Novel Methods and Sources for Regeneration of Oral Mucosa

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Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2022

UNIVERSITY OF BERGEN

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Date of defense: 16.06.2022

Year: 2022

Title: Novel Methods and Sources for Regeneration of Oral Mucosa

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Print: Skipnes Kommunikasjon / University of Bergen

Dedicated to my parents who mean the world to me.

Scientific Environment

The thesis was conducted from May 2018 to April 2022 at the Department of Clinical Medicine (K1), Centre for Excellence in Cancer Biomarkers (CCBIO), and Department of Clinical Dentistry (IKO) at University of Bergen (UiB), and Department of Pathology at Haukeland University Hospital (HUS). Main supervisor was Prof. Daniela Elena Costea at K1, UiB & HUS, and co-supervisors were Prof. Anne Christine Johannessen at K1, UiB & HUS Prof. Mihaela-Roxana Cimpan at IKO, UiB, and Dr Salwa Suliman researcher at IKO, UiB.

The research was funded by UiB, Norway. Additional financial support was provided by the Meltzer Research Foundation, The Research Council of Norway through its Centre of Excellence for Cancer Biomarkers, CCBIO (Grant No. 22325) and Helse Vest (Grant No. 912260/2019).

Acknowledgements

I would like to express my immense gratitude and heartfelt thanks to my supervisors Prof. Daniela Elena Costea, Prof. Anne Christine Johannessen, Prof. Mihaela Roxana Cimpan and Dr Salwa Suliman for their immense support and guidance throughout my PhD. I would not have finished my PhD without your support. I am grateful to my main supervisor Prof. Daniela Elena Costea for supporting me through thick and thin. I have gained peerless knowledge and priceless experiences from all my supervisors and will cherish it all throughout my life.

A special thanks to Dr Robert Whiley at Blizzard Institute, QMUL for introducing me to the field of scientific research and igniting a fire within me.

I would like to acknowledge my co-authors: Maria, Victoria, Keerthi, Ochiba, Evelyn, Olav, Bogdan, Kayoko, Xiao, Masaru, Tarig, Lisa, Ian, Hassan, Helge, Yassin and Harsh.

I would like to thank my colleagues and co-PhDs at Gade laboratory for pathology: Saroj, Harsh, Nuha, Israa, Bryant, Stian, Ramah, Kala Chand, Diana and Tamandeep for their support. I wish you all the best for the future. A special thanks to Jorunn, Rune, Helge and the mischievous Eirik for their fun-filled house parties throughout the years.

Many thanks to the technical staff Siren Fromreide, Randi Hope Lavik, May Britt Kalvenes, Solrun Johanne Steine and Brith Bergum for all their help and support. Also, great appreciation towards the whole team at IKO, especially Ying Xue and Mohammed Samih.

Personal thanks to Prof. Elisabeth Wik for the opportunity to visit Harvard Medical School, Boston as part of the Research-Stay-Abroad programme and Prof. Diane Bielenberg for graciously hosting me in her lab at Boston Children's Hospital, Harvard Medical School, Boston. My sincere thanks also to Prof. Ingeborg Tinhofer-Keilholz at Charité University, Berlin for the opportunity to work in her lab.

Thanks to all the financial supporters (CCBIO, Meltzer foundation, Institute of Clinical Medicine and Faculty of Medicine and Helse-Vest) who gave me this opportunity to

pursue my PhD and present my research at national and international conferences. I extend my thanks to all academic and administrative staff at (K1) and CCBIO.

To all my close friends at UiB and in Bergen specially Janani, thank you for being my family in Norway, and creating many memorable moments in my life.

Without the immense support of my family, my PhD might not have been possible. Each of my parents (my parents and my in-laws) have been the iron pillars of strength on whom I could lean on during the challenging last 4 years. My brother Rahul and my sister-in-law Ananya, thanks for troubling me and being my stress busters. My cousins who have been a great support during the pandemic and throughout my PhD.

Last but not the least, I would like to thank my husband Arnav for supporting me in every way possible. I cannot imagine finishing my PhD without him being by my side.

With much gratitude, love, and respect I dedicate this thesis to my four parents.

Bergen, March 2022
Dr Ridhima Das

Punjabi
ਕਰਮ ਧਰਤੀ ਸਰੀਰੁ ਜੁਗ ਅੰਤਰਿ ਜੋ ਬੋਵੈ ਸੋ ਖਾਤਿ ॥

Hindi
करम धरती सरीरु जुग अंतरि जो बोवै सो खाति ॥

English
Karam d̲har̲tī sarīr jug an̲tar jo bovai so k̲hāt̲.
Sri Guru Granth Sahib, page 78

Translation
The body is the field of karma in this age; whatever you plant, you shall harvest.

Abbreviations

3D OT	Three Dimensional Organotypic
bFGF	Basic Fibroblast Growth Factor
BMP4	Bone Morphogenic Protein 4
BPE	Bovine Pituitary Extract
CK13	Cytokeratin 13
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGF-R	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
ERM	Epithelial Rests of Malassez
ESC	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FFPE	Fresh Frozen Paraffin Embedded
FGF-2	Fibroblast Growth Factor 2
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GTR	Guided Tissue Regeneration
hEGF	Human Epidermal Growth Factor
HERS	Hertwig's Epithelial Root Sheath
HGF	Hepatocyte Growth Factor
HSC	Hematopoietic Stem Cells
ICAM1	Intercellular Adhesion Molecule 1
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-1	Interleukin 1
IL-1α	Interleukin 1alpha
IL-6	Interleukin 6
IL-8	Interleukin 8
iPSC	Induced Pluripotent Stem Cell

ISH	In-situ Hybridization
KGF	Keratinocyte Growth Factor
KSFM	Keratinocyte Serum Free Media
LIF	Leukaemia Inhibitory Factor
mES	Mouse Embryonic Stem Cells
MMP11	Matrix Metallopeptidase 11
MSC	Mesenchymal Stem Cells
NBCS	New-born Calf Serum
NHOM	Normal Human Oral Mucosa
NHS	Normal Human Skin
NOD/SCID	Non-obese diabetic/Severe combined immunodeficiency
NOF	Normal Oral Fibroblasts
NOK	Normal Oral Keratinocytes
NSF	Normal Skin Fibroblasts
NSK	Normal Skin Keratinocytes
PanCK	Pancytokeratin
PCL	Poly(ε-caprolactone)
PDL	Periodontal Ligament
PDLSC	Periodontal Ligament Stem Cells
PL	Platelet Lysate
PLA	Polylactic Acid
RA	Retinoic Acid
RT-qPCR	Reverse Transcription-Quantitative Polymerase Chain Reaction
SCF	Stem Cell Factor
SW	Sandwich Models
TGF- α	Transforming Growth Factor alpha
VCAM1	Vascular Cell Adhesion Molecule 1

Abstract

Background: Common clinical approaches for replacing damaged oral mucosa are represented by autologous skin grafts which have numerous shortcomings and pose serious post-surgical morbidity. Cultured oral mucosal sheets have been developed in academic research laboratories and few models have even been commercialized, but they also present limitations and are not feasible yet for use in clinics. There is a need to develop alternative methods for the regeneration of oral mucosa that employ more robust and efficient sources of epithelial cells for generation of oral mucosal sheets.

Aims: i) to identify the factors responsible for oral epithelial differentiation for generating oral mucosal sheets **(Paper I)**; ii) to isolate and characterize cells derived from human epithelial rests of Malassez (ERM) and compare them with cells derived from matched normal oral mucosa (NHOM) with regards to their ability to generate oral mucosal sheets **(Paper II**); iii) to test whether pluripotent embryonic stem cells (ESC) can be differentiated into oral epithelium **(Paper III)**; iv) to test whether induced pluripotent stem cells (iPSC) derived from adult human fibroblasts can be differentiated into keratinocytes which can further be used for the generation of oral mucosal sheets for regenerative therapies **(Paper IV)**.

Materials and methods: Different sources of cells were used for generation of oral mucosal sheets, *i.e.,* normal oral keratinocytes (NOK) isolated from NHOM **(Paper I)**, ERM cells isolated from human periodontal ligament **(Paper II),** mouse ESC **(Paper III),** and iPSC reprogrammed from human normal oral and dermal fibroblasts **(Paper IV).** The cells were characterized by flow cytometry, immunohistochemistry (**Paper II, III and IV)**, immunofluorescence **(Paper III and Paper IV),** and RT-qPCR **(Paper IV).** Three dimensional organotypic (3D OT) cultures were constructed using these cells and different growth factors, such as granulocyte macrophage-colony stimulating factor (GM-CSF) and keratinocyte growth factor (KGF) **(Paper I),** or exposure to different extracellular matrix (ECM) components were used to differentiate them into epithelial lineages **(Paper III).** In **Papers II and IV**, 3D OTs were constructed using

alternative human sources for oral epithelial cells (ERM and iPSCs), which were compared to the 3D OTs constructed with NOK cells derived from NHOM.

Results: In **Paper I**, it was revealed that major aspects of NOK differentiation into a mature oral epithelium in 3D OTs were regulated by the combination of GM-CSF and KGF. The terminal stage of the differentiation seemed to be however controlled by other yet unidentified fibroblast-derived diffusible factor(s).

In **Paper II**, the ERM cells contained various proportions of PanCK positive cells and showed a network pattern of growth similar to the network of ERM around the root of the tooth, while NOK formed a uniform, continuous sheet of cells. When grown in 3D OT or in collagen gels, ERM cells formed a less differentiated epithelium than NHOM cells, yet expressing PanCK and vimentin.

Data from **Paper III** confirmed that vitamin C alone could enhance the number of mouse ESC expressing epithelial markers, but the ECM synthesized by either oral or skin fibroblasts or keratinocytes was required to induce the expression of a stratified epithelial phenotype. Further 3D OT culture under vital fibroblast instruction was necessary for further differentiation into mature, regionally relevant epithelial structures, and only the ESC firstly 'primed' in 2D on keratinocyte or fibroblast derived ECMs reached a final epithelial maturation stage in 3D OT cultures as revealed by immunohistochemical staining.

In **Paper IV**, iPSC derived from normal oral and skin fibroblasts were differentiated into keratinocytes using both growth factor and ECM-enriched protocols. These keratinocytes were morphologically and phenotypically comparable to normal adult keratinocytes. Furthermore, iPSC could be also differentiated into keratinocytes in xenofree conditions and used for generation of oral mucosal sheets. Taken together these results indicate iPSC from oral and skin adult fibroblasts as a promising to generate oral mucosal sheets for regenerative therapies.

Conclusions: Overall, this thesis provides new knowledge on the mechanisms of differentiation of oral epithelium and the proof of principle for a novel biomedical application of keratinocytes differentiated from alternative sources such as iPSCs to generate oral mucosal sheets for regenerative therapy.

Sammendrag

Bakgrunn: Dersom man skal erstatte skadet munnslimhinne er det vanlig å bruke autolog hudtransplantasjon, men å bruke hud som erstatning for munnslimhinne har flere begrensninger og gir ofte en dårlig tilheling. Man har greid å dyrke kunstig munnslimhinne i laboratoriet, og slik kunstig slimhinne er også tilgjengelig kommersielt. Men også denne har begrensninger og kan ikke brukes klinisk. Derfor er det behov for å utvikle alternative metoder for å få en bedre tilheling i munnslimhinne, med mer robuste epitelceller som også er lettere tilgjengelige.

Mål: i) å identifisere hvilke faktorer som er nødvendige for å utvikle epitel fra munnslimhinne **(Artikkel I);** ii) å isolere og karakterisere celler som er utgått fra Malassezske epitelrester (ERM) og sammenligne dem med celler utgått fra celler fra normal munnslimhinne (NHOM) fra samme person, med hensyn på evne til å utvikle en kunstig munnslimhinne **(Artikkel II)**; iii) å teste om pluripotente embryonale stamceller (ESC) kan differensieres til oralt epitel **(Artikkel III)**; iv) å teste om induserte multipotente stamceller (iPSC) utviklet fra modne humane fibroblaster kan differensieres til keratinocytter, som videre kan nyttes for å utvikle en kunstig munnslimhinne til behandling av skader i munnslimhinnen **(Artikkel IV)**.

Materiale og metoder: Celler fra flere ulike vev ble brukt for å utvikle kunstig munnslimhinne, f.eks. normale keratinocytter fra munnslimhinne (NOK) isolert fra NHOM **(Artikkel I)**, celler isolert fra rothinnespalten **(Artikkel II)**, embryonale stamceller fra mus **(Artikkel III)** og iPSC som var reprogrammert fra humane fibroblaster fra munnslimhinne og hud **(Artikkel IV)**. Cellene ble karakterisert med væskestømcytometri, immunhistokjemi **(Artikkel II, III og IV)**, immunfluorescens **(Artikkel III og IV)** og RT-qPCR **(Artikkel IV)**. Tredimensjonale organotypiske (3D OT) kulturer ble konstruert med de ulike celletypene sammen med ulike vekstfaktorer som granulocytt makrofag koloni-stimulerende faktor (GM-CSF) og keratinocytt vekstfaktor (KGF) **(Artikkel I)**, eller å eksponere dem for ulike medier for å utvikle dem videre til epitelceller **(Artikkel III)**. I **Artikkel II og IV** ble det konstruert 3D OT ved å bruke alternative kilder av epitelceller fra munnslimhinne, og disse ble sammenlignet med kunstig munnslimhinne utviklet fra NHOM.

Resultater: I **Artikkel I** ble det vist at differensiering av NOK til fullt utviklet oralt epitel i 3D OT ble regulert av en kombinasjon av GM-CSF og KGF. Det siste stadiet av utviklingen ble imidlertid kontrollert av en foreløpig ikke identifisert løselig faktor fra fibroblastene.

I **Artikkkel II** ble det vist at cellene isolert fra ERM inneholdt varierende mengder av Pan CK positive celler, og at vekstmønsteret var ulikt sammenlignet med NOK fra NHOM. Når cellene isolert fra ERM ble dyrket i 3D OT eller i kollagengeler, dannet ERM cellene mindre differensiert epitel sammenlignet med celler fra NHOM, men de uttrykte både PanCK og vimentin.

Data fra **Artikkel III** bekreftet at vitamin C alene kunne øke antall celler fra mus, som uttrykte ESC epiteliale markører, men for å danne epiteliale celler var det nødvendig med ektracellulær matriks (ECM) som var syntetisert av fibroblaster fra enten munnslimhinne eller hud. Fibroblastene var også nødvendige for en videre differensiering til modne epiteliale celler, og bare ESC som var først dyrket i 2D på keratinocytt- eller fibroblastderivert ECM oppnådde et fullt utviklet epitel, bekreftet med immunhistokjemi.

I **Artikkel IV** ble iPSC utviklet fra fibroblaster fra normal munnslimhinne og hud differensiert til keratinocytter ved hjelp av ulike vekstfaktorer og ECM-rike medier. Disse keratinocyttene var morfologisk og funksjonelt sammenlignbare med normale keratinocytter fra munnslimhinne og hud. iPSC kunne også bli differensiert til keratinocytter under xenofrie forhold og utviklet videre til kunstig munnslimhinne. Funnene indikerer at iPSC fra fibroblaster fra munnslimhinne og hud kan være en ny mulighet for å utvikle keratinocytter som kan brukes i regenerativ behandling.

Konklusjoner: Samlet gir denne avhandlingen ny kunnskap om differensieringen av epitel fra munnslimhinne og viser prinsippene for hvordan keratinocytter som er differensiert fra alternative kilder kan brukes til å utvikle kunstig munnslimhinne til regenerativ behandling.

List of Publications

Paper 1

Ridhima Das, Maria Justina Roxana Virlan, Victoria Xenaki, Keerthi K. Kulasekara, Ochiba Lukandu, Evelyn Neppelberg, Olav K. Vintermyr, Anne. Chr. Johannessen, Bogdan Calenic, Daniela Elena Costea. Granulocyte macrophage-colony stimulating factor and keratinocyte growth factor control of early stages of differentiation of oral epithelium. **Eur J Oral Sci. 2022 (in press)**

Paper II

Kayoko Kitajima, **Ridhima Das**, Xiao Liang, Evelyn Neppelberg, Anne Christine Johannessen, Daniela Elena Costea, Masaru Igarashi. Isolation and characterization of cells derived from human epithelial rests of Malassez. **Odontology (2019) 107:291–300**.

Paper III

Ridhima Das, Lisa Harper, Kayoko Kitajima, Tarig Osman, Mihaela Roxana Cimpan, Anne Chr. Johannessen, Salwa Suliman, Ian C. Mackenzie, Daniela-Elena Costea. Embryonic stem cells can generate oral epithelia under matrix instruction. **Submitted Manuscript.**

Paper IV

Ridhima Das, Hassan R.W. Ali, Tarig Osman, Mohammed A. Yassin, Kamal Mustafa, Arild Kvalheim, Harsh Dongre, Helge Ræder, Anne Chr. Johannessen, Mihaela Roxana Cimpan, Salwa Suliman, Daniela-Elena Costea. Generating oral keratinocytes for regenerative therapy from induced pluripotent stem cells derived from adult human oral and skin fibroblasts. **Manuscript**

Contents

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1. Introduction

1.1 Background

Defects in oral mucosa due to tumour resections, trauma or chronic infections often leave the patient with major functional and aesthetic deficiencies [1]. These defects have significant consequences on the quality of life of these patients. Like any other mucosal wounds, wounds in the oral cavity heal by granulation and epithelialization with minimum scarring. Large oral mucosal defects are often present post-trauma or after tumour excision, leading to significant challenges [2, 3]. These large wounds heal by formation of granulation tissue followed by contraction of wound and scar formation which requires adequate amount of soft tissue to cover and promote healing [4, 5]. Current treatments for such defects are mainly autologous dermal [6] and epidermal grafts which might lead to severe post-surgical morbidity or the need for subsequent surgeries (Figure 1) [7]. In oral and maxillofacial surgery, there is a great demand for oral mucosal constructs for intra-oral grafting as oral reconstruction after trauma or tumour resections is necessary to preserve function and aesthetics [8, 9].

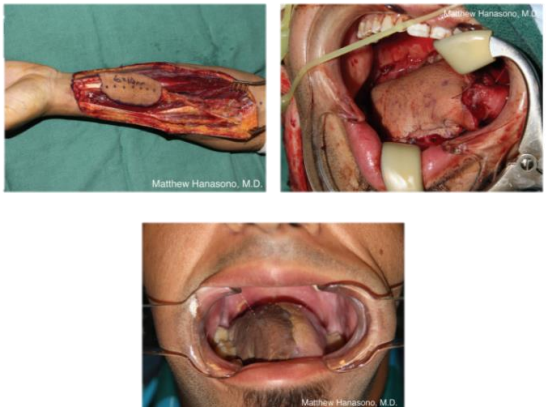


Figure 1. Example of a patient treated with the currently available autologous skin graft method. From Hanasono MM, Advances in Medicine, 2014, Reconstructive Surgery for Head and Neck Cancer Patients [10] (Cited under Creative Commons Attribution License)

1.2 Oral mucosa

1.2.1 Oral mucosa structure and function

Mucosa is the moist lining of the body cavities such as those of gastrointestinal tract including mouth, nasal passages, or of genitourinary tract such as vagina, bladder, *etc*. Oral mucosa is a distinctive habitat where hard and soft tissues along with saliva and the commensal microbiome work in equilibrium [11]. The main function of oral mucosa is to safeguard the underlying structures from various external stimuli. Oral mucosa also plays an important role in mastication, deglutition, and speech. Oral mucosa can be subdivided based on its function into masticatory mucosa, lining mucosa and specialized mucosa. Masticatory mucosa is present on the hard palate and attached gingiva. The lining mucosa is present on the buccal mucosa, labial mucosa, alveolar mucosa, as well as the mucosa lining the ventral surface of the tongue, floor of the mouth, and soft palate. The specialized mucosa is present on the dorsum of the tongue (Figure 2). Oral mucosa structurally consists of a stratified squamous epithelium called the oral epithelium and the underlying connective tissue called lamina propria [12].

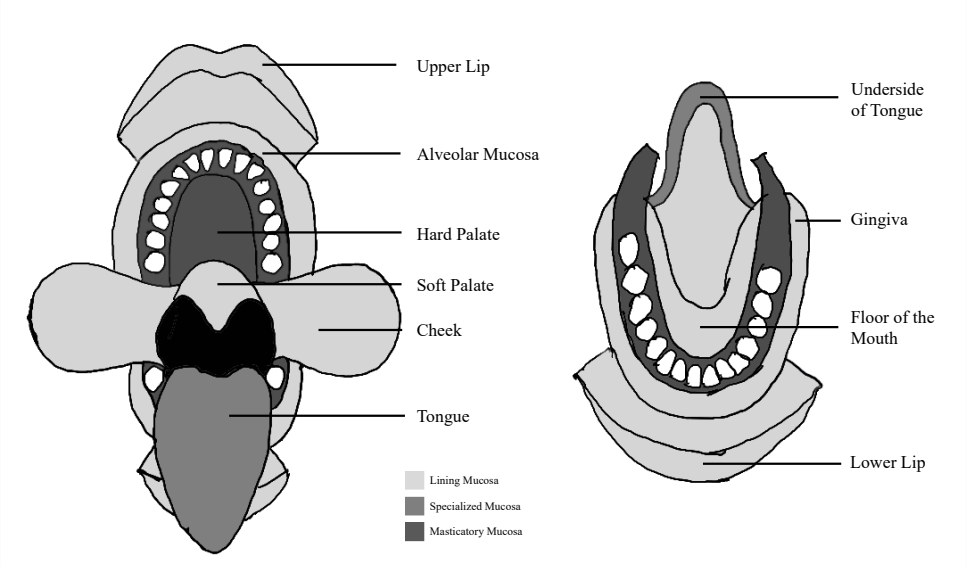


Figure 2. The general anatomy of the oral cavity. Adapted from [12]

The oral mucosa is a self-regenerating tissue where cells from the basal layer (stem cells and transit amplifying cells) undergo mitotic division followed by differentiation and stratification as the cells migrate to the surface (maturing population) and replace cells as they are shed from the surface [13]. The oral mucosa is attached to underlying structures of the oral cavity by a loose connective tissue called the submucosa [12]. In gingiva and some parts of the hard palate, oral mucosa is attached directly to the underlying bone, and together with the periosteum forms a complex structure called mucoperiosteum. The lamina propria of the oral mucosa consists of fibroblasts, blood and lymph vessels, neural elements, fat cells and extracellular matrix containing fibres and mucopolysaccharides. Cancer treatments reduce the rate of cell proliferation in both epithelium and the connective tissue. The ionizing radiations affects the fibroblasts leading to fibrosis, hypo vascularity of the blood vessels and tissue ischaemia. These alterations lead to reduced ability of the oral mucosa to both regenerate and resist infections [11].

The oral cavity is a continuously changing environment with exposure to harmful stimuli, and the oral mucosa essentially defends and protects the underlying tissue. This defensive role is facilitated by the epithelium, the immune cells in the epithelium and the lamina propria. In addition, oral mucosa also has essential sensory functions like pain, touch, temperature, and taste, the last one exclusive for the oral cavity. These sensory functions are executed by a variety of specialised nerve endings, cells (Merkel cells) and cellular structures (taste buds) found in different locations of the oral mucosa [14].

1.2.2 Oral epithelium

The oral epithelium is a stratified squamous epithelium organized in distinct layers (stratums). The oral epithelium can be keratinized or non-keratinized based on location and function. The keratinized oral epithelium contains the basal layer, squamous (prickle) cell layer, granular cell layer, and the keratinized layer. The non-keratinized oral epithelium contains the basal layer, squamous (prickle) cell layer, intermediate cell layer, and the superficial cell layer. The basal and the prickle layer form about two

thirds of the thickness of the oral epithelium. In the keratinized epithelium, the layer after these two layers is the granular layer also called as stratum granulosum where the cells are flattened and contain granules. There are two types of keratinization present in oral mucosa, with or without the persistence of the nuclei in the keratinized layer, known as parakeratinized and orthokeratinized respectively [15]. Figure 3 illustrates the structure of the keratinized and non-keratinized mucosa.

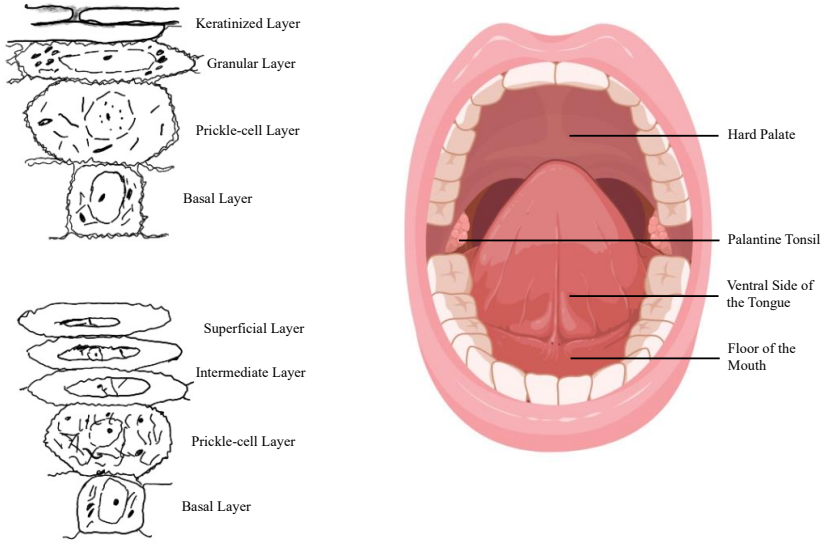


Figure 3. Outline structure of keratinized and non-keratinized mucosa. Regional distribution of functional tissues in the mouth. Adapted from [12] (Created using BioRender.com)

The unattached, mobile alveolar mucosa has a thin, non-keratinized epithelium with loose collagen fibres whereas the attached mucosa has a keratinized epithelium with condensed/tightly arranged collagen fibres in the connective tissue. Hard palate and the attached gingiva have keratinized attached mucosa. It acts as a barrier against various damaging environmental factors like pathogens and chemical substances [16].

Gingiva and hard palate are lined by ortho or para-keratinized squamous epithelium, while the floor of the mouth and the buccal mucosa are lined by non-keratinized stratified squamous epithelium (Figure 4). The specialized mucosa which is present on

the dorsal part of the tongue presents several types of papillae and has both keratinized and non-keratinized epithelium [17].

The structural differences in the oral epithelium are also accompanied by variations in the chemical and physical composition of keratinocytes such as the type of keratins, keratin-associated proteins like for example filaggrin and involucrin, and cell surface carbohydrates. Keratins belong to a family of 20 proteins, and the expression is site specific as well as specific to the differentiation pattern. All the cells in the basal layer of the stratified squamous oral epithelium express cytokeratins 5 and 14 whereas the cells in the suprabasal layers express cytokeratins 4 and 13. The suprabasal cell layers of the keratinized oral epithelium express also cytokeratins 1 and 10, similar to epidermis[11, 14, 18].

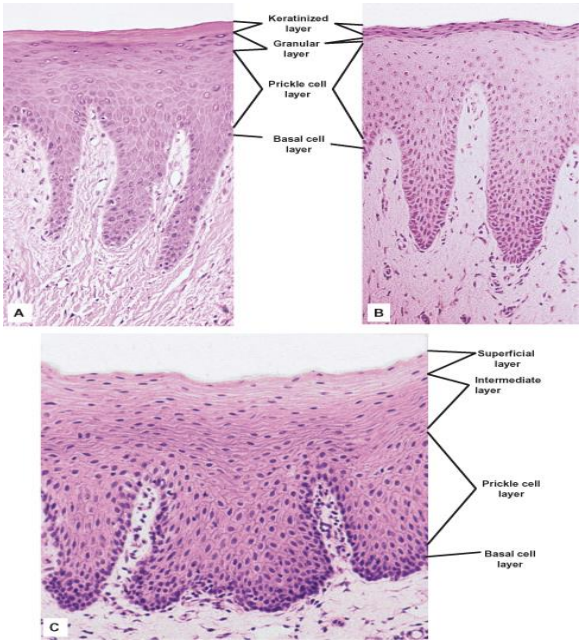


Figure 4. Haematoxylin & eosin Images of keratinized and non-keratinized mucosa (Squier and Finkelstein, 2003, Copyright Mosby)

Stem cell hierarchy of oral mucosa

The homeostasis of oral epithelium is maintained by continuous balancing of the cell loss on the surface of the oral epithelium with cell division every 14 to 21 days due to

the high functional demands of the oral mucosa. The replenishment starts from the basal layer. Like any other epithelia, the oral epithelium is considered to contain three types of cells: adult epithelial stem cells, transit amplifying cells, and post-mitotic terminally differentiating cells [19]. Each stem cell division normally produces one stem cell and one amplifying cell that undergoes a series of further divisions to produce terminally differentiating cells [13]. These cells start moving superficially. They become flattened and sloughed off at the surface [11].

1.2.3 Skin *vs.* oral mucosa

Human skin is made up of epidermis, dermis, and the dermo-epidermal junction which is constituted by the basement membrane [20]. The outermost layer of the skin, epidermis, acts as barrier by protecting the body from various microorganisms and providing resistance to body against injuries, while also preventing the loss of water and other body fluids. The epidermis is mainly made up of keratinocytes.

Skin and the oral mucosa are structurally and functionally similar tissues and are characterized by the presence of a protective stratified squamous epithelium and a supportive underlying connective tissue layer [21]. Even though both have a stratified squamous epithelium and a supportive connective tissue, there are several structural differences between skin and oral mucosa. Skin has hair follicles and sweat glands which are not present in the oral mucosa. The outermost layer of the epidermis, the stratum corneum, consists of 10-20 layers of cornified cells which provide a robust permeability barrier to the skin. Regions of gingiva and the hard palate in oral cavity are also keratinized and have the stratum corneum, but this is present in lower amount hence it is considered as more permeable that the skin epidermis. Also, the oral mucosa has a continuous flow of saliva hence low water gradient and loss of water through the mucosa [22, 23].

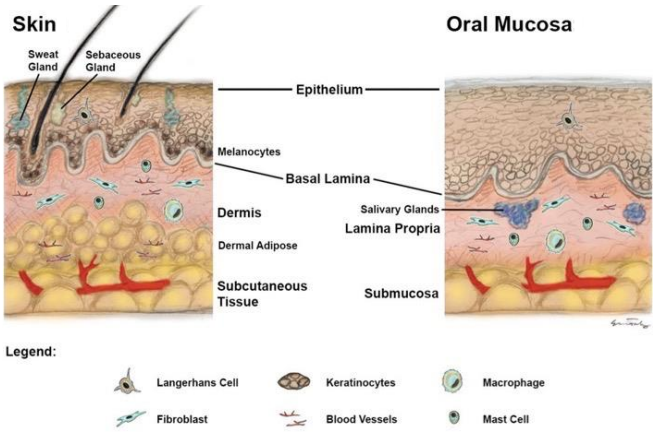


Figure 5. Schematic representation of skin and oral mucosa histology [21]. (Cited under Creative Commons Attribution License)

1.3 Current treatments for oral mucosal defects

To reconstruct large oral mucosal defects and promote healing, the procedures vary from application of various decellularized scaffolds to autologous skin grafts and more recently three-dimensional mucosa sheets reconstructed in the laboratory [24-26]. The current approaches for treatment of oral mucosal defects (Figure 6) can be broadly classified as follows: oral mucosal grafts and tissue-engineered oral mucosa equivalents.

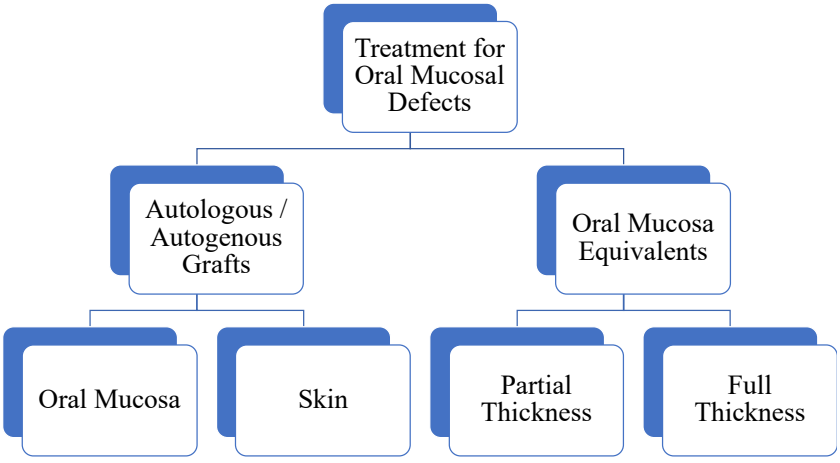


Figure 6. Current approaches for treatment of oral mucosal defects. Adapted from [27].

Autologous skin transplantation is the gold standard used in clinics after major surgeries/trauma with defects of oral mucosa in the oral cavity (Figure 1). Skin grafts have several disadvantages like hair growth, a different keratinization pattern, as well as possible infection due to the wet oral mucosa [5, 28, 29]. Oral mucosa is an excellent source for intraoral grafts, but due to limited amount of graft material available it is difficult to use in treating large defects [4, 5].

By comparison, skin wounds that are deep into the dermis usually are unable to heal on their own, often leading to scarring, cosmetic deformities and thus require a specialized treatment. The gold standard for such cutaneous wounds is autologous skin grafts which have limitations of inadequate donor sites to harvest skin [30]. Skin substitutes have been developed to address some of these limitations and have been widely used for replacement of skin [31]. While none of these existing substitutes can fully replace the damaged skin, they have been used extensively to treat burns and other chronic and non-healing wounds [32-34].

