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3D Printed Tissue Engineered Model for Bone Invasion of Oral Cancer

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Abstract

Recent advances in three-dimensional printing technology have led to a rapid expansion of its applications in tissue engineering. The present study was designed to develop and characterize an in vitro multi-layered human alveolar bone, based on a 3D printed scaffold, combined with tissue engineered oral mucosal model. The objective was to incorporate oral squamous cell carcinoma (OSCC) cell line spheroids to the 3D model at different anatomical levels to represent different stages of oral cancer. Histological evaluation of the 3D tissue model revealed a tri-layered structure consisting of distinct epithelial, connective tissue, and bone layers; replicating normal oral tissue architecture. The mucosal part showed a well-differentiated stratified oral squamous epithelium similar to that of the native tissue counterpart, as demonstrated by immunohistochemistry for cytokeratin 13 and 14. Histological assessment of the cancerous models demonstrated OSCC spheroids at three depths including supra-epithelial level, sub-epithelial level, and deep in the connective tissue-bone interface.

Abbreviations: 3DP, Three-dimensional printing; OSCC, oral squamous cell carcinoma; ABMM, alveolar bone and oral mucosal model; CBMM, cancerous bone oral mucosal model; DMEM, Dulbecco's Modified Eagles Medium; PBS, phosphate buffered saline; OMM, Oral mucosal model; NOM, normal oral mucosa; H & E, haematoxylin and eosin; SEM, scanning electron microscopy.

The 3D tissue engineered composite model closely simulated the native oral hard and soft tissues and has the potential to be used as a valuable in vitro model for the investigation of bone invasion of oral cancer and for the evaluation of novel diagnostic or therapeutic approaches to manage OSCC in the future.

Keywords: Bone, tissue engineering, oral mucosa, 3D printing, oral cancer.

1. Introduction

Tissue engineering owes most of its advances to improvements in novel biomaterial-based technologies that can accurately replicate the heterogeneous nature of native organs and tissues. Three-dimensional printing (3DP) is an innovative technique that offers an entirely new method of reconstructing complex tissues comprising intricate 3D microarchitectures, such as bone, cartilage, skin, and blood vessels (Bose et al., 2018; Lanza et al., 2014). 3DP has also been used to create physiologically-relevant in vitro models which can be applied as alternatives to conventional 2D and animal models in a number of research settings, such as disease modelling and drug screening (Pati et al., 2016; Sean and Anthony, 2014). Numerous methods have been developed to fabricate bone tissue engineering scaffolds including particulate leaching, freeze-drying, and phase separation. Although a high scaffold porosity can be achieved using these methods, numerous properties of the scaffold's internal structure – such as pore size, shape and interconnectivity – are difficult to control (Thavornyutikarn et al., 2014). In contrast, 3DP can produce the desired structure with defined dimensions by utilizing computer-aided design (CAD) technologies (Asa'ad et al., 2016; Bose et al., 2012; Bose et al., 2013; Sears et al., 2016). It enables mimicking bone's hierarchy through construction of multiscale scaffolds with small and large pores and high interconnectivity which in turn directly related to scaffold performance since it influence bone growth and strength (Egan et al., 2017; Wang et al., 2017).

One of the applications by which the advantages of 3DP can be exploited, is fabrication of reproducible constructs to be used for in vitro disease modelling. Oral squamous cell carcinoma (OSCC) is the most common Head & Neck malignancy, and accounts for approximately 90% of all oral and oropharyngeal tumours (Chi et al., 2015). To date, several authors have undertaken 3D in vitro modelling of OSCC using soft tissue-only constructs (Che et al., 2006; Colley et al., 2011; Kataoka et al., 2010). However, OSCC frequently invades the underlying alveolar bone due to close anatomical relationship of these two entities (Ebrahimi et al., 2011; Goda et al., 2010). Indeed, tumours of the tongue, retromolar region, and floor of mouth invade the mandible in 42%, 48%, and 62% of cases, respectively (Brown et al., 2002). Therefore, the absence of a bone-equivalent construct within the soft tissue models limits their validity in translating in vitro findings which are heavily influenced by the presence or absence of bony invasion. A suitable 3D in vitro model which combines both soft and hard tissues is therefore desirable in achieving a more sophisticated model of OSCC progression.

The aim of this study was to use this 3D printed bone scaffold to construct an in vitro tissue engineered composite human alveolar bone and oral mucosal model (ABMM) and examine the potential application of this model in the investigation of oral cancer progression by further developing a cancerous bone oral mucosal model (CBMM).

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma Aldrich, UK unless otherwise stated. Gingival biopsies and bone chips were obtained with written, informed consent, from patients undergoing elective oral surgery at Charles Clifford Dental Hospital, Sheffield, UK, with the appropriate ethical approval from the UK National Research Ethics Services Committee (number 15/LO/0116). The cell line UPCI-SCC090 was received under Material Transfer Agreement from Prof. S. Gollin, University of Pittsburgh School of Public Health, Pittsburgh.

2.2. Isolation and cultivation of primary human gingival and bone cells

Normal human oral keratinocytes and fibroblasts were isolated from oral mucosa biopsies as previously described (Colley et al., 2011). Briefly, biopsies were collected and incubated for 16 hours at 4 °C in 0.25% trypsin-EDTA solution in order to separate the epithelium from connective tissue. Following enzymatic digestion, oral keratinocytes were scraped and plated at a density of $6\times10^4/cm^2$ with an equal number of irradiated mouse 3T3 feeder layers in Green's medium (Rheinwald and Green, 1975). Fibroblasts were isolated from connective tissue by digestion with 0.05% (w/v) collagenase type I (Gibco, USA) at 37 °C for 4 hours. Then cells were centrifuged and cultured in complete DMEM (DMEM- GlutaMAX™ supplemented with 10% foetal bovine serum, 625 ng/mL fungizone, and 100 IU:100 mg ml^{-1} Penicillin/Streptomycin). Both keratinocytes and fibroblasts were fed three times a week until confluency and then used at passage 2.

