Development of Poly(Ethylene Glycol) Hydrogels for Salivary Gland Tissue Engineering Applications

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More than 40,000 patients are diagnosed with head and neck cancers annually in the United States with the vast majority receiving radiation therapy. Salivary glands are irreparably damaged by radiation therapy resulting in xerostomia, which severely affects patient quality of life. Cell-based therapies have shown some promise in mouse models of radiation-induced xerostomia, but they suffer from insufficient and inconsistent gland regeneration and accompanying secretory function. To aid in the development of regenerative therapies, poly(ethylene glycol) hydrogels were investigated for the encapsulation of primary submandibular gland (SMG) cells for tissue engineering applications. Different methods of hydrogel formation and cell preparation were examined to identify cytocompatible encapsulation conditions for SMG cells. Cell viability was much higher after thiol-ene polymerizations compared with conventional methacrylate polymerizations due to reduced membrane peroxidation and intracellular reactive oxygen species formation. In addition, the formation of multicellular microspheres before encapsulation maximized cell–cell contacts and increased viability of SMG cells over 14-day culture periods. Thiol-ene hydrogel-encapsulated microspheres also promoted SMG proliferation. Lineage tracing was employed to determine the cellular composition of hydrogel-encapsulated microspheres using markers for acinar (Mist1) and duct (Keratin5) cells. Our findings indicate that both acinar and duct cell phenotypes are present throughout the 14 day culture period. However, the acinar:duct cell ratios are reduced over time, likely due to duct cell proliferation. Altogether, permissive encapsulation methods for primary SMG cells have been identified that promote cell viability, proliferation, and maintenance of differentiated salivary gland cell phenotypes, which allows for translation of this approach for salivary gland tissue engineering applications.

Introduction

EVERY YEAR, MORE THAN 40,000 patients are diagnosed with head and neck cancers in the United States. Many receive radiation therapy, which leads to irreparable damage of the salivary glands, resulting in a permanent dry mouth, a condition known as xerostomia.¹ Xerostomia can negatively affect speech, diet, and oral hygiene. Current treatments for xerostomia attempt to lubricate the mouth with artificial saliva or via pharmacological stimulation of residual tissue to increase salivary production. However, no current treatment can fully restore or emulate the myriad functions of the salivary gland, leading to oral health deficiencies.^{1,2}

The salivary gland is composed of two major cell types: acinar cells that initiate salivary secretion and duct cells that propel and modify the ionic components of the secretions.³ Although the salivary gland does not regenerate after radiation damage, it exhibits regenerative potential after mild

insults. For example, in a rodent model of salivary gland injury, ligation of the excretory duct results in atrophy of the acinar cells. After removal of the ligation, both the submandibular and parotid glands have restored acinar structures, which supports some inherent but limited gland regeneration.^{4–6} No salivary gland stem cell has been definitively identified as contributing to gland regeneration; however, several duct cell subtypes have been characterized as progenitor cells.^{7–12} Furthermore, although the direct injection of progenitor cell populations, namely c-Kit⁺ salivary progenitor cells^{10,13} or mesenchymal stem cells (MSCs),¹⁴ into irradiated submandibular glands (SMGs) showed some functional improvement, restoration of saliva secretion was incomplete, and highly variable.¹³

To reproducibly promote regeneration and functional recovery of irradiated salivary glands, biomaterial-based approaches for cell transplantation have been explored. Numerous studies have focused on feasibility of using

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nanofibers or hydrogel-based scaffolds.^{15–25} Although a few studies have translated their findings *in vivo*,^{16,17,23} no study to date has demonstrated that the tissue developed on or within biomaterials contributes to restoration of gland function. To overcome these limitations, we propose use of poly(ethylene glycol) (PEG) hydrogels for salivary gland cell transplantation. Unlike hydrogels derived from natural materials (e.g., collagen, fibrin, and hyaluronic acid), PEG hydrogels are inert due to their highly hydrated and uncharged structure, providing a “blank slate” for introduction of growth factors and extracellular matrix (ECM) proteins or mimetics to control cell behavior.²⁶ Furthermore, the stiffness, degradability, and mesh size (typically between 10 and 30 nm) of PEG hydrogels can be controlled by the composition and relative amounts of PEG macromers.²⁷

PEG hydrogels are traditionally formed through radical-mediated photopolymerizations,^{28,29} which allow for rapid fabrication into geometries dictated by custom molds *in vitro* or to match tissue defects *in vivo*.^{30,31} PEG hydrogels have been utilized successfully to culture and control the behavior of various cell types, including MSCs,^{32,33} chondrocytes,^{34–36} osteoblasts,³⁷ pancreatic β -islet cells,^{38,39} and neurons.^{40,41} In addition, recent work has demonstrated the ability of PEG hydrogels to provide spatiotemporal control of MSC delivery *in vivo* to promote bone regeneration.^{31,42,43}

In this work, methods have been explored to encapsulate, culture, and characterize primary SMG cells within PEG hydrogels, with the long-term goal of developing a tissue engineering approach for the salivary gland. Due to the sensitivity of salivary gland cells to reactive oxygen species (ROS),^{44–48} we examined the effects of two forms of radical-mediated hydrogel polymerization: chain addition methacrylate-based polymerizations and step-growth thiol-ene polymerizations on primary SMG cells. PEG hydrogels are bioinert,²⁶ and they lack cell–matrix and cell–cell interactions that are commonly utilized to maintain survivability of sensitive cell types.^{32,38,41,49,50} As cell–cell interactions, in particular, play a vital role in salivary gland cell functions *in vitro* and during gland development,^{20,51–57} we also explored the use of SMG cell aggregation into microspheres to increase long-term viability of hydrogel-encapsulated SMG cells. Finally, we examined the cellular composition and proliferative potential of the encapsulated SMG microspheres. Overall, this work demonstrates that PEG hydrogels provide an approach to culture and expand primary SMG cells *in vitro* for use in salivary gland regenerative therapies.

Methods

Hydrogel macromer synthesis

Materials. PEG-monomethacrylate (PEGMM, 2 kDa, Fig. 1A) and dithiol-functionalized PEG (3.4 kDa, Fig. 3A[i]) were purchased from Dajac Labs and Laysan Bio, respectively. Unfunctionalized PEG (10 kDa) was purchased from Alfa Aesar. Four-arm PEG (20 kDa) was purchased from Jenkem Technologies. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as described.⁵⁸

Dimethacrylate-functionalized PEG synthesis. Linear dimethacrylate-functionalized PEG macromers (PEGDM, Fig. 3A[ii]) were synthesized according to previously es-

tablished protocols.^{28,59,60} Briefly, 5 g of linear 10 kDa PEG was methacrylated with methacrylic anhydride (Alfa Aesar, 5 molar equivalents [meq] per PEG hydroxyl) in a commercially available microwave (120 VAC, 12 A; Sharp Carousel). Solutions of PEG and methacrylic anhydride were microwaved for 5 min, pausing every 30 s to vortex samples. PEGDM was dissolved into dichloromethane (DCM, ~50 mL) in a round bottom flask and precipitated in ~1 L ice-cold diethyl ether.

Precipitated product was collected on filter paper using vacuum filtration and dried under vacuum for 24 h. Percent methacrylation (>90%) was verified through ¹H-NMR using a Bruker Avance 400 MHz spectrometer (¹H NMR [CDCl₃]: 3.5–3.7) (ether protons of PEG, 909H, multiplet), 5.5 (methacrylate vinyl protons, 2H, singlet), 6.2 (terminal vinyl protons, 2H, singlet), and 1.9 (terminal methyl protons, 6H, multiplet).

