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Characterization of Multi-layered Tissue Engineered Human Alveolar Bone and Gingival Mucosa

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Abstract:	Advances in tissue engineering have permitted assembly of multi-layered composite tissue constructs for potential applications in the treatment of combined hard and soft tissue defects and as an alternative in vitro test model to animal experimental systems. The aim of this study was to develop and characterize a novel three-dimensional combined human alveolar bone and gingival mucosal model based on primary cells isolated from the oral tissues. Bone component of the model was engineered by seeding primary human alveolar osteoblasts (HAOBs) into a hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold and culturing in a spinner bioreactor. The engineered bone was then laminated, using an adhesive tissue sealant, with tissue engineered gingival mucosa consisting of air/liquid interface-cultured normal human gingival keratinocytes on oral fibroblast-populated collagen gel scaffold. Histological characterization revealed a structure consisting of established epithelial, connective tissue,

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	<p>and bone layers closely comparable to normal oral tissue architecture. The mucosal component demonstrated a mature epithelium undergoing terminal differentiation similar to that characteristic of native buccal mucosa, as confirmed using cytokeratin 13 (CK13) and cytokeratin 14 (CK14) immunohistochemistry. Ultrastructural analysis confirmed the presence of desmosomes and hemi-desmosomes in the epithelial layer, a continuous basement membrane and newly synthesized collagen in the connective tissue layer. Quantitative PCR (qPCR) assessment of osteogenesis-related gene expression showed a higher expression of genes encoded Collagen I (COL1) and Osteonectin (ON) as well as Osteocalcin (OC), Osteopontin (OPN), and Alkaline phosphatase (ALP). ELISA quantification of collagen I, ON, and OC confirmed a pattern of secretion which paralleled the model's gene expression profile. We demonstrate here that replicating the anatomical setting between oral mucosa and the underlying alveolar bone is feasible and the developed model showed characteristics similar to those of normal tissue counterparts. This tri-layered model therefore offers great scope as an advanced, and anatomically-representative tissue-engineered alternative to animal models.</p>

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3 **Characterization of Multi-layered Tissue Engineered Human Alveolar Bone and**
4
5 **Gingival Mucosa**
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8 Thafar Almela*, Sarmad Al-Sahaf, Robert Bolt, Ian M. Brook, Keyvan Moharamzadeh *

9
10 School of Clinical Dentistry, University of Sheffield, Clarendon Crescent, Sheffield, S10
11
12 2TA UK
13

14
15
16 **Corresponding authors:*
17

18
19 Tkalmela1@sheffield.ac.uk
20

21
22 K.moharamzadeh@Sheffield.ac.uk
23
24
25
26

27 Thafar Almela; BDS, MSc

28
29 University of Sheffield, School of Clinical Dentistry, 19 Clarendon Crescent, Sheffield
30
31 S10 2TA
32

33
34 Telephone: +44 (0) 7476828728
35

36 Email: Tkalmela1@sheffield.ac.uk
37
38
39
40

41 Sarmad Al-Sahaf; BDS, MSc

42
43 University of Sheffield, School of Clinical Dentistry, 19 Clarendon Crescent, Sheffield, S10
44
45 2TA
46

47 Telephone: +44 (0)7425059153
48

49 Email: sshal-sahaf1@sheffield.ac.uk
50
51
52
53

54 Robert Bolt; BDS (Hons), MFDS, MBChB (Hons), MCLinRes PhD, MOralSurg.

55
56 University of Sheffield, School of Clinical Dentistry 19 Clarendon Crescent, Sheffield,
57
58 S10 2TA
59
60

1
2
3 Telephone: +44(0)114 2265463
4

5 Email: r.bolt@sheffield.ac.uk
6
7
8

9 Ian M. Brook; BDS MDS FDSRCS(Eng) PhD
10

11 The School of Clinical Dentistry, University of Sheffield, 19 Claremont Crescent, Sheffield
12 S10 2TA
13

14
15 Telephone: +44(0) 1142717851
16

17 Email: i.brook@sheffield.ac.uk
18
19
20

21 Keyvan Moharamzadeh; BSc, DDS, PhD, FHEA, FDSRCS
22

23 The School of Clinical Dentistry, University of Sheffield, 19 Claremont Crescent, Sheffield
24 S10 2TA
25
26

27
28 Telephone: +44(0) 1142717910
29

30 Email: K.moharamzadeh@sheffield.ac.uk
31
32
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34

35 The study was performed in School of Clinical Dentistry, University of Sheffield, Claremont
36 Crescent, Sheffield, S10 2TA UK.
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Abstract

Advances in tissue engineering have permitted assembly of multi-layered composite tissue constructs for potential applications in the treatment of combined hard and soft tissue defects and as an alternative in vitro test model to animal experimental systems. The aim of this study was to develop and characterize a novel three-dimensional combined human alveolar bone and gingival mucosal model based on primary cells isolated from the oral tissues. Bone component of the model was engineered by seeding primary human alveolar osteoblasts (HAOBs) into a hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold and culturing in a spinner bioreactor. The engineered bone was then laminated, using an adhesive tissue sealant, with tissue engineered gingival mucosa consisting of air/liquid interface-cultured normal human gingival keratinocytes on oral fibroblast-populated collagen gel scaffold. Histological characterization revealed a structure consisting of established epithelial, connective tissue, and bone layers closely comparable to normal oral tissue architecture. The mucosal component demonstrated a mature epithelium undergoing terminal differentiation similar to that characteristic of native buccal mucosa, as confirmed using cytokeratin 13 (CK13) and cytokeratin 14 (CK14) immunohistochemistry. Ultrastructural analysis confirmed the presence of desmosomes and hemi-desmosomes in the epithelial layer, a continuous basement membrane and newly synthesized collagen in the connective tissue layer. Quantitative PCR (qPCR) assessment of osteogenesis-related gene expression showed a higher expression of genes encoded Collagen I (COL1) and Osteonectin (ON) as well as Osteocalcin (OC), Osteopontin (OPN), and Alkaline phosphatase (ALP). ELISA quantification of collagen I, ON, and OC confirmed a pattern of secretion which paralleled the model's gene expression profile. We demonstrate here that replicating the anatomical setting between oral mucosa and the underlying alveolar bone is feasible and the developed model showed characteristics similar to those of normal tissue counterparts. This tri-layered model therefore offers great scope as an advanced, and anatomically-representative tissue-engineered alternative to animal models.

Keywords: Bone engineering, oral mucosa engineering, alveolar bone mucosal model, composite tissue engineering.