Both oral mucosal and skin grafts require two surgical procedures: i) harvesting of the tissue and ii) transplantation of the tissue, which usually results in increased donor site morbidity [4, 5, 35]. Additionally, there may be postoperative pain, scar formation and other surgical complications which altogether limit the use of autologous grafts. There is a need to develop new approaches for reconstruction of oral mucosal defects [36-38]. *In vitro* engineered oral mucosal substitutes are a promising alternative to the use of autologous grafts.

Another alternative for replacement of mucosa is human amniotic membrane. Amniotic membrane is a semipermeable membrane, and it is an immunotolerant structure. The biomechanical guided tissue regeneration (GTR) using amniotic membrane seems to be a good alternative since it not only maintaining the structural and anatomical configuration of regenerated tissues but also contributes to the enhancement of healing through reduction of postoperative scarring and subsequent loss of function [39].

Epidermal cell sheets have been used to treat severe cutaneous defects, such as burns and wounds, and based on this principle, small oral biopsies have been used to develop

autologous oral epithelial cell sheets. However, oral epithelial cell sheets developed so far had some drawbacks: they were very fragile, difficult to handle, and prone to contract [40-44]. There are several other tissues engineered alternatives available which are further discussed in section 1.4.

1.4 Tissue engineering

Tissue engineering is a developing field originally proposed by Langer et al in 1993 that aims at using progenitor cells, biocompatible biomaterials, along with appropriate biochemical and mechanical signals, to repair and regenerate damaged tissues [45, 46]. The standard principle relies on the combination of biomaterial scaffolds, cells, and/or bioactive molecules to form tissue engineered constructs that promote the formation and integration of injured tissue within the host environment (Figure 7) [47].

In addition to the clinical applications of tissue engineering, it can also be used to create tissue-like structures or organs-on-a-chip for drug toxicity and efficacy testing [48].

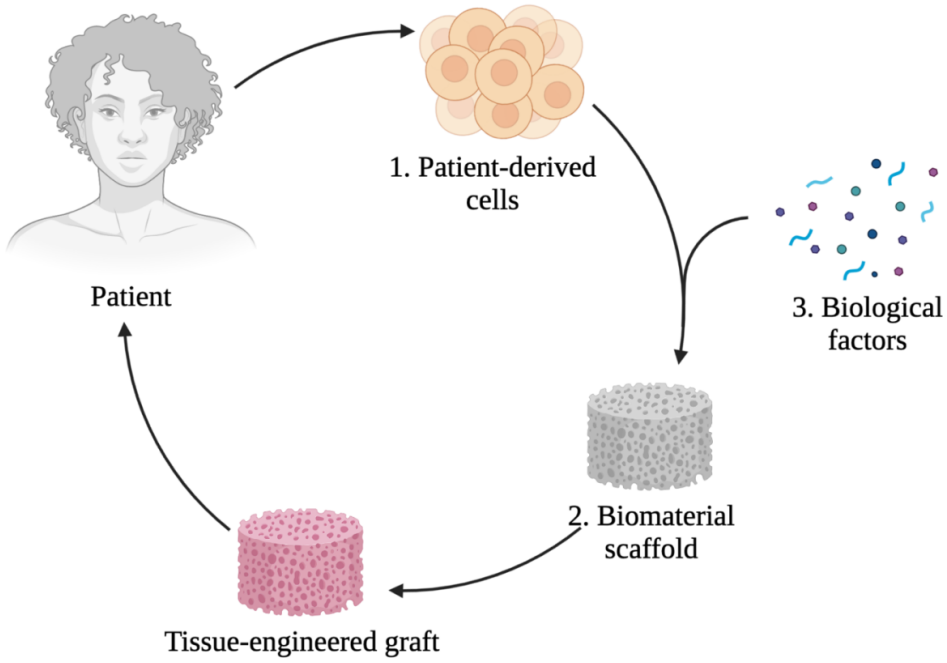


Figure 7. The three key components in tissue engineering, (1) cells; (2) a scaffold for transplantation and support; and (3) biological factors, such as cytokines and growth factors (Created using BioRender.com)

The prerequisite for the *in vitro* fabrication of engineered oral mucosal and dermal equivalents is the primary cultures of fibroblasts and keratinocytes from human oral mucosa and human skin. Rheinwald and Green pioneered this field already in 1975 by establishing techniques for isolating primary keratinocytes from patient samples and expanding them *in vitro*. They first managed to culture keratinocytes on the extracellular matrix synthesized by post-mitotic mouse fibroblasts which acted as a feeder layer. They further developed this technique by eliminating the feeder layers and the use of various recombinant growth supplements in the media like epidermal growth factor (EGF) [49].

Since the development of tissue engineered epithelial sheets by Rheinwald and Green and their use as for example reported by O'Connor et al for grafting [50] many studies have been reported successful fabrication and implantation of tissue engineered epidermal sheets [51, 52]. The two major disadvantages for using these *in vitro* fabricated epithelial sheets were: 1) the possibility of transferring exogenous components to patients undergoing grafting from the feeder layer used for culture of keratinocytes which was comprised by irradiated 3T3 mouse fibroblasts, and 2) the fragility of the sheets while handing and suturing [52].

With the increasing knowledge about the importance of the role of the extracellular matrix (ECM) in tissue regeneration, the tissue engineering approaches were focusing on developing biomimetic biomaterials that mimicked the bioactivity of an ECM while acting as scaffolds for the delivery of the therapeutic cells. They also can act as scaffolds and provisional matrices to support the tissue that is lost until the new tissue grows and replaces the scaffold. The scaffolds provide a suitable ecosystem for the delivery of cells and guidance for regeneration or repair of the tissue [53-55].

1.4.1 Tissue engineering of oral mucosa

Tissue engineering requires progenitor cells from a donor tissue, which are often in limited number. The most common used progenitor cells in mucosal tissue engineering are adult and embryonic stem cells (ESC). Stem cells have two major characteristics:

(*a*) self-renewal and (*b*) pluripotency, or the ability to differentiate into cells of multiple lineages [56].

A wide range of biomaterials both natural and synthetic have been explored in regeneration of the oral and maxillofacial region such as collagen, gelatine, poly (lactic acid), poly(caprolactone), poly (lactic-co-glycolic acid), and poly (glycolic acid) [57-59]. Specifically, in regeneration of oral mucosa, biomaterials should be appropriate for providing the characteristic moist environment of the oral cavity. To accomplish these properties, the most suitable scaffold should mimic the natural ECM [60].

Naturally derived scaffolds are known to have high biocompatibility [61, 62]. Recently, the use of decellularized matrix from the donor tissue have gained popularity in the field of tissue engineering [63]. The decellularized scaffolds are the biological tissues obtained by the different decellularization methods like physical, chemical, and enzymatic methods [64] and posess the structural characteristics of a hydrogel while retaining the function characteristics of ECM.

The studies by Badylak et al. in 2004 have shown that decellularized matrix can be used between the species and hence reduce immune rejection [65]. This method has become lately more widely used for regeneration of oral tissues and skin [66, 67].

Another important consideration in tissue engineering is the signals in the form of growth factors and differentiation factors for the stem/progenitor cells. The frequently used growth factors for tissue engineering of skin and oral mucosa are bone morphogenetic proteins (BMP), basic fibroblast growth factor (bFGF or FGF-2), and recombinant human epidermal growth factor (rhEGF) [68-70]. Several studies have been reported where the growth factors were used in regeneration of the mucosa [71-74] in the past few decades, but still there is lack of adequate techniques specially in relation to regenerative procedures of the oral mucosa.

Numerous sources of cells have been used in tissue engineering of oral mucosa in preclinical and clinical studies such as stem cells, fibroblasts and keratinocytes derived from the same patient, healthy donors or animals [75].

In 2012 Peramo et al. reported a 3D tissue structure that can be used in the repair of lip defects which consisted of a continuous layer with morphological features of a lip,

epidermal skin, vermilion border and oral mucosa [76]. Yoshizawa et al. reported in 2012 the ex-vivo graft produced by the oral mucosa equivalent (EVPOME) with keratinocytes which, when placed on the intraoral wounds, promoted epithelial regeneration of the oral mucosa [52].

In the recent years there has been stupendous advances in the regeneration of oral, dental, and craniofacial structures, but still there is need for improving and optimizing the methods before they are routinely used in the clinic for a favourable outcome.

1.4.2 Tissue engineering of skin

Amongst one of the first tissue-engineered skin constructs was the product developed by Howard Green and colleagues [50, 77, 78]. It contained few layers of cells and no dermis. This led to the development of the first commercially available skin product, Epicel (Genzyme, Cambridge, MA, USA), which contains sheets of autologous keratinocytes [79]. Eugene Bell and co-workers in 1981 fabricated a complex skin substitute reconstituting both dermis and the epidermis. The dermis was made by seeding the fibroblasts in the collagen gel which led to contraction of the gel, and this formed the neodermis. The keratinocytes were grown on top of the neodermis initially in submerged culture and later on were airlifted and exposed to air-liquid interface which lead to differentiation and keratinization [80]. Chai et al in 2012 established a three-dimensional oral mucosal model using primary keratinocytes, fibroblasts and scaffold derived from the skin [81].

Current approaches for skin tissue engineering

The most commonly used method in skin tissue engineering is regeneration of the keratinocyte layer by use of a scaffold containing growth factors and ECM to stimulate the proliferation of the keratinocytes (Figure 8). These methods work efficiently in cases of partial-thickness wounds. They have also been used in cases of full thickness wounds but have not been very successful [62]. The most comprehensive tissue-cultured skin incorporates both living dermis and epidermis, which are usually cultured from allogeneic sources [62].

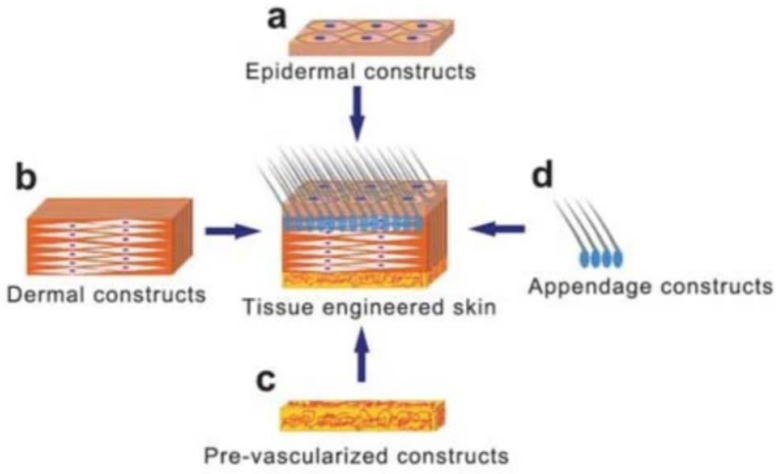


Figure 8. Current approaches to skin tissue engineering [82]. (Cited under Creative Commons Attribution License)

The tissue engineered skin therapies have been commercially available for decades but still have limited clinical use [83, 84] due to lack of competent full thickness skin substitutes that can be vascularized. They usually act as temporary wound dressing until the patient's own skin regenerates and is available for autografts [85]. One of the main limitations of the tissue engineered skin is slow revascularization and poor attachment to the wounds. This leads to increased risk of infection and rejection [86]. The tissue engineered skin also lacks important structures like sebaceous glands, sweat glands and cells like melanocytes, Langerhans cells [87, 88]. Tissue engineered skin models are used in clinical applications as grafts as well as *in vitro* models. Fibre matrices in combination with the hydrogels or alone are developed to deliver the growth factors and the cells to promote healing [89].

The fields of regenerative medicine and tissue engineering are significantly complex and widely interdisciplinary. They require a deep understanding on the intertwined relationship around the development and sustainability of tissues and organs. Those encouraged by early successes in this field on skin regeneration, hoped to succeed by implanting a construct formed from any cell type placed in a matrix [62].

Recent advances in stem cell biology may enable intelligent tissue engineering techniques for replacement and regeneration of skin.

1.5 Stem cells

Cell based therapies for wound healing have gained popularity in the last decade. Stem cells have become an important source for cells in tissue engineering and regeneration. Stem cells are undifferentiated cells and have the ability to renew themselves (self-renewal) [90]. The developmental stage at which the stem cells are isolated determines what type of cells they can differentiate into. The ESC which are isolated from the inner cell mass of the blastocyst are pluripotent as they can differentiate into all three germ layers (endoderm, mesoderm, and ectoderm). The adult stem cells which are isolated from the adult tissue are multipotent and exhibit limited differentiation. Along with this ability to differentiate, as mentioned above stem cells also have the ability to self-renew and maintain tissue homeostasis [91]. This inherent capacity to divide and proliferate makes stem cells a favourable source for regeneration.

Stem cells are the internal repair system of the body. Stem cell activity mainly depends on the organ from which they originate [92]. Stem cells have a great potential in medicine. Due to their abilities, stem cells have become an attractive source for use in the regeneration and repair. The use of stem cells for tissue regeneration can be enhanced using various scaffolds and/or by adding different growth factors which provide the lineage specific cues for the differentiation and proliferation of the stem cells [93].

1.5.1 Stem cell classification based on origin

Stem cells can be divided into 4 broad categories based on their origin: ESCs, fetal stem cells, adult stem cells (ASC), and induced pluripotent stem cells (iPSCs). ESCs and iPSCs are pluripotent, while the ASC are multipotent, oligopotent, or unipotent [94, 95].

ESCs differentiate into any of the three germ layers and become multipotent stem cells, when the potency is limited to the cells of that particular germ layer. ASC include

different types of cells such as haematopoietic stem cells (HSC), mesenchymal stem cells (MSC), neural stem cell, and epithelial stem cells.

The iPSC are reprogrammed from adult somatic cells using 4 transcription factors called the Yamanaka factors Oct3/4, Sox2, c-Myc, and Klf4 (Figure 9) [96].

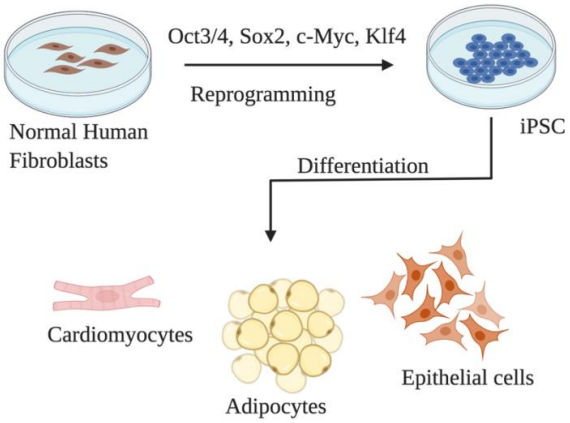


Figure 9. Figure shows how reprogramming of normal human fibroblasts into iPSC is done by retroviral delivery of four genes. (Created using BioRender.com)

1.5.2 Sources of stem cells in oral and maxillofacial tissues

Oral and maxillofacial region is a rich source of adult stem cells. These cells have comparable properties of self-renewal and differentiation *in vitro*. Based on the location, these ASCs are divided into two major categories: dental and non-dental (Figure 10) [97, 98].

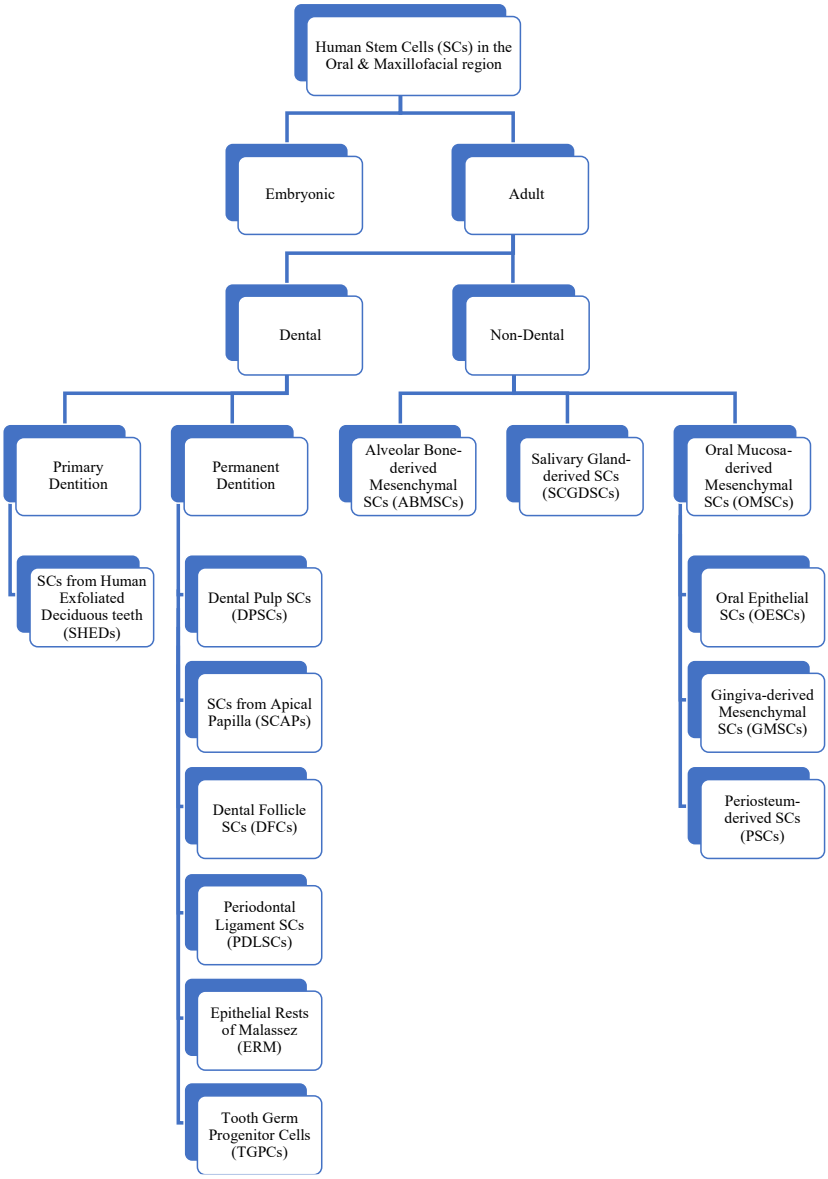


Figure 10. Human stem cells in oral and maxillofacial region. Adapted from [99]

Dental pulp stem cells, are the first human derived dental MSCs found in teeth, and are considered an important source for regenerative procedures [100]. Dental pulp stem cells are extracted from the pulp of deciduous and permanent teeth. They have a capacity to differentiate into odontoblast, osteoblast, myoblast, adipocyte,

cardiomyocyte, neuron-like cell, and hepatocyte-like cells *in vitro* [75, 101, 102]. Periodontal ligament (PDL) stem cells are present on alveolar bone and the root surfaces. They have a definite role in regeneration of cementum and PDL. They can produce cells of mesenchymal origin *in vitro* [103, 104]. Other source of epithelial progenitors from oral mucosa could be the epithelial rests of Malassez (ERM). These are islands and strands of epithelial cells surrounding the dental root, located in the periodontal ligament (Figure 11) [105]. They are derived from the Hertwig's epithelial root sheath (HERS) fragments during advanced dental-root development. The ERM are therefore in a direct lineage from the HERS and are derived from the cervical loop epithelium in the enamel organ. The ERM may contain epithelial progenitor/stem cells with the capacity to generate oral epithelium in a fashion similar to the progenitor epithelial cells from oral mucosa and can be a novel source for regeneration of oral mucosa.

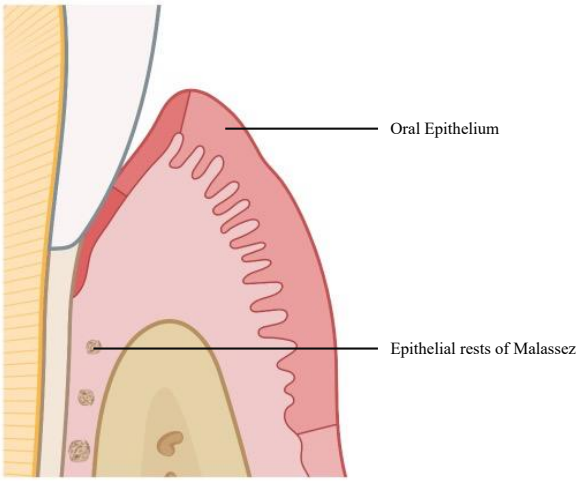


Figure 11. Epithelial rests of Malassez (Created using BioRender.com)

Stem cells derived from apical papilla are of mesenchymal origin. They are found in the immature roots and can be isolated from permanent tooth apical papilla. They have a higher capacity to proliferate as compared to dental pulp stem cells and can be a promising source of stem cells with their ability to differentiate into multiple cell types [106].

Dental follicle stem cells are derived from the dental follicle surrounding the developing tooth. They can differentiate into multiple cell types like osteoblast, cementoblast, alveolar bone, dentin-like tissues, PDL, cementum, adipocyte, chondrocyte, cardiomyocyte, and neuron-like cells [103].

Tooth germ progenitor cells are derived from the dental mesenchyme of the third molar tooth germ in the late bell stage. They have demonstrated high proliferation capacity and can differentiate into adipogenic, chondrogenic, osteogenic, odontogenic, and neurogenic tissue [107, 108].

Stem cells of human exfoliated deciduous teeth are extracted from exfoliated deciduous teeth. They have higher proliferation capacity than dental pulp stem cells and can differentiate into many different tissues and SCs, including adipocytes, osteoblasts, odontoblasts, neural cells, hepatocytes, and endothelial cells. Stem cells of human exfoliated deciduous teeth have a high proliferative capacity, high multipotency, immunosuppressor ability, and least risk of oncogenesis [109].

Alveolar bone-derived mesenchymal stem cells are isolated from the human alveolar bone and are a favourable source of MSCs. They are multipotent cells and can differentiate into osteoblasts, adipocytes, and chondroblasts [98].

Salivary gland-derived stem cells are isolated from salivary glands [110].

Oral mucosa-derived stem cells include oral epithelial stem cells, gingiva-derived mesenchymal stem cells, and periosteum-derived stem cells. Oral mucosa-derived mesenchymal stem cells can differentiate into distinctive mesenchymal lineages and have immunomodulatory properties [110].

1.5.3 Stem cells in regeneration of skin and oral mucosa

The use of stem cells in tissue engineering of the skin and oral mucosa may provide a potential solution to overcome the limitations of current technologies by use of autologous/ allogeneic cells. Use of stem cells has been partly effective, though, the long-term adverse effects and possibility of teratoma formation with the use of pluripotent stem cells should be considered [111, 112].

Bone marrow-derived mesenchymal stem cells (BM-MSC) promote wound repair when applied on cutaneous wounds as the MSC stimulate the fibroblasts to upregulate the expression of integrin alpha 7 and downregulate the expression of MMP11, ICAM1 and VCAM1 [113].

The iPSC reprogrammed from somatic cells are an important source for patient-derived cells for therapeutic applications. The iPSC can be differentiated into a wide variety of cells [114]. It has been experimentally proven that the fibroblasts differentiated from iPSC expressed the specific properties comparable to the parent population and in some cases even exceeded it [114-117]. The ECM produced by the iPSC-derived fibroblasts had an amplified potency as compared to the parent fibroblast [118]. The iPSC-derived fibroblasts may improve effectiveness of regenerative therapies. Human induced pluripotent stem cells are a novel source of autologous cells for dermal and oral regeneration. ESCs have shown controlled differentiation into tissue specific lineages *in vitro* and *in vivo* for regeneration of oral mucosa [119]. Perinatal stem cells like human umbilical cord Wharton's jelly stem cells have been used in regeneration of oral mucosa and skin [120].

Hard tissue engineering in oral and maxillofacial region has evolved rapidly over the recent past, and clinical usage of growth factor, scaffolds and stem cells has been progressively increasing in correcting small and large bone craniofacial defects [121]. On the other hand, the same cannot be stated towards oral soft tissue engineering, where both research and clinical applications for oral mucosa and soft tissues need further studies [27]. Skin substitutes for regeneration have progressed remarkably in the last two decades but there are no efficient models for regeneration of oral mucosa. This lack of efficient methods for regeneration of oral mucosa leads us to the need of this study to explore novel/alternative sources and methods for generation of clinical grade mucosal sheets.

The above-mentioned studies demonstrated that tissue engineering can be used for repair of oral mucosal and skin defects and can overcome the challenges of use of traditional autologous grafts. However, there are some limitations of use of cells, growth factors and scaffolds used in tissue engineering.

1.6 Challenges in tissue engineering

1.6.1 Challenges of scaffolds

An ideal scaffold should be biocompatible, have tuned degradability and mimic the ECM. In the tissue, the ECM should provide functional and structural integrity as well as appropriate conditions for growth of the cells. This complexity of the ECM is difficult to recapitulate by biomaterials [55, 122]. The ECM derived materials have evolved in the form of various shapes, coatings, hydrogels, cell sheets and decellularized tissues over the past decades, but it is still challenging [123]. Native tissues exhibit physiological stiffness to provide adequate environment for growth and function of the cell which is difficult to reproduce by the biomaterials. Synthetic polymers can be easily fabricated with desired stiffness and thickness, but the use of synthetic scaffolds could lead to immune responses and also lack the biological cues required for the cells [55, 124].

1.6.2 Challenges of growth factors

In tissue regeneration the cells in the microenvironment produce various bioactive molecules like cytokines and growth factors. The growth factors released stimulate the various cellular processes like proliferation, differentiation and migration [125]. To be an effective therapeutic agent, growth factors should reach the site of injury without undergoing degradation and should remain long enough to induce regeneration [126]. The growth factors usually provided exogenously are not very effective as they are diffused away from the wounds and often get digested or deactivated. Also, they may fail in induction of tissue regeneration due to low availability and slow penetration due to large size or toxicity if in excess [127, 128].

1.6.3 Challenges of cells

ASC are predominantly used in mucosa and skin tissue engineering which is challenging due to inadequate number of cells. They need lineage specific expansion medium for the cells to retain their phenotype. For clinical use of these cells efficient xeno-free culture conditions need to be followed which often lead to low yield in cells

[129]. MSCs are the most commonly used stem cells [130]. Though MSCs have been used extensively in regenerative therapies, potential tumorigenicity has been reported with the use in clinical therapies [131, 132].

Apart from the stem cells – autologous, allogeneic and xenogeneic somatic or differentiated cells have also been used for tissue engineering. The problem of immune rejection by use of xenogeneic cells has been overcome as demonstrated by various studies, but long-term effects still need to be tested [133-135]. As compared to xenogeneic cells, allogeneic cells are better to overcome the problem of immune rejection [136]. The use of allogeneic cells also has its limitation as these cells may cause ethical problems. Autologous cells are the best source as the problems of immune rejection and other ethical issues will not be faced, but the use is restricted due to limited sources for harvesting these cells.

All these challenges with the use of adult stem cells as well as with the other cell sources mentioned above, call for the need to explore alternative sources of stem cells for regeneration of oral mucosa.

2. Rationale and aims of the study

There is an increasing need for regeneration of oral soft tissues due to tumour resections, injuries such as burns, trauma, and congenital disabilities. Soft tissue defects generally heal on their own by regeneration, but large defects cause permanent loss of tissue and scar tissue. Presently, the restoration of the soft tissue defects in the oral and maxillofacial region remains a clinical challenge due to lack of effective therapies for such defects, especially when they are large [137, 138]. Despite several advancements in tissue engineering over the past decades, there is still a need to develop efficient methods for regeneration of oral mucosa, to overcome the problems associated with the use of conventional autologous grafts for treatment of oral mucosal defects. Tissue engineering of oral mucosa, using various sources of cells together with more innovative differentiation methods, can overcome these problems and address the unmet clinical need for regeneration of the mucosa. More focussed studies are required to study alternative sources of cells for regeneration of oral mucosa. The hypothesis of this study was that alternative sources of epithelial cells can be used for the generation of oral mucosal sheets for regeneration of oral mucosa.

Aims

General Aim

To assess novel methods for regeneration of oral mucosa by using various sources of cells.

Specific Aims

1. To identify the factors responsible for oral epithelial differentiation for generating oral mucosal sheets.
2. To isolate and characterize human ERM cells and compare them with cells derived from matched NHOM for their ability to generate oral mucosal sheets.
3. To test whether pluripotent ESC can be differentiated into oral epithelium.
4. To differentiate iPSC derived from adult human fibroblasts into oral keratinocytes which can then further be used for generation of oral mucosal sheets for regenerative therapies.

3. Methodological considerations

To cover the scope of the thesis, patient tissue samples were used, as well as experimental *in vitro* models and methods that are described in detail in the individual papers, and *in vivo* mouse models. In the following section, particular aspects and rationale for the methods used are discussed.

3.1 Patient samples and ethical approvals

Cells were isolated from normal human skin (NHS), NHOM, and PDL of human extracted teeth, as well as fibroblasts from skin (ear) and oral (buccal) mouse mucosa.

Paper I

NHOM samples were obtained from eighteen healthy donors undergoing wisdom tooth extraction after informed consent. Out of these eighteen samples, seven samples were snapped-frozen in isopentane and six were embedded in paraffin. These served as positive controls for different immunostainings. Five samples were used for primary cell isolation for 2D and 3D organotypic cultures. The study was approved by the Ethics Committee of Western Norway (REK nr.2010/481).

Paper II

Matched samples of tissues from the PDL of extracted tooth and NHOM were collected after informed consent from healthy patients undergoing wisdom tooth extraction. The project was approved by the Ethics Committee of Western Norway (REK nr.2010/481).

Paper III

Normal skin and oral fibroblasts were isolated from mouse ear and buccal mucosa, respectively. This project was approved by Norwegian Food Safety Authority (NFSA) (FOTS ID 2006400).

Paper IV

NHOM and NHS samples were obtained from three healthy donors after informed consent and used for cell isolation in culture. The project was approved by the Ethics Committee of Western Norway (REK nr.80005).

3.2 Choice of methods

The thesis includes specific studies described in **Paper I, II, III and IV** (Figure 12) that included *in vitro* assays to investigate the biological characteristics and behaviour of epithelial and stem cells when exposed to different ECM components, as well as *in vivo* assays as proof of principle for *in vivo* mucosal regeneration (described in the thesis only as a pilot study).

We have previously shown that oral epithelial differentiation is directed by underlying fibroblasts, but the responsible factor(s) has not been identified. We wanted to identify fibroblast-derived factors responsible for oral epithelial differentiation. To study the interactions between fibroblasts and epithelial cells, *in vitro* 3D OT models were constructed using a well-developed protocol used in our lab [139]. **Paper I**

We also wanted to find alternative sources for stem cells for oral mucosal regeneration. In lieu of this we isolated cells from ERM, differentiated mouse ESC, and generated human iPSC by reprogramming of human normal oral and skin fibroblasts. To analyse and characterize epithelial and stem cells obtained from the above-mentioned sources we used several *in vitro* assays including light microscopy, flow cytometry, immunohistochemistry (IHC), western blotting and qPCR. 3D OT models were also used to test the ability of these cells to make oral mucosal equivalents *in vitro* and were compared to 3D models which were made using cells isolated from NHOM. **Paper II, III and IV**

In addition, a mouse model was devised to test *in vivo* the viability of oral mucosal sheets generated by use of iPSC (thesis only).

Challenges in this study

Since this study involved the isolation of primary cells from patient samples, we faced challenges due to bacterial and fungal infections and difficulty in isolation of primary cells from all patient tissues. The iPSCs used were observed carefully and regularly checked for mycoplasma using MycoAlert^{TM} mycoplasma detection kit (Lonza, LT07-318), as per manufacturer's instructions. During the differentiation of iPSC into

keratinocytes the use of antibiotics and antimycotics was avoided in order not to perturb the differentiation process, hence the cells were often infected with fungus and bacteria and had to be discarded while the lengthy differentiation protocols were run.

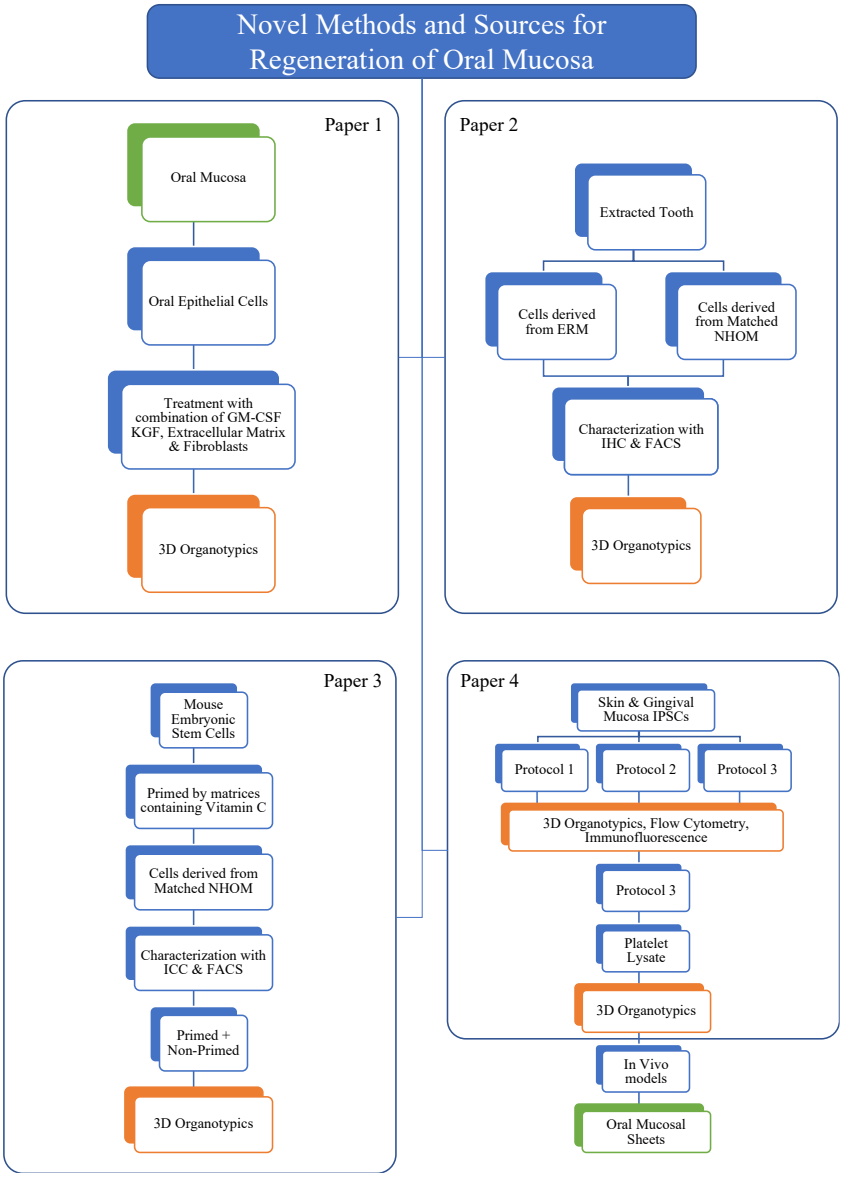


Figure 12. Schematic outline of the work**.** Stepwise presentation of the techniques used in this thesis, divided into the four study groups, starting with the 3D OT standardization in **Paper I** and ending with 3D OT in **Paper IV**. Each step is colour co-ordinated in all the four studies.