Norbornene-functionalized PEG synthesis. Four-arm 20 kDa PEG was functionalized with norbornene (PEG-NORB, Fig. 2A) using *N,N'*-dicyclohexylcarbodiimide (DCC) coupling as previously described.²⁹ Briefly, norbornene carboxylate (10 meq per PEG hydroxyl), DCC (5 meq per PEG hydroxyl), pyridine (1 meq per PEG hydroxyl), and 4-dimethylaminopyridine (DMAP, 0.5 meq per PEG hydroxyl) were dissolved in ~100 mL DCM at room temperature for ~30 min until a white precipitate (dicyclohexylurea) formed, which indicated formation of dinorbornene carboxylic acid anhydride. Five grams of four-arm PEG dissolved in ~50 mL DCM was added drop-wise to the anhydride solution, which was subsequently septa sealed, vented with a needle, and stirred overnight at room temperature.

The resulting solution was vacuum filtered and the filtrate was precipitated in 1 L ice-cold diethyl ether, collected by vacuum filtration, redissolved in 75 mL DCM, and reprecipitated two additional times in 1 L ice-cold diethyl ether. Structure and percent functionalization (>90%) was determined by ¹H-NMR using a Bruker Avance 400 MHz spectrometer (¹H NMR [CDCl₃]: δ =6.0–6.3 [norbornene vinyl protons, 8H, multiplet]), (3.5–3.9 PEG ether protons, 1817H, multiplet). The final product was dialyzed against distilled, deionized water (ddH₂O) for 24 h using 1000 MWCO dialysis tubing (Spectrum Laboratories) and lyophilized.

Peptide synthesis. The peptide KGKGKGKGC (Fig. 2A) was synthesized by standard solid-phase peptide synthesis on Fmoc-Gly-Wang resin (EMD) using a Liberty 1 Microwave-Assisted Peptide Synthesizer (CEM) with UV monitoring as previously described.⁶¹ All amino acids (AAPPTec) were dissolved in *N*-methylpyrrolidone (NMP) at 0.2 M. All amino acids were Fmoc-protected, with cysteine and lysine side chains protected with trityl and tert-butylloxycarbonyl, respectively. Then, 0.5 M Benzo[1,2,3-c:4,5-b']diazole-*N,N,N'*-tetramethyl-uronium-hexafluoro-phosphate (Ana-Spec) in dimethylformamide (DMF) was used as activator, 2 M diisopropylethylamine (Alfa Aesar) in NMP was used as activator base, and 5% piperazine (Alfa Aesar) in DMF was used for deprotection. Peptides were cleaved and deprotected by adding the on-resin 0.25 mmol peptide (0.316 g resin) to a cleavage cocktail composed of 18.5 mL trifluoroacetic acid (TFA, Acros Organic) (Acros Organic), 0.5 mL triisopropylsilane (Sigma Aldrich), 0.5 mL ddH₂O,

and 0.5 mL 3,6 dioxo-1,8-octane dithiol and mixing for 2 h. Cleaved peptide was separated from resin via vacuum filtration, purified via precipitation in ice-cold diethyl ether (~ 180 mL), and collected by centrifugation. The peptide was resuspended in ice-cold diethyl ether, centrifuged twice more, dried under vacuum overnight, dialyzed against ddH₂O using 500 MWCO dialysis tubing (Spectrum Labs) for 48 h, and lyophilized.

Peptide molecular weight was verified using a Bruker AutoflexIII Smartbeam matrix-assisted laser desorption ionization time-of-flight mass spectrometer with 1 mg/mL peptide dissolved in 50:50 acetonitrile:H₂O + 1% TFA added in a 1:1 volumetric ratio of 10 mg/mL α -Cyano-4-hydroxycinnamic acid matrix (Tokyo Chemical Industry) dissolved in 50:50 acetonitrile:H₂O + 1% TFA. Peptide standards (Bruker Peptide Calibration Standard, 206195) were used for calibration. Peptide purity was analyzed via 205 nm absorbance measured in ddH₂O with an Evolution UV/Vis detector (Thermo Scientific) and compared with predicted values using a previously published methodology.⁶²

Cell culture

Isolation and culture of mouse SMG cells. Primary mouse SMG cells were isolated as previously described.⁸ Briefly, female C57BL/6 mice aged 8–24 weeks were euthanized according to animal protocols approved by the University of Rochester University Committee for Animal Resources. SMGs were surgically removed, finely minced with a razor blade, and dissociated by incubation in 5 mL Hank's Balanced Salt Solution (GIBCO) with CaCl₂ and MgCl₂, 1 mg/mL collagenase Type II (GIBCO), and 5 μ g/mL hyaluronidase (Applchem) at 37°C for 40 min with gentle shaking.

Cells were then washed in 5 mL of Dulbecco's phosphate-buffered saline (DPBS; GIBCO) and with 0.05% Trypsin (GIBCO) for 4 min at room temperature. Cells were passed through 40 μ m mesh filters and resuspended in 5 mL complete media (Dulbecco's Modified Eagle Medium (DMEM): F12 [1:1; GIBCO]) with Glutamax (1 $\times$; GIBCO), Antibiotic-Antimycotic solution (100 IU/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B; GIBCO), N₂ supplement (1 $\times$ Invitrogen), 10 μ g/mL insulin (Life Technologies), 1 μ M dexamethasone (Sigma), 20 ng/mL epidermal growth factor (EGF; Life Technologies), and 20 ng/mL basic fibroblast growth factor (bFGF; Life Technologies). Cells were either immediately prepared for encapsulation or seeded at a density of $\sim 1.2 \times 10^6$ cells/mL in 60 mm non-tissue culture treated petri dishes (5 mL total) and incubated in standard tissue culture conditions (37°C, 5% CO₂) for 48 h to allow microsphere formation.

Analysis of SMG cell function after exposure to nongelling polymerizations

Primary SMG cells were placed in PEGMM (Fig. 1A) to expose them to chain polymerization conditions while avoiding hydrogel formation. Polymerization using monofunctional macromers proceeds similar to a gelling polymerization, but the use of monofunctional rather than difunctional macromers prevents crosslinking and hydrogel formation (Fig. 2A). Similarly, nongelling thiol-ene polymerization was performed with a peptide that incorporates one cysteine group, KGKGKGKCG (Fig. 2A), which

reacts with one norbornene functionality of four-arm norbornene-functionalized PEG (PEGNORB) macromers, precluding crosslink formation (Fig. 2A). Nongelling polymerizations were employed for this subset of studies, because the absence of sol-to-gel transitions reduced potential variability due to diffusional constraints. For experiments directly comparing the effects of thiol-ene or methacrylate polymerization, macromer concentrations were chosen so that the concentrations of reacting functionalities (methacrylate, thiol + norbornene groups) were identical.

Nongelling chain and step-growth polymerizations. Freshly isolated primary SMG cells were resuspended in either 4 wt% PEGMM (20 mM methacrylate groups) or 5 wt% four-arm PEG-norbornene + 0.96 wt% of the peptide KGKGKGKCG (10 mM norbornene groups + 10 mM thiol groups). LAP, synthesized as described,⁵⁸ was used as the photoinitiator at a concentration of 0.028 wt%. Polymerization solutions were pipetted into 1 mL syringe molds and exposed to 3 min of 5 mW/cm² 365 nm light. Polymerizations were also performed using different radical propagating group concentrations (e.g., 20–2.5 mM) in the prepolymerization solution (Figs. 1C and 2E).

