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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-hours they exhibited the same level of adherence as those cells seeded on tissue culture treated surfaces, by 4-days 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 they exhibited enhanced mineralisation as determined by alizarin red staining and mineralised marker expression. Supplementation of a 3% alginate hydrogel with pulp ECM components and dental pulp cells followed by differentiation induction in mineralisation 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 indicates 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.

Dental Pulp Cell Behaviour in Biomimetic Environments

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Short title: Dental pulp cell culture with extracellular matrix in 2D and 3D

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-hours they exhibited the same level of adherence as those cells seeded on tissue culture treated surfaces, by 4-days 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 they exhibited enhanced mineralisation as determined by alizarin red staining and mineralised marker expression. Supplementation of a 3% alginate hydrogel with pulp ECM components and dental pulp cells followed by differentiation induction in mineralisation 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 indicates 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.

Key words: Pulp, dentine, extracellular matrix, hydrogel, growth factor, doping

INTRODUCTION

Different populations of mesenchymal stem cells (MSCs) are described within the pulp including dental pulp stem cell (DPSC) (Gronthos et al. 2000), stem cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003) and stem cells from the apical papilla (SCAP) (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 is of a relatively gelatinous consistency and contains significant amounts of collagen (types I and III), proteoglycans and glycosaminoglycans (Tsuzaki et al. 1990), non-collagenous proteins including fibronectin, tenascin, osteonectin and osteopontin and many members of growth factor families. Indeed the regulation of dental tissue regeneration also involves signalling derived from its ECM with members of the TGFβ superfamily being directly implicated in stimulating dentinogenic repair (Smith et al. 2012a).

Several approaches have been utilised to isolate post-natal MSCs from dental and other tissues, with the simplest utilising 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 (FACs) and magnetic activated cell sorting (MACs) 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 (LNGFR) and negative selection for CD31 and CD146 being used to isolate pulp MSCs (Yang et al. 2007; Nakashima et al. 2009; Zhang et al. 2006). Recent reports however demonstrate that MSCs also exhibit selective adhesion to surfaces coated with ECM-derived

molecules and subsequently cell adhesion to fibronectin coated cultureware has been proposed for pulp MSC enrichment (Waddington et al. 2009).

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 DPSCs and SHED on scaffolds which 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 (Chen et al. 2015; Zhang et al. 2006; Zheng et al. 2011).

We subsequently hypothesised 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 hypothesised that dental ECM-supplementation of a hydrogel scaffold may provide a more physiologically relevant environment for 3D dental tissue engineering. Furthermore the generation of robust *in vitro* models may reduce the requirement for *in vivo* experimentation and the associated costs and constraints.

MATERIALS & METHODS

Isolation of Dental Tissue and Cells

Bovine mandibular incisor teeth were from <2-month old male Holstein Friesian calves (Bates' Wholesale Butchers, Birmingham, UK). Maxillary and mandibular incisors were from 100-120g 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 Characterisation of Pulp Extracellular Matrix (pECM)

Bovine incisor pulps were mechanically dissected (0.5–1mm^3 pieces) and combined with 1ml 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, Dorset, UK) and 1.5mM sodium azide (VWR International, Lutterworth, UK). Tissue suspensions were homogenised on ice [Ultra-Turrax T8 homogeniser (IKA Labortechnik, Staufen, Germany)] and agitated for 24 hours at 4°C. Centrifuged supernatants were removed and pellets re-suspended in 1ml 0.5M NaCl (Sigma-Aldrich, Dorset, UK). Re-suspending, homogenisation, agitation and pelleting was repeated in triplicate and supernatants pooled. Pulpal tissue was re-suspended in 1ml cold 0.1M tartaric acid solution (pH 2.0) (Hopkin & Williams Ltd, Birmingham, UK) and subjected to the same homogenisation, stirring and centrifugation protocol as described above (Bellon et al. 1988). NaCl and tartaric acid soluble pECM extracts were dialysed (19mm tubing) against dH_2O for 2-weeks at 4°C with daily water changes prior to lyophilisation.

Characterisation of pECM Components

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

Non-collagenous proteins (NCPs) were assayed using Coomassie Brilliant Blue G-250 (Sigma-Aldrich, Dorset, UK) with absorbance determined at 595nm using a UV/VIS Spectrometer (Philips, Colchester, UK) with a BSA standard (Bradford 1976). The dimethyl methylene blue assay was used to quantify glycosaminoglycans (GAGs) (Farndale et al. 1986). The GAG chain, chondroitin-4-sulphate (Sigma-Aldrich, Dorset, UK), was used as standard with absorbance at 525nm. Collagen was assayed using Sirius Red (VWR International, Lutterworth, UK) in saturated Picric acid (Sigma- Aldrich, Dorset, UK) (Tullberg-Reinert and Jundt 1999). Absorbance values were determined at 490-570nm using an ELX800 Universal Microplate reader (Bio-tex Instruments, USA) with rat tail collagen type-I standard (Sigma- Aldrich, Dorset, UK).

Primary Cell Culture

Culture was in α-MEM (Biosera, Nuaille, France), containing 2mM L-glutamine supplemented with 1% penicillin / streptomycin (all Sigma-Aldrich, Dorset, UK) and 10% foetal calf serum (Biosera, Nuaille, France). Primary pulp cells were isolated from rodent tissue by enzymatic disaggregation (Patel et al. 2009). Cells ($2x10^4$) were seeded on culture surfaces (Sarstedt, Leicester, UK) and viable cells counted using the trypan blue exclusion assay (Sigma-Aldrich, Dorset, UK).

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

Scanning Electron Microscopy (SEM)

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 minutes followed by dehydration by sequential 10 minute treatments in increasing concentrations (v/v) of ethanol followed by exposure to hexamethyl-disilazane (Sigma-Aldrich, Dorset, UK). Culture surfaces were attached to aluminium SEM stubs using Acheson electrodag (Agar Scientific, Stansted, UK) and sputter-coated with gold under vacuum (Emitech K550X). SEM images were obtained using an accelerating voltage of 10kV using a JSM-840A SEM (Joel, Welwyn Garden City, UK).

