Published in final edited form as:

J Tissue Eng Regen Med. 2017 December ; 11(12): 3326–3336. doi:10.1002/term.2246.

Developing a biomimetic tooth bud model

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Abstract

A long-term goal is to bioengineer, fully functional, living teeth for regenerative medicine and dentistry applications. Biologically based replacement teeth would avoid insufficiencies of the currently used dental implants. Using natural tooth development as a guide, a model was fabricated using post-natal porcine dental epithelial (pDE), porcine dental mesenchymal (pDM) progenitor cells, and human umbilical vein endothelial cells (HUVEC) encapsulated within gelatin methacrylate (GelMA) hydrogels. Previous publications have shown that post-natal DE and DM cells seeded onto synthetic scaffolds exhibited mineralized tooth crowns composed of dentin and enamel. However, these tooth structures were small and formed within the pores of the scaffolds. The present study shows that dental cell-encapsulated GelMA constructs can support mineralized dental tissue formation of predictable size and shape. Individually encapsulated pDE or pDM cell GelMA constructs were analysed to identify formulas that supported pDE and pDM cell attachment, spreading, metabolic activity, and neo-vasculature formation with co-seeded endothelial cells (HUVECs). GelMA constructs consisting of pDE-HUVECS in 3% GelMA and pDM-HUVECS within 5% GelMA supported dental cell differentiation and vascular mineralized dental tissue formation *in vivo*. These studies are the first to demonstrate the use of GelMA hydrogels to support the formation of post-natal dental progenitor cell-derived mineralized and functionally vascularized tissues of specified size and shape. These results introduce a novel three-

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Conflict of interest

The authors have declared that there is no conflict of interest.

Supporting information

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dimensional biomimetic tooth bud model for eventual bioengineered tooth replacement teeth in humans.

Keywords

biodegradable hydrogel; cell encapsulation; cell differentiation; biomineralization; progenitor cell; vascularization

1. Introduction

Mature teeth are highly complex mineralized organs that play vital roles in our everyday lives. It has been reported that over 158 million people globally are currently suffering from tooth loss (Vos *et al.* 2012). Synthetic dental implants are a common therapy for tooth loss. However, artificial implants may cause severe complications, such as peri-implantitis, bone loss, receding gums and periodontal tissues, and eventual implant failure (Greenstein *et al.*, 2008; Jung *et al.*, 2008). Fully functional biologically based biomimetic teeth, that are vascularized and innervated, would be an attractive alternative to currently used artificial dental implants (Murray *et al.*, 2007; Yen and Yelick, 2011; Lai *et al.*, 2014).

The present study describes the development and fabrication of a bioengineered, three-dimensional (3D) biologically based tooth bud model, designed to facilitate the dental epithelial (DE) and dental mesenchymal (DM) tissue interactions that occur in natural tooth development (Thesleff *et al.*, 1989). Previously, it was shown that post-natal porcine and rat DE and DM cells, when seeded onto synthetic scaffolds, retained the ability to form small, anatomically correct tooth crowns consisting of enamel, dentin and pulp tissues (Young *et al.*, 2002; Duailibi *et al.*, 2004, 2008; Abukawa *et al.*, 2009). These studies were the first to demonstrate the successful use of adult, post-natal (as opposed to embryonic) dental progenitor cells for whole-tooth tissue engineering applications. However, an important limitation of these bioengineered teeth is that they were very small and of unpredictable size and shape.

A novel biomimetic tooth bud model that employs post-natal dental cells encapsulated within tunable, photopolymerizable gelatin methacrylate (GelMA) hydrogel scaffolds is described. Dental cell-encapsulated GelMA constructs were designed to facilitate organized DE–DE, DM–DM and DE–DM cell interactions leading to amelo-blast and odontoblast differentiation, respectively, and the formation of bioengineered teeth of predictable size and shape. GelMA hydrogels are largely composed of denatured collagen and retain many of collagen's natural properties including Arg-Gly-Asp (RGD) adhesive domains and matrix metalloproteinase (MMP) sensitive sites (Nichol *et al.*, 2010), which are known to enhance cell binding and cell-mediated matrix degradation, respectively. In addition, the physical properties of GelMA hydrogels can be tuned by varying GelMA and/or photoinitiator (PI) concentrations, to create scaffolds exhibiting elastic moduli approximating those of a variety of natural tissues. This versatile hydrogel has been used to successfully bioengineer contractile skeletal muscle, beating cardiac patches, functional vascular networks, and endochondral bone (Chen *et al.*, 2012; Hosseini *et al.*, 2012; Shin *et al.*, 2013; Visser *et al.*, 2015; Nguyen *et al.*, 2016).

To identify GelMA formulas suitable for bioengineered tooth development, individually encapsulated DE or DM cell GelMA constructs were created with elastic moduli similar to those of natural tooth bud-derived enamel organ and pulp organ tissues. Human umbilical vein endothelial cells (HUVECs) were then included in these constructs to facilitate neovasculture formation within the constructs, and *in vivo* integration with host vasculature. The importance of the vasculature in the developing enamel organ and dental pulp has been well documented (Decker, 1967; Yoshida *et al.*, 1989; Manzke *et al.*, 2005; Nait Lechguer *et al.*, 2008); in addition, HUVECS have been demonstrated to promote neovascular formation in a variety of bioengineered tissues, and to facilitate *in vivo* engraftment with host vasculature (Rouwkema *et al.*, 2006; Zhang *et al.*, 2010b). Based on these studies, individual GelMA formula constructs were created that incorporated either porcine dental epithelial (pDE) cells and HUVECs (pDE–HUVECs) alone, or porcine dental mesenchymal (pDM) cells and HUVECS (pDM–HUVECs) alone, respectively, and analysed in 3D *in vitro* culture to monitor cell morphology, metabolic activity, and vascular network formation over time. Based on our promising *in vitro* results, constructs were then fabricated consisting of two different GelMA formulae: Gel 1 for encapsulated DE–HUVECs and Gel 3 for encapsulated DM–HUVECs. The resulting replicate 3D tooth bud constructs were created and cultured *in vitro* in osteogenic media, and subsequently either further studied *in vitro* or implanted and grown subcutaneously in immunocompromised rats. Analyses of explanted *in vivo* tooth bud constructs revealed the formation of highly mineralized and vascularized bioengineered tooth constructs that approximated the size and shape of the original GelMA construct. This appears to be the first report to demonstrate the formation of vascularized biomineralized dental tissues from dental cell-encapsulated GelMA constructs.

