Surrogate Models of Tooth Movement

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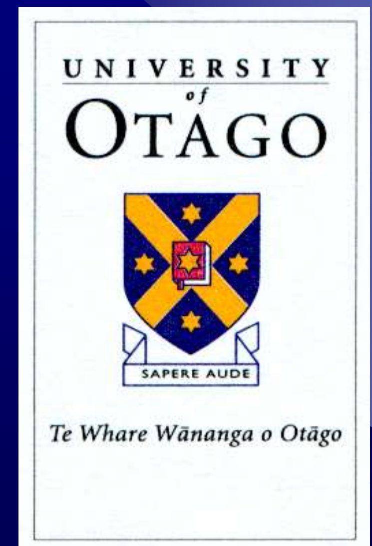
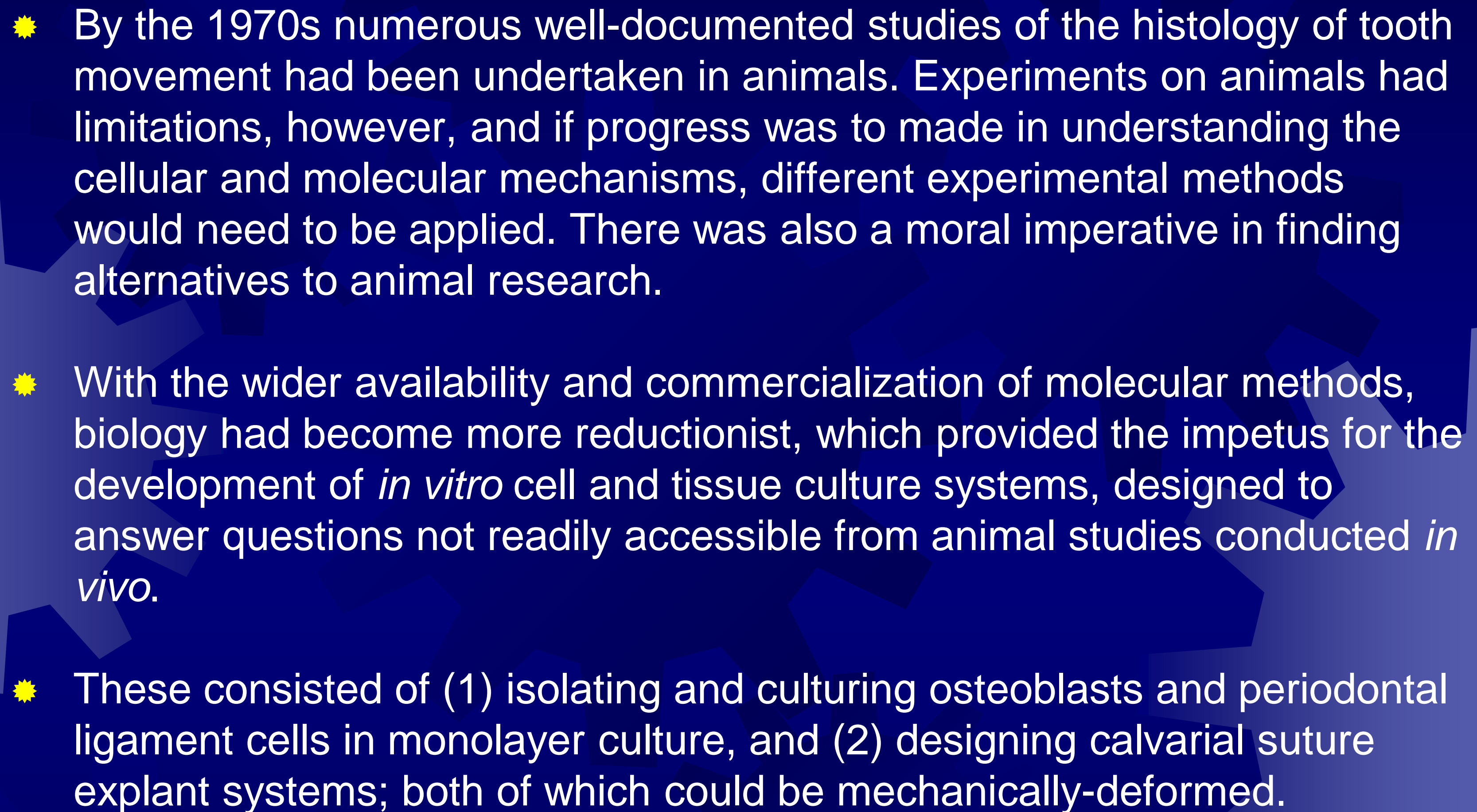
Biological Foundations of Orthodontics and Dentofacial Orthopaedics

Seminar 14

2013

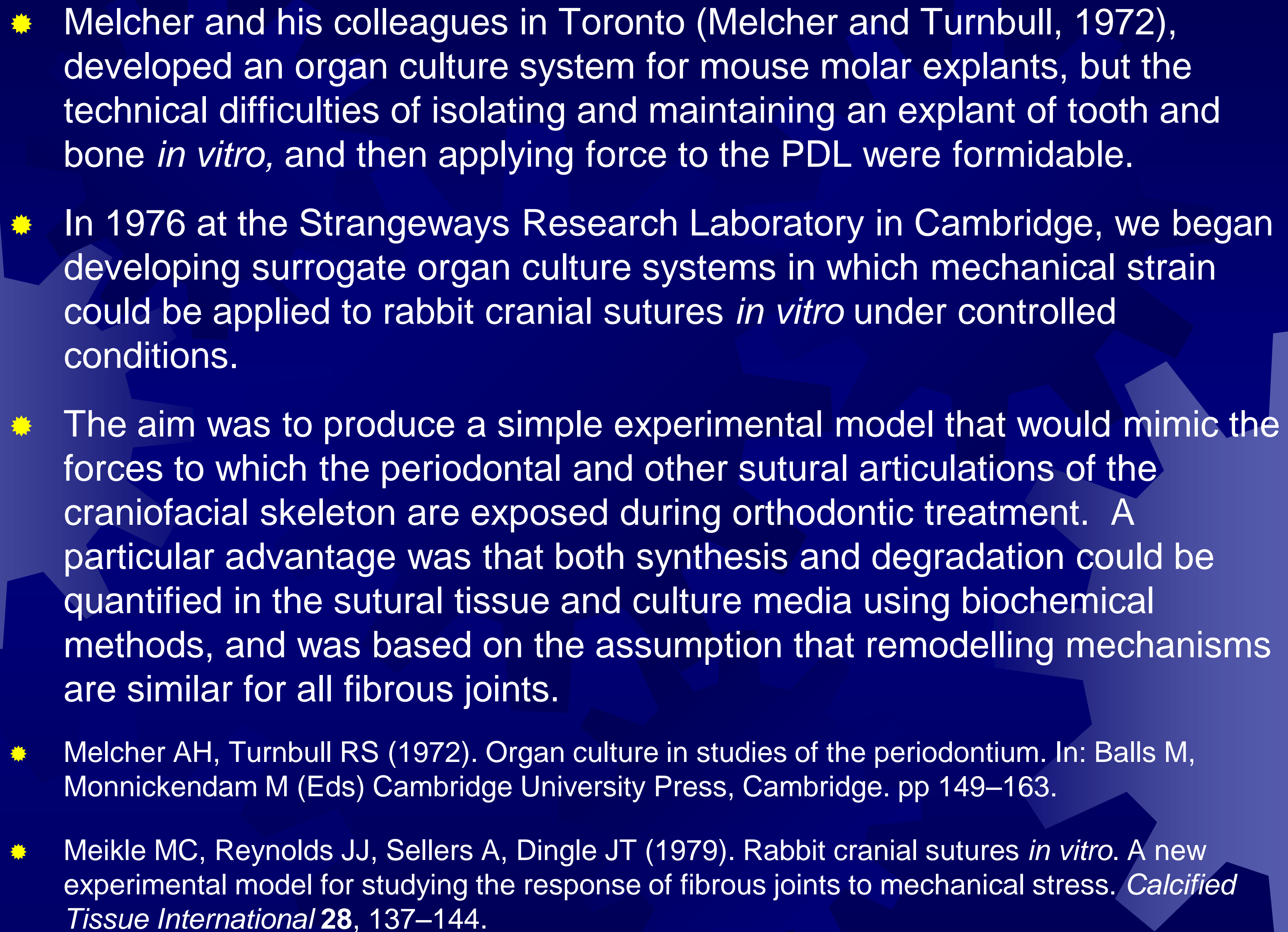




- By the 1970s numerous well-documented studies of the histology of tooth movement had been undertaken in animals. Experiments on animals had limitations, however, and if progress was to made in understanding the cellular and molecular mechanisms, different experimental methods would need to be applied. There was also a moral imperative in finding alternatives to animal research.

- With the wider availability and commercialization of molecular methods, biology had become more reductionist, which provided the impetus for the development of *in vitro* cell and tissue culture systems, designed to answer questions not readily accessible from animal studies conducted *in vivo*.

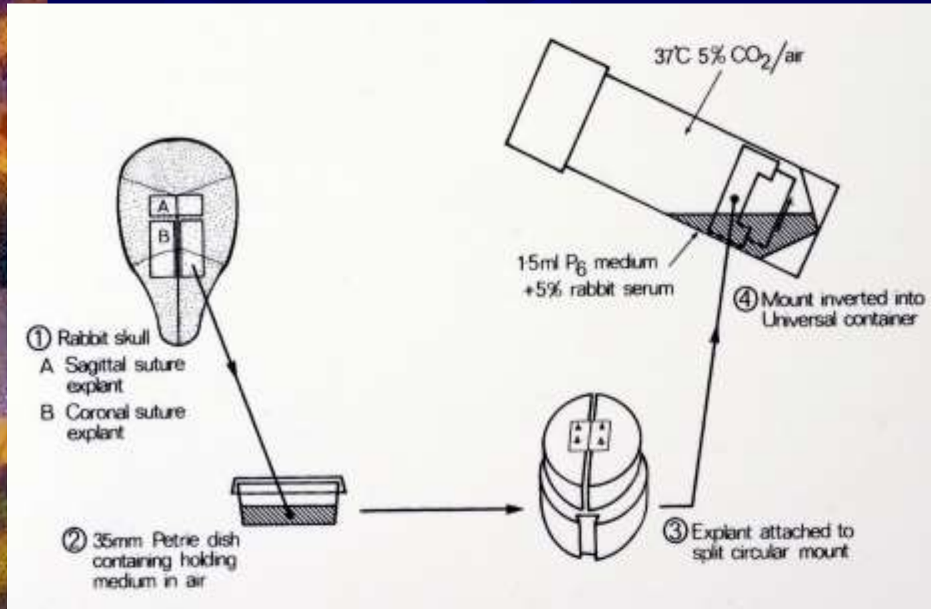
- These consisted of (1) isolating and culturing osteoblasts and periodontal ligament cells in monolayer culture, and (2) designing calvarial suture explant systems; both of which could be mechanically-deformed.

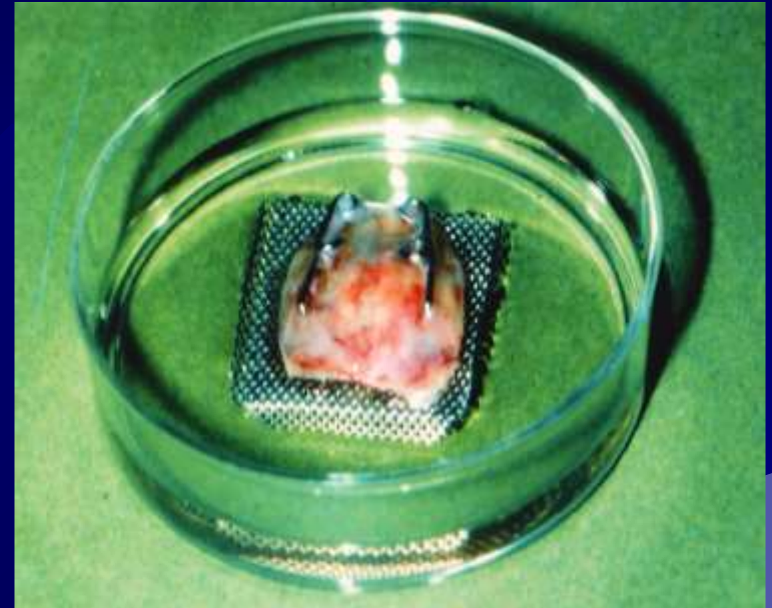


- Melcher and his colleagues in Toronto (Melcher and Turnbull, 1972), developed an organ culture system for mouse molar explants, but the technical difficulties of isolating and maintaining an explant of tooth and bone *in vitro,* and then applying force to the PDL were formidable.
- In 1976 at the Strangeways Research Laboratory in Cambridge, we began developing surrogate organ culture systems in which mechanical strain could be applied to rabbit cranial sutures *in vitro* under controlled conditions.
- The aim was to produce a simple experimental model that would mimic the forces to which the periodontal and other sutural articulations of the craniofacial skeleton are exposed during orthodontic treatment. A particular advantage was that both synthesis and degradation could be quantified in the sutural tissue and culture media using biochemical methods, and was based on the assumption that remodelling mechanisms are similar for all fibrous joints.

- Melcher AH, Turnbull RS (1972). Organ culture in studies of the periodontium. In: Balls M, Monnickendam M (Eds) Cambridge University Press, Cambridge. pp 149–163.
- Meikle MC, Reynolds JJ, Sellers A, Dingle JT (1979). Rabbit cranial sutures *in vitro*. A new experimental model for studying the response of fibrous joints to mechanical stress. *Calcified Tissue International* **28**, 137–144.

Calvarial sutures *in vitro*

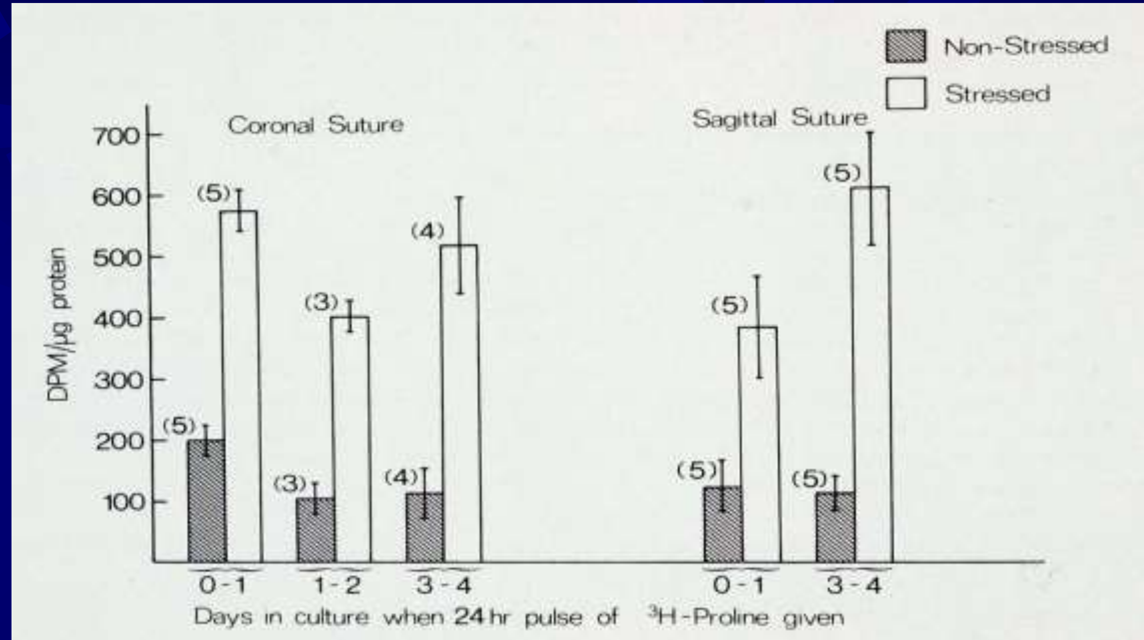




- Experimental procedure for culturing fibrous joints from rabbit calvaria. A tensile strain was applied by a split-circular mount and coil spring to coronal sutures from 1-week-old rabbits, and incubated at 37ºC in a roller culture apparatus rotating at 1 rev/minute. One explant was stressed, the other serving as control.
- Sagittal suture explant cultured on a stainless steel mesh grid in a 5-cm Petri dish; tensile stress was applied with a helical coil spring. The spring could also be adjusted to apply compression.

