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# Engineering three-dimensional constructs of the periodontal ligament in hyaluronan–gelatin hydrogel films and a mechanically active environment

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*Background and Objective:* Periodontal ligament (PDL) cells in stationary two-dimensional culture systems are in a double default state. Our aim therefore was to engineer and characterize three-dimensional constructs, by seeding PDL cells into hyaluronan–gelatin hydrogel films (80–100 μm) in a format capable of being mechanically deformed.

*Material and Methods:* Human PDL constructs were cultured with and without connective tissue growth factor (CTGF) and fibroblast growth factor (FGF)-2 in (i) stationary cultures, and (ii) mechanically active cultures subjected to cyclic strains of 12% at 0.2 Hz each min, 6 h/d, in a Flexercell FX-4000 Strain Unit. The following parameters were measured: cell number and viability by laser scanning confocal microscopy; cell proliferation with the MTS assay; the expression of a panel of 18 genes using real-time RT-PCR; matrix metalloproteinases (MMPs) 1–3, TIMP-1, CTGF and FGF-2 protein levels in supernatants from mechanically activated cultures with Enzyme-linked immunosorbent assays. Constructs from stationary cultures were also examined by scanning electron microscopy and immunostained for actin and vinculin.

*Results:* Although initially randomly distributed, the cells became organized into a bilayer by day 7; apoptotic cells remained constant at approximately 5% of the total. CTGF/FGF-2 stimulated cell proliferation in stationary cultures, but relative quantity values suggested modest effects on gene expression. Two transcription factors (*RUNX2* and *PPARG*), two collagens (*COL1A1*, *COL3A1*), four MMPs (*MMP-1–3*, *TIMP-1*), *TGFB1*, *RANKL*, *OPG* and *P4HB* were detected by gel electrophoresis and Ct values < 35. In mechanically active cultures, with the exception of *P4HB*, *TGFB1* and *RANKL*, each was upregulated at some point in the time scale, as was the synthesis of MMPs and TIMP-1. *SOX9*, *MYOD*, *SP7*, *BMP2*, *BGLAP* or *COL2A1* were not detected in either stationary or mechanically active cultures.

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*Conclusion:* Three-dimensional tissue constructs provide additional complexity to monolayer culture systems, and suggest some of the assumptions regarding cell growth, differentiation and matrix turnover based on two-dimensional cultures may not apply to cells in three-dimensional matrices. Primarily developed as a transitional *in vitro* model for studying cell–cell and cell–matrix interactions in tooth support, the system is also suitable for investigating the pathogenesis of periodontal diseases, and importantly from the clinical point of view, in a mechanically active environment.

Two-dimensional culture systems have been widely used to investigate the pathophysiology and mechanobiology of the periodontal ligament (PDL), a specialized connective tissue that evolved to provide attachment of teeth to the bones of the jaws (1–4). However, two-dimensional systems are limited by the fact that cells are normally embedded in a complex extracellular network of collagens, proteoglycans and non-collagenous proteins, not attached to tissue culture plastic or substrates composed of matrix proteins such as collagen or fibronectin. The PDL also functions in a mechanically active environment, which means that cells in conventional stationary two-dimensional culture systems lack the mechanical stimuli to which they are exposed *in vivo*. In other words, PDL cells in monolayer culture are effectively in a double default state. First, by the absence of the three-dimensional structure of a tissue construct, and second, the lack of biomechanical stimuli provided by mastication and other forms of occlusal loading.

Because they have material properties that resemble naturally occurring extracellular matrices and bridge the gap between two-dimensional cell culture systems and animal models, hydrogels have been widely used to create three-dimensional tissue constructs for use in cell biology, bioengineering and regenerative medicine. Hydrogels are a class of polymer materials that can absorb large amounts of water without dissolving, due to the physical or chemical cross-linkage of the various hydrophilic polymer chains from which they are composed (5,6). Not only do hydrogels mimic more closely the environment experienced by cells *in vivo*, they also confer beneficial effects on gene expression, cell adhesion, morphology and phenotype (7–10).

Collagens are the most abundant proteins in connective tissues, and collagen hydrogels have been widely used as tissue culture scaffolds for numerous cell types since first being described by Elsdale and Bard (11) 40 years ago. Nevertheless, collagen is just one of the major structural macromolecules found in extracellular matrices. The challenge for engineering three-dimensional tissue constructs has been to replicate not only connective tissue complexity with minimal components, but also the mechanical and viscoelastic characteristics of the native tissue – the intention being to enable the incorporated cells reproduce their parent tissue as closely as possible (12,13). To address the limitations posed by two-dimensional cultures, the aim of the present investigation was to engineer and characterize a tissue that more closely resembled the structure of the PDL by seeding cells into Extracel™, a commercially-available hydrogel matrix composed of hyaluronan (HA) and gelatin (14,15), and in a format that could be mechanically deformed. Although primarily developed as a transitional *in vitro* model for studying mechanisms of tooth support, the system is also suitable for investigating the pathogenesis of periodontal diseases.

## Material & methods

### Preparation of periodontal ligament cell/Extracel™ constructs in stationary culture

Primary human PDL fibroblasts were purchased from a commercial source (ScienCell Research Laboratories, Carlsbad, CA, USA; Lot number: 5145). The cells, isolated from human periodontal tissue from several donors are cryopreserved at passage 1, delivered frozen on dry ice, and immediately transferred to liquid nitrogen on receipt. They were subsequently thawed and expanded in Dulbecco minimal Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Singapore) and antibiotic–antimycotic reagents as described below. The expanded cells were harvested, aliquoted and stored in liquid $N_2$ until required; passage three cells were used in all subsequent experiments. Extracel™ was purchased from Glycosan Biosystems (Salt Lake City, UT, USA). The hydrogel is formed when the cross-linking agent, Extralink™ (polyethylene glycol diacetate) is added to a mixture of Glycosil™ (thiol-modified HA) and Gelin-S™ (thiol-modified gelatin). The three components come as lyophilized solids, and DG Water™ (degassed, deionized water) was used to dissolve the Glycosil, Gelin-S and Extralink in individual vials. Glycosil and Gelin-S were mixed 1 : 1 and $2.5 \times 10^6$/mL PDL cells added. Extralink was added to the Glycosil–Gelin-S mix at a ratio of 1 : 4, and after blending in a pipette, 300 μL of the gel mixture was added to 35 mm diameter type I collagen coated six-well plates (Sigma-Aldrich, Singapore) and spread to form a film 80–100 μm in thickness. The constructs were allowed to gel at 37°C, which occurred after about 20 min; PDL/Extracel cultures were then incubated in 3 mL DMEM (Gibco, Invitrogen, Singapore) supplemented with 10% FBS, antibiotic–antimycotic reagent (10,000 units, penicillin, 10,000 μg streptomycin and 25 μg/mL amphotericin B;

Invitrogen), 100 mM L-glutamine (Invitrogen) and Gentamycin (10 mg/mL, Gibco) and cultured at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. The culture medium was changed every 2 d.