3.2.1 *In vitro* cell culture

In vivo models provide an insight into the whole body, while *in vitro* cell models help in studying the biological functions of cells and facilitate functional manipulation of specific proteins and genes to identify affected cellular pathways [140].

Primary cell isolation and culture

Extracted teeth, NHOM and normal human skin samples were transported on ice in transport medium: Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) with 200 IU penicillin per mL (GibcoBRL), 200 µg streptomycin per mL (GibcoBRL), and 0.5 µg amphotericin B per mL (GibcoBRL). Cells were isolated following the standard explant method at 37 °C in 5% CO_2, in a humidified incubator [139]. Briefly, no enzymes were used, and the tissue was cut into smaller pieces on culture dishes. Using this technique, cells remain attached to the ECM as there is no separation of the epithelium from the connective tissue, which helps the cells to recover from stress [141].

The periodontal ligament (PDL) attached to the cervical, middle and apex one-third of the root was removed with a scalpel and collected separately under a dissecting microscope. The PDL and NHOM tissues were cut in approximately 1 mm^3 pieces, allowed to adhere to cell culture dishes (Nunclon™ Delta, Thermo Fisher Scientific, CA, US) and were cultured using the explant method mentioned above [139]. (**Paper II)**

Primary human normal oral fibroblasts (NOF) and keratinocytes (NOK) were isolated from the tissue samples as previously described [139]. Keratinocytes were routinely grown on plastic surfaces (Nunc, Naperville, I.L., USA) with no feeding layers, in keratinocyte serum free medium (KSFM) (Gibco BRL, Grand Island, N.Y., USA) supplemented with 1 ng/ml human recombinant epidermal growth factor (Gibco BRL), 25 µg/ml bovine pituitary extract (Gibco BRL), 2 mM L-glutamine (Gibco BRL), 100 U/ml penicillin (Gibco BRL), 100 µg/ml streptomycin (Gibco BRL), and 0.25 µg/ml amphotericin B (Gibco BRL). Fibroblasts were grown in Minimum Essential Medium Eagle (MEM) (Sigma, St. Louis, M.O., US) supplemented with 10% fetal calf serum

(FCS) (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. (**Paper I)**

Mitomycin C (Sigma)-inactivated 3T3 fibroblasts (10–100 µl/ml of mitomycin C solution per ml of culture medium for 2 h) were added to the dishes planed for isolation of epithelial cells and incubated in keratinocyte serum-free media (KSFM, Gibco BRL) supplemented with 1 ng/ml epidermal growth factor (EGF human recombinant), 25 µg/ml bovine pituitary extract (BPE), 20 µg/ml L-glutamine, 1% AB/AM (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) (all from Gibco BRL). Cells with the same morphology from different explants were then pooled together to eliminate the risk of clonality of isolated cells and further propagated in lineage-specific medium. (**Paper II)**

The feeder independent ESC line E14.2 [142] was primed by growth on various matrices in media containing vitamin C, without any leukaemia inhibitory factor (LIF). The matrices investigated were gelatine, laminin, those formed by skin and oral fibroblasts, and those formed by skin and oral keratinocytes. **(Paper III)**

Primary mouse normal skin and oral fibroblasts were isolated from the tissue samples as previously described [139]. They were grown in Minimum Essential Medium Eagle (MEM) (Sigma, St. Louis, M.O., US) supplemented with 10% fetal calf serum (FCS) (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. (**Paper II)**

Human NOF and NSF were isolated from the tissue samples, as previously described [139]. They were grown in Dulbecco′s Modified Eagle′s Medium – high glucose (DMEM) (D6429, Sigma) supplemented with 10% new-born calf serum (NBCS) (Fischer scientific), 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Gibco). The NOF and NSF were also grown in xenofree conditions using Dulbecco′s Modified Eagle′s Medium – high glucose (DMEM) (D6429, Sigma) supplemented with 5% platelet lysate (PL) (BergenLys (R) Platelet lysate, unfiltered, 4-month storage (PC), 23 October 2019 AIT/IKO, 1% Heparin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Gibco). The NOK and NSF were reprogrammed to gingival and skin iPSC using Yamanaka factors (Oct3/4, Sox2,

Klf4, c-Myc). For reprogramming of fibroblasts, cells were transfected (Nucleofector 2b Device, Lonza, Switzerland) with 1ug each of the three episomal reprogramming plasmids (**hUl***, MYC & LIN28*; **hSk**, *SOX 2 & KLF 4*; **hOCT4/shp53**, *OCT 4 & RNA against p53*) and plated onto a six well plate containing either FBS or PL supplemented with DMEM. After reaching confluency, the cells were passaged onto pre-coated dishes with Geltrex (Gibco, ThermoFisher Scientific, Massachusetts, United States). The following day, the media was changed to StemFlex media (StemFlex Medium, Gibco, ThermoFisher Scientific, Massachusetts, United States) with supplements and 1% AB/AM (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B). Each iPSC colony was cultured individually in StemFlex media until passage 10, after which the cells were characterized. (**Paper IV)**

3.2.2 Differentiation of iPSC into skin and gingival keratinocytes

In the quest to differentiate oral and skin fibroblast-derived iPSC into oral keratinocytes, three different protocols (Table 1), two growth factors enriched and one ECM-enriched, were tested and adapted from previous literature on differentiating skin fibroblast-derived iPSC into skin keratinocytes [143-145].

Skin and gingival fibroblast-derived iPSC were obtained from our collaborators at the Tissue Engineering Group at Department of Clinical Dentistry and Diabetes research Group at Department of Clinical Science at the University of Bergen. Cells obtained from all the three protocols were characterized and based on the obtained results, the ECM-enriched protocol (protocol no.3) [145] was selected to produce oral keratinocytes by growing the iPSC cells on ECM synthesised by human fibroblast inactivated feeder layer, and it was further tested in xeno-free conditions. (**Paper IV)**

Table 1. The protocols tested for differentiation of iPSCs to keratinocytes

Protocols	***Conditions***
Protocol 1 (Zao et al) [143]	hEGF, bFGF, BMP4, RA
Protocol 2 (Bilousva et at) [144]	N2B27 media, BMP4 & RA
Protocol 3 (Yoshida et al) [145]	ECM produced by mitomycin-inactivated human primary fibroblasts, BMP4

3.2.3 Immunohistochemical staining (IHC)

IHC is a technique that uses antibodies to visualize a protein of interest in tissues [146]. This is the most widely used technique for detection of proteins. Immunostaining includes both enzymatic and fluorescent visualization of the proteins. The IHC and immunofluorescence (IF) techniques allow a detailed analysis of protein expression in individual cells within intact tissue sections. Since these techniques involve several steps, substantial optimization is required to limit the false positive and false negative results [147]. In Paper I, II, III and IV we used IHC to assess the expression of various epithelial and mesenchymal markers in 3D organotypic models. The method has been also used to evaluate the phenotype of the epithelium of the mucosal sheets xenotransplanted in the animal model.

Paper I

The immunohistochemical staining was carried out using the DAKO autostainer – Universal Staining System (DAKO-USA, Carpinteria, California, US). Five μm thick FFPE 3D organotypic sections were used. The staining for α6 integrin and E-cadherin was first carried on fresh frozen sections fixed for 30 sec. in 50% cold acetone, then for 5 min. in 100% acetone before washing in distilled water. All sections were then processed as previously reported [139]. Primary antibodies (all IgG1) used in this study were: Ki-67, MIB-1clone, 1:50 (DAKO), cytokeratin 13 (CK13), KS-1A3 clone, 1:400 (Novocastra Laboratories Ltd, Newcastle, UK); β1-integrin, K20 clone, 1:2000 (DAKO), α6-integrin, BQ16 clone, 1:1000 (DAKO), EGF-R, E30 clone, 1:100 (DAKO), E-cadherin, HECD-1 clone, 1:9000 (R&D Systems, Abingdon, U.K), collagen IV, CIV221 clone, 1:25 (DAKO). The presence of antigen was visualised with DAB+ (3,3'-diaminobenzidine, DAKO).

Paper II

Cells isolated from NHOM and PDL were grown on coverslips and fixed in 4% PFA for 20 min. at room temperature and then exposed for 1 hr to a solution containing antibodies against PanCK (DAKO, Glostrup, Denmark). The presence of antigens was then evidenced by adding DAB (3,3′diaminobenzidine, DAKO) for 10 min. The sections treated with antibody diluent instead of primary antibody were used as

negative controls. To stain 3D organotypic cultures and gels, 3 µm sections were cut, deparaffinized and rehydrated by immersion in xylene and diminishing concentrations of alcohol. Sections were then incubated over night at 4 °C with one of the following monoclonal mouse anti-human primary antibodies: anti-pancytokeratin (1:2000, DAKO) and anti-vimentin (1:2000, DAKO). Envision+® anti-mouse (DAKO) was used to detect the site of reaction according to the manufacturer`s instructions. The reaction was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB). Sections were then counterstained with haematoxylin (DAKO), dehydrated and cover slipped.

Paper III

ESC 'primed' first on various matrices in 2D cultures were then grown in 3D organotypic cultures and the tissues obtained were examined to assess their regional specificity in the differentiated phenotype of the epithelium [148]. Keratin pairs 1/10 and 4/13 were used to distinguish keratinized (skin) versus non keratinised (oral) epithelial phenotype. Five µm thick FFPE 3D organotypic sections were used. All sections were processed as previously reported [139]. The presence of antigens was detected using DAB+ (3,3'-diaminobenzidine, DAKO). Formalin-fixed paraffin-embedded (FFPE) blocks of mouse skin and oral mucosa were used as positive controls.

Paper IV

The differentiated iPSC both in FBS and PL were grown in 3D organotypic cultures, and the tissues obtained were examined to assess the epithelium produced by the differentiated iPSC. Primary antibodies anti-pancytokeratin, AE1/AE3 clone (1:100 DAKO), anti-vimentin (1:2000, DAKO), anti-CK10 and anti CK 13 (1:10, CRUK) were used. Four µm thick FFPE 3D organotypic sections were used. All sections were processed as previously reported [139]. The presence of antigens was detected using DAB+ (3,3'-diaminobenzidine, DAKO).

The same panel of antibodies has been used to evaluate the phenotype of the epithelium of the mucosal sheets xenotransplanted in the animal model.

3.2.4 Immunofluorescence (IF)

To quantify the extent of differentiation into epithelial lineages after 'priming' of ESC in 2D cultures, the expression of PanCK (DAKO A/S, Glostrup, Denmark, titration 1:200) as a marker of epithelial differentiation and of embryonic stem cell markers stage-specific embryonic antigen-1 (SSEA-1 or CD15, Clone MC-480, Stem Cell Technologies, Cambridge, UK) and OCT-3/4 (POU5F1, Clone 40, Stem Cell Technologies) were analysed. The cells were grown on cover slips and fixed with 4% PFA for 15 min. Cells were then washed with PBS before being permeabilized using 0.3% Triton for 10 min. and then again washed with PBS. Blocking was done using 5% BSA (Sigma) in PBS for 45 min. followed by primary antibody (1ug/ml) for 60 min. The cells were then washed with PBS and secondary antibody (goat anti-mouse IgG, FITC, Stem Cell technologies) was added for 60 min. in dark. Cells were then mounted in Mounting Medium with DAPI Fluoroshield (Abcam, UK). The IgG1 isotype primary antibody was used as negative control. (**Paper III)**

The NOK, both skin and gingival fibroblast-derived iPSC and differentiated iPSC were cultured on cover slips and fixed with 4% PFA at room temperature. The cells were then permeabilized with 0.1% Triton, blocked using 5% BSA, and immunostaining was done using anti-pancytokeratin, clones AE1/AE3 (1:100 DAKO). Alexa Fluor 488 anti-mouse IgG was used to detect the site of reaction according to the manufacturer`s instructions and were then mounted on the slides with vectashield (Vector Labs), and the images were captured using florescence microscope. (**Paper IV)**

3.2.5 Detection of apoptotic cell death

TUNEL Method

Cell death by apoptosis was detected by the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP *in situ* nick end-labelling) on formalin fixed paraffin embedded sections of 3D organotypic cultures [149]. For positive controls, specimens were treated with 0.5 mg/ml DNase (Roche Diagnostics) in Tris-buffered saline (TBS) for 15 min at 37°C prior to incubation with bovine serum albumin. The specificity of the TUNEL reaction was tested by substituting the

biotinylated dUTP in the TUNEL labelling mixture with unbiotinylated UTP (Roche) in excess. TUNEL positive keratinocytes found within the basal cell layer were considered spontaneously apoptotic cells, while TUNEL positive cells found at the superficial cell layer on top of the epithelium were considered terminally differentiated keratinocytes [150]. (**Paper I)**

3.2.6 ELISA

The enzyme-linked immunosorbent assay (ELISA) is an immunological assay commonly used to detect and quantify antibodies, antigens, proteins, and glycoproteins in biological samples. The antigen is immobilized on a solid surface and then complexed with an antibody that is linked to a reporter enzyme. It is a highly specific antigen-antibody reaction [151]. The conditioned media from NOFs grown in 3D in collagen gels (0.5mil/ml) was analysed for levels of various growth factors and cytokines by using the Widescreen Human Cancer Panel 2 (Novagen, Millipore, US) with Luminex beads (R&D Systems, Inc, Minneapolis, MN). **(Paper I)**

3.2.7 Evaluation of NHOM tissue samples

The NHOM tissue, as previously described, was embedded in paraffin and five μm sections were stained for hematoxylin-eosin. The Ki-67 /proliferation index was used to determine the percentage of proliferating cells in the basal cell layer per 400 μm length of epithelial-mesenchymal interface. The measurements and counts were done at 200-fold magnification using a standard microscope (Leika DMLM, Germany). (**Paper 1)**

3.2.8 Flow cytometry

Flow cytometry is an established method to analyse the expression of cell surface and intracellular molecules and to characterize and define different cell types. It measures the fluorescence intensity produced by fluorescent-labelled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules [152-154].

For fluorescent-activated cell sorting (FACS), cells isolated from the PDL and NHOM were stained with the following antibodies: anti-ESA-APC conjugated (Biomed,

USA), anti-PDGFRB-PE conjugated (CD140b-PE conjugated, R&D Systems, UK), anti-CD44-PE (R&D Systems, USA), anti-CD31-PE conjugated (R&D Systems, USA) with both positive and negative controls. DAPI nuclear dye (Sigma) was used at 1 µg/ml to exclude dead cells. All analyses were performed on the FACS aria SORP (Becton Dickinson, USA). (**Paper II)**

To quantify the differentiation of embryonic stem cells and differentiated iPSC into epithelial lineages, a FACS analysis was carried out to analyse the expression of the epithelial markers using cytokeratins, E-cadherin and pluripotency markers using SSEA-1 and Oct-4 and TRA60. (**Paper III and Paper IV)**

3.2.9 Gene expression analysis using real time PCR

Francis Crick's 1957 statement "DNA makes RNA, and RNA makes protein" is the central dogma of molecular biology and succinctly explains the flow of genetic information within a biological system [155]. Each step in this system is critical for gene expression and even a small change may have a butterfly effect towards the differentiation of stem cells. We used the real-time quantitative PCR (RT-qPCR) technique to study the amplification of the target gene expression. The TaqMan assay is a real-time quantitative PCR (RT-qPCR) method which uses fluorogenic single stranded oligonucleotide probes which bind to the specific target and generate fluorescent signals [156, 157]. To quantify the expression of epithelial and stemness markers for the keratinocytes differentiated from the iPSC we used cytokeratins, OCT3/4, SOX2 and Nanog for qPCR. The cells were gown in a 6-well plate and the lysate was collected using an RNA lysis buffer. The RNA extraction was done using a Qiagen RNeasy Mini Kit as per manufacturer's instructions. Measurement of the total RNA concentration was done using a Nanodrop spectrophotometer. An amount of 100 ng of total RNA were converted to cDNA using the High-capacity cDNA kit as per manufacturer's instructions (Applied Biosystems, Carlsbad, US). TaqMan gene expression assays (Applied Biosystems®) were used to detect the mRNA levels of stemness (OCT3/4, SOX2, Nanog) and epithelial markers (KR1, 5, 13, 18, 19). An RT-qPCR amplification was performed using AB 7500 PCR system for 40 cycles. (**Paper IV)**

3.2.10 Immunoblotting

Immunoblotting is a semi quantitative detection of proteins in a protein mixture, e.g., a cell lysate, which is applied to a gel electrophoresis to sort the proteins based on size. The separated protein bands are then transferred to a carrier membrane, e.g., nitrocellulose, nylon or PVDF. This process is called blotting. The proteins adhere to the membrane in the same pattern as they have been separated due to interactions of charges. The proteins on this immunoblot are then accessible for antibody-binding for detection [158, 159].

The protein expression of PanCK from iPSC-derived epithelial cells was detected using immunoblotting. PanCK was detected for all the cells that differentiated to keratinocytes using protocol 3, in both xenogeneic and xenofree conditions. (**Paper IV)**

3.2.11 3D OT cultures

The conventional two-dimensional (2D) monolayer cultures lack the complex intercellular and cell matrix interactions occurring in real-life physiologic state. To circumvent the limitation of 2D models, 3D OT models have been elaborated [160, 161].

Organotypic models are the *in vitro* representations in 3D of the *in vivo* environment. These models have been used extensively for many years to study the cellular behaviour inside the body [162]. In this thesis, we constructed 3D OT models using epithelial cells and stem cells from different sources and fibroblasts to study the oral mucosa and oral epithelial differentiation.

The 3D OT cultures were obtained by growing epithelial cells on top of fibroblast-populated collagen type I (BD Biosciences) biomatrices, using a protocol well-established in our laboratory[139]. In Paper I, II, III, and IV simple collagen gels were prepared on ice by mixing 7 vol. (3.40 mg/ml) of rat tail collagen type I (Collaborative Biomedical, Bedford, M.A., USA), 2 vol. reconstitution buffer (261 mM NaHCO3, 150 mM NaOH, 200 mM HEPES) pH 8.15, 1 vol. DMEM 10x (Sigma) and 1 vol. FCS. The *3D* organotypic cultures were grown in serum-free FAD medium (DMEM: Ham's

F-12 / 3:1) supplemented with 1 μM hydrocortisone, 0.8 μM insulin, 0.25 mM transferrin, 0.25 mM L-ascorbic acid, 15-30 μM linoleic acid, 15 μM bovine serum albumin, and 2 mM L-glutamine (all from Sigma).

Human growth factors (EGF, KGF, GM-CSF, TGFα, IL-1α, HGF, SCF) (Sigma) were added to the culture media of some of the simple collagen matrix cultures at a range of 0.1-100ng/ml. Sandwich models were prepared by interposing a layer of collagen biomatrix between the epithelial compartment and the fibroblast containing matrix. (**Paper I)**

The epithelial cells isolated from NHOM and PDL were also embedded in collagen gels, formalin fixed, paraffin embedded and sectioned. These 3D OT tissue sections were stained with hematoxylin and eosin. (**Paper II).**

The ESC which were primed on matrices were grown in 3D cultures and were analyzed for regional specificity. **(Paper III).**

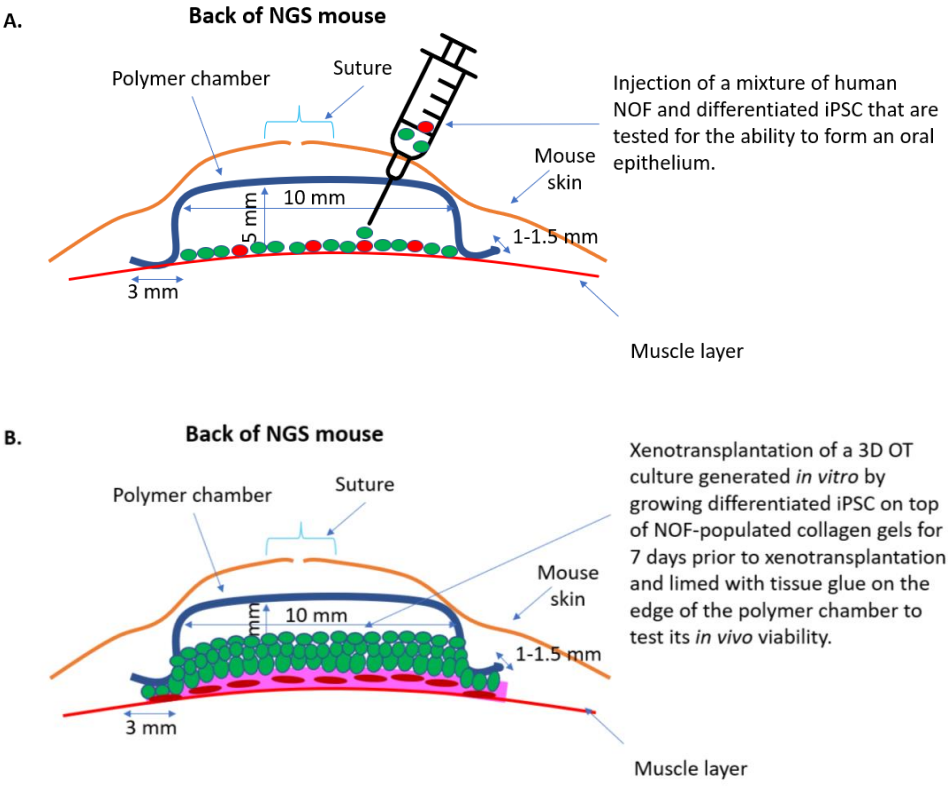
The differentiated iPSC grown in 3D OT cultures were analysed for their ability to form epithelium to be used as oral mucosal sheets in regenerative medicine. (**Paper IV)**

3.2.12 3D printing of Poly ε-caprolactone (PCL) chambers for xenotransplantation of mucosal sheets

A 3D CAD model was designed (dimensions in Figure 13A,B) with Magics software (EnvisionTEC, Germany), then sliced into layers at a slice thickness of 80% of the inner needle diameter (ID). In accordance with the manufacturer's guidelines (3D Bioplotter® RP, EnvisionTEC), a slice thickness of 0.32 mm was applied to a stainless-steel needle 0.4 mm in diameter (ID). Before adding 2.5 g of PCL (MW 45 kDa, melting temperature = 60 °C, Sigma-Aldrich) granules to the cartridge, it was preheated to a preheating temperature of 110°C. The PCL was then kept constant at this temperature during the entire printing time. Chambers were extruded at the predefined designs.

3.2.13 *In vivo* mice models

The study was approved by the Norwegian Food Safety Authority (NFSA) (FOTS ID 22627 and FOTS ID 2006400). A total of 11 NSG mice were used for this study. These mice are immunocompromised and lack B and T lymphocytes, as well as natural killer cells. The differentiated iPSCs were both injected subcutaneously within the pre-implanted PCL chambers (6 mice, Figure 13A), as well as implanted as a mucosal sheet after being pre-assembled *in vitro* in 3D OT models seven days prior to xenotransplantation and then glued to the lower edge of the PCL chambers with tissue glue Histoacryl® (5mice, Figure 13B). The chambers and tissues underneath were harvested after 2 more weeks.



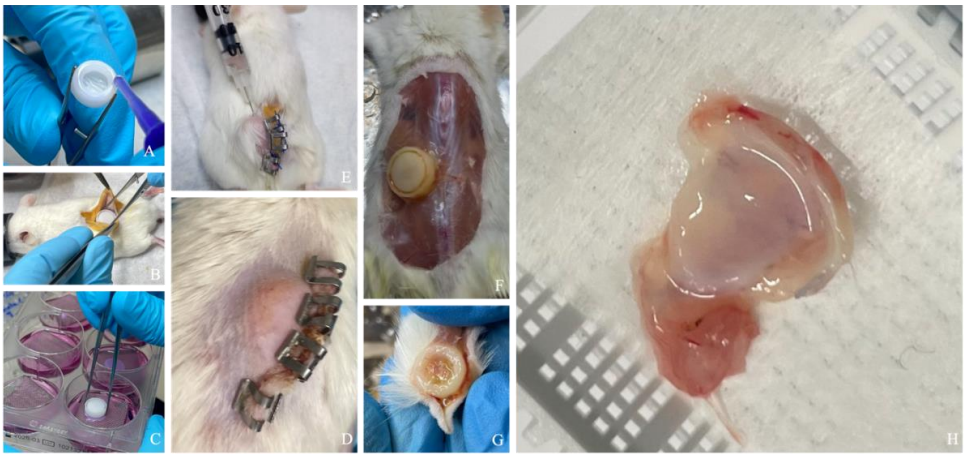


Figure 13. *In vivo* mice models. A) PCL chambers were glued using Histoacryl® either directly on the back of the mice (B) or were glued to the 3D OTs (C). After the chambers were glued to the mice in both the models the pockets were sutured and secured with metal clams (D). In the first animal model the cells were injected under the chambers (E). F shows how the chambers looked on the back of the mice at the day they were harvested. G and H show the macroscopic image of how the chambers and the mucosal sheets looked the day they were harvested and placed in cassettes for processing.

3.2.14 *In situ* hybridization (ISH)

To identify the transplanted human cells, ISH for the human specific repetitive Alu sequence was used. ISH was performed by RNAscope 2.5 High-Definition Brown Assay according to the manufacturer's instructions (all from Advanced Cell Diagnostics, Newark, CA, United States). Briefly, the tissue sections were baked at 60°C for 1 h followed by deparaffinization in 100% xylene and twice for 5 min each and then two times in 100% ethanol. The slides were then treated with an endogenous peroxidase-blocking reagent, for 15 min by boiling in target retrieval buffer and then treated with protease buffer for 30 min at 40°C. The slides were then incubated with the target Alu probe for 2 h at 40°C, followed by signal amplification. For colorimetric detection, 3,3′- Diaminobenzidine (DAB) was used for 5 min at RT followed by counterstaining with hematoxylin for 5 min. A peptidylprolyl isomerase B (PPIB) Positive Control Probe was used to validate the assay.

3.2.15 Statistical analysis

The statistical analysis was performed using the Wilcoxon paired test with a level of significance set at 5% (SPSS 11.0) in **Paper I.**

One-way ANOVA was used to compare the expression of different markers in the isolated cells. All statistical analyses were performed using the statistical package IBM SPSS version 15 (IBM, USA), considering *p* values less than 0.01 statistically significant in **Paper II.**

One-way ANOVA analysis with Tukey's test for multiple comparisons was used to compare the expression of pluripotency and epithelial markers in the differentiated cells. All statistical analysis was performed using GraphPad prism 9, considering *p* values less than 0.05 statistically significant in **Paper IV**.

Overall considerations

Primary human cultures are a source of significant biological variations [163]. We consider that we have reduced variation in 3D cultures due to our tough primary cell isolating procedure. Our method for isolation of cells is based on a combination of enzymatic and mechanical treatment which is much tougher to the cells than the explant method. By using this tough isolation method, we usually obtain cells from fewer donors but during isolation procedure we selected more robust and homogenous cells, that usually propagate well and are giving rise to quite similar epithelium in 3D OT cultures. In addition, the experiments were planned and performed meticulously to limit the unwanted technical variations to reach reproducible conclusions. To reduce experimental variations, the regular practices included consistent seeding densities, coated plates, reproducible experimental setup, adequate number of replicates and appropriate controls and mycoplasma testing.

Data from assays using live cells were normalized to the cell number of the individual cell populations used as estimated by using trypan blue and Annexin V/PI staining. Data from end-point assays performed using cell lysates were normalized to total the protein content or total RNA content estimated spectrophotometrically.

To summarize, by employing adequate technical and biological replicates and appropriate normalization methods, we performed a robust assessment of the oral mucosa, oral epithelial differentiation, and differentiation of embryonic stem cells and iPSC. We were then able to draw overall conclusions of the study from the combination of different differentiation experiments and patient cohorts.

4. Summary of results

Paper I: Granulocyte macrophage-colony stimulating factor and keratinocyte growth factor control early stages of differentiation of oral epithelium

In this study we sought to identify fibroblast derived factors which are responsible for oral epithelial differentiation. The NOK and NOF were isolated from healthy volunteers and used to construct 3D OT cultures. The oral epithelium formed by growing primary NOK on simple collagen gels in absence of fibroblasts displayed a thin, undifferentiated epithelium with low cell proliferation. Presence of fibroblasts in the collagen matrix, either in direct contact with keratinocytes or at distance ('sandwich models - SW') induced a significant increase in epithelial thickness and cell proliferation. Analysis of conditioned medium from 3D gels populated with NOFs showed that NOFs were able to secrete significant amounts of HGF, KGF, GM-CSF and IL-1α when grown in 3D monocellular cultures *in vitro*. Both KGF and GM-CSF at concentrations higher than 1 ng/ml, either alone or in combination, significantly increased cell proliferation in the basal cell layer. EGF, TGFα, IL-1α, HGF or SCF did not alter significantly epithelial thickness or epithelial cell proliferation in 3D OT monocellular cultures of keratinocytes. The IHC for various differentiation markers showed a weak expression of CK13 and strong expression of β1 integrin and EGF-R in all the cell layers and no deposition of collagen IV at the interface of the epithelium and matrix in 3D cultures. When fibroblasts were present in direct contact or at a distance from NOK grown in 3D-OT, strong expression of CK13 was seen throughout the suprabasal cell layers, and a polarization to the basal cell layer of β1 integrin and EGF-R, as well as synthesis and deposition of collagen IV at the interface of the epithelium and matrix were observed. Addition of KGF (0.1-100 ng/ml) did not change the undifferentiated phenotype of the oral epithelium grown on simple collagen gels. Addition of GM-CSF (>1 ng/ml) induced the expression of CK13 in all suprabasal cell layers and polarization of β1 integrin to the basal cell layer. The admixture of GM-CSF and KGF (10 ng/ml each) induced, in addition, polarization of EGFR to the basal cell layer and a fine deposition of collagen IV at the epithelium-matrix interface. None of

the other growth factors tested in the study (EGF, TGFα, IL-1α, HGF, SCF) influenced, when added, the phenotype of the epithelium grown on simple collagen gels.

The 3D OT monocellular cultures of oral keratinocytes displayed TUNEL positive cells randomly distributed within the epithelium. There was no polarization of TUNEL positive cells to the superficial layer, suggesting that cells did not complete the terminal stages of epithelial differentiation in these cultures. Similar pattern of distribution of TUNEL positive cells was also observed in the 3D OT monocellular cultures of oral keratinocytes supplemented with GM-CSF alone or in combination with KGF. Polarization of TUNEL positive cells to the superficial cell layer was observed only when fibroblasts were present in the connective tissue equivalent, either in direct contact or at distance from the epithelium. Addition of an anti-GM-CSF antibody to the culture medium of fibroblast-containing cultures did not impair cell growth or the terminal differentiation of the reconstituted oral epithelium. None of the other added growth factors tested in the study (EGF, TGFα, IL-1α, HGF, SCF) influenced the distribution of TUNEL positive cells within the epithelium grown on simple collagen gels.

Overall, this data suggested that major aspects of oral epithelial differentiation are regulated by GM-CSF in combination with KGF, but its terminal stage is controlled by another yet unidentified fibroblast-derived soluble factor.

Paper II: Isolation and characterization of cells derived from human ERM

In this study we aimed to investigate whether cells isolated from ERM can be used as an alternative source of stem cells for regeneration of normal human oral mucosa. We isolated and characterized the cells derived from ERM surrounding extracted human wisdom teeth and compared them to cells derived from donor-matched normal human oral mucosa. The cells were characterized by immunohistochemistry and flow cytometry for the expression of epithelial markers (PanCK, ESA), mesenchymal cell marker PDGFRB, blood vessel marker CD31 and stem cell marker CD44. The cells with epithelial morphology were isolated from periodontium of cervical, middle, and apical parts of the root, but expressed a considerably lower percentage of ESA and

PanCK-positive cells than cells isolated from NHOM ($p < 0.001$). ERM cells expressed a significantly higher percentage of the stem cell related marker CD44 (cervical 92.93 ± 0.25%, middle 93.8 ± 0.26%, apical 94.36 ± 0.41%) than cells isolated from NHOM (27.8 ± 1.47%, $p < 0.001$). Oral mucosa was reconstructed in 3D OTs by growing the cells isolated from REM on top of collagen gels populated with fibroblasts. The cells isolated from both ERM and the NHOM expressed the epithelial markers (ESA and PanCK), to a certain degree PDGFR which is a marker for mesenchymal phenotype, but not CD31 which is an endothelial cell marker. The ERM cells formed a less differentiated epithelium in 3D organotypic cultures when compared to the epithelium formed by cells from NHOM but expressed PanCK and vimentin.