Primary alveolar human osteoblasts were isolated from aspirated waste bone chips, collected from patients undergoing osteotomy prior to dental implant placement (Clausen et al., 2006; Jonsson et al., 1999; Mailhot and Borke, 1998). Bone fragments were cultured as explants in complete DMEM supplemented with 50µg/ml L-ascorbic acid 2-phosphate. The culture was left undisturbed for 7 days as any dislodgment of explants may impede cell outgrowth. Medium was replaced 2-3 times/week until the culture attained confluency, whereby cells were subcultured, and used in the 3rd passage.

2.3. 3DP bone scaffold fabrication

The detailed method used to print bilayer scaffold replicating the cortico-cancellous alveolar bone architecture has been described previously (Almela et al., 2017). In brief, an injectable β-Tricalcium phosphate paste was prepared (Sigma, US) and plotted using a 3D-bioprinter (EnvisionTEC, Germany) to form a disc of 10 mm × 2 mm thickness. Scaffolds were air-dried

overnight and then sintered at 1100 ^{o}C. Prior to use, scaffolds were autoclaved and prewetted with medium for 24h.

2.4. Construction of normal alveolar bone mucosal models (ABMMs)

Oral mucosal models (OMMs) were constructed as previously described (Dongari-Bagtzoglou and Kashleva, 2006). A solution of 10×DMEM, FBS 8.5% (v/v), L-glutamine 2 mM, reconstitution buffer (22 mg ml^{-1} sodium bicarbonate and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 5 mg ml^{-1} rat-tail type I collagen (R & D system, UK) was prepared and neutralized by 1M sodium hydroxide to pH=7.4. Then, 1 ml of fibroblasts suspension was added at a concentration of 2×10^5 fibroblasts per model and 2 ml of the resultant fibroblast-containing collagen mixture was transferred to cell culture transwell inserts (0.4 µm pore size, Millipore), incubated at 37 °C for 2 hours until solidified, then completely submerged in complete DMEM for 3 days. Following that, 1×10^6 keratinocytes were seeded in each model and kept in submerged culture for 4 days after which OMMs were raised to air/liquid interface and cultured for a further 10 days.

Bone constructs were prepared by dropwise seeding of each scaffold with 2×10^6 osteoblasts. After 24 hours of culture in complete DMEM /ascorbic acid, cell-scaffold constructs were suspended in a spinner bioreactor (Branstead Stem, UK) which continuously spun for 17 days at a rate of 30 rpm. The medium was changed every other day and once the cultures of bone constructs and OMMs were complete, both tissues were combined using a fibrin adhesive sealant (ARTISS, Baxter, UK). The resultant ABMMs were then cultured at air/liquid interface for additional 5 days, after which the models were processed for assessment and characterisation.

2.5. Construction of cancerous bone mucosa model (CBMMs)

Tumour spheroids were generated from UPCI-SCC090 cells using the liquid overlay method as previously described (Carlsson and Yuhas, 1984). A 96-well plate was coated with 1.5% type V agarose (w/v in serum-free DMEM). Then, 100 µl of cell suspension containing a 1×10^4 UPCI-SCC090 were added to each well. The cells were incubated for 4 days and medium changed every 48h. CBMMs were then generated by addition of 30-35 spheroids of UPCI-SCC090 to ABMMs at different steps of model preparation to produce three distinct levels of OSCC. Spheroids were added either to epithelium (carcinoma in situ), epithelium and connective tissue layers, and connective tissue and bone interface.

2.6. Characterization of mucosal part

Frozen sections of OMMs were prepared for histological and immunofluorescent examination. 14 µm sections were stained with haematoxylin and eosin (H&E) and imaged using an Olympus BX51 microscope and Colourview IIIu camera with associated Cell^D software (Olympus Soft Imaging Solutions, GmbH, Münster, Germany).

For immunofluorescent staining, sections were washed with phosphate buffered saline (PBS), permeabilized with 0.2 % (v/v) Triton x-100, and then blocked with 1 % (w/v) bovine serum albumin (BSA) in 0.1 % (v/v) PBS-Tween for 1 hour. Sections were then incubated overnight at 4 ºC with anti-cytokeratin 13 (1:100, Abcam) and anti-cytokeratin 14 (1:100, Abcam). IgG isotype was use in negative control sections. Following that, secondary antibodies were added and images were captured using Carl Zeiss microscope and colour view QI click camera with associated Image-Pro Plus.7.0.1 software (Zeiss Ltd, Germany).

2.7. Characterization of bony part by scanning electron microscopy (SEM)

SEM was performed to observe cellular morphology at the end of culture. Constructs were removed from the culture medium, washed in PBS, fixed with 3% of glutaraldehyde, and dehydrated in gradient concentrations of 50, 60,70%, 80%, 90% and 100% ethanol. Samples

were sputter-coated with gold (~20 nm) and images were then captured at an acceleration voltage of 15 kV using a scanning electron microscope (Philips XL-20, USA).

2.8. Histological examination of ABMMs and CBMMs

Samples were fixed in 4 % (w/v) paraformaldehyde for 24 hours and then embedded in 2-hydroxyethyl methacrylate resin (Technovit 7100, Heraeus Kulzer) according to manufacturer's instructions. Ground sections were prepared for H&E staining by cutting the block into 100-150 µm sections (IsoMet® 1000 precision saw, Buehler UK Ltd, UK) then the thickness was further reduced to 30-35 µm by grinding with silicon carbide papers of P800 and P1200 roughness (Buehler ™ Metaserv, UK).

