindle-shaped cells. In this method, the induction media were aspirated from the cell culture plate and the heterogeneous cells were washed with phosphate buffer saline thrice. After that, approximately 0.5 mL of trypsin-EDTA 0.25% was applied for 2 min, resulting in the detachment of the spindle-shaped cells and leaving several cobblestone-shaped cells attached to the culture plate. These cobblestone-shaped cells were then washed with PBS thrice and further cultured in the induction media to obtain a colony of epithelial-like cells.

CHARACTERISATION OF EPITHELIAL-LIKE CELLS

Proliferation Rate Evaluation

The epithelial-like cells derived from SHED at the density of 10×10^3 cells/well were seeded in 6-well plate. Approximately 100 µL alamarBlue® cell viability reagent was loaded into each well at day 0, 3, 5, 7, 15, and 21, respectively. The culture media were collected after 4 h of incubation and the result was read using an absorbance plate reader at the wavelengths of 570 and 600 nm.

Gene Expression of Epithelial Markers via Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction

The controls (SHED and human keratinocytes) and induced cells were harvested at 1, 3-, 7-, 14-, and 21-days post-induction and the extraction of total RNA was performed using innuPREP RNA Mini Kit (Analytik Jena, Germany). The reverse-transcription of 200 ng of total RNA was later carried out according to the protocol using Tetro cDNA Synthesis Kit (Bioline, UK) containing MMLV Reverse Transcriptase and Oligo $(dT)_{18}$. The generated cDNA was used as a template for cDNA amplification using MyTaq™ HS Mix (Bioline, UK). The gene-specific primers were designed based on the published cDNA sequences in GenBank (Table 1). The stem cell-specific gene markers used were *CD44, CD73,* and *Nestin*, while the epithelial-specific gene markers used were *CK18*, *FLG,* and *KRT14*. Meanwhile, β-*actin* served as the housekeeping gene. The integrity of PCR products using 1% agarose gel electrophoresis was examined and all reactions were done in triplicates. The human keratinocytes were used as the positive control, whereas SHED without any induction was used as the negative control.

TABLE 1. Primer sequences for various genes used in sqRT-PCR

Gene	Primer sequences	Size (bp)	Accession No	Reference
β-actin	F: 5′-TGGCACCCAGCACAATGAA-3′ R: 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′	186	NM_001101.3	Gazaria and Ramírez-García (2017)
CD44	F: 5′-GGCTCACTCAAGCTCTTTAACT-3′ R: 5′-TAGACCTCCCTTATTTCTATCGT-3′	284	NM_000610.3	Zhang et al. (2016)
CD73	F: 5′-CTGCCTCTGTCCTCTTTCTT-3′ R: 5′-TATTTTCTGACCACCCACTCA-3′	311	NM_002526.3	Zhang et al. (2016)
Nestin	F: 5′-CAGCGTTGGAACAGAGGTTGG-3′ R: 5′-TGGCACAGGTGTCTCAAGGGTAG-3′	389	NM_006617.1	Ma et al. (2012)
CK18	F: 5′-CAAGGAGGAGCTGCTCTTCATG-3′ R: 5′-TGGTGCTCTCCTCAATCTGCTG-3′	221	NM_199187.1	Păunescu et al. (2007)
FLG	F: 5′-TGCCATAATTAATCTTTTCAAGCA-3′ R: 5′-TGCTTTCTGTGCTTGTGTCC-3′	285	NM_002016.1	Iwamoto et al. (2012)
KRT14	F: 5′-GACCATTGAGGACCTGAGGA-3′ R: 5′-GGCTCTCAATCTGCATCTCC-3′	224	NM_000526.4	Gschwandtner et al. (2013)

Protein Expression of Epithelial Markers via Flow Cytometry Analysis

The flow cytometry analysis on the epithelial-like cells and controls (human keratinocytes and SHED) at the density of 1×10^6 cells was performed. Following harvesting, the cells were fixed with ice-cold ethanol for 10 min and permeabilised with 0.1% Triton X-100. Then, the blocking reagent was applied for 15 min before primary staining was carried out by incubating the cells with the epithelial primary antibodies: rabbit-anti-E-cadherin (1:5; Santa Cruz Biotechnology Inc., CA), rabbit-anti-p63 (1:100; Biorbyt, USA), and rabbit-anti-pan-cytokeratin (1:100; Santa Cruz Biotechnology Inc., CA) antibodies for 1 h. Thereafter, the cell pellets were incubated with the secondary antibody, namely the goat anti-rabbit IgG (Alexa Fluor® 488) antibody (1:400; Abcam, CA) at room temperature in the dark for 30 min before being investigated using the flow cytometer.