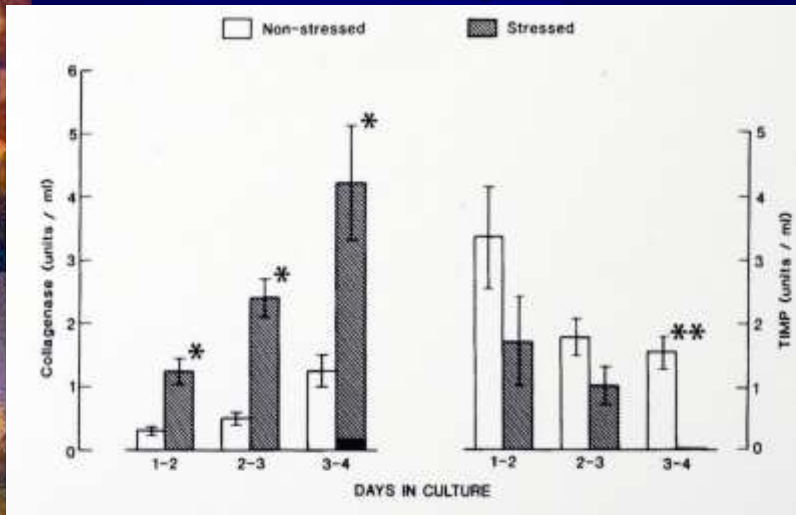
Stimulation of protein and DNA synthesis

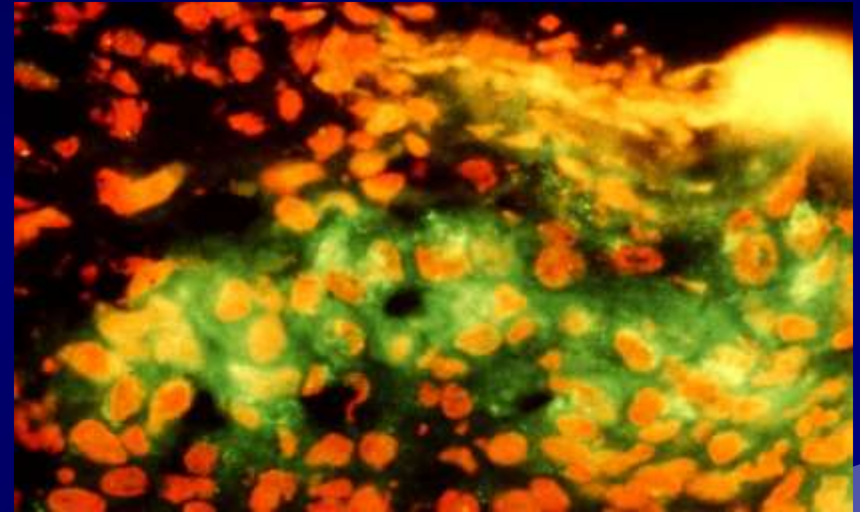
From Meikle *et al*. (1979). *Calcified Tissue International* **28**, 137–144.



- Culturing explants in medium containing ^{3}H-proline resulted in a 2–3–fold increase in suture protein synthesis, and a 2–fold increase in collagen synthesis detectable after 6 h. Control sutures synthesized type I collagen, 20% of newly synthesized collagen in stressed sutures was type III, showing the biomechanical environment is an important determinant of the collagen type. (Meikle *et al. (*1982). *Archives of Oral Biology* **27**, 609–613)
- DNA content of stressed sutures increased 3–fold after 48 h indicating a stimulation in cell proliferation.

Stimulation of matrix degradation





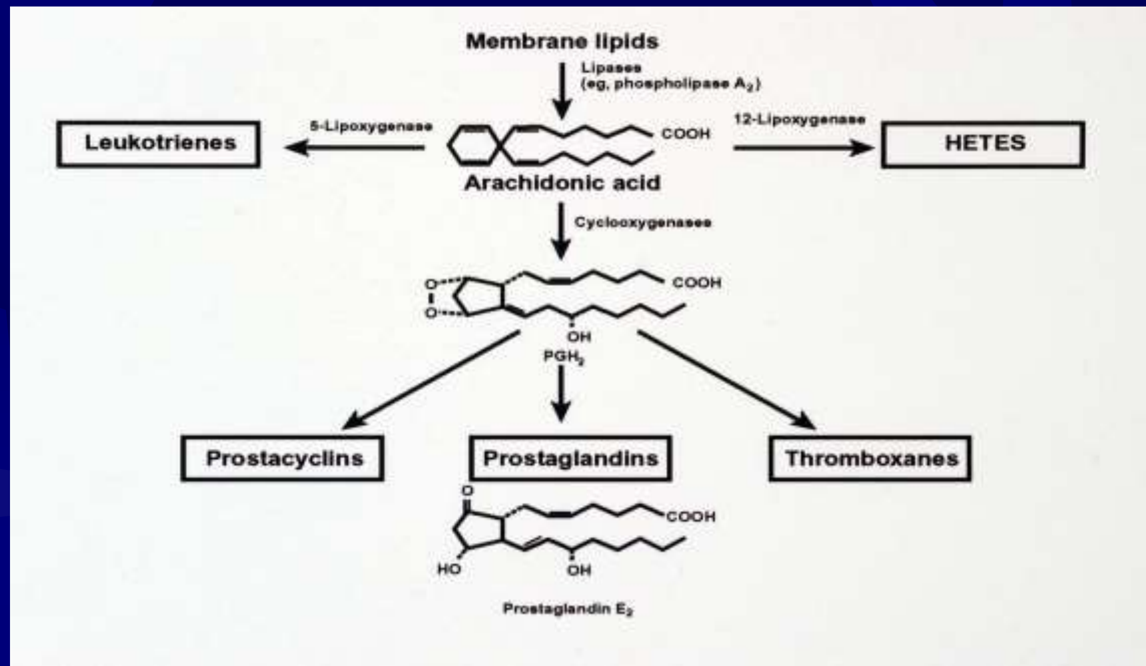
- Mechanically-strained cells did not preferentially synthesize structural proteins. Mechanical deformation also stimulated the synthesis of enzymes responsible for their specific hydrolysis; as in this experiment, which measured changes in the synthesis of collagenase and its inhibitor TIMP (**T**issue **I**nhibitor of **M**etallo **P**roteinases) in the culture media.
- The photomicrograph shows immunolocalization of active collagenase in the suture, suggesting matrix degradation is a prerequisite for cell proliferation to accommodate an increase in the cell population.
- From Green *et al*. (1990). *American Journal of Orthodontics and Dentofacial Orthopedics* **97**, 281–288.

Mechanical strain activates multiple cell signalling pathways

- Animal models have demonstrated many important cellular and biochemical changes associated with externally loading bones *in vivo*. However, bone consists of several differentiated cell types making it difficult to determine the response of individual cells. As a consequence, experimental models have been developed which involve culturing cells in monolayers on a substrate that can be mechanically deformed.

- Connective tissue cells respond to a variety of physical stimuli and there is abundant evidence from *in vitro* models that deformation of the plasma membrane by mechanical means initiates specific biochemical changes within the cells. Alterations in prostaglandins, cyclic nucleotides, inositol phosphates and stretch-activated ion channels have all been reported in mechanically deformed cells. These signalling pathways lie downstream from the initial mechanoreception event at the cell surface.

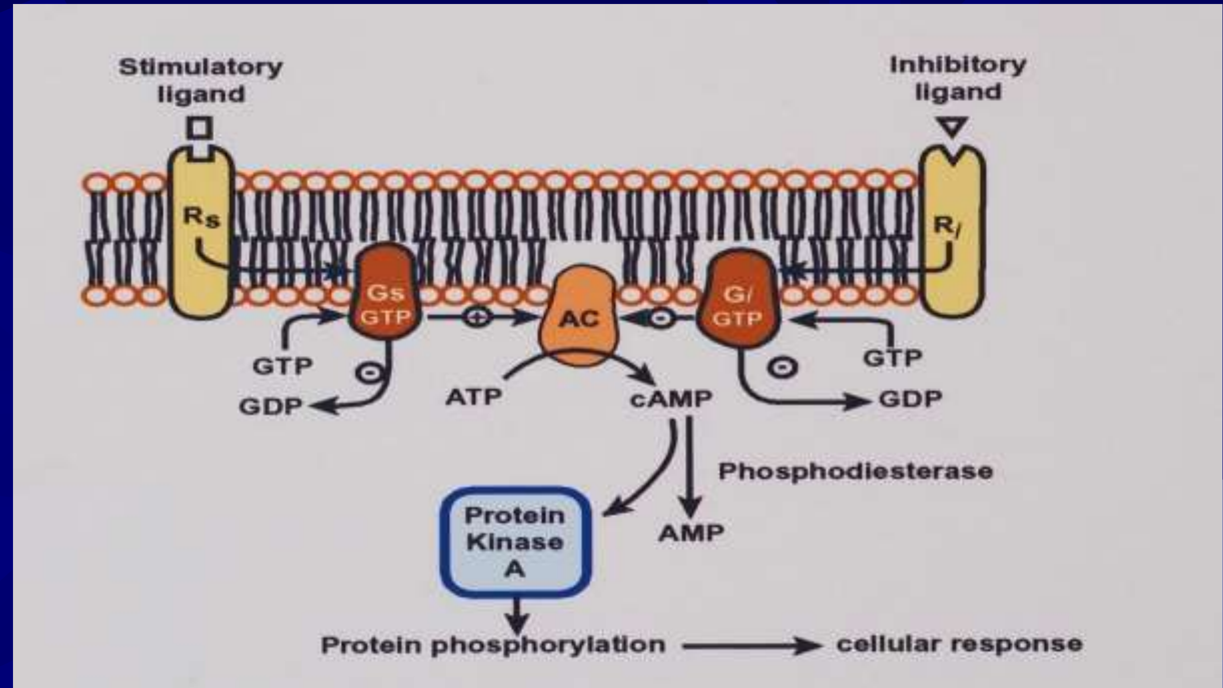
Role of prostaglandins



- PGs are metabolites of arachidonic (eicosatetraenoic) acid, discovered by von Euler (1934) in the prostate gland – hence the name. However, PGs are ubiquitous.
- Arachidonic acid is present in precursor form in membrane lipids from which it is released through the action of phospholipases. Arachidonic acid can be (1) converted to PGs, prostacyclins and thromboxanes via the cyclooxygenase pathway, or (2) acted upon to form leukotrienes and HETEs via the lipoxygenase pathway.

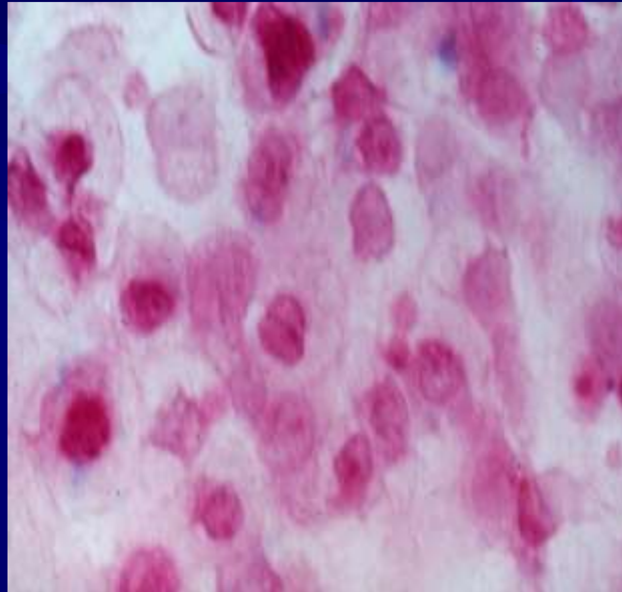
The cyclic AMP pathway

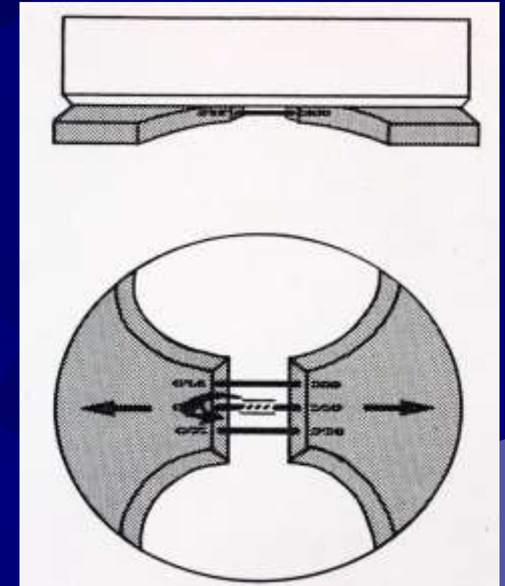
Adapted from Berridge (1985), *Scientific American*.



- External messengers such as hormones and cytokines are regulated by stimulatory (Rs) and inhibitory (Ri) receptors. These interact with G proteins to stimulate or inhibit adenylate cyclase (AC) which converts ATP to cAMP.
- cAMP binds to the regulatory component of its protein kinase to liberate the catalytic component which is then free to phosphorylate specific proteins that regulate the cellular response. Inactivation of cAMP is effected by the enzyme phosphodiesterase.

