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Investigating wound healing characteristics of gingival and skin keratinocytes in organotypic cultures

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ABSTRACT

Objectives: The gingiva heals at an accelerated rate with reduced scarring when compared to skin. Potential well-studied factors include immune cell number, angiogenesis disparities and fibroblast gene expression. Differential keratinocyte gene expression, however, remains relatively understudied. This study explored the contrasting healing efficiencies of gingival and skin keratinocytes, alongside their differential gene expression patterns.
Methods: 3D organotypic culture models of human gingiva and skin were developed using temporarily immortalised primary keratinocytes. Models were wounded for visualisation of re-epithelialisation and analysis of keratinocyte migration to close the wound gap. Concurrently, differentially expressed genes between primary gingival and skin keratinocytes were identified, validated, and functionally assessed.
Results: Characterisation of the 3D cultures of gingiva and skin showed differentiation markers that recapitulated organisation of the corresponding *in vivo* tissue. Upon wounding, gingival models displayed a significantly higher efficiency in re-epithelialisation and stratification versus skin, repopulating the wound gap within 24 hours. This difference was likely due to distinct patterns of migration, with gingival cells demonstrating a form of sheet migration, in contrast to skin, where the leading edge was typically 1-2 cells thick. A candidate approach was used to identify several genes that were differentially expressed between gingival and skin keratinocytes. Knockdown of *PITX1* resulted in reduced migration capacity of gingival cells.
Conclusion: Gingival keratinocytes retain *in vivo* superior wound healing capabilities in *in vitro* 2D and 3D environments. Intrinsic gene expression differences could result in gingival cells being 'primed' for healing and play a role in faster wound resolution.
Clinical Significance Statement: The successful development of organotypic models, that recapitulate re-epithelialisation, will underpin further studies to analyse the oral response to wound stimuli, and potential therapeutic interventions, in an *in vitro* environment.

1. Introduction

The healing of the oral mucosa is often compared to foetal wound healing due to their shared superiority over skin, with both healing at a faster rate and displaying minimal scar formation [1,2]. This superior wound closure has been demonstrated at the tissue level in multiple models including pigs, mice and humans, highlighting a pattern of differential wound resolution [3–5]. Multiple factors have been attributed to this disparity, including; reduced number of immune cells in oral tissue, of which presence can lead to scarring [6,7], lower levels of pro-fibrotic TGFβ1 in oral wounds [8], lower levels of angiogenesis and VEGF expression in oral wounds [7,9] and differential gene expression between fibroblasts of the tissues [10,11]. In comparison to these other factors, the role of keratinocytes remains relatively understudied. However, the superior wound healing of oral keratinocytes has been demonstrated in hard palate cells *in vitro*, potentially due to the presence of wound-activated transcriptional networks in unwounded cells [5,12]. For example, the transcription factor paired-like homeodomain 1

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(*PITX1*) has been shown to be highly expressed in oral keratinocytes compared to skin and when virally induced in skin keratinocytes increases their migratory capacity [5].

The gingiva itself consists of multiple epithelia, differing in keratinisation in order to protect against abrasion (keratinised) or more flexible to aid mastication (non-keratinised junctional epithelium (JE)) (Fig. 1A) [13]. The JE mediates the adhesion of the gingival tissue to the tooth surface through hemidesmosomes with integrin α6β4 providing the specific anchoring function [14]. It is also characterised by the absence of rete ridges and large intercellular spaces. The morphology of the keratinised epithelium of the gingiva is more akin to skin, both expressing KRT1 and KRT10, rather than KRT4 and KRT13 expressed by the JE [13].

3D organotypic models are often used to recreate epithelia although the use of them to recreate gingival tissue has been limited compared to their wide-spread use to model skin (Fig. 1B). However, it has been shown that all three types of gingival epithelia can be reconstituted, depending upon the time incubated at the air-liquid interface [15]. These protocols often call for large numbers of primary cells per model, in the range of 250,000 fibroblasts and up to 700,000 keratinocytes, which can be problematic as large biopsies would be required [16–18]. To overcome this, ROCK inhibitor, most often Y27632, can be used to effectively immortalise primary keratinocytes and rescue those close to replicative senescence without the need for genetic manipulation [19, 20]. The removal of the inhibitor in 3D cultures allows the cells to differentiate at the air-liquid interface [20,21].

Epithelia act as a barrier against infection and fluid loss, essential for tissue function. Consequently, wound healing plays a critical role in the preservation of health and consists of four phases: haemostasis, inflammation, proliferation, and maturation. In brief, haemostasis includes vascular constriction, platelet aggregation and fibrin formation. Inflammation involves the influx of leukocytes and lymphocytes, followed by re-epithelialisation, angiogenesis, collagen synthesis and ECM formation in the proliferation step. Finally, the process ends with maturation, where collagen is remodelled and vascular maturation occurs [22]. Keratinocytes are the main driver of re-epithelialisation, where they migrate and proliferate to cover the wound. Two theories of how this occurs are sliding sheet migration, where basal and suprabasal cells migrate collectively [23], and extending shield migration, where the epithelia migrates in a triangular like format with basal cells protruding with a single cell tip [24].

