Dental Pulp Cell Behavior in Biomimetic Environments

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Abstract

There is emerging recognition of the importance of a physiologically relevant in vitro cell culture environment to promote maintenance of stem cells for tissue engineering and regenerative medicine purposes. In vivo, appropriate cellular cues are provided by local tissue extracellular matrix (ECM), and these are not currently recapitulated well in vitro using traditional cultureware. We therefore hypothesized that better replication of the in vivo environment for cell culture and differentiation could be achieved by culturing dental pulp cells with their associated ECM. Primary dental pulp cells were subsequently seeded onto pulp-derived ECM-coated cultureware. While at up to 24 h they exhibited the same level of adherence as those cells seeded on tissue culture–treated surfaces, by 4 d cell numbers and proliferation rates were significantly decreased in cells grown on pulp ECM compared with controls. Analysis of stem cell and differentiation marker transcripts, as well as Oct 3/4 protein distribution, supported the hypothesis that cells cultured on ECM better maintained a stem cell phenotype compared with those cultured on standard tissue culture–treated surfaces. Subsequent differentiation analysis of cells cultured on ECM demonstrated that they exhibited enhanced mineralization, as determined by alizarin red staining and mineralized marker expression. Supplementation of a 3% alginate hydrogel with pulp ECM components and dental pulp cells followed by differentiation induction in mineralization medium resulted in a time-dependent mineral deposition at the periphery of the construct, as demonstrated histologically and using micro–computed tomography analysis, which was reminiscent of tooth structure. In conclusion, data indicate that culture of pulp cells in the presence of ECM better replicates the in vivo environment, maintaining a stem cell phenotype suitable for downstream tissue engineering applications.

Keywords: dentin, extracellular matrix, hydrogel, growth factors, doping, stem cells

Introduction

Different populations of mesenchymal stem cells (MSCs) are described within the pulp, including dental pulp stem cell (Gronthos et al. 2000), stem cells from human exfoliated deciduous teeth (Miura et al. 2003), and stem cells from the apical papilla (Sonoyama et al. 2006). The environment within the MSC niche is critical for regulating cell homeostasis, proliferation, and differentiation (Burness and Sipkins 2010), and the extracellular matrix (ECM) of the pulp provides both biochemical and biomechanical cues. The dental pulp ECM (pECM) is of a relatively gelatinous consistency and contains significant amounts of 1) collagen (types I and III), proteoglycans, and glycosaminoglycans (Tsuzaki et al. 1990); 2) noncollagenous proteins, including fibronectin, tenascin, osteonectin, and osteopontin; and 3) many members of growth factor families. Indeed, the regulation of dental tissue regeneration also involves signaling derived from its ECM, with members of the TGFβ superfamily being directly implicated in stimulating dentinogenic repair (Smith, Scheven, et al. 2012).

Several approaches have been utilized to isolate postnatal MSCs from dental and other tissues, with the simplest utilizing standard cultureware adherence (Friedenstein et al. 1976). Heterogeneous populations of cells are subsequently isolated with MSC-like properties, including clonogenicity and high proliferative capacity (Gronthos et al. 2000; Miura et al. 2003).

Fluorescence-activated cell sorting and magnetic activated cell sorting are also routinely used for MSC isolation (Zannettino et al. 2007) with positive selection for STRO-1, CD105, c-kit, CD34, and low-affinity nerve growth factor receptor and negative selection for CD31 and CD146 being used to isolate pulp MSCs (Zhang et al. 2006; Yang et al. 2007; Nakashima et al. 2009). Recent reports, however, demonstrate that MSCs also exhibit selective adhesion to surfaces coated with ECM-derived molecules; subsequently, cell adhesion to fibronectin-coated cultureware has been proposed for pulp MSC enrichment (Waddington et al. 2009).

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Dental tissue engineering strategies require biomimetic scaffolds, morphogens, and progenitor cell populations to generate whole tooth or tooth component structures. Studies in vivo have demonstrated tooth tissue formation by seeding dental pulp stem cells and stem cells from human exfoliated deciduous teeth on scaffolds that exhibit similar properties to native pulp tissue, such as collagen and poly-L-lactic acid (Sumita et al. 2006; Cordeiro et al. 2008). Doping of these structures with dental ECM-derived morphogens has also been shown to promote differentiation and mineral deposition of encapsulated MSCs. Notably, however, many of these approaches rely on implantation in animal models to enable engineering dental tissue structures (Zhang et al. 2006; Zheng et al. 2011; Chen et al. 2015).

We subsequently hypothesized that dental ECM-coated cultureware may better recapitulate the in vivo environment and aid selection and maintenance of pulp MSCs from heterogeneous primary pulp cell populations. Furthermore, we hypothesized that dental ECM supplementation of a hydrogel scaffold may provide a more physiologically relevant environment for 3-dimensional dental tissue engineering. Finally, the generation of robust in vitro models may reduce the requirement for in vivo experimentation and the associated costs and constraints.