2. Materials and methods

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

Three cell types were used to create bioengineered 3D GelMA tooth buds: (1) pDE cells, (2) pDM) cells, and (3) HUVECs. Primary pDE and pDM progenitor cells were obtained and cultured as previously described (Young *et al.*, 2002, 2005). Briefly, pDE and pDM progenitor cells were isolated from unerupted tooth buds extracted from 5-month-old porcine jaws. Single-cell suspensions were plated in DE or DM cell selective media and expanded in a humidified environment with 5% CO₂ at 37°C. HUVECs (PSC100010; ATCC, Manassas, VA, USA) were expanded in vascular basal media (PCS100030; ATCC) with vascular endothelial growth factor (VEGF) growth kit (PCS10004; ATCC) in humidified 5% CO₂ at 37°C. All expanded cells were cryopreserved in 10% dimethylsulfoxide (DMSO) in appropriate culture media until use.

2.2. GelMA cell encapsulation

Lyophilized GelMA was fully dissolved in DMEM/F12 media at the desired concentration, and photoinitiator (PI) (Irgacure2959; Sigma, St Louis, MO, USA) was added to create three different GelMA formulas denoted Gel 1 (3% GelMA, 0.1% PI), Gel 2 (3% GelMA, 0.5% PI), and Gel 3 (5% GelMA, 0.1% PI) (see Figure 1a).

For *in vitro* analyses, acellular GelMA Gel 1–3 solutions, each containing either pDE–HUVECs or pDM–HUVECs, were each pipetted into replicate 6 mm diameter polydimethylsiloxane (PDMS) ring molds in 24-well plates. pDE–HUVECs (1:1) or pDM–HUVECs (1:1) (1.2×10^6 total cells per 20 μ l) were suspended in GelMA Gel 1–3 solutions, and layered on top of a respective acellular GelMA layer. The resulting constructs were then photo-crosslinked by exposure to 9.16 W/cm² ultraviolet (UV) light for 30 s using an Omnicure S2000 (Lumen Dynamics Group Inc., Mississauga, ON, Canada). After removal of the PDMS molds, the GelMA constructs were *in vitro*-cultured in osteogenic media [1:1:1 Dulbecco's Modified Eagle Medium (DMEM)/F12–LCH8/vascular basal media] supplemented with 1% Pen-Strep, 10% fetal bovine serum (FBS), 100 nM dexamethasone, 10 mM beta glycerol phosphate, and 0.05 mM ascorbic acid for 1, 2, and 4 weeks.

Based on *in vitro* analyses, GelMA constructs were next created from selected Gel 1 and Gel 3 formulae as follows. pDM–HUVECs (1:1) were suspended in GelMA Gel 3 (0.6×10^6 cells per 20 μ l) and pipetted into PDMS moulds. Next, pDE–HUVECs (1:1) were suspended in GelMA Gel 1 (0.6×10^6 cells in 20 μ l per sample) and layered on top of replicate pDM–HUVEC GelMA Gel 3 layers, followed by photo-crosslinking as described above. The resulting 3D tooth bud constructs were precultured *in vitro* in osteogenic media for 2 weeks. *In vitro*-cultured constructs were subsequently cultured for an additional 1 week or 6 weeks *in vitro*, or implanted subcutaneously in nude rats for 1, 3 and 6 weeks.

2.3. Mechanical testing of natural dental tissues and GelMA constructs

Force volume–atomic force microscopy (FV-AFM) was used to measure the elastic modulus of natural porcine dental tissues compared with UV-crosslinked GelMA formula Gels 1, 2, and 3. Freshly harvested enamel organ and pulp tissues were biopunched to generate 6 mm diameter, 2 mm high tissue samples to size-match fabricated GelMA constructs. Cultured pDE and pDM cells were encapsulated in a 1:1 ratio (3.0×10^4 cells/ μ l) in each of the three GelMA formulae (Gel 1, Gel 2, and Gel 3). Acellular GelMA constructs were also included in these analyses. Whole-mount cell-encapsulated and acellular GelMA constructs were placed in Dulbecco's phosphate-buffered saline (DPBS) (#14190–250; Life Technologies, Carlsbad, CA, USA) and mechanically tested using a Dimension 3100 atomic force microscope (Bruker, Santa Barbara, CA, USA), as described previously (Marturano *et al.*, 2013). Indentation force curves were measured over $10 \times 10 \mu\text{m}^2$ areas at two separate locations using atomic force microscopy (AFM) cantilevers with 0.06 N/m spring constants (Bruker) and 5 μ m radius SiO₂ spherical tips. The slopes of the linear region of the force-displacement curves were converted to elastic moduli using a spherical model of Hertzian indentation mechanics (Oliver and Pharr, 1992). All samples were measured in triplicate. Statistical analyses were performed as described below.

2.4. *In vivo* subcutaneous implantation

All animal surgeries were performed using approved Institutional Animal Care and Use Committee (IACUC) protocols and Mandatory Animal Care and Use (MACU) regulations. Replicate cell-encapsulated and acellular GelMA constructs were implanted subcutaneously onto the backs of immunocompromised 5-month-old female Rowett Nude rats (Charles River Laboratories, Wilmington, MA, USA) and harvested after 1, 3 and 6 weeks of growth.

Each rat host randomly received two cell-seeded and two acellular GelMA constructs. All samples were tested in at least six replicates.

2.5. Bioengineered GelMA tooth bud construct harvest and analyses

Harvested *in vitro* and *in vivo* constructs were fixed in 10% formalin overnight and washed in DPBS. Following X-ray analysis, mineralized constructs were immersed in fresh decalcification solution (22.5% formic acid +10% sodium citrate) every 3 days until fully decalcified. Decalcification was defined by lack of ammonium oxalate–calcium precipitate formation after 20 min. For paraffin sectioning, constructs were dehydrated through graded ethanol and xylene, submerged in molten paraffin for 18 h, embedded in paraffin blocks, and serially sectioned (6 μ m thick) as described previously (Zhang *et al.*, 2010a). Hematoxylin and eosin (H&E) and PicroSirius Red (Polysciences, Warrington, PA, USA) stains were used to analyse selected sections.