The most common method to study wound healing *in vitro* is to analyse migration using a scratch assay [25]. A robust, replicable 2D method allows for comparison between cell types and genetic manipulation of the cells (for example siRNA). An improvement of the traditional 2D method is to use 3D organotypic models to allow stratification, differentiation, and paracrine signalling between cell types. These models are improving rapidly to incorporate immune cells [26,27], demonstrate aspects of wound healing [28], and mimic scarring [29], but are unable to recapitulate all phases of wound healing. They can,

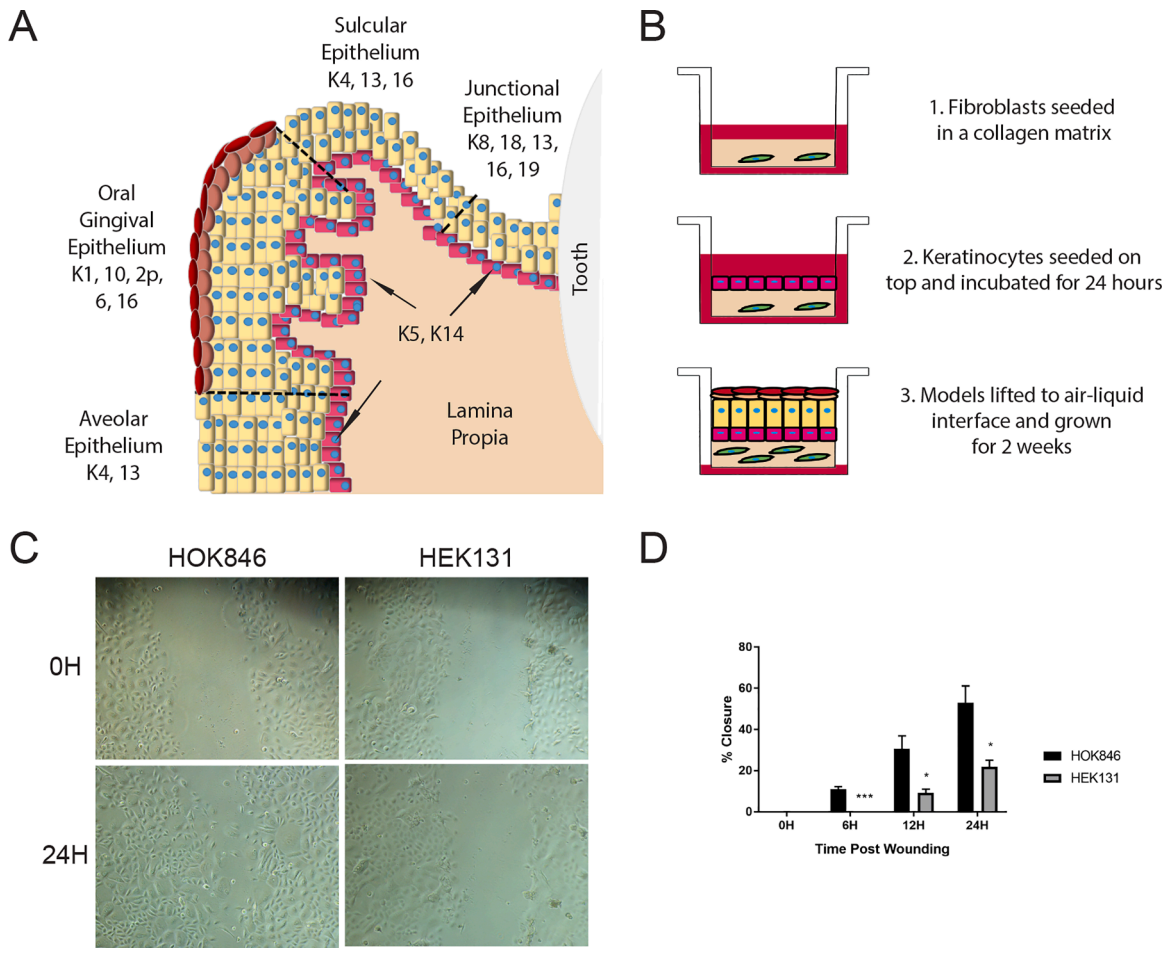

Fig. 1. Wound closure assay of gingival and skin primary keratinocytes.
A. Schematic diagram of the different human gingiva epithelia, detailing the cytokeratins expressed in each. B. Schematic of creating 3D organotypic models of the human skin and gingiva. C. – Cells were grown to confluency and scratched with a p100 pipette tip. Cells were imaged at 0H, 6H, 12H and 24H post wounding. 0H and 24H images are shown. D – Percentage wound closure was calculated using ImageJ and differences were analysed using . (n=3, multiple t tests were performed, a test for each timepoint *p<0.05, ***p<0.001,).

however, be used to study re-epithelialisation after wounding through biopsy punch, ultra-pulsed CO_2 lasers and other wounding techniques [24,28,30].

Here we aim to recreate the human gingiva and skin using 3D organotypic models and subsequently use these models to investigate the different wound healing capacities of gingival and skin keratinocytes. We also aim to investigate the differences in the transcriptional pathways between gingival and skin keratinocytes that could contribute to the superior wound healing of oral tissues observed *in vivo*. Identifying these differences will advance our knowledge of oral wound healing and potentially highlight pathways that could be targeted to improve the efficiency of skin wound healing. Furthermore, the development of 3D organotypic models of the gingiva using primary keratinocytes and ROCK inhibitor will allow broader research to be achieved without the use of animal models.

2. Methods

2.1. Cell culture

Human oral keratinocytes (HOK, gingival keratinocytes extracted from juveniles) and human epidermal foreskin keratinocytes derived from 3 to 6 donors (HEK, Invitrogen) were expanded on feeder fibroblasts in the presence of ROCK inhibitor (10μM) in FAD media (3:1 (v/v) DMEM:F12, 10% FBS, 5 ug/ml insulin, 0.4 ug/ml hydrocortisone, 10^{-10} M cholera enterotoxin, 10 ng/ml EGF and 1% penicillin/streptomycin) to induce indefinite proliferation of keratinocytes [19,31]. HGF (human gingival fibroblasts, ATCC-PCS-201-018) and HDF (human dermal fibroblasts, neonatal foreskin) were grown in DMEM w/ 10% FBS.