Materials and Methods

Isolation of Dental Tissue and Cells

Bovine mandibular incisor teeth were from male Holstein Friesian calves <2 mo old (Bates' Wholesale Butchers, Birmingham, UK). Maxillary and mandibular incisors were from 100- to 120-g Wistar Hannover rats (Charles River Laboratories, Welwyn Garden City, UK). Teeth were dissected from jaws, and pulp was extirpated for ECM extraction and cell isolation.

Extraction and Characterization of pECM

Bovine incisor pulps were mechanically dissected (0.5- to 1-mm^3 pieces) and combined with 1 mL of ice-cold 0.5M NaCl (Sigma-Aldrich, Dorset, UK) extraction solution (pH 11.7) containing protease inhibitors, 25mM EDTA, 1mM phenylmethylsulphonyl fluoride, and 5mM *N*-ethylmaleimide (Sigma-Aldrich) and 1.5mM sodium azide (VWR International, Lutterworth, UK). Tissue suspensions were homogenized on ice (Ultra-Turrax T8 Homogenizer; IKA Labortechnik, Staufen, Germany) and agitated for 24 h at 4 °C. Centrifuged supernatants were removed and pellets resuspended in 1 mL of 0.5M NaCl (Sigma-Aldrich). Resuspending, homogenization, agitation, and pelleting were repeated in triplicate and supernatants pooled. Pulpal tissue was resuspended in 1 mL of cold 0.1M tartaric acid solution (pH 2.0; Hopkin & Williams Ltd, Birmingham, UK) and subjected to the same homogenization, stirring, and centrifugation protocol as described above (Bellon et al. 1988). NaCl- and tartaric acid–soluble pECM extracts were dialyzed (19-mm tubing) against dH$_2$O for 2 wk at 4 °C with daily water changes prior to lyophilization.

Characterization of pECM Components

Lyophilized pECM (0.5 mg) in lithium dodecyl sulphate buffer was denatured (105 °C) with NuPAGE reducing agent. Samples were electrophoresed on NuPAGE 10% Bis-Tris gels and stained with the SilverXpress Silver kit (Life Technologies, East Lothian, UK).

Noncollagenous proteins were assayed with Coomassie Brilliant Blue G-250 (Sigma-Aldrich) with absorbance determined at 595 nm via a UV/VIS Spectrometer (Philips, Colchester, UK) with a bovine serum albumin standard (Bradford 1976). The dimethyl methylene blue assay was used to quantify glycosaminoglycans (Farndale et al. 1986). The glycosaminoglycan chain, chondroitin-4-sulphate (Sigma-Aldrich), was used as standard with absorbance at 525 nm. Collagen was assayed with Sirius Red (VWR International) in saturated Picric acid (Sigma-Aldrich; Tullberg-Reinert and Jundt 1999). Absorbance values were determined at 490 to 570 nm through an ELX800 Universal Microplate reader (BioTex Instruments, Houston, TX, USA) with rat tail collagen type I standard (Sigma-Aldrich).

Primary Cell Culture

Culture was in α-MEM (Biosera, Nuaille, France), containing 2mM L-glutamine supplemented with 1% penicillin/streptomycin (all Sigma-Aldrich) and 10% fetal calf serum (Biosera). Primary pulp cells were isolated from rodent tissue by enzymatic disaggregation (Patel et al. 2009). Cells (2×10^4) were seeded on culture surfaces (Sarstedt, Leicester, UK) and viable cells counted with the trypan blue exclusion assay (Sigma-Aldrich).

One milliliter of pECM (1 mg/mL) dissolved in phosphate-buffered saline (PBS) was used to coat 35-mm^2 culture dishes (Sarstedt) by incubation at 4 °C for 24 h. Surfaces were washed in triplicate with PBS to remove unbound protein. Coating of culture surfaces were assessed with Coomassie Brilliant Blue G-250 (Bradford 1976). To induce mineralizing lineage differentiation, culture medium was supplemented with 10^{-7}M dexamethasone, 10mM β-glycerophosphate, and 50 μg/mL of ascorbic acid (all Sigma-Aldrich; Gronthos et al. 2000).

Scanning Electron Microscopy

For fixation, surfaces were treated with 2.5% glutaraldehyde (Agar Scientific, Stansted, UK) in 0.1M sodium cacodylate buffer (Fisher Scientific, Loughborough, UK) for 30 min, followed by dehydration by sequential 10-min treatments in increasing concentrations (v/v) of ethanol followed by exposure to hexamethyl-disilazane (Sigma-Aldrich). Culture surfaces were attached to aluminium scanning electron microscopy (SEM) stubs with Acheson electrodag (Agar Scientific) and sputter-coated with gold under vacuum (Emitech K550X). SEM images were obtained through an accelerating voltage of 10 kV via a JSM-840A SEM (Joel, Welwyn Garden City, UK).