High Content Cell Analysis (HCA) of OCT 3/4

HCA was performed at Imagen Biotech (Manchester, UK). Pulp cells were fixed (30 minutes) with 10% paraformaldehyde (VWR International, Lutterworth, UK). Cells were incubated with Oct 3/4 primary antibody (Abcam, Milton, UK) diluted 1:100 in 0.1M phosphate buffer

pH7.8 with 0.1% BSA for 1-hour at room temperature. Cells were washed (3x) and incubated with goat anti-rabbit IgG secondary antibody conjugated to an Alexa-Fluor® 488 fluorescent label (VWR International, Lutterworth, UK) for 1-hour. ArrayScan High Content Screening (HCS) Imaging Cytometer and ArrayScan II Data Acquisition and Data Viewer 3.0 software (Fisher, Loughborough, UK) were used for analysis.

Alizarin Red Staining

Cultures were fixed in 10% paraformaldehyde (VWR International, Lutterworth, UK) for 30 minutes, washed with PBS alizarin red solution added (VWR International, Lutterworth, UK) (Gregory et al. 2004). Excess stain was removed with PBS and cultures de-stained in 10mM acetic acid. Stain was quantified at 405nm using an ELX800 Universal Microplate reader (Bio-Tex Instruments Inc, Houston, US) and compared with 40mM alizarin red stock solution.

Bromodeoxyuridine (BrdU) Proliferation Assay

The 5-bromo-2-deoxy-uridine labelling and detection kit II (Roche Life Sciences, Burgess Hill, UK) was used. Medium was removed from cultures and replaced with 500µl BrdU labelling medium. After 60 minutes incubation, BrdU labelling medium was removed and dishes washed in triplicate with PBS. Cells were fixed (30% absolute ethanol/70% 50mM glycine) for 20 minutes at room temperature. Fluid was removed and samples incubated at -20°C for 30 minutes. After PBS washing, 700µl anti-BrdU (kit reagent) was added to each dish for 30 minutes at 37°C, surfaces were PBS washed. 700µl AP conjugate was added for 30 minutes

at 37°C. Dishes were washed with PBS before 700µl colour substrate was added for 30 minutes 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.

Semi-quantitative RT-PCR Analysis

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

PCRs contained 50-100ng of cDNA, 12.5µl 2xREDTaq Ready mix (Sigma-Aldrich, Dorset, UK), 1µl of 1µM forward primer (VWR International, Lutterworth, UK), 1µl of 1µM reverse primer (Invitrogen, UK), and 12.5µl of water (VWR International, Lutterworth, UK). Primer and assay details are in **Supplementary Table 1**. A GeneAmp 2700 Thermocycler (Applied Biosystems, Manchester, UK) was used with initial denaturation of 94°C for 5 minutes, 1 minute cycles of 94°C, denaturation for 20 seconds, annealing at 60/60.5°C for 20 seconds, extension at 68°C for 20 seconds, and a final extension at 72°C for 10 minutes.

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

Cell Encapsulation and Culture in Alginate Gels

Low viscosity sodium-alginate (Sigma-Aldrich, Dorset, UK) 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 $5x10^5$ cells/ml +/- pECM supplementation. Constructs were added dropwise into culture dishes (Sarstedt, Leicester, UK) containing 100mM $CaCl_2$ and incubated at 37˚C for 1 hour to form cross-linked spheres (Hunt et al. 2009). Spheres were washed (x3) in α-MEM and re-suspended in control or lineage inductive media. Cells were released from alginate using 100mM tri-sodium citrate (Sigma-Aldrich, Dorset, UK).

Histological Analysis of Hydrogel Constructs

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

Micro-Computed Tomography (MicroCT)

Alginate constructs were scanned at 80kV, 100µA at an isotropic resolution of 4µm with camera exposure of 200ms, 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 using NRecon 1.6.2 software (Bruker, Coventry, UK). For thresholding a hydroxyapatite mineral phantom was used comprising of tetracalcium phosphate (TTCP)/dicalcium phosphate anhydrous (DCPA) powder and α-tricalcium phosphate (α-TCP) powder (Hofmann et al. 2007).

Statistical Analysis

Paired student T-tests and one-way ANOVA with $p<0.05$ with a Tukey post hoc test was used to determine statistical significance compared with controls.

RESULTS

Pulp cell cultures on pECM

Triplicate pECM isolates demonstrated consistent protein content profiles and yields. Ratios of GAG, NCP and collagen yields for the pECM isolates were also consistent between extractions. Coating of cultureware was confirmed by Coomassie blue staining and SEM analysis (***Figure 1***). This cultureware coating approach enabled comparison with standard culturing approaches. Viable cells at 24 hours post-seeding 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 cells cultured on pECM coated cultureware exhibited significantly decreased proliferation compared with controls (***Figure 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 (***Figure 3***). Nuclear versus cytoplasmic localisation data for the pluripotent transcription factor Oct 3/4 was consistent with gene expression analysis 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 (***Figure 3***).