To summarize, epithelial cells could be isolated from the human periodontium and expanded in culture while expressing stemness and epithelial markers.

Paper III: Differentiation of mouse ESC into epithelial lineages for skin and oral mucosal regeneration

This study aimed to investigate if pluripotent ESC can be induced into epithelial lineages for generation of skin and oral mucosa sheets by exposure to matrix products of connective tissue cells. Feeder-independent mouse ESC, when grown in standard undifferentiating conditions of media containing LIF, formed tight colonies with high expression of SSEA-1 (38.5±3.2%) and no expression of PanCK (less than 1%). When LIF was removed, the ESC became scattered, flattened, and acquired a differentiated morphology, with very few tight colonies and reduced expression of ESC markers SSEA-1 and OCT-3/4. Though, the lack of staining with the PanCK antibody indicated still no differentiation into epithelial phenotypes. Further loss of tight colonies with scattering and flattening of cells with a further decrease in expression of SSEA-1 to 15.5±2.4% was observed with the addition of vitamin C. The percentage of the ESC cells expressing PanCK was less than 1% when grown in presence of BMP4 but rose to 1.7±0.4% with addition of vitamin C. The matrix formed by the ear-derived fibroblasts and the laminin-coated plates in combination with vitamin C were most effective in influencing epithelial differentiation of ESC. PanCK expression

significantly increased to 7.0±0.6% ($p<0.05\%$) when grown on laminin, to 5.3±0.7% ($p<0.05$) when ESCs were grown on oral fibroblasts-derived matrix, and to 8.6±1.3% ($p<0.05$) when ESCs were grown on ear fibroblasts-derived matrix. Keratinocytes derived from oral mucosa and ear skin were the controls and showed high expression (98-100%) of PanCK and low expression (1.7±0.5%) of the SSEA-1.

Both primed and non-primed ESC developed into two morphologically distinct cell populations when grown for 14 days in 3D-OT organotypic cultures on simple collagen gels. More marked differences in differentiation patterns were observed in 3D cultures after 21 days of culture. On 3D-OT gels populated with buccal fibroblasts, ESC primed on buccal fibroblasts fully differentiated into a parakeratinized stratified squamous epithelium, similar to normal buccal epithelium.

ESC primed by ear skin mouse fibroblasts and keratinocytes and further grown on 3D-OT gels populated with ear fibroblasts developed into a typical ortho-keratinized epidermis with structures similar to sebaceous glands.

To summarize, the results indicate that ESC can be a promising source of mucosal sheets which can be used in regenerative medicine.

Paper IV: Generating oral keratinocytes for regenerative therapy from iPSCs derived from normal human skin and oral fibroblasts.

In this study the skin and gingival fibroblast-derived iPSC were investigated for their potential to differentiate into keratinocytes to generate mucosal sheets for regeneration of oral mucosa. All the three protocols tested were able to differentiate the iPSC to keratinocytes. The most efficient protocol was the ECM-enriched protocol (protocol III) and this was chosen for differentiation of iPSC in xeno-free conditions as well. Further characterization using flow cytometry for expression of TRA-60, Oct3/4, Sox2 (pluripotency markers) and E-cadherin (epithelial marker). The keratinocytes differentiated from skin and gingival iPSC in xenogeneic conditions showed lower expression of TRA-60, Oct3/4, Sox2 and higher expression of E-cadherin as compared to the controls undifferentiated iPSC and NOK respectively. However, the

keratinocytes differentiated from skin and gingival iPSC in xeno-free conditions expressed a considerably lower percentage of E-cadherin and higher percentage of TRA-60, Oct3/4, Sox2 than cells in xenogeneic conditions, although not statistically significant. Gene expression analysis using RT-qPCR showed decrease in expression of stemness markers Sox2, Oct3/4, Nanog and increase in expression of epithelial markers cytokeratin 1,5,13,18,19 in both xeno-free and xenogeneic conditions, but to a lesser extent in xenogeneic conditions. Immunoblotting for PanCK was positive for cells in both xenogeneic and xeno-free conditions. Oral mucosa was reconstructed in 3D OTs by growing the differentiated iPSC in both FBS and PL on top of collagen gels populated with fibroblasts. The iPSCs differentiated in FBS-containing conditions formed a cohesive 5-6 multi-layered tissue on top of collagen gels (both those derived from skin and gingival fibroblasts), with a basal compartment with cells perpendicular on the collagen interface and a more superficial compartment with more flattened cells. Cells differentiated in PL formed also a multilayer tissue on top of collagen gels, which in some areas was even thicker (8-10 cell layers) but the tissue formed showed less distinct basal and superficial cell layers. Immunophenotyping of this tissue developed from differentiated iPSCs in FBS-containing conditions showed strong expression of PanCK, indicating differentiation towards stratified squamous epithelium by iPSCs differentiated in FBS (both gingival and skin fibroblast derived iPSCs). The tissues formed on top of the collagen gels by differentiated iPSCs in PL-containing conditions showed only patchy expression of PanCK, indicating a limited differentiation towards stratified squamous epithelium when iPSCs were differentiated in xeno-free conditions (both skin and gingival fibroblast derived iPScs). Differentiated gingival fibroblast-derived iPSCs gave rise to an epithelium that was negative for CK10 but positive for CK13 in the suprabasal layers both in FBS and PL conditions, suggesting differentiation of the epithelium formed in the 3D OTs on top of collagen gels towards an oral phenotype under oral fibroblast directionality. The expression was, however, weaker than in the 3D OT models reconstructed with NOK.

Vimentin was expressed, as expected by the fibroblasts in the collagen matrix but also by few cells in the basal compartment of the tissues regenerated using differentiated iPSCs, similar to the epithelial sheets reconstructed from NOK.

To summarize, epithelial cells could be differentiated from iPSC in both FBS and PL and when cultured in 3D OT cultures expressed epithelial markers and were able to form an oral epithelium under the influence of oral fibroblasts. Based on the results from **Paper I**, GM-SCF (10 ng/ml) was also added to some of the 3D OT cultures. No significant differences were observed in differentiation of the epithelium-like tissue formed by differentiated iPSCs with or without GM-CSF. However, in presence of GM-CSF a thicker epithelium-like tissue was formed on top of the collagen gels, suggesting a higher proliferation of the epithelial-like tissue generated by differentiated iPSCs in presence of GM-CSF, especially when grown in PL conditions.

Viability of *in vivo* xenotransplanted 3D OTs in a mice model as a proof of principle for clinical use of the oral mucosal sheets derived from differentiated iPSCs

As a proof of principle, we first used an animal model based on previous literature [164] in which differentiated iPSCs admixed with fibroblasts were injected into the inner space of the PCL chambers as explained in Figure 13A. The results of that experiment showed inconsistent formation of a single epithelial layer only even in the control mice injected with admixture of NOK and NOF (confirmed with pancytokeratin staining and hAlu ISH – data not shown).

The model has been adapted and improved to xenotransplant differentiated iPSCs already assembled in 3D OT cultures prior to xenotransplantation as explained in Materials and methods (Figure 13B).

For this model, we first constructed 3D OTs using differentiated iPSCs on top of NOF-populated collagen gels and xenoimplanted them in five NGS mice using the PCL chambers (Figure 13). As control of the method, we constructed 3D OT models using NOK and NOF isolated from normal adult human oral mucosa and xenoimplanted them after 7 days of *in vitro* culture on the back of the NGS mice, similar to the other 3D OT cultures (Figure 13). Both gingival and skin fibroblast derived iPSCs grown in

FBS formed a stratified squamous epithelium – like structures facing the inner side of the PCL chambers (Figure 14). Immunophenotyping confirmed epithelial differentiation by showing strong expression of PanCK. Particularly G-iPSC gave rise to an epithelium that was negative for CK10 but expressed CK13, indicating epithelial differentiation towards an oral phenotype (Figure 14).

When differentiated and grown in xeno-free conditions (PL), both gingival and skin fibroblast derived iPSCs formed a stratified multi-layered epithelial-like tissue which was not that cohesive and was more basaloid-like throughout all its thickness, indicating less differentiation towards stratified squamous epithelial phenotype in these conditions. However, the epithelial-like tissues generated from iPSCs differentiated in xeno-free conditions showed expression of PanCK and CK13, although weaker than in FBS containing conditions.

When compared to the epithelium generated by xenotransplanted NOK-containing 3D OTs, the epithelium generated by differentiated iPSCs was less differentiated and expressed weaker CK13, while PanCK expression was comparable for the FBS-containing conditions.

Vimentin was expressed, as expected by the human fibroblasts present in the collagen matrices of the xenotransplanted 3D OTs but also by the cells in the basal compartment of the tissues regenerated using differentiated iPSCs, similar to the epithelial sheets reconstructed from NOK.

The human origin of the tissues analysed was proven by the detection of hALU by ISH (Figure 14).

Taken together, the results from the *in vivo* model corroborate well with the results obtained from the *in vitro* 3D OT tests.

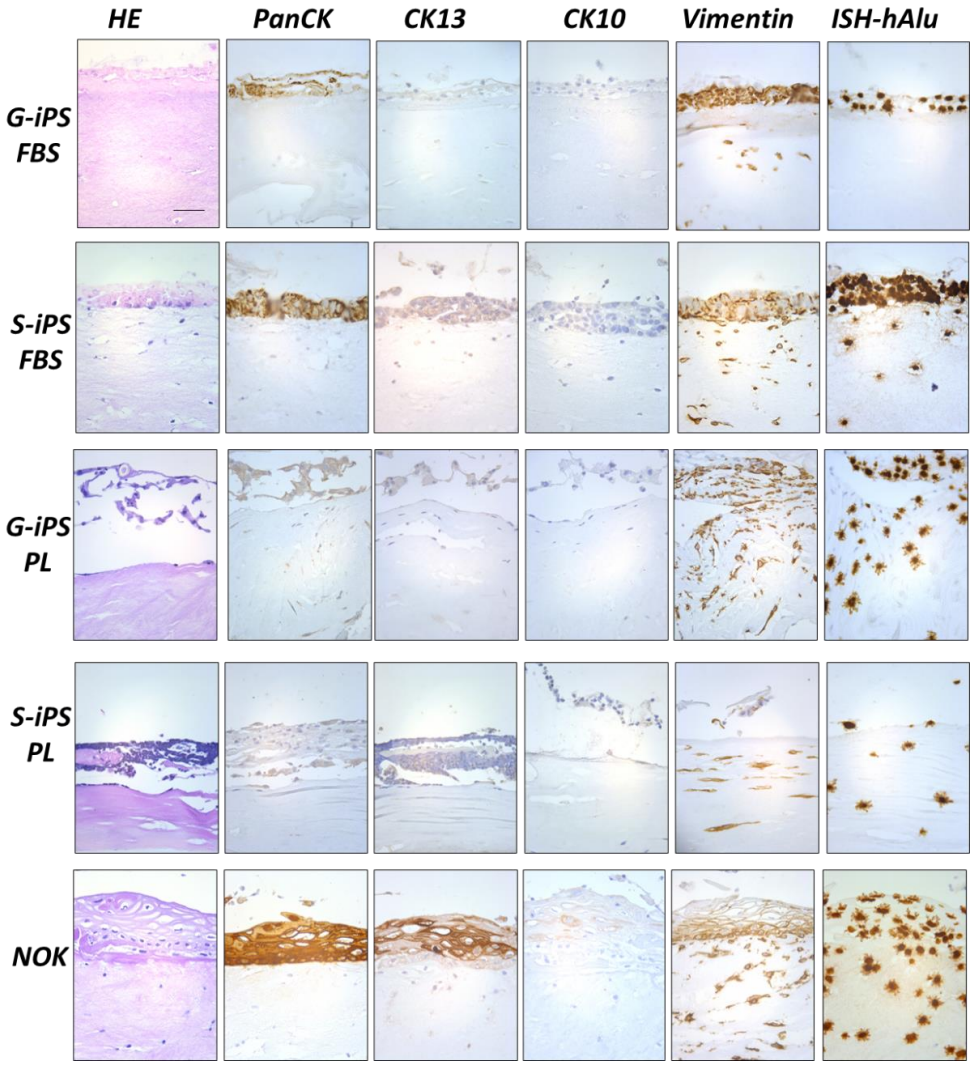


Figure 14. Histological images (haematoxylin-eosin) and representative immunostaining of 3D OT cultures of cells differentiated from gingival and skin fibroblast derived iPSC (G-iPSCs and S-iPSCs) in FBS and PL seeded on top of human oral fibroblast populated collagen matrices for 7 days and then implanted in NGS mice using PCL chambers for 14 more days.

5. Discussion

The dynamic and wet environment of the oral cavity makes the treatment of oral mucosal defects challenging, unlike other external epidermal lesions. In addition, the continuous facial movements during chewing, swallowing and speech, as well as facial expressions cause movements of tongue and oral mucosa that makes local treatment ineffective due to short retention on the mucosal surfaces [165-167]. Various dressing materials to cover the oral wounds have been cited in the literature, but each material comes with its pros and cons [168]. The mucosal grafts are the ideal grafts, but limited availability and donor site morbidity limit their use [169]. Skin grafts have also been used as a biological dressing, but they often fail to retain oral mucosa texture [170]. Recently, *in vitro* culture of skin and oral keratinocytes has been used to generate autologous grafts for these defects. They are a good alternative to the above-mentioned grafts, but the use is still limited due to elective surgeries, as well as the cost of the procedure [9, 168].

5.1 Role of KGF and GM-CSF in proliferation and differentiation of epithelial cells

To generate mucosal grafts with a phenotype as close as possible to the native mucosa and using standardized and xeno-free conditions, one first needs to know what factors are responsible for generating that phenotype. Therefore, this study aimed first at identifying the optimal conditions, *i.e.*, well defined growth and differentiation factors necessary for obtaining *in vitro* a fully differentiated oral mucosa epithelial phenotype. At the time of starting this study, it was known that KGF was able to induce cell proliferation and played a vital role in wound healing [139]. Previous studies have shown that for activation of fibroblasts in wound healing, upregulation of KGF is necessary [171]. Upregulation of KGF has also been observed to be induced by the presence of keratinocytes in the co-culture [172, 173].

GM-CSF is a hematopoietic growth factor, but it was also shown to regulate growth and differentiation of dermal keratinocytes and to play an important role in wound healing [174, 175]. KGF and GM-CSF receptors have been shown to be expressed in

keratinocytes by paracrine regulation mediated by KGF and GM-CSF secreted by fibroblasts, or by autocrine regulation mediated by TGF-α [176].

Studies on skin models have shown that IL-1 produced by keratinocytes induce the fibroblast production of KGF, GM-CSF, TGFα, IL-6, IL-8, IL-1. In return, KGF and GM-CSF promote the proliferation and differentiation of the keratinocytes, in a positive feedback loop. The production of IL-1 by fibroblasts may amplify the production of KGF and GM-CSF [177]. From all the growth factors which were tested in this study only GM-CSF, alone or in combination with KGF had a significant effect on differentiation of the oral epithelium.

5.2 Terminal differentiation of oral epithelium *in vitro* by underlying fibroblasts in 3D OT

The effect of fibroblasts on proliferation of the dermal keratinocytes has been previously investigated and demonstrated by several studies [178-180]. The survival of keratinocytes is enhanced by reduced apoptosis, decreased expression of Bcl2 and upregulation of p53 in the presence of fibroblasts [178, 181, 182]. The role of dermal fibroblasts in proliferation, migration and differentiation of keratinocytes has been studied in detail in both *in vivo* and *in vitro* models [183, 184]. The epidermal homeostasis is mainly mediated by the ECM, growth factors and cytokines [185, 186].

Studies have suggested that the diffusible factors secreted by fibroblasts are sufficient for full epidermal differentiation [182]. Formation and maintenance of mature oral epithelium rely also on a tightly balanced process of keratinocyte proliferation and terminal differentiation [187], but the knowledge about the specific factors involved is more limited than for skin. Our research group has previously developed standardized 3D OT models of the human oral mucosa [188] and has also shown that fibroblasts are essential for the differentiation of oral epithelium [139]. The results of this study further show that the fibroblast-derived diffusible factors can fully restore the differentiated phenotype of *in vitro* oral epithelium, including the fine-tuned terminal stage of epithelial differentiation.

In contrast to reported studies from skin models proving that a combination if KGF and GM-CSF can substitute for the dermal fibroblasts and provide sufficient support for both growth and terminal differentiation of skin keratinocyte in their absence [189-191], our current study shows that the final stages of oral epithelial maturation could not be restored by KGF and GM-CSF only. These differences between skin and oral mucosa morphogenesis might be due to the fact that oral mucosal fibroblasts and adult skin fibroblasts have different origin (the former originates from the neural crest and the latter from the mesoderm) and gene expressions, and consequently, different phenotypes and functions [192]. Oral fibroblasts were proven to express higher levels of KGF and to accelerate much faster the collagen gel contraction than dermal stromal cells [193, 194]. Our data presented here, based on the analysis of conditioned medium collected from 3D monocellular cultures containing NOF only embedded in the collagen matrix, corroborate with previous literature [194-196] by showing that oral fibroblasts synthesize considerable amounts of KGF, GM-CSF and HGF, although oral keratinocytes have also been proven to synthesize GM-CSF [197].

That the mesenchymal cell source has a significant influence on the thickness and ultrastructure of the epithelium has been previously shown [198]. Moreover, cytokeratin expression of the epithelial component was also proven to be strongly influenced by the origin of fibroblasts [199]. The data resulting from this work showed that the fibroblasts derived diffusible factors differentiated the oral epithelium *in vitro*.

The data presented in this study indicate that in contrast to skin, other soluble factors than KGF and GM-CSF released from fibroblasts exert the final tuning of oral epithelial differentiation. In support for this conclusion comes also the observation that addition of neutralizing antibodies against human GM-CSF, previously shown to reduced keratinocyte proliferation and differentiation in skin models [200], did not impair cell proliferation or differentiation in our oral mucosa models. Taken together, the results of this study indicate that major aspects of oral epithelial differentiation are regulated by GM-CSF in combination with KGF, but its terminal stage is controlled by another yet unidentified fibroblast-derived diffusible factor.

5.3 ERM represents a valuable source of stem cells that can be differentiated into oral epithelium

PDL is a highly specialized connective tissue which connects the cementum and the alveolar bone [201]. PDL contains heterogeneous cell populations such as fibroblasts, osteoblasts, PDLSCs, and ERM [202].

The existence of epithelial cell populations in the PDL was first reported by Serres in 1817 [203]. In 1884, Malassez confirmed the presence of epithelial components in PDL and also the fact that ERM are the remains of the Hertwig's epithelial sheath [204]. Different dynamic states of the ERM have been reported in the past studies [205, 206]. Morphologically, ERM are a network of islands and cords of epithelial cells which remain in the mesenchymal matrices throughout the post-natal life whereas the epithelial cells which are present in the other tissues are separated by basal lamina from the underlying connective tissue [105, 207]. It has been shown that ERM plays an important role in maintaining the homeostasis of the periodontium [208] and in prevention of root resorption [209]. Additionally, ERM cells play a functional role in maintenance of periodontal ligament space [210]. Some studies also suggest that ERM plays a role in formation and repair of enamel and cementum [211, 212].

Since the isolation of the stem cells from dental pulp, existence of post-embryonic stem cells in the periodontal tissue has been also suggested [213]. Presence of multipotent stem cells in PDL was reported in 2004 [214]. Postnatal stem cells are known to reside as subpopulations in the tissues and when stimulated undergo proliferation and migration and regenerate the damaged tissues [215, 216].

CD44 is an adhesive molecule that binds with extracellular matrices such as hyaluronic acid and is strongly involved in lymphocyte homing, lymphocyte activation, cell-to-cell adhesion, cell-to-matrix adhesion, and cell movement, as well as cancer cell proliferation and metastasis. It has also been acknowledged as a stem cell marker for in various types of solid cancers, including oral squamous cell carcinoma [217]. CD44 expression was also described in various cell lines, including hematopoietic cells, fibroblasts, epithelial cells, vascular endothelial cells, muscle cells, and neuroglial cells and is either expressed or absent in the differentiation and proliferation of each of these

cell lines. In squamous epithelial mucosa, CD44 expression is enhanced at the basal cell layer which is the proliferating compartment containing the adult epithelial stem cells, while it is weakly expressed or not present on the more superficial layers [218]. Moreover, CD44 may be related to the differentiation and proliferation of hematopoietic stem cells and B cells in the dental pulp [219, 220]. It is significant that our results of FACS analysis revealed that the CD44-positive cell ratio was much higher in cells obtained from PDL than in the oral mucosa.

The results of this study show that when grown in 3D organotypic cultures and in collagen gels the cells derived from PDL formed a less differentiated epithelium that expressed weaker PanCK and stronger vimentin than the matched epithelial cells isolated from normal mucosa, indicating that these cells have a less epithelial phenotype and a more mesenchymal phenotype. That might indicate that those cells are less differentiated and more EMT than the cells derived from NHOM. Accordingly, this might be the reason that they did not form a well-differentiated and keratinized epithelium in 3D organotypic cultures, such as the epithelium formed by the cells derived from NHOM.

When grown inside the gels, the cells derived from NHOM grew more clustered, in groups, while the epithelial cells derived from ERM grew alone in the matrix. This again might indicate that the cells from NHOM are probably more differentiated and express more epithelial cell-to-cell adhesion molecules, while cells derived from ERM are less differentiated.

Our results are in accordance with the results published in 2016 by Tsunematsu et al. [209]. They have isolated odontogenic epithelial cells with epithelial marker-positive and mesenchymal marker-negative features from ERMs in human PDL and reported that they have stem cell-like characteristics. The findings we present here bring new information about the extent of the stemness of the cells derived from ERMs compared to the epithelial cells derived from NHOM.

We anticipate that the cells isolated from ERM will be investigated in more detail in the future for their possible involvement in cyst formation, by developing an experimental model for radicular cyst formation. This model will also provide a

valuable experimental biological system for testing of novel, alternative ways of treatment for radicular cysts.

5.4 Differentiation of ESC into epithelial lineages

It is a well-established method now to culture both murine and human ESC and to propagate them indefinitely in an undifferentiated state without loss of differentiation capacity, both *in vivo* and *in vitro* [221]. ESCs represent a possibly inexhaustible source of any somatic cell type [63].

We show in this study that vitamin C alone was able to enhance the number of cells expressing epithelial markers, but the extracellular matrix synthesized by either oral/skin fibroblasts or keratinocytes was required to induce expression of a stratified epithelial phenotype. Vitamin C plays a critical role in regeneration and wound healing [222]. It regulates the differentiation of skin keratinocytes by regulating the function of AP-1 complexes [223]. A study by Guenou et al. (2009) has shown that hESC induced with vitamin C and BMP4 could be differentiated into cytokeratin 14 positive cells [224]. It is a well-known fact that LIF plays a critical role in maintaining the pluripotency of the embryonic stem cells *in vitro* [225, 226]. This study confirms that with LIF removal in both 2D and 3D cultures, the ESC were more differentiated.

5.5 Differentiation of ESC into epithelial lineages by influence of the underlying ECM

During the process of embryogenesis, ESCs exist in ECMs which regulates migration, proliferation, and differentiation of the ESC. Hence, for optimal ESC differentiation *in vitro* conditions should mimic the *in vivo* microenvironment. Three dimensional culture for ESC differentiation is therefore a valuable tool [227].

Coraux et al. suggested already in 2003 that once primed, ESC can differentiate into epithelial tissues, given sufficient time and continuous instruction from vital fibroblasts [142]. However, to our knowledge, their interesting work has not been replicated by others. The findings presented here support their suggestion. Our data indicate that both fibroblast and keratinocyte-produced matrix, as well as commercially available laminin

enhance the induction of a keratinocyte phenotype. In view of reports suggesting that epithelial differentiation of stem cells occurs within an epithelial context, we also included an additional condition of ESC growth on matrix produced by keratinocytes [228]. Of interest, this was the only condition that generated mucous-producing cells, but it was insufficient to generate a fully differentiated epithelium, with a superficial layer of keratin. Further 3D culture with vital fibroblast instruction for 22 days was necessary for further differentiation into mature, regionally relevant epithelial structures. We showed here, by staining with different keratin markers that a specific keratin pattern can be induced in ESC grown on an extracellular substrate, depending on the region of origin of the cells that generated that substrate. The differences in tissue patterns observed indicate that this may be valuable in generating epithelial tissues with defined regional specificities.

This is to be expected since ESC have been shown to have the ability to differentiate into any type of cell when provided proper induction *in vitro* [229-231]. This characteristic ability of ESC has been used in regenerative medicine for more than a decade now, but the problem is the ethical considerations with the use of human embryonic stem cells. In this study we used the mouse embryonic stem cells, and the results indicate that ESC can be a promising source of skin/mucosal sheets which can be used in regenerative medicine [232], and that regional fibroblasts are essential for the full epithelial maturation. To summarize, the results indicate that ESC can be a

5.6 Differentiated iPSCs reprogrammed from oral and skin fibroblasts in both xenogeneic and xenofree conditions as a novel cell source for regeneration of oral mucosa

Tissue engineering implies providing progenitor cells or cell-derived products to damaged tissues or organs to restore their structure function. In recent years, the use of stem cells has significantly changed the outlook of tissue engineering with their ability of self-renewal and differentiation [233, 234]. The advances of the stem cell biology, combined with tissue engineering, have unlocked new possibilities in the field of regenerative medicine [235]. Since the ground-breaking findings of iPSC by Takahashi

and Yamanaka, a new direction of research in regenerative medicine has been instigated. The use of iPSC is advantageous as they are reprogrammed from adult somatic cells, hence there are no ethical dilemmas. Also, the somatic cells for reprogramming to iPSC are easily harvested from the tissues avoiding the need for invasive surgeries. Lastly, the use of iPSC derived from autologous adult cells evade the immune response and improve the *in vivo* survival [235].

To date, several groups have reported protocols to differentiate mouse ESC and human iPSC to epidermal keratinocytes (cytokeratin14-positive). Metallo et al. [236] used retinoic acid and BMP4 on embryoid bodies in mono-layer culture on collagen IV-coated surface without feeder cells to induce keratinocyte differentiation from human ESC. Kawasaki et al [237] in 2000 developed a method using feeder cells to promote neural differentiation of mouse ESC, and showed that BMP4 addition promotes the initiation of epidermal determination from neuronal ectoderm. Lian et al. [238] in 2012 described a method using small molecule inhibitors of Src family kinases to derive simple epithelial progenitors, which further differentiate into epidermal keratinocytes in serum-free conditions. However, none of these methods have generated epidermal keratinocyte stem cells (cytokeratin 14 and cytokeratin 15 double positive) with a proliferative capacity of more than two population doublings or long-term engraftability.

Our results show that we were able to differentiate our gingival and skin iPSC to epithelial lineages using all the protocols even though we slightly modified the protocols. There have been several studies in which skin iPSCs have been differentiated to keratinocytes [144, 145], but to the best of our knowledge there are no published studies showing differentiation of oral iPSC into oral keratinocytes. But the currently available protocols need more optimization since the protocols used were for differentiation of the skin iPSCs.

Up to now, the use of iPSCs has been used in preclinical studies for treatment of burns and other skin disorders for regenerative therapy and had shown very promising results with a tremendous impact in the field of dermatology for the past decade [239-242]. However, the regeneration of oral mucosa defects has been lagging. The present study

is one of the first where gingival iPSCs were differentiated into oral keratinocytes and employed to produce 3D mucosal sheets *in vitro*.

There is still limited knowledge about the intraoral wound healing post trauma leading to lack of efficient regenerative therapies [243]. Impaired wound healing renders the oral cavity more susceptible to various challenges such as prolonged inflammation and other postoperative complications. Tissue regeneration involving biomaterials along with cells and growth factors has a great potential for better healing of oral mucosal lesions [244].

5.7 *In vivo* models for regeneration of oral mucosa

As a proof of principle, we have devised and optimized an *in vivo* rodent model where with the help of PCL chambers we were able to implant and maintain 3D OTs in the mice. The 3D OT models are very adaptive and cells can be cultured in defined matrix composition and/or mechanical properties that can be modulated to investigate the relationship between cells and the underlying ECM [245]. They were also proved to be more robust for xenotransplantation of differentiated iPSCs and NOK than the injection of admixture of differentiated iPSCs or NOK and NOF.

As mentioned, the results from the *in vivo* model corroborated well with the results obtained from the *in vitro* 3D OT tests and suggest that differentiation of oral or skin fibroblast-derived iPSCs into epithelial lineages for oral mucosal regeneration is a promising avenue for generation of clinical grade oral mucosal sheets. Nevertheless, further work is necessary for improving the robustness and efficiency of the methodology established here as a proof of principle.

Conclusions

The main conclusion of the thesis based on the results of the four studies are:

1. GM-CSF, alone or in combination with KGF has a significant effect in the differentiation of the oral epithelium, however the terminal differentiation of NHOM is directed by underlying fibroblasts through yet unknown factors.
2. The cells derived from the ERM were isolated and characterized. The oral mucosal sheets constructed using these cells showed a certain differentiation towards oral epithelium but were less differentiated when compared to the oral mucosal sheets generated by NOK cells derived from NHOM.
3. ESC were differentiated into epithelial lineages in presence of vitamin C after removal of LIF. Primed ESCs were able differentiate into oral epithelial tissues, given sufficient time and continuous instruction from vital oral fibroblasts.
4. iPSC derived from gingival and skin adult fibroblasts were differentiated towards keratinocytes in both xenogeneic and xenofree conditions and were able to generate oral mucosal sheets under the influence of vital oral fibroblasts. Nevertheless, the oral mucosal sheets were less differentiated in xenofree conditions as compared to those generated in xenogeneic conditions and by NOK cells from NHOM, indicating the need for further work for improvement of this method.

Overall, the work presented in this thesis provides new insights for novel techniques for regeneration of oral mucosa with the use of alternative sources of cells.

6. Future perspectives

Several questions still remain unanswered with respect to alternative sources and methods for regeneration of oral mucosa, and further studies are required to arrive at conclusive results, particularly when it comes to *in vivo* oral mucosal regeneration and generation of clinical grade oral mucosal sheets. Some key future study directions are:

1. Identification of the yet unidentified diffusible factor(s) secreted by the oral fibroblasts in 3D models which are responsible for terminal oral epithelial differentiation.
2. Deeper investigation and optimization of a more rigorous enrichment of epithelial stem-like cell populations in ERM, as an alternative source of oral epithelial cells.
3. Improvement of the xeno-free method for generation of clinical grade oral mucosal sheets by use of iPSCs.
4. Safety in vivo tests for the use of iPScs for generation of clinical grade oral mucosal sheets.
5. Further study of the mechanisms of oral epithelial differentiation of iPSCs by growth factors and ECM secreted by the oral fibroblasts.

References

1. Eckardt, A.M., et al., *Reconstruction of oral mucosal defects using the nasolabial flap: clinical experience with 22 patients.* Head Neck Oncol, 2011. **3**: p. 28.
2. Karamanos, E., et al., *Wound Healing in Plastic Surgery: Does Age Matter? An American College of Surgeons National Surgical Quality Improvement Program Study.* Plastic and Reconstructive Surgery, 2015. **135**(3): p. 876-881.
3. Futran, N.D., *Microvascular Free-Flap Reconstruction in the Head and Neck.* Jama, 2000. **284**(14).
4. Izumi, K., H. Kato, and S.E. Feinberg, *Tissue engineered oral mucosa*, in *Stem cell biology and tissue engineering in dental sciences*. 2015, Elsevier. p. 721-731.
5. Kato, H., et al., *Fabrication of Large Size Ex Vivo-Produced Oral Mucosal Equivalents for Clinical Application.* Tissue Engineering Part C: Methods, 2015. **21**(9): p. 872-880.
6. Shi, L.-J., et al., *Application of Acellular Dermal Matrix in Reconstruction of Oral Mucosal Defects in 36 Cases.* Journal of Oral and Maxillofacial Surgery, 2012. **70**(11): p. e586-e591.
7. Zang, J., et al., *Comparison of xenogeneic acellular dermal matrix and skin grafts in reconstruction of postoperative defects of hypopharyngeal cancer.* Medicine, 2020. **99**(9).
8. Rastogi, S., M. Modi, and B. Sathian, *The efficacy of collagen membrane as a biodegradable wound dressing material for surgical defects of oral mucosa: a prospective study.* J Oral Maxillofac Surg, 2009. **67**(8): p. 1600-6.
9. Izumi, K. and S.E. Feinberg, *Skin and oral mucosal substitutes.* Oral Maxillofac Surg Clin North Am, 2002. **14**(1): p. 61-71.
10. Hanasono, M.M., *Reconstructive Surgery for Head and Neck Cancer Patients.* Advances in Medicine, 2014. **2014**: p. 1-28.
11. Squier, C.A. and M.J. Kremer, *Biology of oral mucosa and esophagus.* J Natl Cancer Inst Monogr, 2001(29): p. 7-15.
12. Cruchley, A.T. and L.A. Bergmeier, *Structure and Functions of the Oral Mucosa*, in *Oral Mucosa in Health and Disease: A Concise Handbook*, L.A. Bergmeier, Editor. 2018, Springer International Publishing: Cham. p. 1-18.
13. Costea, D.E., et al., *Cancer stem cells - new and potentially important targets for the therapy of oral squamous cell carcinoma.* Oral Dis, 2006. **12**(5): p. 443-54.
14. Squier, C. and K.A. Brogden, *Human Oral Mucosa*. 2011.
15. *Oral Mucosa*, in *Ten Cate's Oral Histology*. 2013. p. 278-310.
16. Presland, R.B., et al., *Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris.* J Invest Dermatol, 2000. **115**(6): p. 1072-81.
17. Garant, P.R. and P. Garant, *Oral cells and tissues*. Vol. 35. 2003: Quintessence Publishing Company Chicago, IL, USA:.