1. Introduction

The orofacial region is a highly complex anatomical structure, comprising a large number of tissue types within a relatively small area. Restoration of defects following trauma, excision of pathology, and in the correction of developmental deformities, poses a great challenge due to the lack of suitable donor sites for harvesting grafts capable of accurately replicating the missing tissues. Whilst the need for anatomically-accurate grafting materials offers a niche that could be filled using refined tissue engineered constructs, it also imposes a considerable challenge to current composite tissue engineering techniques in order to create and attach the numerous tissue types required to replicate the normal anatomy of this region.⁽¹⁾ Despite this challenge, 3D *in vitro* screening systems, based on human cells and tissues, have already attracted significant attention, as they offer more robust and predictive experimental data compared to 2D or animal models.⁽²⁾ Limitations of 2D models include the loss of a natural 3D environment, which is in turn reflected in cell behaviour,⁽³⁾ and additionally the absence of a 3D environment excludes important factors such as hypoxic gradients and drug penetration. Furthermore, animal studies may be misleading due to interspecies molecular and physiological differences.⁽⁴⁾ Therefore, the short-term need for anatomically-representative 3D models of the oro-facial region is assured. Future development of these models beyond experimental analysis furthermore offers potential to achieve the longer-term goal of establishing tissue engineered grafting materials capable of improving and simplifying the reconstruction of surgical defects in the orofacial region.

Over the last few decades, there has been a substantial amount of innovation and progress in the engineering of various tissues found in the orofacial region, such as cartilage, bone, mucosa, and periodontium.⁽⁵⁾ This has encouraged researchers to develop more intricately-structured hybrid tissues that differ in the characteristics of their constituent parts, yet comprise a single functional unit. To date, only a few examples of composite tissues have

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3 been engineered which replicate the orofacial region. Recent successes include the
4
5 engineering of osteochondral components of the temporomandibular joint, which comprises
6
7 both articular cartilage and subchondral bone; ^(6, 7) and the engineering of a ligamentous
8
9 interface between tooth and alveolar bone to replicate the bone-periodontal ligament
10
11 complex. ⁽⁸⁾

12
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14 Despite these advances, the three key components of the majority of orofacial tissues are that
15
16 of bone, fibrous connective tissue and an overlying epithelium. Development of an accurate
17
18 alveolar bone-mucosal model therefore represents another important step in the process of
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20 achieving a clinically utilisable tissue engineered orofacial construct. We previously reported
21
22 the feasibility of tissue engineering such a model using cancer and immortal cell lines, ⁽⁹⁾
23
24 although acknowledged that patient-sourced primary cells are essential for such a model to be
25
26 developed to create a more accurate representation of native tissue.
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31 The aim of this study was to develop a novel three-dimensional combined human alveolar
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33 bone and gingival mucosal model based on primary cells isolated from the native human oral
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35 hard and soft tissues and to characterize this model qualitatively and quantitatively to
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37 examine whether it accurately replicates the normal tissues in terms of histology,
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39 ultrastructural appearance, differentiation and phenotype characteristics.
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2. Materials and Methods

2.1. Study design

Gingival biopsies and bone chips were obtained with written, informed consent from patients undergoing elective oral surgery at Charles Clifford Dental Hospital, Sheffield, UK, under appropriate ethical approval from National Research Ethics Services Committee (number 15/LO/0116). The study included simultaneous tissue engineering of oral mucosal model (OMM) and bone model (BM), then combining both to form a composite alveolar bone mucosal model (ABMM) (Figure1). All materials were purchased from Sigma Aldrich, UK unless otherwise stated.

2.2. Isolation and cultivation of primary human gingival cells

Normal human oral keratinocytes (NHOKs) and normal human oral fibroblasts (NHOFs) were isolated from gingival biopsies as previously described with some modifications.⁽¹⁰⁾ Briefly, the gingival tissues were collected and kept for 4-5 hours at 4°C in transport medium consisting of serum free Dulbecco's Modified Eagles Medium (DMEM)-GlutaMAX™ (Gibco, USA) supplemented with 100 IU:100 mg ml⁻¹ penicillin-streptomycin (P/S) and 625 ng ml⁻¹ fungizone. NHOKs were then enzymatically isolated from the biopsies by incubating the tissue in 0.25% trypsin-EDTA for 14-16 hours at 4°C. The epithelial layer was then scraped, cut into small pieces, and plated in 25 cm² tissue culture flask at a density of 1.5×10⁶ with an equal number of i3T3 feeder layer fibroblasts. The keratinocytes were then cultured in a humidified atmosphere of 5 % CO₂ /95 % air at 37 °C in Green's medium.⁽¹¹⁾

NHOFs were isolated from the connective tissue layer by incubating in 0.05% (w/v) collagenase type I (Gibco, USA) in DMEM-GlutaMAX™ containing 10% Fetal bovine serum (FBS) at 37 °C for 4 hours. The digested tissue was then centrifuged at 200 g for 5 minutes, and the resultant pellet re-suspended in complete DMEM (CDMEM; DMEM-

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3 GlutaMAX™ supplemented with 10% FBS, 625 ng/mL fungizone, and 100 IU:100 mg ml⁻¹
4
5 P/S).
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8 Both NHOKs and NHOFs were fed three times a week until confluency and then used at
9
10 passage 2.
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12 13 **2.3. Isolation and cultivation of primary human alveolar osteoblasts**

14
15
16 Primary human alveolar osteoblasts (HAOBs) were isolated from bone chips collected in a
17
18 sterile bone trap during preparation of dental implant sites.^(12, 13) After collection in transport
19
20 medium, the osseous tissues were extensively rinsed in phosphate buffered saline (PBS) and
21
22 vortexed to remove blood components. Bone fragments were cultured as explants in 75 cm²
23
24 flask in CDMEM supplemented with 50 µg/ml L-ascorbic acid 2-phosphate (L-AA) at 37°C
25
26 in a humidified atmosphere of 95% air, 5% CO₂. The culture was left undisturbed for 7 days,
27
28 after which the medium was replaced 2-3 times/week until the culture attained confluency,
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30 whereby cells were detached by trypsin/EDTA (0.25%), sub cultured, and used in the 3nd
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32 passage.
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36 37 **2.4. Engineering the oral mucosal models (OMMs)**