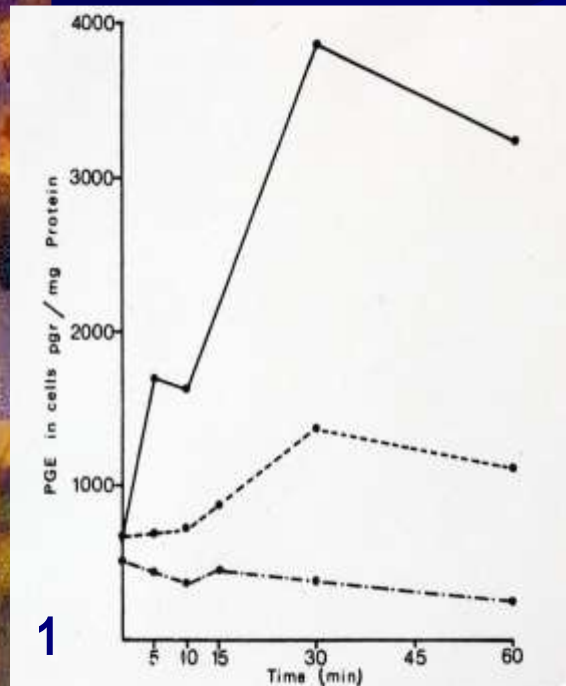
Effect of mechanical stress on cultured bone cells

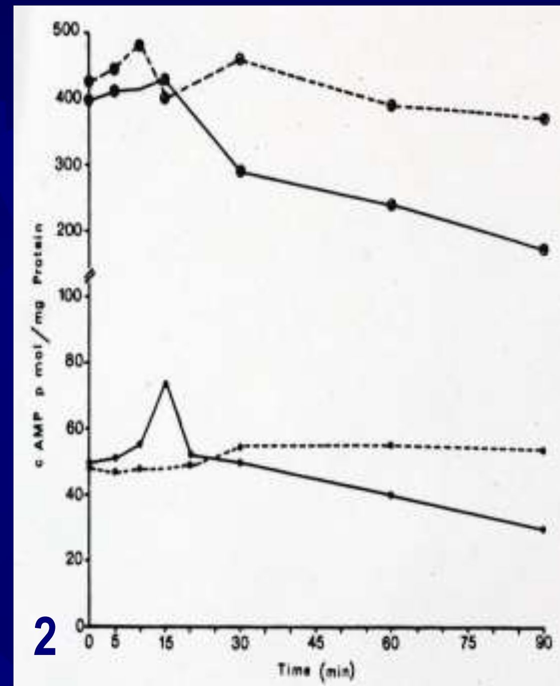


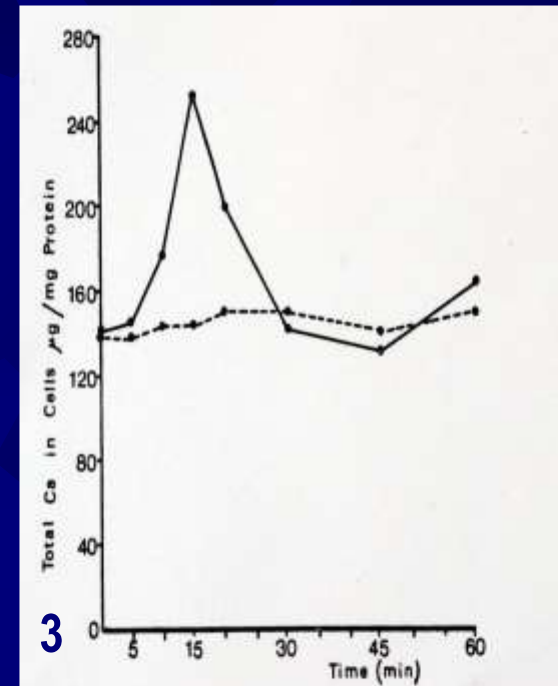


- During the 1970s methods were developed for growing cells *in vitro* which could then be mechanically-deformed. These cells are mouse calvarial osteoblasts in monolayer culture stained for alkaline phosphatase (ALP), an osteoblast marker.
- Harell *et al*. (1977) cultured osteoblasts in Petri dishes, which were deformed by means of an orthodontic screw cemented to the base.
- Diagram from Harell *et al*. (1977). *Calcified Tissue Research* **22**, 202–207..

Prostaglandins, cAMP and calcium



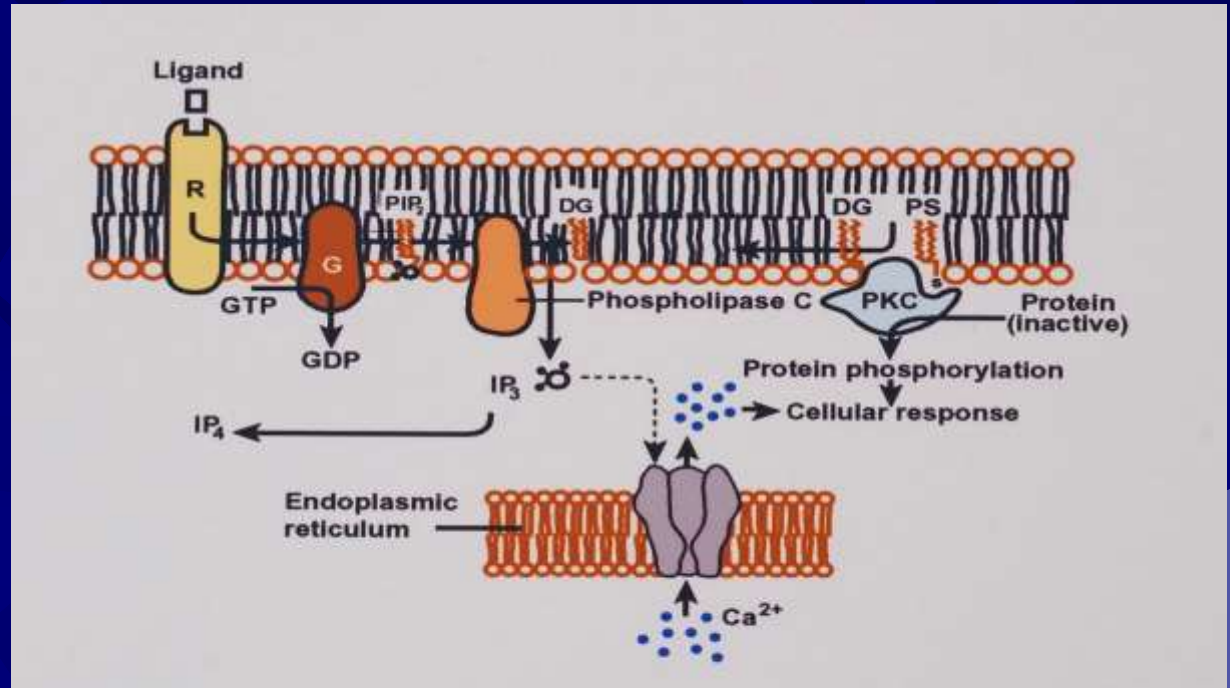




- They found (1) an almost immediate increase in PGE_2 at 5 min, which reached a plateau at 30 min due to *de novo* synthesis being inhibited by the COX inhibitor indomethacin.
- This was followed by (2) an increase in cyclic AMP, which peaked at 15 min, with (3) a simultaneous increase in intracellular [Ca^{2+}] which also peaked at 15 min.
- From Harell *et al*. (1977). *Calcified Tissue Research* **22**, 202–207.

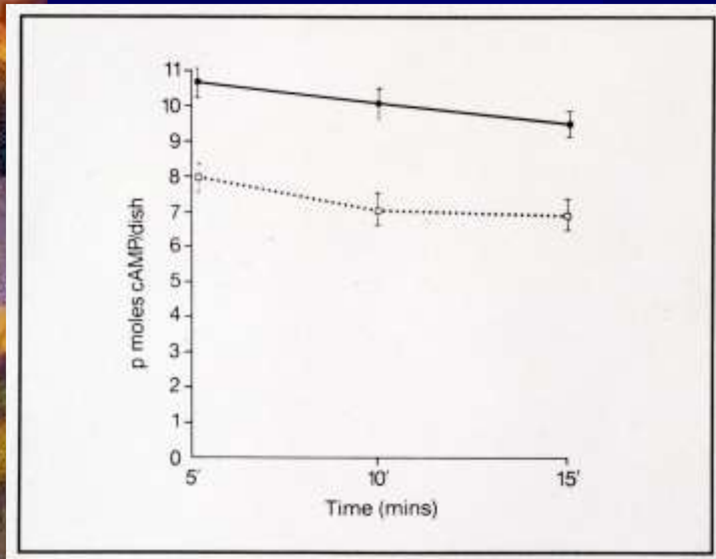
The phosphoinositide pathway

Adapted from Berridge (1985). *Scientific American*.



- External ligands transmit information through a stimulatory G protein (G) to activate phospholipase C which cleaves PIP_2 into diacylglycerol (DG) and inositol triphosphate (IP_3).
- IP_3 is water soluble and diffuses into the cytoplasm where it mobilizes Ca^{2+} from the ER. DG remains within the cell membrane and activates PKC which then phosphorylates a protein. The continued action of these two second messenger pathways accounts for early and sustained cellular responses.

Dual elevation of cAMP and inositol phosphates by mechanical stress



	Control (a)	Mechanical (b)	Mean ratio (b/a)	F
Osteoblasts	20256	33547	1.50	226*
	30732	51147		145**
	19532	21019		9.1*
Osteoblasts + PGE2	14010	18408	1.24	6.7*
	11299	12877		10*

From Sandy *et al.* (1989). *Biochimica et Biophysica Acta.* **1010**, 265–169.

- Left. Time course of cAMP activation by intermittant mechanical stress in osteoblast monolayer cultures.
- Right. Mean accumulation of ^{3}H-inositol phosphates in osteoblast cultures expressed as dpm/dish.
- The PI pathway accounts for many of the changes seen in mechanically-deformed tissues including the elevation of intracellular [Ca^{2+}] and increased DNA synthesis.

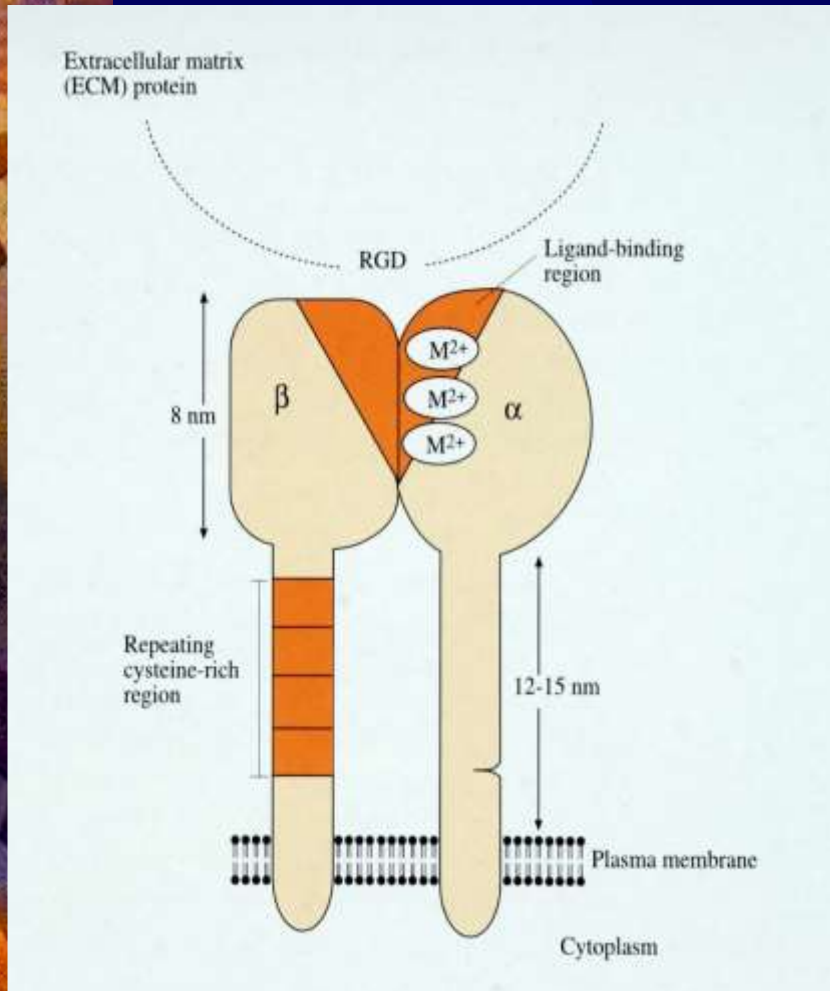
Mechanotransduction through integrins

- Signalling pathways such as cAMP, inositol phosphates and stretch-activated ion channels, lie downstream from the initial mechanotransduction event at focal adhesions, where integrin receptors link the extracellular matrix to the cytoskeleton.

- Integrins function both as cell adhesion molecules and outside-in signalling receptors, and numerous signalling pathways have been shown to be activated by integrins, including the low molecular weight GTPases, Rab and Rho, and MAP (mitogen-activated protein) kinase subtypes. Both are altered in mechanically-stretched periodontal ligament fibroblasts (Basdra *et al*. 1995) and osteoblasts (Peverali *et al*. 2001).

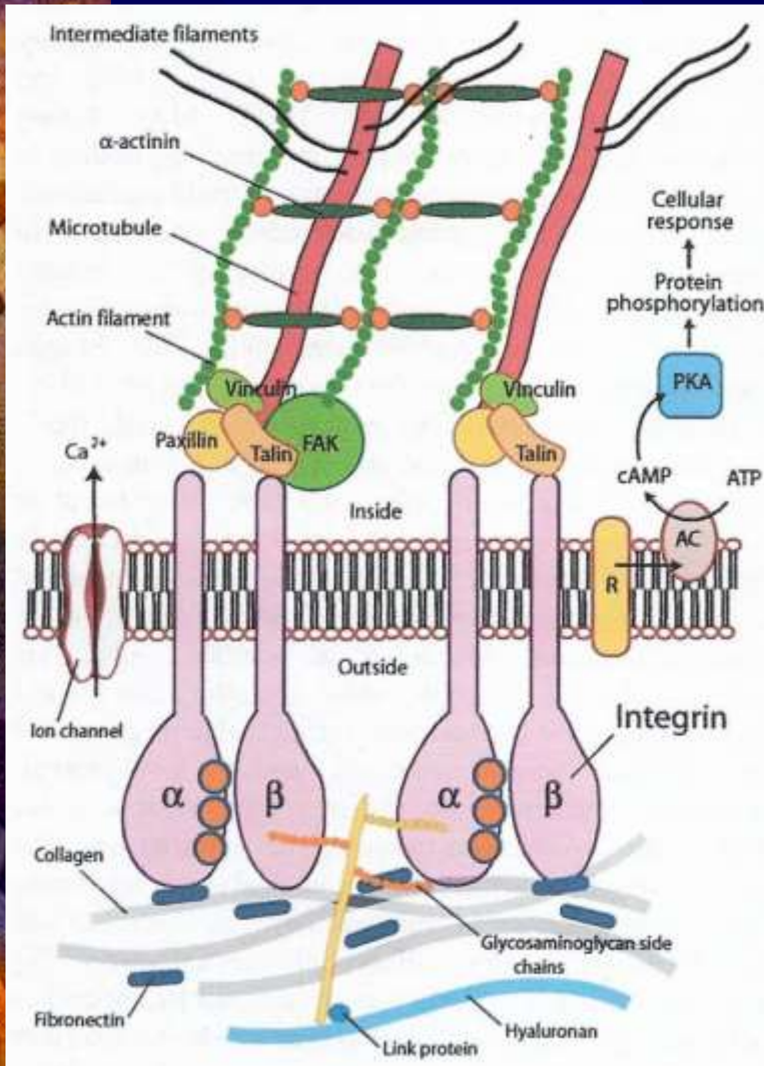
- Basdra *et al.* (1995). *Biochimica et Biophysica Acta* **1268**, 209–213.
- Peverali *et al*. (2001). *Molecular Medicine* **7**, 68–78.