High-Content Cell Analysis of Oct 3/4

High-content cell analysis was performed at Imagen Biotech (Manchester, UK). Pulp cells were fixed (30 min) with 10% paraformaldehyde (VWR International). Cells were incubated with Oct 3/4 primary antibody (Abcam, Milton, UK) diluted 1:100 in 0.1M phosphate buffer (pH 7.8) with 0.1% bovine serum albumin for 1 h at room temperature. Cells were washed (3×) and incubated with goat anti-rabbit IgG secondary antibody conjugated to an Alexa-Fluor 488 fluorescent label (VWR International) for 1 h. ArrayScan high-content screening imaging cytometer and ArrayScan II Data Acquisition and Data Viewer 3.0 software (Fisher Scientific) were used for analysis.

Alizarin Red Staining

Cultures were fixed in 10% paraformaldehyde (VWR International) for 30 min and washed with PBS, with alizarin red solution added (VWR International; Gregory et al. 2004). Excess stain was removed with PBS and cultures de-stained in 10mM acetic acid. Stain was quantified at 405 nm with an ELX800 Universal Microplate Reader (BioTex) and compared with 40mM alizarin red stock solution.

Bromodeoxyuridine Proliferation Assay

The 5-bromo-2-deoxy-uridine Labeling and Detection Kit II (Roche Life Sciences, Burgess Hill, UK) was used. Medium was removed from cultures and replaced with 500 µL of bromodeoxyuridine (BrdU) labeling medium. After 60 min of incubation, BrdU labeling medium was removed and dishes washed in triplicate with PBS. Cells were fixed (30% absolute ethanol / 70% 50mM glycine) for 20 min at room temperature. Fluid was removed and samples incubated at −20 °C for 30 min. After PBS washing, 700 µL of anti-BrdU (kit reagent) was added to each dish for 30 min at 37 °C, and surfaces were PBS washed. Alkaline phosphatase conjugate (700 µL) was then added for 30 min at 37 °C. Dishes were washed with PBS before 700 µL of color substrate was added for 30 min at room temperature. Proliferating cells incorporating BrdU into DNA were detected by positive antibody staining under a Nikon Eclipse TE300 microscope (Nikon, Kingston, UK). A graticule was used to determine percentage of proliferating cells.
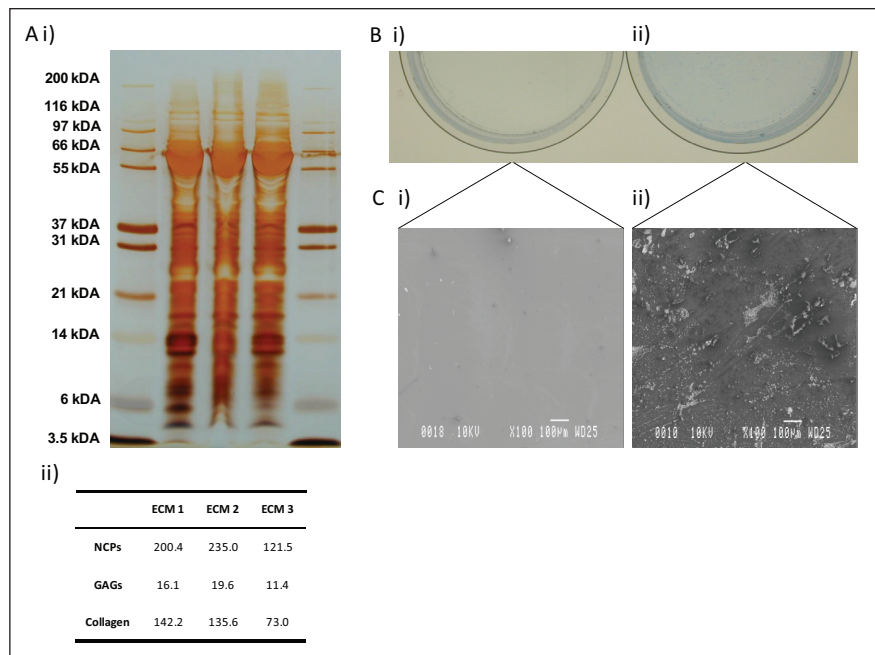
	ECM 1	ECM 2	ECM 3
NCPs	200.4	235.0	121.5
GAGs	16.1	19.6	11.4
Collagen	142.2	135.6	73.0

Figure 1. Characterization of pulp extracellular matrix extracts and coating of cultureware. (**A**) Image of (i) 1D-PAGE analysis of the 3 replicate extracts of pulp extracellular matrix (pECM): 0.125 mg of pECM was loaded in a NuPAGE 10% Bis-Tris gel and visualized with SilverXpress Silver Staining Kit; 10 µL of Mark12 molecular weight ladder was loaded as a weight reference. Protein extracts cover a range of sizes. (ii) Colorimetric analysis of pECM extracts showing quantification (µg/mg of extracellular matrix [ECM]) of noncollagenous proteins (NCPs), glycosaminoglycans (GAGs), and soluble collagen content in the 3 separate pECM extractions. (**B**) Coomassie blue staining of (i) uncoated control and (ii) pECM-treated cultureware surfaces. Positive staining of coated dishes shows protein. (**C**) Secondary electron photomicrographs from scanning electron microscope of (i) untreated culture polystyrene surfaces and (ii) ECM-treated cultureware at ×100 magnification. Scale bars are shown. Images demonstrate a change in the surface appearance following ECM coating, indicating protein adsorption to treated surfaces.

