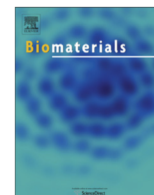
Biomaterials 106 (2016) 167–179

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Dental cell sheet biomimetic tooth bud model

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form
12 August 2016
Accepted 15 August 2016
Available online 17 August 2016

Keywords:
Tooth development
Dental stem cells
Tissue engineering
Regenerative medicine
Biomaterials

ABSTRACT

Tissue engineering and regenerative medicine technologies offer promising therapies for both medicine and dentistry. Our long-term goal is to create functional biomimetic tooth buds for eventual tooth replacement in humans. Here, our objective was to create a biomimetic 3D tooth bud model consisting of dental epithelial (DE) – dental mesenchymal (DM) cell sheets (CSs) combined with biomimetic enamel organ and pulp organ layers created using GelMA hydrogels. Pig DE or DM cells seeded on temperature-responsive plates at various cell densities (0.02, 0.114 and 0.228 cells $10^6/cm^2$) and cultured for 7, 14 and 21 days were used to generate DE and DM cell sheets, respectively. Dental CSs were combined with GelMA encapsulated DE and DM cell layers to form bioengineered 3D tooth buds. Biomimetic 3D tooth bud constructs were cultured *in vitro*, or implanted *in vivo* for 3 weeks. Analyses were performed using micro-CT, H&E staining, polarized light (Pol) microscopy, immunofluorescent (IF) and immunohistochemical (IHC) analyses. H&E, IHC and IF analyses showed that *in vitro* cultured multilayered DE-DM CSs expressed appropriate tooth marker expression patterns including SHH, BMP2, RUNX2, tenascin and syndecan, which normally direct DE-DM interactions, DM cell condensation, and dental cell differentiation. *In vivo* implanted 3D tooth bud constructs exhibited mineralized tissue formation of specified size and shape, and SHH, BMP2 and RUNX2and dental cell differentiation marker expression. We propose our biomimetic 3D tooth buds as models to study optimized DE-DM cell interactions leading to functional biomimetic replacement tooth formation.

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1. Introduction

Tooth loss due to genetic disorders or microbial diseases, iatrogenic, traumatic, or therapeutic insults, patient negligence and poor oral hygiene continues to affect most adults at some point during their lives [3,4]. It is estimated that approximately 150 million adults currently suffer from tooth loss, and that over 10 Million new cases of edentulism will arise during this decade [3]. Dental clinical procedures such as root canal treatment and dental implants are commonly used as tooth repair/replacement therapies [5,6]. However, root canal therapy results in loss of tooth sensitivity and vitality, hence, the tooth cannot respond immunologically to subsequent infections, and also become brittle over time [5]. Dental implants, such as titanium implants, are not equivalent to natural teeth, either in function nor aesthetics, because they lack periodontal and cementum tissues which function to cushion and modulate the mechanical stress of mastication, while at the same time promoting healthy alveolar bone turnover [7,8]. These disadvantages have prompted an ongoing search for alternative methods that would avoid the need for root canal and for dental implants. Examples of proposed approaches to engineer biological teeth include: tissue engineering scaffolds; stimulation of third dentition formation; cell-tissue recombination; chimeric tooth tissue engineering; and gene-manipulated tooth regeneration [8–11].

To date, our group has been using tissue engineering approaches to identify optimal scaffold materials and designs that promote dental epithelial (DE) and mesenchymal (DM) cell interactions leading to replacement tooth formation [4,12,13]. For the study presented here, we have used DE and DM progenitor cells isolated from un-erupted molar tooth buds extracted from 5 to 6 month old

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http://dx.doi.org/10.1016/j.biomaterials.2016.08.024
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porcine jaws, which consisted of enamel organ, dental papilla, and dental follicle tissues and cells. The enamel organ is derived from the ectoderm, while the dental papilla and dental follicle are derived from the neural crest (also called ectomesenchyme) [14]. Briefly, harvested tooth bud tissues were used to create single cell suspensions, which were then cultured *in vitro* as previously described [4,15]. Differentiated DE and DM cells die during this process, resulting in highly enriched populations of undifferentiated DE and DM cells. The rationale for using pig teeth at these developmental stages was to obtain sufficient numbers of dental progenitor cell populations, also known as dental stem cells (DSCs), that can be used to generate teeth and supporting tissues [16]. The progenitor DE cells generated in this manner will differentiate into enamel forming ameloblasts, while the DM cells will give rise to odontoblasts, dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP) and dental follicle precursor cells (DFPCs) [17]. In many instances, our prior published reports have shown that instead of creating bioengineered teeth that adopted the size and shape of the scaffold, we observed that many small tooth crowns formed throughout the implant, suggesting that these models lacked the proper ECM molecule gradients present in naturally formed teeth, which provide essential cues for proper tooth development, and for periodontal tissue and surrounding alveolar bone formation [4,12,13,15].