For immunofluorescent (IF) analysis, paraffin sections were blocked for 20 min in 5% bovine serum albumin (BSA) and incubated for 1 h with primary antibodies: mouse anti-Factor 8 (ab20721, 1:50; Abcam, Cambridge, MA, USA); and rabbit anti-cytokeratin 14 (ab53115, 1:25; Abcam); or rabbit anti-vimentin (BS-0756R, 1:25; Bioss, Woburn, MA, USA). Sections were incubated for an additional hour with fluorescent-conjugated secondary antibodies anti-mouse IgG-Alexa Fluor 488 (515–545-003, 1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and anti-rabbit IgG-Alexa Fluor 568 (A11011, 1:100; Invitrogen, Carlsbad, CA, USA). Sections were cover-slipped with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (H-1500; Vector Laboratories, Burlingame, CA, USA).

For immunohistochemical (IHC) analyses, sections were incubated for 1 h with primary antibody: [mouse anti-hCD31 (ab187377, 1:20; Abcam; and LS-B5577, 1:20; LifeSpan BioSciences, Seattle, WA, USA); rabbit anti-E-cadherin (ABIN1858334, 1:20; Antibodies Online, Atlanta, GA, USA); mouse anti-vimentin (sc-6260, 1:4000; Santa Cruz Biotechnology, Dallas, Texas, USA); mouse anti-osteocalcin (ab13418, 1:400; Abcam); rabbit anti-dentine sialophosphoprotein (GTX60194, 1:50; Genetex, Irvine, CA, USA); or rabbit anti-Amelogenin (ABT260, 1:500–1:750, Millipore, Billerica, MA, USA)]. Sections were incubated for 45 min with biotin-SP conjugated secondary antibody anti-mouse immunoglobulin G (IgG) (711–065–150, 1:500; Jackson ImmunoResearch) or anti-rabbit IgG (711–065–152, 1:500; Jackson ImmunoResearch), 45 min in ABC reagent (PK-4000; Vector Laboratories), and 5 min in 3,3-diaminobenzidine (DAB) (D4293, Sigma, St Louis, MO, USA, USA). All slides were counterstained with 0.1% fast green (F7252; Sigma).

2.6. The MMP activities of cell-encapsulated GelMA tooth bud constructs

The activity of MMP was measured using a Sensolyte 390 Generic MMP Activity Kit (Anaspec, Fremont, CA, USA) following manufacturer's suggested protocol. Briefly, MMP substrate was added to each replicate sample ($n = 3$) and incubated for 1 h at 37°C. Fluorescence was then measured using a MDC SpectraMax M5 Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.7. Statistical analyses

GraphPad Prism5 Software (GraphPad Software, San Diego, CA, USA) was used to determine statistical significance via one-way ANOVA followed by the Sidaks multiple comparison test for the AFM results.

3. Results

3.1. Mechanical properties of GelMA constructs and natural dental tissue

The GelMA formulas (Gels 1, 2 and 3) were created by varying the GelMA and/or photoinitiator (PI) concentrations as indicated in Figure 1a. The elastic moduli of GelMA Gels 1, 2, and 3, and of natural porcine dental pulp and enamel organ tissue samples were measured using

FV-AFM (Figure 1b–d). The results showed that acellular GelMA Gel 1 exhibited the lowest elastic modulus (2.33 ± 0.19 kPa), while acellular GelMA Gel 2 and Gel 3 exhibited elastic moduli of 4.19 ± 0.55 kPa and 4.47 ± 0.74 kPa, respectively (Figure 1b). The AFM results also demonstrated that pDM cell seeded GelMA Gel 3 constructs exhibited elastic moduli similar to that of natural dental pulp tissue, 2.45 ± 0.03 kPa and 2.39 ± 0.26 kPa, respectively (Figure 1c,d). The elastic moduli of dental cell-encapsulated GelMA Gels 1 and 2, and of pDE enamel organ tissue, were all below the limits of detection using this approach (Figure 1c,d). Together, these results indicated that the elastic moduli of selected GelMA formulas were within a range similar to those of natural dental tissues.

3.2. *In vitro* characterization of bioengineered GelMA constructs

3.2.1. Gross and cellular morphologies of 3D bioengineered GelMA constructs

Bright field microscopy was used to monitor the gross morphology of individual pDE-HUVEC-encapsulated, or pDM-HUVEC-encapsulated GelMA constructs over 4 weeks of *in vitro* culture. As previously reported, it was observed that dental cell-encapsulated Gel 1 and Gel 3 GelMA constructs shrunk in size over time in culture (Smith *et al.*, 2014), likely the result of encapsulated cell attachment and force exerted on the surrounding hydrogel (Ulijn *et al.*, 2007; Ahearne *et al.*, 2009; Chung *et al.*, 2012; Ahearne, 2014). Shrinkage of pDE-HUVEC-encapsulated constructs appeared somewhat greater in Gel 1 compared with Gels 2 and 3, while pDM-HUVEC-encapsulated constructs exhibited the greatest contraction in Gel 3 (Smith *et al.*, 2014). All acellular GelMA constructs and cell-seeded Gel 2 constructs retained their size and shape over time in culture (Smith *et al.*, 2014).

In addition, distinct morphological changes were observed in GelMA Gel 1 and Gel 3 encapsulated cells, including cell spreading and clustering (see the Supplementary material online, Figure S1A,B, arrowheads). In contrast, GelMA Gel 2 encapsulated cells remained as individual, round cells over time in culture (see the Supplementary material online, Figure S1A,B, arrows). These results signify that pDE-HUVECs and pDM-HUVECs encapsulated in GelMA Gels 1 and 3 exhibited cell attachment and spreading, while those in Gel 2 did not.