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40 Collagen-based OMM was constructed according to the technique described by Dongari-
41
42 Bagtzoglou and Kashleva.⁽¹⁴⁾ A solution of 10 × DMEM 13.8 mg ml⁻¹, FBS 8.5% (v/v), L-
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44 glutamine 2 mM, reconstitution buffer (22 mg ml⁻¹ sodium bicarbonate and 20 mM HEPES),
45
46 and 5 mg ml⁻¹ rat-tail type I collagen (R & D system) was prepared on ice and neutralized by
47
48 1M sodium hydroxide to pH=7.4.
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51 Finally, a cell suspension of NHOFs in CDMEM at a concentration of 2×10⁵ cells per model
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53 was added to the solution. The resultant fibroblast-containing collagen was transferred into
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55 tissue culture inserts (0.4 µm pore size, 30 mm diameter, Millipore) and incubated at 37°C
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57 for 2 hours until solidified. 1.5 ml CDMEM was then added inside and outside the insert.
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3 After 3 days, 1×10^6 NHOK cells (per model) suspended in 50 μ l Green's medium were
4 seeded on the gel surface and allowed to adhere for 3 hours. 2 ml Green's medium was then
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6 gently added into the insert and incubated at 37 °C, 5% CO₂ for 4 days. When epithelial cells
7
8 reached confluency, the culture was raised to air/liquid interface and fed every other day for
9
10 10 days.
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13 14 15 **2.5. Engineering the bone models (BMs)**

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17 10 sterile ceramic discs (2mm \times 10mm) of Hydroxyapatite/Tricalcium phosphate (HA/TCP)
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19 (60%/40%) (Ceramisis LTD, UK) with total porosity of 78.9 % were used as a scaffold. The
20
21 discs were placed in 24- well plates and pre-wetted with CDMEM 24 hours before cell
22
23 seeding. 2×10^6 HOBs cells suspended in 15 μ l CDMEM /L-AA were then added dropwise to
24
25 each scaffold. The cells were allowed to adhere for 2 hours, and then BMs completely
26
27 covered with 2 ml CDMEM /L-AA and incubated overnight. After 24 hours, BMs were
28
29 suspended in a spinner bioreactor (Branstead Stem, UK) and spun at a rate of 30 rpm. The
30
31 medium was changed every 2 days for 17 days.
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36 **2.6. Cell viability assessment using PrestoBlue (PB) live assay**

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39 The assessment of cell viability within BMs and OMMs was performed using the PB assay
40
41 (Invitrogen, USA) at day 17 before the combination of the two constructs. BMs and OMMs
42
43 were separately placed in a 12- well tissue culture plate, washed with PBS, and then a
44
45 mixture of 900 μ l of CDMEM and 100 μ l of PB reagent was added to each well. Three
46
47 acellular discs and collagen gel were included as negative controls for BMs and OMMs,
48
49 respectively. After 3 hours incubation at 37°C, the fluorescence values (excitation/emission:
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51 560/590 nm) of 200 μ l aliquot were measured in triplicates using spectrophotometric plate
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53 reader (Infinite® M200, TECAN, USA). For calculation, the average fluorescence values of
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3 the medium containing the scaffolds without cells were subtracted from the averaged sample
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5 readings.
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14 **2.7. Engineering the composite alveolar bone mucosal models (ABMMs)**

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16 ABMMs were constructed as previously described.⁽⁹⁾ At day 17, BM and OMM models were
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18 combined using a biocompatible fibrin-based adhesive sealant (ARTISS, Baxter, UK). In
19
20 brief, BMs were retrieved and placed on a sterile culture plate containing 10 ml CDMEM.
21
22 The fibrin adhesive was prepared in a pre-filled syringe according to manufacturer's
23
24 instructions, and a thin layer of the mixed fibrinogen-thrombin sealer applied to the non-
25
26 epithelial side of an OMM. The OMM was then immediately attached to the surface of a BM
27
28 and held in the desired position with gentle compression for a minimum of 60 seconds to
29
30 ensure the adhesive material had completely set, and that both models were firmly adhered to
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32 each other. The ABMMs were then further cultured for 5 days, after which the analyses were
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34 performed.
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39 **2.8. Histological examination of ABMM**

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41 ABMMs were fixed in 4% paraformaldehyde (PFA) for 24 hours and embedded in 2-
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43 hydroxyethyl methacrylate resin (Technovit 7100, Heraeus Kulzer) according to
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45 manufacturer's instructions. Briefly, samples were dehydrated in graded ethanol series 75%,
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47 95%, 100% for at least 2 hours, then pre-infiltrated with equal parts of basic solution and
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49 100% ethanol overnight on a rotating mixer. Infiltration was performed with infiltration
50
51 medium consisting of 100 ml basic solution and 1 g Hardener1. Finally, samples were
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53 polymerized in suitable molds with premixed 15 ml infiltration medium and 1 ml Hardener-
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55 2. Polymerisation was completed over the period of 2 hours at room temperature.
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3 For routine haematoxylin and eosin (H&E) staining (Leica Microsystems), the ground block
4 was first sectioned into 100-150 μm thickness with a cutting machine (IsoMet[®] 1000
5 precision saw, Buehler UK Ltd, UK). The section was then adhered to a glass slide using
6 cyanoacrylate adhesive (Loctite[®] glass bond UV curing, UK). The thickness was then further
7 reduced to 30-35 μm by grinding the sections with silicon carbide papers of P800 and P1200
8 roughness in a grinder-polisher machine (Buehler[™] Metaserv, UK).
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16 17 **2.9. Histological, Immunofluorescent (IF), and Transmission Electron Microscopy** 18 **(TEM) Examinations of OMMs.** 19

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21
22 OMMs were processed for histological, IF, and TEM examination at the end of culture
23 period. Frozen sections were prepared as previously described.⁽¹⁵⁾ OMM were fixed with 4%
24 PFA for 24 hours, followed by an overnight incubation in 18% sucrose in PBS. The samples
25 were frozen in OCT Compound (Thermofisher) and sectioned to 14 μm , and sections were
26 then either stained with H&E for examination under inverted microscope (Olympus, Japan),
27 or were subjected to IF staining.
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36 For IF staining, the sections were washed with PBS for 5 minutes, permeabilized with 0.2 %
37 Triton x-100 for 5 minutes, and then blocked with 1% bovine serum albumin (BSA) in 0.1 %
38 PBS-Tween for 1 hour. The sections were then incubated overnight at 4 °C with conjugated
39 anti-Cytokeratin 13 (Abcam, UK, ab198584) and anti-Cytokeratin 14 (Abcam, UK,
40 ab192055) antibodies at a working dilution of 1:100. After washing, slides were mounted
41 using DAPI-containing mounting medium (Thermofisher, UK) and viewed using IF
42 microscopy (Zeiss Ltd, Germany). Normal oral mucosa (NOM) was used as a positive
43 control, while negative control was OMM stained with IgG isotype (1:100) (ebioscience,
44 UK), followed by goat anti rabbit secondary antibodies (1:200) (Abcam, UK, ab150083).
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3 For TEM analysis, 4mm sections of OMM were fixed in 3% glutaraldehyde in 0.1 M sodium
4 cacodylate buffer for 2 hours at 4°C, and post fixed in 1% osmium tetroxide for 2 hours.