To promote fibroblast proliferation and differentiation, gel constructs were cultured with and without human recombinant connective tissue growth factor (CTGF; 100 ng/mL; BioVendor Laboratories, Guangzhou, China) and human recombinant fibroblast growth factor (FGF)-2 (10 ng/mL; Gibco). Growth factors were incorporated into both the gel matrix and culture media.

### Scanning electron microscopy

To examine the tissue constructs by scanning electron microscopy, culture media was aspirated from the 35 mm culture dishes and the constructs fixed in 2.5% glutaraldehyde at 4°C overnight, washed in phosphate-buffered saline (PBS) twice and then post-fixed for 2 h at room temperature in 2% osmium tetroxide. They were then dehydrated in a graded series of ethanols and dried by transferring the specimens in 100% ethanol to a critical point drying apparatus (Leica EM CPD030, Singapore) and ethanol exchanged for liquid $CO_2$ under pressure. After six cycles of this exchange, the temperature of the chamber was raised to 40°C, a point at which the liquid changes into a gas and drying occurs. The dried specimens were mounted on to aluminium specimen stubs with conductive tape and sputter coated with gold–palladium alloy for 120 s, placed in a vacuum and viewed in a scanning electron microscope (Philips/FEI XL30 FEG scanning electron microscopy, Singapore) at an accelerating voltage of 10 kV.

### Laser scanning confocal microscopy

Laser scanning confocal microscopy was used for the following.

*Analysis of cell viability*— The effect on cell viability of incorporating cells into Extracel was determined by the fluorescein diacetate (FDA)–propidium iodide (PI) method (16). At the end of each time point, culture supernatants were discarded and the cells stained with 0.1 mL (2 μg) FDA and 0.3 mL (6 μg) PI from stock solutions (Sigma-Aldrich) and viewed with a Carl Zeiss, LSM 510 META confocal imaging system – viable cells fluoresce bright green, nonviable cells bright red. Three fields (120 μm × 120 μm), one in the centre and two approximately 1 cm on either side were chosen for analysis, and the number of viable and nonviable cells in three wells at each time point counted using Image-Pro Plus (version 6.1.0.346) software (Media Cybernetics, Bethesda, MD, USA).

*Immunostaining*— The cells were washed with PBS and fixed in 4% formaldehyde at room temperature for 15 min. The Extracel gel constructs were permeabilized with 0.1% Triton X-100 in PBS, and blocked with 5% normal goat serum or FBS for 1 h at room temperature. The cells were then triple-stained with an Actin Cytoskeleton/Focal Adhesion Staining Kit (Merck Millipore, Singapore) as follows: first, a mouse antivinculin monoclonal antibody (1 mg/mL) diluted with blocking solution in the ratio of 1 : 150 was incubated with prepared cells at 4°C overnight. The constructs were washed with PBST (0.1% Tween-20 in PBS) for 2 h and a secondary antibody, goat antimouse IgG conjugated to fluorescein isothiocyanate (2 mg/mL) added at a dilution of 1 : 300. Second, orientation of the actin filaments was mapped by adding tetramethyl rhodamine isothiocyanate-conjugated phalloidin (60 μg/mL) at a dilution of 1 : 250, and after 60 min incubation the cells washed three times with PBST. Finally, the constructs were incubated with 4′,6-diamidino-2-phenylindol (0.1 mg/mL) to stain nuclei. Cells were viewed with an Olympus Fluoview FV 1000 (Olympus, Japan) confocal imaging system at 60 × magnification and processed using Imaris version 6.1.5 software.

### Application of cyclic mechanical strain to constructs of the periodontal ligament

PDL/Extracel constructs 80–100 μm in thickness were formed on type I collagen-coated silicone membranes in six-well, 35 mm flexible-bottomed Tissue Train® culture plates (Flexcell International Corporation, Hillsborough, NC, USA) fitted with a bonded foam perimeter to improve cell attachment. The constructs were subjected to an in-plane biaxial cyclic strain of 12% for 5 s (0.2 Hz) every 60 s for 6 h/d, using a square wave-form around circular loading posts in a standard Bioflex baseplate linked to a Flexercell FX-4000T Tension Plus Strain Unit (Flexcell International). The strain value of 12% was based on data derived from a finite element model, which suggested that maximal PDL strains for horizontal displacements of a human maxillary central incisor under physiological loading lies close to 8–25% depending upon the apico-crestal position (17). We have previously used the figure of 12% corresponding to the mid-root position, to deform PDL cells in two-dimensional cultures (3,4,18).

### Cell recovery from Extracel

At the end of the experimental period the culture medium was aspirated from each well and the hydrogel surface washed with PBS; 1.5 mL trypsin-EDTA (Invitrogen) was added and incubated at 37°C for 3 h. After a further wash, 1.5 mL of 10 × collagenase/hyaluronidase in DMEM (StemCell Technologies, Singapore) was added and incubated overnight at 37°C. This was based on a protocol developed and recommended by Glycosan Biosystems, the manufacturers of Extracel. At the end of the second incubation, 3 mL DMEM supplemented with 10% FBS was added to the hydrolysed gel and the cell suspension centrifuged at 290 *g* for 5 min. The resulting pellet was resuspended in 1.5 mL DMEM and centrifuged again at 6708 *g* for 2 min.

### RNA extraction

Extraction of RNA was carried out using an RNeasy Plus Mini kit (Qiagen, Singapore) according to the manufacturer's instructions. This yielded RNA with an A260/280 value > 1.95 and an A230/280 value > 1.2 in a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), indicative of a pure sample. Integrity of the RNA was assessed by gel electrophoresis on an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA); the RIN (RNA Integrity Number) values were in the range 9.0–9.9.