Protein Expression of Epithelial Markers via Immunofluorescence Staining

The epithelial-like cells and controls were cultured on #1.5 (.27 mm) thickness, 12 mm circular glass coverslip in a 24-well plate. Then, they were cultivated when they reached 70% confluence and fixed with ice-cold methanol for 10 min at -20 °C. The permeabilisation and blocking were carried out using 0.1% Triton X-100/PBS and normal goat serum, respectively. The primary staining involved three specific epithelial primary antibodies similar to those used in the abovementioned flow cytometry, namely rabbit-anti-E-cadherin (1:50), rabbit-anti-p63 (1:100), and rabbit-anti-pan-cytokeratin (1:50). After the incubation of cells with the primary antibodies at 4 ºC overnight, the secondary antibody, namely goat anti-rabbit IgG (Alexa Fluor® 488) antibody (1:400), was applied at RT for 2 h. The cells were incubated in DAPI (Sigma-Aldrich, USA) for 5 min to counterstain the nuclei, whereas fluorescent mounting medium (DAKO, USA) was applied to mount the slides before they were viewed under a fluorescent microscope.

STATISTICAL ANALYSIS

Statistical analysis using Repeated Measures Analysis of Variance (ANOVA) ($p<0.05$) was carried out to analyse the cellular proliferation rate, whereas One-Way ANOVA ($p<0.05$) was performed to analyse the gene expression of epithelial markers since the data were normally distributed. All data are indicated as the mean value of triplicates.

RESULTS AND DISCUSSION

Stem cell differentiation phenomena are regulated by numerous aspects like intercellular contact, physical forces, ECM-cell contact, mitogen presence (e.g., growth factors and cytokines) (Clause et al. 2010). In this work, we assessed the efficacy of the growth factor mixture to stimulate SHED epithelial differentiation. This cocktail comprises HGF, KGF, IGF-II, and EGF. It is known that several cells like epidermal keratinocytes, epithelial, hepatocytes, and intestinal epithelial cells express KGF (Yen et al. 2014). This factor acts as a mitogen for several epithelial cell types. A study by Shangar et al. (2020) also found that using KGF in conjunction with collagen type 4 to transdifferentiate dental pulp stem cells (DPSCs) into cornea epithelial like cells (CEC) holds promise. IGF-II and EGF are known to stimulate cell differentiation, migration, proliferation, extracellular matrix component production, and basement lining creation to facilitate faster healing in corneal and epidermal injuries (Bergman et al. 2013; Schultz et al. 1992). EGF has been frequently used in investigations involving dental stem cells for many years, as in treating cultured dental follicle cells with EGF for more than 6 days boosted their development compared to controls (Lin et al. 1996). In the study involving dental stem cells, IGF-II is a less explored growth factor. One study found that releasing this growth factor from the dentine matrix by pulp-capping agents increased pulp tissue repair-related activities (Tomson et al. 2016). Similarly, HFG is another factor that induces morphogenesis, angiogenesis, epithelial proliferation, and motility (Nakamura & Mizuno 2010). In addition to the aforementioned tasks, HGF is also employed for differentiation in dental stem cells. Previous research found that this growth factor might drive DPSCs to develop into hepatocyte-like cells (Ishkitiev et al. 2010).