Semiquantitative Reverse Transcription Polymerase Chain Reaction Analysis

RNA was isolated via the QIAGEN RNeasy Minikit (Qiagen, Manchester, UK). Cells were lysed in RLT buffer, and 70% (v/v) ethanol was added, vortexed, and added to an RNeasy mini-column. Bound RNA was washed with ethanol kit buffer prior to centrifugal drying. DNase-treated RNA was collected in molecular-grade water.

The QIAGEN Omniscript RT Kit (Qiagen) was used to reverse transcribe 1.5 to 2 µg of RNA using oligo-dT primer (Life Technologies, Paisley, UK), Omniscript reverse transcriptase and buffer, deoxynucleoside triphosphates, with RNase inhibitor (Promega, Southampton, UK) at 37 °C for 1 h. Synthesized cDNA was cleaned on Microcon YM-30 spin baskets (Millipore, Livingston, UK). RNA and cDNA concentrations were determined with a Biophotometer (Eppendorf, Stevenage, UK).

Polymerase chain reactions contained 50 to 100 ng of cDNA, 12.5 µL of 2× REDTaq Ready Mix (Sigma-Aldrich), 1 µL of 1µM forward primer (VWR International), 1 µL of 1µM reverse primer (Invitrogen, UK), and 12.5 µL of water
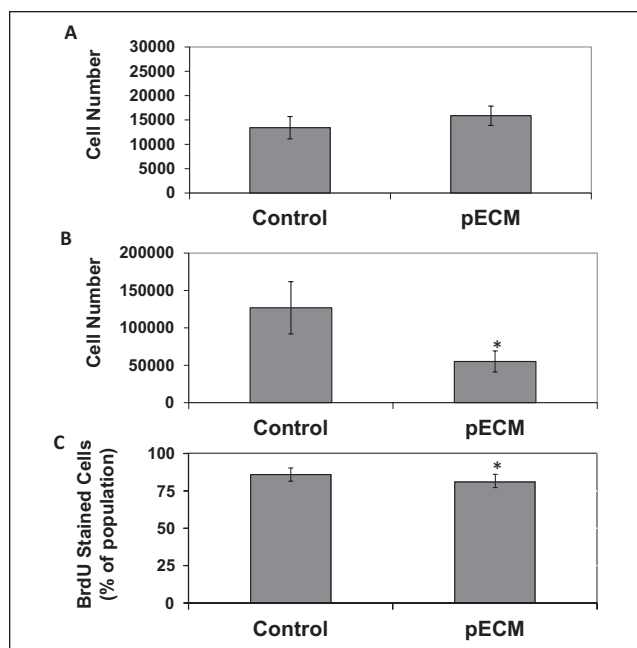
Figure 2. Data showing the number of adherent primary dental pulp cells (**A**) at 24-h and (**B**) at 4-d culture. Cells were seeded on control and pulp extracellular matrix (pECM)–coated plasticware. No statistical differences were found in cell adhesion numbers after 24 h. (**C**) Bromodeoxyuridine (BrdU) proliferation assay on primary dental pulp cell cultures. Cells were counted in 10 fields of view in replicate plates representing >50% of the total stained area per culture. Data indicated a significant decrease in the percentage of cells showing active proliferation in the pulp cell population cultured on pECM-coated versus noncoated cultureware. Standard deviation bars plotted. *$P < 0.05$.

(VWR International). Primer and assay details are in the Appendix Table. A GeneAmp 2700 Thermocycler (Applied Biosystems, Manchester, UK) was used with initial denaturation of 94 °C for 5 min, 1-min cycles of 94 °C, denaturation for 20 s, annealing at 60/60.5 °C for 20 s, extension at 68 °C for 20 s, and a final extension at 72 °C for 10 min.

Amplified products were analyzed on 1.5% (w/v) agarose gels (Web Scientific, Crewe, UK) with images analyzed with GeneTools software (Syngene, Cambridge, UK). Target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase.

Cell Encapsulation and Culture in Alginate Gels

Low-viscosity sodium alginate (Sigma-Aldrich) was prepared at 1%, 3%, and 5% w/v in PBS/α-MEM (1:1) and autoclaved at 121 °C. Pulp cells were dispersed by pipetting throughout the alginate at 5×10^5 cells/mL ± pECM supplementation. Constructs were added dropwise into culture dishes (Sarstedt) containing 100mM $CaCl_2$ and incubated at 37 °C for 1 h to form cross-linked spheres (Hunt et al. 2009). Spheres were washed (×3) in α-MEM and resuspended in control or lineage-inductive media. Cells were released from alginate using 100mM trisodium citrate (Sigma-Aldrich).