Integrins: cell-surface receptors



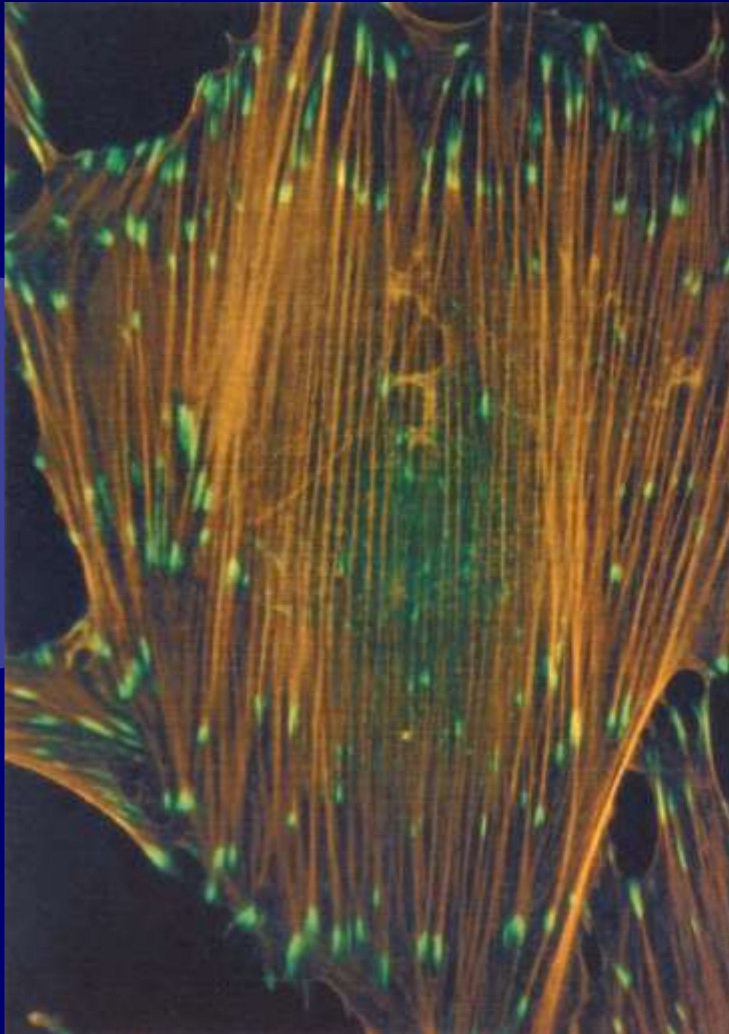
- Each integrin receptor consists of two glycosylated polypeptides, the α and β chains, whose *Mr* varies from 100,000 to 140,000.
- The α chain has several binding sites for divalent cations [M^{2+}] which stabilizes the connection to the β chain; each β chain has four cysteine-rich repeated sequences.
- The extracellular domain binds to ligands such as the RGD (arg-gly-asp) sequence and the cytoplasmic domain of the β chain is connected to actin associated cytoskeletal proteins.
- Adapted from Hynes RO (1992). *Cell* **69**, 11–25.

Integrin receptors and focal adhesions



- Focal adhesions are sites where integrins link actin-associated cytoskeletal proteins (talin, vinculin, α-actinin) and signaling molecules such as FAK (focal adhesion kinase) and paxillin to the structural macromolecules of the extracellular matrix, such as collagen, fibronectin and proteoglycans.
- Firm attachment to focal adhesions enables mechanical deformation of the cell to be recognized by (1) the cytoskeleton and intracellular signaling pathways, and (2) mechanosensitive ion channels, phospholipids and G-protein-coupled receptors in the plasma membrane.
- AC, adenylate cyclase; PKA, protein kinase A; R, G-protein-coupled receptor.

Focal adhesions



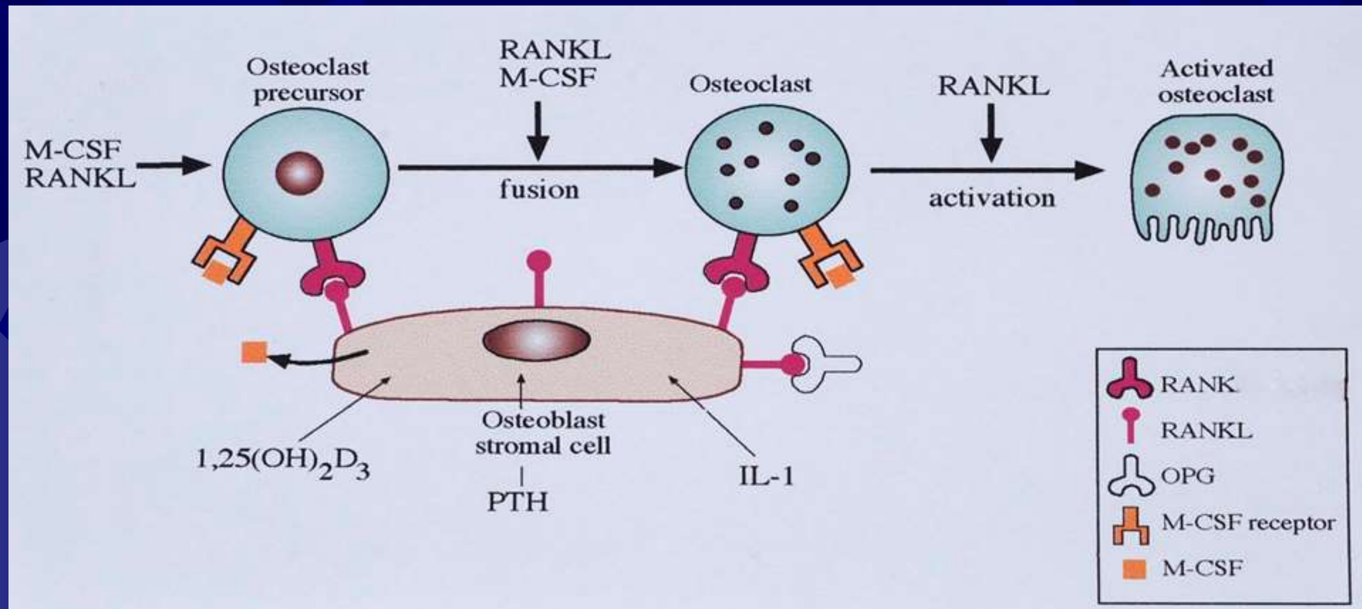
- Focal adhesions localize at the end of actin stress fibres. In this figure a rat embryo fibroblast has been immunostained for the focal adhesion protein vinculin (green) and for actin (brown).
- Adhesion to the substrate is mediated by integrins and stimulation of contractility by serum factors has generated isometric tension resulting in alignment of actin filaments to form stress fibres.
- From Sastry and Burridge (2000). *Experimental Cell Research*. **261**, 25–36.

Cytokines: cell–cell signalling molecules

- One of the most significant advances in connective tissue biology during the 1980s was the demonstration that many cytokines, in addition to mediating the host immunological response to exogenous antigens, were also produced by connective tissue cells such as fibroblasts and osteoblasts and were clearly involved in normal physiological turnover and bone remodelling.

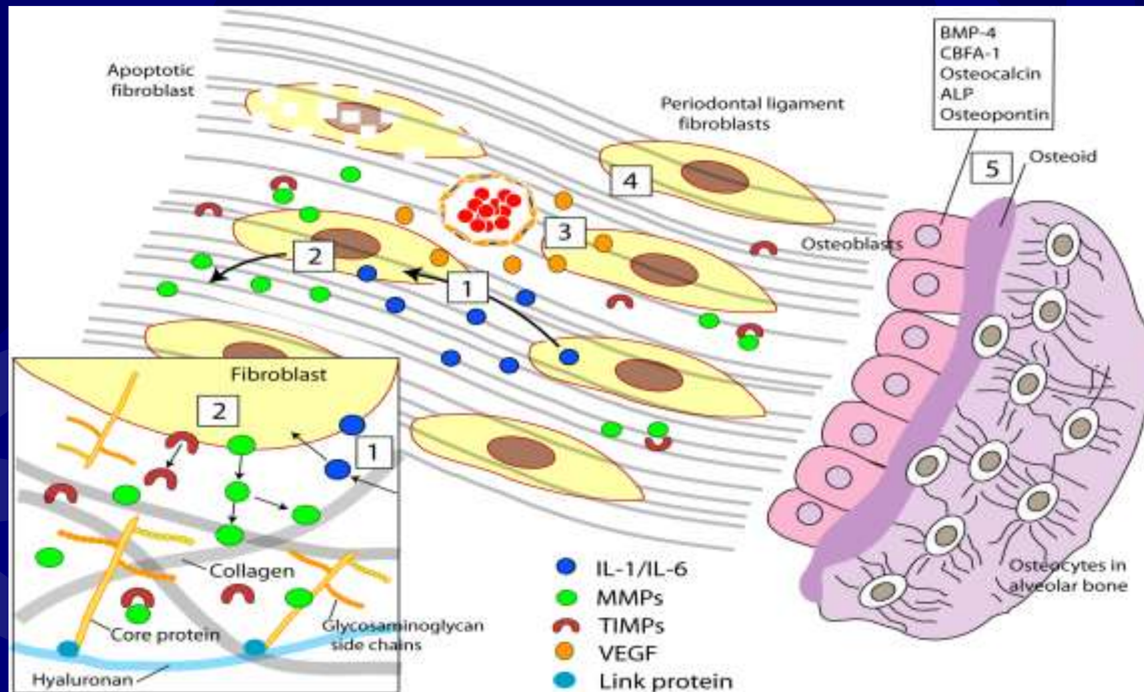
- Cytokines are small molecular weight signalling molecules (mw<25kDa) produced by cells that regulate or modify the action of other cells in an autocrine (acting on the cell of origin) or paracrine (acting on adjacent cells) manner through cell surface receptors. The definition includes the interleukins, tumour necrosis factors, interferons, growth factors and colony stimulating factors.

RANK, RANKL and OPG



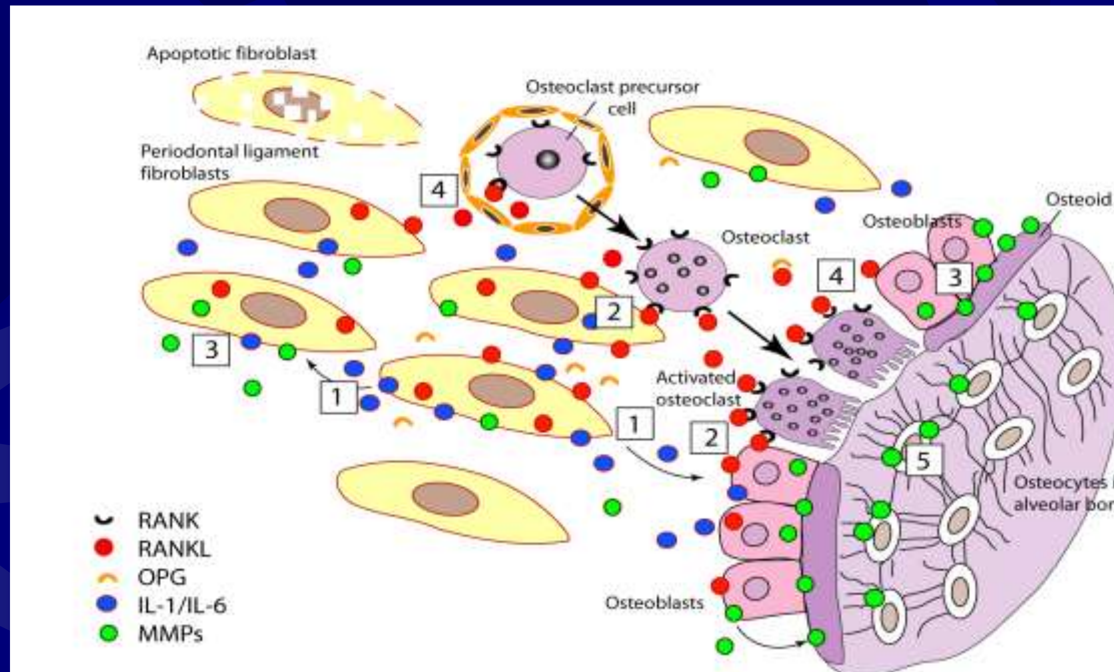
- Receptors for bone resorptive agents such as PTH, 1,25 $(OH)_2vitD_3$ and IL-1 are expressed on osteoblasts, not osteoclasts.
- These stimulate the synthesis of RANKL (receptor activator of NFκB ligand) by osteoblasts, which promotes osteoclast differentiation and function by binding to RANK expressed on osteoclasts and their precursor cells. OPG (osteoprotegerin) acts as an inhibitor of osteoclast formation by competing with RANKL for the membrane receptor.
- From Meikle (2002). *Craniofacial Development, Growth and Evolution*.

Hypothetical model–tension side



- (1) PDL fibroblasts under tensile strain synthesize IL-1 and IL-6, (2) these in turn stimulate MMP and inhibit TIMP synthesis by PDL cells.
- (3) VEGF secretion promotes angiogenesis, and degradation of the ECM by MMPs facilitates cell proliferation and capillary growth.
- (4) PDL cells synthesize collagens and proteoglycans, and (5) osteoblasts synthesize structural proteins and new bone matrix.

Hypothetical model–pressure side

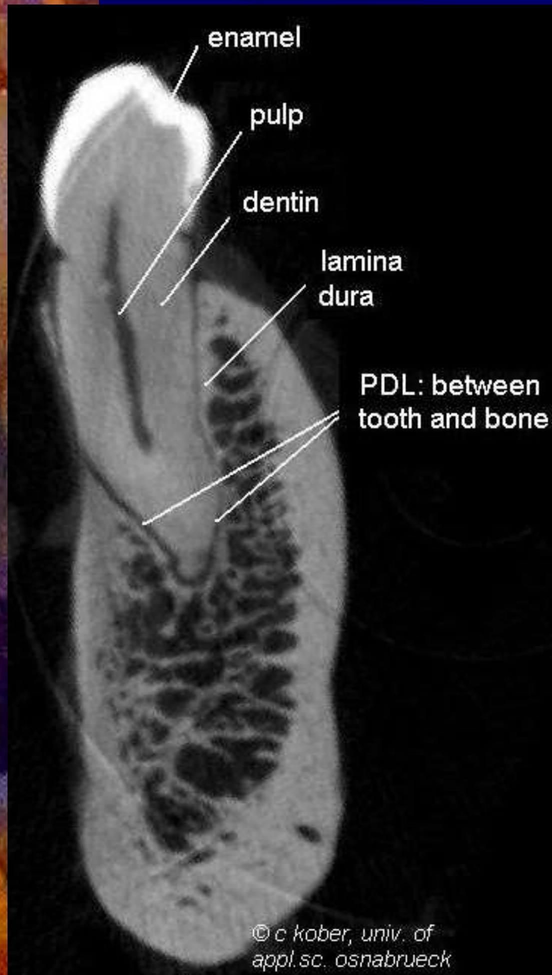


- (1) Compression of PDL cells results in the synthesis of IL-1 and IL-6, which in turn stimulates RANKL (2) and MMP (3) synthesis by PDL cells and osteoblasts.
- (4) RANKL stimulates osteoclast formation and function, while MMPs produced by osteoblasts degrade the osteoid layer enabling osteoclasts to access the mineralized matrix; (5) deformation of alveolar bone up-regulates MMP expression by osteocytes in the adjacent bone.

Response of human PDL cells to mechanical strain *in vitro*

- One of the advantages of the commercialization of molecular biology was that clinical departments could study genomics and proteomics without a large well-founded laboratory, and limited financial resources and technical help.
- The following discussion covers work by DClinDent orthodontic postgraduate and doctoral research students at the University of Otago, and National University of Singapore, in which human PDL cells were subject to cyclic strain and the RNA extracted. The samples were then screened by Superarray Bioscience Corporation (Frederick, Maryland, USA) for the expression of genes of interest using real time RT-PCR, technology that enabled interacting cytokine networks to be studied instead of just individual mediators.
- Although significant progress is being made, our understanding of a very complex biological process remains far from complete. This is true of the remodelling dynamics of both the PDL and alveolar bone.

The PDL and bone represent two different functional domains



- The PDL evolved to provide for the eruption and attachment of teeth to the bones of the jaws, and in addition, also prevents the fusion or ankylosis of teeth to the bone.
- Together with the supporting bone, the PDL develops from the dental follicle, a connective tissue composed of ectomesenchymal cells surrounding the developing roots, derived from the cephalic neural crest.
- From a biomechanical point of view the PDL and bone can be regarded as two distinct functional domains, and our strategy to date has been to correlate data from (1) cultured osteoblasts, and (2) *in vitro* models of the PDL, with histological and serum data from rat models of tooth movement.