The experiment started with the addition of a single growth factor, i.e., KGF (10 ng/mL) to the induction media. This method, however, did not result in the development of SHED into epithelial-like cells. The following attempts were made with KGF (10 ng/mL) and EGF (20 ng/mL), both of which failed to result in effective cell differentiation. Further attempts were made as per Păunescu et al. (2007), in which their study used various growth factors in the culture media, particularly KGF (10 ng/mL), EGF (20 ng/mL), HGF (10 ng/mL), and IGF-II (60 ng/mL). However, there were no cobblestone/cuboidal shaped cells observed throughout the 21 days of incubation. This contradicted finding might be due to different cells (their study induced human bone marrow-

derived MSCs) and culture environments (they used DMEM medium) employed between the current study and Păunescu et al. (2007). Therefore, the approach was slightly modified by increasing the concentrations of the growth factors to 25%, thus making the new concentrations to be 12.5 ng/mL of KGF, 25 ng/mL of EGF, 12.5 ng/mL of HGF, and 75 ng/mL of IGF-II. With the increased concentration of these factors, there was emergence of several cuboidal/cobblestone-shaped cells in the culture at day 7, similar to the typical appearance of the epithelial cells, denoting that the differentiation of SHED into epithelial-like cells started to occur. Hence, this approach was thought to successfully offer the necessary environment for SHED activation and to be responsible for SHED differentiation into epithelial-like cells. Taken together, all of the efforts suggest that, while SHED could be easily differentiated into many types of cells, it was quite difficult to induce these cells into epithelial lineage, which required multiple combinations of growth factors at different concentrations to achieve the maximum induction of the cells.

MORPHOLOGICAL CHANGES AND PROLIFERATION PROPERTIES

Cell monolayers grew adequately on the culture plate. Photomicrographs indicated numerous epithelial-like cells growing on the induction culture on the 7th day; these cells look like human keratinocytes. Induced cells transform from a spindle-like appearance to a cobblestone shape. These differentiated cells appear relatively less confluent than SHED. AlamarBlue® cell viability reagent used employed for proliferation assay at specific periods. It indicated that proliferation was completed for all cells with extended incubation time. Between days 3 and 7, epithelial-like cells proliferated significantly, followed by a subsequent decline at day 15 onwards, compared to SHED. Group-specific one-way ANOVA tests (one-way ANOVA, post hoc Bonferroni test, $p<0.05$) and other major differences indicate how several growth factors act together and enhance proliferation and differentiation (Figure 1).

These outcomes show that the growth factor mixture initiated a morphological transformation where spindle-shaped SHED changed to epithelial-like cells. Observations also indicated a more rapid proliferation between days three and five than the control SHED sample, suggesting that differentiation started after the seventh day. Morphological assessments were made along with these observations. Epithelial cells had a cobblestone shape on the seventh day, indicating that differentiation and proliferation shared an inverse correlation. These observations align with Ruijtenberg and van den Heuvel (2016), who stated that cells differentiate after proliferation stagnates, reduces, or stops. Epithelial-like cells had lower proliferation rates between days 15 and 21, suggesting that the culture augmented using growth factors did not lead to cell quiescence.

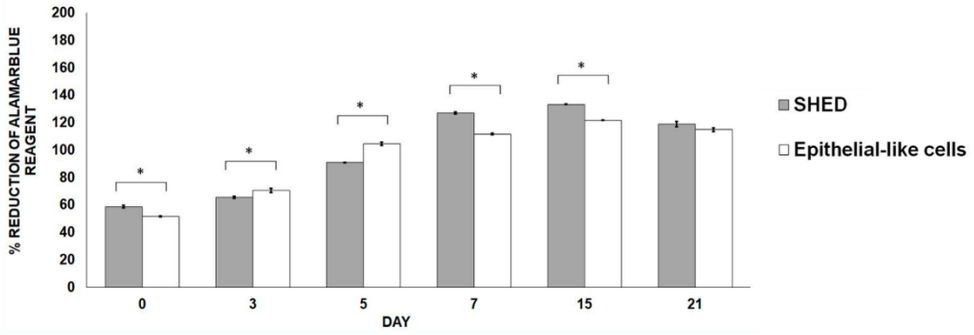

FIGURE 1. Proliferation rate between epithelial-like cells and SHED at 10 × 10^3 cells/well of seeding density incubated for 0, 3, 5, 7, 15 and 21 days. *indicates a significant difference between epithelial-like cells and SHED (Repeated Measures ANOVA, $p<0.05$)

GENE EXPRESSION OF EPITHELIAL MARKERS

Samples having 1, 3, 7, 14, and 21-day induction periods were identified. RNA was extracted, and electrophoresis was performed using 1% agarose gel. Observations indicated the presence of 18S and 28S bands, suggesting an adequate RNA extract preservation level. The samples were suitable for downstream sqRT-PCR assessment (data not indicated). Stem cell marker expression for *CD44* (284 bp), *CD73* (311 bp), and *Nestin* (389 bp) suggested the preserved SHED stemness for the entire

differentiation duration (data not indicated). In contrast, epithelial-linked gene expressions for *FLG* (285 bp), *CK18* (221 bp), and *KRT14* (224 bp) were evaluated for human keratinocytes, epithelial-like cells, and SHED, respectively. Data were normalised based on the housekeeping gene (β-actin) and benchmarked using gene expression ratios, which indicated average densities.