2.2. Scratch Assay

HOKs and HEKs were grown to confluency in a 24 well plate in keratinocyte serum free media (KSFM) (Gibco. #10744019). Once confluent, cells were scratched with a p200 tip, washed with PBS and left to migrate in fresh KSFM. Percentage closure was calculated using ImageJ to measure the wound gap in each image and the wound gap reduction was calculated over the timepoints [25]. Samples were blinded and analysed independently. Percentage wound closure was compared between HOKs and HEKs and analysed using t tests at each time point.

2.3. 3D Organotypic cultures

Collagen was diluted to 4mg/ml (w/v) in DMEM fibroblast media and neutralised with NaOH. 350μl of cell/collagen solution, containing 2×10^5 fibroblasts, was then added to each transwell insert (Millipore, MCHT12H48) and left to polymerise for 1 hour. 3×10^5 Keratinocytes in 500μl of FAD media were seeded on each collagen matrix (ROCK inhibitor was not supplemented to allow epithelial differentiation). 2ml of FAD media was added beneath the insert and models were incubated at 37°C overnight. After 24 hours, models were lifted to the air-liquid interface and grown for 14 days before cryopreservation.

2.4. Immunofluorescence and H&E

Frozen sections of organotypics were stained with haematoxylin and eosin or immunolabeled with antibodies (Supplementary data). Stained tissue was then imaged using a Leica Epi DM5000 microscope equipped with a DFC350 FX digital camera using the Metamorph software and processed using ImageJ.

2.5. Biopsy punch method

Organotypics were wounded with a 2mm biopsy punch and placed on top of a fibroblast seeded collagen matrix, constructed as above. Models were incubated for up to 72 hours before cryopreservation.

2.6. Microarray Analysis

Previously published microarray data was analysed to select genes that showed a greater than 2-fold increase in RNA extracted from oral tissue compared to skin with a false discovery rate (FDR) of $p<0.01$ [12, 32].

2.7. qPCR and Immuno-blotting

The 2D scratch assay was repeated with RNA and protein being extracted from cells pre wounding and at 6, 12 and 24 hours post wounding using a Qiagen RNeasy kit (Qiagen 74104) and RIPA buffer respectively. qPCR was performed on an ABI 7500 (standard) machine using KAPA SYBR Fast Mix (KAPA KK4602). Western blots were performed and analysed using chemi-luminescence and a developer. Primers and antibodies available in supplementary data.

2.8. siRNA

siRNA knockdown in HOK cells was achieved by forward transfection using Dharmafect 1 and PITX1 ONTARGET PLUS smartpool and a non-targeting control (reagents in supplementary). Knockdown was validated through qPCR and immuno-blotting. siPITX1 and siC HOK cells were scratched 4 days after transfection and were imaged at 0, 6, 12 and 24 hours post wounding.

2.9. Statistical Analysis

All statistical analysis was conducted using Graphpad Prism 7 (Graphpad software Inc. Ca, USA). All tests are detailed in the figure legends for the corresponding figures. For all figures; $*p<0.05$, $**p<0.01$, $***p<0.001$ $****p<0.0001$.

3. Results

3.1. Gingival keratinocytes display superior wound closure

Hard palate keratinocytes have previously been shown to possess enhanced migration following wounding, compared to skin keratinocytes [12]. Here it was analysed whether gingival keratinocytes from a juvenile donor possessed superior migratory potential compared to epidermal keratinocytes from a neonate. A standard monolayer scratch assay showed HOKs closed the gap more rapidly than HEKs and percentage closure was significantly higher at 24 hours (Fig. 1C-D). This indicated HOKs possessed superior wound resolution compared to HEKs. Furthermore, this superiority was demonstrated despite the difference in age of the donor keratinocytes, suggesting that keratinocytes maintain these intrinsic differences.

3.2. Gingival organotypics are distinct from skin models

To assess whether this superiority was repeated when keratinocytes were grown in a 3D environment, organotypic cultures of the human gingiva and skin were constructed and analysed for their differentiation and protein expression (Fig. 2). H&E staining of the models displayed a greater level of cornification in the HEK models, indicative of a terminal differentiation that was absent in the HOK models. This was further supported by immunolabelling of differentiation markers. KRT10, a marker of keratinised epithelia, was absent in HOK models, but present in HEK models. The converse was evident for the non-keratinised epithelia marker, KRT13. Furthermore, the apparent higher expression of terminal differentiation marker involucrin in HEK models supported the findings of different levels of keratinisation between the models. The keratinisation observed in HEK models was similar to that of skin *in vivo*