SMG cell viability after exposure to nongelling polymerizations. SMG cells were resuspended at 25×10^6 cells/mL in nongelling polymerization solution, and 45 μ L samples ($N=3-8$) were pipetted into syringe molds. Before polymerizations, 10 μ L of cell suspension was removed and diluted in 156 μ L of phosphate-buffered saline (PBS) in a 1.5 mL tube as the untreated control sample. Dilution was performed to ensure that the concentration of cells was within the linear range of the CellTiterGlo ATP assay from Promega (final cell concentration was $\sim 80,000$ cells per well). After polymerization, 10 μ L samples were removed and diluted in 156 μ L PBS. One hundred fifty-six microliter of CellTiterGlo reagent mix was added to each sample, which was then distributed into wells of white 96-well plates (100 μ L/well in triplicate). Samples were incubated at room temperature for 10 min and placed on an orbital shaker for 2 min. Sample luminescence was measured in triplicate on a SynergyMix (BioTek) luminescence plate reader and compared with an ATP standard curve (EMD Millipore) to quantitate sample ATP.

Most primary cell types experience a significant increase in oxygen tension when prepared for primary cell culture,⁶³ which results in an increase in intracellular oxidative stress. Thus, for all measurements of intracellular oxidative stress,^{64–66} cells were allowed to incubate in suspension culture for 24 h before analysis. To detect intracellular ROS, SMG cells were treated with 5 μ M CellRox and 2 μ M Calcein AM (Life Technologies) for 30 min, washed with 5 mL DPBS, and resuspended at a concentration of 4.0×10^6 cells/mL in 50 μ L/well aliquots in black 96-well plates. Samples ($N=4$ for each treatment) were resuspended in either methacrylate (20 mM methacrylate) or thiol-ene (10 mM thiol + 10 mM norbornene) nongelling polymerization solution with 0.028 wt% LAP or in DPBS (No Treatment). Samples were exposed to ~ 5 mW/cm² 365 nm light for 3 min. Measurements of nongelling polymerization solution CellRox fluorescence ($\lambda_{\text{Ex}}/\lambda_{\text{Em}}=630/670$ nm) were taken at 0, 0.5, 1.5, and 3.0 min, utilizing equivalent gain at each measurement. CellRox signal was normalized to Calcein

AM fluorescence ($\lambda_{\text{Ex}}/\lambda_{\text{Em}}$ 485/530 nm) at time 0 to account for variations in cell number. All measurements were performed using a Tecan Infinite M200 plate reader.

The malonyldialdehyde (MDA; Sigma-Aldrich) assay was used to analyze lipid peroxidation of SMG cells. Primary SMG cells were resuspended at 2.5×10^7 cells/mL in either thiol-ene or methacrylate nongelling solutions (detailed earlier), DPBS (PBS), or 2 mM hydrogen peroxide (positive control, H_2O_2) in complete media for 2 h. Samples (55 μL , $N=3$ [H_2O_2 , PBS], six [all others]) were pipetted into syringe molds. Twenty microliter samples were removed at $t=0$, 3, and 10 min (methacrylate only) of UV exposure and added to 300 μL of MDA lysis buffer with 3 μL butylated hydroxytoluene. Mixed samples were incubated on ice for 30 min and then gently pipetted to mix and lyse all cells. Samples were centrifuged at 13,000 g for 10 min, and 200 μL of each was added to 600 μL of thiobarbituric acid (TBA) dissolved in 100% acetic acid in 1.5 mL tubes. Fluorescent TBA-MDA adducts formed during 60-min incubation at 95°C and were measured in triplicate in black 96-well plates using a Tecan Infinite M200 plate reader ($\lambda_{\text{Ex}}/\lambda_{\text{Em}}$ 530/560 nm). Fluorescent signal was quantified using standard curves based on provided MDA standards.

Hydrogel encapsulation of primary SMG cells

The viability of SMG cells encapsulated within methacrylate or thiol-ene hydrogels was compared using LIVE/DEAD staining. The concentration of radical generating or propagating species (LAP and methacrylate, norbornene, and thiol, respectively) was minimized to maximize cell viability. However, the number of radical-propagating species was kept constant between the chain addition and step growth polymerizations, similar to described elsewhere.³⁹ Thus, the conditions of 0.028 wt% LAP and 8.4 wt% PEGDM (16.8 mM methacrylate) or 4.2 wt% PEGNORB + 1.3 wt% PEG-dithiol (8.4 mM norbornene + 8.4 mM thiol) were used as they were the lowest wt% of LAP and macromer (and hence lowest concentration of radical-generating and propagating species) in which hydrogels could be formed reliably. Cell concentrations (10^7 cells/mL of Primary SMG cells and 5×10^6 for microspheres) used for encapsulated cell LIVE/DEAD and ATP analysis were lowered to decrease cell debris aggregates, which interfere with image analysis.

Chain addition methacrylate-based PEG hydrogels. For hydrogel encapsulation within methacrylate polymerized systems, SMG cells (10^7 cells/mL) were resuspended in 8.4 wt% PEGDM dissolved in DPBS. LAP was used as the photoinitiator at a concentration of 0.028 wt%. Cell suspensions (30 μL each) were pipetted into 1 mL syringes with tips removed and polymerized via exposure to $5 \text{ mW}/\text{cm}^2$ 365 nm UV light for 10 min to form cylindrical hydrogels with heights of 2 mm and diameters of 4 mm. Immediately after polymerization, hydrogels were placed in complete media (1 mL/hydrogel) in 24-well plates.

Step growth thiol-ene PEG hydrogels. For thiol-ene encapsulations, primary SMG cells (10^7 cells/mL) or microspheres (5×10^6 cells/mL) were resuspended in 4.2 wt% PEGNORB + 1.3 wt% PEG-dithiol with 0.028 wt% LAP in

DPBS. Thirty microliter of cell suspension was pipetted into 1 mL syringes with tips removed and polymerized via exposure to $5 \text{ mW}/\text{cm}^2$ 365 nm UV light for 3 min, as thiol-ene polymerizations are more rapid than methacrylate polymerizations and undergo complete polymerization in less than 3 min.²⁹ Immediately after polymerization, hydrogels were placed in complete media (1 mL each) in 24-well plates.

Encapsulation of SMG cells within collagen hydrogels. Collagen gels were formed as previously described.^{67,68} Briefly, liquid collagen type I from rat tail (4 mg/mL; BD Bioscience) was added to $2 \times$ DMEM at a 1:1 volumetric ratio on ice. The pH of Collagen/DMEM solution was raised to 7.4 through addition of 0.1 M NaOH in 10 μL aliquots. pH was monitored using pH strips 5 min after addition of each aliquot. Once pH reached 7.4, $1 \times$ DMEM was added to dilute the total amount of collagen to 1.0 mg/mL. Primary SMG cells were resuspended at 10^7 cells/mL and 30 μL of the cell/collagen solution was pipetted into 1 mL syringes with tips removed. Samples were incubated for 1 h at 37°C and 5% CO_2 to allow complete gelation of collagen. Gels were added to complete media (1 mL) in 24-well plates.

Analysis of hydrogel-encapsulated SMG cell viability. After polymerizations, polymerized hydrogels with encapsulated primary SMG cells or microspheres were placed in 1 mL of complete media and incubated for 1 h at 37°C with 5% CO_2 . Calcein AM and ethidium homodimer (Life Technologies) were added directly to the media to a final concentration of 2 and 4 μM , respectively, and incubated for 30 min at 37°C with 5% CO_2 . Samples ($N=6$ [thiol-ene] or 3 [all others]) were placed in glass-bottom microwell dishes (Maktech) and imaged with an FV1000 laser scanning confocal microscope (Olympus).