Preparation of human PDL cells

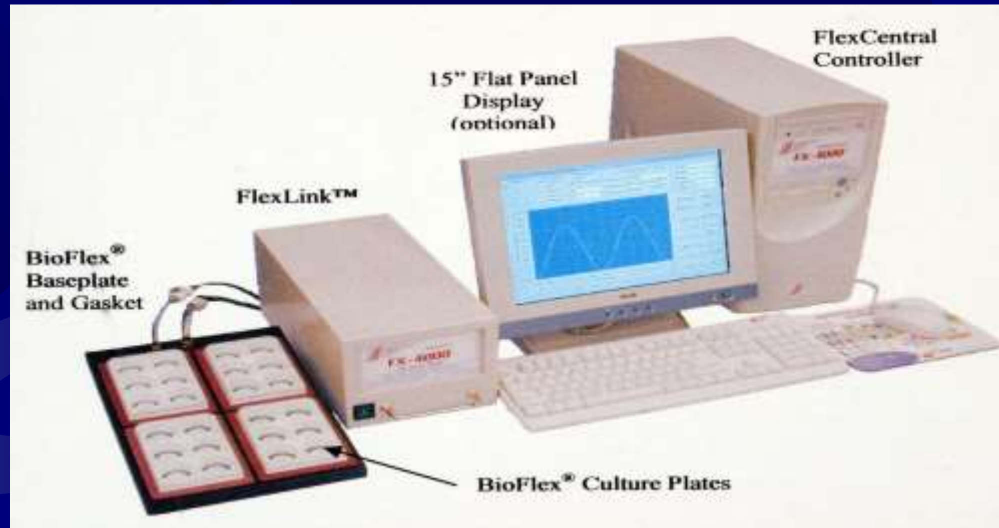
- Human PDL cells are obtained by scraping remnants of the PDL from the roots of newly extracted premolar teeth for orthodontic reasons as described by Somerman *et al*. (1988). Human PDL cells can also be purchased commercially.
- The explants are then plated out onto 30–50 mm Petri dishes and cultured in DMEM (Dulbecco's modification of Eagle's medium), plus serum and antibiotics at 37°C. Cells grow out from the explants and after a few days reach confluence.

- Somerman *et al.* (1988*). Journal of Dental Research* **67**, 66–70.

Expansion of PDL cells

- Once the cells have reached confluence they are removed by trypsinization, and then expanded in Falcon flasks to obtain sufficient cells for experimental purposes.
- Normally 3rd to 4th passage cells (P3–P4) are used since all cultured cells become progressively senescent with each passage.

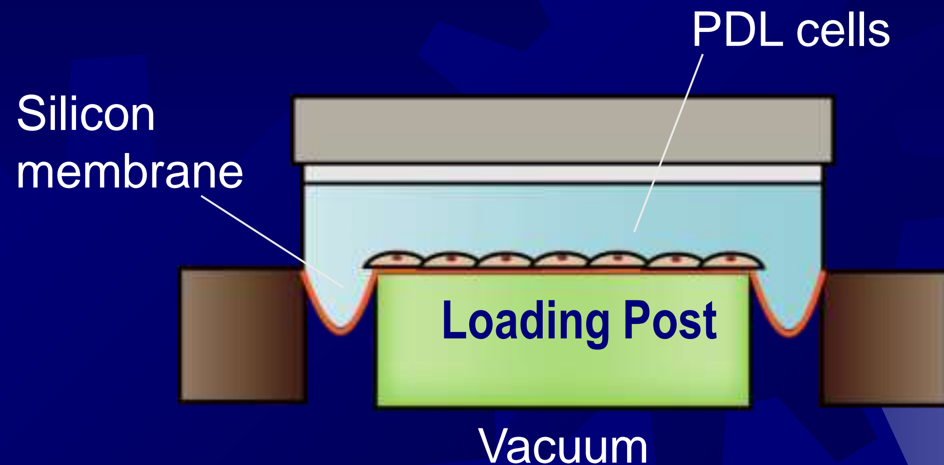
Flexercell FX-4000 strain unit



- In the early days investigators including ourselves used a variety of Heath Robinson contraptions to mechanically deform cells. Commercial strain units and accessories are now available and transformed the field.
- The instrument we have used with some success for several years in a number of investigations is the Flexercell FX-4000™ Strain Unit, a computerized instrument able to apply controlled, static or variable cyclic tension or compression to cells *in vitro*. Such set-ups don't come cheap, but are a valuable addition to the research laboratory of university orthodontic departments for staff and post-graduate student research.

In-plane deformation of PDL cells

Uniflex culture plate



- To evaluate the osteogenic potential of PDL cells, a 12% uniaxial cyclic tensile strain was applied for 5 sec (0.2 Hertz) every 90 sec for 6–24 h to cultured human PDL cells in monolayer culture and the RNA extracted. Vacuum pressure is applied to suck down the edges of the membrane. Owing to the Poisson effect there is no such thing as a purely tensile strain.
- Samples were screened for the expression of 78 genes of osteogenic significance by Superarray Bioscience, Maryland, USA, using the RT² Profiler PCR Array System. Expression profiles of the target genes were measured relative to the mean critical threshold (CT) values of 5 different calibrator genes (GAPDH, β-2-microglobulin, β-actin, HPRT1 and RPLI3A).

Altered gene expression

From Wescott *at al.* (2007). *Journal of Dental Research* **86**, 1212–1216.

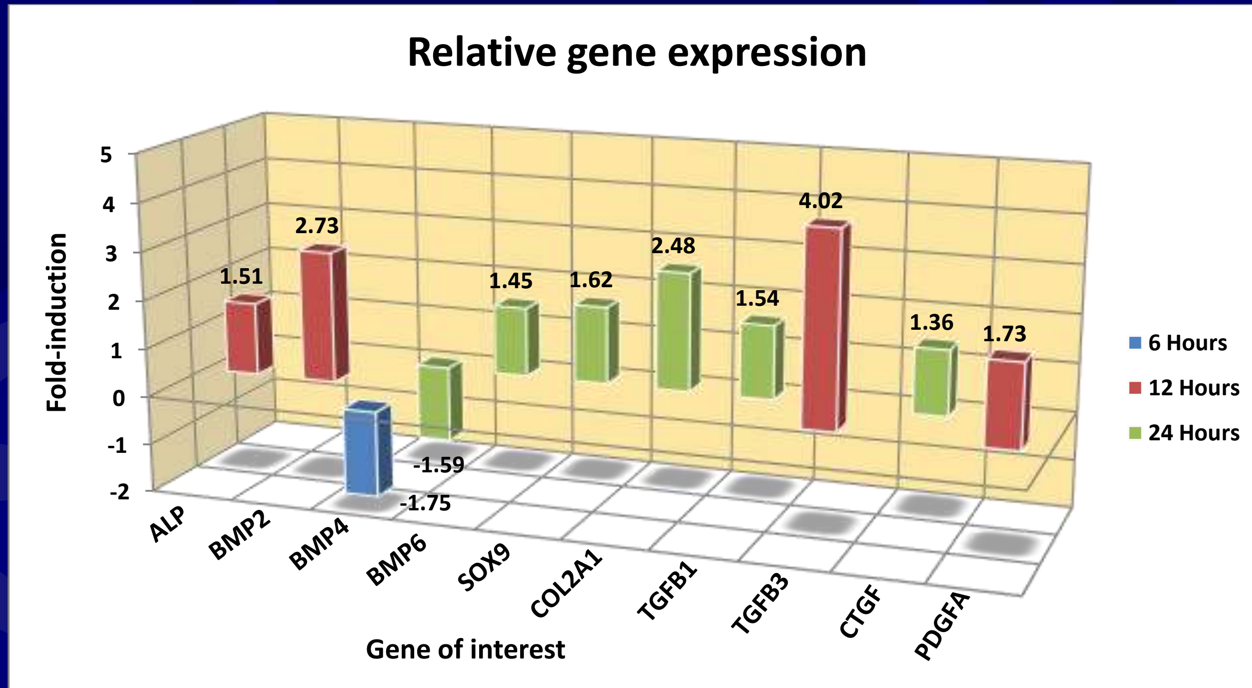
Table. Alterations in Gene Expression by Cultured Human Periodontal Ligament Cells Following Intermittent Tensile Mechanical Strain

		Fold Up- or Down-regulation (exp/control)					
Name of Gene	Description	6 hrs	p-value	12 hrs	p-value	24 hrs	p-value
ALPL	Alkaline phosphatase (liver/bone/kidney)	2.15*	0.2863	1.51	0.0140	-1.00	0.9977
ANXA5	Annexin A5	-1.01	0.9549	-1.08	0.4419	-1.14	0.0400
BMP2	Bone morphogenetic protein 2	1.64	0.2167	2.73	0.0412	-1.28	0.5302
BMP4	Bone morphogenetic protein 4	-1.75	0.0335	-1.33	0.3609	-1.58	0.0118
BMP5	Bone morphogenetic protein 5	2.72*	0.1075	ND	ND	ND	ND
BMP6	Bone morphogenetic protein 6	1.05	0.7308	1.12	0.4089	1.45	0.0152
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	-2.21*	0.1647	1.09	0.2399	-1.11	0.5445
COL11A1	Collagen, type XI, alpha 1	-1.38	0.4942	-1.18	0.1561	-1.65	0.0237
COL2A1	Collagen, type II, alpha 1	1.07	0.9252	2.48	0.0127	1.71	0.4639
COL3A1	Collagen, type III, alpha 1	-1.19	0.3269	-1.13	0.0033	-1.32	0.1299
EGF	Epidermal growth factor	-1.01	0.9731	-1.76	0.0182	-1.69	0.0102
ICAM1	Intercellular adhesion molecule 1	1.09	0.8571	3.25	0.0067	1.29	0.2927
IGF1	Insulin-like growth factor 1	1.40	0.5808	2.46*	0.2323	-1.50	0.5736
ITGB1	Integrin, beta 1	-1.30	0.6177	-1.17	0.0367	-1.03	0.7270
MSX1	Msh homeobox homolog 1	1.15	0.2071	-1.27	0.0356	-1.17	0.1314
PHEX	Phosphate-regulating endopeptidase homolog	2.14	0.0057	1.20	0.4715	-1.77	0.2055
SMAD1	mothers against DPP homolog 1 (*Drosophila*)	1.36	0.2384	1.03	0.6335	-1.38	0.0099
SOX9	SRY (sex-determining region Y)-box 9	-1.10	0.7717	1.17	0.4990	1.62	0.0218
VEGFA	Vascular endothelial growth factor	1.12	0.4987	1.03	0.9272	1.41	0.0309

A grey box indicates a statistically significant change. $P < 0.05$.
* Exp/control ratio greater than ± 2, but not statistically significant. $P < 0.05$.
ND, Not detected.

- There was an element of ‘fishing’ in these investigations that is inevitable in screening out ‘genes of interest’ from a vast number of candidate genes.
- A gene was regarded as being constitutively expressed if it was detected at a CT of < 35. The 19 above genes showed either a statistically significant difference in expression, or a T/C ratio of ± 2.

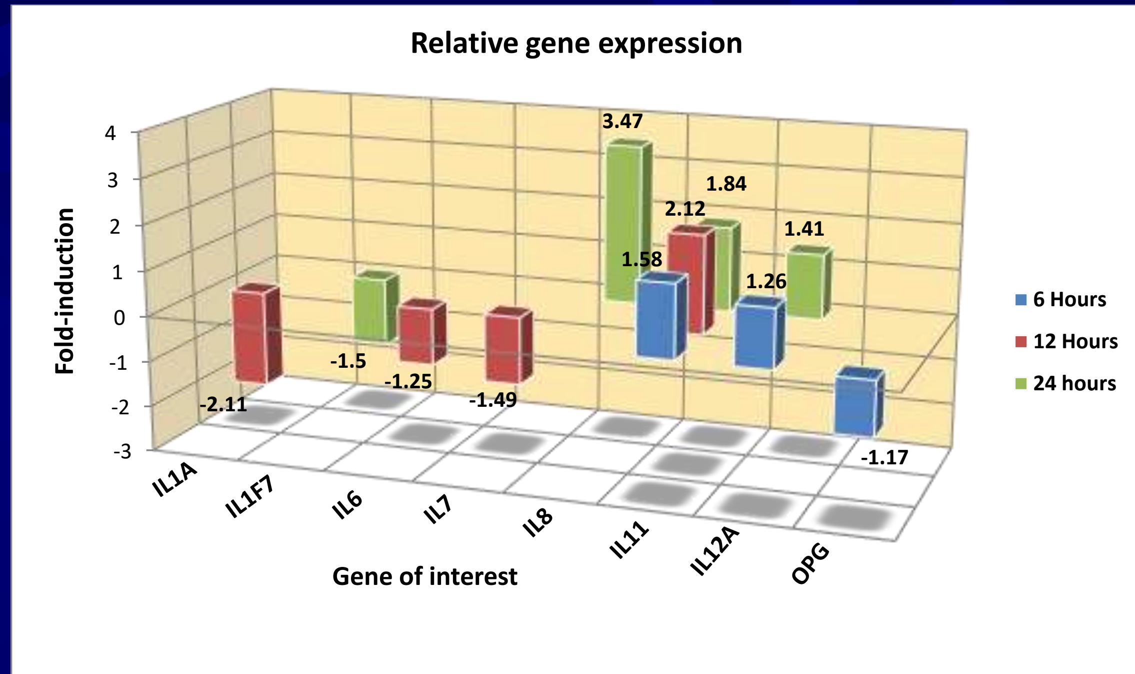
Mechanoresponsive genes



- A 3-D profile of genes showing a significant ($P < 0.05$) up or down-regulation of mRNA expression. See Wescott *et al.* (2007) for discussion.
- The up-regulation of these genes is consistent with an osteogenic (*ALP*, *BMP2*, *BMP6*); chondrogenic (*SOX9*, *COL2A1*) and fibroblastic (*TGFB1*, *TGFB3, CTGF*) response by the PDL cells. PDGF is an important external signal for cell division in fibroblasts.
- Wescott *at al*. (2007). *Journal of Dental Research* **86**, 1212–1216.

Mechanoresponsive interleukins

Pinkerton *et al.* (2008) *Journal of Periodontal Research* **43**, 343–351.



- In another study using the same regimen, 79 cytokine and growth factor genes were targeted; 41 were detected at CT values < 35, and of these 15 showed a significant change in relative expression, including seven multifunctional (pleiotropic) interleukins.
- Among these were: *IL1A*, *IL1F7*, *IL6* and *IL7* (down) and *IL8*, *IL11* and *IL12* (up). Alterations ranged from a 2.11-fold downregulation of *IL1A* to a 3.37-fold upregulation of *IL8* at 24 h. *OPG* (osteoprotegerin) is an important inhibitor of bone resorption was downregulated.

Is orthodontic tooth movement an inflammatory process?