3.2.2. Cell activity characterization of *in vitro*-cultured cell-encapsulated GelMA constructs

—It was previously reported that both pDE–HUVEC and pDM–HUVEC GelMA encapsulated cells exhibited highest metabolic activity in Gel 1 and Gel 3, respectively, and exhibited only basal metabolic activity in GelMA Gel 2 (Smith *et al.*, 2014). Because of the importance of MMP activity in dental cell differentiation and mineralized dentin and enamel matrix secretion (Bartlett and Simmer, 1999; Randall and Hall, 2002; Yoshida *et al.*, 2003), the MMP activity of *in vitro*-cultured GelMA encapsulated cells were measured (see the Supplementary material online, Figure S1C, D). Slightly increased MMP activity was found in pDE–HUVEC-encapsulated GelMA Gel 1 and Gel 3 constructs over time in culture (see the Supplementary material online, Figure S1C). In contrast, GelMA Gel 2 encapsulated pDE–HUVECs exhibited a sharp decrease in MMP activity over time in culture (see the Supplementary material online, Figure S1C). The pDM–HUVEC-encapsulated GelMA Gel 3 constructs exhibited increased MMP activity over time in culture, while pDM–HUVECs GelMA Gel 1 constructs exhibited decreased MMP activity (see the Supplementary material online, Figure S1D). pDM–HUVECs exhibited only a basal level of MMP activity in GelMA Gel 2 throughout the 4 weeks of *in vitro* culture. Together, these results indicate that GelMA Gels 1 and 3 supported cell attachment, morphology, and metabolic and MMP activities of encapsulated pDE–HUVEC and pDM–HUVEC, respectively, while, in contrast, GelMA Gel 2 did not.

3.2.3. Capillary-like network formation within *in vitro*-cultured GelMA constructs

—A well-defined, functional vascularized network is required for the long-term survival of bioengineered tissues, and for proper integration with the recipient host vasculature (Lovett *et al.*, 2009; Novosel *et al.*, 2011). In natural tissues and organs, blood vessels are composed of a luminal endothelial cell layer, surrounded by a layer of smooth muscle cells (Melero-Martin *et al.*, 2008; Hahn and Schwartz, 2009; Drummond *et al.*, 2011; Chen *et al.*, 2012). Based on published reports that mesenchymal stem cells (MSCs) and endothelial cells exhibit the ability to self-organize into capillary-like networks after encapsulation in GelMA hydrogel *in vitro* and *in vivo* (Chen *et al.* 2012; Lin *et al.* 2013), immunofluorescent (IF) histochemical analyses was used to examine neo-vessel formation and organization within *in vitro*-cultured pDM–HUVEC-encapsulated GelMA constructs (Figure 2). An elaborate and well-defined capillary-like network formation was identified in pDM–HUVEC GelMA Gel 3 constructs (Figure 2a), which appeared quite similar to vascular networks found in naturally formed dental pulp tissue (Nait Lechguer *et al.*, 2008). Confocal analyses revealed pDM and HUVEC cell organization within these networks (Figure 2b). In contrast, capillary-like network formation was not observed in pDE–HUVEC constructs (Figure 2c).

3.3. GelMA tooth bud constructs

Based on the promising preliminary *in vitro* analyses of DE–HUVECs and DM–HUVECs encapsulated within individual GelMA formulations, *in vitro* and *in vivo* analyses of 3D tooth bud GelMA constructs consisting of a GelMA Gel 1 encapsulated DE–HUVECs combined with GelMA Gel 3 encapsulated DM–HUVEC were performed (Figure 3a). The resulting cell-seeded GelMA constructs were then cultured in osteogenic media for 2 weeks,

and then either cultured *in vitro* for an additional 1 week or 6 weeks, or implanted subcutaneously into nude rat hosts *in vivo* for 1, 3 or 6 weeks (Figure 3b).

3.3.1. In vitro-cultured GelMA tooth bud constructs—Histological analyses of H&E-stained *in vitro*-cultured dental cell–HUVEC-encapsulated GelMA constructs revealed the presence of discrete and interconnected cell clusters throughout the constructs (see the Supplementary material online, Figure S2A1,A2, arrows). In addition, H&E-stained dental cell-secreted extracellular matrix appeared to increase over time in *in vitro* culture, which was indicative of dental cell differentiation (see the Supplementary material online, Figure S2A1,A2, arrowheads). The immunohistochemical analyses identified E-cadherin (Ecad)-expressing pDE cells, vimentin (Vm)-expressing pDM cells, and CD31-expressing HUVEC cell populations present throughout the *in vitro*-cultured constructs (see the Supplementary material online, Figure S2B–D). Organized clusters of CD31-positive HUVECs increased in number and size over time in culture, and were localized throughout the constructs after 6 weeks of *in vitro* culture.

Analyses of tooth and bone differentiation marker expression in sectioned *in vitro*-cultured GelMA constructs (see the Supplementary material online, Figure S3) revealed robust expression of the DM differentiation marker dentine sialophosphoprotein (DSPP) after both 1 week and 6 weeks of culture (see the Supplementary material online, Figure S3A1,A2, arrows). The osteoblast differentiation marker osteocalcin (OC) was not detected in 1-week *in vitro* constructs, and only faintly detected in 6-week *in vitro*-cultured constructs (see the Supplementary material online, Figure S3B1,B2, arrows). The pDE cell differentiation marker amelogenin (AM) was only faintly detected in both 1-week and 6-week *in vitro*-cultured constructs (see the Supplementary material online, Figure S3C1,C2, arrows). Together, these results indicated that both pDE and pDM cells exhibited robust proliferation and dental cell differentiation marker expression in 3D tooth bud constructs after 6 weeks of *in vitro* culture. In contrast, bone differentiation marker expression was weak, even after 6 weeks of *in vitro* culture.