Differentiation analysis of pulp cells on ECM

Two-weeks cell culture on pECM surfaces in the presence of mineralisation supplements resulted in an enhanced mineralising phenotype (***Figure 4***). Cell count data indicated that differences in quantitative staining were not due to variations in cell numbers. Similar profiles were observed at 3-weeks (data not shown). Gene expression analyses using markers of dental and mineralising cell differentiation indicated differential gene expression between control and differentiation conditions (***Figure 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 weeks, while cell numbers did not increase, there was no significant loss of viability (**Figure S1**). Subsequently, pulp cells were encapsulated in 3% hydrogels containing pECM components which had previously demonstrated influence on MSC behaviour and exposed to mineralisation medium for up to 5-weeks while controls were cultured in unsupplemented medium. Inspection of 3D cultures indicated that mineralisation medium exposed cultures appeared visually opaque and microCT analysis confirmed deposition of a radiodense layer at the construct periphery. H&E analysis indicated increased protein deposition at the construct surfaces exposed to mineralisation medium that became more distinct with time and likely contributed to an increased mineralised matrix deposition (***Figure 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 their self-renewal, proliferation, differentiation, mobilization and homing (Yin and Li 2006). Within healthy tissues, progenitor/MSC niches are reportedly quiescent and slow cycling MSCs are located in close proximity to ECM rich regions which regulate their behaviour (Booth and Potten 2000; Bi et al. 2007). We therefore hypothesised 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 which 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 signalling pathways exist between different MSC-types.

To characterise pECM extracts we have performed proteomic mass spectrometry and identified over 90 proteins present in these preparations (data not shown). Several molecules previously utilised 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 we observed, it is likely that more complex ECM interactions occur which generate a more relevant and comprehensive environment for regulating MSC behaviour. Our culture approach may

therefore provide a MSC biomimetic environment. Previously we have found that when pECM was subjected to enzymatic degradation, modulation of cell proliferation was abolished (Smith et al. 2012b) indicating its integrity is important in regulating cell behaviour. 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 (Celiz et al. 2014; Fu et al. 2010; Trappmann et al. 2012). Indeed future experiments which isolate physical effects on cells from biological effects could be performed by coating culture plasticware with inert materials which solely change cultureware surface topography in a similar manner to that observed due to ECM coating. Furthermore comprehensive identification of factors important in the ECM which maintain MSC-potency may have significant application in future tissue regenerative and engineering strategies.

The present study also demonstrated that mineralisation 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 re-seeded 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 previously 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 (Hunt et al. 2009; Smith et al. 2007; Kong et al. 2003). Furthermore we have previously 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 mineralised tissue which is consistent with our previous reports (Smith et al. 2012c). These outer surface effects could be due to differences in oxygen tension, reduced diffusion of mineralisation signals, and/or physical restriction of the more deeply encapsulated cells.

Data presented here indicates the potential importance and utility of generating biomimetic environments *in vitro,* both in 2D and 3D, for tissue engineering purposes. Further studies are however required to confirm the importance of the role of pulp ECM signalling within 3D 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 having future clinical application.

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FIGURE LEGENDS

Figure 1. Ai) Image of 1D-PAGE analysis of the three replicate extracts of pulp ECM (pECM). 0.125mg of pECM was loaded in a NuPAGE 10% Bis-Tris gel, and visualised using SilverXpress Silver staining kit. 10µl of Mark12 molecular weight (MW) ladder was loaded as a weight reference. Protein extracts cover a range of sizes. **ii)** Colourimetric analysis of pECM extracts showing quantification (µg/mg ECM) of non-collagenous proteins (NCPs), glycosaminoglycans (GAGs) and soluble collagen content in the three 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 SEM of **i)** untreated culture polystyrene surfaces and **ii)** ECM treated cultureware at x100 magnification. Scale bars are shown. Images demonstrate a change in the surface appearance following ECM coating indicating that protein adsorption to treated surfaces.

Figure 2. Data showing the number of adherent primary dental pulp cells **A)** at 24-hours and **B)** at 4-days culture. Cells were seeded on control and pulp ECM (pECM) coated plasticware. No statistical differences were found in cell adhesion numbers after 24 hours. **C)** BrdU proliferation assay in primary dental pulp cell cultures. Cells were counted in 10-fields of view in replicate plates representing over 50% of the total stained area per culture. Data showed a significant decrease in the percentage of cells showing active proliferation in the pulp cell population cultured on pECM coated compared with non-coated cultureware. Standard deviation bars plotted, * = $P<0.05$.

Figure 3. A) Gene expression analysis for **i)** dentinogenic markers, **ii)** mesenchymal / general stem cell markers, **iii)** pluripotent stem cell markers. Densitometric intensities were normalised against GAPDH and plotted as relative gene expression. Data demonstrated there were statistically significant lower levels of the dentinogenic markers DMP and DSPP when cultured on pulp ECM (pECM) compared with uncoated cultureware in pulp cell populations. The expression of mesenchymal / general and pluripotent stem cell markers was generally increased in cultures on pulp ECM compared with uncoated cultureware. **B)** ArrayScan HCS imaging cytometer (Cellomics, UK) high content analysis of primary pulp cells stained for OCT 3/4 after 4-days 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)** Graphical representation showing the percentage of cells in the population that stained positive for OCT3/4. Semi-quantitative data shows increased percentage levels of the population staining positive for OCT 3/4 after culture on pulp ECM compared with control uncoated cultureware surfaces. This work was undertaken at Imagen Biotech, Manchester, UK (http://www.imagen-biotech.com/).

Figure 4. Analysis of pulp cell behaviour at two weeks under different culture conditions. **A)** Representative images of alizarin red stained cultures and following quantification are provided. Parentheses with * indicate between groups which demonstrate statistically significant differences. N=4. **B)** Semi-quantitative gene expression analysis for mineralisation associated transcripts. Levels were normalised to the GAPDH housekeeping gene and densitometric values plotted. Cell cultures analysed were: Unc.+CM = uncoated cultureware with control medium; Unc.+MM = uncoated culture cultureware with mineralisation

medium; pECM+CM = pulp ECM coated cultureware with control medium; pECM+MM = pulp ECM coated cultureware with mineralisation medium. Standard deviation bars plotted. * = P<0.05 as determined by ANOVA with a Tukey post hoc test. N=2.