5
6 Tissue was dehydrated with 70%, 95%, and 100% ethanol and then embedded in resin.
7
8 Samples were left for 48 hours in the oven at 60 °C, and thereafter examined using a
9
10 transmission electron microscope (FEI tecnai 12 Bio-twin, 120Kv TEM).
11
12

13 14 **2.10. Quantitative polymerase chain reaction (qPCR) examination**

15
16 For gene expression analysis, ABMMs were snap frozen, grinded, and digested with lysis
17 buffer. RNA was isolated using isolate II RNA Mini Kit (BioLine, UK) according to
18 manufacturer's instructions. 500 ng total RNA was reverse transcribed using a High-Capacity
19 RNA to Complementary DNA (cDNA) kit (Life Technologies, UK).
20
21

22
23 0.5 µl cDNA was amplified using 5 µl TaqMan gene expression master mix, 3.5 µl nuclease
24 free water and 0.5 µl TaqMan gene probes (Applied Biosystems). Genes encoding the
25 following markers were then interrogated; ALP, Osteopontin (OP), Osteonectin (ON),
26
27 Osteocalcin (OC), Collagen I (COL1), Ki 67, Cytokeratin 10 (CK10), and Cytokeratin 13
28 (CK13). B-2-Microglobulin was used as a reference control gene (All Applied biosystem,
29 UK).
30
31

32
33 Cycling conditions were holding at 95°C for 10 minutes, then 95°C for 10 seconds, followed
34 by 60 °C for 45 seconds and the cycle was repeated 40 times (QIAGEN, Germany). The
35 threshold cycle (Ct) was normalized against the reference gene (Δ Ct) and the expression
36 relative to it was calculated.
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39 40 **2.11. Enzyme-linked immunosorbent assay (ELISA)**

41
42 Commercially available ELISA kits for COL1, ON (R&D systems, UK), and OC (Abcam,
43 UK) were used according to manufacturer's instructions in order to quantify these proteins in
44 ABMM after 24 hours incubation in serum free tissue culture conditioned medium. The
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3 solutions were read at absorbance 450 nm using a microplate reader (Infinite® M200,
4
5 TECAN, USA).

6 7 **2.12. Statistics**

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9 All data were presented in terms of mean \pm SD of three independent experiments performed
10
11 in triplicate. One-way ANOVA complemented by Tukey's multiple comparisons test was
12
13 performed using GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA), and differences
14
15 were considered significant when $p < 0.05$.

16 17 **3. Results**

18 19 **3.1. Viability assay**

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21 Cell viability testing using the PB assay revealed that HAOBs within the bone model as well
22
23 as NHOFs and NHOKs in the OMM remained vital throughout the experiment (Figure 2).
24
25

26 27 **3.2. Histological assessment of ABMM**

28
29 Histological observation revealed that the ABMM had a structure consisting of epithelial,
30
31 connective tissue and bony layers which were comparable to the histological architecture of
32
33 the soft and hard tissues of the oral cavity (Figure 3A). The model's surface displayed a
34
35 continuous stratified epithelial layer, and a connective tissue layer densely populated with
36
37 viable fibroblasts (Figure 3B). The hard-soft tissue interface showed a thin band of cell-
38
39 infiltrated sealant adhering both layers (Figure 3C). Viable cells evenly populated the
40
41 scaffold porosities with a secreted matrix partially or completely filling the pores of the
42
43 scaffold (Figure 3D).
44
45

46 47 **3.3. OMM assessments**

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49 Histologically, the OMM showed a proliferating basal layer and well-differentiated stratified
50
51 squamous oral epithelium of 12-14 NHOKs thickness, which mimicked that of NOM (Figure
52
53 4). The epithelium consisted of four distinct layers that included equivalents to basal, spinous,
54
55 intermediate, and superficial cells, respectively. The uppermost aspect of the superficial layer
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3 had cells of a flattened appearance, while cells in the basal layer remained rounded. Glycogen
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5 granules were occasionally observed in the intermediate layer. NHOFs were found dispersed
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7 homogeneously in the connective tissue.
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9
10 Figure 5 shows the keratin expression of NOM and OMM, as assessed by immunofluorescent
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12 staining for CK13 and CK14. The suprabasal cells of the OMM strongly expressed CK13,
13
14 consistent with the staining of intermediate and superficial cells in para-keratinized stratified
15
16 epithelium.⁽¹⁶⁾ CK14 was expressed throughout the entire epithelium.
17

18
19 Ultrastructural analysis of the OMM demonstrated the presence of numerous desmosomes
20
21 between adjacent epithelial cells (Figure 6A). A continuous and intact basement membrane
22
23 was formed, anchoring the epithelium firmly to the connective tissue by means of
24
25 hemidesmosomal attachments (Figure 6B). In the sup-epithelial layer, a high amount of
26
27 newly synthesized collagen was observed (Figure 6C).
28

29 30 **3.4. qPCR assessment**

31
32 Figure (7) summarises gene expression for the composite model, including both HAOB and
33
34 NHOK cell components. Of all genes evaluated, COL1 demonstrated the highest level of
35
36 expression. Bone-specific genes such as ALP, OC, ON, and OP were detected, with ON
37
38 expressed in high quantities. Genes encoding CK10 and CK13 were expressed, and were not
39
40 significantly different.
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43 44 **3.5. ELISA**

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46 ELISA results for COL1, ON, and OC were consistent with the qPCR findings. COL1
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48 demonstrated a significantly increased protein concentration in comparison to ON and OC.
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50 ON, in turn, was higher than OC (Figure 8).
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53 54 **4. Discussion**