3.3.2. In vivo implanted GelMA tooth bud constructs—It was previously reported that *in vivo* implanted dental cell-encapsulated GelMA constructs exhibited hard tissue formation (Smith *et al.*, 2014). In the present study, bright field and corresponding X-ray images of explanted *in vivo* constructs are provided (Figure 3c–j). The X-ray analyses revealed distinct areas of radio-opacity indicative of mineralized tissue formation (Figure 3c'–j'). Mineralized tissues were present in 6/11 (55%) of the 3-week explants, and in 9/13 (69%) of 6-week explants. In many cases, mineralized tissue formation appeared to adopt the size and shape of the GelMA construct. All of the dental cell-encapsulated GelMA constructs grown *in vivo* for only 1 week, and all of the acellular GelMA constructs, appeared negative for mineralized tissue formation by X-ray. Following X-ray analyses, mineralized 3D tooth bud constructs were decalcified using weekly changes of decalcification solution, which were monitored for calcium precipitate (see the Supplementary material online, Figure S4). The majority of the 6-week *in vivo* constructs took longer to decalcify compared with the 3-week *in vivo* constructs, which was indicative of increased biomineralization of implanted constructs over time for *in vivo* growth.

The H&E-stained paraffin sectioned *in vivo* explants exhibited distinct GelMA tooth bud constructs surrounded by host tissue (see the Supplementary material online, Figure S5A,B,D). The cellularity of the cell-seeded *in vivo* implanted constructs was observed to increase between 3 weeks and 6 weeks, and distinct neovascularization and the formation of bone-like tissues was observed throughout the implants over time (see the Supplementary material online, Figure S5A,B,D). Polarized light microscopy of H&E-stained sections revealed increased collagen matrix secretion by GelMA encapsulated cells over *in vivo* implantation time (see the Supplementary material online, Figure S5B2,D2, arrows). Paraffin sectioned *in vivo* 3-week and 6-week explants were also examined using PicroSirius Red staining, which specifically detects collagen (red) under normal light microscopy, and under polarized light distinguishes between collagen type I (thick, yellow/orange) and collagen type III (thin, green). The *in vivo* 3-week and 6-week week GelMA explants showed significant collagen content, consisting of both collagen type I and collagen type III fibres, indicative of newly formed collagen matrix (see the Supplementary material online, Figure S5C2, E2). Collagen fibre alignment within the GelMA constructs appeared to be oriented perpendicular to the encapsulating host tissue (see the Supplementary material online, Figure S5C2,E2), clearly defining collagen secretion by the encapsulated cell seeded GelMA constructs as opposed to that of rat host tissue.

Host red blood cells (RBCs) were observed in blood vessels located throughout the cell-seeded constructs. The presence of RBCs within the implanted constructs (see the Supplementary material online, Figure S6), which can only come from the host circulatory system, confirmed the functionality of bioengineered GelMA tooth bud vascular networks. Functional vasculature, defined as ‘vasculature containing RBCs’ (Levenberg *et al.*, 2005; Chen *et al.*, 2012), is required to support bioengineered tooth integration and growth after *in vivo* implantation.

Immunohistochemistry was used to identify pDE, pDM, and HUVEC cell populations within *in vivo* implanted GelMA constructs (Figure 4). Ecad-positive pDE cells, VM-positive pDM cells, and CD31-positive HUVECS were clearly present throughout the entire construct in 1-, 3-, and 6-week explants, indicating mixing of cells throughout the construct (Figure 4d–l). Organized CD31-expressing neovasculature was apparent after 3 weeks and 6 weeks of *in vivo* growth (Figure 4,k’,l’, arrows). As the CD31 antibody used in these analyses is human specific (see the Supplementary material online, Figure S7), these results indicated GelMA encapsulated HUVEC (as opposed to host cell) contribution to the neovasculature that formed within GelMA tooth bud constructs.

Dental cell differentiation marker expression within harvested *in vivo* GelMA constructs was examined next (Figure 5). Distinct and robust DSPP and OC expression was detected in sectioned 3-week and 6-week *in vivo* implanted constructs, which was indicative of both DM cell-derived odontoblast and osteoblast cell differentiation (Figure 5a–f). This observation supports previous reports from this group (Xu *et al.*, 2008; Zhang *et al.*, 2011, 2014), and those of others (Yu *et al.*, 2007; Dimitrova-Nakov *et al.*, 2014), which have shown that DM cells exhibit the capacity to develop into osteodentin-producing cells that can express both DSPP and OC.

Similarly, AM expression was clearly observed in 1-, 3- and 6-week *in vivo* tooth bud constructs, which is indicative of DE cell-derived ameloblast differentiation (Figure 5g–i). Together, these results support the successful generation of 3D biomimetic tooth constructs exhibiting mineralized osteodentin-like and enamel matrix formation.

4. Discussion

The ability to successfully create bioengineered replacement teeth that resemble and function as well as natural teeth, would provide distinct advantages over currently used synthetic dental implants (Yen and Yelick, 2011). The present study has described the design and characterization of dental and endothelial cell-encapsulated GelMA constructs as 3D biomimetic tooth bud models. Encapsulated cell behavior is highly influenced by the architecture and mechanical properties of the scaffold (Engler *et al.*, 2006; Trappmann and Chen, 2013), which in turn direct cell differentiation, fate, and function (Lutolf and Hubbell, 2005; Engler *et al.*, 2006; Brandl *et al.*, 2007; Wells, 2008; Sun *et al.*, 2012; Hazeltine *et al.*, 2013). Based on previous reports, the present study sought to identify GelMA formulas that exhibited elastic moduli similar to those of natural tooth bud enamel organ and pulp organ tissues. The FV-AFM analyses of the elastic moduli of dental cell-encapsulated GelMA Gel 3 constructs closely matched that of fresh pDM tissue. In contrast, dental cell-seeded GelMA Gel 1 constructs were below the level of detection by FV-AFM, as was natural tooth bud enamel organ tissue (Figure 1c,d). Characterization of *in vitro*-cultured individually encapsulated pDE–HUVEC and pDM–HUVEC GelMA constructs provided insight to cellular morphology and metabolic activity in each GelMA formula. Based on the results of the present study, pDM–HUVEC cell-seeded GelMA Gel 3, and pDE–HUVEC cell-seeded GelMA Gel 1 were selected to create 3D biomimetic tooth bud constructs. The GelMA Gel 2 formula did not support cell proliferation or metabolic activity of encapsulated cells, likely because of toxicity introduced by high photoinitiator concentration.