### Gene expression using real-time polymerase chain reaction

A known amount (500 ng) of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) and a MyCycler™ thermal cycler (Bio-Rad). Real-time PCR was performed in triplicate using Fast SYBR® Green Master Mix (Applied Biosystems, Singapore) with a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Samples were screened for the expression of 18 genes; the primer sequences (First Base, Singapore) used and the annealing temperatures are listed in Table 1. The amplification was carried out via the first step at 95°C for 10 min, followed by 40 cycles with 15 s at 95°C, 10 s at the particular annealing temperature, and 20 s at 72°C. The fluorescence signal was acquired at 72°C. The relative expression of these genes was normalized to the expression of two calibrator genes: *ACTB* (β-actin) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) using the standard curve method. Critical threshold (Ct) values were calculated using the StepOnePlus™ Version2.1 software

*Table 1.* Genes and primer sequences used for real-time RT-PCR

| HGNC agreed gene symbol | Description | Primer sequences | Annealing temperature |
|---|---|---|---|
| *ACTB* | Actin, beta | F: CCAAGGCCAACCGCGAGAAGATGAC<br>R: AGGGTACATGGTGGTGCCGCCAGAC | 58 |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | F: ACCACAGTCCATGCCATCAC<br>R: TCCACCACCCTGTTGCTGTA | 60 |
| *P4HB* | Prolyl-4-hydroxylase, beta subunit | F: GTCTTTGTGGAGTTCTATGCCC<br>R: GTCATCGTCTTCCTCCATGTCT | 62 |
| *RUNX2* | Runt-related transcription factor 2 | F: TGAGAGCCGCTTCTCCAACC<br>R: GCGGAAGCATTCTGGAAGGA | 58 |
| *SOX9* | SRY (sex determining region Y)-Box 9 | F: GAACGCACATCAAGACGGAG<br>R: TCTCGTTGATTTCGCTGCTC | 58 |
| *PPARG* | Peroxisome proliferator-activated receptor-gamma | F: ATTGACCCAGAAAGCGATTC<br>R: CAAAGGAGTGGGAGTGGTCT | 62 |
| *MYOD* | Myogenic differentiation antigen 1 | F: CGGCGGAACTGCTACGAAG<br>R: GCGACTCAGAAGGCACGTC | 60 |
| *COL1A1* | Collagen type I, Alpha-1 | F: GAACGCGTGTCATCCCTTGT<br>R: GAACGAGGTAGTCTTTCAGCAACA | 60 |
| *COL2A1* | Collagen type II, Alpha-1 | F: TTCAGCTATGGAGATGACAATC<br>R: AGAGTCCTAGAGTGACTGAG | 58 |
| *COL3A1* | Collagen Type III, Alpha-1 | F: AACACGCAAGGCTGTGAGACT<br>R: GCCAACGTCCACACCAAATT | 60 |
| *MMP1* | Matrix metalloproteinase 1; collagenase 1 | F: GGGAGATCATCGGGACAACTC<br>R: GGGCCTGGTTGAAAAGCAT | 60 |
| *MMP2* | Matrix metalloproteinase 2; gelatinase A (72 kDa) | F: TGATCTTGACCAGAATACCATCGA<br>R: GGCTTGCGAGGGAAGAAGTT | 60 |
| *MMP3* | Matrix metalloproteinase3; stromelysin 1 | F: TGGCATTCAGTCCCTCTATGG<br>R: AGGACAAAGCAGGATCACAGTT | 60 |
| *TIMP1* | Tissue inhibitor of metalloproteinases 1 | F: CTGTTGTTGCTGTGGCTGATA<br>R: CCGTCCACAAGCAATGAGT | 60 |
| *TGFB1* | Transforming growth factor, beta 1 | F: GCAACAATTCCTGGCGATACCTC<br>R: AGTTCTTCTCCGTGGAGCTGAAG | 60 |
| *BGLAP* | Gamma carboxyglutamic acid protein; Osteocalcin | F: ATGAGAGCCCTCACACTCCTC<br>R: GCCGTAGAAGCGCCGATAGGC | 60 |
| *SP7* | Transcription factor Sp7; Osterix | F: TGGCGTCCTCTCTGCTTGA<br>R: TCAGTGAGGGAAGGGTGGGT | 58 |
| *BMP2* | Bone morphogenetic protein 2 | F: CAGAGACCCACCCCCAGCA<br>R: CTGTTTGTGTTTGGCTTGAC | 58 |
| *TNFSF11* | Tumour necrosis factor ligand superfamily, member 11; RANKL | F: TCCCATCTGGTTCCCATAAA<br>R: GGTGCTTCCTCCTTTCATCA | 60 |
| *TNFRSF11B* | Tumour necrosis factor receptor superfamily member 11B; Osteoprotegerin | F: TTCCGGAAACAGTGAATCAA<br>R: CGCTGTTTTCACAGAGGTCA | 60 |

(Applied Biosystems); a gene was excluded if it could not be detected by agarose gel electrophoresis or had a threshold value exceeding 33–35.

### Agarose gel electrophoresis

The PCR products were checked by electrophoresis with 1.5% agarose gel stained with 1 × SYBR® Safe DNA Gel Stain (Invitrogen). Two microlitres of 6 × loading dye (10 mM Tris-HCl, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA; Fermentas, Singapore) was mixed with 8 μL of the PCR products and loaded against 8 μL of 100 bp DNA ladder (Fermentas). The gels were run for 55 min at 75V in 1 × TBE buffer and observed by ultraviolet transillumination on a BioRad imaging system using Quantity One® v 4.6 software (BioRad, Hercules, CA, USA).

### MTS assay for cell proliferation

The laser beam in confocal microscopy does not penetrate the silicone membrane used to support cell cultures in Flexcell Bioflex plates. For the mechanical stress experiments, cell proliferation was therefore measured by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Singapore), a colorimetric assay based on the ability of viable cells to reduce the novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] with an electron coupling reagent (phenazine ethosulphate) by mitochondrial NADPH or NADH dehydrogenase into a coloured formazan product (19). At the end of each time point, the supernatant was removed and 1.2 mL of MTS solution (200 μL of CellTiter 96® AQueous One Solution reagent/mL of DMEM) was added to each well of the tissue train plates. The plates were incubated at 37°C in 5% $CO_2$/95% air for 30 min. Absorbance was measured at 490 nm by an uQuant microplate spectrophotometer (Biotek, Singapore) the formazan product being directly proportional to the number of viable cells.

### Measurement of enzymes and growth factors in culture supernatants by Enzyme-linked immunosorbent assays

Conditioned media from the 24 h period before each end-point were harvested. Enzyme-linked immunosorbent assays (ELISAs) were used to quantitate levels of a selection of proteolytic enzymes used by fibroblasts to remodel their extracellular matrices: the panel included matrix metalloproteinase MMP-1 (collagenase-1); MMP-2 (gelatinase-A; 72 kDa); MMP-3 (stromelysin-1); and TIMP-1 (tissue inhibitor of metalloproteinases-1). All were assayed using Quantikine® colorimetric sandwich ELISAs (R&D Systems China, Shanghai, China) accor ding to the manufacturer's instructions. We also assayed the culture media for CTGF (Aviscera Bioscience, Santa Clara, CA, USA) and FGF-2 (R&D Systems China).