Histologic Analysis of Hydrogel Constructs

Encapsulated cells were fixed within alginate by submersion in 10% v/v paraformaldehyde (VWR International) for 30 min. Fixed gels were progressively dehydrated in increasing concentrations of alcohol for 15 min each. Gels were submerged in 2 changes of xylene (VWR International) for 15 min. Gels were embedded in paraffin wax (Sakura, Thatcham, UK) and cooled to 5 °C, and 5-μm sections were stained with hematoxylin and eosin (Surgipath Europe Ltd, Peterborough, UK).

Micro–computed tomography

Alginate constructs were scanned at 80 kV, 100 μA, at an isotropic resolution of 4 μm with camera exposure of 200 ms, a rotation step of 0.3°, frame averaging of 4, and omission of an X-ray filter, using a Skyscan 1172 MicroCT system (Bruker, Coventry, UK). Images were reconstructed with NRecon 1.6.2 software (Bruker). For thresholding, a hydroxyapatite mineral phantom was used composed of tetracalcium phosphate / dicalcium phosphate anhydrous powder and α-tricalcium phosphate powder (Hofmann et al. 2007).

Statistical Analysis

Paired Student's *t* tests and 1-way analysis of variance with $P < 0.05$ and a Tukey post hoc test were used to determine statistical significance as compared with controls.

Results

Pulp Cell Cultures on pECM

Triplicate pECM isolates demonstrated consistent protein content profiles and yields. Ratios of glycosaminoglycan, noncollagenous protein, and collagen yields for the pECM isolates were also consistent between extractions. Coating of cultureware was confirmed by Coomassie blue staining and SEM analysis (Fig. 1). This cultureware-coating approach enabled comparison with standard culturing approaches. Viable cells at 24 h postseeding on uncoated control and pECM-coated cultureware demonstrated no significant differences. Day 5 cell numbers were significantly lower on pECM-coated cultureware compared with control. Consistent with this, BrdU data indicated that cells cultured on pECM-coated cultureware exhibited significantly decreased proliferation compared with controls (Fig. 2).