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3 There is an increasingly recognized need to engineer tissue equivalents for both clinical
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5 and experimental applications. In this study, we have developed and characterized an *in vitro*
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7 3D human alveolar osteomucosal construct that replicates the natural anatomical relationship
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9 of oral mucosal tissue and the underlying bone.
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12 In 3D culture, cell viability is influenced by several factors including the scaffold and culture
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14 environment. Calcium phosphate and collagen constitute the main elements of the
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16 extracellular matrix of bone and oral mucosa, respectively.⁽¹⁷⁾ Therefore, due to their
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18 biocompatibility, they have been widely used in tissue engineering as scaffolds to support
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20 cells growth without compromising their vitality.⁽¹⁸⁾ The culture environment, on the other
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22 hand, that promotes mass transfer and nutrient delivery is another important factor to
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24 maintain cell vitality. In our study, growing OMM in a static culture raised no problem due to
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26 the hydrophilic, high water-containing networks of collagen hydrogel that enhance
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28 permeability for oxygen, nutrients and water-soluble metabolites.⁽¹⁹⁾ Ceramic-based bone
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30 model, by contrast, required a dynamic flow to improve nutrient and waste diffusion because
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32 the static culture is only sufficient to nourish the thin superficial layer, approximately 100-
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34 200 μ m, contacting the medium.⁽²⁰⁾ As the cells increase in number, so does their metabolic
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36 demand and the build-up of waste products. Consequently, the deeper cells in the tissue
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38 interior can be deprived of oxygen and nutrients source in long-term static culture conditions.
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40 For this reason, the total culture time of the composite tissue after combining the hard and
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42 soft tissue constructs was limited to an additional 5 days at the static air/liquid interface in
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44 this study.
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51 We have shown that the histological structure and expression of key markers of epithelial
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53 differentiation in OMM were comparable to those of its normal tissue counterpart. OMM
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55 displayed the characteristics of a para-keratinized epithelium, which suggests that the
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3 keratinocytes used in this model retain the para-keratinized status of the original oral mucosa
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5 from which they were derived.⁽²¹⁾
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8 The control of keratinocyte in proliferation and differentiation is multifactorial. Several
9
10 studies have confirmed the role of fibroblasts in epithelial development through the
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12 stimulation of keratinocyte proliferation, migration, and keratin expression.^(22, 23) Fibroblasts
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14 establish such growth-promoting roles through paracrine cross talk between NHOFs and
15
16 NHOKs via cytokines such as heparin-binding EGF-like growth factor (HB-EGF),
17
18 Interleukin 1 alpha (IL-1 α) and Transforming growth factor beta 1 (TGF- β 1).⁽²⁴⁾ This
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20 function requires an optimal fibroblast density, as the presence of either excessive or
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22 inadequate numbers of fibroblast will adversely affect epithelium morphogenesis, leading to
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24 differentiation markers being inappropriately expressed.⁽²⁵⁾ In our models, optimal fibroblast
25
26 seeding density in order to support an anatomically representative epithelial layer was found
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28 to be 2×10^5 cells per model. Keratinocyte senescence may also impact on the capacity to
29
30 achieve anatomically representative epithelia in tissue culture models. Due to sustained
31
32 telomerase expression within primary cells, sequential sub culturing may restrict the capacity
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34 of keratinocytes to divide once seeded into a 3D model, and therefore it is recommended that
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36 the use of keratinocytes in tissue substitute constructs is limited to passage 3 or less.⁽²⁶⁾
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42 The proper functionality of keratinocytes is important not only for epithelial layer formation,
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44 but also for epithelial-connective tissue attachment and cell-cell adhesion, which are essential
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46 for achieving an accurate, functional mucosal substitute. Our data, consistent with data from
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48 other studies, has confirmed that cells within the 3D models actively synthesised the
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50 ultrastructural components including desmosomes, hemidesmosomes, and basement
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52 membrane required for this structural stability.^(27, 28)
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3 Although collagen I is a non-specific marker of osteogenesis, relatively high levels were
4 expressed in our model compared to other osteogenic genes; this may be due to a number of
5 reasons. Firstly, this marker constitutes the most abundant component of bone extracellular
6 matrix (90% of the organic component) and becomes upregulated during bone formation.⁽²⁹⁾
7
8 Secondly, ascorbic acid was added as a component of the culture medium in order to
9 maintain the osteoblastic phenotype of bone-derived cells; ascorbic acid is known to
10 stimulate of cell growth and collagen synthesis in osteoblasts.⁽³⁰⁾ The hydroxylation of
11 proline residues of procollagen is increased to approximately 40% by ascorbate, which is
12 known to stabilize the collagen triple helix.⁽³¹⁾ Thirdly, although collagen I is the main
13 organic component secreted by osteoblasts, it is also produced in abundance by fibroblasts^{(32,}
14³³⁾, and therefore the fibroblastic component of the model may have contributed to the
15 collagen levels observed.
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29 We undertook analysis of further osteoblastic markers, including Osteocalcin, Osteopontin,
30 Osteonectin and ALP. ALP, like Collagen I, is expressed in tissues other than bone and
31 therefore its expression cannot be considered specific, although it is synthesised by
32 osteoblasts and has been used to assess osteoblast phenotype and matrix mineralization.⁽³⁴⁾
33
34 Conversely, Osteocalcin, Osteopontin, and Osteonectin are major non-collagenous, bone-
35 specific proteins that play profound roles in bone formation.⁽²⁹⁾ Osteonectin, in particular, is
36 localized to mineralized bone trabeculae and is present at higher concentrations within
37 extracellular matrix than in osteocytes. It selectively binds to collagen I and the resultant
38 osteonectin-collagen complexes initiate mineral phase deposition by binding synthetic apatite
39 crystals and free calcium ions.⁽³⁵⁾ Interestingly, Osteonectin can be demonstrated in active
40 osteoblasts and osteoprogenitor cells as well as in young osteocytes, but not in aged,
41 quiescent osteocytes; the protein is therefore a reliable marker of functional osteoblasts.⁽³⁶⁾
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The simultaneous expression of Osteonectin and its encoding gene, SPARC, strongly

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3 suggests that HOBs maintained their phenotypic characteristics in spite of a protracted culture
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5 period of 2 months.
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7 The expression of all osteoblast-associated molecules varies over the different stages of
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9 bone development, and therefore the expression profiles observed in our models, which
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11 were cultured over a relatively short period compared to that of normal human bone
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13 turnover may vary somewhat to that observed *in vivo*. For example, ALP increases in the
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15 initial stages of bone formation, yet decreases as mineralization progresses, whereas
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17 osteopontin is first detected in young bone, whilst Osteocalcin appears towards the end of
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19 the mineralization process.⁽³⁷⁾
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27 **5. Conclusion**

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29 This study demonstrates that tissue engineered tri-layered reconstructs based on primary
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31 human alveolar osteoblasts cultured in porous hydroxyapatite/tricalcium phosphate scaffolds
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33 adhered to collagen-embedded gingival fibroblasts overlain with primary gingival
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35 keratinocytes cultured at the air/liquid interface, were able to mimic the native alveolar bone
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37 and overlaying full-thickness mucosal structures. The engineered combined hard and soft
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39 tissue provides scope to act as a valuable alternative to 2D and animal models for various *in*
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41 *vitro* and potential *in vivo* applications. Further experiments are underway to assess the
42
43 suitability of this 3D model for *in vitro* evaluation of bone invasion of oral cancer as well as
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45 development of *in vitro* tissue engineered models of osseointegrated dental implants using the
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47 3D composite bone mucosal system.
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54 **Acknowledgement**

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5
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7
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9
10 LTD, UK for providing the bone scaffolds.
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12