18. Lindberg, K. and J.G. Rheinwald, *Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts.* Differentiation, 1990. **45**(3): p. 230-41.
19. Costea, D.E., et al., *Epithelial stem cells and malignancy.* Journal of Anatomy, 2008. **213**(1): p. 45-51.
20. Arda, O., N. Göksügür, and Y. Tüzün, *Basic histological structure and functions of facial skin.* Clinics in Dermatology, 2014. **32**(1): p. 3-13.
21. Griffin, M.F., et al., *Understanding Scarring in the Oral Mucosa.* Advances in Wound Care, 2021.
22. Diaz-del Consuelo, I., et al., *Comparison of the lipid composition of porcine buccal and esophageal permeability barriers.* Archives of Oral Biology, 2005. **50**(12): p. 981-987.
23. Slominski, A.T., et al., *Intrinsic Differences between Oral and Skin Keratinocytes.* PLoS ONE, 2014. **9**(9).
24. Kutz, M., *Biomedical engineering and design handbook*. Vol. 1. 2009: McGraw-Hill New York, NY, USA:.
25. Goldberg, V.M. and S. Stevenson, *Natural history of autografts and allografts.* Clin Orthop Relat Res, 1987(225): p. 7-16.
26. Aghaloo, T.L. and D. Hadaya, *Basic Principles of Bioengineering and Regeneration.* Oral and Maxillofacial Surgery Clinics of North America, 2017. **29**(1): p. 1-7.
27. Masson-Meyers, D.S., L.E. Bertassoni, and L. Tayebi, *Oral mucosa equivalents, prevascularization approaches, and potential applications.* Connective Tissue Research, 2022: p. 1-16.
28. Meyer, U., et al., *Fundamentals of tissue engineering and regenerative medicine*. 2009: Springer.
29. Perez-Amodio, S., et al., *Hypoxia preconditioning of tissue-engineered mucosa enhances its angiogenic capacity in vitro.* Tissue Engineering Part A, 2011. **17**(11-12): p. 1583-1593.
30. Föhn, M. and H. Bannasch, *Artificial Skin*, in *Tissue Engineering*. 2007. p. 167-182.
31. Damanhuri, M., J. Boyle, and S. Enoch, *Advances in tissue-engineered skin substitutes.* Wounds Int, 2011. **2**(1): p. 27-34.
32. Papini, R., *Management of burn injuries of various depths.* Bmj, 2004. **329**(7458): p. 158-160.
33. Herman, A.R., *The history of skin grafts.* Journal of drugs in dermatology: JDD, 2002. **1**(3): p. 298-301.
34. Boyce, S.T., *Design principles for composition and performance of cultured skin substitutes.* Burns, 2001. **27**(5): p. 523-533.
35. Kriegebaum, U., et al., *Tissue engineering of human oral mucosa on different scaffolds: in vitro experiments as a basis for clinical applications.* Oral Surg Oral Med Oral Pathol Oral Radiol, 2012. **114**(5 Suppl): p. S190-8.
36. Llames, S., et al., *Tissue-Engineered Oral Mucosa for Mucosal Reconstruction in a Pediatric Patient with Hemifacial Microsomia and Ankyloglossia.* The Cleft Palate-Craniofacial Journal, 2014. **51**(2): p. 246-251.

37. Heller, M., et al., *The impact of intercellular communication for the generation of complex multicellular prevascularized tissue equivalents.* Journal of Biomedical Materials Research Part A, 2019. **108**(3): p. 734-748.
38. Nishita, M., et al., *Ror2 signaling regulates Golgi structure and transport through IFT20 for tumor invasiveness.* Scientific Reports, 2017. **7**(1).
39. Odet, S., et al., *Surgical Application of Human Amniotic Membrane and Amnion-Chorion Membrane in the Oral Cavity and Efficacy Evaluation: Corollary With Ophthalmological and Wound Healing Experiences.* Frontiers in Bioengineering and Biotechnology, 2021. **9**.
40. Bodner, L. and N. Grossman, *Autologous cultured mucosal graft to cover large intraoral mucosal defects: A clinical study.* Journal of Oral and Maxillofacial Surgery, 2003. **61**(2): p. 169-173.
41. Nishiyama, K., et al., *Construction of Vascularized Oral Mucosa Equivalents Using a Layer-by-Layer Cell Coating Technology.* Tissue Engineering Part C: Methods, 2019. **25**(5): p. 262-275.
42. Kinikoglu, F.B., *Tissue engineering of full-thickness human oral mucosa*. 2010, Lyon 1.
43. Moharamzadeh, K., *Oral mucosa tissue engineering*, in *Biomaterials for Oral and Dental Tissue Engineering*. 2017. p. 223-244.
44. Tra, W.M.W., et al., *Tissue-engineered mucosa is a suitable model to quantify the acute biological effects of ionizing radiation.* International Journal of Oral and Maxillofacial Surgery, 2013. **42**(8): p. 939-948.
45. Langer, R. and J.P. Vacanti, *Tissue engineering.* Science, 1993. **260**(5110): p. 920-6.
46. *Origins.* Tissue Engineering Part A, 2009. **15**(7): p. 1449-1450.
47. Lanza, R., et al., *Principles of tissue engineering*. 2020: Academic press.
48. Berthiaume, F., T.J. Maguire, and M.L. Yarmush, *Tissue Engineering and Regenerative Medicine: History, Progress, and Challenges.* Annual Review of Chemical and Biomolecular Engineering, 2011. **2**(1): p. 403-430.
49. Rheinwald, J.G. and H. Green, *Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes.* Nature, 1977. **265**(5593): p. 421-4.
50. O'Connor, N., et al., *Grafting of Burns with Cultured Epithelium Prepared from Autologous Epidermal Cells.* The Lancet, 1981. **317**(8211): p. 75-78.
51. Izumi, K., J. Song, and S.E. Feinberg, *Development of a tissue-engineered human oral mucosa: from the bench to the bed side.* Cells Tissues Organs, 2004. **176**(1-3): p. 134-52.
52. Yoshizawa, M., et al., *Keratinocytes of tissue-engineered human oral mucosa promote re-epithelialization after intraoral grafting in athymic mice.* J Oral Maxillofac Surg, 2012. **70**(5): p. 1199-214.
53. Bissell, M.J., H.G. Hall, and G. Parry, *How does the extracellular matrix direct gene expression?* Journal of Theoretical Biology, 1982. **99**(1): p. 31-68.
54. Marçal, H., et al., *A comprehensive protein expression profile of extracellular matrix biomaterial derived from porcine urinary bladder.* Regenerative Medicine, 2012. **7**(2): p. 159-166.
55. Engler, A.J., et al., *Matrix Elasticity Directs Stem Cell Lineage Specification.* Cell, 2006. **126**(4): p. 677-689.

56. Dabelsteen, S. and I. Mackenzie, *The stem cell concept in oral mucosa and in cancer.* Nor Tannlegeforen Tid, 2006. **116**(32): p. 6.
57. Li, W.-J., et al., *Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold.* Biomaterials, 2005. **26**(25): p. 5158-5166.
58. Vacanti, C.A., et al., *Tissue-engineered growth of bone and cartilage.* Transplant Proc, 1993. **25**(1 Pt 2): p. 1019-21.
59. Ishaug-Riley, S.L., et al., *Student research award in the doctoral degree candidate category, Society for Biomaterials 23rd Annual Meeting, New Orleans, LA, April 30-May 4, 1997: Ectopic bone formation by marrow stromal osteoblast transplantation using poly(DL-lactic-co-glycolic acid) foams implanted into the rat mesentery.* Journal of Biomedical Materials Research, 1997. **36**(1): p. 1-8.
60. Marin, E., F. Boschetto, and G. Pezzotti, *Biomaterials and biocompatibility: An historical overview.* J Biomed Mater Res A, 2020. **108**(8): p. 1617-1633.
61. Chevallay, B. and D. Herbage, *Collagen-based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy.* Med Biol Eng Comput, 2000. **38**(2): p. 211-8.
62. Berthiaume, F., T.J. Maguire, and M.L. Yarmush, *Tissue engineering and regenerative medicine: history, progress, and challenges.* Annu Rev Chem Biomol Eng, 2011. **2**: p. 403-30.
63. Jungebluth, P., et al., *Structural and morphologic evaluation of a novel detergent–enzymatic tissue-engineered tracheal tubular matrix.* The Journal of Thoracic and Cardiovascular Surgery, 2009. **138**(3): p. 586-593.
64. Gilbert, T., T. Sellaro, and S. Badylak, *Decellularization of tissues and organs.* Biomaterials, 2006.
65. Badylak, S.F., *Xenogeneic extracellular matrix as a scaffold for tissue reconstruction.* Transplant Immunology, 2004. **12**(3-4): p. 367-377.
66. Almansoori, A.A., et al., *Mesenchymal Stem Cell Therapy in Submandibular Salivary Gland Allotransplantation: Experimental Study.* Transplantation, 2019. **103**(6): p. 1111-1120.
67. Sarmin, A.M. and J.T. Connelly, *Fabrication of Human Skin Equivalents Using Decellularized Extracellular Matrix.* Current Protocols, 2022. **2**(3).
68. Zhao, J., et al., *Sustained local application of epidermal growth factor to accelerate reepithelialization of tracheal grafts.* The Journal of Thoracic and Cardiovascular Surgery, 2010. **140**(1): p. 209-215.
69. Park, J.H., et al., *A novel tissue-engineered trachea with a mechanical behavior similar to native trachea.* Biomaterials, 2015. **62**: p. 106-115.
70. Ben Amara, H., et al., *Healing kinetics of oral soft tissue wounds treated with recombinant epidermal growth factor: Translation from a canine model.* Journal of Clinical Periodontology, 2019. **46**(1): p. 105-117.
71. Hayashi, K., et al., *Nerve growth factor ?/pro-nerve growth factor and their receptors in normal human oral mucosa.* European Journal of Oral Sciences, 2007. **115**(5): p. 344-354.
72. Dally, J., et al., *Hepatocyte Growth Factor Mediates Enhanced Wound Healing Responses and Resistance to Transforming Growth Factor-β1-Driven*

Myofibroblast Differentiation in Oral Mucosal Fibroblasts. International Journal of Molecular Sciences, 2017. **18**(9).
73. Yu, R., et al., *IL-22 mediates the oral mucosal wound healing via STAT3 in keratinocytes.* Archives of Oral Biology, 2016. **72**: p. 14-20.
74. Keswani, S.G., et al., *Role of salivary vascular endothelial growth factor (VEGF) in palatal mucosal wound healing.* Wound Repair and Regeneration, 2013. **21**(4): p. 554-562.
75. Har, A. and J.C. Park, *Dental Stem Cells and Their Applications.* Chin J Dent Res, 2015. **18**(4): p. 207-12.
76. Peramo, A., C.L. Marcelo, and S.E. Feinberg, *Tissue engineering of lips and muco-cutaneous junctions: in vitro development of tissue engineered constructs of oral mucosa and skin for lip reconstruction.* Tissue Eng Part C Methods, 2012. **18**(4): p. 273-82.
77. Rheinwatd, J.G. and H. Green, *Seria cultivation of strains of human epidemal keratinocytes: the formation keratinizin colonies from single cell is.* Cell, 1975. **6**(3): p. 331-343.
78. Green, H., O. Kehinde, and J. Thomas, *Growth of cultured human epidermal cells into multiple epithelia suitable for grafting.* Proceedings of the National Academy of Sciences, 1979. **76**(11): p. 5665-5668.
79. Green, H., *The birth of therapy with cultured cells.* BioEssays, 2008. **30**(9): p. 897-903.
80. Bell, E., et al., *Living Tissue Formed in Vitro and Accepted as Skin-Equivalent Tissue of Full Thickness.* Science, 1981. **211**(4486): p. 1052-1054.
81. Chai, W.L., et al., *Development of a Novel Model for the Investigation of Implant–Soft Tissue Interface.* Journal of Periodontology, 2010. **81**(8): p. 1187-1195.
82. Jin, Y. and H. Bi, *Current progress of skin tissue engineering: Seed cells, bioscaffolds, and construction strategies.* Burns & Trauma, 2013. **1**(2).
83. Franklin, S.E., et al., *Comparison of the Friction Behavior of Occluded Human Skin and Synthetic Skin in Dry and Moist Conditions.* Tribology Transactions, 2016. **60**(5): p. 861-872.
84. Slominski, A.T., et al., *Tissue Engineered Skin Substitutes Created by Laser-Assisted Bioprinting Form Skin-Like Structures in the Dorsal Skin Fold Chamber in Mice.* PLoS ONE, 2013. **8**(3).
85. MacNeil, S., *Progress and opportunities for tissue-engineered skin.* Nature, 2007. **445**(7130): p. 874-880.
86. Tremblay, P.-L., et al., *Inosculation of Tissue-Engineered Capillaries with the Host's Vasculature in a Reconstructed Skin Transplanted on Mice.* American Journal of Transplantation, 2005. **5**(5): p. 1002-1010.
87. Jahoda, C.A.B., et al., *Human Hair Follicle Regeneration Following Amputation and Grafting into the Nude Mouse.* Journal of Investigative Dermatology, 1996. **107**(6): p. 804-807.
88. Cuono, C., R. Langdon, and J. McGuire, *Use of cultured epidermal autografts and dermal allografts as skin replacement after burn injury.* The Lancet, 1986. **327**(8490): p. 1123-1124.

89. Kumbar, S.G., et al., *Electrospun nanofiber scaffolds: engineering soft tissues.* Biomedical Materials, 2008. **3**(3).
90. Shcheglovitov, A., M. Rao, and A. Yoo, *Special issue on stem cell and tissue engineering in development, disease, and repair.* Developmental Dynamics, 2019. **248**(1): p. 7-9.
91. Lagarkova, M.A., *Such Various Stem Cells.* Biochemistry (Moscow), 2019. **84**(3): p. 187-189.
92. Hartenstein, V., *Stem cells in the context of evolution and development.* Development Genes and Evolution, 2012. **223**(1-2): p. 1-3.
93. Martinotti, S., et al., *Epidermal Stem Cells in Regenerative Medicine*, in *Cell Biology and Translational Medicine, Volume 10*. 2020. p. 17-21.
94. Zakrzewski, W., et al., *Stem cells: past, present, and future.* Stem Cell Research & Therapy, 2019. **10**(1).
95. Clevers, H., *What is an adult stem cell?* Science, 2015. **350**(6266): p. 1319-1320.
96. Takahashi, K. and S. Yamanaka, *Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors.* Cell, 2006. **126**(4): p. 663-676.
97. Egusa, H., et al., *Stem cells in dentistry – Part I: Stem cell sources.* Journal of Prosthodontic Research, 2012. **56**(3): p. 151-165.
98. Cao, C., S. Tarlé, and D. Kaigler, *Characterization of the immunomodulatory properties of alveolar bone-derived mesenchymal stem cells.* Stem Cell Research & Therapy, 2020. **11**(1).
99. Matichescu, A., et al., *Advanced Biomaterials and Techniques for Oral Tissue Engineering and Regeneration—A Review.* Materials, 2020. **13**(22).
100. Pisciotta, A., et al., *Human Dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations.* BMC Developmental Biology, 2015. **15**(1).
101. Hollands, P., D. Aboyeji, and M. Orcharton, *Dental pulp stem cells in regenerative medicine.* Br Dent J, 2018.
102. Tsutsui, T.W., *Dental Pulp Stem Cells: Advances to Applications.* Stem Cells Cloning, 2020. **13**: p. 33-42.
103. Zakrzewski, W., et al., *Stem cells: past, present, and future.* Stem Cell Res Ther, 2019. **10**(1): p. 68.
104. Sharpe, P.T., *Dental mesenchymal stem cells.* Development, 2016. **143**(13): p. 2273-80.
105. Xiong, J., et al., *Epithelial Cell Rests of Malassez Contain Unique Stem Cell Populations Capable of Undergoing Epithelial–Mesenchymal Transition.* Stem Cells and Development, 2012. **21**(11): p. 2012-2025.
106. Csete, M., et al., *Mesenchymal Stem Cell-Mediated Functional Tooth Regeneration in Swine.* PLoS ONE, 2006. **1**(1).
107. Chalisserry, E.P., et al., *Therapeutic potential of dental stem cells.* Journal of Tissue Engineering, 2017. **8**.
108. Yalvac, M.E., et al., *Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-*

vascularization, osteo-, adipo- and neurogenesis. The Pharmacogenomics Journal, 2010. **10**(2): p. 105-113.
109. Miura, M., et al., *SHED: Stem cells from human exfoliated deciduous teeth.* Proceedings of the National Academy of Sciences, 2003. **100**(10): p. 5807-5812.
110. Grawish, M.E., *Gingival-derived mesenchymal stem cells: An endless resource for regenerative dentistry.* World J Stem Cells, 2018. **10**(9): p. 116-118.
111. Li, Y., et al., *Long-term Safety and Efficacy of Human-Induced Pluripotent Stem Cell (iPS) Grafts in a Preclinical Model of Retinitis Pigmentosa.* Molecular Medicine, 2012. **18**(9): p. 1312-1319.
112. Lin, T., et al., *A chemical platform for improved induction of human iPSCs.* Nature Methods, 2009. **6**(11): p. 805-808.
113. Smith, A.N., et al., *Mesenchymal stem cells induce dermal fibroblast responses to injury.* Experimental Cell Research, 2010. **316**(1): p. 48-54.
114. Yamanaka, S., *Induced Pluripotent Stem Cells: Past, Present, and Future.* Cell Stem Cell, 2012. **10**(6): p. 678-684.
115. Kang, L., et al., *Induced pluripotent stem cells (iPSCs)—a new era of reprogramming.* Journal of Genetics and Genomics, 2010. **37**(7): p. 415-421.
116. Pera, M., et al., *Telomere Dynamics in Human Cells Reprogrammed to Pluripotency.* PLoS ONE, 2009. **4**(12).
117. Klymkowsky, M., et al., *Mitochondrial Rejuvenation After Induced Pluripotency.* PLoS ONE, 2010. **5**(11).
118. Shamis, Y., et al., *iPSC-derived fibroblasts demonstrate augmented production and assembly of extracellular matrix proteins.* In Vitro Cellular & Developmental Biology - Animal, 2012. **48**(2): p. 112-122.
119. Shamis, Y., et al., *Fibroblasts derived from human embryonic stem cells direct development and repair of 3D human skin equivalents.* Stem Cell Research & Therapy, 2011. **2**(1).
120. Garzón, I., et al., *Wharton's Jelly Stem Cells: A Novel Cell Source for Oral Mucosa and Skin Epithelia Regeneration.* Stem Cells Translational Medicine, 2013. **2**(8): p. 625-632.
121. Shanbhag, S., et al., *Xeno-Free Spheroids of Human Gingiva-Derived Progenitor Cells for Bone Tissue Engineering.* Front Bioeng Biotechnol, 2020. **8**: p. 968.
122. Rho, K.S., et al., *Electrospinning of collagen nanofibers: Effects on the behavior of normal human keratinocytes and early-stage wound healing.* Biomaterials, 2006. **27**(8): p. 1452-1461.
123. Choi, J.S., et al., *Full-Thickness Skin Wound Healing Using Human Placenta-Derived Extracellular Matrix Containing Bioactive Molecules.* Tissue Engineering Part A, 2013. **19**(3-4): p. 329-339.
124. Gilmartin, D.J., et al., *Integration of Scaffolds into Full-Thickness Skin Wounds: The Connexin Response.* Advanced Healthcare Materials, 2013. **2**(8): p. 1151-1160.
125. Ren, X., et al., *Growth Factor Engineering Strategies for Regenerative Medicine Applications.* Frontiers in Bioengineering and Biotechnology, 2020. **7**.

126. Giannobile, W.V., S.J. Hollister, and P.X. Ma, *Future Prospects for Periodontal Bioengineering Using Growth Factors.* Clinical Advances in Periodontics, 2011. **1**(2): p. 88-94.
127. Andreadis, S.T. and D.J. Geer, *Biomimetic approaches to protein and gene delivery for tissue regeneration.* Trends in Biotechnology, 2006. **24**(7): p. 331-337.
128. Tayalia, P. and D.J. Mooney, *Controlled Growth Factor Delivery for Tissue Engineering.* Advanced Materials, 2009. **21**(32-33): p. 3269-3285.
129. Rady, D., et al., *Mesenchymal Stem/Progenitor Cells: The Prospect of Human Clinical Translation.* Stem Cells International, 2020. **2020**: p. 1-45.
130. Bianco, P., et al., *Bone Marrow Stromal Stem Cells: Nature, Biology, and Potential Applications.* Stem Cells, 2009. **19**(3): p. 180-192.
131. Mohseny, A.B., et al., *Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2.* The Journal of Pathology, 2009. **219**(3): p. 294-305.
132. Tarte, K., et al., *Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation.* Blood, 2010. **115**(8): p. 1549-1553.
133. Mohiuddin, M.M., et al., *One-Year Heterotopic Cardiac Xenograft Survival in a Pig to Baboon Model.* American Journal of Transplantation, 2014. **14**(2): p. 488-489.
134. Iwase, H., et al., *Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date.* Xenotransplantation, 2015. **22**(4): p. 302-309.
135. Sun, X., et al., *The assessment of xenogeneic bone immunotoxicity and risk management study.* BioMedical Engineering OnLine, 2019. **18**(1).
136. Goyer, B., et al., *Immune tolerance of tissue-engineered skin produced with allogeneic or xenogeneic fibroblasts and syngeneic keratinocytes grafted on mice.* Acta Biomaterialia, 2019. **90**: p. 192-204.
137. Herford, A.S., et al., *Use of a Porcine Collagen Matrix as an Alternative to Autogenous Tissue for Grafting Oral Soft Tissue Defects.* Journal of Oral and Maxillofacial Surgery, 2010. **68**(7): p. 1463-1470.
138. Roh, J.-L., et al., *Promotion of oral surgical wound healing using autologous mucosal cell sheets.* Oral Oncology, 2017. **69**: p. 84-91.
139. Costea, D.E., et al., *Crucial effects of fibroblasts and keratinocyte growth factor on morphogenesis of reconstituted human oral epithelium.* J Invest Dermatol, 2003. **121**(6): p. 1479-86.
140. Jiménez, G., et al., *Models of Disease*, in *Osteochondral Tissue Engineering*. 2018. p. 331-350.
141. Hendijani, F., *Explant culture: An advantageous method for isolation of mesenchymal stem cells from human tissues.* Cell Prolif, 2017. **50**(2).
142. Coraux, C., et al., *Reconstituted Skin from Murine Embryonic Stem Cells.* Current Biology, 2003. **13**(10): p. 849-853.
143. Zhao, A., et al., *Generation of keratinocyte stem-like cells from human fibroblasts via a direct reprogramming approach.* Biotechnol Prog, 2020. **36**(3): p. e2961.

144. Kogut, I., D.R. Roop, and G. Bilousova, *Differentiation of human induced pluripotent stem cells into a keratinocyte lineage.* Methods Mol Biol, 2014. **1195**: p. 1-12.
145. Yoshida, S., et al., *Generation of stratified squamous epithelial progenitor cells from mouse induced pluripotent stem cells.* PLoS One, 2011. **6**(12): p. e28856.
146. Vijayaraj, P., *Immunochemistry Analysis Using on Tissue Sections*, in *Single-Cell Protein Analysis*. 2022. p. 1-16.
147. Dou, Z., et al., *Optimized protocols for in situ hybridization, immunohistochemistry, and immunofluorescence on skeletal tissue.* Acta Histochemica, 2021. **123**(5).
148. Dabija-Wolter, G., et al., *In vitroreconstruction of human junctional and sulcular epithelium.* Journal of Oral Pathology & Medicine, 2012. **42**(5): p. 396-404.
149. Loro, L.L., et al., *Apoptosis and expression of Bax and Bcl-2 in snuff- and non-snuff associated oral squamous cell carcinomas.* Anticancer Res, 2000. **20**(5A): p. 2855-60.
150. Loro, L.L., O.K. Vintermyr, and A.C. Johannessen, *Cell death regulation in oral squamous cell carcinoma: methodological considerations and clinical significance.* J Oral Pathol Med, 2003. **32**(3): p. 125-38.
151. Aydin, S., *A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA.* Peptides, 2015. **72**: p. 4-15.
152. McKinnon, K.M., *Flow Cytometry: An Overview.* Current Protocols in Immunology, 2018. **120**(1).
153. Radcliff, G. and M.J. Jaroszeski, *Basics of Flow Cytometry*, in *Flow Cytometry Protocols*. 1997. p. 1-24.
154. Verschoor, C.P., et al., *An Introduction to Automated Flow Cytometry Gating Tools and Their Implementation.* Frontiers in Immunology, 2015. **6**.
155. Cobb, M., *60 years ago, Francis Crick changed the logic of biology.* PLOS Biology, 2017. **15**(9).
156. Kralik, P. and M. Ricchi, *A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything.* Frontiers in Microbiology, 2017. **8**.
157. Tajadini, M., M. Panjehpour, and S. Javanmard, *Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes.* Advanced Biomedical Research, 2014. **3**(1).
158. Janes, K.A., *An analysis of critical factors for quantitative immunoblotting.* Science Signaling, 2015. **8**(371).
159. Magi, B. and S. Liberatori, *Immunoblotting Techniques*, in *Immunochemical Protocols*. 2005. p. 227-253.
160. Seubert, A.C., M. Krafft, and K. Kretzschmar, *Generation and Characterization of Murine Oral Mucosal Organoid Cultures.* Journal of Visualized Experiments, 2021(173).

161. Lu, E.M.C., et al., *Development of an in vitro model of the dentogingival junction using 3D organotypic constructs.* Journal of Periodontal Research, 2020. **56**(1): p. 147-153.
162. Chioni, A.-M., R.T. Bajwa, and R. Grose, *3D Organotypic Culture Model to Study Components of ERK Signaling*, in *ERK Signaling*. 2017. p. 255-267.
163. Hirsch, C. and S. Schildknecht, *In Vitro Research Reproducibility: Keeping Up High Standards.* Frontiers in Pharmacology, 2019. **10**.
164. Kataoka, K., et al., *Participation of Adult Mouse Bone Marrow Cells in Reconstitution of Skin.* The American Journal of Pathology, 2003. **163**(4): p. 1227-1231.
165. Lamont, R.J., H. Koo, and G. Hajishengallis, *The oral microbiota: dynamic communities and host interactions.* Nature Reviews Microbiology, 2018. **16**(12): p. 745-759.
166. Hearnden, V., et al., *New developments and opportunities in oral mucosal drug delivery for local and systemic disease.* Advanced Drug Delivery Reviews, 2012. **64**(1): p. 16-28.
167. Zhang, W., et al., *Promoting Oral Mucosal Wound Healing with a Hydrogel Adhesive Based on a Phototriggered S-Nitrosylation Coupling Reaction.* Advanced Materials, 2021. **33**(48).
168. Ahmad, F. and J. Lata, *Collagen matrix membrane as a biological dressing in defects of the oral mucosa.* National Journal of Maxillofacial Surgery, 2021. **12**(2).
169. Rastogi, S., M. Modi, and B. Sathian, *The Efficacy of Collagen Membrane as a Biodegradable Wound Dressing Material for Surgical Defects of Oral Mucosa: A Prospective Study.* Journal of Oral and Maxillofacial Surgery, 2009. **67**(8): p. 1600-1606.
170. Izumi, K. and S.E. Feinberg, *Skin and oral mucosal substitutes.* Oral and Maxillofacial Surgery Clinics of North America, 2002. **14**(1): p. 61-71.
171. Werner, S., T. Krieg, and H. Smola, *Keratinocyte–Fibroblast Interactions in Wound Healing.* Journal of Investigative Dermatology, 2007. **127**(5): p. 998-1008.
172. Wallâ€ , I.B., et al., *Force generation and protease gene expression in organotypic co-cultures of fibroblasts and keratinocytes.* Journal of Tissue Engineering and Regenerative Medicine, 2009. **3**(8): p. 647-650.
173. Tandara, A.A., et al., *Hydrated keratinocytes reduce collagen synthesis by fibroblasts via paracrine mechanisms.* Wound Repair and Regeneration, 2007. **15**(4): p. 497-504.
174. Maas-Szabowski, N., et al., *Organotypic Cocultures with Genetically Modified Mouse Fibroblasts as a Tool to Dissect Molecular Mechanisms Regulating Keratinocyte Growth and Differentiation.* Journal of Investigative Dermatology, 2001. **116**(5): p. 816-820.
175. Mann, A., et al., *Granulocyte–Macrophage Colony-Stimulating Factor Is Essential for Normal Wound Healing.* Journal of Investigative Dermatology Symposium Proceedings, 2006. **11**(1): p. 87-92.

176. Maas-Szabowski, N., A. Stärker, and N.E. Fusenig, *Epidermal tissue regeneration and stromal interaction in HaCaT cells is initiated by TGF-α.* Journal of Cell Science, 2003. **116**(14): p. 2937-2948.
177. Russo, B., N.C. Brembilla, and C. Chizzolini, *Interplay Between Keratinocytes and Fibroblasts: A Systematic Review Providing a New Angle for Understanding Skin Fibrotic Disorders.* Frontiers in Immunology, 2020. **11**.
178. Fernandez, T.L., et al., *In VitroInvestigations on the Effect of Dermal Fibroblasts on Keratinocyte Responses to Ultraviolet B Radiation.* Photochemistry and Photobiology, 2014. **90**(6): p. 1332-1339.
179. Chowdhury, S.R., B.S. Aminuddin, and B.H. Ruszymah, *Effect of supplementation of dermal fibroblasts conditioned medium on expansion of keratinocytes through enhancing attachment.* Indian J Exp Biol, 2012. **50**(5): p. 332-9.
180. Pani, G., et al., *Enhanced Keratinocyte Proliferation and Migration in Co-culture with Fibroblasts.* PLoS ONE, 2012. **7**(7).
181. Peura, M., et al., *Paracrine factors from fibroblast aggregates in a fibrin-matrix carrier enhance keratinocyte viability and migration.* Journal of Biomedical Materials Research Part A, 2010. **95A**(2): p. 658-664.
182. El Ghalbzouri, A. and M. Ponec, *Diffusible factors released by fibroblasts support epidermal morphogenesis and deposition of basement membrane components.* Wound Repair and Regeneration, 2004. **12**(3): p. 359-367.
183. Billingham, R.E. and W.K. Silvers, *Studies on the Conservation of Epidermal Specificities of Skin and Certain Mucosas in Adult Mammals.* Journal of Experimental Medicine, 1967. **125**(3): p. 429-446.
184. Ralston, et al., *The requirement for basement membrane antigens in the production of human epidermal/dermal composites in vitro.* British Journal of Dermatology, 1999. **140**(4): p. 605-615.
185. Čabrijan, L. and J. Lipozenčić, *Adhesion molecules in keratinocytes.* Clinics in Dermatology, 2011. **29**(4): p. 427-431.
186. Mackenzie, I.C. and N.E. Fusenig, *Regeneration of Organized Epithelial Structure.* Journal of Investigative Dermatology, 1983. **81**(1): p. S189-S194.
187. Calenic, B., et al., *Oral keratinocyte stem/progenitor cells: specific markers, molecular signaling pathways and potential uses.* Periodontol 2000, 2015. **69**(1): p. 68-82.
188. Costea, D.E., et al., *The phenotype of in vitro reconstituted normal human oral epithelium is essentially determined by culture medium.* J Oral Pathol Med, 2005. **34**(4): p. 247-52.
189. Maas-Szabowski, N., H.J. Stark, and N.E. Fusenig, *Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced keratinocyte growth factor expression in resting fibroblasts.* J Invest Dermatol, 2000. **114**(6): p. 1075-84.
190. El Ghalbzouri, A. and M. Ponec, *Diffusible factors released by fibroblasts support epidermal morphogenesis and deposition of basement membrane components.* Wound Repair Regen, 2004. **12**(3): p. 359-67.
191. Florin, L., et al., *Delayed wound healing and epidermal hyperproliferation in mice lacking JunB in the skin.* J Invest Dermatol, 2006. **126**(4): p. 902-11.