Gene expression analysis for mesenchymal and pluripotent stem cell markers indicated that in general markers of stem cell phenotype were more abundantly expressed in cultures maintained on pECM compared with controls. Expression of the odontogenic cell fate markers DSPP and DMP were relatively more abundant in pulp cells cultured on uncoated cultureware compared with cells grown on pECM (Fig. 3). Nuclear versus cytoplasmic localization data for the pluripotent transcription factor Oct 3/4 was consistent with gene expression analysis
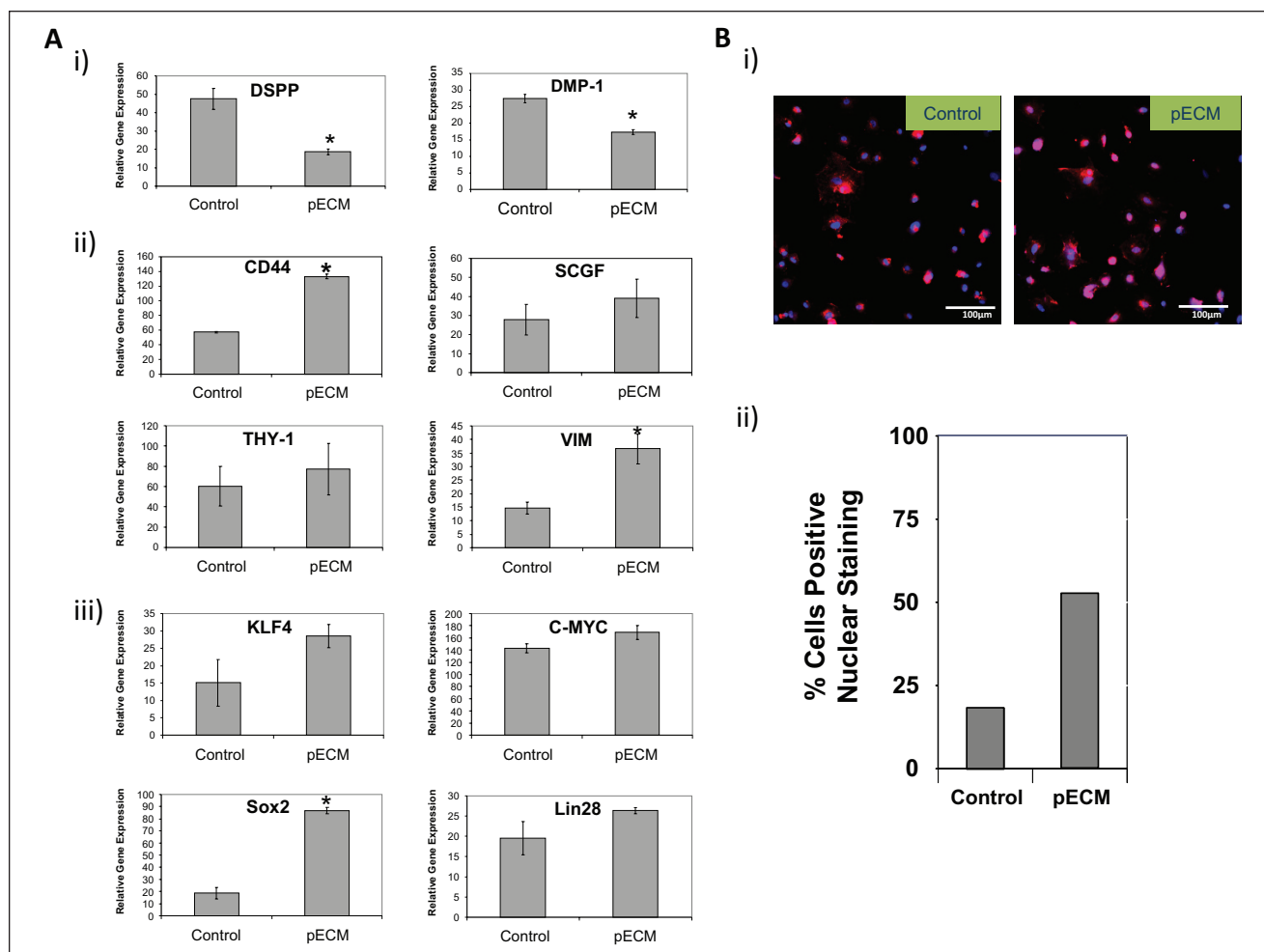
Figure 3. Gene and protein expression analysis for dentinogenic and stem cell markers. (**A**) Gene expression analysis for (i) dentinogenic markers, (ii) mesenchymal / general stem cell markers, and (iii) pluripotent stem cell markers. Densitometric intensities were normalized against GAPDH and plotted as relative gene expression. Data indicated statistically significant lower levels of the dentinogenic markers DMP and DSPP when cultured on pulp extracellular matrix (pECM) as compared with uncoated cultureware in pulp cell populations. The expression of mesenchymal/general and pluripotent stem cell markers was generally increased in cultures on pECM versus uncoated cultureware. (**B**) ArrayScan high-content screening imaging cytometer (Fisher Scientific, UK) analysis of primary pulp cells stained for Oct 3/4 after 4-d culture on uncoated control and pECM-coated cultureware. *$P < 0.05$, $n = 3$. (i) Representative images for Oct 3/4–stained images. $n = 10$. (ii) Percentage of cells in the population that stained positive for Oct 3/4. Semiquantitative data show increased percentage levels of the population staining positive for Oct 3/4 after culture on pECM compared with control uncoated cultureware surfaces. This work was undertaken at Imagen Biotech, Manchester, UK (http://www.imagen-biotech. com/).

