Bioengineered Tooth Buds Exhibit Features of Natural Tooth Buds

Journal of Dental Research
2018, Vol. 97(10) 1144–1151
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for Dental Research 2018
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DOI: 10.1177/0022034518779075
journals.sagepub.com/home/jdr

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Abstract

Tooth loss is a significant health issue currently affecting millions of people worldwide. Artificial dental implants, the current gold standard tooth replacement therapy, do not exhibit many properties of natural teeth and can be associated with complications leading to implant failure. Here we propose bioengineered tooth buds as a superior alternative tooth replacement therapy. We describe improved methods to create highly cellularized bioengineered tooth bud constructs that formed hallmark features that resemble natural tooth buds such as the dental epithelial stem cell niche, enamel knot signaling centers, transient amplifying cells, and mineralized dental tissue formation. These constructs were composed of postnatal dental cells encapsulated within a hydrogel material that were implanted subcutaneously into immunocompromised rats. To our knowledge, this is the first report describing the use of postnatal dental cells to create bioengineered tooth buds that exhibit evidence of these features of natural tooth development. We propose future bioengineered tooth buds as a promising, clinically relevant tooth replacement therapy.

Keywords: tissue engineering, odontogenesis, stem cell, ameloblast, odontoblast, regeneration

Introduction

Synthetic dental implants are susceptible to peri-implantitis, gingival recession, and bone resorption at the implant site, leading to implant failure (Chrcanovic et al. 2014, 2016; Esposito et al. 2012; Greenstein et al. 2008). To create vitalized teeth for human tooth replacement, bioengineered tooth regeneration has emerged as an innovative field of translational dentistry. Studies have shown that postnatal dental stem cells (DSCs) retain the ability to form small, anatomically correct whole tooth crowns in animal models (Duailibi et al. 2004; Young et al. 2005), supporting the feasibility of this approach.

Currently, a variety of biodegradable scaffolds are being tested for utility in whole-tooth regeneration therapies (Smith and Yelick 2016). In particular, gelatin methacryloyl (GelMA) hydrogel scaffolds were shown to support DSCs and human umbilical vein endothelial cell (HUVEC) differentiation into mineralized dental tissues of specified size and shape (Smith et al. 2017). To improve upon this model, here we investigated 3 ways to optimize GelMA tooth bud constructs to facilitate their use in humans. First, we tested whether sequentially photocrosslinking GelMA bilayers would better maintain distinct dental epithelial (DE) and dental mesenchymal (DM) cell layers. Next, to optimize initial cell seeding densities, we compared 3.0×10^7 , 6.0×10^7 , and 9.0×10^7 cells/mL and examined dental cell differentiation. Last, we tested whether extended culture in normal growth media, prior to culture in osteogenic differentiation media, resulted in improved tooth bud construct cellularity and mineralized dental tissue formation.

Materials and Methods

Dental and Endothelial Cell Culture

DE and DM cells were isolated from porcine tooth buds and cultured as previously described (Smith et al. 2017). Briefly, unerupted early bell stage tooth buds were extracted from 5-mo-old porcine jaws. The enamel and pulp organs were dissected apart and used to prepare single-cell suspensions of DE and DM cells, respectively, which were cultured and expanded in vitro in appropriate media. HUVECs (PCS100010; ATCC) were grown in vascular basal media (PCS100030; ATCC) with vascular endothelial growth factor (VEGF) growth kit (PCS10004; ATCC). All cells were expanded in 5% CO₂ at 37°C and cryopreserved in 10% dimethyl sulfoxide (DMSO) until use.

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A supplemental appendix to this article is available online.