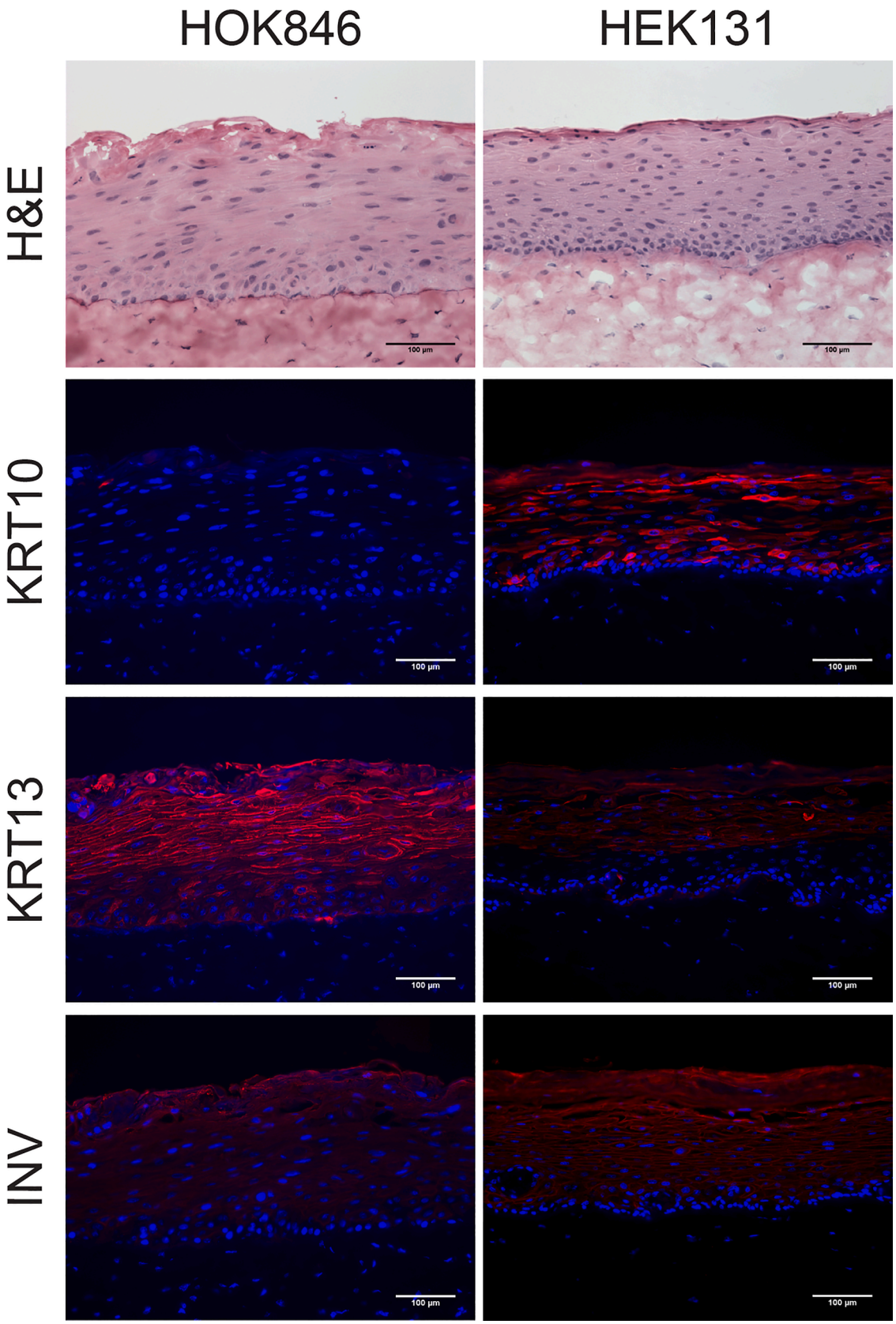

Fig. 2. H&E and immunofluorescent comparison of human oral equivalents and epidermal equivalents.
Primary human oral and epidermal keratinocytes (HOK846 and HEK131) were grown on a type I collagen matrix populated with primary gingival or dermal fibroblasts respectively at the air-liquid interface for 14 days to create a 3D organotypic culture. Models were stained with haematoxylin and eosin and were immunofluorescently labelled for differentiation markers keratin 10, keratin 13 and terminal differentiation marker involucrin. Nuclei were labelled with DAPI (blue). Scale bar = 100 µm.

whereas the non-keratinised HOK models appeared to recapitulate the JE of the gingiva [13]. Taken together these results demonstrate that the models were representative of *in vivo* gingival JE and epidermis.

3.3. Gingival models display superior re-epithelialisation

Once models were demonstrated to recapitulate *in vivo* tissue and be distinct from one another they were wounded using a biopsy punch and wound resolution was assessed (Fig. 3A). At 24 hours post-wounding, HOK models had migrated to almost close the wound, however, HEK models had only migrated down the wound margin to the underlying matrix. At 48 hours, HOKs had fully repopulated the wound, whilst HEK models had migrated to close the wound gap with little stratification present. This vertical wound closure was not improved upon at 72 hours. Re-epithelialisation was quantified by measuring the stratification height of the keratinocytes at the centre of the wound or the leading edge (Fig. 3B). Stratification of HOK models was significantly greater than HEK models at 24 and 72 hour time points, emphasising the superior wound resolution. This difference indicated that superior wound healing demonstrated by oral mucosa *in vivo* and by gingival keratinocytes in monolayer *in vitro* was indeed maintained in the 3D environment.

3.4. Wound healing genes are differentially expressed between gingival and skin keratinocytes

To select genes that could play a role in superior wound healing, published microarrays on mouse RNA were analysed [12,32]. Our analysis of Turabelidze et al. (2014), found that out of 10,097 genes, 7, 529 were less than 2-fold different either way between unwounded oral (hard palate) and skin keratinocytes, 1,058 were upregulated in oral keratinocytes and 1,510 in skin (Fig. 4A). Of the 1,058 genes upregulated in oral cells, some were structural proteins involved in the differentiation of epithelia, such as KRT4 and KRT13, which would be expected in oral keratinocytes.

Analysis of the microarray data published by Chen et al. (2010) found there was greater difference in expression between unwounded oral and skin tissues. Of 5,196 genes, 1,756 were upregulated oral tissue, 2,112 in skin and 1,328 showed no difference (2-fold difference threshold) (Fig. 4B). In this study, RNA was extracted from multiple cell types within the tissue, potentially explaining the disparity between the number of differentially expressed genes compared to that seen in Turabelidze et al. (2014), where RNA was extracted solely from keratinocytes.