regarding molecular pluripotent cell phenotype. Cells cultured on pECM demonstrated increased levels (~50%) of nuclear Oct 3/4 compared with controls (~20%), supporting the notion that cell-ECM interactions maintain an undifferentiated phenotype (Fig. 3).

Differentiation Analysis of Pulp Cells on ECM

Two-week cell culture on pECM surfaces in the presence of mineralization supplements resulted in an enhanced mineralizing phenotype (Fig. 4). Cell count data indicated that differences in quantitative staining were not due to variations in cell numbers. Similar profiles were observed at 3 wk (data not shown). Gene expression analyses using markers of dental and mineralizing cell differentiation indicated differential gene

expression between control and differentiation conditions (Fig. 4B).

Pulp Cells Cultured in Hydrogels Containing pECM

Studies of pulp cells encapsulated in 1%, 3%, and 5% hydrogels with or without pECM indicated that at 2 wk, while cell numbers did not increase, there was no significant loss of viability (Appendix Fig.). Subsequently, pulp cells were encapsulated in 3% hydrogels containing pECM components that had previously demonstrated influence on MSC behavior and were exposed to mineralization medium for up to 5 wk while controls were cultured in unsupplemented medium. Inspection of 3-dimensional cultures indicated that mineralization medium–exposed cultures appeared visually opaque, and micro–computed tomography
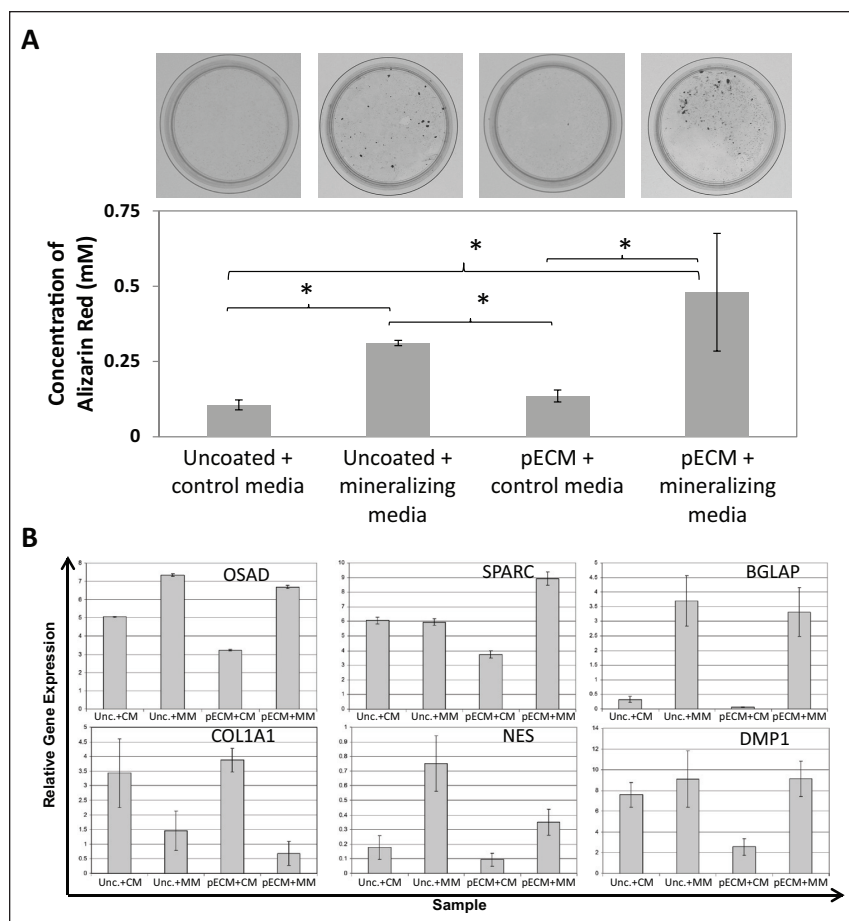
We therefore hypothesized that culture of pulp cells with pECM may better recreate the niche environment. While data potentially supported this hypothesis, the enhanced stem cell phenotype may also be due to pECM attracting a specific population of progenitor cells. Alternatively, the results obtained may reflect the pECM maintaining the dental pulp cells in a more quiescent and undifferentiated state, which may relate to the lower cell-cycling rates that decrease cell densities and associated cell-cell interactions. Interestingly, we have also found that bone marrow MSCs exhibit similar adherent and growth profiles on pECM (unpublished data). These data could indicate that the effect exerted by ECM may not be cell type specific and that common regulatory signaling pathways exist among different MSC types.

To characterize pECM extracts, we have performed proteomic mass spectrometry and identified >90 proteins present in these preparations (data not shown). Several molecules previously utilized for culture surface coating for MSC selection, including fibronectin and collagen type I (He et al. 2014; Waddington et al. 2009), were identified as being present. While these molecules may contribute to the cellular affects that we observed, it is likely that more complex ECM interactions occur that generate a more relevant and comprehensive environment for regulating MSC behavior. Our culture approach may therefore provide a MSC biomimetic environment. Previously, we found that when pECM was subjected to enzymatic degradation, modulation of cell proliferation was abolished (J.G. Smith et al. 2012), indicating that its integrity is important in regulating cell behavior. Conceivably, this may reflect the role of the ECM in vivo during healing whereby enzymatic activities of bacterial and host proteases during disease (Cooper et al. 2014) may lead to the release of MSCs from their niche, enabling their proliferation and differentiation (Schedin et al. 2000). This notion is also supported by previous studies demonstrating that during standard culture expansion, MSC phenotype is lost (Patel et al. 2009). Surface properties such as chemistry, topography, and elastic modulus elicit biomechanical forces on cells, and substrates that exert these effects have been shown to be important in regulating cellular events (Fu et al. 2010; Trappmann et al. 2012; Celiz et al. 2014). Indeed, future experiments that isolate physical effects on cells from biological effects could be performed by coating culture plasticware with inert materials that solely change cultureware surface topography in a similar manner to that observed due to

Figure 4. Analysis of pulp cell behavior at 2 wk under different culture conditions. (**A**) Representative images of alizarin red–stained cultures and following quantification are provided. Parentheses with asterisks indicate statistically significant differences between groups. $n = 4$. (**B**) Semiquantitative gene expression analysis for mineralization-associated transcripts. Levels were normalized to the GAPDH housekeeping gene and densitometric values plotted. Cell cultures analyzed: uncoated cultureware with control medium (Unc.+CM), uncoated cultureware with mineralization medium (Unc.+MM), pulp extracellular matrix (pECM)–coated cultureware with control medium (pECM+CM), and pECM-coated cultureware with mineralization medium (pECM+MM). Standard deviation bars plotted. *$P < 0.05$ as determined by analysis of variance with a Tukey post hoc test. $n = 2$.

analysis confirmed deposition of a radiodense layer at the construct periphery. Hematoxylin and eosin analysis indicated increased protein deposition at the construct surfaces exposed to mineralization medium, which became more distinct with time and likely contributed to an increased mineralized matrix deposition (Fig. 5).

Discussion

Several stem/progenitor cell niches are reported within the postnatal dental pulp, including sites within the central pulp stroma and in perivascular regions. The heterogeneous nature of these niches implicates local tissue signals being important for self-renewal, proliferation, differentiation, mobilization, and homing of cells (Yin and Li 2006). Within healthy tissues, progenitor/MSC niches are reportedly quiescent, and slow-cycling MSCs are in close proximity to ECM-rich regions that regulate their behavior (Booth and Potten 2000; Bi et al. 2007).