During the current research, changes in CK18 gene expression were detectable during the complete differentiation course (Figure 2). *CK18* expression was found for epithelial-like cells that were prepared using induction substances even on the first day. This marker had significantly low expression levels than SHED-specific values corresponding to days 1, 3, and 7. *CK18* expression suggested that epithelial-like cells had intermediate filaments (Kulesh & Oshima 1988). Moreover, stem cells also expressed this marker (Li et al. 2011). The early expression of *CK18* supported the theory that this cytokeratin is among the first epithelial structural protein that is expressed during the differentiation into epithelial cells (Griesche et al. 2012). *CK18* expression levels in epithelial-like cells peaked on the 14th day compared to that in SHED and HEK. This phenomenon could be due to the probability of mesenchymal-epithelial transitions (MET), where the mesenchymal cells start to lose their motility and migratory properties and acquire the adhesion and polarity of epithelial cells. In other words, the mesenchymal cell migratory and motility characteristics are reduced, and they start gaining polarity and adhesion characteristics matching epithelial cells (Li et al. 2011). Human keratinocytes had more significant *CK18* expression than SHED or epithelial-like cells for the entire 21-day period.

In contrast, *FLG* expression patterns suggest that this epithelial-correlated marker indicated varying human keratinocytes and epithelial-like cell expression levels. *FLG* expression for epithelial-like cells starts at day seven (Figure 2). Gene expression levels for epithelial-like cells were significantly more than human keratinocytes, measured at days 7, 14, and 21. *FLG* expression declined on the 14th day for epithelial-like cells but rose on the 21st day. However, *FLG* expression was more minor for human keratinocytes than epithelial-like cells regardless of the observation period. *FLG* expression was absent