Figure 5. Deposition of mineral on outer surfaces of alginate hydrogels containing pulp ECM components and cells after cultures exposure to control and mineralisation media. **A i)** Representative photographs, and microCT images for the **ii)** sagittal plane in 2D and **iii)** 3D reconstructions of the alginate gels, after 5-weeks culture. **B)** Analysis showing number of pixels above the intensity of the hydroxyapatite phantom mineral threshold in microCT scanned images of alginate gels. Data demonstrated that culture in mineralisation medium resulted in an increase in the number of pixels above the hydroxyapatite phantom mineral threshold density. **C)** Representative photomicrographs of histological sections of the 3% alginate gels containing pulp ECM components and primary pulp cells stained with haemotoxylin and eosin after culture in control medium and mineralisation medium for **i)** 2 weeks, **ii)** 3 weeks, **iii)** 4 weeks, and **iv)** 5 weeks. Images show increased staining on the outer surfaces of gels exposed to mineralisation medium. Scale bars are shown.

Figure S1. Viable cells released from different concentrations of alginate hydrogels in the presence or absence of pulp ECM molecules over a two week period. **A)** Photomicrographs of released primary pulp cells after culture for one week in different alginate gel conditions. **i)** 1% alginate gel, **ii)** 1% alginate gel with pECM molecules, **iii)** 3% alginate gel, **iv)** 3% alginate gel with pECM molecules, **v)** 5% alginate gel, and **vi)** 5% alginate gel with pECM

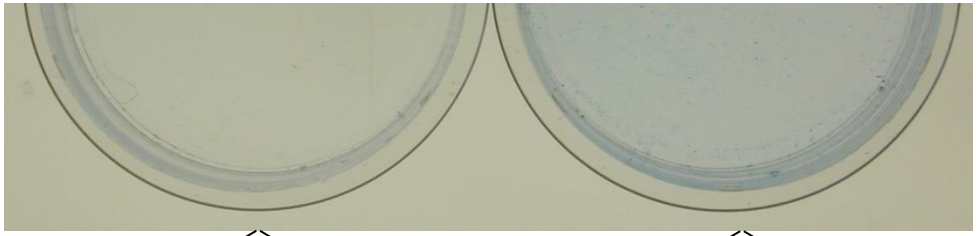
molecules. Phase contrast images are representative of cells released from all six gel conditions showing that released cells could adhere to polystyrene surfaces and retained a morphological appearance similar to that of controls. Scale bars are shown. **B)** The number of viable cells released from the gels was determined by Trypan blue staining and standard cell counts remained relatively constant over the two week period for all six culture conditions. There was no statistical difference identified between viable cell numbers under the culture conditions analysed. Standard deviation bars are shown. N=4.

A i)

ii)

	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

B i) ii)



C i) ii)