### Statistical methods

Differences between the groups were determined by Student's *t*-test (two-tailed) using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) with the level of significance set at $p < 0.05$.

## Results

Our initial experiments were carried out on stationary cultures in 35 mm six-well culture plates – these were chosen because the wells are the same diameter as the Tissue Train plates, with the added advantage that laser scanning confocal microscopy can be used to visualize the cells. On being encapsulated into the HA–gelatin matrix, PDL cells remained rounded over the first few days, gradually acquiring a fibroblastic phenotype, and although initially distributed evenly throughout the gel, with cell growth the central two-thirds of the wells became densely populated with the peripheral one-third less so. When the constructs were viewed in profile, the cells were found to be initially distributed randomly throughout the gel, but by the second wk had became organized into a double cell layer, one at the gel–substrate interface, and the other near the surface (Fig. 1).

Laser scanning confocal microscopy and FDA/PI staining proved effective at distinguishing viable and nonviable cells (Fig. 2). A progressive increase in the number of cells in stationary cultures occurred both with and without the addition of growth factors, which plateaued about 2 wk, the number of nonviable cells remaining fairly constant at approximately 5%. However, the addition of CTGF and FGF-2 resulted in a significant increase in the number of cells per field compared to controls (Table 2). We also found that maintaining the cultures for up to 6 wk had no significant effect on the number of viable cells, but increased the number undergoing apoptosis to about 14% in cultures with growth factors, and 12% in those without (data not shown).

Lack of stiffness of the gel was a disadvantage when attempting to examine the tissue constructs by scanning electron microscopy, the specimens collapsing during the critical point drying stage of preparation. Nevertheless, adequate images of the cells and the dense network of HA and gelatin fibrils were still obtained, although the density of the fibrillary matrix is likely to be exaggerated by shrinkage during dehydration (Fig. 3). Laser scanning confocal microscopy and triple staining for vinculin, actin and DNA, however, enabled images of the morphology and organization of the cells within the hydrogel matrix to be captured. At the gel–substrate interface the cells assumed an amoeboid-like morphology, and near the surface the characteristic elongated fusiform appearance of fibroblasts in two-dimensional culture (Fig. 4).

We next examined the effects of CTGF and FGF-2 on gene expression by cells in the stationary cultures. Of the panel of 18 genes screened (Table 1), 12 were detected at Ct values < 35 (Table 3), and subsequently confirmed by agarose gel electrophoresis (Fig. 5). We failed to detect *SOX9*, *MYOD*, *SP7* (osterix), *BMP2*, *BGLAP* (osteocalcin) or *COL2A1* in either stationary or mechanically

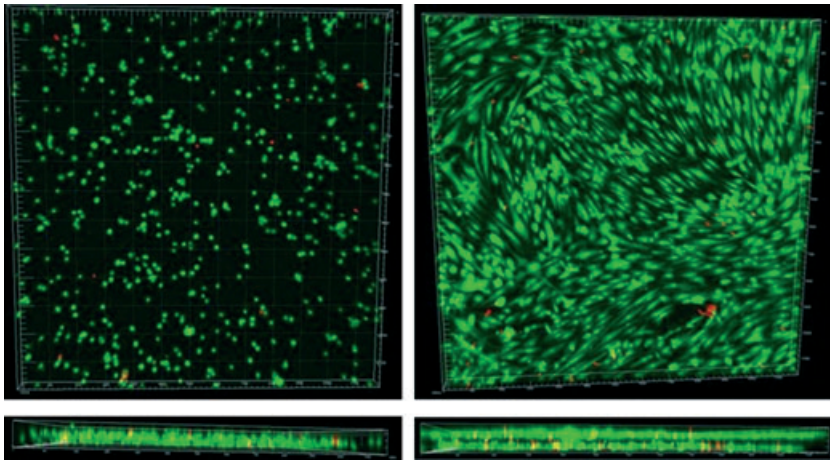

*Fig. 1.* Human periodontal ligament cells in hyaluronan–gelatin hydrogel films (80–100 μm in thickness) in 35 mm diameter type I collagen coated six-well plates, cultured with connective tissue growth factor (100 ng/mL) and fibroblast growth factor-2 (10 ng/mL). Left: 24 h. Right: 2 wk. The cells were stained with 0.1 mL (2 μg) fluorescein diacetate and 0.3 mL (6 μg) propidium iodide and viewed with a Carl Zeiss, LSM 510 META confocal imaging system; viable cells fluoresce bright green, non-viable cells bright red. Images are viewed from above and in profile. Profile view shows that cells are initially distributed randomly throughout the gel, but as they proliferate they become organized into a double cell layer; the field measures 120 × 120 μm.

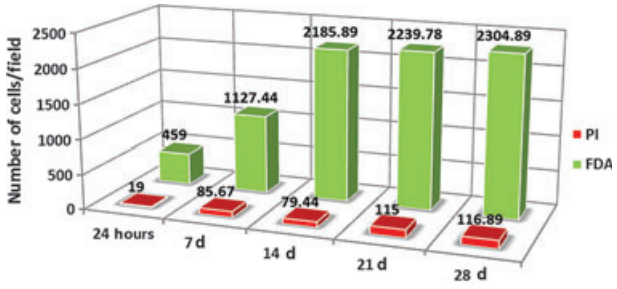


*Fig. 2.* Viability of human periodontal ligament cells incorporated into Extracel films (80–100 μm thickness) cultured in 35 mm diameter type I collagen coated six-well plates in the presence of connective tissue growth factor (100 ng/mL) and fibroblast growth factor-2 (10 ng/mL). At the end of each time-point the cells were stained with fluorescein diacetate and propidium iodide and viewed with a Carl Zeiss, LSM 510 META confocal imaging system; viable cells (green) and non-viable cells (red). Three fields were selected from three wells and the number of viable and nonviable cells counted using Image-Pro Plus (version 6.1.0.346) software.

activated cultures. The relative quantity (RQ) values suggested addition of growth factors had modest effects on gene expression, apart from *RUNX2*, *COL1A1* and *TGFB1*, which were upregulated at RQ values > 2.00 at some point in the time-scale; *MMP1* was upregulated across all three time-points (Table 3).

The effect of mechanical stress on gene expression with and without growth factors is shown in Table 4. A higher number of genes were expressed at RQ values > 2.00, particularly *COL3A1*, *MMP3*, *TIMP1* and *OPG*. However, we were unable to detect any significant additive or synergistic effects between the effects of mechanical stress alone and CTGF/FGF-2 with the sole exception of *PPARG*. Protein levels of MMP-1–3 and TIMP-1 in supernatants from cultures that had been mechanically stressed in the absence of CTGF/FGF-2 were significantly upregulated at various points in the time-scale, with MMP-3 and TIMP-1 across all three time-points (Fig. 6).