192. Mah, W., et al., *Human gingival fibroblasts display a non-fibrotic phenotype distinct from skin fibroblasts in three-dimensional cultures.* PLoS One, 2014. **9**(3): p. e90715.
193. Cichon, A.C., et al., *AKT in stromal fibroblasts controls invasion of epithelial cells.* Oncotarget, 2013. **4**(7): p. 1103-16.
194. Shannon, D.B., et al., *Phenotypic differences between oral and skin fibroblasts in wound contraction and growth factor expression.* Wound Repair Regen, 2006. **14**(2): p. 172-8.
195. Stephens, P., et al., *Phenotypic variation in the production of bioactive hepatocyte growth factor/scatter factor by oral mucosal and skin fibroblasts.* Wound Repair Regen, 2001. **9**(1): p. 34-43.
196. YANG., H.-S.K.S.-G.L.K.-H.K.U.-K.K.J.-R.K.I.-K.C.D.-K., *Production Of Gm-Csf And Tgf-beta 1 In Irradiated Human Gingival Fibroblasts Cultured With Lipopolysaccharide.* Journal of the Korean Association of Oral and Maxillofacial Surgeons 2002: p. 169-174.
197. Gonzalez, O.A., J.L. Ebersole, and C.B. Huang, *Supernatants from oral epithelial cells and gingival fibroblasts modulate human immunodeficiency virus type 1 promoter activation induced by periodontopathogens in monocytes/macrophages.* Mol Oral Microbiol, 2010. **25**(2): p. 136-49.
198. Kinikoglu, B., et al., *Influence of the mesenchymal cell source on oral epithelial development.* J Tissue Eng Regen Med, 2012. **6**(3): p. 245-52.
199. Smola, H., et al., *Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture.* Exp Cell Res, 1998. **239**(2): p. 399-410.
200. Maas-Szabowski, N., A. Shimotoyodome, and N.E. Fusenig, *Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism.* J Cell Sci, 1999. **112 (Pt 12)**: p. 1843-53.
201. Beertsen, W., C.A.G. McCulloch, and J. Sodek, *The periodontal ligament: a unique, multifunctional connective tissue.* Periodontology 2000, 1997. **13**(1): p. 20-40.
202. LeBlanc, A.R.H., et al., *Tooth attachment and pleurodont implantation in lizards: Histology, development, and evolution.* Journal of Anatomy, 2020. **238**(5): p. 1156-1178.
203. Serres, E.R.A., *Essai sur l'anatomie et la physiologie des dents, ou nouvelle théorie de la dentition*. 1817: Méquignon-Marvis.
204. Malassez, L., *Sur l'existence de masses epithelailes dans ligament alveolodentaire chez l'homme adulte et a l'etat normal.* Comptes Rendus Societe de Biologie, 1884. **36**: p. 241-244.
205. Kvam, E. and O. Gilhuus-Moe, *Uptake of 3H-Thymidine by an Epithelial Rest in the Periodontal Membrane a Preliminary Report.* Acta Odontologica Scandinavica, 1970. **28**(1): p. 143-146.
206. Main, D., *Odontogenic epithelial residues aroud rabbit mandibular cheek teeth.* J. Dent. Res., 1972. **51**: p. 841.
207. Ten Cate, A.R., *The role of epithelium in the development, structure and function of the tissues of tooth support.* Oral Dis, 1996. **2**(1): p. 55-62.

208. Nam, H., et al., *Expression profile of the stem cell markers in human Hertwig's epithelial root sheath/Epithelial rests of Malassez cells.* Molecules and Cells, 2011. **31**(4): p. 355-360.
209. Tsunematsu, T., et al., *Human odontogenic epithelial cells derived from epithelial rests of Malassez possess stem cell properties.* Laboratory Investigation, 2016. **96**(10): p. 1063-1075.
210. Lindskog, S., L. Blomlof, and L. Hammarstrom, *Evidence for a role of odontogenic epithelium in maintaining the periodontal space.* Journal of Clinical Periodontology, 1988. **15**(6): p. 371-373.
211. Hasegawa, N., et al., *Immunohistochemical characteristics of epithelial cell rests of Malassez during cementum repair.* Journal of Periodontal Research, 2003. **38**(1): p. 51-56.
212. Shinmura, Y., et al., *Quiescent epithelial cell rests of Malassez can differentiate into ameloblast-like cells.* Journal of Cellular Physiology, 2008. **217**(3): p. 728-738.
213. Owen, M. and A.J. Friedenstein, *Stromal Stem Cells: Marrow-Derived Osteogenic Precursors*, in *Ciba Foundation Symposium 136 - Cell and Molecular Biology of Vertebrate Hard Tissues*. 2007. p. 42-60.
214. Seo, B.-M., et al., *Investigation of multipotent postnatal stem cells from human periodontal ligament.* The Lancet, 2004. **364**(9429): p. 149-155.
215. Azizi, S.A., et al., *Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts.* Proceedings of the National Academy of Sciences, 1998. **95**(7): p. 3908-3913.
216. Ferrari, G., et al., *Muscle Regeneration by Bone Marrow-Derived Myogenic Progenitors.* Science, 1998. **279**(5356): p. 1528-1530.
217. Adnan, Y., et al., *High CD44 Immunoexpression Correlates with Poor Overall Survival: Assessing the Role of Cancer Stem Cell Markers in Oral Squamous Cell Carcinoma Patients from the High-Risk Population of Pakistan.* International Journal of Surgical Oncology, 2022. **2022**: p. 1-14.
218. Fox, S.B., et al., *Normal human tissues, in addition to some tumors, express multiple different CD44 isoforms.* Cancer Res, 1994. **54**(16): p. 4539-46.
219. Kansas, G.S., M.J. Muirhead, and M.O. Dailey, *Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans.* Blood, 1990. **76**(12): p. 2483-92.
220. Miyake, K., et al., *Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures.* Journal of Experimental Medicine, 1990. **171**(2): p. 477-488.
221. Rippon, H.J., et al., *Embryonic Stem Cells as a Source of Pulmonary Epithelium In Vitro and In Vivo.* Proceedings of the American Thoracic Society, 2008. **5**(6): p. 717-722.
222. Jagetia, G.C., et al., *Augmentation of wound healing by ascorbic acid treatment in mice exposed toγ-radiation.* International Journal of Radiation Biology, 2009. **80**(5): p. 347-354.
223. Savini, I., et al., *Characterization of Keratinocyte Differentiation Induced by Ascorbic Acid: Protein Kinase C Involvement and Vitamin C Homeostasis11The*

authors declared not to have a conflict of interest. Journal of Investigative Dermatology, 2002. **118**(2): p. 372-379.
224. Guenou, H., et al., *Human embryonic stem-cell derivatives for full reconstruction of the pluristratified epidermis: a preclinical study.* The Lancet, 2009. **374**(9703): p. 1745-1753.
225. Mathieu, M.-E., et al., *LIF-Dependent Signaling: New Pieces in the Lego.* Stem Cell Reviews and Reports, 2011. **8**(1): p. 1-15.
226. Hirai, H., P. Karian, and N. Kikyo, *Regulation of embryonic stem cell self-renewal and pluripotency by leukaemia inhibitory factor.* Biochemical Journal, 2011. **438**(1): p. 11-23.
227. Chen, S.S., et al., *Differentiation of Rhesus Monkey Embryonic Stem Cells in Three-Dimensional Collagen Matrix*, in *Embryonic Stem Cell Protocols*. 2006. p. 431-444.
228. Shen, C.-N., Z.D. Burke, and D. Tosh, *Transdifferentiation, Metaplasia and Tissue Regeneration.* Organogenesis, 2014. **1**(2): p. 36-44.
229. Li, N., et al., *Reconstitution of male germline cell specification from mouse embryonic stem cells using defined factors in vitro.* Cell Death & Differentiation, 2019. **26**(10): p. 2115-2124.
230. Agarwal, S., K.L. Holton, and R. Lanza, *Efficient Differentiation of Functional Hepatocytes from Human Embryonic Stem Cells.* Stem Cells, 2008. **26**(5): p. 1117-1127.
231. Guan, K., J. Rohwedel, and A.M. Wobus, Cytotechnology, 1999. **30**(1/3): p. 211-226.
232. Li, Y., et al., *Efficacy of stem cell therapy for burn wounds: a systematic review and meta-analysis of preclinical studies.* Stem Cell Research & Therapy, 2020. **11**(1).
233. van der Sanden, B., et al., *Optimizing stem cell culture.* Journal of Cellular Biochemistry, 2010. **111**(4): p. 801-807.
234. Keller, G., *Embryonic stem cell differentiation: emergence of a new era in biology and medicine.* Genes & Development, 2005. **19**(10): p. 1129-1155.
235. Shukla, A.K., G. Gao, and B.S. Kim, *Applications of 3D Bioprinting Technology in Induced Pluripotent Stem Cells-Based Tissue Engineering.* Micromachines, 2022. **13**(2).
236. Metallo, C.M., et al., *Directed Differentiation of Human Embryonic Stem Cells to Epidermal Progenitors*, in *Epidermal Cells*. 2010. p. 83-92.
237. Kawasaki, H., et al., *Induction of Midbrain Dopaminergic Neurons from ES Cells by Stromal Cell–Derived Inducing Activity.* Neuron, 2000. **28**(1): p. 31-40.
238. Lian, X., et al., *Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions.* Nature Protocols, 2012. **8**(1): p. 162-175.
239. Soares, E. and H. Zhou, *Pluripotent Stem Cell Differentiation Toward Functional Basal Stratified Epithelial Cells*. 2020.
240. Yan, Y., et al., *Effect of iPSCs-derived keratinocytes on healing of full-thickness skin wounds in mice.* Experimental Cell Research, 2019. **385**(1).

241. Choudhury, S., N. Surendran, and A. Das, *Recent advances in the induced pluripotent stem cell-based skin regeneration.* Wound Repair and Regeneration, 2021. **29**(5): p. 697-710.
242. Itoh, M., et al., *Generation of keratinocytes from normal and recessive dystrophic epidermolysis bullosa-induced pluripotent stem cells.* Proceedings of the National Academy of Sciences, 2011. **108**(21): p. 8797-8802.
243. Toma, A.I., et al., *Oral wound healing models and emerging regenerative therapies.* Translational Research, 2021. **236**: p. 17-34.
244. Nauta, A., G.C. Gurtner, and M.T. Longaker, *Wound healing and regenerative strategies.* Oral Diseases, 2011. **17**(6): p. 541-549.
245. Milligan, D.A., E.J. Tyler, and C.L. Bishop, *Tissue engineering to better understand senescence: Organotypics come of age.* Mechanisms of Ageing and Development, 2020. **190**.

Publications not included in the thesis

Asma Almazyad, Yao Gao, Shokoufeh Shahrabi Farahani, A Gartung, L Sui, **Ridhima Das**, Daniela Elena Costea, Randolph Watnick, Dipak Panigrahy, Rosalyn M Adam, Diane R Bielenberg. Novel neuropilin 2-targeting biologic for the treatment of oral squamous cell carcinoma. July 2021, Oral Surgery Oral Medicine Oral Pathology and Oral Radiology 132(1): e5

Zhuoyuan Zhang, Zhenjie Gao, Saroj Rajthala, Dipak Sapkota, Harsh Dongre, Himalaya Parajuli, Salwa Suliman, **Ridhima Das**, Longjiang Li, Laurence A. Bindoff, Daniela Elena Costea & Xiao Liang. Metabolic reprogramming of normal oral fibroblasts correlated with increased glycolytic metabolism of oral squamous celland precedes their activation into carcinoma associated fibroblasts. March 2020, Cellular and Molecular Life Sciences 77(1).

Dan Columbo, **Ridhima Das**, Asma Almazyad, Yao Gao, Diane R Bielenberg. Tumor Growth in the Skin during Anagen and Telogen Phases of the Mouse Hair Cycle, April 2020, The FASEB Journal.

Asma Almazyad, Yao Gao, Shokoufeh Shahrabi-Farahani, Lufei Sui, Allison Gartung, **Ridhima Das**, Daniela Elena Costea, Randy Watnick, Dipak Panigrahy, Rosalyn M. Adam, Diane R. Bielenberg. Neuropilin 2 Drives Tumor Lymphangiogenesis in Oral Squamous Cell Carcinoma, April 2020, The FASEB Journa

Paper I

Granulocyte macrophage-colony stimulating factor and keratinocyte growth factor control of early stages of differentiation of oral epithelium

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HOW TO CITE: Das R, Virlan MJR, Xenaki V, Kulasekara KK, Lukandu O, Neppelberg E, et al. Granulocyte macrophage-colony stimulating factor and keratinocyte growth factor control of early stages of differentiation of oral epithelium. Eur J Oral Sci. 2022

ABSTRACT

Oral epithelial differentiation is known to be directed by underlying fibroblasts, but the responsible factor(s) have not been yet identified. We aimed here to identify fibroblast-derived factors responsible for oral epithelial differentiation. Primary normal human oral keratinocytes and fibroblasts were isolated from healthy volunteers after informed consent (n=5) and 3D-organotypic (3D-OT) cultures were constructed. Various growth factors were added at a range of 0.1-100ng/ml. 3D-OTs were harvested after ten days and assessed histologically, by immunohistochemistry and the TUNEL method. Epithelium developed in 3D-OT without fibroblasts showed an undifferentiated phenotype. Addition of granulocyte macrophage-colony stimulating factor (GM-CSF) induced expression of cytokeratin 13 in suprabasal cell layers. Admixture of GM-CSF and keratinocyte growth factor (KGF) induced, in addition, polarization of epidermal growth factor receptor and β1-integrin to basal cell layer and collagen IV deposition. Terminal differentiation with polarization of TUNEL-positive cells to superficial layers ocured only in the presence of fibroblasts in collagen gels either in direct contact or at distance from normal oral keratinocytes. Taken together, these results show that major aspects of oral epithelial differentiation are regulated by the synergic combination of GM-CSF and KGF. However, the terminal stage seems to be controlled by other yet unidentified fibroblast-derived diffusible factor(s).

KEYWORDS: mouth mucosa; cell proliferation; cell differentiation.

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INTRODUCTION

It is well established that the molecular interactions between epithelium and mesenchyme is essential for keratinocyte proliferation, differentiation and repair in both skin and oral mucosa (1-4). Previous studies on three dimentional organotypic (3D-OT) *in vitro* models, including our own, demonstrated that fibroblasts were essential for the resemblance of the tissues reconstructed 3D *in vitro* with the *in vivo* human oral mucosa (1, 5, 6). Several studies have tested various fibroblast-derived factors for their role on oral epithelium differentiation; keratinocyte growth factor (KGF) was found to stimulate proliferation of oral keratinocytes but not influence their differentiation when added alone to 3D-OT models contructed with keratinocyte only (3D-OT monocultures) (1). This was in contrast to the effect of KGF on dermal keratinocytes in 3D-OT cultures (7). The hematopoietic granulocyte macrophage-colony stimulating factor (GM-CSF) which is synthesized by macrophages, T cells, mast cells, natural killer cells, endothelial cells and fibroblasts and which normally functions as a cytokine facilitating development of the immune system and promoting the defense against infections was found to also regulate dermal keratinocyte growth and differentiation (8). Fibroblast-keratinocyte co-cultures in fetal skin models strongly enhanced the expression of GM-CSF by fetal skin cells (9), while dermal keratinocyte-released interleukin 1α (Il-1α) induced the expression of both KGF and GM-CSF in dermal fibroblasts (10). These studies indicated GM-CSF as a growth factor involved in epithelial-mesenchymal interactions, but its effect on oral epithelial morphogenesis has not been tested so far. Much of the knowledge on epithelial-mesenchymal interactions comes from studies on skin models, but there are distinctive signals for epithelial differentiation of oral and dermal fibroblasts (11) and there is a gap of knowledge on how fibroblasts regulate the differentiation of oral epithelium. The aim of this study was to identify the fibroblast-derived factors responsible for oral epithelial differentiation, and for this purpose several growth factors were tested, such as epidermal growth factor (EGF), KGF, GM-CSF, transforming growth factor α (TGFα), IL-1α, hepatocyte growth factor (HGF), and stem cell factor (SCF). The present study presents data in support for the control of oral epithelial differentiation by the underlying mesenchyme via soluble factors synthesized by oral fibroblasts. GM-CSF, alone or in combination with KGF, was able to control several steps of differentiation, except its terminal stages. This indicates that other yet unidentified fibroblast-derived soluble factor(s) may be responsible for regulation of terminal differentiation in oral epithelia.

MATERIALS AND METHODS

Human subjects: Eighteen samples of normal human oral mucosa were obtained from healthy donors undergoing wisdom tooth extraction (details in Table 1). Seven samples were snap-frozen in isopentane and six samples were formalin-fixed and embedded in paraffin. Cells successfully isolated and propagated from five samples were used for growing of 3D-OT cultures. The study was approved by the Ethics Committee of the University of Bergen (REK 2010/481) and the samples were collected after informed consent.

Primary cell cultures: Primary human normal oral fibroblasts and keratinocytes were isolated as previously described (1). Normal oral keratinocytes were routinely grown on plastic surfaces (Nunc) with no feeding layers, in keratinocyte serum free medium supplemented with 1 ng/ml human recombinant epidermal growth factor (GibcoBRL), 25 μg/ml bovine pituitary extract (GibcoBRL), 2 mM L-glutamine (GibcoBRL), 100 U/ml penicillin (GibcoBRL), 100 μg/ml streptomycin (GibcoBRL), 0.25 μg/ml amphotericin B (GibcoBRL). Normal oral fibroblasts were grown in Minimum Essential Medium Eagle (Sigma) supplemented with 10% foetal calf serum (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B. To reduce the variability, one single batch of foetal calf serum has been used throughout the studies.

3D-OT cell culture procedures: Simple collagen gels (700 μl for each culture) were prepared on ice by mixing 7 vol. (3.40 mg/ml) of rat tail collagen type I (Collaborative Biomedical), 2 vol. reconstitution buffer (261 mM NaHCO3, 150 mM NaOH, 200 mM HEPES) pH 8.15, 1 vol. Dulbecco's Modified Eagle Medium (DMEM) 10x (Sigma) and 1 vol. foetal calf serum. Fibroblast-containing collagen matrices were prepared by mixing 1 vol. foetal calf serum containing $0.5x10^6$/ml normal oral fibroblasts in passages 2-4. Seven hundred μl of the prepared matrix was pipetted in 24 well plates and let for 30 min in the incubator to gellify. Normal oral fibroblasts growth medium (1ml/well) was then added ove the matrices. After 24h, the medium on top of the gels was removed and normal oral keratinocytes ($0.5x10^6$ cells/culture) at second passage were added in 1ml of their growth medium(1). After 24 to 48h, the cultures were lifted on the air-liquid interface. The flow of procedures for construction of 3D-OT cultures is presented in Figure 1. The suspended 3D organotypic cultures were grown in serum free medium comprising DMEM and Ham's F-12 nutrient mix in 3:1, supplemented with 1 μM hydrocortisone, 0.8 μM insulin, 0.25 mM transferrin, 0.25 mM L-ascorbic acid, 15-30 μM linoleic acid, 15 μM bovine serum albumin,

2 mM L-glutamine (all from Sigma). Sandwich models were manufactured by interposing a layer of collagen biomatrix (500 μl) between the epithelial compartment and the fibroblast containing matrix. Human growth factors (EGF, KGF, GM-CSF, TGFα, IL-1α, HGF, SCF – Sigma) were added to the culture media of some of the collagen simple matrix cultures at a range of 0.1-100 ng/ml, as summarized in Table 2. All cultures were maintained at 37°C in 5% CO2 incubators for the whole duration of the experiment. All cultures were harvested on day 10 of co-culture. One half of each culture was snap frozen in isopentane pre-chilled in liquid nitrogen and the other fixed in 4% buffered formalin pH 7.15 and embedded in paraffin. Experiments (run in duplicates) were repeated 5 times, each time with primary cells isolated from different patients (n=5 donors).

ELISA: Conditioned media was collected from normal oral fibroblasts cells (n=5 donors) maintained in monocellular (normal oral fibroblasts only) 3D cultures at similar passages, and analyzed for levels of various growth factors and cytokines by using the Widescreen Human Cancer Panel 2 (Novagen) with Luminex beads (R&D Systems).

Immunohistochemical staining: The immunohistochemical staining was carried out using the DAKO autostainer – Universal Staining System (DAKO). Five μm thick fresh or formalin fixed, paraffin embedded sections were used. The staining for E-cadherin was carried on fresh frozen sections fixed for 30 sec in 50% cold acetone, and afterwards for 5 min in 100% acetone before washing in distilled water. All sections were processed then as previously reported(1). The sections were incubated with the primary antibody for 60 min, and afterwards with the secondary antibody conjugated with horseradish peroxidase labelled polymer (EnVision+ System; DAKO) for 30 min. Primary antibodies (all IgG1) and titrations used in this study were as follows: Ki-67, MIB-1clone, 1:50 (DAKO); cytokeratin 13 (CK13), KS-1A3 clone, 1:400 (Novocastra Laboratories); β1-integrin, K20 clone, 1:2000 (DAKO), EGF-R, E30 clone, 1:100 (DAKO), E-cadherin, HECD-1 clone, 1:9000 (R&D Systems) , collagen IV, CIV221 clone, 1:25 (DAKO). Presence of antigen was visualised with DAB+ (3,3'-diaminobenzidine, DAKO). Biopsies of normal human oral mucosa served as reference controls (those marked as frozen and formalin fixed and embedded in paraffin in Table 1). Specimens incubated with antibody diluent (DAKO) or CD 3 antibody (having the same isotype as the antibodies tested in the study) instead of primary antibody were used as negative controls.

TUNEL method: Cell death was detected by the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP*in situ* nick end-labelling) on formalin fixed paraffin embedded sections (12). For positive controls, specimens were treated with 0.5 mg/ml DNase (Roche Diagnostics) in tris-buffered saline for 15 min at 37°C prior to incubation with bovine serum albumin. The specificity of the TUNEL reaction was tested by substituting the biotinylated dUTP in the TUNEL labelling mixture with unbiotinylated dUTP (Roche) in excess. TUNEL positive keratinocytes found within the basal cell layer were considered spontaneously apoptotic cells, while TUNEL positive cells found at the superficial cell layer on top of the epithelium were considered terminally differentiated keratinocytes (13).

Evaluation of samples and statistical analysis: ELISA results are presented with values normalized for 10^6 cells; data were analysed using t-test with a level of significance set at 5% (SPSS 11.0). The data is presented as mean +/- SD. Tissue sections (5μm) from paraffin embedded specimens, stained with Haematoxylin-eosin, were morphometrically analysed by a computer based optical image analyser (analySIS 11.0 Pro Soft Imaging System). Ki-67 /proliferation index was determined as the percentage of positive cells among all cells of the basal cell layer per 400μm length of the epithelial-mesenchymal interface. The measurements and counts were done at 200 fold magnification on a standard microscope (LeikaDMLM) on 6 consecutive fields situated 200 μm apart. Statistical analysis was performed using Wilcoxon paired test with a level of significance set at 5% (SPSS 11.0).

RESULTS

Effects of KGF and GM-CSF, alone or in combination, on epithelial cell proliferation and thickness of *in vitro* reconstructed normal human oral epithelium

The oral mucosa formed by growing primary normal human buccal keratinocytes on simple collagen gels in absence of fibroblasts displayed a thin epithelium (Figure 2A) with low cell proliferation (Figure 3). Presence of fibroblasts in the collagen matrix, either in direct contact with keratinocytes (Figure 2J) or at distance (in the‘sandwich models’- Figure 2K) induced an increase in cell proliferation (Figure 3). No differences between cell proliferation indices or the phenotypes of reconstructed oral epithelia could be detected between cultures with direct keratinocyte-fibroblast contact and cultures with keratinocytes at distance from fibroblasts (Figure 3). Analysis of conditioned medium from 3D gels populated with fibroblasts showed that fibroblasts secreted HGF, KGF, GM-CSF and IL-1α when grown in 3D cultures *in vitro* (Figure 4). Both KGF and GM-CSF at concentrations higher than 1 ng/ml, either alone or in

combination, increased cell proliferation in the basal cell layer (Figure 3). EGF, TGFα, IL-1α, HGF or SCF did not alter epithelial thickness (Figure 2B, E-H) or epithelial cell proliferation in 3D-OT monocultures of keratinocytes (Figure 4).

Effects of GM-CSF alone or in combination with KGF on oral epithelial differentiation of *in vitro* reconstituted normal human oral epithelium

When grown in 3D monocultures on simple collagen gels, normal oral keratinocytes formed an epithelium with an undifferentiated phenotype (Figure 5, Table 2). Immunohistochemistry for various differentiation markers of these cultures revealed a weak, scattered expression of cytokeratin 13 (CK13, Figure 5A), strong expression of β1-integrin (Figure 5F) and epidermal growth factor receptor (EGF-R, Figure 5K) throughout all cell layers with no deposition of collagen IV at the epithelium-matrix interface (Figure 5P).

The presence of fibroblasts either in direct contact or at distance from the epithelium promoted formation of a fully maturated human buccal epithelium (Table 2) similar to the *in vivo* oral mucosa as judged after the panel of differentiation markers used in this study: uniform and strong expression of CK13 throughout all suprabasal epithelial cell layers (Figure 5D,E), polarization to the basal cell layer of β1 intergin, (Figure 5I,J) and EGF-R (Figure 5N,O), as well as synthesis and deposition of collagen IV at the epithelium-matrix interface (Figure 5S,T). Addition of KGF (0.1-100ng/ml) did not change the undifferentiated phenotype of the oral epithelium grown on simple collagen gels, as previously reported by our group (1). Addition of GM-CSF (>1ng/ml) induced the expression of CK13 in all suprabasal cell layers (Figure 5B), and polarization of β1 integrin (Figure 5G) to the basal cell layer. The admixture of GM-CSF and KGF (10ng/ml each) induced, in addition, polarization of EGF-R (Figure 5M) to the basal cell layer and a fine deposition of collagen IV at the epithelium-matrix interface (Figure 5R). This analysis shows that GM-CSF alone or in combination with KGF was able to induce major aspects of oral epithelial differentiation of *in vitro* reconstituted normal human oral epithelium. None of the other growth factors tested in the study (EGF, TGFα, IL-1α, HGF, SCF) did influence, when added, the phenotype of the epithelium grown on simple collagen gels.

Effects of KGF and GM-SCF on terminal differentiation of *in vitro* reconstituted normal human oral epithelium

The 3D monocultures of oral keratinocytes displayed TUNEL positive cells randomly distributed within the epithelium (Figure 5U). There was no polarization of TUNEL positive cells to the superficial layer, suggesting that cells did not complete the terminal stages of epithelial differentiation in these cultures. Similar pattern of distribution of TUNEL positive cells was also observed in the *3D* monocultures of oral keratinocytes supplemented with GM-CSF alone or in combination with KGF (Figure 5V,X respectively). Polarization of TUNEL positive cells to the superficial cell layer was observed only when fibroblasts were present in the connective tissue equivalent, either in direct contact or at distance from the epithelium (Figure 5Y,Z). Addition of an anti-GM-CSF antibody to the culture medium of fibroblast-containing cultures did not impair cell growth or the terminal differentiation of the reconstituted oral epithelium (Figure 2L). None of the other growth factors tested in the study (EGF, TGFα, IL-1α, HGF, SCF) did influence, when added, the distribution of TUNEL positive cells within the epithelium grown on simple collagen gels. This analysis shows that terminal differentiation of *in vitro* reconstituted normal human oral epithelium was induced by underlying fibroblasts through diffusible factors, but not by the combination of KGF and GM-CSF.

DISCUSSION

Formation and maintenance of mature oral epithelium rely on a tightly balanced process of keratinocyte proliferation and terminal differentiation (14), but the knowledge about the specific factors involved is limited. Previously, we have developed a highly standardised serum free organotypic 3D-OT model of human oral mucosa (15) and showed that fibroblasts are essential for differentiation of oral epithelium (1). Data presented here further demonstrate that fibroblast-derived diffusible factors are able to fully restore the differentiated phenotype of *in vitro* oral epithelium, including the fine-tunned terminal stage of epithelial differentiation. From all the growth factors tested in the present study, alone or in various combinations, only GM-CSF, alone or in combination with KGF, had a significant effect on the phenotype of oral epithelium. Previous reports from similar '3D organotypic' models of skin morphogenesis and homeostasis (3, 16, 17) identified also GM-CSF, alone or in combination with KGF, as a factor that induces a significant effect on the phenotype of epithelium. Of note, the skin 3D organotypic cultures supplemented with KGF only, displayed delays in expression of differentiation markers (18, 19). Other reports showed that dermal keratinocytes treated with KGF exhibited increased proliferation as well, and inhibited differentiation, while reduced KGF levels restored the expression of differentiation markers

(2). One possible explanation is that the secretion of high doses of KGF by fibroblasts might influence the choice between proliferation and differentiation (19). A limitation of the present study is that we did not test the effect of neutralizing antibodies for KGF, such that we could not infer more on the importance of this growth factor for the fine tunning of differentiation in oral epithelium.

The hematopoietic growth factor GM-CSF is another growth factor that was found to regulate dermal keratinocyte growth and differentiation (8), playing an important role during the process of wound healing (20). Fibroblast-keratinocyte interactions in skin models strongly enhanced the expression of GM-CSF (9), KGF and its receptor (21), while dermal keratinocyte-released IL-1 induced the expression of both KGF and GM-CSF (10). The regulatory mechanism of these two factors in skin homeostasis is a feedback loop within the multiple other epithelial-mesenchymal interactions: skin keratinocytes release IL-1α and IL-1β, which stimulate the release of KGF and GM-CSF by dermal fibroblasts. Then in turn, these two growth factors synthesized by dermal fibroblasts act on skin keratinocytes regulating their differentiation and proliferation (3, 7, 10).

In contrast to these reported observations from skin models proving that a combination if KGF and GM-CSF can substitute for the dermal fibroblasts and provide sufficient support for both growth and differentiation of skin keratinocyte in their absence (3, 16, 17), our current study shows that the final stages of oral epithelial maturation could not be restored by KGF and GM-CSF only. These differences between skin and oral mucosa morphogenesis might be due to the fact that oral mucosal fibroblasts and adult skin fibroblasts have different origin (the former originates from the neural crest and the latter from the mesoderm) and gene expressions, and consequently, different phenotypes and functions (22). Oral fibroblasts were proven to express higher levels of KGF and to accelerate much faster the collagen gel contraction than dermal stromal cells (23, 24). The results presented here, based on analysis of conditioned medium collected from oral fibroblasts maintained in *3D* monocellular cultures containing fibroblasts collagen matrices, show that oral fibroblasts synthesise considerable amounts of KGF, GM-CSF and HGF. These results corroborate with previous literature showing the oral fibroblasts to be the major producers of these growth factors (24-26), although oral keratinocytes have also been proven to synthesise GM-CSF (27).

That the mesenchymal cell source has a significant influence on the thickness and ultrastructure of the epithelium has been previously shown (28). Moreover, cytokeratin expression of the epithelial component was also proven to be strongly influenced by the origin of fibroblasts (29).

The data presented in this study indicate that in contrast to skin, other soluble factors than KGF and GM-CSF released from fibroblasts exert the final tuning of oral epithelial differentiation. In support for this conclusion comes also the observation that addition of neutralizing antibodies against human GM-CSF, previously shown to reduced keratinocyte proliferation and differentiation in skin models (7), did not impair cell proliferation or differentiation in our oral mucosa models. Taken together, the results of this study indicate that major aspects of oral epithelial differentiation are regulated by GM-CSF in combination with KGF, but its terminal stage is controlled by another yet unidentified fibroblast-derived diffusible factor.

ACKNOWLEDGEMENTS

The authors thank Mrs. Gunnvor Øijordsbakken (Gade Laboratory for Pathology) for support with histology and immunohistochemistry.

FUNDING INFORMATION

This work was funded by the Sectorial Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund, Romanian Government under the contract number SOP HRD/159/1.5/S/135760, Bergen Medical Research Foundation (Grant no. 20/2009), The Norwegian Cancer Research Association(Grant No. 515970/2011), Helse Vest (Grant No. 912260/2019), The Research Council of Norway through its Centers of Excellence funding scheme, (Grant No. 22325), and The Norwegian Agency for International Cooperation and Quality Enhancement in Higher Education (project number CPEA-LT-2016/10106).

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REFERENCES

1. Costea DE, Loro LL, Dimba EA, Vintermyr OK, Johannessen AC. Crucial effects of fibroblasts and keratinocyte growth factor on morphogenesis of reconstituted human oral epithelium. J Invest Dermatol. 2003;121:1479-86.
2. Pickard A, Cichon AC, Menges C, Patel D, McCance DJ. Regulation of epithelial differentiation and proliferation by the stroma: a role for the retinoblastoma protein. J Invest Dermatol. 2012;132:2691-9.
3. Maas-Szabowski N, Stark HJ, Fusenig NE. Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced keratinocyte growth factor expression in resting fibroblasts. J Invest Dermatol. 2000;114:1075-84.
4. Santosh AB, Jones TJ. The epithelial-mesenchymal interactions: insights into physiological and pathological aspects of oral tissues. Oncol Rev. 2014;8:239. doi: 10.4081/oncol.2014.239.
5. Dongari-Bagtzoglou A, Kashleva H. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. Nat Protoc. 2006;1:2012-8.
6. Calenic B, Ishkitiev N, Yaegaki K, Imai T, Costache M, Tovaru M, et al. Characterization of oral keratinocyte stem cells and prospects of its differentiation to oral epithelial equivalents. Rom J Morphol Embryol. 2010;51:641-5.
7. Maas-Szabowski N, Shimotoyodome A, Fusenig NE. Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism. J Cell Sci. 1999;112:1843-53.
8. Maas-Szabowski N, Szabowski A, Stark HJ, Andrecht S, Kolbus A, Schorpp-Kistner M, Angel P, Fusenig NE. Organotypic cocultures with genetically modified mouse fibroblasts as a tool to dissect molecular mechanisms regulating keratinocyte growth and differentiation. J Invest Dermatol. 2001; 116:816-20.
9. Zuliani T, Saiagh S, Knol AC, Esbelin J, Dreno B. Fetal fibroblasts and keratinocytes with immunosuppressive properties for allogeneic cell-based wound therapy. PLoS One. 2013;8:e70408. doi: 10.1371/journal.pone.0070408.
10. Maas-Szabowski N, Starker A, Fusenig NE. Epidermal tissue regeneration and stromal interaction in HaCaT cells is initiated by TGF-alpha. J Cell Sci. 2003;116:2937-48.