To address this, we recently established a 3D biomimetic tooth bud model using photopolymerizable gelatin methacrylamide (GelMA) hydrogel formulas, designed to facilitate DE and DM cell interactions leading to ameloblast and odontoblast differentiation, respectively, and the formation of bioengineered teeth of predictable size and shape [18,19]. GelMA hydrogels exhibit many properties that make it an attractive material for tissue engineering applications including [20,21]: i) it is largely composed of denatured collagen and is relatively inexpensive; ii) it retains collagen's natural RGD adhesive domains and MMP sensitive sites which are known to enhance cell binding and cell-mediated matrix degradation, respectively; iii) the physical properties of GelMA hydrogels can be tuned by varying GelMA and/or photoinitiator (PI) concentrations; and iv) GelMA is suitable for cell encapsulation at 37 °C, and promotes cell viability and proliferation. In these reports we identified GelMA formulas that exhibited elastic moduli similar to those of natural tooth bud derived enamel organ and pulp organ tissue, and in addition to DE and DM cells we incorporated Human Umbilical Vein Endothelial Cells (HUVECs) to promote neovasculature formation to facilitate *in vivo* engraftment with host tissues [18,19]. Our published 3D tooth bud model consisted of a biomimetic enamel organ layer (DE-HUVEC encapsulated in 3% GelMA) and biomimetic pulp organ (DM-HUVEC encapsulated in 5% GelMA). *In vitro* culture and *in vivo* implantation studies showed that the 3D GelMA biomimetic tooth bud constructs supported DE and DM cell attachment, spreading, metabolic activity, neovasculature formation, and mineralized tissue formation of specified size and shape *in vivo* [19]. However, limitations to this model included cell mixing between GelMA layers, and lack of distinct enamel or dentin layers.

Here, we aimed to improve our 3D tooth bud model, and overcome such limitations by using successive photocrosslinking of individual dental cell-seeded GelMA layers, and to increase DE-DM cell interactions by introducing DE-DM cell sheet (CSs) layers between the biomimetic enamel and pulp organs of our 3D tooth bud model (Fig. 1). Our results demonstrate the successful creation of multilayered DE-DM cell sheet containing GelMA (CSG) 3D biomimetic tooth bud constructs. We also show that *in vivo* CSG implanted constructs exhibited distinct biomimetic enamel and pulp layers, and that DE and DM cells express dental cell differentiation marker expression (DSPP, OC, and AM). We propose this novel 3D bioengineered tooth bud model as a means to study DE and DM cell interactions leading to biomimetic replacement tooth formation.

2. Materials and methods

2.1. Primary dental cell isolation, in vitro culture and expansion

Porcine DE and DM progenitor cells were obtained and cultured as previously published [4,15]. Briefly, DE and DM progenitor cells were isolated from un-erupted tooth buds extracted from 5 month old porcine jaws. Single cell suspensions of DE and DM tooth bud cells were prepared, seeded in T175 cm^2 flasks (Corning Inc., Corning, NY, USA), and expanded using epithelial medium [LHC-8 (GIBCO), 10% FBS, 1% PSA, 0.5 g/mL Epinephrine] or mesenchymal medium [Advanced DMEMF12 (GIBCO), 10% FBS, 25 μg/mL Ascorbic Acid, 1% PSA, 1% Glutamax], respectively, in 5% CO_2 at 37 °C. Expanded cells were cryopreserved in 10% DMSO in appropriate culture media until use.

2.2. Dental cell sheet fabrication

DE and DM tooth bud cells were recovered from cryopreservation and expanded in T175 cm^2 flasks. DE and DM cells were trypsinized and seeded on UpCell thermo-responsive plates (CellSeed, Tokyo, Japan) at densities of 0.02, 0.114 and 0.228 $\times$ 10^6 cells/cm^2 for 7, 14 and 21 days, in 5% CO_2 at 37 °C. Cell sheets (CSs) were detached from the thermo-responsive plates by decreasing the temperature to 20 °C and harvested by gentle flushing with media. The formation of layered CS was made by careful stacking of the CSs [22].

2.3. Creation of 3D tooth bud constructs

Biomimetic 3D tooth bud constructs were fabricated as depicted in Fig. 1. DE and DM cell sheets were harvested after 14 days at plating cell densities of 0.228 and 0.114 $\times$ 10^6 cells/cm^2, respectively. Lyophilized GelMA was fully dissolved in DMEM/F12 media (w/v), and photo-initiator (Irgacure2959, Sigma, St. Louis, MO) was added to create 3 and 5% GelMA formulations, denoted as 3% GelMA and 5% GelMA. Dental cell containing GelMA bilayers were created by sequential photo-crosslinking via exposure to 9.16 W/cm2 UV light for 20 s using an Omnicure S2000 (Lumen Dynamics Group Inc., Mississauga (ON) Canada). For *in vitro* studies, harvested cell sheets were layered over dental cell encapsulated and acellular GelMA constructs and cultured in osteogenic media (DMEM/F12:LCH8 basal media supplemented with, 1%PSA, 10% FBS, 100 nM Dexamethasone, 10 mM beta Glycerol Phosphate, 0.05 mM Ascorbic Acid) for 24 h, and for 4, 7 and 12 days, in 5% CO2 at 37 °C (Fig. 1).

2.4. In vivo implantation of biomimetic tooth bud constructs

For *in vivo* analyses, bioengineered 3D CS-GelMA constructs were cultured in osteogenic media for 4 days in 5% CO2 at 37 °C, and randomly implanted subcutaneously onto the backs of immunocompromised 5 month old female Rowett Nude rats (Charles River Laboratories, Willmington, MA). All animal surgeries were performed using Tufts University approved Institutional Animal Care and Use Committee (IACUC) protocols and Mandatory Animal Care and Use (MACU) regulations. Four replicate bioengineered 3D tooth buds and acellular GelMA constructs were implanted and harvested after 3 weeks (Fig. 1).

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