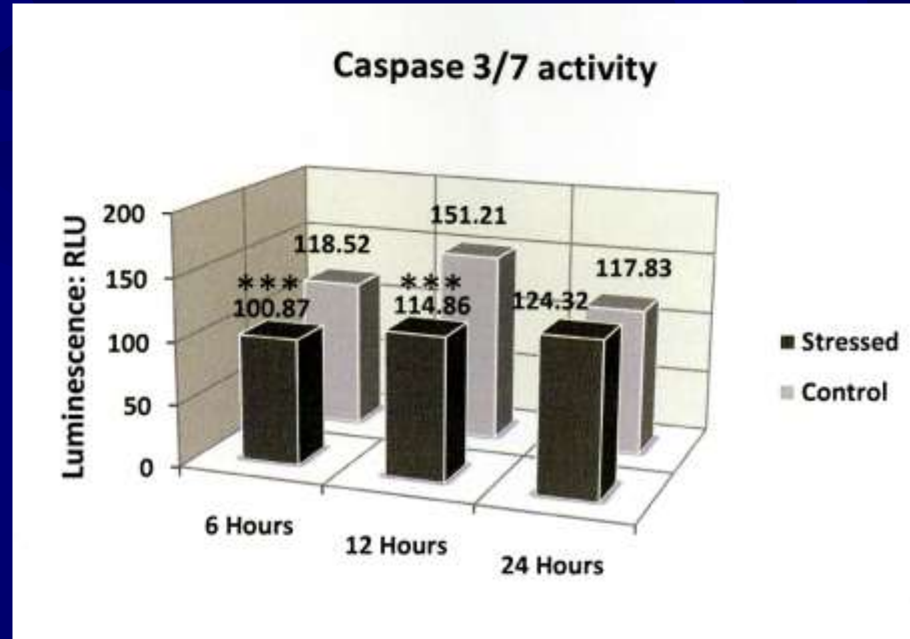
- The demonstration that cytokines, frequently referred to as inflammatory mediators are involved in remodelling the PDL has revived the concept tooth movement is an inflammatory process, and raises two questions: first, are cytokines unique to inflammation and second, to what extent can tooth movement be regarded as an inflammatory event?
- The answer to the first question is no. The naming of cytokines has been based on the biological activity by which it was first identified. Before being sequenced, IL-1 was known by several names (lymphocyte-activating factor, endogenous pyrogen, catabolin, mononuclear cell factor) depending on the bioassay in which it was active.
- They were not referred as cytokines at the time, and many including IL-1 and TNF-α, were eventually shown to be produced by numerous cell types including connective tissue cells.
- The opinion that tooth movement an inflammatory process because it is diminished by NSAIDs, merely shows that arachidonic acid metabolites are involved, and their activity can be blocked by COX inhibitors.

Mechanical strain and cell adhesion

- Cell adhesion plays important roles in maintaining the structural integrity of connective tissues, and sensing changes in the biomechanical environment of cells.
- Cells in culture are not uniformly attached to their substrate, but ‘tack-welded’ at focal adhesions where integrin receptors physically link actin-associated cytoskeletal proteins (talin, vinculin, α-actinin, paxillin), as well as to adhesion molecules on the surface of adjacent cells.
- Integrin-mediated adhesive interactions play key roles in cell migration, proliferation and differentiation, and also regulate intracellular signal transduction pathways that control adhesion-induced (outside-in) changes in cell physiology.
- In a study of 84 adhesion-related genes the following parameters were measured: (1) cell viability by the MTT assay; (2) the activity of two ‘executioner’ caspases 3 and 7, and (3) the expression of 84 genes encoding adhesion-related molecules using real-time RT-PCR microarrays.
- Saminathan *et al.* (2012). *Journal of Periodontal Research* **47**, 212–221.

Mechanical strain and apoptosis

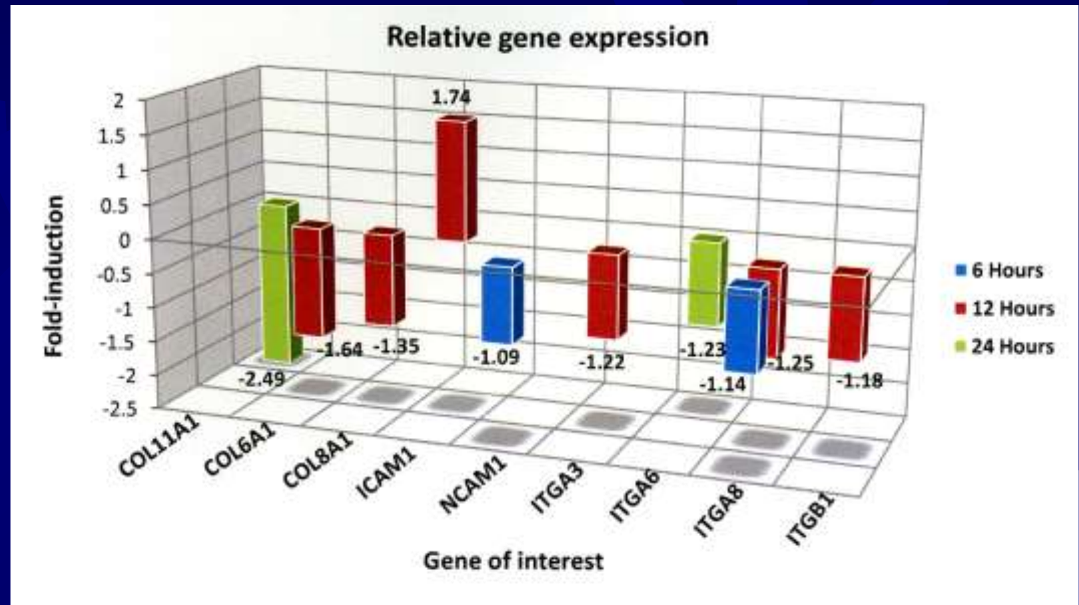
From Saminathan *et al.* (2012). *Journal of Periodontal Research* **47**, 212–221



- An in-plane cyclic strain of 12% to PDL cells resulted in a small reduction in metabolic activity and delivered a transient anti-apoptosis signal to the cells at 6 h and 12 h; this contrasts with reports showing a more vigorous out-of-plane cyclic strain of 20% to PDL cells transiently increases apoptosis (Zhong *et al*. (2008). *Oral Diseases* **14**, 270–276).
- Induction of apoptosis appears to be related to the magnitude and frequency of the applied strain, and likely to be reflected in real life, where the PDL is more heavily stressed during mastication and bruxism.

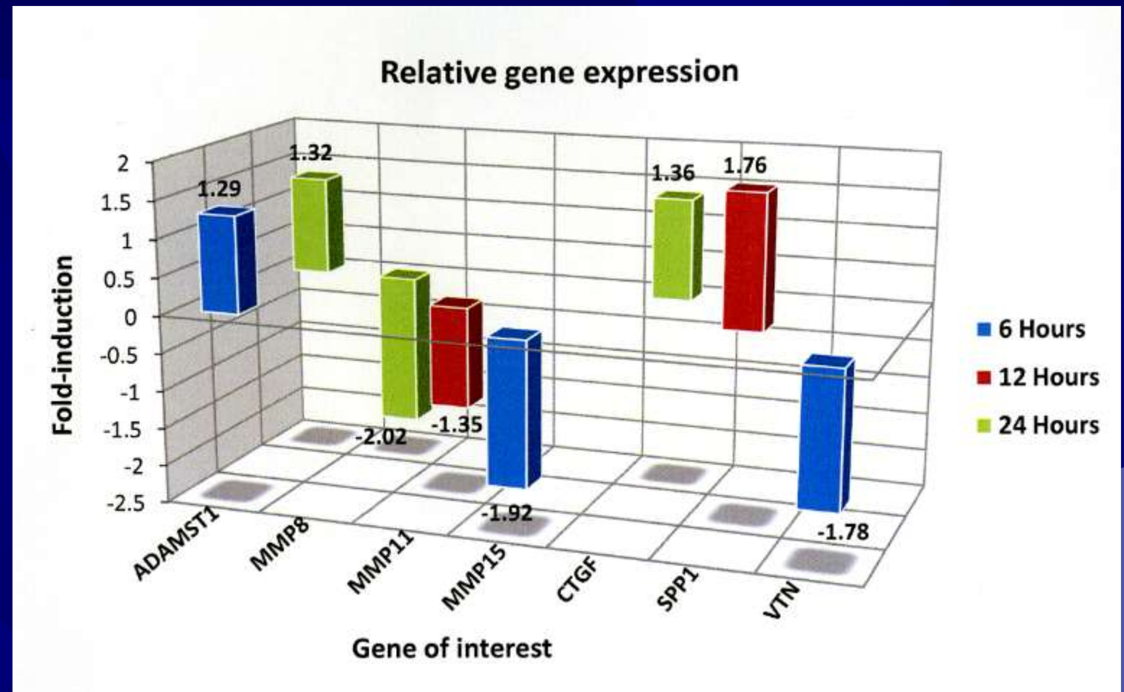
Cyclic strain and adhesion-related genes - I

From Saminathan *et al.* (2012). *Journal of Periodontal Research* **47**, 212–221



- Fold induction (T/C ratio) of 9 genes showing a significant change in mRNA expression: Three coding collagen alpha chains *COL11A1*, *COL6A1*, *COL8A1*; two cell-adhesion molecules *ICAM1*, *NCAM1*; and four integrin subunits *ITGA3, ITGA6*, *ITGA8*, *ITGB1*. All were down-regulated with the exception of the transmembrane protein *ICAM1,* upregulated 1.74-fold.
- Alterations in expression suggests cell adhesion molecules are mostly downregulated to facilitate cellular reorientation. Some are also involved in mechanosensing, and the activation of mechanotransduction pathways.

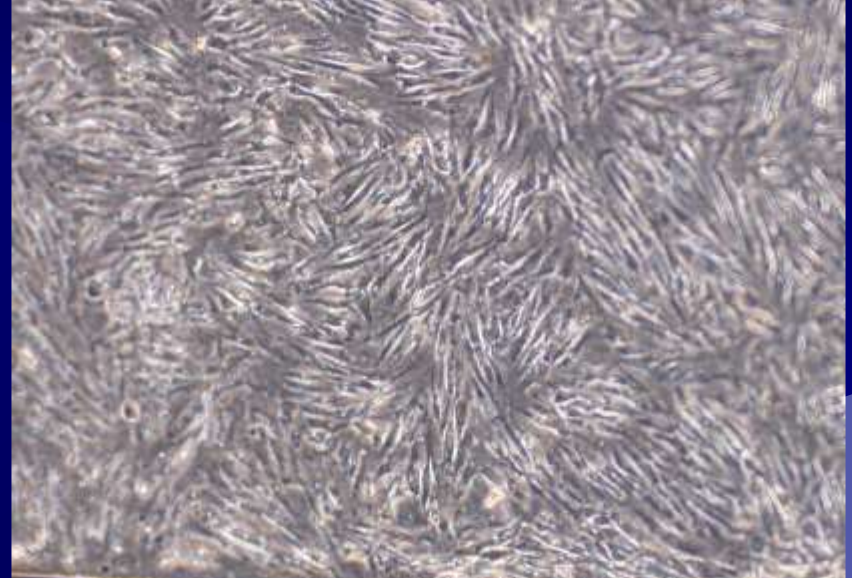
Cyclic strain and adhesion-related genes - II



From Saminathan *et al.* (2012). *Journal of Periodontal Research* **47**, 212–221

- Three-dimensional profile showing statistically significant alterations ($p < 0.05$) in mRNA expression (T/C ratio) of the following seven genes encoding four members of the MMP family of proteolytic enzymes: the fibroblast differentiation gene, CTGF, and the extracellular matrix proteins, SPP1 (osteopontin) and VTN. ADAMST1 was one of only two genes altered across two time points.

Reorientation of PDL cells under cyclic strain



- The application of an in-plane deformation of 12% for 5 sec (0.2 Hz) every 90 sec, using a square waveform, around rectangular ArcTangle™ loading posts resulted in reorientation of the cells. Both images were taken at the edge of the centrally located type I collagen strip.
- Left, control culture, the cells adopt a swirling pattern. Right, stressed culture after 6 h stained by the Giemsa method. Cells become aligned at varying angles to the long axis of the applied strain.

Engineering 3-dimensional constructs of the PDL

- Although PDL cells in 2-D culture systems have been widely used to investigate the mechanobiology of the periodontal ligament, the cells are normally embedded in a complex 3-D extracellular matrix composed of collagens, proteoglycans and noncollagenous proteins – not attached to tissue culture plastic or substrates of type I collagen or fibronectin. In other words, the cells are in a functional default state, and with the absence of mechanical stimuli a double-default state.

- To address the deficiencies of conventional 2-D systems, hydrogels have been widely used as scaffolds to create a variety of 3-D tissue constructs. Not only do hydrogels have material properties that mimic more closely the environment experienced by cells *in vivo*, they also confer beneficial effects on gene expression, cell adhesion and phenotype.

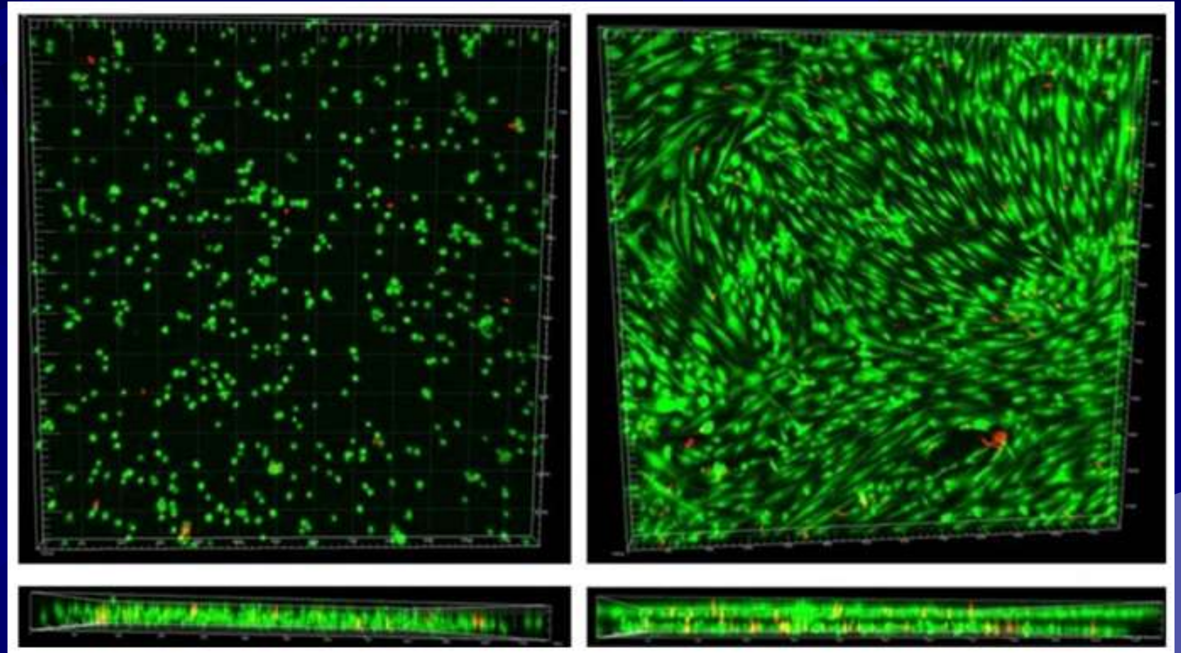
- Nevertheless, there are some formidable problems to overcome in engineering 3-D tissue constructs, including not only the replication of tissue complexity, but also the mechanical and viscoelastic characteristics of the native tissue.

PDL cells in 3-dimensional Extracel

- To create tissue constructs more closely resembling the structure of the PDL *in vivo*, we seeded human PDL cells into Extracel, a commercially-available hydrogel composed of hyaluronan (HA) and gelatin (GLN) in a format for use with the Flexercell system for mechanically deforming cells (Saminathan *et al*. 2013).
- For these experiments primary human PDL cells were purchased from a commercial source (ScienCell Research Laboratories, Carlsbad, CA, USA; Lot number: 5145), isolated from several donors.
- While thin films (80-100 μm) of PDL/HA-GLN constructs are suitable for studies of tensile mechanical strain, thicker constructs are required for investigating the effects of compression.