2.9. Quantitative real-time PCR (qRT-PCR) examination of ABMMs

To evaluate the osteogenic and epithelial differentiation of ABMM, the expression of the osteogenesis and epithelial associated markers were assessed using qRT-PCR (Motor gene Q, QIAGEN, Germany). Following snap freezing and grinding of ABMMs, RNA extraction (Isolate II RNA, BioLine, UK) and complementary DNA (cDNA) (Life Technologies, UK) were performed according to manufacturer's instructions. Genes encoding the following markers were investigated; Alkaline phosphatase (ALP), Osteopontin (OP), Osteonectin (ON), Osteocalcin (OC), Collagen I (COL1), Cytokeratin 10 (CK10), and Cytokeratin 13 (CK13). B-2-Microglobulin was used as a reference control gene (All Applied biosystem, UK). The positive controls of bone and epithelial component were alveolar osteoblasts and keratinocytes, respectively while the negative control was OMM for bone and no cDNA sample for epithelium. The threshold cycle (Ct) was normalized against the reference gene (Δ Ct) and the expression relative to it was calculated.

2.10. Statistics

Data are representative of three independent experiments. All the measurements were conducted in triplicate (n=3) and the results were reported as mean ± SD using GraphPad Prism v7.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Histological and immunofluorescent examination of mucosal part.

OMMs displayed a multi-layered well-developed stratified squamous oral epithelium consisting of distinct basal, intermediate, and superficial non-keratinized layers overlying collagen-populated fibroblasts in a way mimicking NOM (Fig. 1A and B). Oral keratinocytes showed flattened appearance as they reached the superficial layer while the basal layer had cuboidal cells. Cytokeratin (CKs) analysis showed strong expression of CK13 throughout the entire epithelium of OMM and NOM (Fig. 1C and D). CK14 was strongly expressed in the basal layer while it downregulated in the intermediate layer and disappeared in the superfacial layer (Fig. 1F and G).

3.2. SEM evaluation of the bony part.

Fig. 2A and 2B demonstrated that porous and compact side of a cellular scaffold, respectively. SEM imaging of bony component of ABMM revealed the macroporous scaffold structure that supported the cell adhesion, penetration, and growth on both surfaces of the scaffold. The proliferated cells in the porous side showed elongation and orientation along the scaffold strands which indicates that the osteoblasts spread and align along the surface microstructures (Fig. 2C). The pores and strands in the compact side, however, were completely covered by cellular layer (Fig. 2D).

3.3. Histological assessment of ABMMs and CBMMs

ABMMs demonstrated a histological structure consisting of oral mucosa adhered to the underlying bone scaffold, simulating the native oral hard and soft tissues (Fig. 3A). The uppermost surface displayed a continuous stratified epithelium covering a connective tissue densely populated with fibroblasts. A thin band of cell infiltrated fibrin was revealed at the bone mucosa interface. The pores of 3DP scaffold containing two apparently viable cell layers; a central mass of fusiform cells partially-aligned with each other, and a separate mural monolayer of rounded cells within a more eosinophilic matrix along the inner pore surfaces (Fig. 3B). Such cellular alignment was noticed in native oral bone (Fig. 3C).

CBMMs displayed a combined bone and oral mucosal structure with clearly visible tumour spheroids located at different depths. Fig. 4A illustrates the histological pattern of OSCC in which tumour cells located in the epithelium (carcinoma in situ). Fig. 4B represents OSCC in the epithelium and connective tissue while Fig. 4C demonstrates tumour cells at the connective tissue in direct contact with bone.

3.4. qRT-PCR assessment of ABMMs

Of all genes investigated, COL1 and ON had the highest expression within ABMMs. Bone-specific genes including ALP, OC, and OP as well as epithelial markers; CK10 and CK13 were detected with CK13 showing higher level. The trend of gene expression in ABMM was observed in the osteoblasts (positive control) although the expression of OC and OP was minimal. Undetectable OC and OP as well as the negligible amount of ALP (0.0002) in the mucosal part of the model (negative control) indicated the osteogenic specificity of these markers. Conversely, COL1 and ON were detected in mucosa which demonstrated that these markers can be expressed by cells other than bone cell (Fig. 5).

4. Discussion

The demand for engineering human tissue equivalents for both clinical and experimental purposes is increasing. In the oral and maxillofacial area, several promising studies have replicated various orofacial tissues such as cartilage, skin, bone, and periodontium (Pallua and Suschek, 2010). Tissue engineering of bone (Ferracane et al., 2014; Thavornyutikarn et al., 2014) and oral mucosa (Kinikoglu et al., 2015; Moharamzadeh et al., 2012) in particular, has been extensively investigated using different biomaterials and techniques. However, a truly representative model of the complex tissues native to the orofacial region requires a combination of multiple cells and/or tissues in a single composite construct. Here, we have successfully developed and characterized an in vitro, 3DP, human alveolar bone-mucosal model resembling the native oral hard and soft tissues, and furthermore demonstrated the feasibility of using this model to investigate OSCC progression.

3D printing holds a remarkable promise particularly for tissue engineering by providing a robust and rapid approach to fabricate and assemble tissue with a structural control at macro, micro, and even nanoscale with lower cost and higher flexibility and efficiency. The main advantage of 3D printing technologies is their capability to produce complex 3D objects rapidly from a computer model with varying internal and external structures. Two strategies are involved in this technology; the first one includes cell seeding of complex printed scaffold while the second strategy aims to deliver cells and structure simultaneously using scaffold-based or scaffold-less approach (Zhu, 2016).