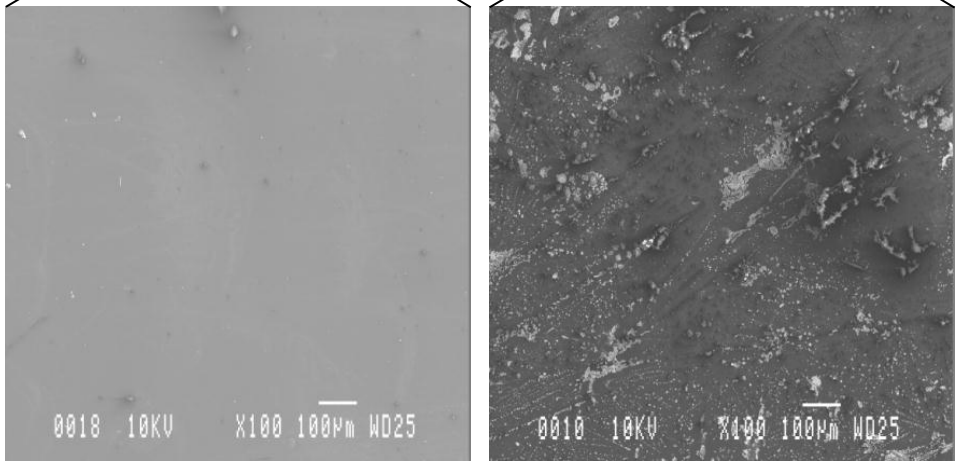


Fig 1

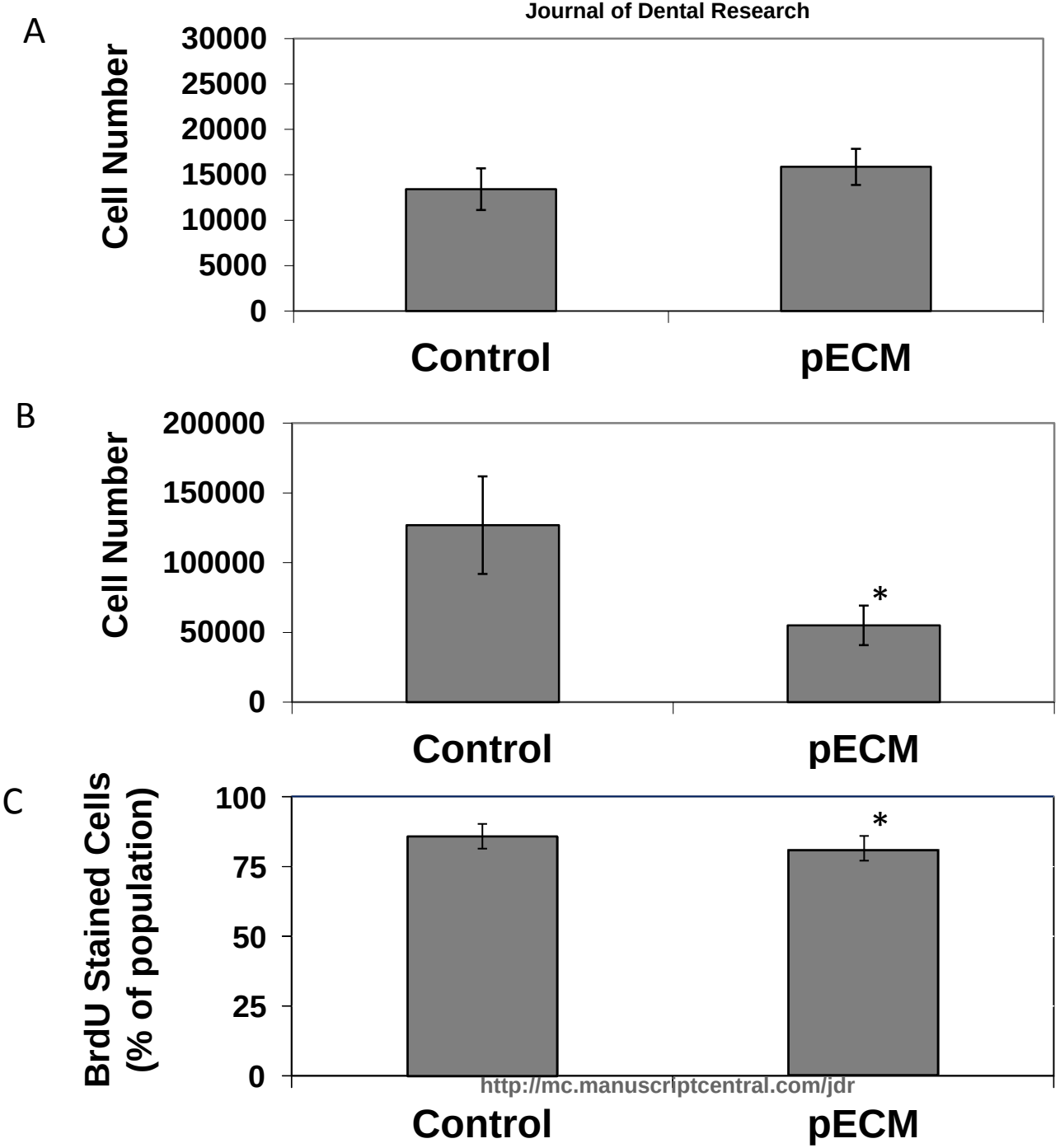


Fig 2

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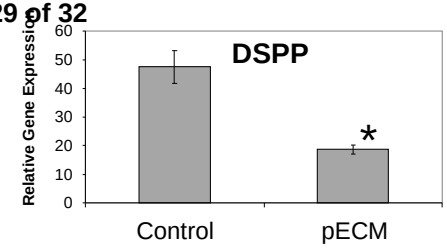

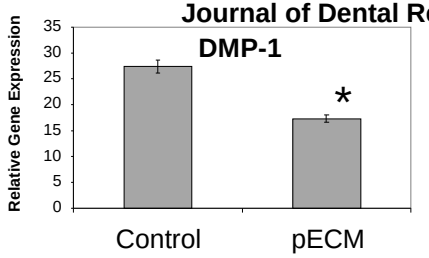

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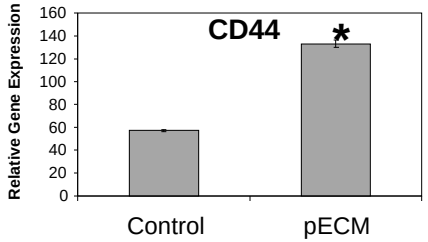

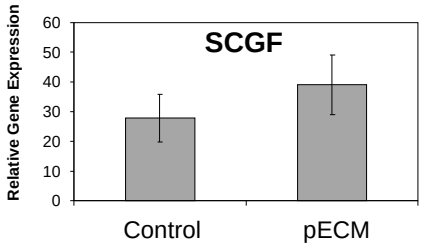

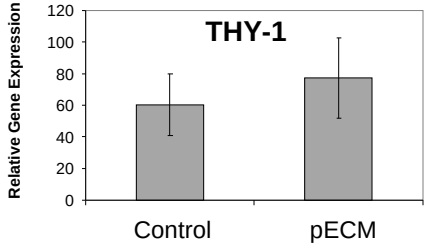

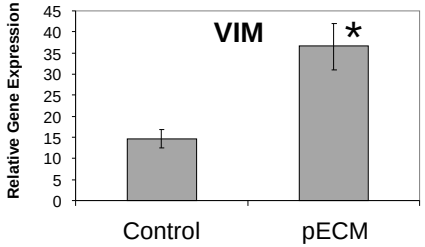

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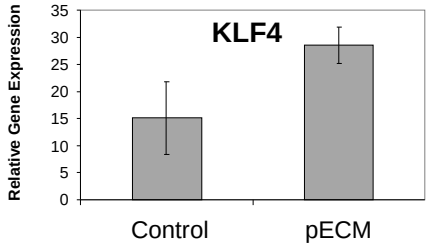

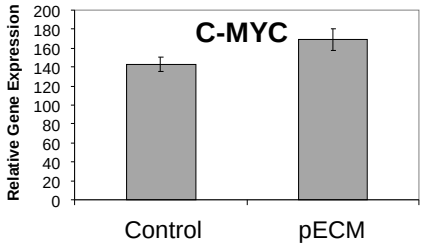

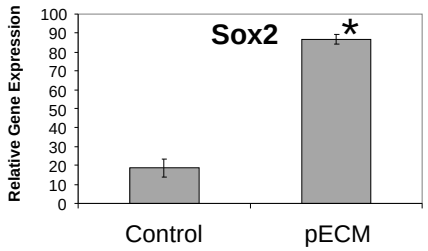

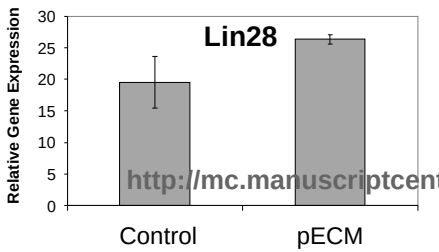

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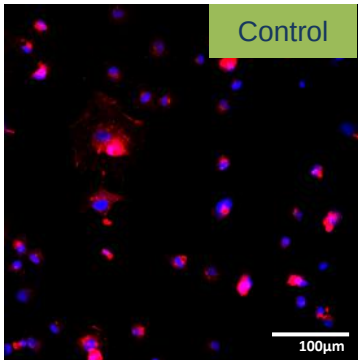