Three 100 μm z-stacks were taken at three separate locations at bottom of hydrogels at $100\times$ magnification. Each 100 μm z-stack consisted of 10 images separated by 10 μm . The number of living and dead cells was quantified in $100\times$ z-stacks using VisioPharm software. Briefly, cells were categorized as live or dead based on Bayesian classification of the relative amounts of Calcein AM and Ethidium Homodimer staining. In addition, the majority of the cross-sectional area of hydrogels were imaged in singular lower power ($40\times$) 315 μm z-stacks consisting of 12 slices separated by 28.7 μm and the percent within the field of view (FOV) area in the $40\times 315 \mu\text{m}$ z-stacks containing living (CalceinAM positive) cells was quantified using ImageJ.

To further quantify viability in thiol-ene hydrogels, encapsulated primary SMG cells or microspheres were cultured for indicated times with media changes every 48 h, and new EGF and FGF were added to complete media stocks every 4 days. LIVE/DEAD analysis, as described earlier, was performed on thiol-ene polymerized hydrogels 4, 7, and 14 days after encapsulation. For whole hydrogel ATP analysis, thiol-ene polymerized hydrogels at indicated timed points were incubated in 500 μL of 50% CellTiterGlo Reagent/50% DPBS for 45 min on an orbital shaker at room temperature. One hundred microliter triplicates were added to a white 96-well plate, and sample luminescence was measured on a SynergyMix (Bio-Tek) luminescence plate reader. ATP was quantified using a standard curve as described in section "SMG cell viability after exposure to nongelling polymerizations".

Assessment of encapsulated cell lineage. The *Mist1^{CreERT2}* mice (*Mist1Cre*) were produced as described⁶⁹ and generously donated by Dr. Stephen Konieczny (Purdue University). *Mist1^{CreERT2}* mice were mated to the R26^{tm9(CAG-tdTomato)Hze} reporter strain, from Jackson Laboratories. Activation of *Mist1Cre* in the presence of tamoxifen results in genetic labeling of a high proportion of acinar cells by expression of the tomato red reporter gene. Labeling of duct cells was done by crossing the same reporter strain with a transgenic line in which the keratin 5 promoter drives CreER (*K5Cre*) (Jovov *et al.*⁷⁰; donated by Dr. Jianwen Que, University of Rochester). Both strains were maintained on a C57BL/6 background.

Tamoxifen (60 mg/mL dissolved in corn oil) was administered by gavage (0.25 mg/g body weight) on four consecutive days in the week before gland harvest for *Mist1Cre* mice and 1 day before harvest for *K5Cre* mice. SMG glands were harvested, cultured as microspheres, and encapsulated as described earlier. Three 100 μ m z-stacks were taken at three separate locations at bottom of hydrogels at 100 $\times$ magnification at day 0, 4, 7, and 14 days post-encapsulation. Each 100 μ m z-stack consisted of 10 images separated by 10 μ m. The percentage of cross-sectional area containing tdTomato-expressing cells within Calcein AM-positive microspheres was quantified using VisioPharm and an in-house developed application. The application quantified the total area circumscribed within Calcein AM-expressing microspheres as well as the total area of tdTomato fluorescence using image thresholding. Percentages of *Mist1* and *K5* cells were calculated by dividing the total area of tdTomato fluorescence by the total microsphere area determined by Calcein AM staining.

Edu labeling of encapsulated microspheres. SMG microspheres were incubated in 10 μ M EdU for 3 h and fixed with 1 mL 4.0% paraformaldehyde for 30 min, washed twice with 1 mL 3% bovine serum albumin (BSA) in DPBS, and permeabilized with 1 mL of 0.5% of Triton X-100 for 30 min. Samples were then washed twice with 1 mL 3% BSA in DPBS and fluorescently labeled with AlexaFluor 647 using the Click-iT[®] Plus EdU AlexaFluor[®] 647 imaging kit (Life Technologies) with Hoechst 33342 as a nuclear counterstain. All samples were imaged with a FV1000 laser scanning confocal microscope (Olympus) at 200 $\times$ magnification.

Disruption of cell–cell contacts with trypsinization. SMG microspheres were washed with DPBS, resuspended in 2 mL of 0.05% Trypsin/ethylenediaminetetraacetic acid (GIBCO), and incubated at 37°C for 10 min. Trypsin was diluted by adding 2 mL of sphere media, and the entire volume was passed through a 40 μ m strainer. Trypsinized SMG cells were then pelleted and washed with 5 mL DPBS. Trypsinized SMG cells were encapsulated and characterized as described with both LIVE/DEAD staining and ATP quantification immediately and at 7 days after encapsulation.

Statistical analysis

Statistical significance was determined with Prism (Version 6.02; Graphpad) using either one-way analysis of variance (ANOVA) with Tukey's HSD *posthoc* analysis or two-way ANOVA with Sidak *posthoc* analysis. All data and error bars are represented as averages and standard deviations.

Results and Discussion

Exposure to methacrylate-based chain addition polymerizations reduced SMG cell viability via increased intracellular ROS mediated by lipid membrane peroxidation

The overarching objective of this work is the eventual use of PEG hydrogels to transplant SMG cells *in vivo* to restore salivary gland function after irradiation. Therefore, initial experiments focused on identification of hydrogel encapsulation methods that maintain primary SMG cell survivability. Initially, methacrylate-functionalized PEG macromers were used due to their ease of synthesis and successful use with myriad cell types.^{32–34,37,38,40,71} To screen SMG cytocompatibility, nongelling polymerizations were used to alleviate potential issues with diffusional constraints^{28,59} by using macromers with only one functionality (Fig. 1A). SMG cells were exposed to the reactions necessary to form hydrogels, including radical generation by cleavage of photoinitiators and propagation through methacrylate-based carbon–carbon double bonds, but not to the sol-to-gel formation consistent with hydrogel formation. Similar experiments have been used to study the effects of hydrogel polymerization on a β -cell line.³⁹

Primary SMG cells were resuspended in PEGMM and the photoinitiator, LAP. Nongelling methacrylate polymerizations in the presence of 20 mM PEGMM + LAP resulted in a $\sim 70\%$ reduction in viability of primary SMG cells, as assessed by decreased ATP levels (Fig. 1B). Cellular ATP has a rapid turnover ($t_{1/2} \sim$ minutes), and dead cells experience a rapid drop in ATP levels,^{72–74} making ATP an appropriate surrogate analyte for viability.³⁹ Immediately after exposure to chain-addition methacrylate polymerization conditions, SMG cells experienced a significant decrease in viability, suggesting acute cell death (Fig. 1B).

Cell viability exhibited an inverse proportional relationship with PEGMM macromers present within the reaction. Macromer concentrations of 5 mM or less did not result in detectable cytotoxicity (Fig. 1C). Neither LAP alone nor 2.5 mM PEGMM caused decreases in cell viability (Fig. 1C), supporting the idea that polymerization-associated cytotoxicity is directly related to PEGMM macromer concentration. Somewhat surprisingly, exposure to 365 nm light alone significantly increased ATP levels by $\sim 35\%$. Although 365 nm light has not previously been found to affect cellular viability,³⁹ we hypothesize that the increase in ATP levels may be related to mixing that increases overall dissolved oxygen in the cell/macromer suspension. Increases in dissolved oxygen have been shown to increase cellular ATP levels, perhaps through increased cellular metabolism.^{75,76}

In contrast to fibroblasts,⁷¹ primary osteoblasts,⁷⁷ primary chondrocytes,³⁴ and human MSCs,³² primary SMG cells exhibit poor viability when exposed to methacrylate-based chain-addition polymerization reactions (Fig. 1B). The SMG cell population is heterogeneous and consists of terminally differentiated cells, many of which undergo apoptosis after isolation.^{8,10} However, the process of apoptosis takes ~ 3 h,⁷⁸ which is in stark contrast to the ~ 60 – 80% of SMG cell death observed after only minutes of exposure to chain-addition polymerizations (Fig. 1B). We speculated that ROS produced by the interference of oxygen during the methacrylate polymerization process⁷⁹ may cause the