The histological structure and expression of key epithelial markers associated with stratification, differentiation, and keratinization were similar between normal and engineered mucosa. This is consistent with the findings of recent studies characterizing engineered oral mucosa and comparing it with normal human oral mucosa (Buskermolen et al., 2016; Jennings

et al., 2016). Similar to other OMMs (Chai et al., 2010; Kinikoglu et al., 2009), the model used in this study showed the characteristics of a para-keratinized epithelium, as demonstrated by the weak expression of CK10 and strong expression of CK13, two established biomarkers of suprabasal cells in keratinized and non-keratinized stratified epithelium, respectively (Reibel et al., 1989). In contrast, other studies revelaed strong expression of CK10 in superficial layer (Moharamzadeh et al., 2008; Tra et al., 2012). Such a difference between these oral mucosal models may be attributed to the fact that oral keratinocytes appear to maintain the properties of their original donor epithelia, which may be either keratinized (gingiva and palate) or non-keratinized (buccal mucosa) (de Luca et al., 1990). Regardless of keratinization status, the epithelium in our model appeared well-differentiated, with discernible superficial and basal layers. Such enhancement of epithelial maturation may be a result of the interactions between fibroblasts and keratinocytes via soluble factors, such as interleukin-1beta secreted by keratinocytes, which in turn regulates the expression of keratinocyte growth factor in fibroblasts (Okazaki et al., 2003; Rakhorst et al., 2006; Witte and Kao, 2005). Regarding gene expression, although all the marker encoded genes were detected, COL1 and ON expressions were higher than the remaining markers. The high expression of COL1 in our model was expected, as collagen is the most abundant component of bone extracellular matrix, constituting 90% of its organic component (Allori et al., 2008), and moreover due to further collagen secretion by fibroblasts present in the mucosal component of the model (Schwarz, 2015). Osteonectin is a major non-collagenous extracellular matrix component of bone. This Calcium binding glycoprotein is present mainly in active osteoblasts, osteoprogenitor cells and immature osteocytes, whereas it is absent in mature osteocytes and therefore it is considered an osteogenic differentiation marker indicating bone formation (Jundt et al., 1987). The main role of ON in active bone mineralisation is to selectively bind newly-secreted collagen fibrils with apatite crystals. By implication, the presence of ON in high levels suggests that abundant

collagenous matrix will also be concomitantly secreted, as was observed in our model (Termine et al., 1981). However, the co-expression of ON with collagen has been reported in cells other than osteoblasts such as fibroblasts and pulp cells which have a high rate of collagen turnover (Shiba et al., 1995). This may explain the concomitant expression of ON and COL1 in OMM (negative control) while all other bone specific markers were un detectable.

This gene expression pattern can be understood within the context of the gradual cell and tissue changes that occur over the steady process of bone formation, including proliferation, matrix maturation, and mineralization. In the matrix formation, cells synthesize significant levels of collagen and growth factors to support matrix formation and their own proliferation. The subsequent mineralization stage is marked by the expression of proteins that have affinity to mineral phases, such as OC, OP, and ON (Lian et al., 1998).

Regarding CBMM, many studies have shown that once the OSCC has invaded the mandible, it may progress through the bone in an erosive, infiltrative or mixed pattern (Slootweg and Muller, 1989). In this study, we may determine the interaction between different kind of tumour microenvironment layers that influence the cancer growth, progression and metastasis. The effect of the gravity could be excluded, as it was noticed dissemination of the tumour cells on the roof of bone marrow as seen in fig. 4, and the SCC spheroids were seeded on vertical direction on the top of the model. However, it should be kept in mind that the size of spheroids and the length of their culture, are limited by the absence of angiogenesis; the innermost tumour cells may therefore become quiescent and ultimately apoptose or necrose. The lack of oxygen & nutrients, as well as the accumulation of waste products and decreased pH can result in a central necrotic core when the spheroid's size exceeds 500 – 600 μm (Friedrich et al., 2007). Although present on a more macroscopic scale, changes in pH, oxygen tension and nutrient availability also occur in vivo as a result of cancer growth outstripping vascular supply, and

therefore these features within our model may replicate those properties of cancer which contribute to tumour resistance to therapy and cytokine release.

Although it is appreciated that the total culture period of 22 days is relatively shorter compared to the in vivo implantation studies that are usually extended for months, such experimental design was necessary. First, finite life span and limited proliferative potential of primary cells impeding culture for long time because cells undergo apoptosis (Jilka et al., 1998). Second, the composite model is cultured at a static air/liquid interface condition after the incorporation of the soft and hard tissues on final days, extended static culture may deprive the deeper parts of the multilayer tissue from oxygen and nutrients causing cell death and tissue necrosis.

Nevertheless, establishment of a tissue engineered in vitro 3D oral cancer model by co-culturing cancer spheroids and multiple types of normal human cells within appropriate multi-layered scaffolds represents a promising approach to simulate in vivo tumour microenvironment and the clinical situation as closely as possible.

5. Conclusion

Our data suggest that the normal human alveolar bone and mucosa relationship can be replicated in vitro. The developed model has the potential to provide a more reliable human cell-based alternative to 2D or animal models for various in vitro applications. In addition, the development of 3D multi-layered oral cancer model provides a representative tool to engineer and study oral cancer at different anatomical levels. It has the potential to be further developed and characterized to be used for the assessment of novel diagnostic or therapeutic approaches to manage OSCC in the future.

Conflicts of interest statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

Allori, A., Sailon, A., Warren, S., 2008. Biological Basis of Bone Formation, Remodeling, and Repair-- Part II: Extracellular Matrix, in: Allori, A. (Ed.), pp. 275-284.

Almela, T., Brook, I.M., Khoshroo, K., Rasoulianboroujeni, M., Fahimipour, F., Tahriri, M., Dashtimoghadam, E., El-Awa, A., Tayebi, L., Moharamzadeh, K., 2017. Simulation of cortico-cancellous bone structure by 3D printing of bilayer calcium phosphate-based scaffolds. Bioprinting 6, 1-7.

Asa'ad, F., Pagni, G., Pilipchuk, S.P., Gianni, A.B., Giannobile, W.V., Rasperini, G., 2016. 3D-Printed Scaffolds and Biomaterials: Review of Alveolar Bone Augmentation and Periodontal Regeneration Applications. Int J Dent 2016, 1239842.