The design of the 3D biomimetic tooth bud constructs fabricated was intended to facilitate cross-talk between DE–DE cells and DM–DM cells, and between adjacent pDM–HUVEC and pDE–HUVEC cell layers, while at the same time maintaining distinct pulp organ and enamel organ, respectively. However, subsequent immunohisto-chemical analysis of harvested *in vitro*-cultured and *in vivo*-grown implants showed that pDE and pDM cells were not maintained in separate layers, but rather were present throughout the constructs even after only 1 week of culture and/or implantation. These results likely indicate mixing of the dental cell/GelMA solutions before photocrosslinking. Future studies will employ successive photocrosslinking of individual cell-seeded GelMA layers to avoid mixing.

Another limitation of this bioengineered 3D tooth bud model, and a consequence of the above-mentioned cell mixing, is that no distinct enamel or dentin layers were observed. Sequential photocrosslinking combined with the inclusion of additional dental cell differentiation growth factors will likely improve this model to better support dentin and enamel formation. In addition, the inclusion of bioreactor culture conditions that can deliver mechanical stimulation, followed by longer *in vivo* implantation times, may also help to achieve our goal to create functional, biological tooth substitutes.

It is notable that the *in vivo*-cultured biomimetic 3D tooth bud constructs formed robust mineralized tissues that largely adopted the size and shape of the original constructs – an important criterion for an effective biomimetic tooth bud model. In addition, 3D dental cell-encapsulated GelMA tooth bud constructs expressed dental epithelial and dental mesenchymal differentiation markers indicative of ameloblast- and odontoblast-specific cell differentiation, respectively. Also noteworthy is the fact that *in vivo* implanted dental cell-encapsulated GelMA tooth bud constructs exhibited functional vascularization, demonstrated by CD31-expressing HUVEC-derived neovasculature and the presence of circulating host RBCs (see the Supplementary material online, Figure S6). Furthermore, bioengineered capillary-like networks present in both *in vitro*-cultured and *in vivo* implanted constructs resembled natural neovasculature organization (Schematic shown in Figure 6a, cross-section; Figure 6b shows longitudinal section). Together, these results suggest the potential to eventually create viable, vascularized, functional bioengineered tooth replacements.

In conclusion, the results presented validate GelMA hydrogel constructs as promising scaffold materials for whole-tooth engineering and craniofacial reconstruction. This appears to be the first study to successfully demonstrate that dental cell-encapsulated GelMA hydrogels can be used to create bioengineered 3D tooth buds that support dental cell differentiation, functional neovasculature and dental tissue-derived mineralized tissue formation. Future studies will focus on refining the 3D biomimetic tooth bud model to better facilitate DE and DM cell interactions, while preserving DE- and DM-derived enamel organ and pulp organ tissue formation and differentiation. Biomimetic 3D GelMA tooth bud constructs provide a promising model for the eventual development of functional, bioengineered replacement teeth of specified size and shape.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank all members of the Yelick Laboratory, and in particular Betsy Vazquez for her expertise and advice in immuno-histochemistry and microscopy, and Sung Woo (Nathan) Cho who assisted with paraffin sectioning. The authors also thank all members of the Khademhosseini Laboratory. This work was supported by NIH/NIDCR R01DE016132 (PCY). The authors also acknowledge funding support by the NIH Training in Education and Critical Research Skills (K12GM074869) postdoctoral program (to N.R.S.).

References

- Abukawa H, Zhang W, Young CS et al. 2009; Reconstructing mandibular defects using autologous tissue-engineered tooth and bone constructs. *J Oral Maxillofac Surg* 67: 335–347. [PubMed: 19138608]
- Ahearne M 2014; Introduction to cell–hydrogel mechanosensing. *Interface Focus* 4:1–12. doi: 10.1098/rsfs.2013.0038pmid:24748951.
- Ahearne M, Liu K-K, El Haj AJ et al. 2009; Online monitoring of the mechanical behavior of collagen hydrogels: influence of corneal fibroblasts on elastic modulus. *Tissue Eng Part C Methods* 16: 319–327.
- Bartlett JD, Simmer JP. 1999; Proteinases in developing dental enamel. *Crit Rev Oral Biol Med* 10: 425–441. [PubMed: 10634581]