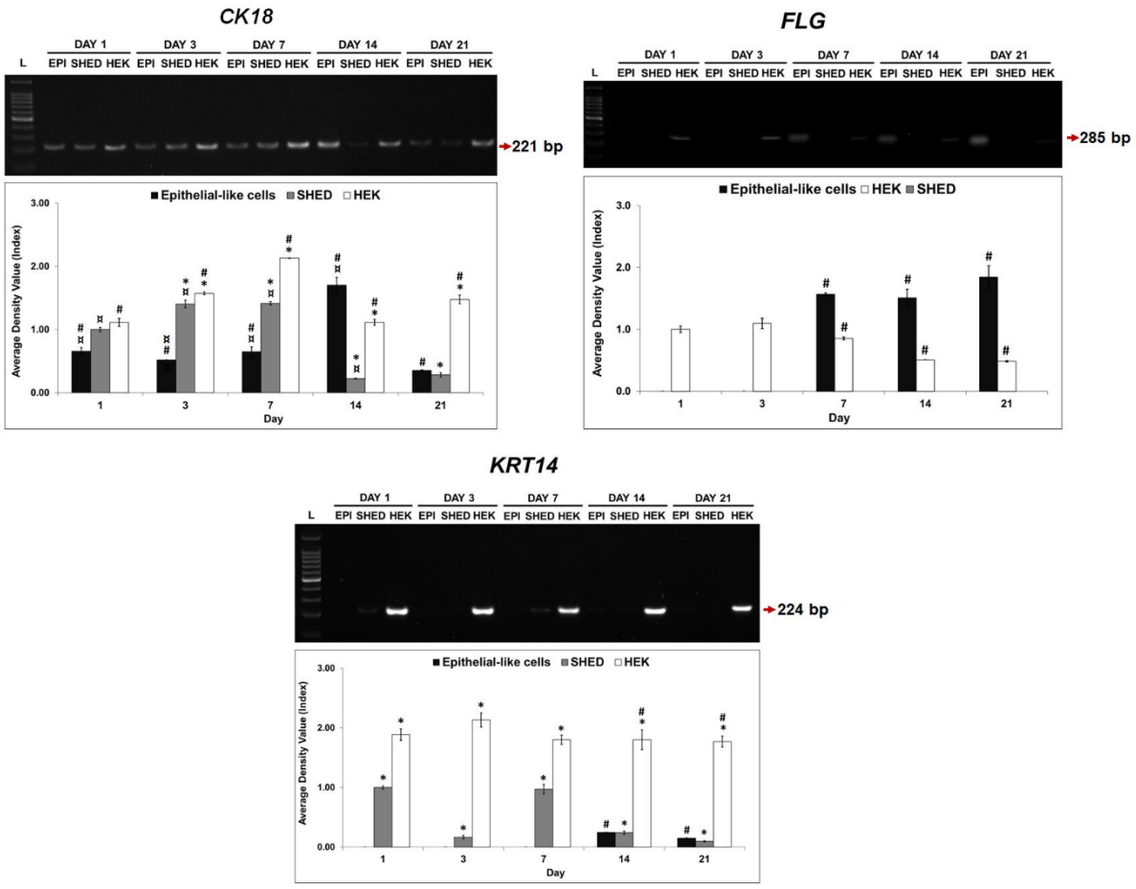

FIGURE 2. Images of agarose gel electrophoresis of epithelial gene markers expressed in SHED treated with growth factors cocktail. The graphs represent the average density values of gene expression. ¤ indicates significant difference between epithelial-like cells and SHED, # indicates significant difference between epithelial-like cells and human keratinocytes, and * indicates significant difference between SHED and human keratinocytes (one-way ANOVA, post-hoc Bonferroni test, $p<0.05$). HEK, human keratinocytes; L, 100 bp DNA ladder 100 bp Plus

for SHED throughout the 21-day incubation period. *FLG* expression on the 7th day suggested the production of an intermediate filament related protein. On day 21, *FLG* expression increased, indicating that epithelial-like cells were in the final differentiation phase; it happened concurrently with *FLG* marker presence indicating keratinocytes undergoing terminal differentiation (Senshu et al. 1996).

In the case of *KRT14* (Figure 2), gene expression for this biomarker was detectable only on the 14th day for epithelial-like cells. Expression levels did not have extensive variation concerning 14th and 21st-day SHED and epithelial-like cells. SHED had gene expression for the complete 21-day incubation phase. Nevertheless, SHED witnessed reduced expression levels on the 14th and 21st days, less pronounced than epithelial-like cells. In the case of human keratinocytes, *KRT14* expression levels were significant for the day 1 to day 21 period *KRT14* expression was first detectable on the 14th day; this gene prevents epidermal cells from losing normal shape (Coulombe & Omary 2002). These observations are aligned with morphological information where cobblestone-shaped cells started appearing between days 7 and 14. Epithelial-like cells did not have a significant gene expression variation between days 14 and 21. Gene expression outcomes of the cells treated with the combination of growth factors indicate that SHED could have developed into epithelial-like cells.

PROTEIN EXPRESSION OF EPITHELIAL MARKERS FROM IMMUNOFLUORESCENCE STAINING AND FLOW CYTOMETRY

We evaluated various epithelial biomarkers that validated the presence of epithelial-like cells. These markers include pan-cytokeratin, E-cadherin, and p63 seven days after induction. Flow cytometry assessment was used because cobblestone-shaped cells were identifiable on days 1 and 3. These epithelial-like cells exhibited significant pan-cytokeratin (78.50%), p63 (90.50%), and E-cadherin (99.35%) expression. Moreover, these markers were highly expressed in the human keratinocytes control samples, i.e., 79.32% pan-cytokeratin, 97.28% p63, and 91.24% E-cadherin. However, SHED samples had relatively mild gene expression levels (1.11% pan-cytokeratin and E-cadherin, and 27.42% of p63) (Figure 3).