Bose, S., Ke, D., Sahasrabudhe, H., Bandyopadhyay, A., 2018. Additive manufacturing of biomaterials. Progress in Materials Science 93, 45-111.

Bose, S., Vahabzadeh, S., Bandyopadhyay, A., 2013. Bone tissue engineering using 3D printing. Materials Today 16, 496-504.

Brown, J.S., Lowe, D., Kalavrezos, N., Souza, J., Magennis, P., Woolgar, J., 2002. Patterns of invasion and routes of tumor entry into the mandible by oral squamous cell carcinoma. Head & Neck 24, 370-383.

Buskermolen, J.K., Reijnders, C.M.A., Spiekstra, S.W., Steinberg, T., Kleverlaan, C.J., Feilzer, A.J., Bakker, A.D., Gibbs, S., 2016. Development of a Full- Thickness Human Gingiva Equivalent Constructed from Immortalized Keratinocytes and Fibroblasts. 22, 781-791.

Carlsson, J., Yuhas, J.M., 1984. Liquid-overlay culture of cellular spheroids. Recent Results Cancer Res 95, 1-23.

Chai, W.L., Moharamzadeh, K., Van Noort, R., Brook, I.M., Emanuelsson, L., Palmquist, A., 2010. Development of a novel model for the investigation of implant- soft tissue interface. Journal of Periodontology 81, 1187-1195.

Che, Z.M., Jung, T.H., Choi, J.H., Yoon, D.J., Jeong, H.J., Lee, E.J., Kim, J., 2006. Collagen-based co- culture for invasive study on cancer cells- fibroblasts interaction. Biochemical and Biophysical Research Communications 346, 268-275.

Chi, A.C., Day, T.A., Neville, B.W., 2015. Oral cavity and oropharyngeal squamous cell carcinoma--an update. CA Cancer J Clin 65, 401-421.

Clausen, C., Hermund, N.U., Donatsky, O., Nielsen, H., 2006. Characterization of human bone cells derived from the maxillary alveolar ridge. Clinical Oral Implants Research 17, 533-540.

Colley, H.E., Hearnden, V., Jones, A.V., Weinreb, P.H., Violette, S.M., Macneil, S., Thornhill, M.H., Murdoch, C., 2011. Development of tissue- engineered models of oral dysplasia and early invasive oral squamous cell carcinoma. British Journal of Cancer 105, 1582.

de Luca, M., Albanese, E., Megna, M., Cancedda, R., Mangiante, P.E., Cadoni, A., Franzi, A.T., 1990. Evidence that human oral epithelium reconstituted in vitro and transplanted onto patients with defects in the oral mucosa retains properties of the original donor site. Transplantation 50, 454-459.

Dongari-Bagtzoglou, A., Kashleva, H., 2006. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. Nat Protoc 1, 2012-2018.

Ebrahimi, A., Murali, R., Gao, K., Elliott, M.S., Clark, J.R., 2011. The prognostic and staging implications of bone invasion in oral squamous cell carcinoma. Cancer 117, 4460-4467.

Egan, P., Ferguson, S.J., Shea, K., 2017. Design of Hierarchical Three- Dimensional Printed Scaffolds Considering Mechanical and Biological Factors for Bone Tissue Engineering. J. Mech. Des. 139.

Ferracane, J.L., Giannobile, W.V., Tevlin, R., McArdle, A., Atashroo, D., Walmsley, G.G., Senarath-Yapa, K., Zielins, E.R., Paik, K.J., Longaker, M.T., Wan, D.C., 2014. Biomaterials for Craniofacial Bone Engineering. Journal of Dental Research 93, 1187-1195.

Friedrich, J., Ebner, R., Kunz-Schughart, L.A., 2007. Experimental anti-tumor therapy in 3-D: spheroids--old hat or new challenge? Int J Radiat Biol 83, 849-871.

Goda, T., Shimo, T., Yoshihama, Y., Hassan, N.M., Ibaragi, S., Kurio, N., Okui, T., Honami, T., Kishimoto, K., Sasaki, A., 2010. Bone destruction by invading oral squamous carcinoma cells mediated by the transforming growth factor-beta signalling pathway. Anticancer Res 30, 2615-2623.

Jennings, L.R., Colley, H.E., Ong, J., Panagakos, F., Masters, J.G., Trivedi, H.M., Murdoch, C., Whawell, S., 2016. Development and Characterization of In Vitro Human Oral Mucosal Equivalents Derived from Immortalized Oral Keratinocytes. Tissue Eng Part C Methods 22, 1108-1117.

Jonsson, K.B., Ljunghall, S., Ljunggren, Ö., Frost, A., Nilsson, O., 1999. Three isolation techniques for primary culture of human osteoblast- like cells. A comparison. Acta Orthopaedica Scandinavica 70, 365-373.

Jundt, G., Berghäuser, K., Termine, J., Schulz, A., 1987. Osteonectin — a differentiation marker of bone cells. Cell and Tissue Research 248, 409-415.

Kataoka, T., Umeda, M., Shigeta, T., Takahashi, H., Komori, T., 2010. A new in vitro model of cancer invasion using AlloDerm, a human cadaveric dermal equivalent: a preliminary report. The Kobe journal of medical sciences 55, E106.

Kinikoglu, B., Auxenfans, C., Pierrillas, P., Justin, V., Breton, P., Burillon, C., Hasirci, V., Damour, O., 2009. Reconstruction of a full- thickness collagen- based human oral mucosal equivalent. Biomaterials 30, 6418-6425.

Kinikoglu, B., Damour, O., Hasirci, V., 2015. Tissue engineering of oral mucosa: a shared concept with skin. J Artif Organs 18, 8-19.

Lanza, R.P., Langer, R.S., Vacanti, J., Langer, R.S., Lanza, R.P., Lanza, R., 2014. Principles of tissue engineering, Fourth edition. ed. Amsterdam : Academic Press, 2014.