Genes associated with wound healing were upregulated in oral keratinocytes/tissue in both datasets including *Pax9* (6.7*/51$^{\#}$), *Nos1*

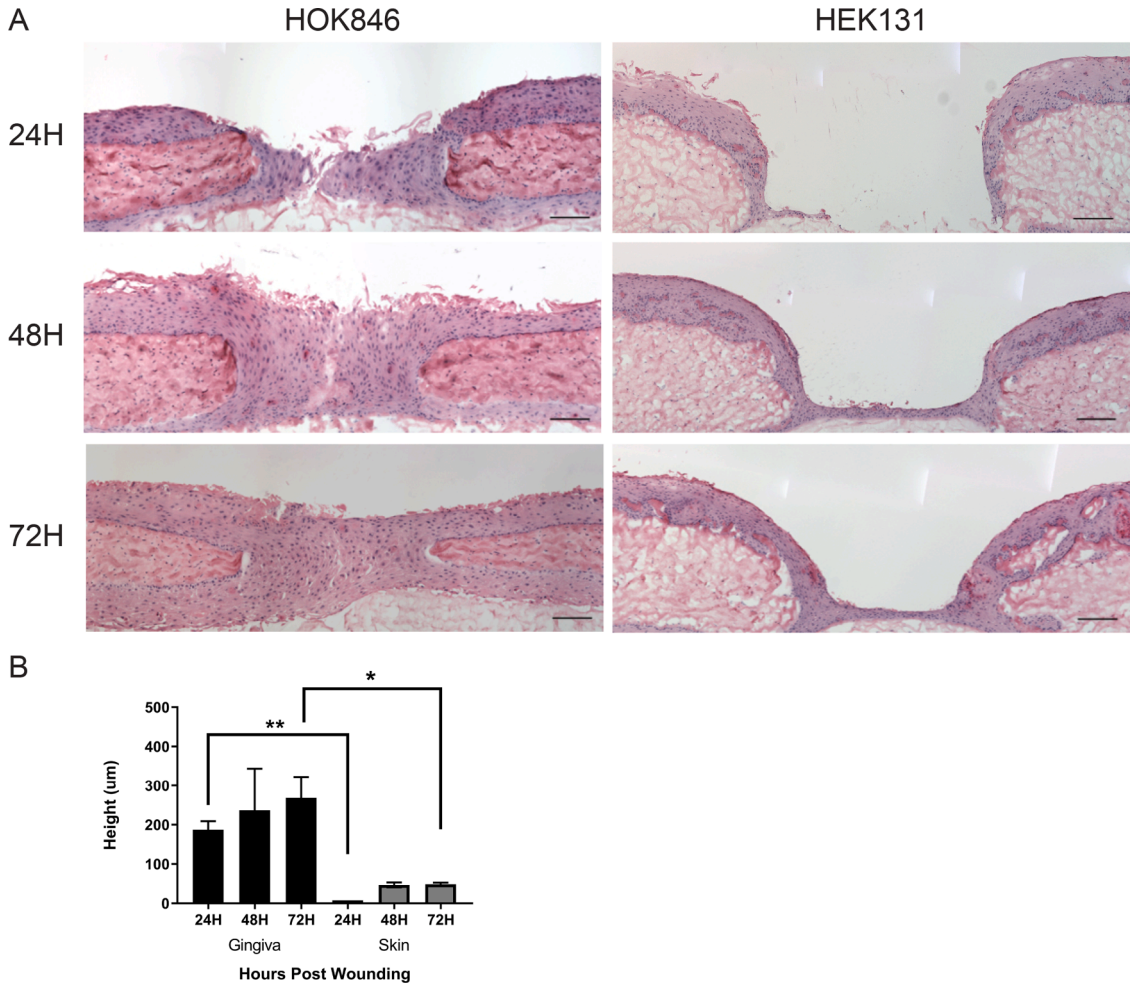

Fig. 3. Re-epithelialisation of wounded gingival and skin models.
A - Haematoxylin and eosin staining of wounded HOK846 with HGF and HEK131 with HDF models. Models were grown at the air-liquid interface for 14 days prior to wounding, after which they were placed on corresponding fibroblast populated collagen matrices for 24, 48 or 72 hours. Models were subsequently frozen and sectioned through to the wound where sections from different models were stained at similar distances from the wound margin. Scale bar = 200 μm. B - Stratification of epithelium at the central point or leading edge was measured in um for each model. Distances were averaged and statistically analysed using a t test at each time point (n=3 *$p<0.05$, **$p<0.01$).