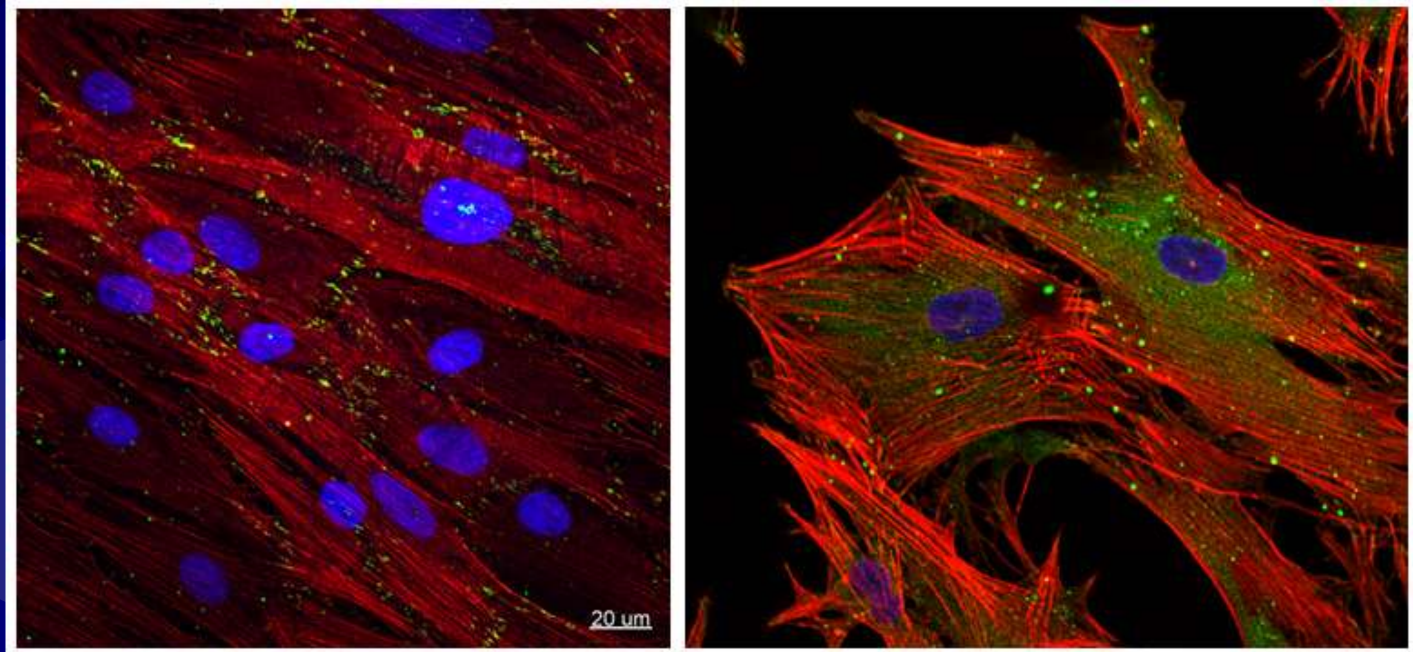
Human PDL cells in HA-GLN Extracel

From Saminathan *et al.* (2013). *Journal of Periodontal Research* **48**, 790–801.



- Human PDL cells in HA–GLN hydrogel films (80–100 μm in thickness) on 35 mm collagen I coated 6-well plates, cultured with CTGF (100 ng/mL) and FGF-2 (10 ng/mL). Left: 24 h. Right: 2 wks.
- Cells were stained with fluorescein diacetate and propidium iodide and viewed with a confocal imaging system; viable cells fluoresce green, nonviable red. Profile view shows that cells are initially distributed randomly throughout the gel, but as they proliferate become organized into a double cell layer; the field measures 120 x 120 μm.

Triple-stained PDL cells in Extracel

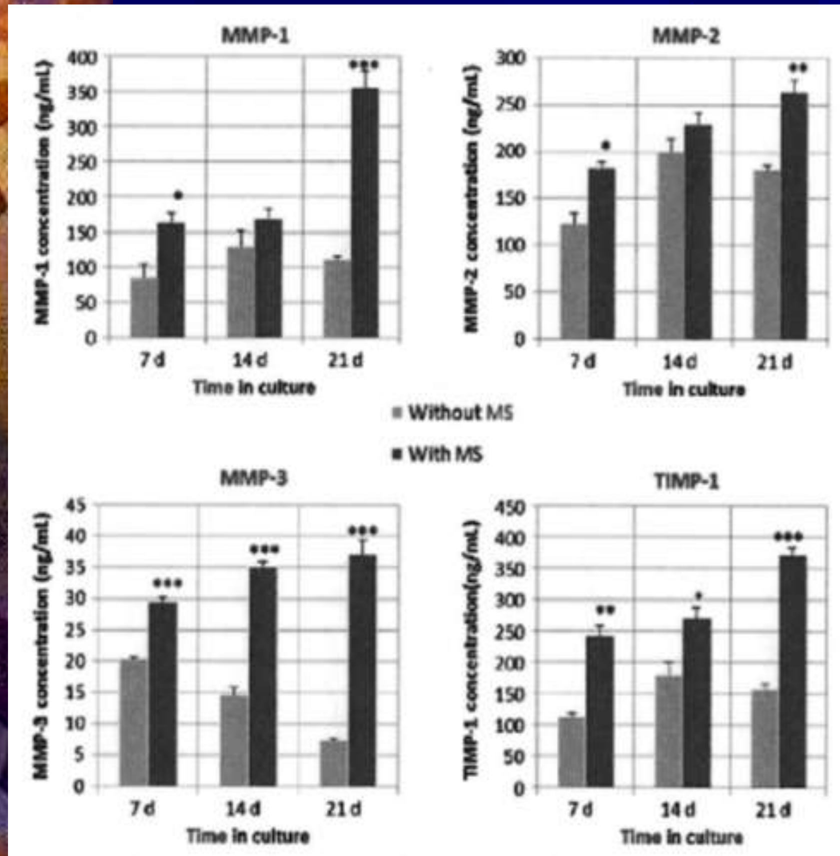


- Confocal immunofluorescent images of human PDL cells in 3-dimensional Extracel films. Left: Cells near gel surface. Right: Cells at gel substrate interface. From Saminathan *et al.* (2013). *Journal of Periodontal Research* **48**, 790–801.
- Cells were fixed, permeabilized and triple stained with: (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).

Viability of human PDL cells in Extracel

- Viability of human PDL cells incorporated into Extracel films (80–100 µm thickness) cultured in 35 mm diameter type I collagen coated six-well plates with CTGF (100 ng/mL) and FGF-2 (10 ng/mL).
- At the end of each time point the media were discarded and the cells stained with 0.1 mL (2µg) FDA (fluorescein diacetate) and 0.3 mL (6µg) PI (propidium iodide) and viewed in the dark with a Carl Zeiss, LSM 510 META confocal imaging system.

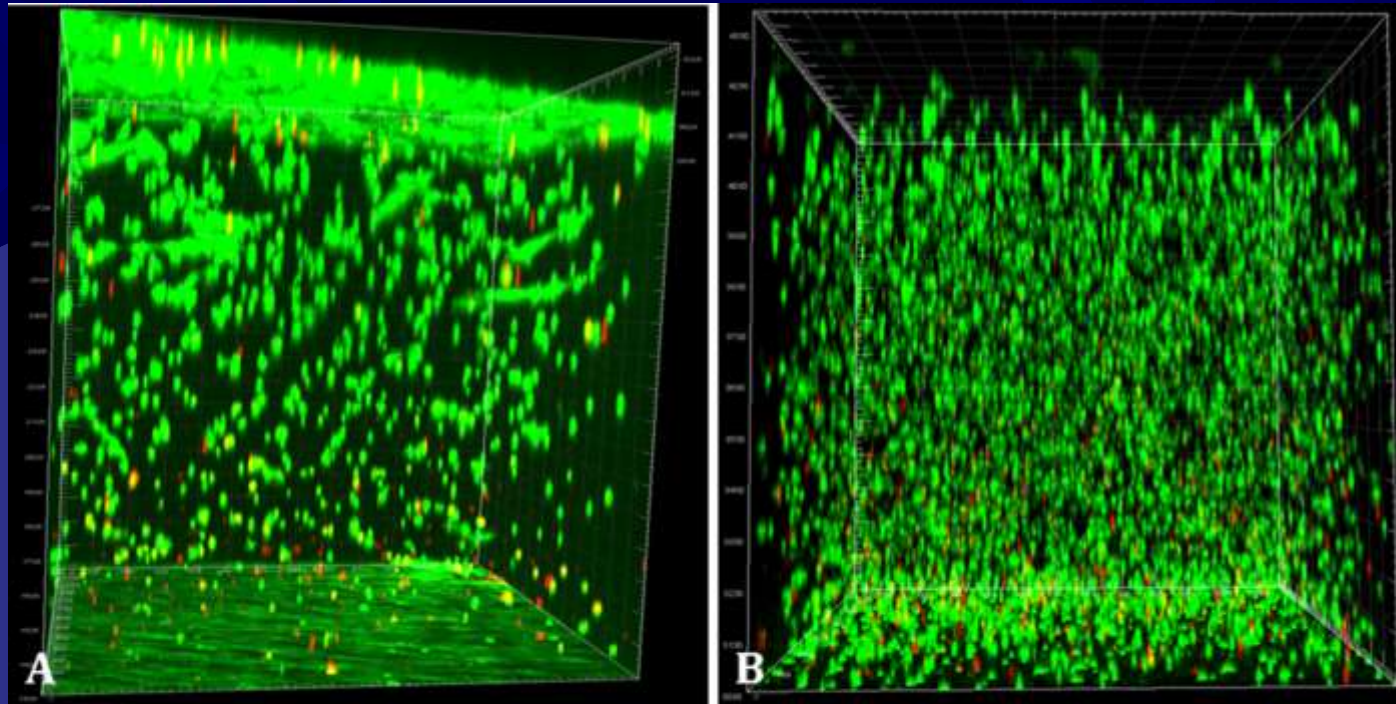
MMPs and TIMP-1 synthesis by PDL cells in Extracel



From Saminathan *et al.* (2013). *Journal of Periodontal Research* **48**, 790–801.

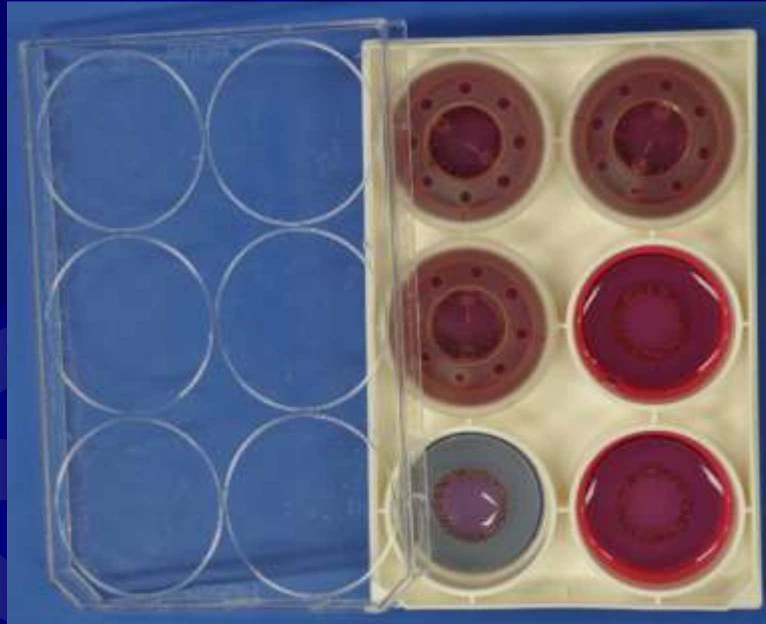
- Protein levels of MMP-1 (collagenase), MMP-2 (gelatinase), MMP-3 (stromelysin) and their inhibitor TIMP-1, measured by ELISAs in supernatants from cultures that had been mechanically stressed in the absence of CTGF/FGF-2, were significantly upregulated at various points in the time-scale, with MMP-3 and TIMP-1 across all three time-points.
- 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. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Human PDL cells in HA-GLN-COL1



- Profile images of PDL cells captured with a Carl Zeiss confocal imaging system. (A) Human PDL cells in hyaluronan-gelatin constructs; the body of the matrix is sparsely populated with most cells aggregating at the gel surface and gel-substrate interface.
- (B) With addition of type I rat tail collagen to provide additional RGD (Arg-Gly-Asp) binding sites, the cells are more evenly distributed throughout the gel matrix. Viable cells fluoresce bright green, dead cells red.

Compressing 3-dimensional constructs

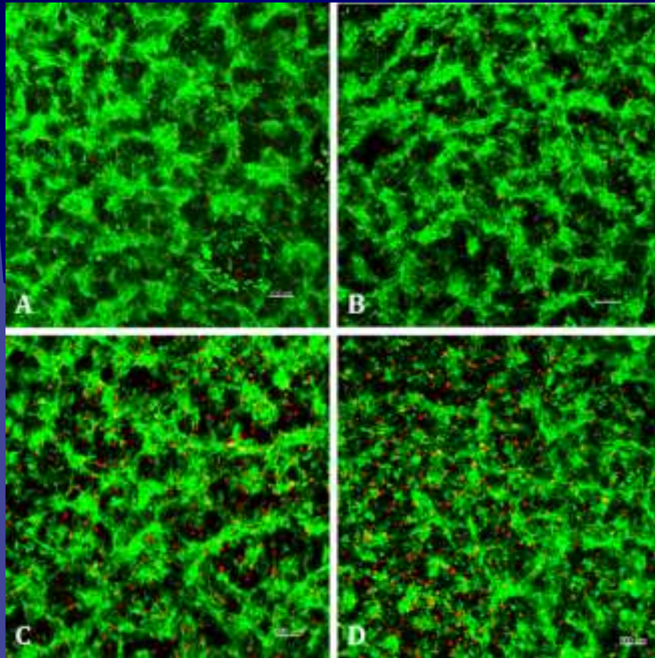


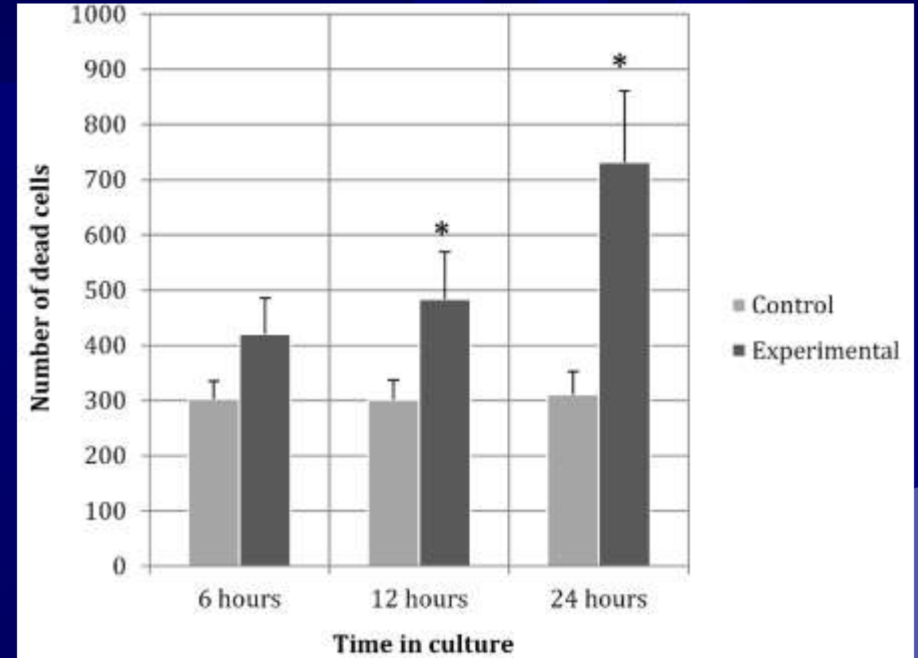
Biopress culture plate for applying compression to cell and tissue samples. Constructs are cultured in 5 ml medium and compressed from below by compressed air, the constructs being constrained by a stationary platen as shown in the top two and middle left wells.

- To apply compression to 3-D constructs, human PDL cells were incorporated into a matrix of hyaluronan, gelatin and type I collagen in sample holders (13 x 1 mm) of six-well Biopress culture plates. The loading dynamics of the PDL were mimicked by applying a cyclic compressive strain of 33.4 kPa to the constructs for 1 sec every 60 sec for 6, 12 and 24 h in a Flexercell strain unit.