- Brandl F, Sommer F, Goepferich A. 2007; Rational design of hydrogels for tissue engineering: Impact of physical factors on cell behavior. *Biomaterials* 28: 134–146.
- Chen Y-C, Lin R-Z, Qi H et al. 2012; Functional human vascular network generated in photocrosslinkable gelatin methacrylate hydrogels. *Adv Funct Mater* 22: 2027–2039. [PubMed: 22907987]
- Chung C, Anderson E, Pera RR et al. 2012; Hydrogel crosslinking density regulates temporal contractility of human embryonic stem cell-derived cardiomyocytes in 3D cultures. *Soft Matter* 8: 10141–10148. [PubMed: 23226161]
- Decker JD. 1967; The development of a vascular supply to the rat molar enamel organ: an electron microscopic study. *Arch Oral Biol* 12: 453–IN412.
- Dimitrova-Nakov S, Baudry A, Harichane Y et al. 2014; Pulp stem cells: implication in reparative dentin formation. *J Endod* 40: S13–S18. [PubMed: 24698687]
- Drummond GR, Selemidis S, Griendling KK et al. 2011; Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat Rev Drug Discov* 10: 453–471.
- Duailibi MT, Duailibi SE, Young CS et al. 2004; Bioengineered teeth from cultured rat tooth bud cells. *J Dent Res* 83: 523–528. [PubMed: 15218040]
- Duailibi SE, Duailibi MT, Zhang W et al. 2008; Bioengineered dental tissues grown in the rat jaw. *J Dent Res* 87: 745–750. [PubMed: 18650546]
- Engler AJ, Sen S, Sweeney HL et al. 2006; Matrix elasticity directs stem cell lineage specification. *Cell* 126: 677–689. [PubMed: 16923388]
- Greenstein G, Cavallaro J, Romanos G et al. 2008; Clinical recommendations for avoiding and managing surgical complications associated with implant dentistry: a review. *J Periodontol* 79: 1317–1329. [PubMed: 18672980]
- Hahn C, Schwartz MA. 2009; Mechanotransduction in vascular physiology and atherogenesis. *Nat Rev Mol Cell Biol* 10: 53–62. [PubMed: 19197332]
- Hazeltine LB, Selekmán JA, Palecek SP. 2013; Engineering the human pluripotent stem cell microenvironment to direct cell fate. *Biotechnol Adv* 31: 1002–1019. [PubMed: 23510904]
- Hosseini V, Ahadian S, Ostrovidov S et al. 2012; Engineered contractile skeletal muscle tissue on a microgrooved methacrylated gelatin substrate. *Tissue Eng Part A* 18: 2453–2465. [PubMed: 22963391]
- Jung RE, Pjetursson BE, Glauser R et al. 2008; A systematic review of the 5-year survival and complication rates of implant-supported single crowns. *Clin Oral Implants Res* 19: 119–130. [PubMed: 18067597]
- Lai W-F, Lee J-M, Jung H-S. 2014; Molecular and engineering approaches to regenerate and repair teeth in mammals. *Cell Mol Life Sci* 71: 1691–1701. [PubMed: 24270857]
- Levenberg S, Rouwkema J, Macdonald M, et al. 2005; Engineering vascularized skeletal muscle tissue. *Nat Biotechnol* 23: 879–884. [PubMed: 15965465]
- Lin R-Z, Chen Y-C, Moreno-Luna R et al. 2013; Transdermal regulation of vascular network bioengineering using a photopolymerizable methacrylated gelatin hydrogel. *Bio-materials* 34: 6785–6796.
- Lovett M, Lee K, Edwards A et al. 2009; Vascularization strategies for tissue engineering. *Tissue Eng Part B Rev* 15: 353–370. [PubMed: 19496677]
- Lutolf MP, Hubbell JA. 2005; Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol* 23: 47–55. [PubMed: 15637621]
- Manzke E, Katchburian E, Faria FP et al. 2005; Structural features of forming and developing blood capillaries of the enamel organ of rat molar tooth germs observed by light and electron microscopy. *J Morphol* 265: 335–342. [PubMed: 16094655]
- Marturano JE, Arena JD, Schiller ZA et al. 2013; Characterization of mechanical and biochemical properties of developing embryonic tendon. *Proc Natl Acad Sci U S A* 110: 6370–6375. [PubMed: 23576745]
- Melero-Martin JM, De Obaldia ME, Kang S-Y et al. 2008; Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ Res* 103: 194–202. [PubMed: 18556575]

- Murray PE, Garcia-Godoy F, Hargreaves KM. 2007; Regenerative endodontics: a review of current status and a call for action. *J Endod* 33: 377–390. [PubMed: 17368324]
- Nait Lechguer A, Kuchler-Bopp S, Hu B et al. 2008; Vascularization of engineered teeth. *J Dent Res* 87: 1138–1143. [PubMed: 19029082]
- Nguyen D, Akay YM, Akay M. 2016; Investigating glioblastoma angiogenesis using a 3D in vitro gelma microwell platform. *IEEE Trans Nanobioscience* 3: 289–293.
- Nichol JW, Koshy ST, Bae H et al. 2010; Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* 31: 5536–5544. [PubMed: 20417964]
- Novosel EC, Kleinhans C, Kluger PJ. 2011; Vascularization is the key challenge in tissue engineering. *Adv Drug Deliv Rev* 63: 300–311. [PubMed: 21396416]
- Oliver WC, Pharr GM. 1992; An improved technique for determining hardness and elastic modulus using load and displacement sensing indentation experiments. *J Mater Res* 7: 1564–1583.
- Randall LE, Hall RC. 2002; Temporospatial expression of matrix metalloproteinases 1, 2, 3, and 9 during early tooth development. *Connect Tissue Res* 43: 205–211. [PubMed: 12489160]
- Rouwkema J, De Boer J, Van Blitterswijk CA. 2006; Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. *Tissue Eng* 12: 2685–2693. [PubMed: 16995802]
- Shin SR, Jung SM, Zalabany M et al. 2013; Carbon-nano-tube-embedded hydrogel sheets for engineering cardiac constructs and bioactuators. *ACS Nano* 7: 2369–2380. [PubMed: 23363247]
- Smith EE, Yelick PC, Khademhosseini A. 2014; Optimization of a biomimetic model for tooth regeneration Bioengineering Conference (NEBEC) 2024 40th Annual. Wiley-Liss, INC: Northeast City; 1–2. doi: 10.1002/jmor.10363
- Sun Y, Chen CS, Fu J. 2012; Forcing Stem cells to behave: a biophysical perspective of the cellular microenvironment. *Annu Rev Biophys* 41: 519–542. [PubMed: 22404680]
- Thesleff I, Vainio S, Jalkanen M, 1989; Cell–matrix interactions in tooth development. *Int J Dev Biol* 33, 91–97. [PubMed: 2485706]
- Trappmann B, Chen CS. 2013; How cells sense extracellular matrix stiffness: a material's perspective. *Curr Opin Biotechnol* 24: 948–953. [PubMed: 23611564]
- Ulijn RV, Bibi N, Jayawarna V et al. 2007; Bioresponsive hydrogels. *Mater Today* 10: 40–48.
- Visser J, Gawlitta D, Benders KEM et al. 2015; Endochondral bone formation in gelatin methacrylamide hydrogel with embedded cartilage-derived matrix particles. *Biomaterials* 37: 174–182. [PubMed: 25453948]
- Vos T, Flaxman AD, Naghavi M et al. 2012; Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380: 2163–2196. [PubMed: 23245607]
- Wells RG. 2008; The role of matrix stiffness in regulating cell behavior. *Hepatology* 47: 1394–1400. [PubMed: 18307210]
- Xu W-P, Zhang W, Asrican R et al. 2008; Accurately shaped tooth bud cell-derived mineralized tissue formation on silk scaffolds. *Tissue Eng Part A* 14: 549–557 [PubMed: 18352829]
- Yen AH, Yelick PC. 2011; Dental tissue regeneration – a mini-review. *Gerontology* 57: 85–94. [PubMed: 20453484]
- Yoshida N, Yoshida K, Stoetzel C et al. 2003; Temporospatial gene expression and protein localization of matrix metal-loproteinases and their inhibitors during mouse molar tooth development. *Dev Dyn* 228: 105–112. [PubMed: 12950084]
- Yoshida S, Ohshima H, Kobayashi S. 1989; Vascularization of the enamel organ in developing molar teeth of rats -scanning electron microscope study of corrosion casts. *Okajimas Folia Anat Jpn* 66: 99–111. [PubMed: 2812679]
- Young CS, Terada S, Vacanti JP et al. 2002; Tissue engineering of complex tooth structures on biodegradable polymer scaffolds. *J Dent Res* 81: 695–700. [PubMed: 12351668]
- Young CS, Abukawa H, Asrican R, et al. 2005; Tissue-engineered hybrid tooth and bone. *Tissue Eng* 11:
- Yu J, Wang Y, Deng Z, et al. 2007; Odontogenic capability: bone marrow stromal stem cells versus dental pulp stem cells. *Biol Cell* 99: 465–474. [PubMed: 17371295]