Lian, J.B., Stein, G.S., Stein, J.L., Van Wijnen, A.J., 1998. Osteocalcin gene promoter: Unlocking the secrets for regulation of osteoblast growth and differentiation. Journal of Cellular Biochemistry, 62-72.

Mailhot, J.M., Borke, J.L., 1998. An isolation and in vitro culturing method for human intraoral bone cells derived from dental implant preparation sites. Clinical Oral Implants Research 9, 43-50.

Moharamzadeh, K., Brook, I., Noort, R., Scutt, A., Smith, K., Thornhill, M., 2008. Development, optimization and characterization of a full-thickness tissue engineered human oral mucosal model for biological assessment of dental biomaterials. J Mater Sci: Mater Med 19, 1793-1801.

Okazaki, M., Yoshimura, K., Suzuki, Y., Harii, K., 2003. Effects of subepithelial fibroblasts on epithelial differentiation in human skin and oral mucosa: heterotypically recombined organotypic culture model. Plastic and reconstructive surgery 112, 784.

Pallua, N., Suschek, C.V., 2010. Tissue engineering: from lab to clinic. Springer, London

Berlin.

Pati, F., Gantelius, J., Svahn, H.A., 2016. 3D Bioprinting of Tissue/ Organ Models, pp. 4650-4665.

Rakhorst, H., Tra, W., Van Neck, J.W., Van Osch, G., Hovius, S., El Ghalbzouri, A., Hofer, S.O.P., 2006. Fibroblasts Accelerate Culturing of Mucosal Substitutes. Tissue Engineering 12, 2321-2331.

Reibel, J., Clausen, H., Dale, B.A., Thacher, S.M., 1989. Immunohistochemical analysis of stratum corneum components in oral squamous epithelia. Differentiation 41, 237-244.

Rheinwald, J.G., Green, H., 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6, 331-334.

Schwarz, R.I., 2015. Collagen I and the fibroblast: High protein expression requires a new paradigm of post- transcriptional, feedback regulation. Biochemistry and Biophysics Reports 3, 38-44.

Sears, N.A., Seshadri, D.R., Dhavalikar, P.S., Cosgriff-Hernandez, E., 2016. A Review of Three- Dimensional Printing in Tissue Engineering. Tissue Engineering Part B: Reviews 22, 298-310.

Shiba, H., Nakamura, S., Shirakawa, M., Nakanishi, K., Okamoto, H., Satakeda, H., Noshiro, M., Kamihagi, K., Katayama, M., Kato, Y., 1995. Effects of basic fibroblast growth factor on proliferation, the expression of osteonectin (SPARC) and alkaline phosphatase, and calcification in cultures of human pulp cells. Dev Biol 170, 457-466.

Slootweg, P.J., Muller, H., 1989. Mandibular invasion by oral squamous cell carcinoma. J Craniomaxillofac Surg 17, 69-74.

Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L., Martin, G.R., 1981. Osteonectin, a bone- specific protein linking mineral to collagen. Cell 26, 99-105.

Thavornyutikarn, B., Chantarapanich, N., Sitthiseripratip, K., Thouas, G.A., Chen, Q., 2014. Bone tissue engineering scaffolding: computer- aided scaffolding techniques. Progress in Biomaterials 3, 61-102.

Tra, W.M.W., Van Neck, J.W., Hovius, S.E.R., Perez-Amodio, S., Van Osch, G.J.V.M., 2012. Characterization of a three- dimensional mucosal equivalent: Similarities and differences with native oral mucosa. Cells Tissues Organs 195, 185-196.

Wang, C., Zhao, Q., Wang, M., 2017. Cryogenic 3d printing for producing hierarchical porous and rhbmp- 2- loaded ca- p/ plla nanocomposite scaffolds for bone tissue engineering. Biofabrication 9, 025031.

Witte, R.P., Kao, W.J., 2005. Keratinocyte- fibroblast paracrine interaction: the effects of substrate and culture condition. Biomaterials 26, 3673-3682.

Figure legends:

Fig. 1. Mucosa part of ABMM display similar characteristics to normal oral mucosa (NOM). Engineered oral mucosa were generated by culturing oral keratinocytes on top of a fibroblast-populated collagen scaffold and compared with human oral mucosa. Image showing H &E histological section **(A and B)** and immunofluorescent analysis for cytokeratin 13 **(C-E)**, cytokeratin 14 **(F-H)** Representative images are from three independent experiments (Scale bars = 100 µm).

Fig. 2. SEM micrographs of bony component of ABMM. Image showing the porous and compact sides of 3DP scaffold without cells **(A and B)** and bony part of ABMM **(C and D)** (Scale bars = 25 µm).

Fig. 3. H&E stained histological ground section of 3DP ABMM. The image showing full-thickness, multi-layered 3DP bone mucosal construct consisting of a stratified oral epithelium, connective tissue layer adherent to the underlying 3DP bone **(A)**. The bony part showing the pores of the scaffold containing two apparently viable cell layers; a central mass of fusiform cells partially-aligned with each other (red arrows), and a separate mural monolayer of rounded cells (blue arrows) **(B)**. This cellular alignment was noticed in natural alveolar bone **(C)** (Scale bars: A = 500 µm; B and C = 100 µm).

Fig. 4. H&E stained histological ground sections of CBMM representing OSCC spheroids with different anatomical level. Red arrows indicate to tumor spheroids in epithelium **(A);** the epithelium and connective tissue **(B);** and connective tissue layer in direct contact with the bone **(C)** (Scale bars =200 µm).

Fig. 5. qRT-PCR analysis of the osteogenic and epithelial gene expression ABMM. The osteogenic genes; OC, ALP, OP, ON, and COL1 and epithelial genes; CK10 and CK13 were detected in the composite model and positive controls (human oral osteoblasts and keratinocytes for bone and epithelial components, respectively). Negative control for bone (OMM) revealed undetectable osteogenic genes except ON and COL1 which can be expressed by non-bony cells.