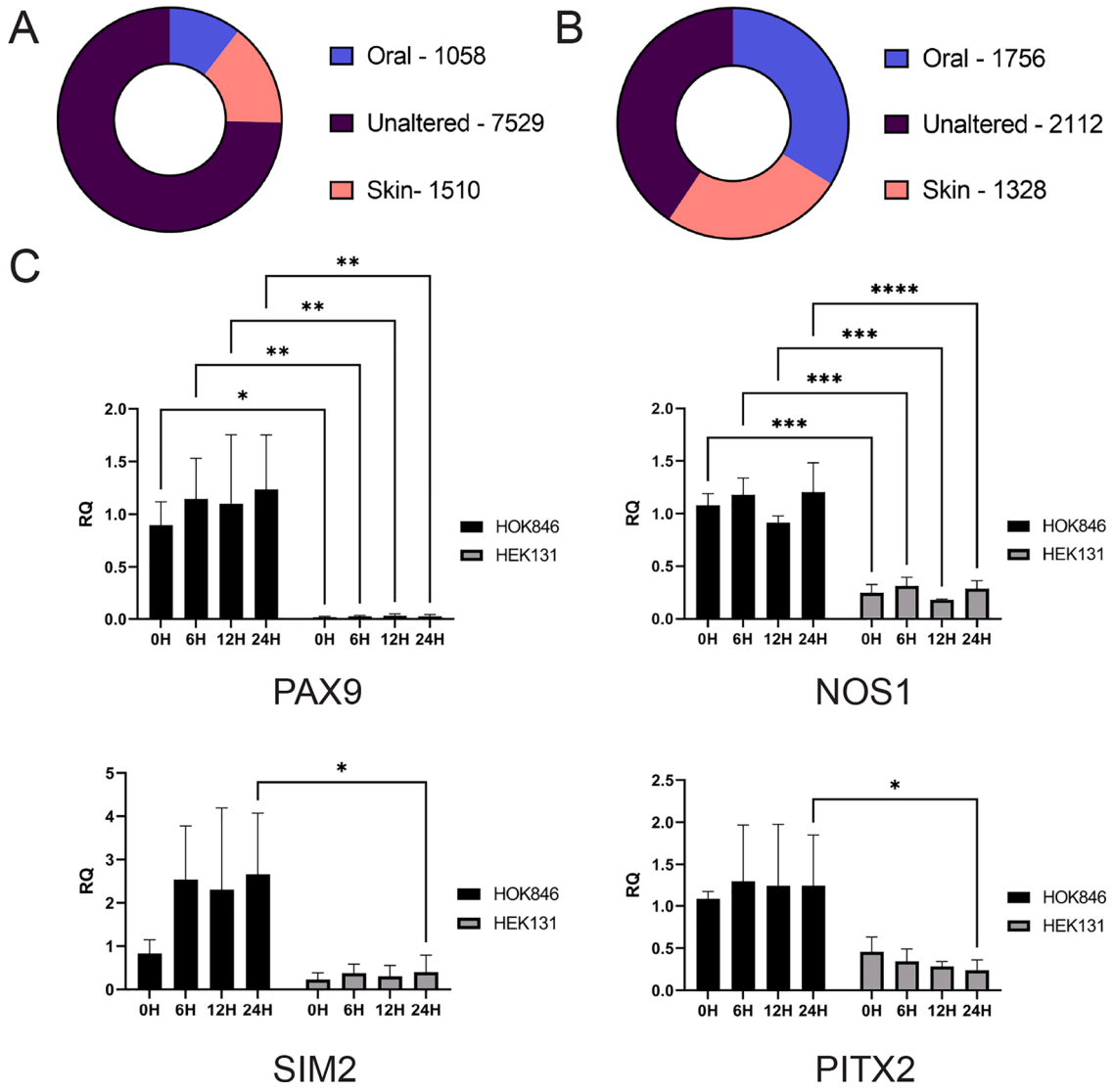

Fig. 4. Differential gene expression between oral and skin keratinocytes.
A – The number of genes upregulated greater than 2-fold in oral keratinocytes compared to skin keratinocytes, vice versa, and the number of genes not significantly altered between the cell types. Data derived from Turabelidze et al. (2014) and calculated with an FDR of $p<0.01$. B – The number of genes upregulated greater than 2 fold in tongue tissue compared to skin tissue, vice versa, and the number of genes not significantly altered between the tissues. Data derived from Chen et al. (2010) and calculated with an FDR of $p<0.01$. C - qPCR analysis of candidate genes for mouse microarray validation, calculated to have >2 fold increase in unwounded oral keratinocytes/tissue compared to unwounded skin keratinocytes/tissue data. RNA was extracted from primary human gingival and skin keratinocytes at 0, 6, 12 and 24 hours post scratch in monolayer. (n=3, One-way ANOVA with a Sidak multiple comparison test; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

(6.9$^{\#}$), *Sim2* (82*/47$^{\#}$), *Pitx2* (243*, 25.9$^{\#}$) and *Pitx1* (69.2*/19.7$^{\#}$) (*fold increase in Turabelidze et al, $^{\#}$fold increase in Chen et al) (*Nos1* did not pass the FDR in Turableidze et al).

As both microarrays were conducted on mice, genes were validated in human primary keratinocytes. This was achieved with scratch assays in monolayer and RNA was extracted at 0, 6, 12 and 24 hours post wounding. qPCR analysis showed *PAX9* and *NOS1* RNA expression was significantly higher in HOKs compared to both unwounded and wounded HEKs (Fig. 4C). *SIM2* and *PITX2* showed no difference between unwounded cells but were upregulated upon wounding and were significantly more highly expressed at 24 hours post wounding. This highlighted these genes as potential regulators of the wound healing process.

Expression of *PITX1* at the RNA level was significantly upregulated in HOKs at all-time points compared to HEKs, where expression did not significantly change over time (Fig. 5A). Protein was extracted in a similar manner to RNA and densitometry analysis displayed significantly higher expression in HOKs at all-time points compared to HEKs (Fig. 5B). *PITX1* was therefore significantly more highly expressed in HOKs at both the RNA and protein level in both wounded and unwounded cells.

PITX1 was subsequently transiently knocked down in HOKs to analyse its effect on wound resolution. Validation of the knockdown demonstrated that *PITX1* expression was still significantly knocked down at the RNA level at 7 days post transfection (Fig. 5C) but was only significantly lowered at the protein level up to 5 days post transfection (Fig. 5D). HOKs were transfected in a 12 well plate format and scratched four days after transfection to allow cells to reach confluency and remain sufficiently PITX1 deficient (Fig. 5E). Analysis of the percentage closure showed more closure of siC cells at 12 hours after transfection compared to si*PITX1* cells although this was not significant ($p = 0.27$) (Fig. 5F). These results taken together could indicate that differential gene expression between keratinocytes could influence wound healing rates.