- Zhang W, Ahluwalia IP, Yelick PC. 2010a; Three dimensional dental epithelial–mesenchymal constructs of predetermined size and shape for tooth regeneration. *Biomaterials* 31: 7995–8003. [PubMed: 20682455]
- Zhang W, Ahluwalia IP, Literman R et al. 2011; Human dental pulp progenitor cell behavior on aqueous and hexafluoroisopropanol (HFIP) based silk scaffolds. *J Biomed Mater Res A* 97: 414–422. [PubMed: 21484985]
- Zhang W, Vázquez B, Yelick PC. 2014; Bioengineered postnatal recombinant tooth bud models. *J Tissue Eng Regen Med* 33: 377–390.
- Zhang X, Yang J, Li Y et al. 2010b; Functional neovascularization in tissue engineering with porcine acellular dermal matrix and human umbilical vein endothelial cells. *Tissue Eng Part C Methods* 17: 423–433. [PubMed: 21062229]

Figure 1.

Comparative elastic moduli of gelatin methacrylate (GelMA) constructs and natural porcine dental tissues. (a) GelMA Gel formulae with corresponding GelMA and photoinitiator concentrations (% w/v). Elastic moduli of (b) unseeded GelMA constructs, (c) porcine dental epithelial (pDE)–porcine dental mesenchymal (pDM) cell-encapsulated GelMA constructs, and (d) natural porcine dental tissues. Dental cell-seeded Gel 3 had similar elastic modulus to that of pDM tissue. Bar graphs represent average $\pm$ SD ($n = 3$). ND, not determined (elastic modulus below detection level). *** $p < 0.001$; ANOVA followed by Sidak's comparison

Figure 2.

Capillary-like network formation within *in vitro*-cultured porcine dental mesenchymal (pDM)–human umbilical vein endothelial cells (HUVECs) gelatin methacrylate (GelMA) constructs. (a,b) pDM–HUVEC Gel 3 construct and (c) porcine dental epithelial (pDE)–HUVEC Gel 3 construct. Vascular network formation was observed in pDM–HUVEC GelMA Gel 3 constructs after 4 weeks of *in vitro* culture (a, arrows). Confocal analyses revealed organized pDM–HUVEC structures (b). No capillary-like formation was observed in pDE–HUVEC constructs (c). Bar: (a,c) 50 μ m (b) 10 μ m.

a GelMA Tooth Bud Constructs

b Experimental Timeline

Figure 3.

Parallel *in vitro* and *in vivo* bioengineered three-dimensional gelatin methacrylate (GelMA) tooth bud constructs. (a) Schematic of construct fabrication. (b) Experimental timeline. (c–j) Harvested *in vivo* implanted GelMA tooth bud constructs. Representative bright field images of replicate *in vivo* GelMA constructs harvested after 3 weeks (c–f) or 6 weeks (g–j) implantation. (c'–j') Radiographic images of corresponding bright field images indicate mineralized tissue formation (arrows) in 3-week and 6-week constructs. Bar: 2 mm. DE, dental epithelial cell; DM, dental mesenchymal cell; HUVEC, human umbilical vein endothelial cell.

Figure 4.

Dental cell and human umbilical vein endothelial cell (HUVEC) distribution within *in vivo* gelatin methacrylate (GelMA) tooth bud constructs. (a–c) Hematoxylin and eosin (H&E) staining revealed high cellularity and the development of bone-like tissue over time. E-cadherin (Ecad)-expressing porcine dental epithelial (pDE) cells (d–f, d'–f' arrows) and vimentin (VM)-expressing porcine dental mesenchymal (pDM) cells (g–i, g'–i' arrows) were detected throughout the constructs. CD31-expressing HUVECs were also detected throughout the constructs (j–l, j'–l') and contributed to vascular networks in 3-week and 6-

week *in vitro*-cultured constructs (k',l' arrows). (d'-l') Higher magnifications of boxed regions in d-l. Bar: (a-l) 200 μm , (d'-l') 50 μm .

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Figure 5.

Dental cell differentiation within *in vivo* gelatin methacrylate (GelMA) tooth bud constructs. A–i Immunohistochemical analyses of tooth and bone specific markers in 1-, 3- and 6-week *in vivo* constructs. The odontoblast differentiation marker dentin sialophosphoprotein (DSPP) was detected throughout the constructs at each time-point (a–c, a'–c'). Odontoblast/osteoblast differentiation marker osteocalcin (OC) expression increased overtime *in vivo* (d–f, d'–f'). Ameloblast differentiation marker amelogenin (AM) was detected throughout the

constructs at all times (g-i, g'-i'). (a'-i') Higher magnification images of boxed regions in a-i. Bar: (a-i) 200 μm , (a'-i') 50 μm .

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Figure 6.

Schematic of bioengineered neovascular formation in gelatin methacrylate (GelMA) tooth bud constructs. (a) Cross-sectional and (b) longitudinal schematic along with a (c) color-coded key depicting the organization of normal blood vessel, *in vitro*-cultured GelMA construct capillary network formation, and neovascularization and mineralization of *in vivo* implanted GelMA constructs. AM, amelogenin; DSPP, dentin sialophosphoprotein; HUVEC, human umbilical vein endothelial cell; OC, osteocalcin; pDE, porcine dental epithelial cell; pDM, porcine dental mesenchymal cell.