Allori, A., Sailon, A., Warren, S., 2008. Biological Basis of Bone Formation, Remodeling, and Repair--Part I: Biochemical Signaling Molecules, in: Allori, A. (Ed.), pp. 259-274.
Almela, T., Brook, I.M., Khoshroo, K., Rasoulianboroujeni, M., Fahimipour, F., Tahriri, M., Dashtimoghadam, E., El-Awa, A., Tayebi, L., Moharamzadeh, K., 2017. Simulation of cortico-cancellous bone structure by 3D printing of bilayer calcium phosphate-based scaffolds. Bioprinting 6, 1-7.
Asa'ad, F., Pagni, G., Pilipchuk, S.P., Giannì, A.B., Giannobile, W.V., Rasperini, G., 2016. 3D- Printed Scaffolds and Biomaterials: Review of Alveolar Bone Augmentation and Periodontal Regeneration Applications. International Journal of Dentistry 2016.
Bose, S., Ke, D., Sahasrabudhe, H., Bandyopadhyay, A., 2018. Additive manufacturing of biomaterials. Progress in Materials Science 93, 45-111.
Bose, S., Roy, M., Bandyopadhyay, A., 2012. Recent advances in bone tissue engineering scaffolds. Trends in Biotechnology.
Bose, S., Vahabzadeh, S., Bandyopadhyay, A., 2013. Bone tissue engineering using 3D printing. Materials Today 16, 496-504.
Brown, J.S., Lowe, D., Kalavrezos, N., Souza, J., Magennis, P., Woolgar, J., 2002. Patterns of invasion and routes of tumor entry into the mandible by oral squamous cell carcinoma. Head & Neck 24, 370-383.
Buskermolen, J.K., Reijnders, C.M.A., Spiekstra, S.W., Steinberg, T., Kleverlaan, C.J., Feilzer, A.J., Bakker, A.D., Gibbs, S., 2016. Development of a Full- Thickness Human Gingiva Equivalent Constructed from Immortalized Keratinocytes and Fibroblasts. 22, 781-791.
Chai, W.L., Moharamzadeh, K., Van Noort, R., Brook, I.M., Emanuelsson, L., Palmquist, A., 2010. Development of a novel model for the investigation of implant- soft tissue interface. Journal of Periodontology 81, 1187-1195.
Che, Z.M., Jung, T.H., Choi, J.H., Yoon, D.J., Jeong, H.J., Lee, E.J., Kim, J., 2006. Collagen- based co-culture for invasive study on cancer cells- fibroblasts interaction. Biochemical and Biophysical Research Communications 346, 268-275.
Chi, A.C., Day, T.A., Neville, B.W., 2015. Oral cavity and oropharyngeal squamous cell carcinoma--an update. CA Cancer J Clin 65, 401-421.
Clausen, C., Hermund, N.U., Donatsky, O., Nielsen, H., 2006. Characterization of human bone cells derived from the maxillary alveolar ridge. Clinical Oral Implants Research 17, 533-540.
Colley, H.E., Hearnden, V., Jones, A.V., Weinreb, P.H., Violette, S.M., Macneil, S., Thornhill, M.H., Murdoch, C., 2011. Development of tissue- engineered models of oral dysplasia and early invasive oral squamous cell carcinoma. British Journal of Cancer 105, 1582.
de Luca, M., Albanese, E., Megna, M., Cancedda, R., Mangiante, P.E., Cadoni, A., Franzi, A.T., 1990. Evidence that human oral epithelium reconstituted in vitro and transplanted onto patients with defects in the oral mucosa retains properties of the original donor site. Transplantation 50, 454-459.
Dongari-Bagtzoglou, A., Kashleva, H., 2006. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. Nat Protoc 1, 2012-2018.
Ebrahimi, A., Murali, R., Gao, K., Elliott, M.S., Clark, J.R., 2011. The prognostic and staging implications of bone invasion in oral squamous cell carcinoma. Cancer 117, 4460-4467.
Egan, P., Ferguson, S.J., Shea, K., 2017. Design of Hierarchical Three- Dimensional Printed Scaffolds Considering Mechanical and Biological Factors for Bone Tissue Engineering. J. Mech. Des. 139.
Ferracane, J.L., Giannobile, W.V., Tevlin, R., McArdle, A., Atashroo, D., Walmsley, G.G., Senarath-Yapa, K., Zielins, E.R., Paik, K.J., Longaker, M.T., Wan, D.C., 2014. Biomaterials for Craniofacial Bone Engineering. Journal of Dental Research 93, 1187-1195.
Friedrich, J., Ebner, R., Kunz-Schughart, L.A., 2007. Experimental anti-tumor therapy in 3-D: spheroids--old hat or new challenge? Int J Radiat Biol 83, 849-871.
Goda, T., Shimo, T., Yoshihama, Y., Hassan, N.M., Ibaragi, S., Kurio, N., Okui, T., Honami, T., Kishimoto, K., Sasaki, A., 2010. Bone destruction by invading oral squamous carcinoma cells mediated by the transforming growth factor-beta signalling pathway. Anticancer Res 30, 2615-2623.