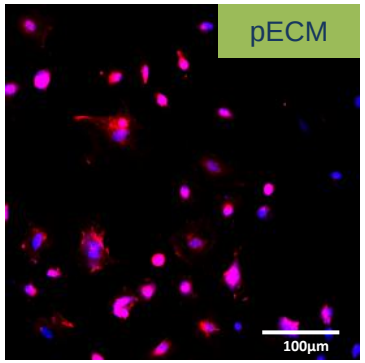

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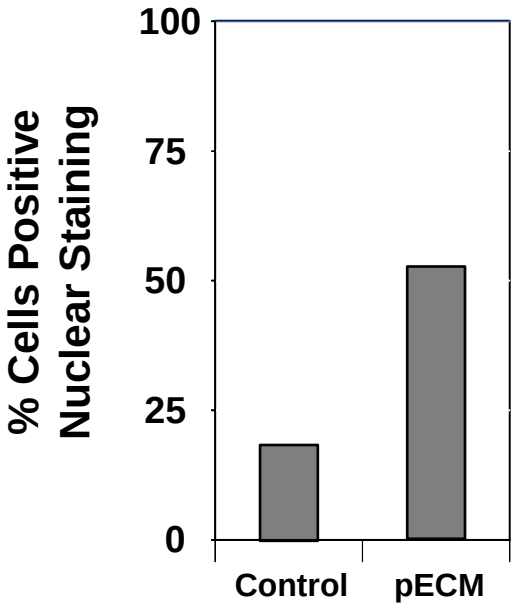
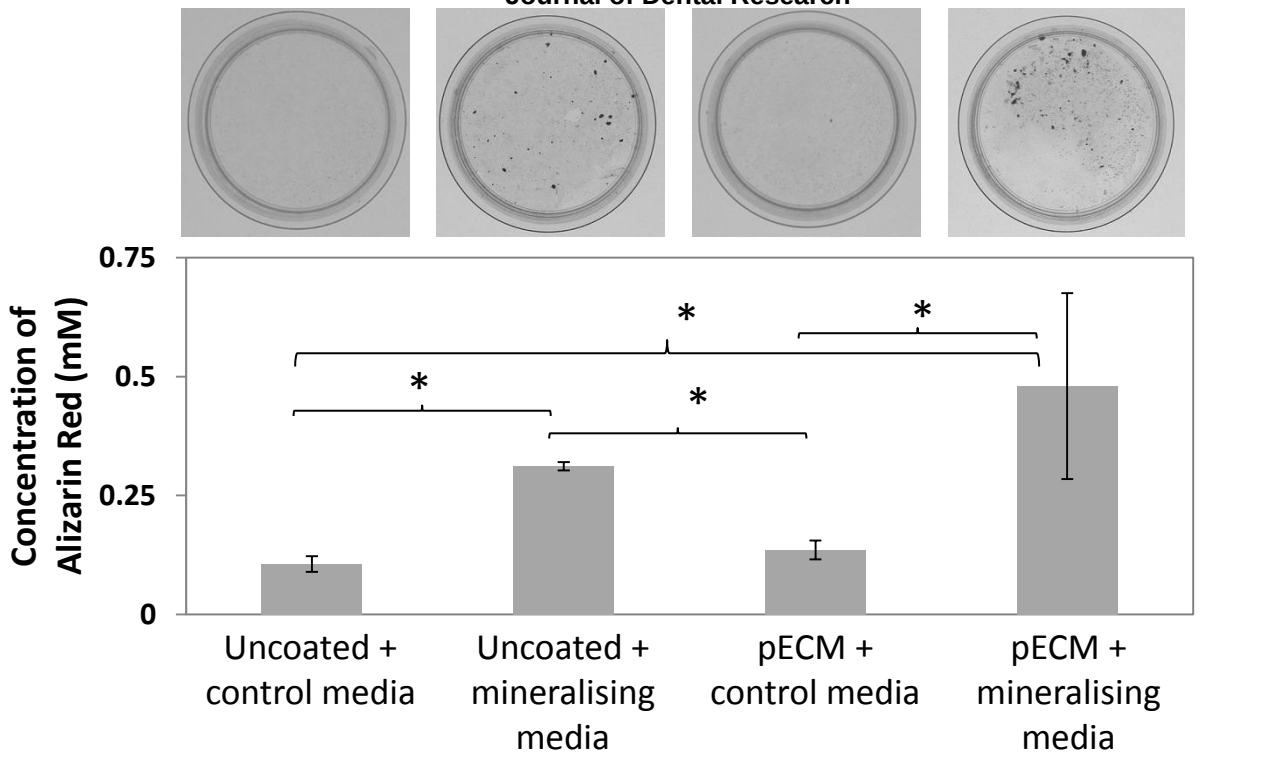