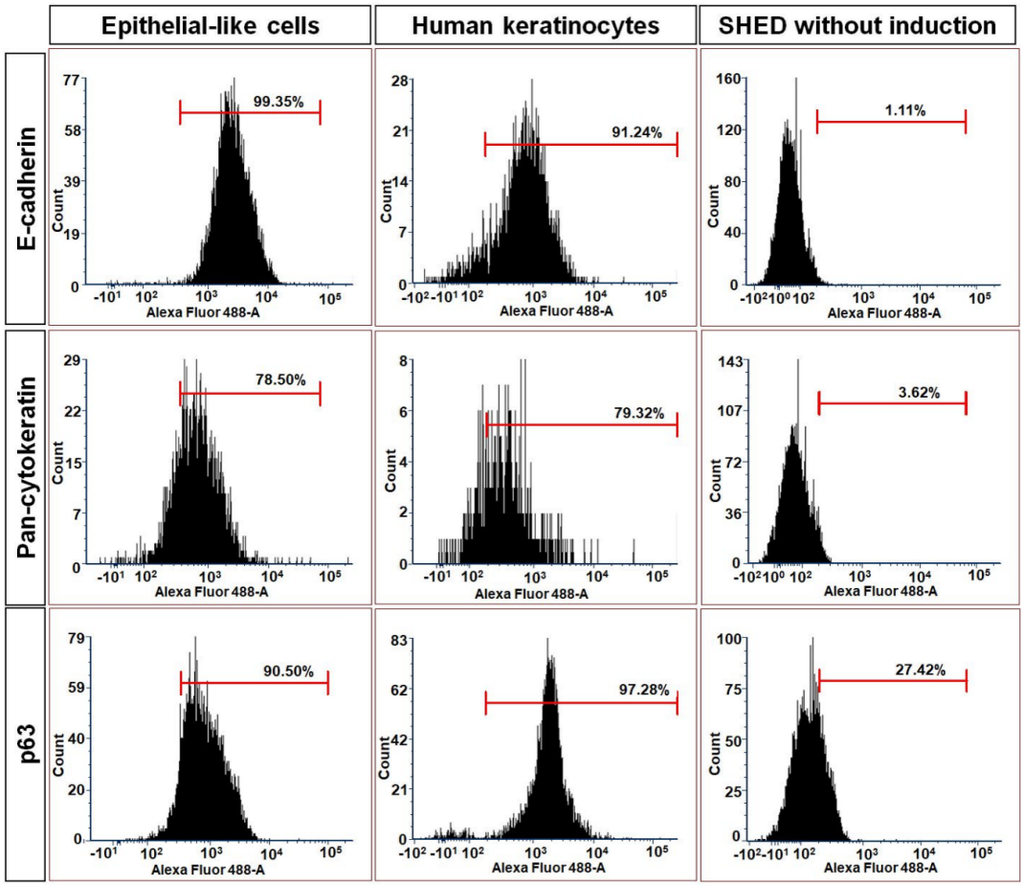

FIGURE 3. The expression of epithelial protein markers expressed of SHED treated with growth factors cocktail at day 7 by flow cytometry. The epithelial-like cells displayed high expression of E-cadherin (99.35%), pan-cytokeratin (78.50%), and p63 (90.50%). SHED with very low detection served as the negative control while human keratinocytes with high expression served as positive control cells

Outcomes from flow cytometry assessment were reinforced by the phenotype-based properties of human keratinocytes, epithelial-like cells, and SHED using a monolayer culture for qualitative immunofluorescence staining. Results showed that epithelial-like cells demonstrated staining against p63, pan-cytokeratin, and E-cadherin which is similar to those for human keratinocytes. Nevertheless, SHED marker expressions were not demonstrated independent of growth factor stimulation, validating the staining phenomena (Figure 4). Concerning the protein expression assessment of epithelial biomarkers, these markers were identified based on three categories, i.e., cytoplasmic (pan-cytokeratin), nuclear (p63), and cell membrane protein (E-cadherin) (Akimoto et al. 2011; de Paiva et al. 2005; Nam & Lee 2009).