Finally, we performed an experiment to exclude the possibility that increases in gene and protein expression in mechanically stressed cultures were due to an increase in cell population. As Flexercell plates are unsuitable for use with laser scanning confocal microscopy, cell growth was measured by the MTS (tetrazolium) assay; this showed that mechanical stress did not have a significant effect on cell number (Table 5). Assays of the supernatants from these cultures showed that CTGF was constitutively synthesized by the cells in picogram quantities; however, no significant differences in CTGF synthesis could be

*Table 2.* Effect of growth factors on the proliferation of periodontal ligament cells

| | 7 d | 14 d | 21 d | 28 d |
|---|---|---|---|---|
| Control | 288.44 ± 47.04 | 1619.67 ± 234.50 | 1543.00 ± 76.36 | 1731.67 ± 117.89 |
| Experimental | 1127.44 ± 59.22*** | 2185.89 ± 156.14*** | 2239.78 ± 44.52*** | 2304.89 ± 52.43*** |

Human periodontal ligament cells were incorporated into Extracel films and cultured with and without connective tissue growth factor (100 ng/mL) and fibroblast growth factor-2 (10 ng/mL). At the end of each time-point the cells were stained with fluorescein diacetate and propidium iodide and viewed with a Carl Zeiss, LSM 510 META confocal imaging system. Three fields were chosen from three wells and the number of viable and non-viable cells counted using Image-Pro Plus (version 6.1.0.346) software. The data are cross-sectional and expressed as means ± SEM.
***$p < 0.001$.

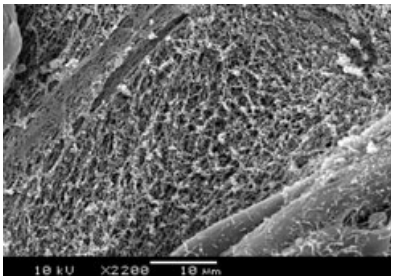

*Fig. 3*. Scanning electron micrograph showing the ultrastructure of the dense network of cross-linked hyaluronan and gelatin fibrils of the hydrogel matrix. This is likely to be exaggerated to some extent by dehydration during specimen preparation. In the lower right-hand corner are three periodontal ligament cells.

detected between control and mechanically stressed cultures (Fig. 7). FGF-2 was also detected in the media in picogram quantities, but at levels (12–17 pg/mL) close to the detection limit of the assay.

## Discussion

A number of attempts to engineer three-dimensional constructs of the PDL *in vitro* by incorporating ligament cells into various natural and synthetic polymers have been reported. Collagen has been most commonly used for obvious reasons given that collagens are the major structural proteins in connective tissues (20–25), but one disadvantage of collagen constructs is a tendency for fibroblast-populated collagen gels to contract over time unless cultured in the presence of MMP inhibitors (26,27).

We therefore carried out preliminary experiments to compare the suitability of 2% agarose gels with the commercially available preparation, Extracel. Agarose, a naturally occurring linear polysaccharide composed of galactose subunits obtained from agar, has been widely used in orthopaedic research due its ability to support the chondrocyte phenotype (28–30). The attraction of Extracel was that it is composed of two major structural molecules, HA and collagen (albeit in denatured form). It soon became apparent, however, that a marked difference existed between the two polymers in terms of cell survival; after 24 h in agarose almost 50% of the ligament cells were non-vital compared to about 5% in Extracel (unpublished findings). There was an improvement in cell growth in agarose cultures over a 4 wk time-course, but the added difficulty of spreading agarose into films that could be mechanically deformed led to our focus on Extracel.

Our reason for adding growth factors to the constructs was based on reports that CTGF is mitogenic for fibroblasts, and able to direct fibroblast differentiation from PDL progenitor cells and mesenchymal stem cells (31,32), while FGF-2 is a mitogen for PDL cells (33). Originally identified as a polypeptide growth factor and downstream mediator of transforming growth factor-β (34,35), CTGF has been reclassified as a matricellular protein, residing in the pericellular matrix and a member of the CCN family, the nomenclature of which is based on Cysteine-rich protein 61 (Cyr61/CCN1), CTGF/CCN2 and Nephroblastoma overexpressed (Nov/CCN3). In addition to being a mitogen, FGF-2 has been shown to regulate the expression of another matricellular protein osteopontin in PDL cells (36); also known as bone sialoprotein 1 and secreted phosphoprotein 1, as the name implies it was originally discovered in bone cells but is synthesized by many cell types. Both CTGF and osteopontin in common with other matricellular proteins do not play a structural role in the extracellular matrix, but serve to regulate cell–matrix interactions and cell function (37). This is achieved via direct binding to specific integrin receptors and heparin sulphate proteoglycans, triggering signal transduction in a wide variety of events such as cell adhesion, migration, proliferation, differentiation and apoptosis (38).

Previous investigations, including our own, have shown that in monolayer culture, *CTGF* is a mechanoresponsive gene (18,39–41). However, we were unable to confirm this finding. The present data further suggest that the addition of exogenous CTGF or FGF-2 to either stationary or mechanically stressed constructs was of limited benefit. In retrospect this is perhaps not surprising, given that real-time RT-PCR microarrays (3,4) have shown that PDL cells *in vitro* constitutively express numerous cytokines and growth factors that exhibit overlapping biological activities (redundancy), as well as multiple biological effects (pleiotropy); it would seem that endogenous production of growth factors by the cells was sufficient to promote growth. Another unexpected finding was that while mechanical stress upregulated the expression of multiple genes, we were

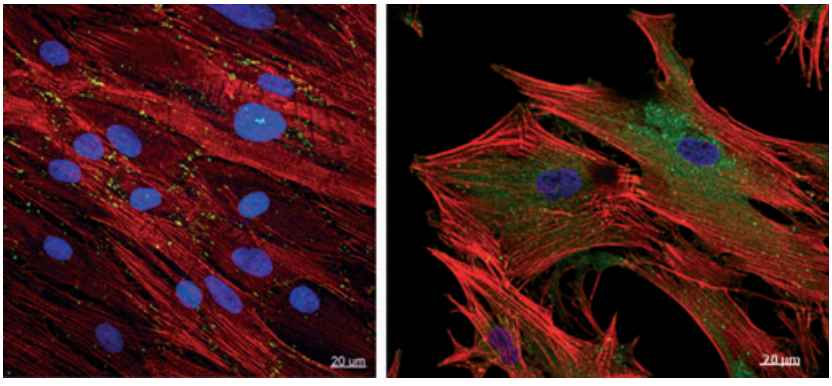

*Fig. 4*. Confocal immunofluorescent images of human periodontal ligament cells in three-dimensional Extracel films. Left: Cells near the gel surface. Right: cells at the gel substrate interface. Cells were fixed, permeabilized and triple stained with: (i) an antibody against vinculin (green), which links integrin receptors at focal adhesions to the actin cytoskeleton; (ii) rhodamine (TRITC) conjugated-phalloidin, which binds actin filaments (F-actin; red); and (iii) DAPI, a fluorescent stain that binds strongly to DNA (nuclei, blue).