11. Kazimi BPS, Lewis MP. Oral and dermal fibroblast organotypic co-cultures show differences in phenotype. IADR 83rd General Session and Exhibition Baltimore,October 2005, Conference Abstract 0273. 2005.
12. Loro LL, Vintermyr OK, Ibrahim SO, Idris AM, Johannessen AC. Apoptosis and expression of Bax and Bcl-2 in snuff- and non-snuff associated oral squamous cell carcinomas. Anticancer Res. 2000;20:2855-60.
13. Loro LL, Vintermyr OK, Johannessen AC. Cell death regulation in oral squamous cell carcinoma: methodological considerations and clinical significance. J Oral Pathol Med. 2003;32:125-38.
14. Calenic B, Greabu M, Caruntu C, Tanase C, Battino M. Oral keratinocyte stem/progenitor cells: specific markers, molecular signaling pathways and potential uses. Periodontol 2000. 2015;69:68-82.
15. Costea DE, Dimba AO, Loro LL, Vintermyr OK, Johannessen AC. The phenotype of in vitro reconstituted normal human oral epithelium is essentially determined by culture medium. J Oral Pathol Med. 2005;34:247-52.
16. El Ghalbzouri A, Ponec M. Diffusible factors released by fibroblasts support epidermal morphogenesis and deposition of basement membrane components. Wound Repair Regen. 2004;12:359-67.
17. Florin L, Knebel J, Zigrino P, Vonderstrass B, Mauch C, Schorpp-Kistner M, et al. Delayed wound healing and epidermal hyperproliferation in mice lacking JunB in the skin. J Invest Dermatol. 2006;126:902-11.
18. Andreadis ST, Hamoen KE, Yarmush ML, Morgan JR. Keratinocyte growth factor induces hyperproliferation and delays differentiation in a skin equivalent model system. FASEB J. 2001;15:898-906.
19. Lotti LV, Rotolo S, Francescangeli F, Frati L, Torrisi MR, Marchese C. AKT and MAPK signaling in KGF-treated and UVB-exposed human epidermal cells. J Cell Physiol. 2007;212:633-42.
20. Mann A, Niekisch K, Schirmacher P, Blessing M. Granulocyte-macrophage colony-stimulating factor is essential for normal wound healing. J Investig Dermatol Symp Proc. 2006;11:87-92.
21. Di CP, Sun Y, Zhao L, Li L, Ding C, Xu Y, et al. Effect of nifedipine on the expression of keratinocyte growth factor and its receptor in cocultured/monocultured fibroblasts and keratinocytes. J Periodontal Res. 2013;48:740-7.

22. Mah W, Jiang G, Olver D, Cheung G, Kim B, Larjava H, et al. Human gingival fibroblasts display a non-fibrotic phenotype distinct from skin fibroblasts in three-dimensional cultures. PLoS One. 2014;9:e90715.
23. Cichon AC, Pickard A, McDade SS, Sharpe DJ, Moran M, James JA, et al. AKT in stromal fibroblasts controls invasion of epithelial cells. Oncotarget. 2013;4:1103-16.
24. Shannon DB, McKeown ST, Lundy FT, Irwin CR. Phenotypic differences between oral and skin fibroblasts in wound contraction and growth factor expression. Wound Repair Regen. 2006;14:172-8.
25. Stephens P, Hiscox S, Cook H, Jiang WG, Zhiquiang W, Thomas DW. Phenotypic variation in the production of bioactive hepatocyte growth factor/scatter factor by oral mucosal and skin fibroblasts. Wound Repair Regen. 2001;9:34-43.
26. Kim HS, Lee SG, Kim KH, Kim UK, Kim JR, Chung IK, Yang DK. Production of GM-CSF and TGF-beta 1 in Irradiated Human Gingival Fibroblasts Cultured with Lipopolysaccharide. Journal of the Korean Association of Oral and Maxillofacial Surgeons 2002;28:169-74.
27. Gonzalez OA, Ebersole JL, Huang CB. Supernatants from oral epithelial cells and gingival fibroblasts modulate human immunodeficiency virus type 1 promoter activation induced by periodontopathogens in monocytes/macrophages. Mol Oral Microbiol. 2010;25:136-49.
28. Kinikoglu B, Rovere MR, Haftek M, Hasirci V, Damour O. Influence of the mesenchymal cell source on oral epithelial development. J Tissue Eng Regen Med. 2012;6:245-52.
29. Smola H, Stark HJ, Thiekotter G, Mirancea N, Krieg T, Fusenig NE. Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. Exp Cell Res. 1998;239:399-410.

FIGURE LEGENDS

Figure 1. Step-by-step laboratory procedures for construction of three dimensional organotypic cultures using primary cells isolated from normal human oral mucosa.

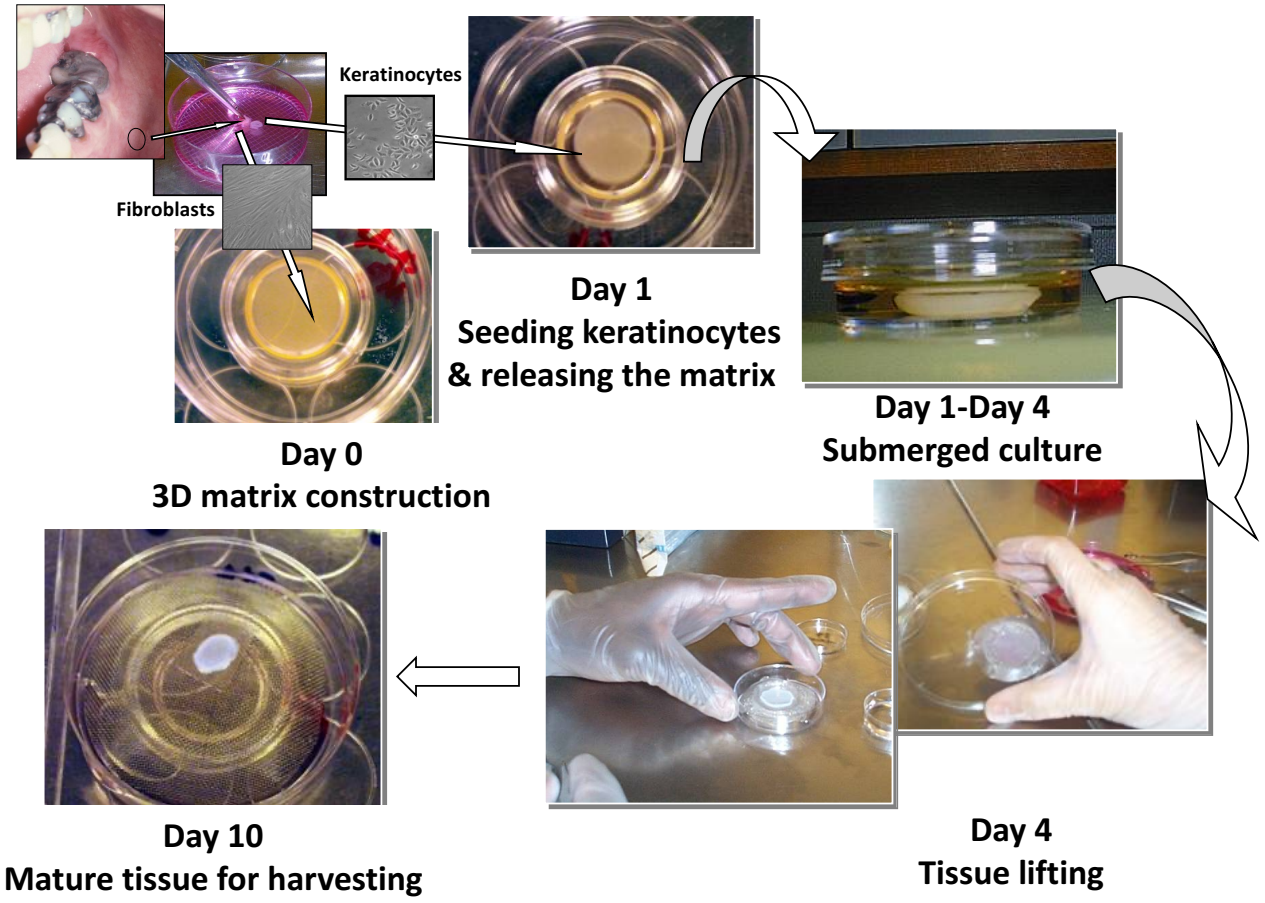
Figure 2. Effects of fibroblast-derived diffusible factors, granulocyte macrophage-colony stimulating factor (GM-CSF) alone or in combination with keratinocyte growth factor (KGF), and an antibody against GM-CSF on tissue morphology in *in vitro* reconstituted human oral epithelium. Three dimensional organotypic cultures were constructed with primary normal human oral keratinocytes on top of either simple collagen type I gels (A-I) and the cultures were grown in medium with various growth factors at 10ng/ml: epidermal growthfactor (B), KGF (C), GM-CSF (D), transforming growth factor alpha (E), interleukin 1 alpha (F), hepatocyte growthfactor (G), stem cell factor (H), or a combination of growth factors KGF and GM-CSF (I). Other three dimensional models were constructed by seeding normal oral keratinocytes on top of human fibroblast-containing collagen gels (J-L) either in direct contact (J and L) or at distance through a layer of simple collagen layer (sandwich models - K). An antibody against GM-CSF (L) was added to three dimensional cultures with fibroblast-containing collagen type I gels. All cultures were harvested on day 10 of co-culture. One half of each culture was fixed in 4% buffered formalin pH 7.15 and embedded in paraffin. Sections of representative cultures stained with haematoxylin & eosin are shown. Scale bar = 100 μm.

Figure 3. The effect of fibroblast-derived soluble factors on oral epithelial cell proliferation in *in vitro* reconstituted human oral epithelium. Human oral epithelium was *in vitro* reconstituted on simple collagen matrix (-Fibs) or on collagen gels populated with fibroblasts in direct contact (+Fibs) or at distance from the epithelial compartment in sandwich models and immunohistochemistry for Ki67 was performed in order to detect the proliferating cells. Immunohistochemistry pictures showing normal oral keratinocytes cells from the same patient grown on top of collagen matrices without any additional growth factors (A), with 10ng/ml keratinocyte growth factor (KGF) (B), with 10ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) (C), with a combination of 10ng/ml KGF and 10ng/ml GM-CSF (D), in sandwich models (E) and on top of fibroblasts-populated collagen gels (F). Bars (mean of duplicate three dimensional cultures constructed with cells from n=5 donors) and standard

deviations show the percentage of Ki67 positive cells among the cells of the basal cell compartment (G).

Figure 4. Quantification of growth factors synthesized by normal oral fibroblasts in three dimensional monocellular cultures. Graph showing secretion of various growth factors and cytokines determined by ELISA for normal oral fibroblasts grown in three dimensional biomatrices. Bars (mean of triplicate 3D cultures containing fibroblasts only in collagen gels, constructed with cells from n=5 donors) and standard deviations are shown (n=5).

Figure 5. The effect of fibroblast-derived diffusible factors (sandwich model) and granulocyte macrophage-colony stimulating factor alone or in combination with keratinocyte growth factor on the phenotype of *in vitro* reconstituted normal human oral epithelium. The cultures were grown for 10 days in the absence (A, B, C, F, G, H, K, L, M, P, Q, R, U, V, X) or presence of fibroblasts in direct contact (E, J, O, T, Z) or at distance through a layer of simple collagen layer (sandwich models – (D, I, N, S, Y) in the collagen matrix. Granulocyte macrophage-colony stimulating factor alone (B, G, L, Q, V) or in combination with keratinocyte growth factor (C, H, M, R, W has been added to some of the three dimensional organotypic cultures in absence of fibroblasts. Immunohistochemistry for cytokeratin 13 (A-E), β1-integrin (F-J), epidermal growth factor receptor (K-O), collagen IV (P-T), and the TUNEL method (U-Z) are shown. Scale bar = 50μm.



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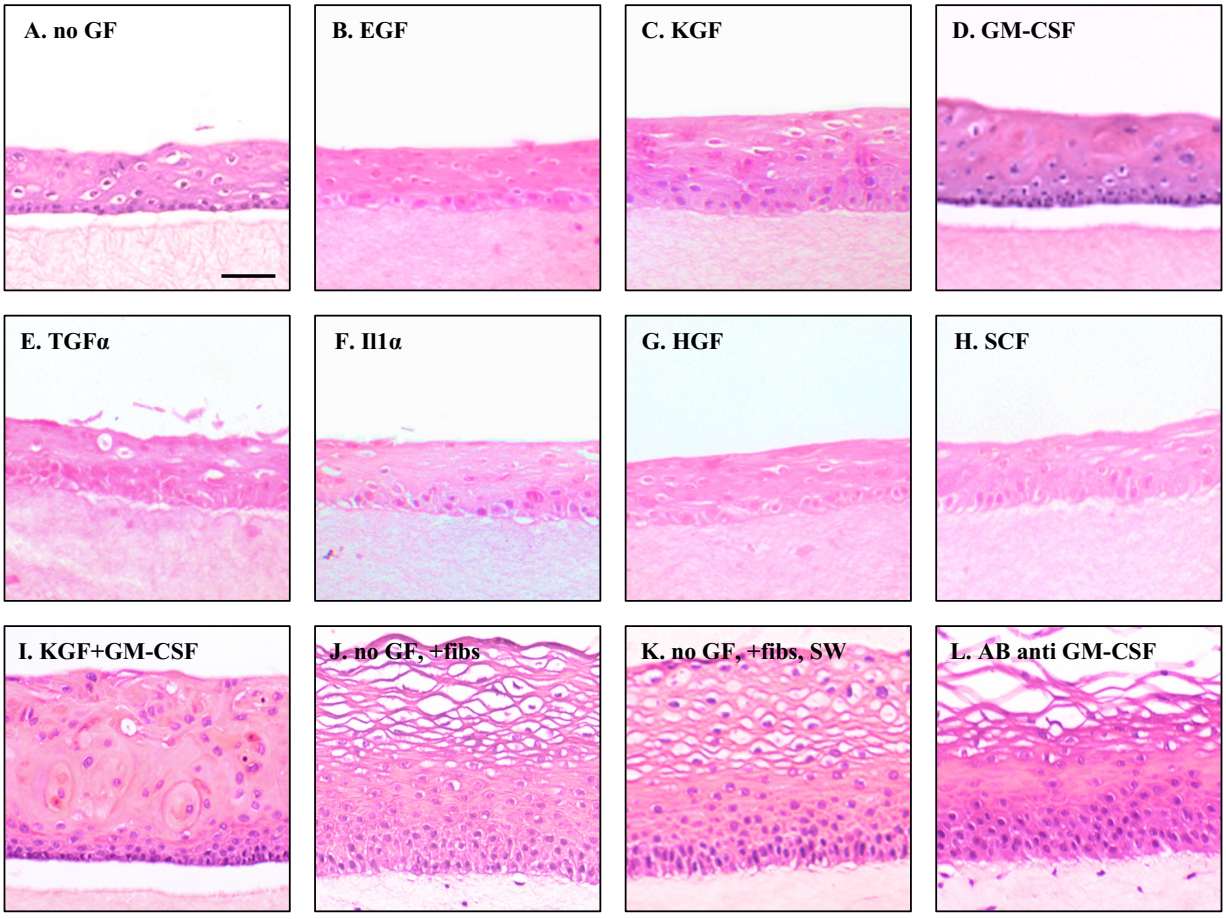

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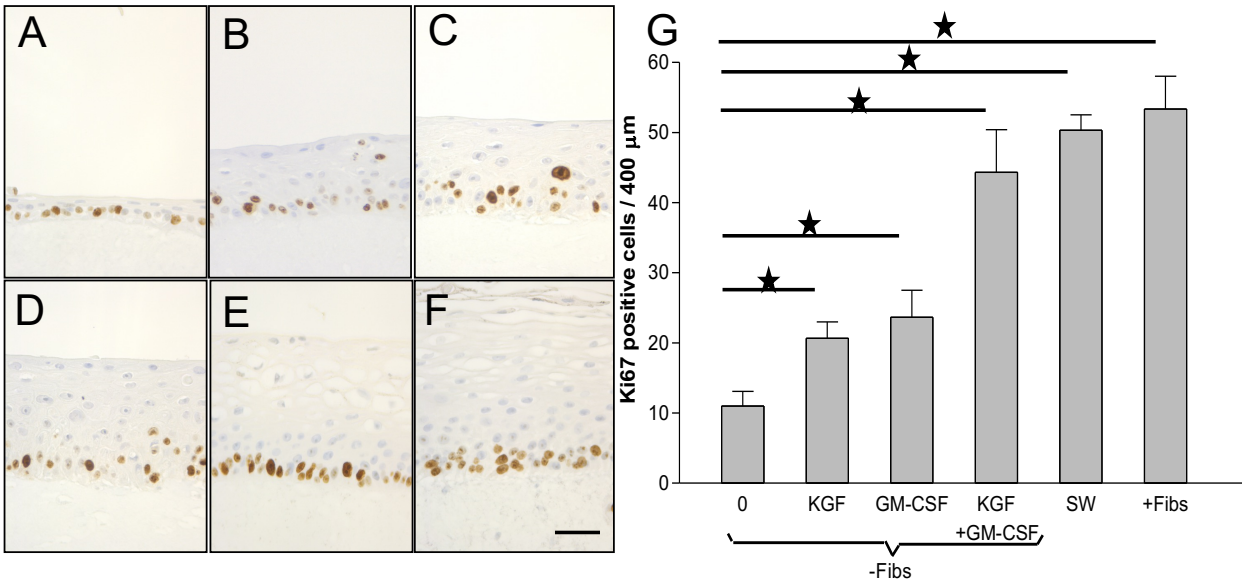

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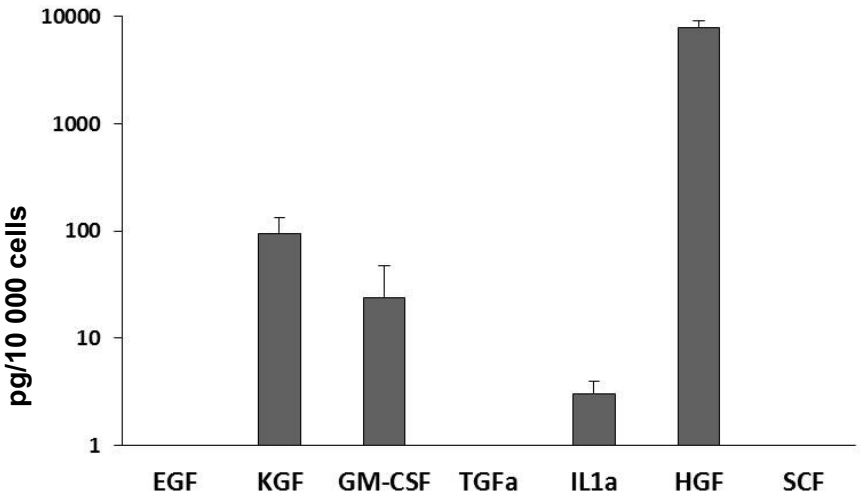

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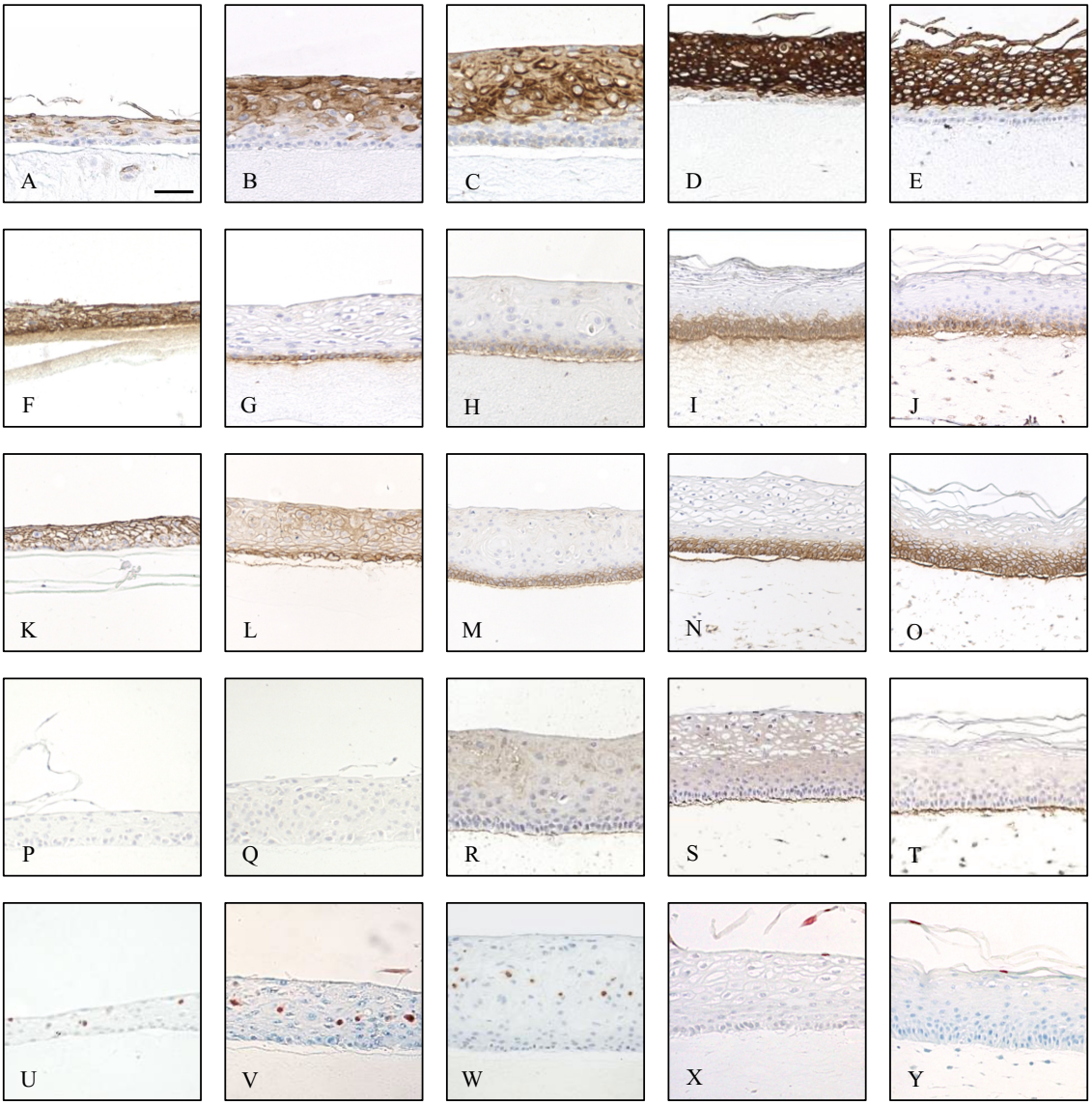

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Table 1. Demographics (age, gender, tobacco use) of the donors included in the study and the usage of the tissues harvested.

Donor ID	Age	Gender	Tobacco use	Usage
41	44	m	no	frozen
42	24	m	no	frozen
49	20	m	no	frozen
62	20	f	no	frozen
77	35	f	no	frozen
92	34	m	no	frozen
98	23	m	no	frozen
43	20	f	no	FFPE
44	35	m	no	FFPE
45	31	m	no	FFPE
55	23	m	no	FFPE
57	26	f	no	FFPE
59	22	m	no	FFPE
48	25	f	no	isolating cells
60	22	m	no	isolating cells and FFPE
63	24	f	no	isolating cells
80	24	f	no	isolating cells
93	25	m	no	isolating cells

Abbreviation: FFPE = formalin fixed and paraffin embedded.

Table 2. Table showing the growth factors added to the 3D-OT cultures and the outcomes of the different combinations in terms of the presence of different epithelial cell layers: presence of spinous cell layer indicates that the cells underwent early differentiation in those culture conditions; presence of superficial cell layer indicates that the cells underwent full differentiation in those culture conditions.

Growth factor/fibs	**Concentration (ng/ml)**	**Basal cell layer**	**Spinous cell layer**	**Superficial cell layer**
none	-	yes	no	no
EGF	10	yes	no	no
EGF	10			
+ KGF	0.1	yes	no	no
	1	yes	no	no
	10	yes	no	no
	100	yes	no	no
EGF	10			
+ GM-CSF	10	yes	no	no
EGF	10			
+ HGF	10	yes	no	no
EGF	10			
+TGFα	10	yes	no	no
+IL-1α	10	yes	no	no
KGF	0.1	yes	no	no
	1	yes	no	no
	10	yes	no	no
	100	yes	no	no
KGF	10			
+ GM-CSF	0.1	yes	no	no
	1	yes	no	no
	10	yes	yes	no
	100	yes	yes	no
KGF	10			
+ HGF	10	yes	no	no
KGF	10			
+ GM-CSF	10			
+ TGFα	10	yes	yes	no

GM-CSF	0.1	yes	no	no
	1	yes	no	no
	10	yes	yes	no
	100	yes	yes	no
GM-CSF	10			
+ HGF	10	yes	yes	no
GM-CSF	10			
+ TGFα	10			
+ HGF	10	yes	yes	no
TGFα	10	yes	no	no
TGFα	10			
+ HGF	10	yes	no	no
IL-1α	10	yes	no	no
HGF	10	yes	no	no
SCF	10	yes	no	no
All growth factors	10	yes	no	no
fibs	-	yes	yes	yes
sandwich	-	yes	yes	yes

Abbreviations: EGF=epidermal growth factor; KGF=keratinocyte growth factor; GM-CSF= granulocyte macrophage-colony stimulating factor; HGF=hepatocyte growth factor; TGFα= transforming growth factor α; SCF=stem cell factor

Paper II

Odontology (2019) 107:291–300
https://doi.org/10.1007/s10266-018-0397-7

ORIGINAL ARTICLE

Isolation and characterization of cells derived from human epithelial rests of Malassez

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Received: 19 March 2018 / Accepted: 14 October 2018 / Published online: 26 November 2018

Abstract
The epithelial rests of Malassez (ERMs) might represent a valuable source of oral epithelial cells with stem cell properties. The purpose of this study was to isolate and characterize cells derived from human ERM, and compare them with cells derived from matched normal oral mucosa (NOM). Matched tissue specimens of the periodontal ligament of extracted tooth and NOM were collected. Cells were isolated in culture, then characterized by immunohistochemistry and flow cytometry for expression of pancytokeratin, ESA, PDGFRB, CD31 and CD44. 3D organotypic cultures were constructed by growing epithelial cells on top of fibroblast-populated collagen gels. Both ERM and NOM-isolated cells expressed the markers of epithelial lineage (ESA and pancytokeratin), and to some extent PDGFR, an indicator of a more mesenchymal phenotype, but not the endothelial cell marker CD31. Cells with epithelial morphology were isolated from periodontium of cervical, middle and apical parts of the root, but contained a significantly lower percentage of ESA and pancytokeratin-positive cells than when isolating cells from NOM ($p < 0.001$). ERM cells expressed a significantly higher percentage of the stem cell-related molecule CD44 (cervical $92.93 \pm 0.25\%$, middle $93.8 \pm 0.26\%$, apical $94.36 \pm 0.41\%$) than cells isolated from NOM ($27.8 \pm 1.47\%$, $p < 0.001$). When grown in 3D organotypic cultures and in collagen gels, ERM cells formed a less differentiated epithelium than NOM cells, but expressing pancytokeratin and vimentin. In conclusion, epithelial cells could be isolated from human periodontium and grown in culture; their in vitro characterization indicates that they have a less differentiated phenotype compared with cells derived from normal oral epithelium.

Keywords Rests of Malassez · Periodontal ligament · Immunohistochemistry · Human · Oral mucosa

Introduction

Hertwig's epithelial sheath is the origin of the epithelial rests of Malassez (ERMs) and contributes to the growth of roots. ERMs are considered to participate in the development of radicular cysts. Hertwig's epithelial sheath and ERMs thus play important roles, in both physiological and pathological root-related processes, but their characterization of and participation in these processes are not yet known.

Mutual epithelial–mesenchymal interactions are thought to play an important role in tooth growth and morphogenesis. When tooth root formation starts, the internal and external enamel epithelium, which have completed crown formation, bend at the tooth cervix and extend its epithelial tip to differentiate into Hertwig's epithelial sheath, which separates the dental papilla and dental sac, resulting in the formation of the tooth root [1, 2].

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Once tooth root formation is complete, Hertwig's epithelial sheath contracts, but a part of it remains in the periodontal ligament space as ERMs. Orban and Weinmann [3] reported that ERMs near the tooth cervix bind with the junctional epithelium and convert to pocket epithelium. It also appears that ERMs that are normally in a static state start to proliferate and form the epithelial lining of radicular cysts when infectious antigenic substances resulting from dental pulp diseases are discharged from the root canal through the apical foramen [4, 5]. Apical periodontal diseases include suppurative apical periodontitis and chronic inflammation, the latter of which may be classified into periapical granuloma or radicular cyst. Clinically, radicular cysts are more likely to be refractory than the former two conditions, which may be related to the fact that the inner wall of the cyst is lined with epithelium.

There are many unanswered questions, such as why do ERMs remain around the tooth apex after completing their role in root formation, and what is the mechanism of the switch from the quiet, normal state, to the proliferative state leading to the formation of epithelium on radicular cysts as a result of stimulation?

We hypothesized that our understanding of the mechanisms of radicular cyst development will advance by isolating cells from ERMs and learning about their characteristics.

The purpose of this study was to characterize the cells derived from human ERM and compare them with cells derived from matched normal oral mucosa (NOM).

Materials and methods

Tissue specimens and primary cell isolation

Matched tissue specimens from the periodontal ligament (PDL) of extracted tooth and NOM were collected after informed consent from healthy patients undergoing wisdom tooth extraction ($N=3$). Cells were isolated in culture following the standard explant method at 37 °C in 5% CO_2, in a humidified incubator. Cells were used from the second or third passage.

Extracted teeth and NOM samples were transported on ice in transport medium: Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) with 2% antibiotics–antimycotics (GibcoBRL, Grand Island, NY, USA). After transport, the extracted teeth and biopsy were washed twice, 5 min each time, with fresh transport medium.

The PDL attached to the cervical, middle and apex one-third of the root was removed with a scalpel and collected separately under a dissecting microscope. PDL and NOM tissues were cut in approximately 1 mm^3 pieces, allowed to adhere to cell culture dishes (Nunclon™ Delta, Thermo Fisher Scientific, CA, US) by letting them for 3–5 min to air dry opened in the sterile hood. After that, culture medium was gently added to the dish, avoiding detachment of the tissue explants. The culture medium used was FAD-FBS medium: DMEM/HAM's F12: 3/1 with 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 20 µg/ml transferrin, 50 µg/ml L-ascorbic acid (all from Sigma). Mitomycine C (Sigma)-inactivated 3T3 fibroblasts (10–100 µl/ml of mitomycin C solution per milliliter of culture medium for 2 h) were added to the dishes planed for isolation of epithelial cells and incubated in keratinocyte serum-free media (KSFM, GibcoBRL) supplemented with 1 ng/ml epidermal growth factor (EGF human recombinant), 25 µg/ml bovine pituitary extract (BPE), 20 µg/ml L-glutamine, 1% AB/AM (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) (all from GibcoBRL). The dishes planed for isolation of fibroblasts were incubated in fibroblast-specific medium (DMEM supplemented with 10% FBS, Sigma, 20 µg/ml L-glutamine, and 1% AB/AM). Outgrowths of cells from tissue explants were morphologically assessed. Despite incubation in lineage-specific medium, some outgrowths of the other cell type could be observed sporadically in the dishes. For proper separation, the epithelial- or fibroblast-looking outgrowths were separately detached from dishes using plastic cloning rings (Sigma) attached with Vaseline (Sigma) on the dishes around individual explants with a specific cellular morphology. Trypsin 10× (Sigma) was added inside the clonal rings, and the cells surrounding an explant with a uniform morphology of either epithelial, or fibroblastic phenotype were detached. Cells with the same morphology from different explants were then pooled together to eliminate the risk of clonality of isolated cells and farther propagated in lineage-specific medium.

All cells were used in their third to fourth passage (split ratio of 1:4), at a viability more than 80%, kept in a humidified atmosphere with 5% CO_2 at 37 °C.

Immunohistochemistry and flow cytometry

Cells were grown on 16 mm^2 cover-slips in 12-well plates, in their respective growth medium. After 5 days, cells were fixed in 4% formalin for 20 min at room temperature (RT) and kept in PBS at 4 °C until used. Antibody against pancytokeratin (DAKO, Glostrup, Denmark) was used for 1 h. Afterwards, the Envision + system-HRP stain system (DAKO) was used following manufacturer's instructions, for 30 min. The presence of antigen was visualized with DAB (3,3′diaminobenzidine, DAKO) for 10 min. The slides were counterstained with haematoxylin (DAKO), dehydrated through an ascending

graded series of alcohol, xylene and then mounted with an alcohol soluble mounting medium (Eukit, DAKO). Sections treated with antibody diluent instead of primary antibody were used as negative controls. For staining of 3D organotypic cultures and gels, 3 µm sections were cut, deparaffinized and rehydrated by immersion in xylene and diminishing concentrations of alcohol. Retrieval of the epitope was performed by heating the sections in a microwave oven in a pH 6.0 target retrieval buffer (DAKO). For pancytokeratin staining, sections were incubated with 1× proteinase K for 10 mins at room temperature. Endogenous enzyme activity and unspecific binding were blocked using peroxidase block and 10% normal goat serum respectively (both from DAKO). Sections were then incubated over night at 4 °C with one of the following monoclonal mouse anti-human primary antibodies: anti-pancytokeratin (1:2000, DAKO), and anti-vimentin (1:2000, DAKO). Envision+® anti-mouse (DAKO) was used to detect the site of reaction according to the manufacturer`s instructions. And, the reaction was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB). Incubation with primary antibody was omitted for negative control sections, and normal human oral mucosa samples have been used as a positive control. Sections were then counterstained with hematoxylin (DAKO), dehydrated and cover-slipped.

For fluorescent activated cell sorting (FACS), cells were detached using trypsin-EDTA 2.5% (Sigma), then stained with the following antibodies: anti-ESA-APC conjugated (Biomed, USA), anti-PDGFRB-PE conjugated (CD140b-PE conjugated, R&D Systems, UK), anti-CD44-PE (R&D Systems, USA), anti-CD31-PE conjugated (R&D Systems, USA), and isotype control IgG2ak-PE and IgG2ak-APC (R&D Systems, USA) at 1:100 dilution in phosphate-buffered saline (PBS) (Invitrogen). DAPI nuclear dye (Sigma) was used at 1 µg/ml to exclude dead cells. All analyses were performed on the FACS aria SORP (Becton Dickinson, USA).