Jennings, L.R., Colley, H.E., Ong, J., Panagakos, F., Masters, J.G., Trivedi, H.M., Murdoch, C., Whawell, S., 2016. Development and Characterization of In Vitro Human Oral Mucosal Equivalents Derived from Immortalized Oral Keratinocytes. Tissue Eng Part C Methods 22, 1108-1117.
Jilka, R.L., Weinstein, R.S., Bellido, T., Parfitt, A.M., Manolagas, S.C., 1998. Osteoblast Programmed Cell Death (Apoptosis): Modulation by Growth Factors and Cytokines. Journal of Bone and Mineral Research 13, 793-802.
Jonsson, K.B., Ljunghall, S., Ljunggren, Ö., Frost, A., Nilsson, O., 1999. Three isolation techniques for primary culture of human osteoblast- like cells. A comparison. Acta Orthopaedica Scandinavica 70, 365-373.
Jundt, G., Berghäuser, K., Termine, J., Schulz, A., 1987. Osteonectin — a differentiation marker of bone cells. Cell and Tissue Research 248, 409-415.
Kataoka, T., Umeda, M., Shigeta, T., Takahashi, H., Komori, T., 2010. A new in vitro model of cancer invasion using AlloDerm, a human cadaveric dermal equivalent: a preliminary report. The Kobe journal of medical sciences 55, E106.
Kinikoglu, B., Auxenfans, C., Pierrillas, P., Justin, V., Breton, P., Burillon, C., Hasirci, V., Damour, O., 2009. Reconstruction of a full- thickness collagen- based human oral mucosal equivalent. Biomaterials 30, 6418-6425.
Kinikoglu, B., Damour, O., Hasirci, V., 2015. Tissue engineering of oral mucosa: a shared concept with skin. J Artif Organs 18, 8-19.
Lanza, R.P., Langer, R.S., Vacanti, J., Langer, R.S., Lanza, R.P., Lanza, R., 2014. Principles of tissue engineering, Fourth edition. ed. Amsterdam : Academic Press, 2014.
Lian, J.B., Stein, G.S., Stein, J.L., Van Wijnen, A.J., 1998. Osteocalcin gene promoter: Unlocking the secrets for regulation of osteoblast growth and differentiation. Journal of Cellular Biochemistry, 62-72.
Mailhot, J.M., Borke, J.L., 1998. An isolation and in vitro culturing method for human intraoral bone cells derived from dental implant preparation sites. Clinical Oral Implants Research 9, 43-50.
Moharamzadeh, K., Brook, I., Noort, R., Scutt, A., Smith, K., Thornhill, M., 2008. Development, optimization and characterization of a full-thickness tissue engineered human oral mucosal model for biological assessment of dental biomaterials. J Mater Sci: Mater Med 19, 1793-1801.
Moharamzadeh, K., Colley, H., Murdoch, C., Hearnden, V., Chai, W.L., Brook, I.M., Thornhill, M.H., MacNeil, S., 2012. Tissue- engineered Oral Mucosa. Journal of Dental Research 91, 642-650.
Okazaki, M., Yoshimura, K., Suzuki, Y., Harii, K., 2003. Effects of subepithelial fibroblasts on epithelial differentiation in human skin and oral mucosa: heterotypically recombined organotypic culture model. Plastic and reconstructive surgery 112, 784.
Pallua, N., Suschek, C.V., 2010. Tissue engineering: from lab to clinic. Springer, London

Berlin.
Pati, F., Gantelius, J., Svahn, H.A., 2016. 3D Bioprinting of Tissue/ Organ Models, pp. 4650-4665.
Rakhorst, H., Tra, W., Van Neck, J.W., Van Osch, G., Hovius, S., El Ghalbzouri, A., Hofer, S.O.P., 2006. Fibroblasts Accelerate Culturing of Mucosal Substitutes. Tissue Engineering 12, 2321-2331.
Reibel, J., Clausen, H., Dale, B.A., Thacher, S.M., 1989. Immunohistochemical analysis of stratum corneum components in oral squamous epithelia. Differentiation 41, 237-244.
Rheinwald, J.G., Green, H., 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6, 331-334.
Schwarz, R.I., 2015. Collagen I and the fibroblast: High protein expression requires a new paradigm of post- transcriptional, feedback regulation. Biochemistry and Biophysics Reports 3, 38-44.
Sean, V.M., Anthony, A., 2014. 3D bioprinting of tissues and organs. Nature Biotechnology 32, 773.
Sears, N.A., Seshadri, D.R., Dhavalikar, P.S., Cosgriff-Hernandez, E., 2016. A Review of Three-Dimensional Printing in Tissue Engineering. Tissue Engineering Part B: Reviews 22, 298-310.
Shiba, H., Nakamura, S., Shirakawa, M., Nakanishi, K., Okamoto, H., Satakeda, H., Noshiro, M., Kamihagi, K., Katayama, M., Kato, Y., 1995. Effects of basic fibroblast growth factor on proliferation,

the expression of osteonectin (SPARC) and alkaline phosphatase, and calcification in cultures of human pulp cells. Dev Biol 170, 457-466.
Slootweg, P.J., Muller, H., 1989. Mandibular invasion by oral squamous cell carcinoma. J Craniomaxillofac Surg 17, 69-74.
Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L., Martin, G.R., 1981. Osteonectin, a bone- specific protein linking mineral to collagen. Cell 26, 99-105.
Thavornyutikarn, B., Chantarapanich, N., Sitthiseripratip, K., Thouas, G.A., Chen, Q., 2014. Bone tissue engineering scaffolding: computer- aided scaffolding techniques. Progress in Biomaterials 3, 61-102.
Tra, W.M.W., Van Neck, J.W., Hovius, S.E.R., Perez-Amodio, S., Van Osch, G.J.V.M., 2012. Characterization of a three- dimensional mucosal equivalent: Similarities and differences with native oral mucosa. Cells Tissues Organs 195, 185-196.
Wang, C., Zhao, Q., Wang, M., 2017. Cryogenic 3d printing for producing hierarchical porous and rhbmp- 2- loaded ca- p/ plla nanocomposite scaffolds for bone tissue engineering. Biofabrication 9, 025031.
Witte, R.P., Kao, W.J., 2005. Keratinocyte- fibroblast paracrine interaction: the effects of substrate and culture condition. Biomaterials 26, 3673-3682.
Zhu, W., 2016. 3D Printing of Functional Biomaterials for Tissue Modeling.