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Fabrication of 3-Dimensional GelMA Hydrogel Tooth Bud Constructs

Cells were encapsulated within GelMA hydrogel at total cell densities of 3.0×10^7 , 6.0×10^7 , or 9.0×10^7 total cells/mL (1 $\times$, 2 $\times$, or 3 $\times$, respectively). DM/HUVECs (1:1) were resuspended in 5% GelMA with 0.1% photoinitiator (PI, Irgacure2959; Sigma-Aldrich), aliquoted (20 μ L) into 6-mm diameter polydimethylsiloxane (PDMS) molds, and photo-crosslinked (18 s) using an OmniCure S200 (Lumen Dynamics Group, Inc.). DE/HUVECs (1:1) were resuspended in 3% GelMA with 0.1% PI, pipetted (20 μ L) on top of photopolymerized DM-HUVEC 5% GelMA layers, and photo-crosslinked (12 s). Bilayered tooth bud constructs were cultured for 1 or 7 d in normal media (Dulbecco's modified Eagle's medium [DMEM]/F12; LCH8: vascular basal media [1:1:1]), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.5 μ g/mL epinephrine, 25 μ g/mL ascorbic acid, 2 mM GlutaMax, and endothelial cell growth kit (ATCC PCS-999-003), followed by 7-d culture in osteogenic media (OM, 1:1:1 culture media + 100 nM dexamethasone, 10 mM β -glycerol phosphate, 50 μ g/mL ascorbic acid).

Live/Dead Assay

The Live/Dead assay (Molecular Probes) was used to determine cell viability in replicate (3) in vitro tooth bud constructs as recommended.

In Vivo Implantation of Tooth Bud Constructs

All animals used in this study were handled in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols. Replicate (4) tooth bud constructs and acellular controls were randomly implanted subcutaneously in 5-wk-old female Rowett Nude rats (120 to 150 g) (RNU; Charles River Laboratories) and grown for 2 or 4 wk.

Tooth Bud Construct Harvest and Micro-Computed Tomography Analysis

Harvested constructs were fixed overnight in 10% formalin, washed in phosphate-buffered saline (PBS), and imaged using a Skyscan 1176 In vivo Micro-CT (Bruker). Analyses were performed using a SkyScan NRecon, Bruker MicroCT CT Analyzer software (Bruker MicroCT) and Avizo software (FEI).

Histological and Immunohistochemical Analyses

Decalcified constructs were paraffin embedded, sectioned (6 μ m), and stained using hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was performed as described (Smith et al. 2016) using primary antibodies: mouse anti-Vimentin (sc-6260, 1:4,000; Santa Cruz Biotechnology), rabbit anti-E-cadherin (ABIN1858334, 1:40; Antibodies Online), mouse anti-hCD31 (ab187377, 1:20; Abcam), rabbit anti-hCD31 (LS-B5577, 1:200; LifeSpan BioSciences), mouse anti-Osteocalcin (ab13418, 1:100; Abcam), rabbit anti-dentin

sialophosphoprotein (GTX60194, 1:150; GeneTex), rabbit anti-Amelogenin (ABT260, 1:500; Millipore), rabbit anti-Ki67 (ab15580, 1:200; Abcam), and rabbit anti-Caspase-3 (ab4051, 1:20; Abcam), followed by secondary antibodies anti-mouse IgG (711-065-150, 1:500; Jackson ImmunoResearch) or anti-rabbit IgG (711-065-152, 1:500; Jackson ImmunoResearch). Sections were then treated with ABC reagent (PK-4000; Vector Laboratories) and DAB (D4293; Sigma-Aldrich) and counterstained with 0.2% fast green (F7252; Sigma-Aldrich).

Immunofluorescent (IF) analyses were performed as published (Smith et al. 2016) using primary antibodies: mouse anti-Fibroblastic Growth Factor 3 (ab10830 1:50 to 1:100; Abcam), rabbit anti-Sonic Hedgehog (sc-9024, 1:50; Santa Cruz Biotechnology), mouse anti- β -catenin (LS-C172582, 1:50; LifeSpan BioScience), rabbit anti-E-cadherin (1:40, ABIN1858334; Antibodies online), mouse anti-Sox2 (1:50, ab79351; Abcam), rabbit anti-Ki67 (ab15580, 1:50; Abcam), mouse anti-Fibrillin 1 (1:50, gift), rabbit anti-Fibrillin 2 (1:100, gift), mouse anti-dentin sialophosphoprotein (SC-73632, 1:100; Santa Cruz Biotechnology), rabbit anti-Osteocalcin (1:50, gift), and rabbit anti-Amelogenin (ABT260, 1:100; Millipore). Secondary antibodies (Invitrogen) included goat anti-mouse IgG-Alexa Fluor 568 (A-11031, 1:100), goat-rabbit IgG-Alexa Fluor 488 (A-11034, 1:100), goat anti-mouse IgG-Alexa Fluor 488 (A-11001, 1:100), and goat-rabbit IgG-Alexa Fluor 568 (A-11011, 1:100).