*Table 3*. Effect of growth factors on gene expression by human periodontal ligament cells

| | 7 d | 14 d | 21 d |
|---|---|---|---|
| *P4HB* | 1.41 ± 0.73 | 0.72 ± 0.25 | 0.44 ± 0.19 |
| *RUNX2* | 0.94 ± 0.27 | 2.01 ± 0.77 | 2.21 ± 0.82 |
| *PPARG* | 1.48 ± 0.84 | 0.95 ± 0.30 | 0.53 ± 0.25 |
| *COL1A1* | 0.81 ± 0.35 | 3.35 ± 0.49 | 1.27 ± 0.82 |
| *COL3A1* | 0.89 ± 0.37 | 1.35 ± 0.25 | 0.61 ± 0.27 |
| *MMP1* | 4.02 ± 1.18 | 4.62 ± 1.42 | 3.16 ± 0.86 |
| *MMP2* | 1.21 ± 0.82 | 0.47 ± 0.26 | 1.45 ± 1.03 |
| *MMP3* | 0.42 ± 0.18 | 0.31 ± 0.09 | 1.15 ± 0.46 |
| *TIMP1* | 0.78 ± 0.26 | 0.30 ± 0.19 | 0.66 ± 0.57 |
| *TGFB1* | 0.97 ± 0.35 | 0.82 ± 0.38 | 3.07 ± 0.71 |
| *RANKL* | 1.06 ± 0.16 | 1.14 ± 0.28 | 0.61 ± 0.22 |
| *OPG* | 0.67 ± 0.37 | 1.29 ± 1.08 | 0.83 ± 0.23 |

Human periodontal ligament cells were cultured in three-dimensional hyaluronan/gelatin films ± connective tissue growth factor (100 ng/mL) and fibroblast growth factor-2 (10 ng/mL) and gene expression quantified by real-time RT-PCR. The data are cross-sectional, expressed as relative quantity ± SEM, and represents the mean of four separate determinations. Relative quantity values > 1.00 signify an increase in gene expression by growth factor-treated cultures over controls.

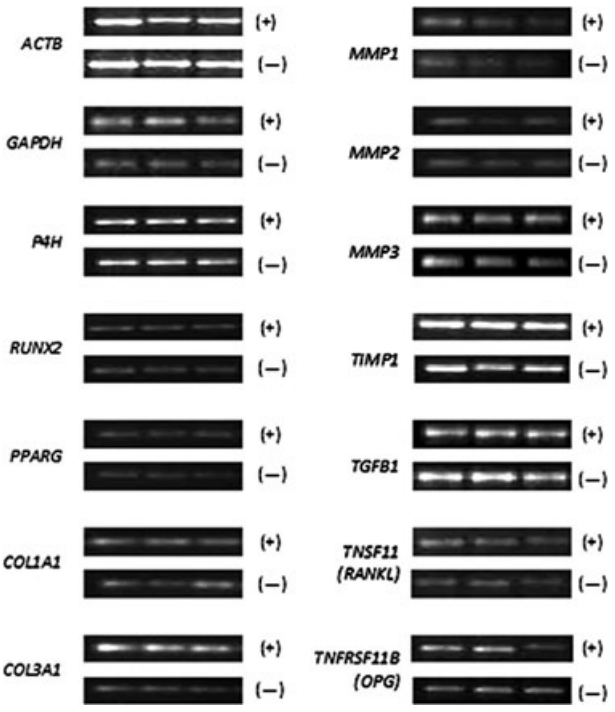


*Fig. 5*. Agarose gel electrophoresis of amplified real-time polymerase chain reaction products following reverse transcription of RNA extracted from periodontal ligament/Extracel constructs cultured with (+) and without (−) connective tissue growth factor (100 ng/mL) and fibroblast growth factor-2 (10 ng/mL) for 7, 14 and 21 d in 35 mm six-well plates. The images are representative examples of the results of four independent experiments. For description of gene symbols please see Table 1.

unable to detect a significant increase in cell proliferation as previously reported for epithelial cells and osteoblasts strained in monolayer culture (42,43).

While predominantly fibroblastic in phenotype, cells cultured from the PDL and expanded *in vitro* constitute a heterogeneous cell population that includes STRO-1-positive mesenchymal stem cells with the potential to differentiate into osteoblasts, chondroblasts, myoblasts and adipocytes (44–46). The osteoblast-specific transcription factor *RUNX2* was expressed by the cells and upregulated by growth factors and mechanical stress and given its location one might expect to find precursors for osteogenic and cementogenic cells. However, none of the downstream mediators of osteogenesis such as *SP7* (Osterix), *BMP2* or *BGLAP* (Osteocalcin) were detected, which suggests that *RUNX2*-expressing osteoprogenitor cells were part of the mesenchymal stem cell pool, but remained just that. Unlike *SOX9* and *MYOD*, which we failed to detect, expression of the adipocyte-related transcription factor *PPARG* and its responsiveness to mechanical strain was intriguing. PPAR-γ ligands induce bone marrow stem cell adipogenesis but also inhibit osteogenesis (47), and the secretion of adipocyte hormones such as leptin, which affect bone development, goes some way to explaining the poorly understood pathophysiological association between fat and bone (48). The reasons for this in the present context are not immediately obvious, but with the PDL bordered on one side by a surface lined with osteoblasts and on the other by cementoblasts, inhibition of osteogenesis by peroxisome proliferator-activated receptor -γ ligands may form part of a mechanism preventing ossification of the ligament.