- Saminathan *et al*., (2015). Engineering the periodontal ligament in hyaluronan-gelatin-type I collagen constructs: upregulation of apoptosis and alterations in gene expression by cyclic compressive strain. *Tissue Engineering Part A* **21**(3–4) 518–529.

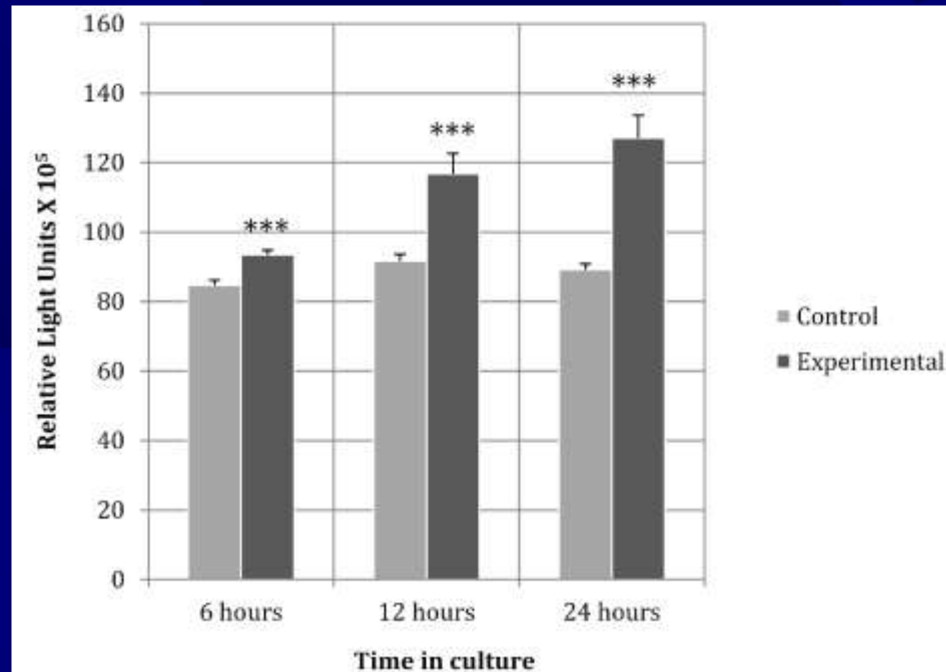
Effect of compression on cell viability





- Viable cells fluoresce bright green, non-viable cells bright red. Each field measures 1200 x 1200 µm. (A) Control; (B) 6 h compression; (C) 12 h compression; (D) 24 h compression.
- A compressive force of 33.4 kPa (340.6 gm/cm^2) was applied for 1 sec (1 Hertz) every 60 sec for 6, 12 and 24 h in 6-well Biopress plates. Three fields were selected from three wells and the number of dead cells counted using Image-Pro Plus software. Data expressed as mean ± SEM $^*p<0.05$.
- From Saminathan *et al.*, (2015). *Tissue Engineering Part A* **21**(3–4) 518–529.

Effect of compression on caspase 3/7



- Effect of cyclic compression on the activity of two executioner caspases (caspase 3 and caspase 7). A compressive force of 33.4 kPa (340.6 gm/cm^2) was applied for 1 sec (1 Hertz) every 60 sec for 6, 12 and 24 h in 6-well Biopress plates.
- Data is cross-sectional and expressed in relative light units as mean ± SEM for six wells. ***$p<0.001$.
- From Saminathan *et al*., (2015). *Tissue Engineering Part A* **21**(3–4) 518–529.

Effect of Compression on gene expression

	6 hours	12 hours	24 hours
P4HB	1.05 ± 0.14	0.96 ± 0.11	0.54 ± 0.05
RUNX2	0.43 ± 0.07	0.96 ± 0.09	ND
SOX9	0.76 ± 0.19	1.60 ± 0.18	ND
PPARG	ND	ND	ND
MYOD	ND	ND	ND
COL1A1	2.89 ± 0.54	1.08 ± 0.15	0.93 ± 0.29
COL2A1	ND	ND	ND
COL3A1	1.61 ± 0.23	1.20 ± 0.09	0.31 ± 0.04
MMP1	2.20 ± 0.28	0.45 ± 0.12	1.12 ± 0.23
MMP2	1.51 ± 0.20	0.81 ± 0.16	1.23 ± 0.21
MMP3	2.41 ± 0.98	1.08 ± 0.19	1.30 ± 0.42
TIMP1	0.71 ± 0.16	0.78 ± 0.13	0.59 ± 0.06
TGFB1	0.50 ± 0.14	1.41 ± 0.66	0.47 ± 0.06
BGLAP	ND	ND	ND
SP7	ND	ND	ND
BMP2	1.41 ± 0.08	1.17 ± 0.07	0.34 ± 0.08
RANKL	1.39 ± 0.39	0.82 ± 0.23	ND
OPG	0.42 ± 0.16	1.13 ± 0.51	0.38 ± 0.11
CTGF	3.15 ± 0.56	3.09 ± 0.97	1.92 ± 0.56
FGF2	1.56 ± 0.23	1.77 ± 0.63	0.63 ± 0.21

- Human PDL cells in 3-dimensional constructs were subjected to a compressive strain of 32.4 kPa (340.6 gm/cm^2) for 1.0 sec (1 Hertz) every 60 sec for 6, 12 and 24 h, and gene expression quantified by real-time RT-PCR.
- *RUNX2* and its chondrogenic equivalent *SOX9* were initially expressed, and in the case of *SOX9* significantly upregulated at 12 h; however, neither were detected at 24 h.
- Most genes were upregulated at some point with RQ values > 1.00 after 6 h and/or 12 h. All were downregulated at 24 h except for the *MMPs*, and *CTGF.*

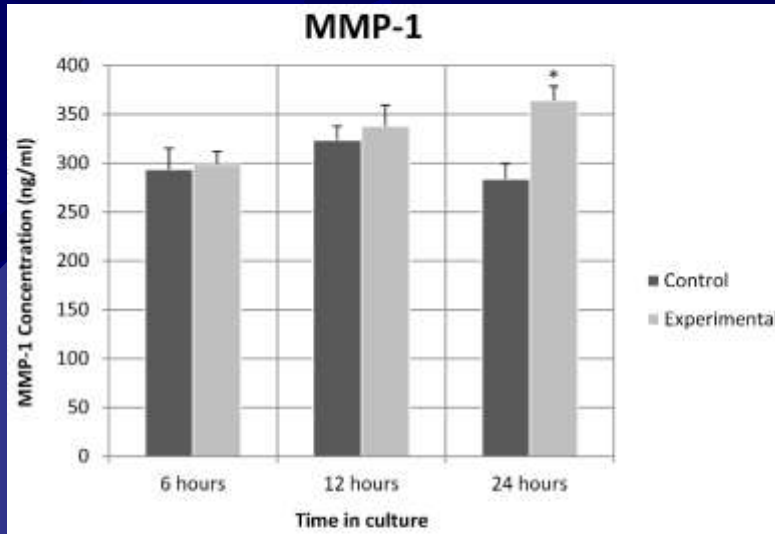
From Saminathan *et al.* (2015). *Tissue Engineering Part A* **21**(3–4) 518–529.

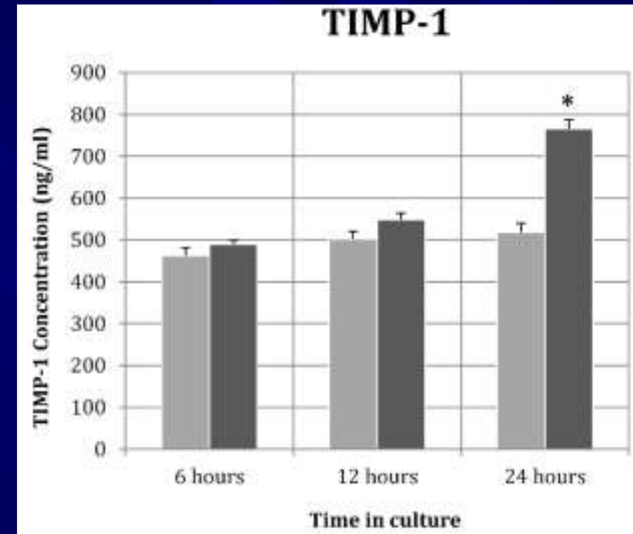
Effect of compression on apoptosis-related genes

	6 hours	12 hours	24 hours
TNF	0.28	1.56	0.24
TNFRSF1A	0.26	0.38	1.24
TNFRSF1B	ND	ND	ND
TNFSF10	0.81	0.70	2.45
IL1A	0.64	0.52	2.00
IL1B	0.39	1.17	0.74
IL6	1.17	0.60	0.71
CASP1	0.53	1.41	1.76
CASP2	0.68	0.96	1.48
CASP3	0.35	0.73	0.89
CASP6	0.23	1.08	1.17
CASP7	0.40	0.49	1.03
CASP8	0.67	0.55	1.59
CASP9	0.49	0.20	1.11
CASP10	0.24	0.68	2.18

- Human PDL cells in 3-dimensional constructs were subjected to a compressive strain of 32.4 kPa (340.6 gm/cm^2) for 1.0 sec (1 Hertz) every 60 sec for 6, 12 and 24 h, and gene expression quantified by real-time RT-PCR.
- The data are cross-sectional; Relative quantity values > 1.00 signify an increase in gene expression by compressed cells over controls.
- Each of the eight caspases (**c**ysteine-dependent **asp**artate-directed protein**ases)** involved in the apoptosis signalling cascade showed an increase in expression.

MMP-1 and TIMP-1 synthesis





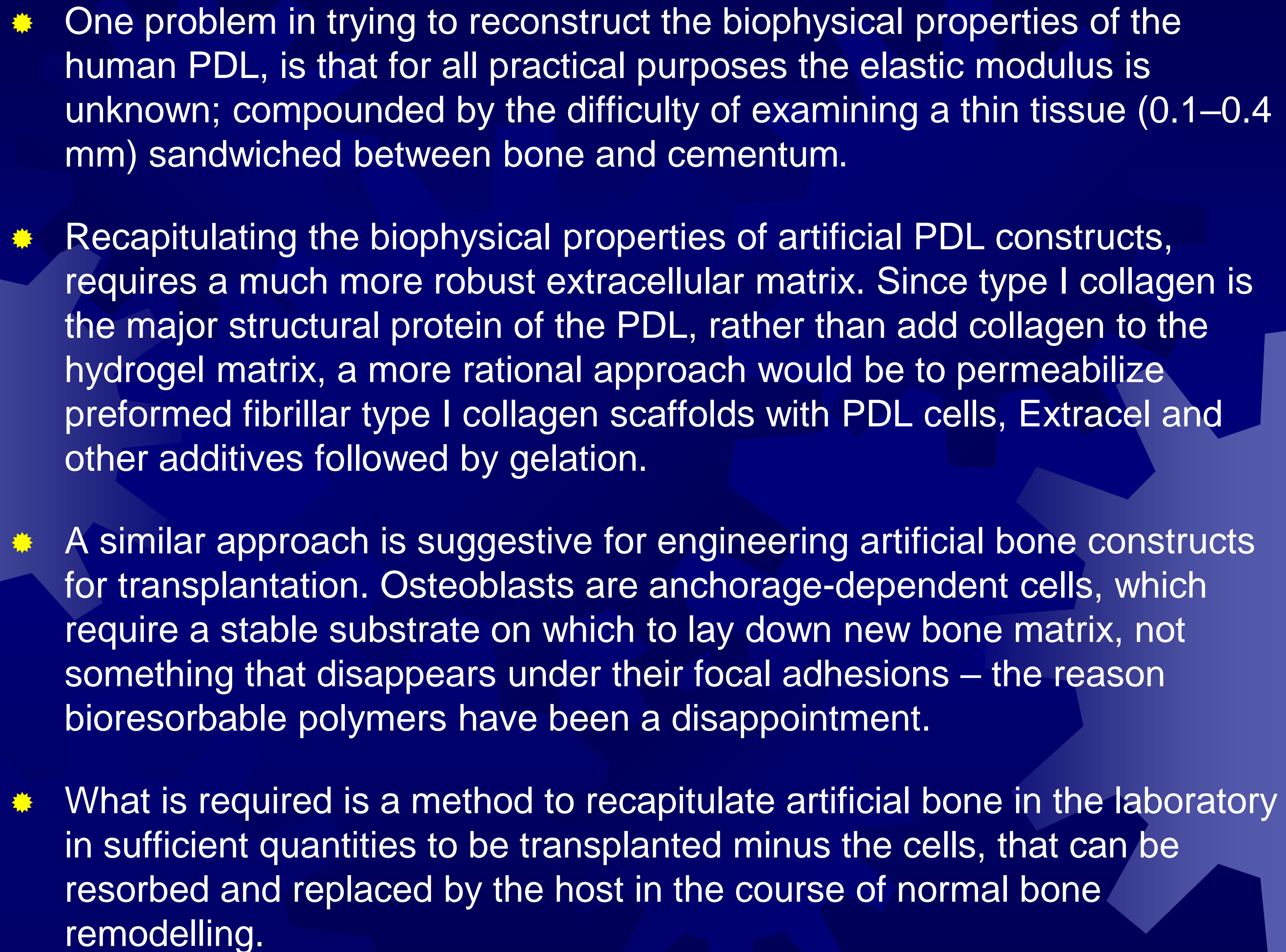
- All three MMPs plus TIMP-1 were identified by ELISAs in culture media from experimental and control cultures, although concentrations of MMP-2 and MMP-3 were low in comparison with MMP-1 and TIMP-1.
- Both were significantly upregulated by cyclic compressive strain, although the concentration of free TIMP-1 was more than twice that of the pro-form of MMP-1 at 24 h, despite *TIMP-1* expression remaining unchanged.
- Because the ELISAs only recognize latent proMMPs and free TIMP-1, we cannot say what proportion of the enzymes are in the active, latent, or complexed forms.

Summary

- Two-dimensional *in vitro* culture systems have provided valuable information regarding cell behaviour at its most reductionist level. However, they lack the physical and other environmental signals provided by 3-dimensional microenvironments that are important determinants of phenotype, proliferation, gene expression, and matrix turnover.

- However, information derived from 2-dimensional models does not reflect the complex physiology of the cells *in vivo.* There is clearly a difference between culturing cells on rigid 2-dimensional substrates, and enclosed within a 3-dimensional microenvironment.

- The challenge in engineering 3-dimensional constructs is to replicate not only tissue complexity, but also the mechanical and viscoelastic behaviour (resistance to elastic deformation or stiffness) of the native tissue, and further improvements in the composition of the present constructs are required.



- One problem in trying to reconstruct the biophysical properties of the human PDL, is that for all practical purposes the elastic modulus is unknown; compounded by the difficulty of examining a thin tissue (0.1–0.4 mm) sandwiched between bone and cementum.
- Recapitulating the biophysical properties of artificial PDL constructs, requires a much more robust extracellular matrix. Since type I collagen is the major structural protein of the PDL, rather than add collagen to the hydrogel matrix, a more rational approach would be to permeabilize preformed fibrillar type I collagen scaffolds with PDL cells, Extracel and other additives followed by gelation.
- A similar approach is suggestive for engineering artificial bone constructs for transplantation. Osteoblasts are anchorage-dependent cells, which require a stable substrate on which to lay down new bone matrix, not something that disappears under their focal adhesions – the reason bioresorbable polymers have been a disappointment.
- What is required is a method to recapitulate artificial bone in the laboratory in sufficient quantities to be transplanted minus the cells, that can be resorbed and replaced by the host in the course of normal bone remodelling.