Results

Initial Cell Seeding Density Positively Correlated with In Vitro Cultured Tooth Bud Construct Cell Density

Bilayered constructs were fabricated at 3 cell seeding densities: 1 $\times$, 2 $\times$, and 3 $\times$ (Fig. 1A), by sequential photo-polymerization (Fig. 1B). Tooth bud constructs were cultured in normal media for 1 d (1D) or 7 d (7D), followed by 7D culture in OM (1D–7D and 7D–7D, respectively) and implanted for 2 or 4 wk (Fig. 1C). OM was used based on our published reports showing dental cell differentiation in OM culture (Zhang et al. 2009; Monteiro et al. 2016; Smith et al. 2017).

H&E-stained sectioned in vitro cultured constructs showed distinct bilayer formation in all constructs (Appendix Fig. 1A, B). Bilayers were present in all in vitro cultured constructs but were less obvious after in vivo growth. Initial cell seeding densities positively correlated with cell densities of all in vitro cultured constructs (Appendix Fig. 1A, B).

Cell proliferation, assessed via Ki67 expression, increased with cell seeding density in 1D–7D constructs (Appendix Fig. 1A) but appeared similar at all 7D–7D cell seeding densities (Appendix Fig. 1B). Apoptosis, assessed via Caspase 3 expression, was lower in 1D–7D constructs compared to 7D–7D constructs (Appendix Fig. 1A, B). Although there appeared to be more cells in the 1D–7D versus 7D–7D constructs, Live/Dead staining revealed high cell viability (~90% to 98%) in all 1D–7D and 7D–7D constructs and low levels of dead cells (~10% to 2%) (Appendix Fig. 1A, B). Together, these results showed

Figure 1. Bioengineered tooth bud construct fabrication. Schematic of tooth bud construct fabrication. Initial cell seeding densities (**A**). Preparation of 5% gelatin methacryloyl (GelMA)-encapsulated dental mesenchymal (DM) cells/human umbilical vein endothelial cells (HUVECs) and 3% GelMA-encapsulated dental epithelial (DE) cells/HUVECs. Sequential photo-crosslinking method (**B**). Timeline for in vitro cultured and in vivo implanted tooth bud constructs (**C**). Constructs were grown in normal media for 1 or 7 d (1D or 7D) and then in osteogenic for an additional 7 d (7D), creating 1D–7D and 7D–7D constructs. Constructs were implanted subcutaneously for 2 wk (2W) or 4 wk (4W).

that in vitro cultured 1D–7D constructs exhibited robust cell proliferation and low apoptosis, while 7D–7D constructs showed relatively reduced cell proliferation but similar viability to 1D–7D constructs.

Dental Cell Localization in In Vitro Cultured Constructs

Distinct E-cadherin (ECAD)-expressing DE cells were restricted to the top layer of bilayered in vitro tooth bud constructs, vimentin (VM)-expressing DM cells were located in the bottom layer of all constructs, and CD31-positive neovascular-like structures were present throughout both layers of all in vitro cultured constructs (Fig. 2).

Classification of In Vivo Grown Tooth Bud Constructs

Bioengineered constructs were harvested after 2-wk (2W) and 4-wk (4W) in vivo growth. First, micro-computed tomography (CT) imaging of all replicate constructs was used to identify those containing detectable mineralized tissue formation. Next, paraffin-sectioned constructs were characterized using histological and IHC approaches (Fig. 2). Every replicate construct was sectioned entirely, and at least 10 sections spanning the entire construct were analyzed. Constructs were classified as “mineralized” if they contained any detectable amount of mineralized tissue by micro-CT. DE rosettes were easily identified by H&E staining and robust ECAD expression and generally

appeared as a single, large structure surrounded by VM-positive DM tissue (Fig. 2). Based on these features, we classified constructs into 1 of 4 types: 1) mineralized tissue and DE rosette, 2) mineralized tissue and no rosette, 3) no mineralized tissue with rosette, and 4) no mineralized tissues and no rosette (Appendix Table).