13 14 15 16 **Conflict of interests**

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19 The authors declare that there is no conflict of interests regarding the publication of this
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21 paper.
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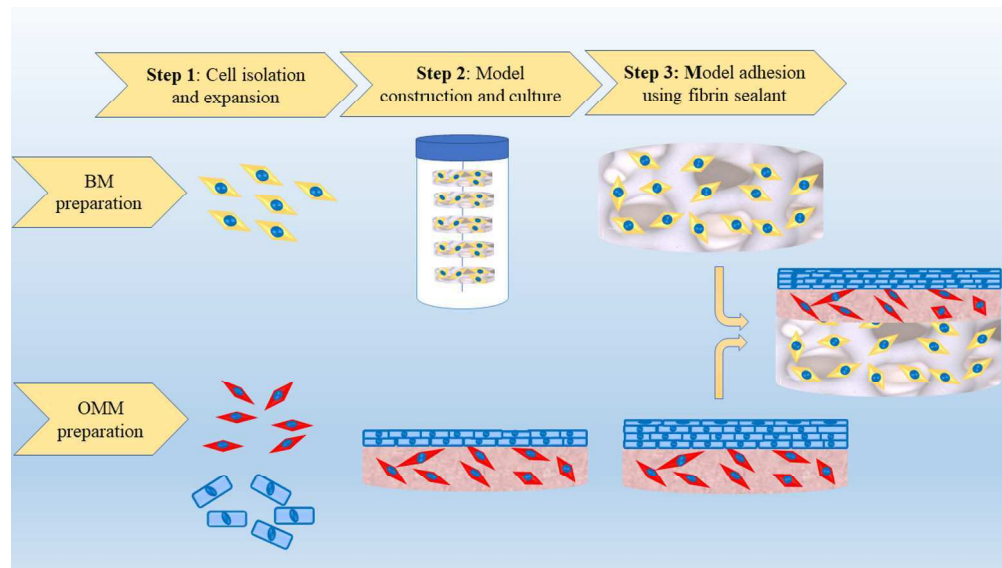


Figure 1. Schematic illustration of the preparation of OMM, BM, and ABMM. The procedure involved 3 main steps. First; HAOBs, NHOFs, and NHOKs were isolated from oral tissues and cultivated in monolayer culture. Second; BMs were prepared by seeding HAOBs in HA/TCP scaffold and cultured in spinner bioreactor while OMMs were prepared from fibroblast-populated collagen gel and air liquid interface culture oral keratinocytes. Third; Combination of BM and OMM using adhesive fibrin to form ABMM.

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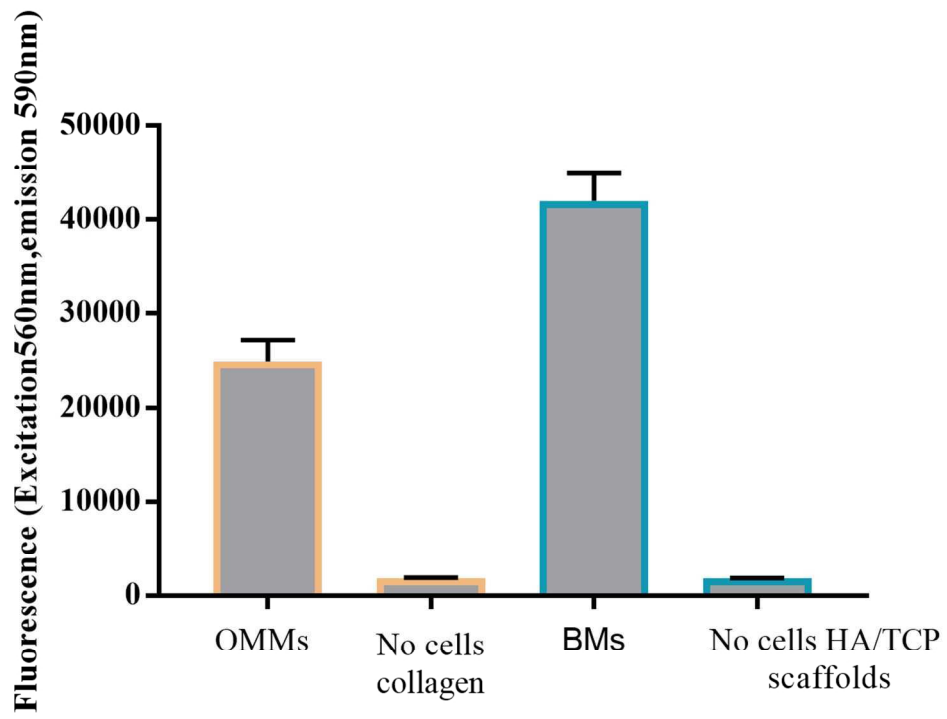


Figure 2. Vitality assessment of OMMs and BMs. The figure shows the activity of HAOBs within HA/TCP scaffold as well as NHOFs and NHOKs in OMMs after 17 days of culture.

107x83mm (300 x 300 DPI)

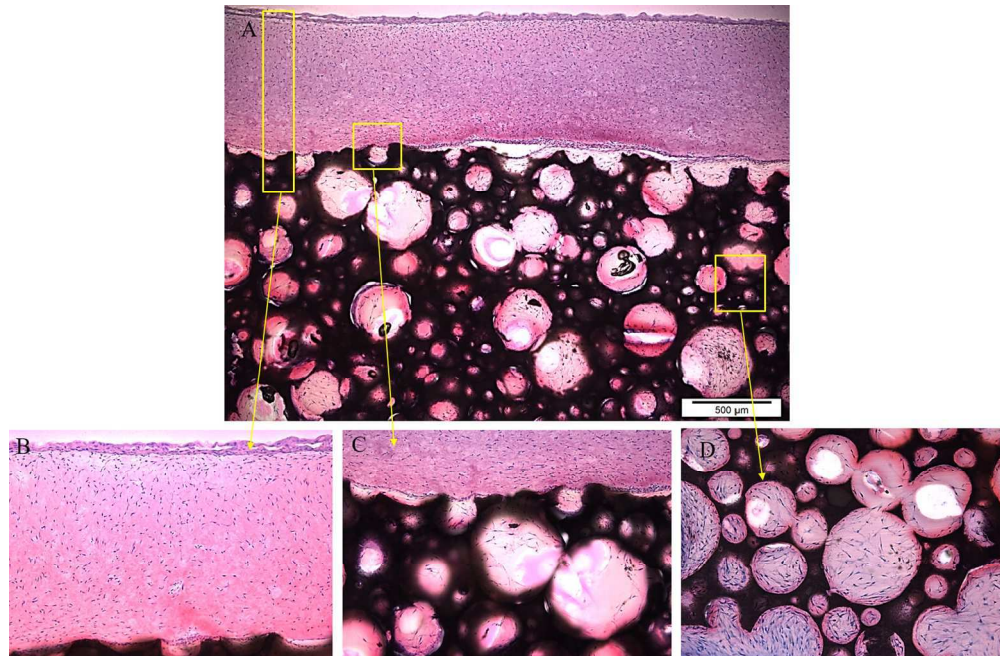


Figure 3. H&E-stained histological sections of ABMM showing (A) full-thickness multi-layered bone and mucosa; and magnified images of (B) oral mucosa part; (C) bone-mucosal interface; and (D) bony part showing the pores of the scaffold filled with osteoblasts and extracellular matrix (Original magnification A \times 4; B, C, and D \times 10).