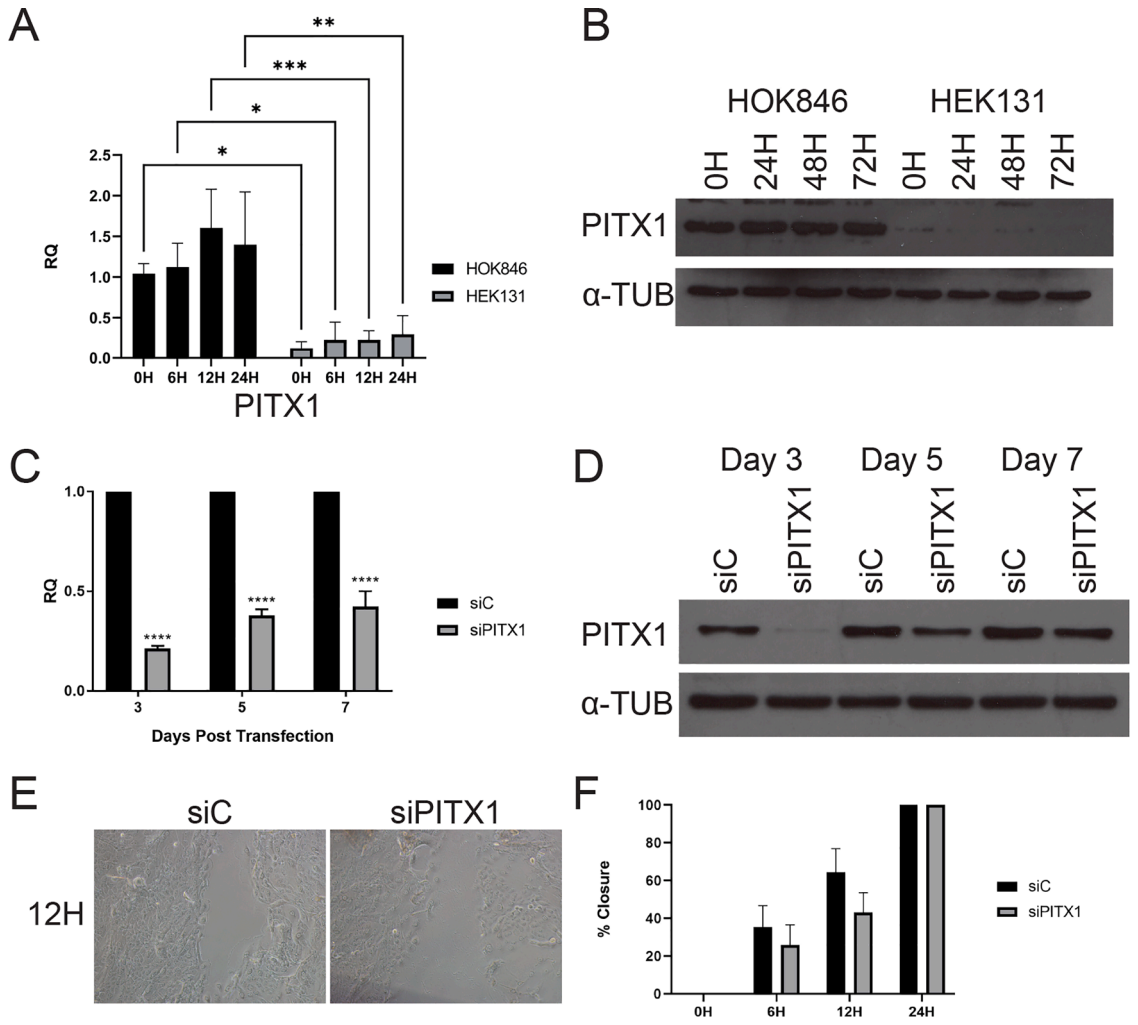

Fig. 5. PITX1 expression and knockdown in primary oral keratinocytes.
A – qPCR analysis of PITX1 expression in gingival and skin keratinocytes (graph displayed in same format as previous) . (n=3, One-way ANOVA with a Sidak multiple comparison test; *p<0.05, **p<0.01, ***p<0.001). B – Western blot analysis of PITX1 expression in gingival and skin keratinocytes. Protein was extracted from primary human gingival and skin keratinocytes at 0, 6, 12 and 24 hours post scratch in monolayer. C&D – qPCR and western blot validation of knockdown of PITX1 expression in HOK846 cells. (n=3, One-way ANOVA with Tukey's multiple comparison test; ****p<0.0001). E – Scratch assay of siC and siPITX1 HOK846 cells, images were taken at 12H post wounding. F – Quantification of wound closure of siC cells compared to siPITX1 cells (n=3, multiple t tests were performed, a test for each timepoint, no significance found).

4. Discussion

We have shown gingival keratinocytes demonstrate superior wound closure in monolayer compared to skin keratinocytes, following the pattern previously demonstrated with hard palate keratinocytes [12]. Furthermore, the use of 3D organotypic models of gingiva and skin demonstrate gingival keratinocytes maintain superior wound resolution in the *in vitro* 3D environment. These findings support previous evidence that keratinocytes contribute to faster wound resolution in oral tissues over skin [5,12,32].

The sliding sheet migration model of wound healing dictates that the epithelium undergoes collective migration because of the cells maintaining their strong desmosomal attachments and, consequently, as the basal cells migrate across the matrix, the entire epidermis moves as a sheet. In *in vivo* tissue this migration is supported by a proliferative region of unwounded epithelium situated about 500 μm back from the wound edge [23,33]. It is possible that the 3D gingival organotypic models are undergoing this style of migration that allows them to close the wound so rapidly.