At 2W, 30% of in vivo implanted 1D–7D constructs exhibited only rosettes, 10% contained both mineralized tissues and rosettes, and 60% contained neither mineralized tissue nor rosettes (Appendix Table). In contrast, 75% of the 2W in vivo implanted 7D–7D constructs contained only rosettes, while the remaining 25% contained neither mineralized tissue nor rosettes (Appendix Table). These results suggested that 7D–7D culture conditions facilitated DE cell rosette formation in in vivo implanted constructs.

At 4W, increased mineralized tissue formation and fewer rosettes were present in both 1D–7D and 7D–7D constructs. Only 10% of 4W 1D–7D constructs contained only rosettes (as compared to 30% at 2W), and 30% were mineralized non-rosette containing. The only mineralized

tissue with rosette construct (10%) was a 1D–7D 3× (similar to the 2W group). The remaining 4W 1D–7D constructs (50%) were nonmineralized, nonrosette containing. Together, these results showed that DE rosettes were more frequently found in 7D–7D 2W in vivo implanted constructs, indicating that extended time in normal media facilitated the formation of rosette structures at 2W. In natural tooth development, founder FGF8+ DE cells form rosettes and migrate to the site of tooth initiation (Prochazka et al. 2015). These rosettes are transient structures, similar to those found in other developing tissues (Harding et al. 2014). In addition, at 4W, a higher percentage of mineral containing constructs were found in 7D–7D (40%) compared to the 1D–7D (29%) group.

Neovasculature Formation Was Localized to DE Rosettes and Mineralized Tissues in In Vivo Constructs

Rosette-containing 1D–7D and 7D–7D constructs exhibited well-defined, ECAD-positive rosettes surrounded by VM-positive DM cells (Fig. 2). CD31-positive cells were present in both layers, within and surrounding the rosettes (Fig. 2). Constructs containing mineralized tissue exhibited very few ECAD-positive cells, with VM and CD31 expression within and surrounding mineralized tissue (Fig. 2, arrows). These results suggested that CD31-positive neovasculature correlated with rosette and mineralized tissue formation in 2W and 4W 1D–7D and 7D–7D in vivo implanted constructs.

Figure 2. Neovascularization localizes with dental epithelial (DE) rosettes and mineralized tissues. Bilayers were obvious in all in vitro cultured constructs, where E-cadherin (ECAD)-positive DE cells were identified in the top layer and vimentin (VM)-positive dental mesenchymal (DM) cells were located in the bottom layer. CD31 vascular-like structures were located throughout both layers of in vitro constructs. ECAD-positive DE cells formed rosette-like structures, which were surrounded by VM-positive DM cells in 2-wk (2W) 1-d (1D)–7-d (7D), 2W 7D–7D, and 4-wk (4W) 1D–7D in vivo constructs. ECAD-positive DE cells and/or VM-positive DM cells were also detected within and around mineralized tissues of 2W 1D–7D, 4W 1D–7D, and 4W 7D–7D in vivo constructs. CD31-expressing human umbilical vein endothelial cells (HUVECs) colocalized to regions of DE rosette-like structures and mineralized tissue formation. Scale bars: 50 μ m.