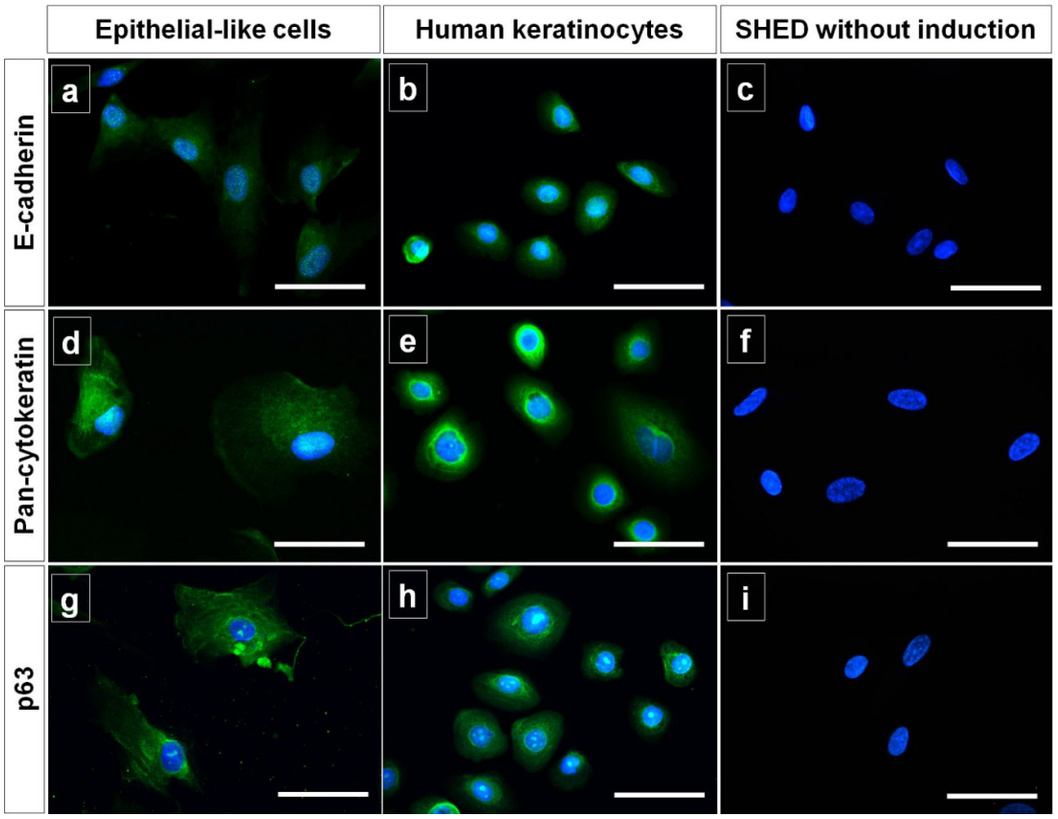

FIGURE 4. The expression of epithelial protein markers expressed of SHED treated with growth factors cocktail at day 7 by immunofluorescence staining. Human keratinocytes (positive control) and SHED (negative control). E-cadherin (a, b, c); pan-cytokeratin (d, e, f); p63 (g, h, i). The epithelial-like cells were positively stained for all markers. Similar pattern of expressions was also observed in human keratinocytes. SHED exhibited no signal supporting the validity of staining. The nuclei were counterstained with DAPI. Scale bar = 100 µm. Magnification is 200 ×

Studies report E-cadherin expression for epidermal and oral keratinocytes (Garlick & Fenjves 1996; Presland & Dale 2000). Also, pan-cytokeratin indicates epithelial cell lineage because it has a transitional filament type in every epithelial cell (Painter et al. 2010). Biomarker p63 is identified in stratified epithelial tissues; it also controls epidermis development (Soares & Zhao 2018). This research determined that epithelial-like cells had expressions of pan-cytokeratin, p63, and E-cadherin. Staining characteristics concerning epithelial protein biomarkers validated the outcomes of gene expression assessment. Hence, signifying that SHED differentiate into epithelial-like cells when stimulated using the mixture of growth factors.

Conclusions

The present study depicted how SHED cells respond to a four growth-factor mixture (EGF, IGF-II, KGF, and HGF) and successfully differentiate into epithelial-like cells. The outcomes indicate that these growth factors are suitable to offer a stimulating growth medium needed to induce SHED to differentiate into epithelial-like cells. This study approach could offer to enable additional research concerning SHED and SHED-differentiated epithelial cells. Such phenomena are vital to recreating tissue, specifically related to the oral cavity.

Acknowledgements

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