Fig 3



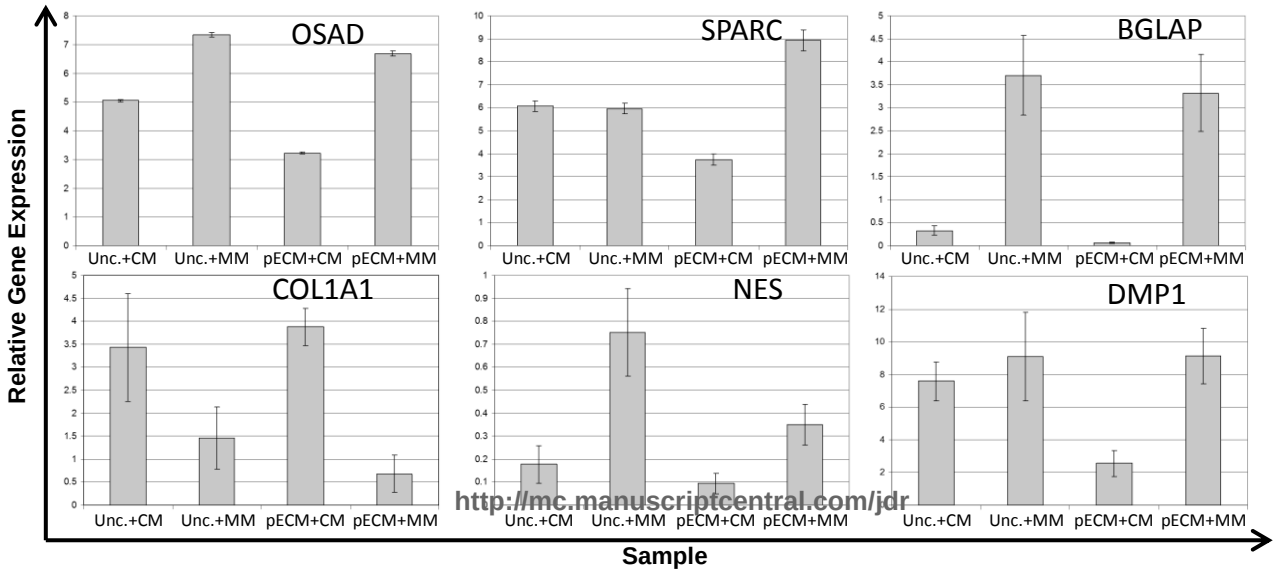
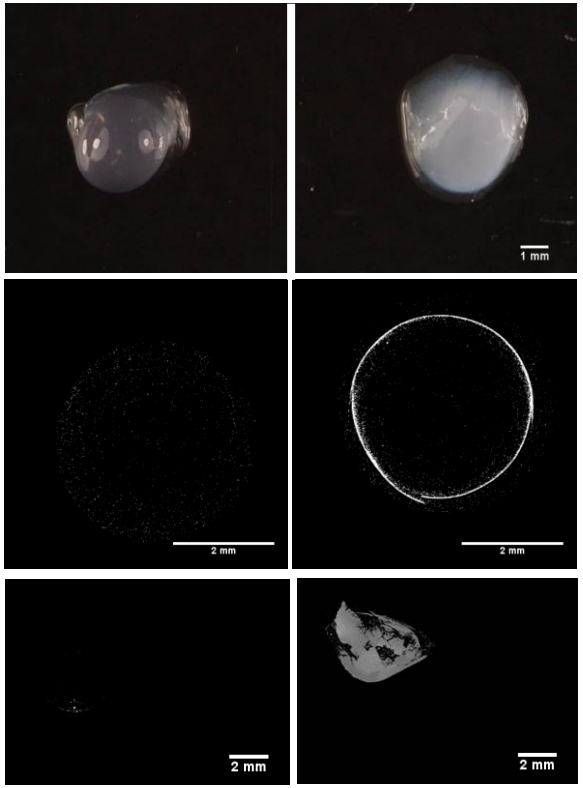
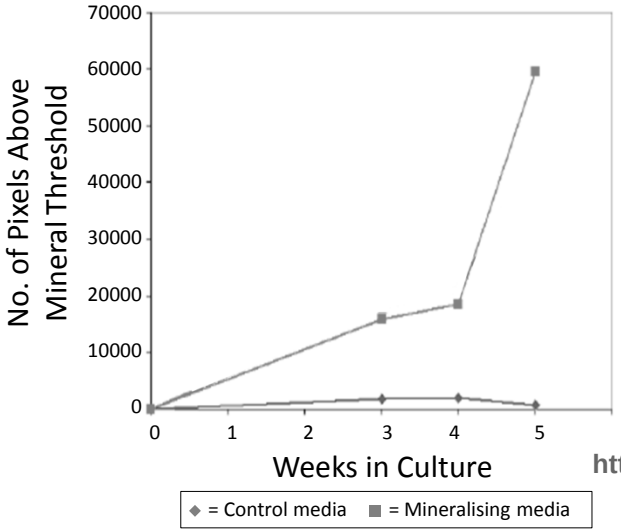


Fig 4

A


Control Media
Mineralisation Media
i)
ii)
iii)
1 mm
2 mm
2 mm
2 mm
2 mm

B


No. of Pixels Above Mineral Threshold
70000
60000
50000
40000
30000
20000
10000
0
0
1
2
3
4
5
Weeks in Culture
◆ = Control media
■ = Mineralising media