FIG. 1. Nongelling chain polymerizations using poly(ethylene glycol) (PEG)-monomethacrylate (PEGMM) result in decreased submandibular gland (SMG) cell viability and increased reactive oxygen species. **(A)** PEGMM ($n = 44$) is dissolved in Dulbecco's phosphate-buffered saline (DPBS) with the photoinitiator and primary SMG cells. Due to the monofunctionality of PEG, aggregates of macromers are formed rather than hydrogels. Illustration is not to scale. **(B)** Cellular ATP levels of SMG exposed to nongelling polymerizations (SMG cells + lithium phenyl-2,4,6-trimethylbenzoylphosphine [LAP] + 20 mM PEGMM). ATP levels are expressed as a % of non UV-exposed SMG cell controls. **(C)** Cellular ATP levels of SMG cells in the presence of decreasing amounts of PEGMM with LAP after exposure to UV light. ATP levels are expressed as % of ATP in SMG cells in PBS. **(D)** Measurement of intracellular oxidative stress using CellROX normalized to levels at time 0, and comparing cells exposed to methacrylate with untreated cells. **(E)** Lipid peroxidation measured via analysis of malonyldialdehyde (MDA) concentration in lysates of SMG cells exposed to methacrylate nongelling polymerization conditions. All methacrylate MDA values were normalized to cells exposed to photoinitiator and macromers but not to UV, while cells treated with 2 mM H₂O₂ were normalized to untreated cells in PBS. Error bars represent $\pm$ standard deviation. $N = 3-8$ polymerizations per treatment. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared with non-UV-exposed samples (**B**, **E**) or Dulbecco's phosphate-buffered saline (DPBS) (**C**, **D**), determined by two-way analysis of variance (ANOVA) with Sidak *posthoc* analysis (**B**) or one-way ANOVA with Tukey's HSD *posthoc* analysis.

polymerization-associated cell death. To investigate the link between increased intracellular oxidative stress and PEG macromer polymerization conditions, the commercially available CellROX dye (Life Technologies) was used to measure intracellular oxidative stress. Lipid peroxidation was also analyzed indirectly by measuring MDA accumulation (MDA; Sigma), as MDA is a product of unsaturated lipid degradation by ROS. Increases in MDA suggest peroxidation of plasma membranes,^{80,81} which can also lead to increases in intracellular oxidative stress.^{19,36}

SMG cells underwent a significant 1.7-fold increase in intracellular oxidative stress compared with SMG cells in DPBS (Fig. 1D). Cells in PBS exposed to UV experience a 1.2-fold increase in intracellular ROS, which suggests that exposure to UV alone causes some increase in intracellular ROS (Fig. 1D). In addition, SMG cells exposed to 3 and 10 min of methacrylate-based polymerizations exhibited statistically significant increases (~1.5- and ~2.2-fold, respectively) in MDA levels compared with untreated cells (Fig. 1E). The increase in MDA was similar to SMG cells treated with H₂O₂ as a positive control. As MDA is a by-product of membrane peroxidation,^{80,81} the increases suggest that methacrylate-based polymerization causes membrane peroxidation. The increased levels of intracellular ROS (Fig. 1D) also support membrane peroxidation, as it is unlikely that the photoinitiator LAP, which exists as a salt,⁵⁸ or the large PEG macromers could cross the cell membrane to directly generate ROS intracellularly.

A rise in oxidative stress associated with membrane oxidation has also been observed for primary chondrocytes encapsulated within methacrylate-based hydrogels.³⁶ The salivary gland has long been known to exhibit a paradoxically acute sensitivity to radiation therapy.^{47,82,83} The loss of terminally differentiated secretory cells after radiation has been suggested to occur through radical-mediated damage of secretory granules⁸⁴ or the plasma membrane.⁴⁴ A process common to both of these phenomena is the formation of hydroxyl radicals from the irradiation of water, which subsequently abstracts hydrogens from unsaturated fatty acids in the plasma membrane and generates peroxyl radicals that propagate through adjacent fatty acids molecules, resulting in membrane destabilization.⁴⁶ We believe that primary SMG cells are exhibiting a similar sensitivity to ROS here and hypothesize that ROS generated by methacrylate-based chain addition polymerizations damage the SMG cell membrane lipids, leading to increased intracellular oxidative stress and cell death.

In contrast to MIN-6 cells, a pancreatic β -cell line,³⁹ exposure to photoinitiator alone did not affect viability of primary SMG cells. We speculate that this may be due to differences in the method of cell preparation. MIN-6 cells are trypsinized into single cell suspensions, while SMG cells are enzymatically degraded into small clumps that likely include some residual ECM. Pericellular ECM has been shown to decrease intracellular ROS generated by exposure to UV and activation of photoinitiator.³⁶ Thus, it is possible that SMG cells may be protected in the presence of photoinitiator alone by residual pericellular ECM, but the combination of photoinitiator and methacrylate species raises ROS concentrations to levels that adversely affect SMG cells. Polymerization of (meth)acrylated PEG macromers impairs the diffusion of radical-generating species (e.g., photoinitiator) and exponentially increases local concentra-

tions of radicals, a process known as auto-acceleration.^{27,85} Increased radical concentrations attributed to auto-acceleration resulted in increases in ROS levels that detrimentally affected PEG hydrogel-encapsulated chondrocytes,³⁶ similar to methacrylate-based polymerization-associated SMG cell death in the studies presented here.

The link between ROS and cell viability was also tested by treating SMG cells with 10 mM of the antioxidant Trolox (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/tea) for 2 h before exposure to nongelling methacrylate polymerizations. Trolox is an analog of vitamin E, which protects lipid membranes from oxidation and has been shown to reduce ROS-mediated cell death in neurons and cardiomyocytes.^{86,87} Ten micromolar Trolox treatment was sufficient to rescue cell viability as measured by ATP levels (Supplementary Fig. S1B) after exposure to nongelling methacrylate polymerizations, further suggesting that ROS generated from methacrylate polymerization causes decreased SMG cell viability. However, antioxidant treatment can also scavenge radicals that are required for crosslink formation, thus inhibiting hydrogel formation, as shown by decreased compressive moduli of hydrogels formed in the presence of both 1 and 10 mM Trolox (Supplementary Fig. S1C). In addition, antioxidants may introduce potential dose-dependent pleiotropic effects, further complicating their use.⁸⁸ We conclude that ROS produced by methacrylate-based PEG polymerization reactions negatively affect SMG cells by increasing lipid peroxidation and intracellular oxidative stress, which subsequently leads to decreased cell viability.