ECM coating. Furthermore, comprehensive identification of factors important in the ECM that maintain MSC potency may have significant application in future tissue regeneration and engineering strategies.

The present study also demonstrated that mineralization was enhanced when cells were cultured in a physiologically relevant ECM environment. While this may be due to the initial maintenance of the MSC phenotype, we have also noted that when cells originally cultured on ECM were reseeded on standard cultureware, they did not demonstrate enhanced alizarin red staining. Similarly, the differentiation of pulp cells along adipogenic lineages on standard cultureware compared with those maintained on ECM demonstrated minimal differentiation differences (data not shown). These data also indicate the potential importance of the ECM in providing additional signals for differentiation.

Alginate hydrogels have been used to viably encapsulate many cell types, and the manipulation of its modulus can influence dental cell differentiation; as such, it was selected as a suitable material for the generation of an in vitro model of dental tissue engineering (Kong et al. 2003; Smith et al. 2007; Hunt et al. 2009). Furthermore, we have shown that alginate hydrogels can provide a conducive environment in which dental repair can occur in vitro (Dobie et al. 2002). In our studies, only cells adjacent to the outer surface of the construct contributed to a mineralized tissue, which is consistent with our previous reports (A.J. Smith, Smith, et al. 2012). These outer-surface effects could be due to differences in oxygen tension, reduced diffusion of mineralization signals, and/or physical restriction of the more deeply encapsulated cells.

Data presented here indicate the potential importance and utility of generating biomimetic environments in vitro, in 2 and 3 dimensions, for tissue engineering purposes. Further studies are, however, required to confirm the important role of pECM signaling within 3-dimensional environments, as well as its relevance in animal model systems. Nevertheless, our findings indicate that application of ECM may aid MSC-phenotype maintenance and subsequent differentiation and that alginate hydrogel scaffolds doped with ECM may provide suitable models to study tooth development and regeneration, as well as future clinical application.

Author Contributions

J.G. Smith, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript; A.J.
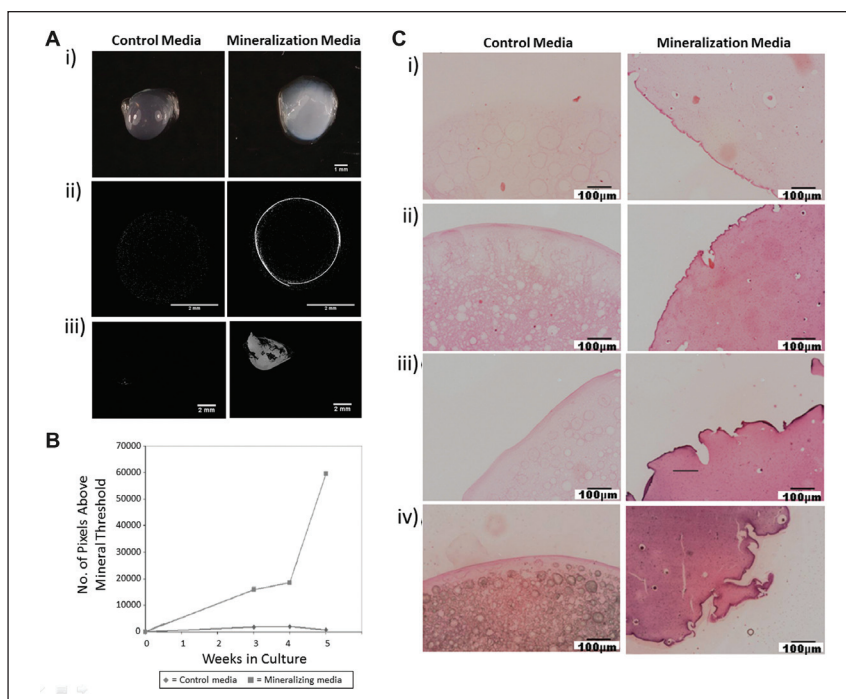

Figure 5. Deposition of mineral on outer surfaces of alginate hydrogels containing pulp extracellular matrix components and cells after cultures' exposure to control and mineralization media. (**A**) Representative (i) photographs and micro–computed tomography images for the (ii) sagittal plane in 2- and (iii) 3-dimensional reconstructions of the alginate gels, after 5-wk culture. (**B**) Analysis showing number of pixels above the intensity of the hydroxyapatite phantom mineral threshold in micro–computed tomography scanned images of alginate gels. Data show that culture in mineralization medium resulted in an increase in the number of pixels above the hydroxyapatite phantom mineral threshold density. (**C**) Representative photomicrographs of histologic sections of the 3% alginate gels containing pulp extracellular matrix components and primary pulp cells stained with hematoxylin and eosin after culture in control medium and mineralization medium for (i) 2 wk, (ii) 3 wk, (iii) 4 wk, and (iv) 5 wk. Images show increased staining on the outer surfaces of gels exposed to mineralization medium. Scale bars are shown.

Smith, R.M. Shelton, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; P.R. Cooper, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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