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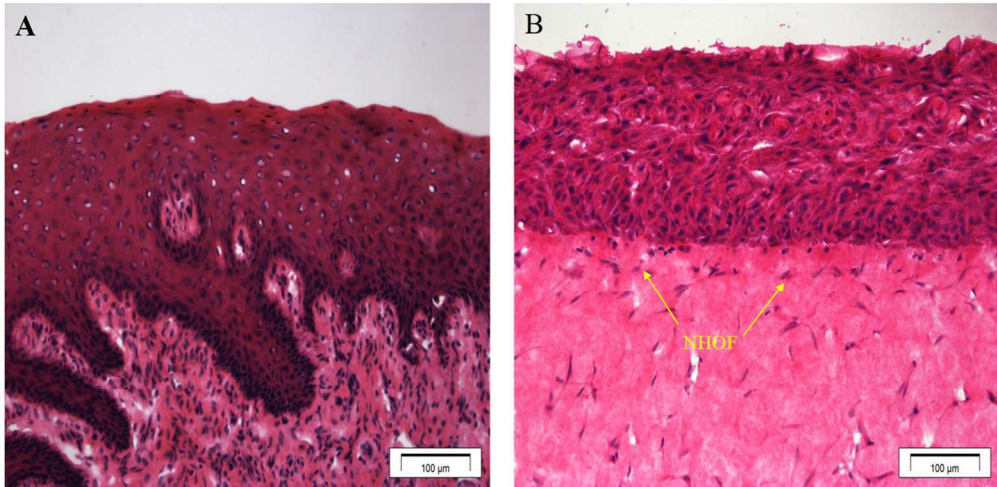


Figure 4. H&E-stained histological sections of (A) NOM; and (B) OMM showing a well-differentiated stratified squamous epithelial layer with viable fibroblasts scattered in the connective tissue layer (Original magnification $\times 20$).

123x60mm (300 x 300 DPI)

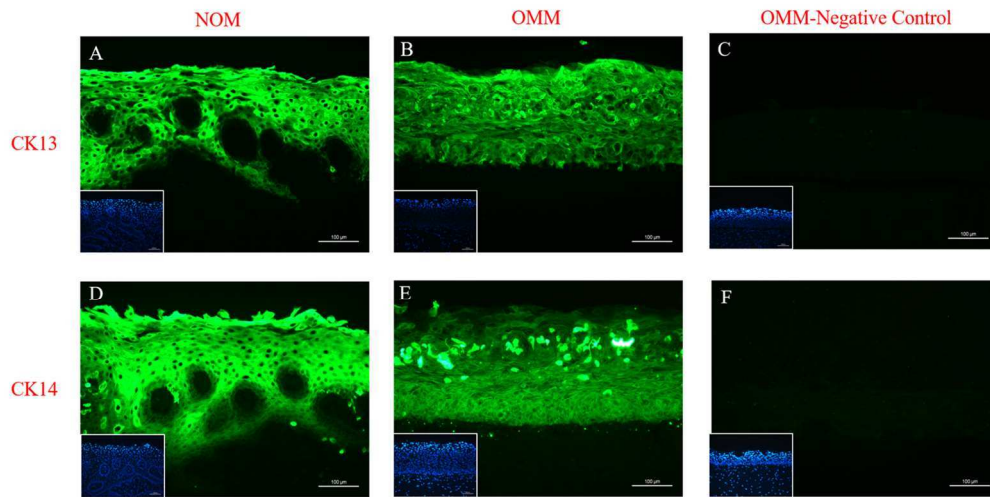


Figure 5. Immunofluorescent staining of the oral mucosa showing (A and D) both epithelial markers CK13 and CK14 were well detected in the NOM; (B and E) strong expression of CK13 and CK14 in the OMM; and (C, F) negative control (Original magnification $\times 20$; the lower left boxes are DAPI stain).

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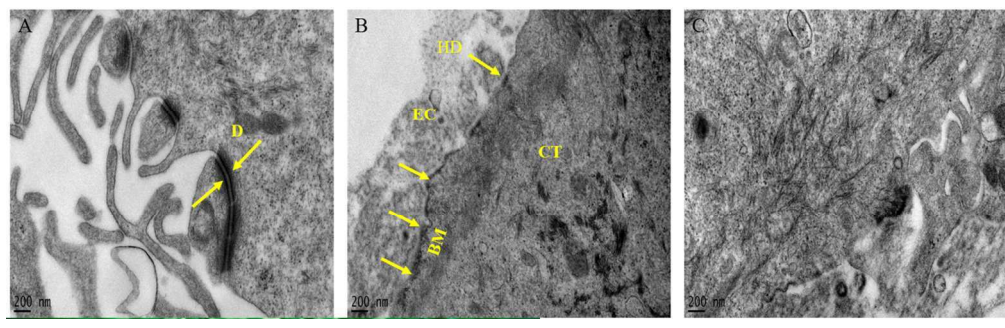


Figure 6. Ultrastructural analysis of the OMM by transmission electron microscopy showing (A) numerous desmosomes (D) between adjacent epithelial cells; (B) basement membrane (BM) formed all along the interface between the epithelial cell (EC) and connective tissue (CT) with the presence of hemidesmosomes (HD); and (C) newly synthesized collagen I fibrils in the connective tissue (Original magnification $\times 50000$).