Exposure to thiol-ene polymerizations preserved SMG cell viability by lowering intracellular ROS levels associated with polymerization

Having established a relationship between chain-addition polymerization, increased ROS, and decreased viability of SMG cells, an alternative polymerization method was examined. Thiol-ene polymerizations are known to result in significantly less overall ROS exposure due to the rapid consumption of generated ROS by free thiol groups.⁷⁹ Furthermore, PEG hydrogels formed using step-growth, thiol-ene polymerizations have excellent cytocompatibility for the encapsulation of ROS-sensitive cell types. For example, MIN-6 cells exhibit high (>90%) viability after encapsulation within thiol-ene hydrogels compared with ~15% viability when encapsulated within chain-polymerized hydrogels.³⁹

Beyond cytocompatibility, it is important to determine and minimize cellular oxidative stress, as *in situ* hydrogel polymerizations could have pleiotropic effects on cell fate and function. ROS have been implicated in a variety of processes such as reducing lifespan of hematopoietic stem cells,⁸⁹ inducing insulin resistance in adipocytes,⁹⁰ and regulating Notch signaling in epidermal cell differentiation.⁹¹ Recent work comparing chain addition and thiol-ene polymerizations for encapsulation of primary chondrocytes shows that increased ROS exposure resulting from chain addition polymerizations leads to hypertrophic chondrocyte phenotypes similar to pathologic chondrocytes observed in osteoarthritis. In contrast, the phenotypes of chondrocytes encapsulated using a thiol-ene polymerization resemble those present in hyaline cartilage.³⁵

To investigate the effects of thiol-ene polymerizations on SMG cell viability, nongelling, thiol-ene polymerizations were performed using four-arm PEG-norbornene and the peptide KGKGKGKCG (Fig. 2A). The single cysteine residue participates in the polymerization but does not act as a crosslinker for hydrogel formation (Fig. 2A). SMG cells exposed to 3 min of thiol-ene polymerization conditions, which is required to reach 100% conversion,²⁹ exhibited significant changes in neither intracellular oxidative stress (Fig. 2B) nor MDA levels (Fig. 2C) compared with untreated cells. This suggests that SMG cells exposed to thiol-ene polymerization conditions do not suffer from lipid peroxidation and subsequent oxidative stress as opposed to SMG cells exposed to methacrylate polymerization.

In contrast to the ~60% decrease in cell viability after methacrylate polymerization, SMG cells exposed to thiol-ene polymerization exhibited no measurable losses in viability (Fig. 2D). In addition, SMG cells exposed separately to components of thiol-ene reactions (e.g., 16 mM of thiol or norbornene [ene]) exhibited no losses in viability (Fig. 2E), further demonstrating the cytocompatibility of thiol-ene hydrogel components. We conclude that the alternative use of thiol-ene polymerizations, which results in minimal ROS, is a favorable strategy for the encapsulation of SMG cells.

Results from the nongelling polymerization studies were verified in gelling polymerizations (Fig. 3A). LIVE/DEAD staining of SMG cells encapsulated within PEG hydrogels formed using methacrylate or thiol-ene polymerization was performed immediately after encapsulation. In addition, SMG cells were encapsulated within collagen hydrogels to provide a comparison to hydrogels that do not rely on radical-based reactions. Under standard suspension culture conditions, control SMG cells had viabilities (% of cells expressing LIVE marker out of total number of cells LIVE + DEAD) of ~66% (Fig. 3B). SMG cells encapsulated in PEGDM exhibited viabilities of ~15% (Fig. 3E). In contrast, SMG cells encapsulated in thiol-ene gels had viabilities of ~57% (Fig. 3C), statistically similar to untreated controls (Fig. 3B).

Somewhat surprisingly, SMG cells encapsulated within thiol-ene hydrogels had slightly higher viabilities than those encapsulated in collagen gels, which exhibited viabilities of ~50% (Fig. 3D). This might be attributed to temperature fluctuations required for collagen hydrogel formation, compounded by 1 h gelation times during which cells are concentrated within a small volume and may experience metabolic stress. Thus, under both nongelling and gelling conditions, the thiol-ene hydrogels provide a suitable environment for SMG cell encapsulation.

Cell-cell interactions are essential for long-term viability of encapsulated SMG cells within thiol-ene polymerized PEG hydrogels

With the identification of a cytocompatible hydrogel polymerization process for SMG cell encapsulation, we next sought to characterize viability over therapeutically relevant time scales. After ductal ligation, SMG gland regeneration occurs within 14 days.⁶ In addition, in previous transplantation studies using MSCs, hydrogels with an *in vivo* degradation time of 2 weeks were employed to improve femoral allograft healing.⁴³ Together, these studies supported our choice of 14 days as a therapeutic timeframe over which to maintain via-

bility *in vitro*. SMG cells were isolated, encapsulated in thiol-ene hydrogels and cell viability was measured using LIVE/DEAD staining (Fig. 4A–D). Results of LIVE/DEAD staining were quantified both as the percentage of cells stained with the LIVE marker out of the total cell (LIVE + DEAD) population and as the percentage of a low power (40×) FOV that is occupied by cells stained with LIVE marker. Measuring the amount of total FOV area occupied by LIVE cells accounts for total changes in cell density of the hydrogel, as only viable cells are analyzed.⁹² Therefore, we measured both the percentage of living cells, which is accurate for short-term culture, and percentage of FOV area to obtain longitudinal measurements of cell viability.

SMG cells encapsulated within thiol-ene hydrogels showed a significant decrease in the percentage of living cells (from ~60% to 35%) by day 4, which was consistent from days 4 to 14 postencapsulation (Fig. 4A, B, E). The % of FOV with living cells also decreased substantially from 10% immediately after encapsulation to 5% at 4 days after encapsulation (Fig. 4F). The decrease in total live cells is due to loss of single cells and smaller cell clusters within the first 4 days after encapsulation (Fig. 4B). By 7 and 14 days after encapsulation, spherical clusters of cells ~50 µm in diameter are the only viable cells observed within gels (Fig. 4C, D). Thus, a substantial proportion of encapsulated SMG cells die during the 14-day culture period, although larger clusters remain viable till day 14 (Fig. 4D).

To counter the loss of single SMG cells after encapsulation, we analyzed cell viability of encapsulated microspheres preformed in suspension culture. After 2–3 days of suspension culture, dissociated SMG cells form microsphere cell clusters (>50 µm in diameter) that include both duct and acinar cells.^{8,10} The viability of microspheres encapsulated within thiol-ene polymerized PEG hydrogels was analyzed using LIVE/DEAD imaging at days 0, 4, 7, and 14 after encapsulation (Fig. 5A–F) and by measuring whole hydrogel cellular ATP levels (Fig. 5I). The heterogeneity in microsphere size is evident with LIVE/DEAD imaging immediately after encapsulation (Fig. 5A). Over the 14-day culture period, there is a significant increase in microsphere size (Fig. 5B–D).

In contrast to the low viability of single cells after encapsulation, the percentage of living cells in microspheres was >70% and comparable to nonencapsulated cells at each time point analyzed (Fig. 5E). Similarly, the FOV area occupied by living cells was ~17% at day 0 and remained statistically equivalent over 14 days (Fig. 5F). Total cellular ATP also increased ~3.5-fold over the 14-day culture period, indicating maintenance of SMG cell viability (Fig. 5I). To confirm the presence of proliferating cells within microspheres, EdU labeling was employed.⁹³ Microspheres at both days 3 (Fig. 5G) and 8 (Fig. 5H) included high numbers of EdU-positive cells, consistent with cell proliferation. Together with the increased measure of cellular ATP (Fig. 5I), this result supports the conclusion that encapsulated microspheres contain viable, proliferative cells.

Due to cellular proliferation, it would be expected that increases in cell density would be observed; however, results measuring the percentage of FOV area containing LIVE cells showed no measurable increases for encapsulated spheres. This could be attributable to hydrolytic degradation and subsequent network swelling,⁹⁴ which would lead to overall decreases in cell density. This phenomenon

FIG. 2. Nongelling step-growth thiol-ene polymerization preserves SMG cell viability and reduces oxidative stress. **(A)** Mono-thiol functionalized peptides (grey dashed lines) participate in radical-mediated polymerization by reacting first with LAP radicals. Resulting thiol radicals then react with four-arm norbornene-functionalized PEG (PEG-NORB, $q = 113$, black crosses) without forming crosslinks. Circles represent cells, illustration is not to scale. **(B)** Measurement of intracellular oxidative stress using CellROX normalized to levels at time 0 and comparing cells exposed to thiol-ene with untreated cells. **(C)** Lipid peroxidation measured via analysis of MDA concentration in lysates of SMG cells exposed to methacrylate and thiol-ene nongelling polymerization conditions. All thiol-ene MDA values were normalized to cells exposed to photoinitiator and macromers but not to UV, while cells treated with 2 mM H₂O₂ were normalized to untreated cells in PBS. **(D)** Measurement of cellular ATP levels in cells exposed to either step-growth thiol-ene (10 mM thiol + 10 mM norbornene) or methacrylate-based chain addition polymerizations (20 mM methacrylate). **(E)** Effects of macromer type on the cellular ATP levels of SMG cells exposed to thiol-ene nongelling polymerization conditions. Error bars represent standard deviation. $N = 8-12$ (**B**), 4 (**C**, **D**), and 6 (**E**). * $p < 0.05$, **** $p < 0.0001$ determined by two-way ANOVA with Sidak *posthoc* analysis, n.s. = not significant.