2W In Vivo Bioengineered Tooth Buds Exhibit Similarities to Naturally Developing Tooth Buds

In natural tooth development, the DE stem cell (DESC) niche expresses the transcription factor Sox-2 (Chavez et al. 2012; Juuri et al. 2012), which is important for DE stem cell maintenance, proliferation, and competence (Juuri et al. 2012, 2013; Seo et al. 2011; Sun et al. 2016). Distinct Sox-2-positive DE rosette-like structures were present in in vitro 1D–7D and 7D–7D (Appendix Fig. 2A, A1, B, B1) and also 2W in vivo cultured 1D–7D and 7D–7D constructs (Appendix Fig. 2 C, C1, D, D1). At 2W, all in vivo implanted bioengineered constructs exhibited a single, prominent Sox-2-positive and ECAD-positive rosette structure (Fig. 3A, B and Appendix Fig. 2). In contrast, in vitro cultured constructs expressed ECAD and Sox-2 DE cells that were distributed throughout the DE cell layer (Appendix Fig. 2). The 2W 2 $\times$ and 3 $\times$ in vivo implanted constructs also expressed the DESC niche marker, LEF-1 (Sasaki et al. 2005; Huang and Qin 2010; Sun et al. 2016) (Fig. 3C), and the cell proliferation marker Ki67 (Fig. 3D), suggesting the formation of a putative bioengineered DESC niche. The bioengineered DESC niche was surrounded by condensing VM-positive DM cells (Fig. 3E) that also expressed the known DM signaling molecule fibroblast growth factor 3 (FGF3)

(Fig. 3F) (Kettunen et al. 2000; Wang et al. 2007) and basement membrane markers collagen IV and laminin (Fig. 3G). All no-primary controls were negative (Fig. 3H).

Bioengineered tissues displayed distinct extracellular matrix (ECM) morphology, visible via H&E staining (Fig. 3I), and DM tissues strongly expressed the DM ECM marker fibrillin 2 (Fib2), while bioengineered DE tissues only weakly expressed the DE ECM marker fibrillin 1 (Fib1) (Fig. 3J), consistent with expression patterns observed in early stage natural tooth buds. Robust expression of β -catenin and loss of ECAD within clusters of DE cells (Fig. 3K) may indicate the presence of putative transit-amplifying (TA) cells, which in natural tooth development migrate from the DESC niche to form enamel-secreting ameloblasts (Li et al. 2012). Importantly, clusters of DE cells coexpressed FGF3 and Sonic hedgehog (SHH), suggesting the formation of putative enamel knots (EKs) (Fig. 3L), signaling centers that direct natural tooth cusp formation (Ahtiainen et al. 2016). Finally, DE-expressed β -catenin and SHH, adjacent to DM-expressed FGF3, indicated activation of canonical Wnt signaling (Fig. 3M, solid arrows) as seen in developing tooth buds (Kratochwil et al. 2002; Åberg et al. 2004; Chang et al. 2013; Tamura and Nemoto 2016). Together, these results demonstrated that bioengineered GelMA tooth bud constructs exhibited certain hallmark features of natural tooth development.

Figure 3. Two-week (2W) in vivo bioengineered tooth buds exhibit many features of naturally developing tooth buds. All rosette-like structures strongly expressed E-cadherin (ECAD) (A), Sox-2 (B), Lef-1 (C), and Ki67 (D), suggesting the formation of a putative dental epithelial stem cell (DESC) niche. The putative DESC niche was surrounded by condensing dental mesenchymal (DM) cells that expressed vimentin (VM) (E) and fibroblast growth factor 3 (FGF3) (F) and was confined within a basement membrane (BM) that expressed collagen IV (Col IV) and laminin (Lam) (G). Negative controls showed no fluorescent signal (H). Hematoxylin and eosin (H&E) staining revealed distinct dental epithelial (DE) and DM extracellular matrix (ECM) morphologies (I), with fibrillin 1 (Fib1) expression localized to the DE layer and fibrillin 2 (Fib2) enriched in the DM layer (J). Lack of (K) ECAD expression in β -catenin (β cat)—expressing cells suggests the presence of putative transit-amplifying (TA) cells. (L) A cluster of DE cells coexpressed Sonic hedgehog (SHH) and FGF3, suggesting the formation of a bioengineered enamel knot signaling center. (M) Schematic demonstrating the temporal expression of early tooth bud markers expressed in bioengineered tooth bud, including those of the DESC niche (Sox2 and Lef-1), EK (FGF3 and SHH), and dental mesenchyme (FGF3). Wnt pathway members were also present in their respective dental cell layer (arrows indicate signaling) (M). Scale bars: 50 μ m.