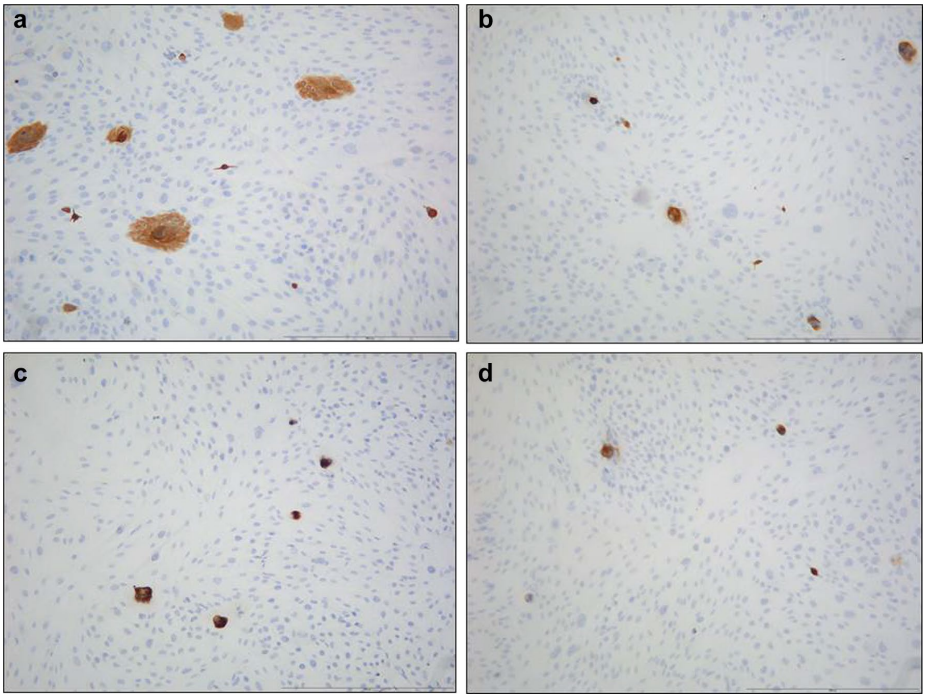

Fig. 1 Pancytokeratin staining of cells isolated from NOM and ERM grown in monolayer. **a** Primary gingival keratinocytes from NOM. **b** Primary cells isolated from ERM at cervical part of the root(REM-C). **c** Primary cells isolated from ERM at middle part of the root(REM-M). **d** Primary cells isolated from ERM at apical part of the root (REM-A) (original magnification ×100, scale bar 100 µm). Cells with epithelial morphology and expressing pancytokeratin could be isolated from both ERM and NOM periodontium. However, the number of pancytokeratin-positive cells isolated from PDL at all root levels was very low, significantly lower than when isolating cells from NOM ($p < 0.001$)

3D assays

3D organotypic cultures were obtained by growing epithelial cells on top of fibroblast-populated collagen type I (BD Biosciences) biomatrices, using a protocol well-established in our laboratory [6]. The organotypic cultures were grown in serum-free FAD medium without addition of EGF. The cultures were lifted at air–liquid interface at day 4 and harvested after 10 days, formalin fixed and paraffin embedded or fresh frozen, as previously described.

The epithelial cells were also incubated in collagen gels, formalin fixed, paraffin embedded and sectioned. These 3D sections were stained hematoxylin and eosin.

Statistical analysis

Data were presented as mean ± SD. One-way Anova was used to compare the expression of different markers in the isolated cells. At least three repeats were performed. All statistical analyses were performed using the statistical package IBM SPSS version 15 (IBM, USA). p values less than 0.01 were considered statistically significant.

Results

Cells with epithelial morphology and expressing pancytokeratin could be isolated (with a similar success rate) from periodontium of cervical (REM-C), middle (REM-M) and apical (REM-A) parts of the root (Fig. 1). However, the number of pancytokeratin-positive cells isolated from PDL at all root levels was very low, significantly lower than when isolating cells from NOM ($p < 0.001$) (Fig. 1).The pattern of growth in culture was also different, with ERM cells forming a network of cellular strands while NOM cells formed a uniform, continuous sheet of monolayer cells (Fig. 2).

Both ERM and NOM cells expressed the markers of epithelial lineage ESA (Fig. 3) and pancytokeratin (Fig. 1), and to some extent PDGFR (CD140b), an indicator of a more mesenchymal phenotype (Fig. 4), but not the endothelial cell

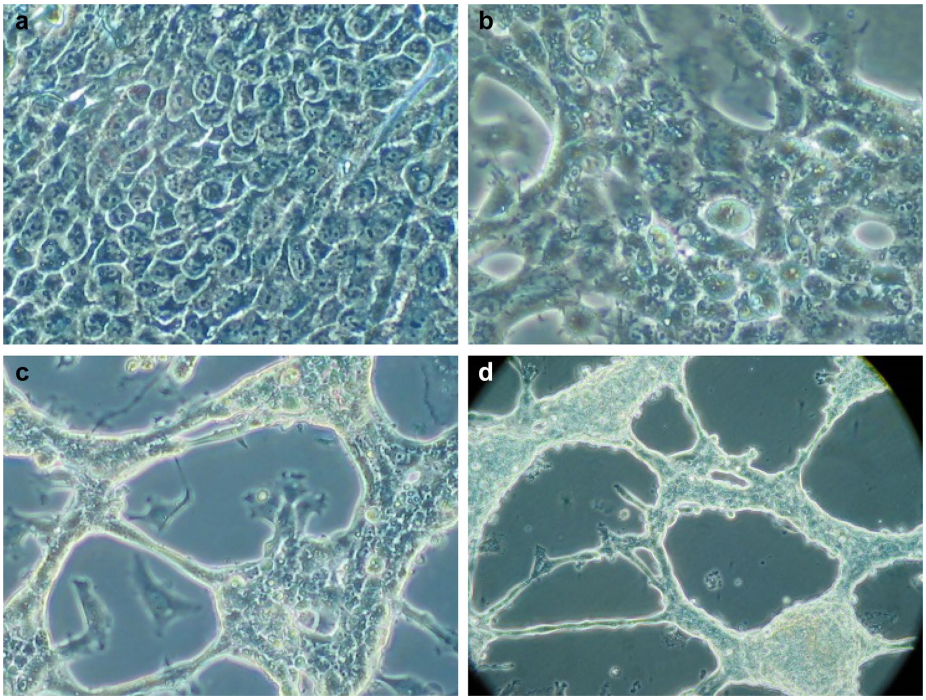

Fig. 2 The pattern of growth in culture from human NOM and ERM grown in monolayer. **a** Primary gingival keratinocytes from NOM. **b** Primary cells isolated from ERM-C. **c** Primary cells isolated from ERM-M. **d** Primary cells isolated from ERM-A. The pattern of growth in culture was also different, with ERM cells forming a network of cellular strands while NOM cells formed a uniform, continuous sheet of monolayer cells (original magnification ×400 for **a** and **b**, ×200 for **c** and ×100 for **d**)

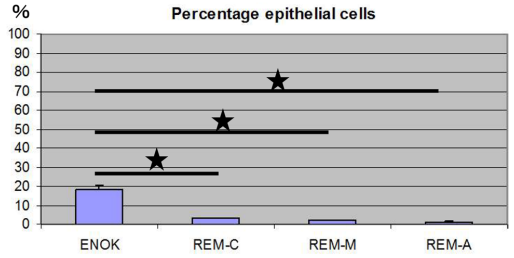

Fig. 3 Percentage of epithelial cells (ESA positive cells) by flow cytometry. Both ERM and NOM(ENOK) cells expressed the markers of epithelial lineage ESA. The statistical significant difference was accepted between NOM and REM-C, NOM and REM-M and NOM and REM-A

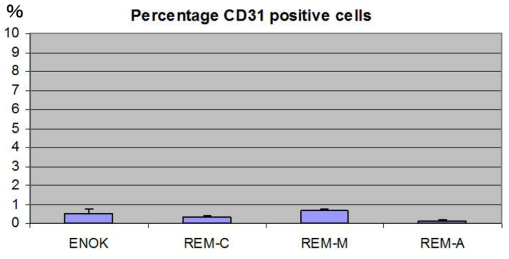

Fig. 5 Percentage of CD31 positive cells by flow cytometry. ERM and NOM(ENOK) cells did not express the endothelial cell marker CD31 so much. There was no significant difference in each cell which appeared to be statistical

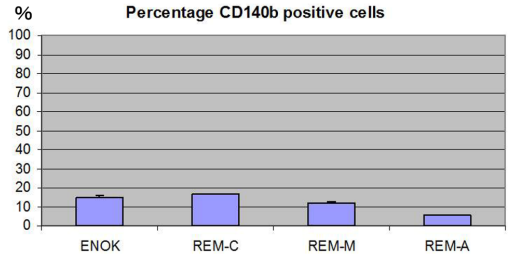

Fig. 4 Percentage of PDGFR positive cells by flow cytometry. Both ERM and NOM(ENOK) cells expressed to some extend PDGFR (CD140b), an indicator of a more mesenchymal phenotype. There was no significant difference in each cell which appeared to be statistical

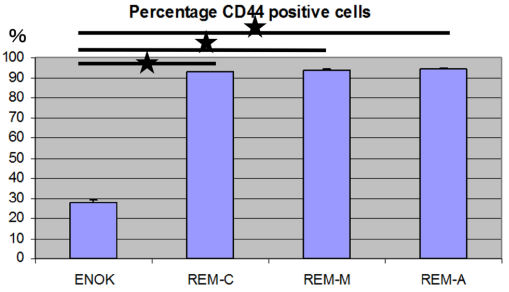

Fig. 6 Percentage of CD44 positive cells by flow cytometry. ERM cells expressed a significantly higher percentage of the stem cell-related adhesion molecule CD44 (cervical 92.93 ± 0.25%, middle 93.8 ± 0.26%, apical 94.36 ± 0.41%) than cells isolated from NOM (27.8 ± 1.47%, $p < 0.001$). The statistical significant difference was accepted between NOM and REM-C, NOM and REM-M and NOM and REM-A

marker CD31 (Fig. 5). ERM cells expressed a significantly higher percentage of the stem cell-related adhesion molecule CD44 (cervical 92.93 ± 0.25%, middle 93.8 ± 0.26%, apical 94.36 ± 0.41%) than cells isolated from NOM (27.8 ± 1.47%, $p < 0.001$) (Fig. 6).

When grown in 3D organotypic cultures (Fig. 7) and in collagen gels (Fig. 8), ERM formed a less differentiated epithelium. ERM cells grown in 3D organotypic culture did not show any signs of differentiation. The cells forming the epithelium had a basaloid appearance throughout the whole epithelial thickness, in contrast to the epithelium formed by the cells isolated form NOM, that showed a distinct basal cell layer and upper, more differentiated cell layers.

When grown in 3D, but imbedded within collagen gels and not on top of the collagen gels, the NOM cells formed small islands with central differentiation. ERM cells did not agglomerate; the ERM cells grew alone, as individual cells, detached from each other.

Epithelium formed by both NOM and ERM cells when grown in 3D organotypic cultures showed positive staining for pancytokeratin (Fig. 9). The intensity of the staining gradually decreased from NOM and REM-C till REM-A, which showed the weakest expression of pancytokeratin. Of note, the pancytokeratin-positive cells in the basal layer of the epithelium formed by REM-A in 3D organotypic cultures displayed an elongated, mesenchymal-like morphology. The small islands formed by NOM cells when grown in 3D gels showed also an intense expression of pancytokeratin (Fig. 10). ERM cells grown in gels showed as well pancytokeratin positivity but much weaker. Interestingly, and most predominantly observed in REM-A gels, the pancytokeratin-positive cells displayed a mixture of shapes, from rounded, epithelial morphology to elongated, mesenchymal morphology.

Staining for vimentin showed that epithelium formed by NOM in 3D organotypic cultures showed scattered positive cells localized mainly to the basal cell layer (Fig. 11). Epithelium formed by REM cells showed

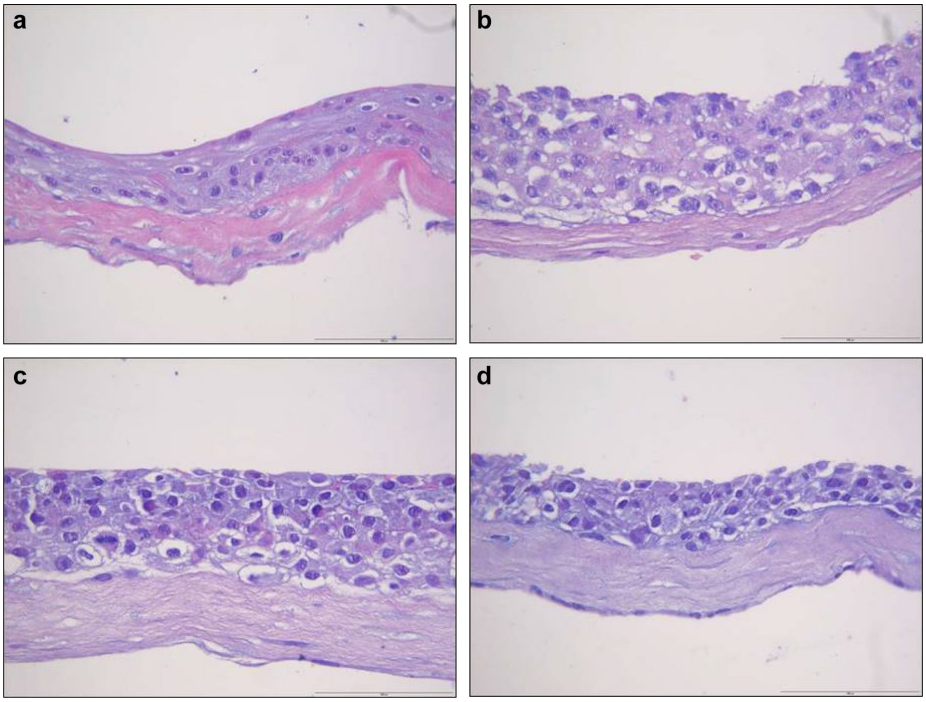

Fig. 7 NOM and ERM cells grown in 3D organotypic culture. **a** NOM. **b** REM-C. **c** REM-M. **d** REM-A (original magnification ×200, scale bar 100 μm). ERM formed a less differentiated epithelium. ERM cells grown in 3D organotypic culture did not show any signs of differentiation. The cells forming the epithelium had a basaloid appearance throughout the whole epithelial thickness, in contrast to the epithelium formed by the cells isolated form NOM, that showed a distinct basal cell layer and upper, more differentiated cell

intense vimentin staining throughout all cell layers, indicating less epithelial differentiation of these cells. All cells showed intense vimentin staining when cultured in gels (data not shown).

Discussion

Serres [7] first reported that epithelial cell populations existed in PDL tissue. Subsequently, in 1884, Malassez [8] confirmed the existence of epithelial components as well as the idea that these components were the remains of Hertwig's epithelial sheath within the PDL spread, garnering ERMs much attention.

It has been reported that ERMs are often composed of several cell populations [9, 10] and have elliptical, funicular, or tufted shapes; they may also be reticulate and wrap around the tooth root or form a network with the junctional epithelium [11–14].

Static, proliferative, degenerative, and differentiated states of ERM dynamics have been observed, with some reports also demonstrating division [15, 16].

Ten Cate [17] and Gilhuus-Moe and Kvam [18] reported that these cells proliferate under certain conditions according to radioactive isotopes, electron microscopy, and histological investigations.

It has been reported that ERMs remain near the tooth cervix, bind with the junctional epithelium, and form pocket epithelium [3]. This, along with the idea that infectious antigenic substances resulting from dental pulp diseases are discharged from the root canal through the apical foramen, the proliferation of normally static ERMs is started, thereby causing them to form the lining epithelium of radicular cysts, which is also supported by the fact that ERMs start to proliferate as a result of culture conditions and stimulation [4, 19, 20].

Reported ERM functions include maintaining PDL space width [21, 22], stimulating dental cement formation [23], protecting root resorption [24], controlling ankylosis, tooth instability, and alveolar resorption [25], and being involved

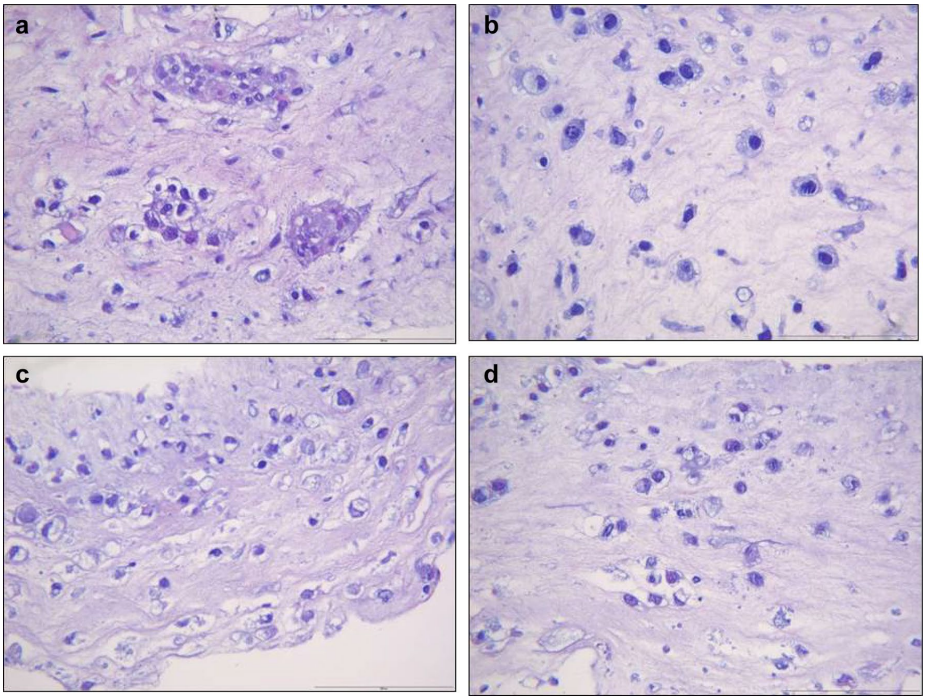

Fig. 8 NOM and ERM cells grown in collagen gels. **a** NOM. **b** REM-C. **c** REM-M. **d** REM-A (original magnification ×200, scale bar 100 μm). When grown in 3D, but imbedded within collagen gels and not on top of the collagen gels, the NOM cells formed small islands with central differentiation. ERM cells did not agglomerate; the ERM cells grew alone, as individual cells, detached from each other

in alveolar resorption from marginal periodontitis caused by prostaglandin and non-prostaglandin bone resorption activity factors [26].

Since stem cells were isolated from dental pulp, it has been suggested that post-embryonic stem cells might also exist in the periodontal tissue of human adults [27]. However, the presence of stem cells in PDL received a large amount of attention in 2004 after Seo et al. [28] reported multipotent cells in human PDL. CD44 is an adhesive molecule that binds with extracellular matrices such as hyaluronic acid and is strongly involved in lymphocyte homing, lymphocyte activation, cell-to-cell adhesion, cell-to-matrix adhesion, and cell movement, as well as cancer cell proliferation and metastasis. Thus, it has also been acknowledged as a stem cell marker for various types of solid cancers. In normal tissue, CD44 is distributed throughout various cell lines, including hematopoietic cells, fibroblasts, epithelial cells, vascular endothelial cells, muscle cells, and neuroglial cells and is either expressed or absent in the differentiation and proliferation of each of these cell lines. In squamous epithelial mucosa, CD44 expression is enhanced at the base where proliferation is strong, while it is weakly expressed or not present on the surface areas [29], Moreover, it may be related to the differentiation and proliferation of hematopoietic stem cells and B cells in the dental pulp [30, 31]. Thus, it appears that CD44 is involved in morphogenesis, wound healing, and tumor progression as an extracellular matrix for cell movement. It is noteworthy that the results of FACS analysis revealed that the CD44-positive cell ratio was much higher in cells obtained from PDL than in the oral mucosa.

When grown in 3D organotypic cultures and in collagen gels the cells derived from PDL formed a less differentiated epithelium that expressed weaker pancytokeratin and stronger vimentin, indicating that these cells have a less epithelial phenotype and a more mesenchymal phenotype. That might indicate that those cells are less differentiated and more EMT than the cells derived from NOM. Accordingly, this might be the reason that they did not form a well-differentiated and keratinized epithelium in 3D organotypic cultures, such as the epithelium formed by the cells derived from NOM.

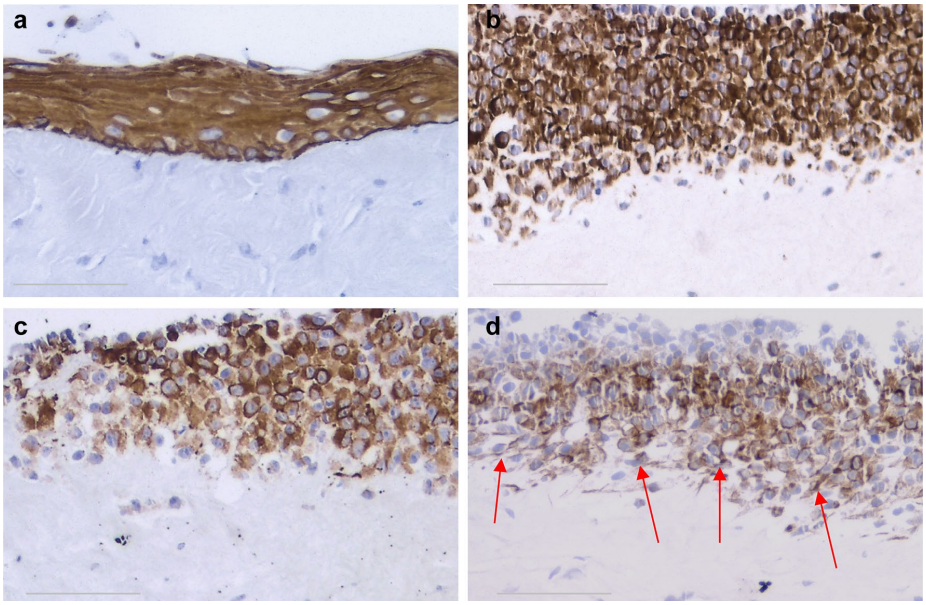

Fig. 9 Pancytokeratin staining of NOM and ERM cells grown in 3D organotypic culture. **a** NOM. **b** REM-C. **c** REM-M. **d** REM-A (original magnification ×100, scale bar 50 µm). Epithelium formed by both NOM and ERM cells showed positive staining for pancytokeratin. However, the intensity of the staining gradually decreased from NOM and REM-C till REM-A, which showed the weakest expression of pancytokeratin. Note the pancytokeratin-positive cells in the basal layer of the epithelium formed by REM-A that display an elongated, mesenchymal morphology (arrows)

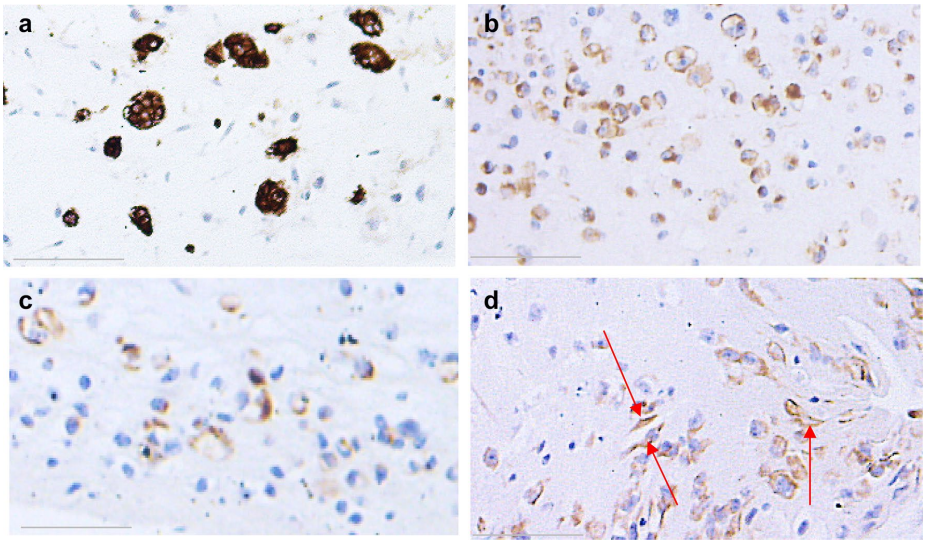

Fig. 10 Pancytokeratin staining of NOM and ERM cells grown in collagen gels. **a** NOM. **b** REM-C. **c** REM-M. **d** REM-A (original magnification ×100, scale bar 100 µm). The small islands formed by NOM cells showed intense pancytokeratin staining. ERM cells grown in gels showed also pancytokeratin positivity but much weaker. Note that the REM-A pancytokeratin-positive cells display a mixture of shapes, from rounded, epithelial morphology to elongated, mesenchymal morphology (arrows)

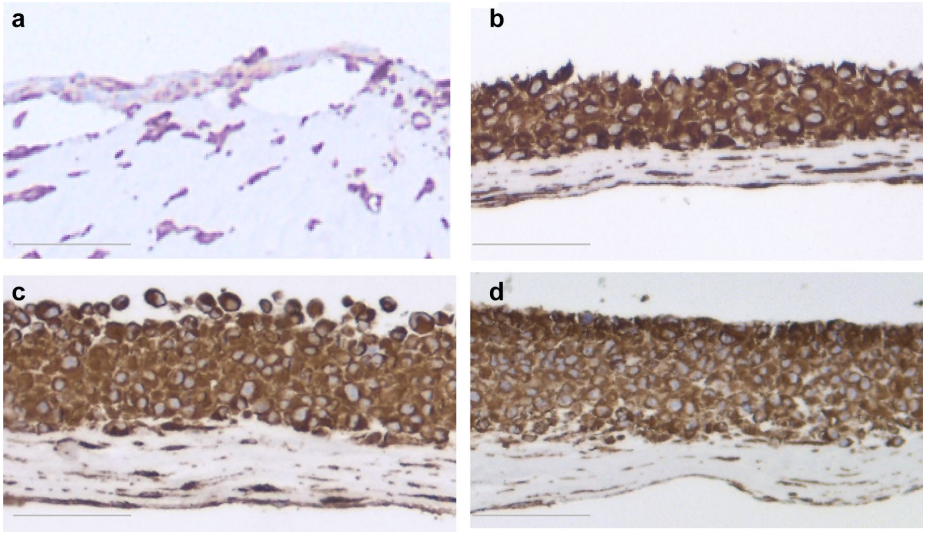

Fig. 11 Vimentin staining of NOM and ERM cells grown in 3D organotypic culture. **a** NOM. **b** REM-C. **c** REM-M. **d** REM-A (original magnification ×100, scale bar 50 μm). Epithelium formed by showed scattered positive cells localized mainly to the basal cell layer. Epithelium formed by REM cells showed intense vimentin staining throughout all cell layers

When grown inside the gels, the cells derived from NOM grew more clustered, in groups, while the epithelial cells derived from REM grew alone in the matrix. This again might indicate that the cells from NOM are probably more differentiated and express more epithelial cell-to-cell adhesion molecules, while cells derived from ERM are less differentiated.

Our results are in line with the results published in 2016 by Tsunematsu et al. [32]. They have isolated odontogenic epithelial cells with epithelial marker-positive and mesenchymal marker-negative features from ERMs in human PDL and reported that they have stem cell-like characteristics. The findings we present here bring new information about the extent of the stemness of the differentiation abilities of the cells derived from ERMs compared to the epithelial cells derived from NOM.

We anticipate that the cell groups isolated here will be investigated in more detail in the future for their possible involvement in cyst formation, by developing an experimental model for radicular cyst formation. This model will also provide a valuable experimental biological system for testing of novel, alternative ways of treatment for radicular cysts.

Conclusions

Epithelial cells could be isolated from the REM existent in adult human periodontium and grown in culture. Their in vitro characterization indicates that cells derived from ERM have a less differentiated phenotype compared with cells derived from normal oral epithelium.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The project was approved by the Committee for Ethics in Health Research of West Norway (REK nr.2010/481); the study was performed in accordance with the Declaration of Helsinki.

References

1. Avery JK. Oral development and histology. Baltimore: Waverly Press, Inc., William & Wilkins; 1994.
2. Ten Cate AR. Oral histology. 2nd ed. St. Louis: The C.V. Mosby Company; 1986.
3. Orban B, Weinmann JP. Diffuse atrophy of the alveolar bone (periodontosis). J Periodontol. 1942;13:13.
4. Ten Cate AR. The epithelial rests of Malassez and the genesis of the dental cyst. Oral Surg. 1972;34:956–64.
5. Xiong J, Gronthos S, Bartold PM. Role of the epithelial cell rests of Malassez in the development, maintenance and

regeneration of periodontal ligament tissue. Periodontology 2000. 2013;63:217–33.
6. Costea DE, Loro LL, Dimba EA, Vintermyr OK, Johannessen AC. Crucial effects of fibroblasts and keratinocyte growth factor on morphogenesis of reconstructed human oral epithelium. J Investig Dermatol. 2003;121:1479–86.
7. Serres A. Essai sur l'anatomie et la physiologie des dents, ou nouvelle theorie de la dentition. 1st ed. Paris: Mequignon-Marvis; 1817.
8. Malassez L. Sur l'existence de masses epithelailes dans le ligament alveolodentaire chez 1′ homme adulte et al'etat normal. Comptes Rendus-Societe de Biologie. 1884;36:241–244.
9. Reeve MR, Wentz FM. The prevalence, morphology and distribution of epithelial rests in the human periodontal ligament. Oral Surg Oral Med Oral Pathol. 1962;15:785–93.
10. Valderhaug J, Zander HA. Relationship of "epithelial rests of Malassez" to other periodontal structures. Periodontics. 1967;5:254–8.
11. Cutress, Crigger. Cell rests of the sheep periodontium. NZ Dent J. 1974;70:39–49.
12. Spouge JD. A new look at the rest of Malassez. A review of their embryological origin, anatomy, and possible role in periodontal health and disease. J Periodontol. 1980;51:437–44.
13. Spouge, JD. The rests of Malassez and chronic marginal periodontitis. J Clin Periodontol. 1984;11:340–7.
14. Spouge JD. A method of schematic three-dimensional reconstruction for studying the gross morphology of epithelial residues in periodontal ligament. Arch Oral Biol. 1986;29:253–5.
15. Kvam E, Gilhuus-Moe O. Uptake of ^{3}H-thymidine by an epithelial rest in the periodontal membrane. A preliminary report. Acta Odontol Scand. 1970;28:143–6.
16. Main DMG. Odontogenic epithelial residues around rabbit mandibular cheek teeth. J Dent Res. 1972;51:841.
17. Ten Cate AR. The histochemical demonstration of specific oxidative enzymes and glycogen in the epithelial rests of Malassez. Arch Oral Biol. 1965;10:207–13.
18. Gilhuus-Moe O, Kvam E. Behavior of the epithelial remnants of Malassez following experimental movement of rat morals. Acta Odontol Scand. 1972;30:139–49.
19. Moskow BS. Origin, histogenesis and fate of calcified bodies in the periodontal ligament. J Periodontol. 1971;42:131–43.
20. Valderhaug J. A histologic study of experimentally induced radicular cysts. Int J Oral Surg. 1972;1:137–47.
21. Hodges GM. Evolution of the mammalian periodontium. In: Melcher AH, Bowen WH, editors. Biology of the periodontium. New York: Academic Press; 1969.
22. Lindskog S, Blomlof I, Hammarstrom L. Evidence for a role of odontogenic epithelium in maintaining the periodontal space. J Clin Periodontol. 1988;15:371–3.
23. Orban B. The epithelial network in the periodontal membrane. J Am Dent Assoc. 1952;44:632–5.
24. Loe H, Waerhaug J. Experimental replantation of teeth in dogs and monkeys. Arch Oral Biol. 1961;3:176–84.
25. Spouge JD. A study of epithelial odontogenic residues in the pig. J Periodontol. 1961;57:1164–71.
26. Brunette DM, Heershce JNM, Purdon AD, Sodek J, Moe HK, Assuras JN. In vitro cultural parameters and protein and prostaglandin secretion of epithelial cells derived from porcine rests of Malassez. Arch Oral Biol. 1979;24:199–203.
27. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp. 1988;136:42–60.
28. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 2004;364:149–55.
29. Fox SB, Fawcett J, Jackson DG, Collins I, Gatter KC, Harris AL, Gearing A, Simmons DL. Normal human tissues, in addition to some tumors, express multiple different CtD44 isoforms. Cancer Res. 1994;54:4539–46.
30. Kansas GS, Muirhead MJ, Dailey MO: Expression of the CDII/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation humans. Blood. 1990;76:2483–92.
31. Miyake K, Medina KL, Hayashi S, Ono S, Hamaoka T, Kincade PW. Mono-clonal antibodies to Pgp-1/CD44 block lymphopoiesis in long-term bone marrow cultures. J Exp Med. 1990;171:477–88.
32. Tsunematsu T, Fujiwara N, Yoshida M, Takayama Y, Kujiraoka S, Qi G, Kitagawa M, Kondo T, Yamada A, Arakaki R, Miyauchi M, Ogawa I, Abiko Y, Nikawa A, Murakami S, Takata T, Ishimaru N, Kudo Y. Human odontogenic epithelial cells derived from epithelial rests of Malassez possess stem cell properties. Lab Investig. 2016;96:1063–75.

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230853153 (print)
9788230851852 (PDF)