A i Thiol-ene-based hydrogels:

ii Methacrylate-based hydrogels:

FIG. 3. SMG encapsulation using step-growth thiol-ene photopolymerization maintains high cell viability. **(A)** Schematic representation of gelling PEG hydrogel polymerization, illustrations are not to scale. Thiol-ene macromers **(i)** consisting of four-arm PEG norbornene ($q = 113$, left) and two-arm PEG-dithiol ($p = 74$, right) or methacrylate macromers **(ii)** consisting of PEG-dimethacrylate were dissolved in DPBS with primary SMG cells and polymerized to form the hydrogel network. Representative 100 μm z-stacks at 100 $\times$ magnification (living cells = green, dead cells = red) of control, un-encapsulated SMG cells **(B)**, SMG cells encapsulated in collagen gels **(C)**, SMG cells encapsulated in step-growth thiol-ene gels **(D)**, and SMG cells encapsulated in methacrylate-based chain addition gels **(E)**. Quantification of cell viability is expressed as a percentage of living cells divided by total cell number **(F)**. $N = 3-6$, * $p < 0.05$ compared with untreated control cells, & $p < 0.05$ compared with all samples determined using one-way ANOVA Tukey's HSD *posthoc* analysis. Color images available online at www.liebertpub.com/tea

FIG. 4. Primary SMG cells exhibit poor viability within thiol-ene polymerized PEG hydrogels. Representative 100 μm z-stacks at 100× magnification (living cells = green, dead cells = red) of primary SMG cells at 0 (A), 4 (B), 7 (C), and 14 (D), after encapsulation. (E) Quantification of cell viability from 100× images expressed as a percentage of living cells divided by total cell number. (F) Percentage of 40× field of view that stains positive for living cells. $N=3$, error bars $\pm$ standard deviation, $*p < 0.05$, compared with day 0 determined using one-way ANOVA with Tukey's HSD *posthoc* analysis. Color images available online at www.liebertpub.com/tea

was observed with human MSCs encapsulated within thiol-ene hydrogels despite exhibiting high viability determined via LIVE/DEAD staining.⁹² Furthermore, as the mesh size of most PEG hydrogels is several orders of magnitude smaller than cells,^{27,95,96} the ability of proliferating cell populations to expand throughout the hydrogel is slowed in the absence of cell-based degradability.⁹⁷ Thus, it is probable that measuring cell density by calculating FOV area occupied by LIVE cells does not fully capture actual overall increases in cell number, as suggested by the whole hydrogel ATP data (Fig. 5I) and EdU staining (Fig. 5G, H).

Cell-cell interactions are essential for *in vitro* salivary gland cell culture and *in vivo* gland development. E-cadherin plays an important role in the development of the SMG,^{51–55} in particular to provide survival signals to maturing duct cells.⁵⁵ Recent studies have shown that cells dispersed from salivary gland tissues in mice and humans can self-assemble to acinar-like structures *in vitro* and display characteristics of native gland structures.^{20,56,57} To investigate whether the increased viability of microspheres within PEG hydrogels is due to increased cell-cell interactions or to an enrichment of more proliferative cells, microspheres were treated with trypsin to form single cell suspensions before encapsulation.

LIVE/DEAD staining showed that both encapsulated microspheres (Fig. 6A) and single cells (Fig. 6C) had

comparable viability at day 0, indicating that trypsin treatment did not cause initial cell death. However, after 7 days, the viability of encapsulated single cells (Fig. 6D) decreased from ~87% to ~10% (Fig. 6B) while microsphere viability remained at ~80% through day 7 (Fig. 6E). In concert, cellular ATP levels declined in single cells by 70% from day 0 to 7, but increased ~250% in microspheres (Fig. 6F). These data support the idea that cell-cell interactions are critical for viability of primary SMG cells, and are in agreement with a previous report showing that cell viability is lost when microspheres are dissociated.¹⁰ Preserving cell-cell interactions has been found to promote the viability of other cell types such as pancreatic β -cells and motor neurons when encapsulated within PEG hydrogels,^{38,41} supporting the rationale for the assembly of these multicellular “microtissues” before encapsulation.

Encapsulated microspheres display markers of acinar cell lineage

The phenotype of cells within encapsulated microspheres was characterized by immunohistochemistry. Microspheres from suspension culture included cells that expressed both acinar (Supplementary Fig. S2A) and duct cell (Supplementary Fig. S2B) markers, in agreement with previous

FIG. 5. SMG microspheres show high and sustained viability within thiol-ene polymerized PEG hydrogels. Representative 100 μm z-stacks at 100 $\times$ magnification (living cells = green, dead cells = red) of primary SMG cells at 0 (A), 4 (B), 7 (C), and 14 days (D), after encapsulation. (E) Quantification of cell viability from 100 $\times$ images expressed as a percentage of living cells divided by total cell number. (F) Percentage of 40 $\times$ field of view that stains positive for living cells. EdU labeling (Blue = 4',6-diamidino-2-phenylindole, Red = EdU) of microspheres at days 3 (G) and 8 after encapsulation (H) to detect cellular proliferation. (I) Quantification of cellular ATP levels at 0, 4, 7, and 14 days after encapsulation. $N=3$, error bars $\pm$ std deviation, * $p < 0.05$, compared with day 0 determined using one-way ANOVA with Tukey's HSD *posthoc* analysis. Color images available online at www.liebertpub.com/tea

reports.^{8,10} To directly track the fate of acinar cells within the hydrogels, a CreLoxP-ROSA26 lineage tracing system was used. In this system, an inducible Cre recombinase has been introduced into the *Mist1* gene locus, which encodes a transcription factor specifically expressed in the secretory acinar cells.⁹⁸ This mouse strain was crossed with the R26 tdTomato reporter strain. Administration of tamoxifen activates the Cre recombinase, which mediates recombination, leading to expression of the tdTomato reporter specifically in acinar cells (Supplementary Fig. S3). Other cell lineages, which can also be labeled with calcein AM, are not tdTomato positive (Supplementary Fig. S2E, F).