In Vivo Mineralized Tissue Formation Increased with Initial Cell Seeding Density

Micro-CT analyses revealed mineralized tissue that roughly adopted the size and shape of the construct in 7 of 35 constructs (Fig. 4A1–A4). Mineralized tissue formation was not homogeneous in either bioengineered DE or DM layers but rather appeared nodular, as assessed via whole micro-CT (Fig. 4). Bioengineered constructs appeared to form mineralized tissues in the center of the constructs, at the DE/DM cell layer interface. The highest mineral density was located in the center of the mineralized tissue, surrounded by intermediate and then lowest mineral density at the periphery (Fig. 4A3, A4, Appendix

Videos 1, 2). Increased cell seeding density trended with increased maximum and average mineral densities (Fig. 4B) and volumes (Fig. 4C). Importantly at 4W, 2 $\times$ 1D–7D and 7D–7D constructs exhibited the greatest volume of the highest mineral density range measured (Fig. 4D).

Ameloblast, Odontoblast, and Osteoblast Differentiation in Mineralized In Vivo Implanted Constructs

Mineralized tissue differentiation marker expression was examined in all mineralized 4W in vivo constructs. We

Figure 4. Mineralized tissue formation in in vivo implanted tooth bud constructs. Bright-field (**A1**) and micro-computed tomography (CT) (**A2–A4**) images of in vivo constructs. Maximum and average mineral densities values correlated with initial cell seeding densities (**B**). The 2× 1D–7D and 2× 7D–7D constructs exhibited highest mineralized tissue density. Initial cell seeding density also correlated with mineralized tissue volume (**C**). The 2× 1D–7D and 2× 7D–7D constructs also exhibited the greatest percent volume of highest mineral density range measured (**D**). Low-density mineralized tissue was located around the periphery, while intermediate- and high-density mineralized tissues were located in the center (**A3**, **A4**). **A4** is the side view of **A3**. Scale bar: 200 μ m.

observed every possible combination of expression of markers Amelogenin (AM), Dentin sialophosphoprotein (DSPP), and Osteocalcin (OC): 1× 1D–7D expressed AM, DSPP, but no OC; 2× (2) 1D–7D constructs only expressed OC; 3× 1D–7D expressed AM and OC; 1× 7D–7D expressed AM only, and 2× 7D–7D expressed all 3 markers (Fig. 5). Together, these results showed that 1D–7D conditions were somewhat biased to OC-expressing bone differentiation, while 7D–7D promoted odontogenic tissue differentiation. Unfortunately, the number of implants that mineralized was too low in these studies to obtain statistically significant results.

Discussion

The purpose of this study was to create a more robust bioengineered tooth bud model. We found that sequential photo-cross-linking resulted in reliable and distinct DE and DM layers present in all in vitro cultured constructs but were less obvious after in vivo implantation and growth. We found that 2× initial cell seeding density resulted in greater mineralized tissue formation.

Prominent features of 2W in vivo implanted constructs included the formation of rosette structures, more common in 7D–7D constructs, and lack of mineralization, resembling natural

initiation stage teeth. In 4W bioengineered tooth constructs, rosette structures were less common, while more mineralized tissue formation was observed. Bioengineered rosette structures expressed DESC niche markers Sox-2 and Lef-1, indicative of early initiation stage tooth DE, and appeared to be transient in that they were less frequently found in 4W in vivo implants. In turn, more 4W in vivo implanted constructs contained mineralized tissue. The correlation of these 2 features—and whether rosette structures promoted later mineralized tissue formation—is an interesting topic for future studies. The 2W in vivo DE rosette structures were surrounded by condensed DM tissue, clearly identified using ECAD and VM immunostaining (Fig. 2). It is unclear whether these DM cells were implant or host derived—further studies are needed to test this possibility.