The genes for RANKL and OPG, two cytokines customarily synthesized by osteoblasts and stromal cells were expressed, *OPG* being highly responsive to mechanical stress. RANKL, which exists in both membrane-bound and soluble forms stimulates the differentiation and function of osteoclasts, an effect mediated by RANK, a member of the TNF receptor family expressed primarily on cells of the monocyte/macrophage lineage, including osteoclasts and their precursor cells (49). OPG is a secreted protein that inhibits bone resorption by acting as a decoy receptor, binding to and neutralizing both cell-bound and soluble RANKL (50). The RANK/RANKL/OPG triad thus constitutes a

*Table 4*. Effect of mechanical stress on gene expression by human periodontal ligament cells

| | No growth factors ± mechanical stress | | | Growth factors ± mechanical stress | | |
|---|---|---|---|---|---|---|
| | 7 d | 14 d | 21 d | 7 d | 14 d | 21 d |
| *P4HB* | 0.78 ± 0.21 | 0.84 ± 0.15 | 1.08 ± 0.10 | 0.85 ± 0.10 | 0.91 ± 0.42 | 1.02 ± 0.22 |
| *RUNX2* | 0.86 ± 0.29 | 1.13 ± 0.12 | 2.62 ± 0.48 | 1.20 ± 0.41 | 1.66 ± 0.17 | 1.38 ± 0.37 |
| *PPARG* | 0.49 ± 0.15 | 1.00 ± 0.39 | 3.00 ± 0.41* | 1.47 ± 0.36 | 1.78 ± 0.18 | 1.00 ± 0.26 |
| *COL1A1* | 0.87 ± 0.21 | 1.53 ± 0.45 | 1.40 ± 0.09 | 0.85 ± 0.08 | 2.01 ± 0.73 | 0.84 ± 0.15 |
| *COL3A1* | 2.02 ± 0.92 | 0.98 ± 0.19 | 3.14 ± 1.15 | 1.49 ± 0.24 | 1.35 ± 0.52 | 3.46 ± 0.45 |
| *MMP1* | 0.44 ± 0.18 | 1.48 ± 0.67 | 4.31 ± 1.46 | 1.06 ± 0.26 | 2.05 ± 1.13 | 5.41 ± 1.70 |
| *MMP2* | 1.00 ± 0.25 | 1.44 ± 0.58 | 1.83 ± 0.17 | 0.88 ± 0.27 | 2.34 ± 0.65 | 2.11 ± 0.33 |
| *MMP3* | 2.66 ± 0.47 | 2.56 ± 0.26 | 13.47 ± 2.27 | 0.77 ± 0.10 | 1.53 ± 0.62 | 13.00 ± 4.49 |
| *TIMP1* | 0.88 ± 0.23 | 2.10 ± 0.17 | 2.71 ± 0.62 | 1.17 ± 0.22 | 3.45 ± 0.62 | 5.54 ± 1.70 |
| *TGFB1* | 0.78 ± 0.27 | 1.13 ± 0.48 | 1.03 ± 0.14 | 0.63 ± 0.11 | 1.39 ± 0.22 | 1.05 ± 0.46 |
| *RANKL* | 0.12 ± 0.01 | 0.56 ± 0.38 | 1.12 ± 0.40 | 0.12 ± 0.05 | 0.05 ± 0.02 | 0.63 ± 0.59 |
| *OPG* | 1.26 ± 0.11 | 2.31 ± 0.84 | 5.02 ± 1.37 | 0.37 ± 0.13 | 2.58 ± 1.06 | 2.28 ± 0.31 |

Human periodontal ligament cells were cultured in three-dimensional hyaluronan/gelatin films ± connective tissue growth factor (100 ng/mL) and fibroblast growth factor-2 (10 ng/mL), ± cyclic mechanical strain for 6 h/d and gene expression quantified by real-time RT-PCR. The data are cross-sectional, expressed as relative quantity ± SEM, and represents the mean of four separate determinations. Relative quantity values > 1.00 signify an increase in gene expression by mechanically active cultures over the corresponding controls.
*$p < 0.05$.

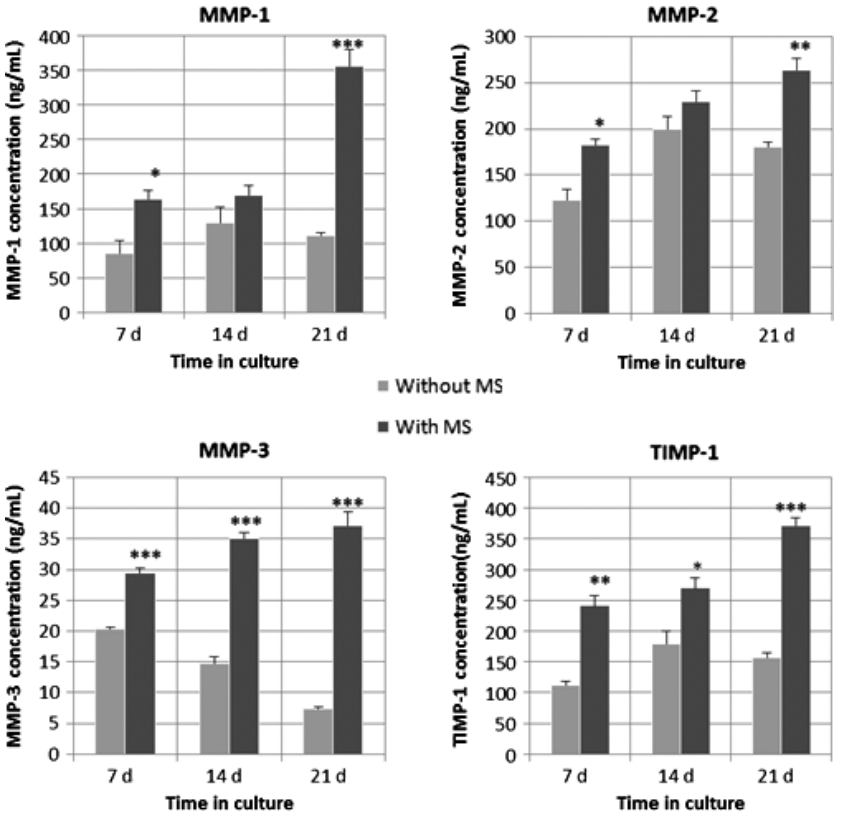


*Fig. 6*. Matrix metalloproteinases (MMP)-1–3 and TIMP-1 synthesis by human periodontal ligament cells in Extracel films subjected to an in-plane biaxial cyclic strain of 12% for 5 s (0.2 Hz) every 60 s for 6 h/d. At each end-point the culture media from six wells was pooled in pairs and assayed in triplicate. The antibodies to MMP-1 recognize pro-MMP-1 only, not active enzyme or MMP-1 complexed to TIMP. The TIMP-1 immunoassay only recognizes natural TIMP-1. The data are cross-sectional and expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

ligand–receptor system that directly regulates the final steps of the bone resorptive cascade, irrespective of the initiating signal (51). The finding that PDL cells also express *RANKL* and *OPG* has led to their being widely investigated *in vitro*, both genes being upregulated by compressive and tensile mechanical strain (52–54). Again, the reasons for this are not clear, but again may be related to limiting ossification of the ligament; the clinical observation that most ankylosed teeth are lower deciduous molars out of occlusion (55) is perhaps relevant.