This lineage tracing system was utilized to study the cellular composition of encapsulated microspheres. Directly after encapsulation, a majority of cells were positive for tdTomato, indicating that the encapsulated microspheres comprised acinar cells (Fig. 7A). This is supported by immunofluorescence imaging of microspheres before encapsulation, which show high expression of the serous acinar cell marker, *Nkcc1* (Supplementary Fig. S2A). In the encapsulated spheres, the percentage of acinar cells decreased from $\sim 80\%$ to $\sim 45\%$ of total cells by day 4 (Figs. 7B and 7I). After 7 and 14 days postencapsulation, the percentage of acinar cells remains stable at 40% (Fig. 7C, D, I),

FIG. 6. Disruption of SMG cell–cell contacts results in cell death. Representative 100 μm z-stacks at 100 $\times$ magnification (living cells = green, dead cells = red) of SMG microspheres (A, B) or single cells after trypsinization (C, D), immediately and 7 days after encapsulation. (E) Quantification of cell viability from 100 $\times$ images expressed as a percentage of living cells divided by total cell number. (F) Quantification of cellular ATP levels at 0, 4, 7, and 14 days after encapsulation. $N=3$ (E) 4 (F), error bars $\pm$ standard deviation, $*p<0.05$, $****p<0.0001$ compared to day 0 by one-way ANOVA with Tukey's HSD *posthoc* analysis (F) or two-way ANOVA with Sidak *posthoc* analysis (E). Color images available online at www.liebertpub.com/tea

demonstrating that viable acinar cells constitute a significant proportion of cells within encapsulated microspheres.

These data suggest either a decrease in the number of acinar cells or an expansion of nonacinar-derived populations or a combination thereof. To assess alternative lineages that may be present, Keratin5 (K5) lineage tracing was employed. K5-expressing cells, found primarily in the myoepithelium and the ducts, have been shown to have progenitor-like properties during development⁹⁹ and to participate in gland healing after duct ligation.¹⁰⁰ Mice carrying Cre at the K5 gene locus and the R26 tdTomato reporter were injected with tamoxifen before gland harvest. On encapsulation of SMG microspheres, only $\sim 4\%$ of cells are K5 positive (Fig. 7E). However, the percentage of K5-lineage-labeled cells increased to $\sim 38\%$ of the total cellular population by 4 days after encapsulation (Fig. 7F) and re-

mained between 30% and 40% of total cells at 7 and 14 days postencapsulation (Fig. 7G, H, I). These data suggest that K5-lineage cells expand significantly within hydrogel-encapsulated SMG microspheres.

Cre-based lineage tracing in microspheres entrapped within PEG hydrogels offers a way to relate the *in vivo* role of cells to that observed in suspension cultures *in vitro*. By analyzing Mist1 and K5-positive cells, we were able to describe 70–80% of the total cellular content of microspheres over a 14 day culture period and capture changes in the encapsulated cell populations. Previous studies^{8,10} have characterized microspheres based on cell morphology and expression of marker proteins, but neither study examined from which lineage the cells originate. As previously mentioned, K5 cells have been shown to have progenitor-like qualities *in vivo*,^{99,100} thus partially explaining the

FIG. 7. Encapsulated SMG microspheres express markers of both acinar and duct cell lineages. One hundred micrometer z-stacks at 100 $\times$ magnification of Mist1-Cre/tdTomato microspheres co-labeled with Calcein AM (green=living, red=lineage of Mist1-expressing cells) at 0 (A), 4 (B), 7(C), and 14 (D) days after encapsulation. One hundred micrometer z-stacks at 100 $\times$ magnification of K5-Cre/tdTomato microspheres co-labeled with Calcein AM (green=living, red=lineage of K5-expressing cells) at 0 (E), 4 (F), 7 (G), and 14 (H) days postencapsulation. (I) Quantification of Mist1-lineage cells and K5-lineage cells. $N=3$, error bars $\pm$ standard deviation, * & $p < 0.05$ compared with day 0 for Mist1 and K5, respectively. Color images available online at www.liebertpub.com/tea

substantial increase in K5-lineage-labeled cells observed over the 14 days postencapsulation.

The decrease in the number of Mist1-lineage labeled cells is supported by absence of cells with acinar-like morphology observed 3 days after sphere culture by others.¹⁰ In contrast, however, our data show that 30–40% of the total encapsulated cell population comprises Mist1-lineage-labeled cells and remains stable from 7 to 14 days after encapsulation, suggesting that acinar-derived cells form a stable population *in vitro*. Taken together, these results demonstrate that encapsulated microspheres are composed of both acinar and duct cells. The expansion of the K5-duct cell population supports their role as a progenitor cell population, while the maintenance of the Mist1-acinar cell

population shows that acinar cells are involved in the formation of microspheres, which has not been observed in previous studies.^{8,10} Moreover, this approach provides a direct method for characterizing the cellular composition of SMG microspheres *in vitro*.

We aim at developing a cell transplantation system for SMG cells that directly contributes to gland regeneration by supporting proliferation and differentiation of transplanted cells. As *in vitro* culture of salivary gland cells under adherent conditions leads to loss of secretory cell characteristics and reduced proliferation,^{101–103} we have adopted the use of microspheres, which have been shown to contribute to gland function after transplantation.^{10,13} It is generally acknowledged that the maintenance and expansion of

functional salivary gland cells requires a biomaterial scaffold.^{21,24,104,105} Here, we have examined the feasibility of using PEG hydrogels. We have established permissive encapsulation procedures for multicellular SMG microspheres using thiol-ene polymerization.

In contrast to threedimensional (3D) studies performed with fibrin²² and hyaluronic acid,^{20,23,25} PEG is a bio-inert material²⁶ that can be modified to provide specific biological cues in a novel “bottom up” approach to tissue engineering. Numerous ECM proteins that are important to salivary gland development and differentiation have been identified,¹⁰⁶ and some of these such as laminin-111²¹ and perlecan²³ have been incorporated into biomaterials. The inclusion of such matrix cues in PEG hydrogels will enable investigation of the specific effects of these modifications on encapsulated microsphere proliferation, cell composition and differentiation, and secretion of salivary factors. Previous studies used either immortalized cell lines or cells expanded from host tissues *in vitro*. These two sources of cells have translational limitations, as cell lines are unlikely to achieve regulatory approval for therapeutic applications, and mature salivary acinar cells have reduced proliferative capacity *in vitro*.¹⁰² Primary cells cultured *in vitro* also rapidly lose both polarity and secretory functions.¹⁰¹

As previously mentioned, microsphere preparation maintains the proliferative and differentiation capacity of salivary gland progenitor cells *in vitro* and has shown the ability to induce regeneration with *in vivo* models of radiation-induced salivary gland damage.^{8,10} One of the challenges of using cultured SMG microspheres is that the increasing microsphere size causes sedimentation and adherence to nontreated tissue culture plastic. PEG hydrogels were used to culture SMG cells till 14 days, providing a practical scaffold for the expansion and study of the fundamental characteristics of SMG microspheres over longer times than in typical suspension cultures.

In addition to providing bottom-up approaches to *in vitro* 3D cultures, PEG hydrogels can be photopolymerized *in situ* to transplant SMG cells to the gland. Previous work using a PEG hydrogel periosteum mimetic to deliver MSCs significantly improved bone healing in a segmental defect model.^{31,42} Critical to this approach was the localization and temporal release of MSCs provided by the PEG hydrogel,⁴³ as MSCs seeded directly on an allograft have little effect on healing.^{107–109} In previous studies, transplantation of primary SMG cells into irradiated glands was performed using intraglandular ~5–10 μ L injections.^{10,12,13} We speculate that *in situ* polymerization of a hydrogel to surround damaged salivary glands may provide a direct means of delivering SMG cells for regeneration.

Conclusions

Methacrylate-based polymerizations that are commonly employed to form PEG hydrogels increase intracellular ROS and plasma membrane oxidation of SMG cells, resulting in significant losses in cell viability. Thiol-ene polymerizations represent a more favorable means for reproducible, cyto-compatible hydrogel encapsulation of SMG cells. Similarly, the formation of multicellular microspheres is essential for the long-term viability of encapsulated SMG cells, which proliferate and comprise both acinar and duct cell lineages.

Future studies will be focused on the use of thiol-ene hydrogels to transplant and direct SMG cell functions to regenerate salivary gland tissues.

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Disclosure Statement

No competing financial interests exist.

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