Bioengineered tooth buds exhibited many features characteristic of natural tooth buds, although in a less reproducible and less organized manner. These included a putative, Sox2-positive DESC niche; putative Shh-expressing EK signaling centers; putative β -catenin-expressing and ECAD-negative TA cells; and activated canonical WNT signaling. In natural teeth, Sox-2 and Lef-1 cooperate with Pitx2 to regulate DE stem cell maintenance (Huang and Qin 2010; Petersson et al. 2011; Sun et al. 2016). Sox-2-positive cells migrate and

Figure 5. Ameloblast, odontoblast, and osteoblast differentiation in in vivo implanted tooth bud constructs at 4 wk (4W). The expression of both tooth-specific differentiation markers Amelogenin (AM) and Dentin sialophosphoprotein (DSPP) was localized around and within mineralized tissues of 1× 1D–7D and 2× 7D–7D in vivo constructs. Robust expression of bone-specific differentiation marker Osteocalcin (OC) was detected in 2× 1D–7D and 3× 1D–7D in vivo constructs and only weakly in 2× 7D–7D in vivo constructs. Scale bars: 50 μ m.

become TA cells that terminally differentiate into ameloblasts (Harada et al. 1999; Li et al. 2015). Although most commonly characterized in the continuously erupting rodent incisor model, TA cells are also found transiently in molar teeth (Li et al. 2015). Coexpression of SHH and FGF3 in tightly packed clusters of DE cells in our bioengineered constructs is indicative of putative EK signaling centers. In the future, additional approaches including DE stem cell lineage tracing, stem cell transplantation assays, and the expression of additional EK markers such as p21, BMP, and Wnt will be examined to further validate these findings. Interestingly, bioengineered tooth buds also expressed tooth-specific ECM markers Fib1 and Fib2, which in natural teeth are expressed in later stage DE and early stage DM (Kira-Tatsuoka et al. 2015).

With respect to mineralized tissue formation, the 4W 2× cell constructs showed the greatest mineralized tissue volume and density, consistent with reports on cell seeding density influencing mesenchymal cell fate (Kim et al. 2009; Luo et al. 2013; Namkoong et al. 2016). Most cells expressed DSPP, OC, or AM—very few expressed both DSPP and OC or DSPP and AM (Appendix Fig. 3).

A key factor for bioengineered mineralized tissue formation was the presence of CD31-positive HUVEC-derived neovasculature. Neovasculature was present in 2W and 4W rosette-like structures and in mineralized tissues in in vivo implanted 1D–7D and 7D–7D constructs (Fig. 2), consistent with natural (Decker 1967; Manzke et al. 2005) and bioengineered tooth development (Rouwkema et al. 2006; Nait Lechguer et al. 2008; Smith et al. 2017). We previously reported that neovasculature contributed to bioengineered mineralized tissue formation (Smith et al. 2017) and that HUVEC survival in bioengineered constructs and blood vessel formation in natural bone development are important for mineralized tissue formation (Greenhill 2014). In in vivo implanted constructs,

CD31-positive HUVECs were located within and around the mineralized tissue, and constructs lacking strong CD31 expression formed less mineralized tissue.

Although these results demonstrate improvements to our previous bioengineered tooth bud model, certain limitations still exist. We observed variability within replicate groups, likely due to extensive time required to spot-cure individual samples, indicating the need for improved methods. We found little evidence of DE or DM cell polarization or distinct dentin and enamel crystal structures, all classic features of natural teeth. These results suggest the lack of sufficient cues within the GelMA constructs as they are currently made.

Despite these limitations, here we have validated new methods to bioengineer tooth buds, created from postnatal dental cells, which resemble features of natural tooth buds. We propose this model as an important step forward toward eventual clinical applications in regenerative dentistry in humans.

Author Contributions

E.E. Smith, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; S. Angstadt, N. Monteiro, contributed to data analysis, drafted and critically revised the manuscript; W. Zhang, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; A. Khademhosseini, P.C. Yelick, contributed to conception and design, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

We thank members of the Yelick Lab for expert advice and support. We also thank all members of the Khademhosseini Lab. Fibrillin 1 and fibrillin 2 antibodies were generous gifts from Dr. Lynn Saki and Dr. Francesco Ramirez. This research was

supported by NIH/NIDCR/NIBIB R01DE016132 (PCY), NIH/NIDCR/NIBIB R01DE026731 (PCY), and F31DE026361 (EES). Any underlying research materials can be available upon request. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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