127x39mm (300 x 300 DPI)

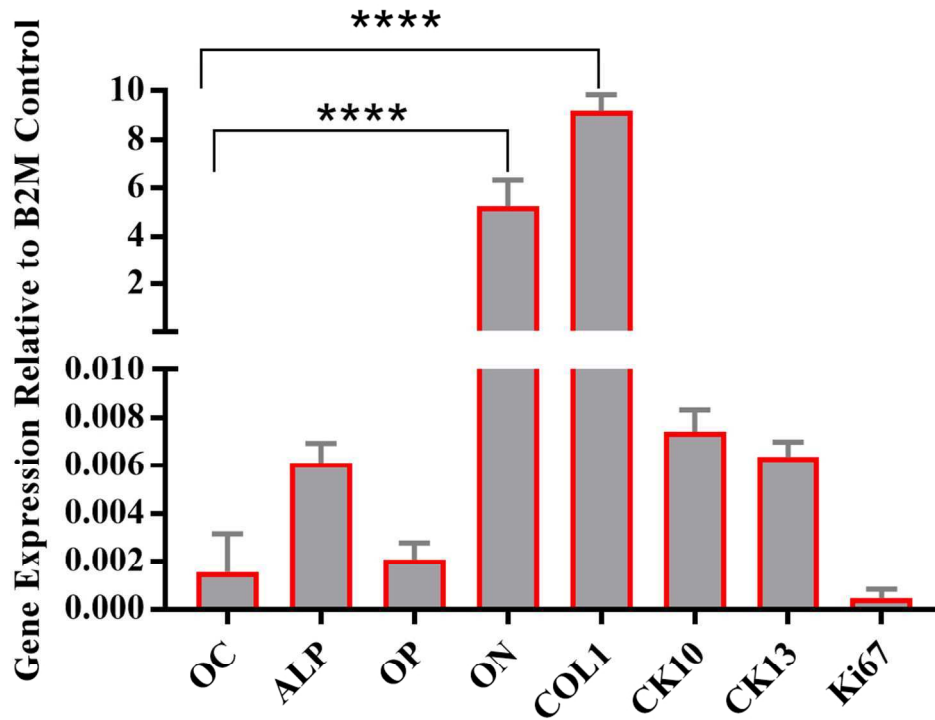


Figure 7. The gene expression of HAOBs and NHOKs in the ABMM as evaluated with PCR analysis. The osteogenic markers; OC, ALP, OP, ON, and COL1, in addition to the epithelial markers; CK10, CK13 were detected. Statistical significance was determined as $p < 0.05$ from control (**** $P < 0.0001$).

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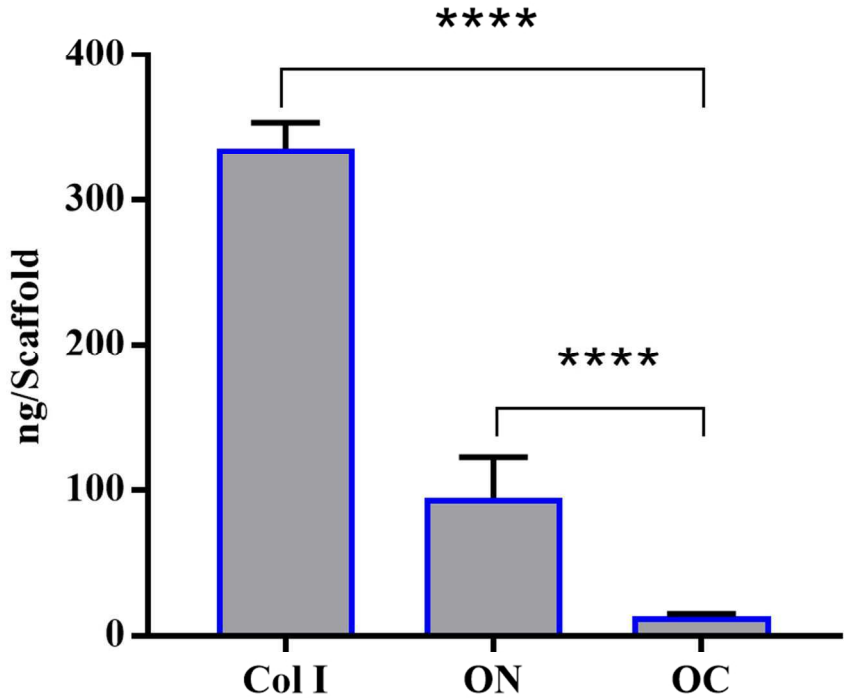


Figure 8. Protein expression of Collagen I, Osteonectin, and Osteocalcin in ABMM analyzed by ELISA. Statistical significance was determined as $p < 0.05$ (**** $P < 0.0001$).

87x69mm (300 x 300 DPI)

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3 **Figure legends:**

4 **Figure 1. Schematic illustration of the preparation of OMM, BM, and ABMM.** The
5 procedure involved 3 main steps. First; HAOBs, NHOFs, and NHOKs were isolated from
6 oral tissues and cultivated in monolayer culture. Second; BMs were prepared by seeding
7 HAOBs in HA/TCP scaffold and cultured in spinner bioreactor while OMMs were prepared
8 from fibroblast-populated collagen gel and air liquid interface culture oral keratinocytes.
9 Third; Combination of BM and OMM using adhesive fibrin to form ABMM.
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18 **Figure 2. Vitality assessment of OMMs and BMs.** The figure shows the activity of HAOBs
19 within HA/TCP scaffold as well as NHOFs and NHOKs in OMMs after 17 days of culture.
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24 **Figure 3. H&E-stained histological sections of ABMM** showing (A) full-thickness multi-
25 layered bone and mucosa; and magnified images of (B) oral mucosa part; (C) bone-mucosal
26 interface; and (D) bony part showing the pores of the scaffold filled with osteoblasts and
27 extracellular matrix (Original magnification A \times 4; B, C, and D \times 10).
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33 **Figure 4. H&E-stained histological sections of (A) NOM;** and (B) OMM showing a well-
34 differentiated stratified squamous epithelial layer with viable fibroblasts scattered in the
35 connective tissue layer (Original magnification \times 20).
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40 **Figure 5. Immunofluorescent staining of the oral mucosa** showing (A and D) both
41 epithelial markers CK13 and CK14 were well detected in the NOM; (B and E) strong
42 expression of CK13 and CK14 in the OMM; and (C, F) negative control (Original
43 magnification \times 20; the lower left boxes are DAPI stain).
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49 **Figure 6. Ultrastructural analysis of the OMM by transmission electron microscopy**
50 showing (A) numerous desmosomes (D) between adjacent epithelial cells; (B) basement
51 membrane (BM) formed all along the interface between the epithelial cell (EC) and
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3 connective tissue (CT) with the presence of hemidesmosomes (HD); and (C) newly
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5 synthesized collagen I fibrils in the connective tissue (Original magnification $\times 50000$).
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8 **Figure 7. The gene expression of HAOBs and NHOKs in the ABMM as evaluated**
9 **with PCR analysis.** The osteogenic markers; OC, ALP, OP, ON, and COL1, in addition to
10 the epithelial markers; CK10, CK13 were detected. Statistical significance was determined
11 as $p < 0.05$ from control (**** $P < 0.0001$).
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17 **Figure 8.** Protein expression of Collagen I, Osteonectin, and Osteocalcin in ABMM analyzed
18 by ELISA. Statistical significance was determined as $p < 0.05$ (**** $P < 0.0001$).
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