The MMPs (matrixins) are a family of proteolytic enzymes synthesized in either secreted forms or bound to the plasma membrane, that play key roles in cell–cell and cell–matrix interactions during growth, morphogenesis and pathophysiological remodelling (56). MMPs function at neutral pH and are released as latent proforms, activation involving the loss of a propeptide of about 80 residues (57). TIMPs, of which TIMP-1 is the major form (58), closely regulate MMP activity by forming high affinity, essentially irreversible complexes with the activated enzyme to prevent uncontrolled resorption. The three major secreted MMPs, MMP-1 (collagenase-1), MMP-2 (gelatinase-A) and MMP-3 (stromelysin-1), as well as

*Table 5*. Effect of mechanical stress on periodontal ligament cell proliferation

| | 7 d | 14 d | 21 d |
|---|---|---|---|
| Control | 0.281 ± 0.017 | 0.864 ± 0.136 | 1.104 ± 0.105 |
| Experimental | 0.225 ± 0.015 | 0.838 ± 0.110 | 0.996 ± 0.100 |

The effect on cell proliferation of an in-plane, biaxial cyclic mechanical strain of 12% for 5 s (0.2 Hz) every 60 s for 6 h/d measured by the colorimetric MTS (tetrazolium) assay to detect living cells; absorbance was measured at 490 nm. Data are expressed as means ± SEM for six wells from three separate experiments. Mechanical strain did not have a significant effect on cell number.

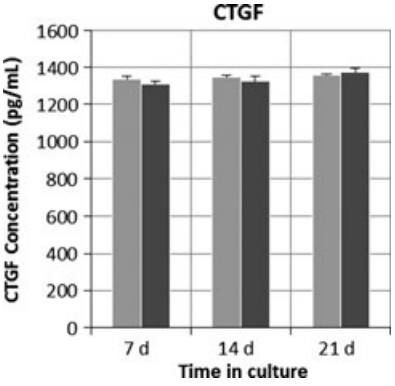


*Fig. 7*. The effect of an in-plane cyclic mechanical strain of 12% for 5 s (0.2 Hz) every 60 s for 6 h/d on the synthesis of CTGF by human periodontal ligament cells. At each end-point the culture media from six wells was pooled in pairs and assayed in triplicate. The data are cross-sectional and expressed as means ± SEM. There was no statistically significant difference between control and mechanically stressed cultures. CTGF, connective tissue growth factor.

their inhibitor TIMP-1, were all released into the culture media as well as being stimulated by mechanical stress at various points in the time-scale. However, whether they were involved in degrading the pericellular matrix, the supporting scaffold or both is unclear and additional research using activity assays and gelatin and casein zymography will be necessary to establish whether the enzymes are in the active, latent or complexed forms; the MMP-1 ELISA, for example, only recognizes proMMP-1. We have previously reported that human PDL cells in monolayer culture constitutively expressed 16 members of the MMP family at Ct values < 35 (18). The list included seven *MMPs*, three *MT-MMPs* (membrane type-MMPs) and three *ADAMTSs* (a disintegrin and metalloproteinase with thrombospondin motifs), a family of proteinases anchored to the extracellular matrix whose actions include cleavage of the matrix proteoglycans aggrecan and versican (59), plus three *TIMPs*. There are therefore plenty of alternative enzymes available for matrix degradation depending on the substrate, and MMP-1 in addition to cleaving native collagen can also act as a gelatinase (60).

Engineering three-dimensional tissue constructs is rather more complicated than it might first appear, and the challenge is to replicate not only the complexity, but also the mechanical and viscoelastic characteristics (resistance to elastic deformation or stiffness) of the native tissue. One of the disadvantages of hydrogels is their poor mechanical properties, resulting in tissue constructs with significantly poorer mechanical strength than the real tissue (12). In an analysis of the rheological properties of cross-linked HA–gelatin hydrogels for use in soft tissue engineering, the elastic moduli ranged from 11 Pa to 3.5 kPa depending on the concentration of HA (61); Extracel has a shear elastic modulus of about 70 Pa (Glycosan Biosystems; personal communication), which is similar to the 90 Pa reported recently for a three-dimensional collagen gel populated with PDL cells (24). For the purposes of the present investigation this enabled the gel to be mechanically deformed, but for tissue engineering applications a much higher stiffness is required. One problem in trying to reconstruct the biophysical properties of the human PDL, a complex fibre-reinforced tissue that responds to mechanical loading in a viscoelastic and nonlinear manner (62), is that the elastic modulus is, for all practical purposes, unknown. The difficulty of examining thin tissue sandwiched between bone and cementum has resulted in a lack of consistency regarding its elastic properties, highlighted by a recent systematic review where Young's modulus in 23 studies using finite element analysis, was found to range from 10 kPa to 1750 MPa, a difference approaching six orders of magnitude (63).

Culturing cells in three dimensions complicates the perennial question of the strain profile experienced by the cells. As discussed previously (18), this is difficult enough to determine with the methods commonly used to deform cells in two dimensions. When a substrate is deformed, irrespective of whether in-plane or out-of-plane, uniaxial or biaxial, the cells will be exposed to a combination of tensile, compressive and shear strains; the amount of deformation will also vary with the position of the cells within the field, and in the Flexercell system deformation of the substrate will only be about half that programmed into the computer (64,65). In the present study, the strain experienced by the cells at the gel–substrate interface, will clearly be different from that experienced by the cells of the surface layer or within the body of the gel. The stress profile is therefore likely to be similar to the complexity revealed by the finite element analysis of the von Mises and principal stresses generated in the PDL when multirooted teeth are mechanically loaded *in vivo* (66). One thing is certain, understanding the strain distribution within the hydrogel matrix and its effect on the biomechanical behaviour of the cells will require sophisticated three-dimensional finite element modelling.

In summary, we have demonstrated that the addition of a third dimension provides another level of complexity to the existing two-dimensional culture systems designed to investigate the mechanobiology of the PDL. While thin films are suitable for studies of tensile strain, thicker constructs will be needed for investigating the

effects of compression. This will require increasing the complexity of the scaffold by incorporating additional structural molecules such as type I collagen and fibronectin into the matrix to provide extra RGD (Arg-Gly-Asp) binding sites; these will aid cell attachment, proliferation and function, enabling cells to populate the entire gel, while the triple helices of intact collagen will stiffen the matrix. Although primarily developed as a transitional *in vitro* model for studying cell–cell and cell–matrix interactions in tooth support, the system is also suitable for investigating the pathogenesis of periodontal diseases, and importantly from the clinical point of view, in a mechanically active environment.

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