Keratinocytes in the 3D skin organotypic models, however, appear to follow a different model of migration. Here, basal keratinocytes move to close the gap, whilst suprabasal cells remain *in situ.* This supports recent findings that re-epithelialisation of skin wounds occurs in sheets of migrating basal keratinocytes [34]. Whilst the multi-layered sliding sheet method results in the sliding of the entire epithelium, the extending shield mechanism consists of an advancing margin of a single epithelial cell, while the epithelium behind it is two to four cells deep [24]. Migration, however, is a dynamic process and it is difficult to analyse the mechanism based upon snapshots in time.

Proliferation is also important in the re-epithelialisation process. As with migration, there is also debate about how migrating epithelial tongues are supported during re-epithelisation. Two theories are that proliferation either occurs in the migrating keratinocytes themselves or in a proliferative zone approximately 500 μm back from the wound edge [24,33]. Analysis of the proliferation of the keratinocytes in the models could be key in understanding the ability of 3D gingival organotypic models to re-epithelialise at such a greater rate.

Intrinsic fibroblast differences could also contribute to the disparity in healing. Gingival fibroblasts proliferate and migrate faster than their skin counterparts, whilst also expressing higher levels of factors involved in the regulation of inflammation and angiogenesis. Skin fibroblasts, however, display significantly higher expression of factors

leading to fibrosis [11]. Furthermore, the addition of gingival fibroblasts or gingival fibroblast conditioned media have also been shown to improve dermal wounds in mice, associated with reduced inflammation, increased angiogenesis and increased collagen deposition [35]. Other oral fibroblasts, buccal fibroblasts, produce significantly higher levels of keratinocyte growth factor and hepatocyte growth factor than skin fibroblasts when cultured in collagen and in the presence of keratinocytes [36]. Taken together, the paracrine signalling of these gingival fibroblasts could result in an increased rate of re-epithelialisation and contribute to the results observed in this study.

The JE of the gingival tissue is characterised *in vivo* by its exceptionally high rate of cellular turnover, as well as a high rate of migration. This migration is facilitated by a relatively small number of desmosomes and gap junctions that connect the cells of the epithelia [37]. The skin conversely has a slower turnover rate and stronger cell-cell junctions. It is, therefore, possibly of little surprise that models of the junctional epithelia re-epithelialise faster than skin equivalents, when cultures consist only of keratinocytes and fibroblasts. Upon wounding *in vivo*, stem cells become active, undergo rapid asymmetric cell fate transitions and generate new stem cells and progenitors promoting tissue expansion and repair [33,38]. Therefore, it is important to remember that although 3D organotypic models provide an important tool to study wound healing in 3D, they do not possess the complexity of *in vivo* tissue.

From the disparity in wounding responses between models it is evident that there are intrinsic differences between the keratinocytes. This was confirmed by the differential gene expression of wound healing related genes. All genes investigated in this study have been linked to either migration, proliferation or wound healing. *SIM2* promotes migration of glioma cells [39], *PAX9* regulates proliferation and migration [40], *NOS1* produces nitric oxide which is involved in scarless embryonic healing [41] and *PITX2* promotes proliferation and migration [42]. All genes, with the exception of *SIM2* and *PITX2*, displayed significantly higher baseline expression in HOKs compared to HEKs (Fig. 4C). Upon wounding, *SIM2* and *PITX2* expression also displayed higher expression. These results indicate that HOKs could be programmed for faster wound healing, supporting previously published evidence [5]. Furthermore, an increase in expression indicates fundamental differences between the keratinocytes in their reaction to wounding stimuli.

Due to the differential expression of *PITX1* it was investigated whether higher expression levels did indeed contribute to superior wound healing. The knockdown of *PITX1* appears to have a negative impact on wound healing in monolayer scratch assays, although not significant (Fig. 5F), supporting previously published data [5]. It has previously been demonstrated that the loss of *PITX1* results in a diminished expression of *p53* which can decrease cell migration in keratinocytes [43,44]. These findings could explain the reduced migration observed in si*PITX1* cells. However, as a tumour suppressor gene, the loss of *PITX1* has been more commonly associated with promoting proliferation and malignancy [45–47]. Therefore, knockdown of PITX1 in gingival keratinocytes requires further classification.

To further investigate the effect of PITX1 knockdown on migration, it would be interesting to construct organotypics with siPITX1-transfected cells. 3D organotypics models have been previously used as a good method to study cells with modified gene expression in a 3D environment. However, our data demonstrates that the protein expression of PITX1 returns to normal after 7 days in monolayer cell culture and therefore, the siRNA induced knockdown would likely not remain after 14 days growth at the air-liquid interface. A possibility would be to optimise a ROCK inhibition protocol to allow for shRNA transfection using primary cells. This would allow for the prolonged knockdown of PITX1. However, continued exposure to the inhibitor could alter cell behaviour due to increased TERT and c-MYC expression for a prolonged period.

Overall, we present through 2D and 3D assays that there is a gingival keratinocytes possess wound healing superiority in compared to skin keratinocytes. This is believed to be due to both contrasting migration methods undertaken by the cells and differential gene expression of the keratinocytes from different sources.

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CRediT authorship contribution statement

Chris J. Smith: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **Eric K. Parkinson:** Resources, Writing – review & editing. **Jingjuin Yang:** Resources, Conceptualization, Writing – review & editing. **Jonathan Pratten:** Resources, Conceptualization, Writing – review & editing. **Edel A. O'Toole:** Resources, Methodology, Writing – review & editing. **Matthew P. Caley:** Resources, Methodology, Writing – review & editing. **Kristin M. Braun:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Conflict of Interest Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jdent.2022.104251.

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