C

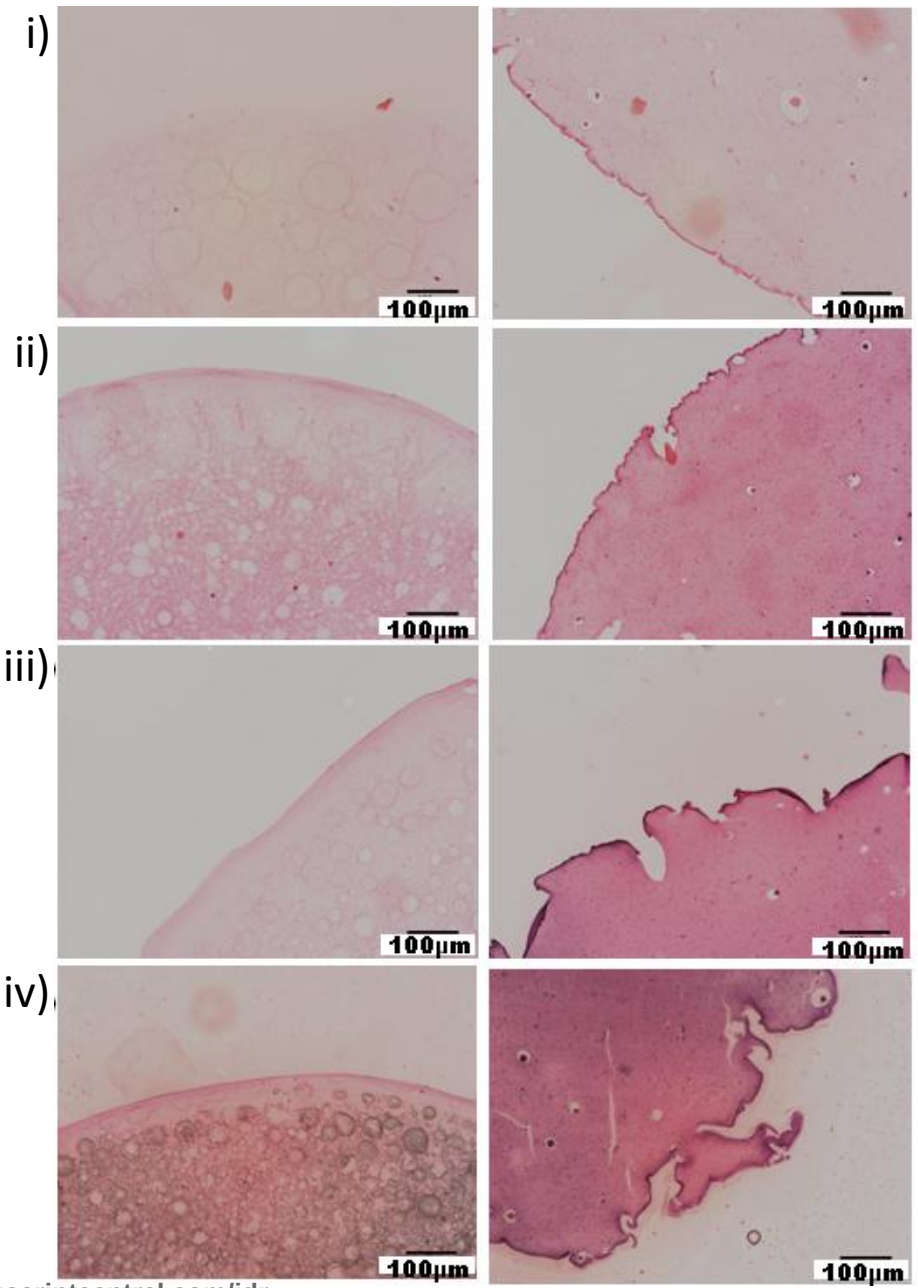

Control Media
Mineralisation Media
i)
ii)
iii)
iv)
100µm

Fig 5

Supplementary Table. Rat gene identifiers and primer sequences used in PCR assays. Primers were designed from Genbank sequences using the Primer Blast programme; http://www.ncbi.nlm.nih.gov/tools/primer-blast/. Sequences were purchased from Invitrogen, UK. Forward (F) and reverse (R) primer.

Gene	Gene Symbol	Genbank Accession Number	Primer Sequences (5'→3')
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	NM_017008.4	F-CCC ATC ACC ATC TTC CAG GAG C R-CCA GTG AGC TTC CCG TTC AGC
Dentin sialophosphoprotein	DSPP	NM_012790.2	F-TGC ATT TTG AAG TGT CTC GC R-CCT CCT GTC TTG GTG TGG TT
Dentine matrix protein 1	DMP-1	NM_203493.3	F-CGG CTG GTG GTC TCT CTA AG R-CAT CAC TGT GGT GGT CCT TG
Oesteocalcin	Bglap	NM_013414.1	F-TCC GCT AGC TCG TCA CAA TTG G R-CCT GAC TGC ATT CTG CCT CTC T
Osetonectin	SPARC	NM_012656.1	F-AAA CAT GGC AAG GTG TGT GA R-AGG TGA CCA GGA CGT TTT TG
Osteoadherin	Osad	NM_031817.1	F-AAC CTT AGC CAC AAC AAA ATT AA R-TTG CTT CAG TTT GTT ATG TCC
Nestin	Nes	NM_012987.1	F-CAT TTA GAT GCT CCC CAG GA R-AAT CCC CAT CTA CCC CAC TC
Collagen type I alpha	COL1A1	BC133728	F-GGG CAA GAC AGT CAT CGA AT R-TTG GTT TTT GGT CAC GTT CA
SRY-related HMG-box-2	Sox-2	NM_001109181.1	F-ATA CAA GGG AAT TGG GAG GG R-AAA CCC AGC AAG AAC CCT TT
Lin-28 homolog A	Lin28	NM_001109269	F-TTT CTT GTT TCC CCC AAA TG R-AGA GGG GCT GGT TGT AAG GT
Kruppel-like factor 4	Klf4	NM_053713	F-ATC ATGGTCAAGTTCCCAGC R-ACC AAG CAC CAT CGT TTA GG
Cellular Myelocytomatosis Oncogene	C-myc	BC091699	F-CTT ACT GAG GAA ACG GCG AG R-GCC CTA TGT ACA CCG GAA GA
Thymus cell antigen 1	Thy-1 / CD90	NM_012673.1	F-AAC CAG AAC CTT CGA CTG GA R-AGG AAG GAG AGG GAA AGC AG
Vimentin	Vim	NM_031140	F-AGA TCG ATG TGG ACG TTT CC R-GCA GGT CCT GGT ATT CAC G
Cluster of Differentiation 44	CD44	NM_012924.2	F-TGG GTT TAC CCA GCT GAA TC R-CTT GCG AAA GCA TCA ACA AA
Stem cell growth factor	SCGF	NM_001012459	F-CCT TTG AGC AGA GGA AGC AA R-TCA AGG TGC AGG TTG CAC TA

Figure S1