# Effects of Mechanical Strain on Hyaluronan Metabolism of Synovial Cells from Osteoarthritic Knees

A thesis submitted to the University of Wales

for the degree of Doctor of Philosophy.

by

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January 2004

# Summary

Development of the synovial joint is complex involving a sequence of precisely controlled events. Separation of the cartilage anlagen involves differential matrix turnover and requires mechanical stimuli, the loss of mechanical stimuli results in joint fusion. Synthesis of hyaluronan at the developing joint line occurs and its continued synthesis is necessary for maintenance of frictionless movement in the adult joint.

Hyaluronan is synthesised by synovial fibroblasts and as a component of the synovial fluid and synovium, helps maintain a consistent volume and viscosity of synovial fluid when the joint is flexed. Reduction in hyaluronan concentration within the synovial fluid is associated with osteoarthritis and rheumatoid arthritis. Joints become painful to move and mobility is lost. The study aimed to investigate whether synovial cells from osteoarthritic knees were able to respond to mechanical stimuli and if so, were cells able to modulate hyaluronan synthesis. Results at the cellular level would be the first step in considering exercise based therapies as a treatment for osteoarthritis.

A four point bending mechanical loading jig was used to apply mechanical strain to synovial cells. Results indicate that synovial cells from osteoarthritic knees are capable of responding to a brief period of mechanical stimuli and modulate hyaluronan release. Differential expression of the three *HAS* genes was also observed upon mechanical strain. It was clear however, that the response to strain varied depending on cell passage. As a result, the development of an immortalised synovial cell line was investigated. Data obtained however, showed that ectopic hTERT expression alone was not sufficient to immortalise synovial cells.

Development of exercise based therapies for the treatment of osteoarthritis will require a considerable amount of further work. This study however, has demonstrated that alterations in hyaluronan synthase expression and subsequent hyaluronan synthesis by synovial cells occurs upon mechanical stimuli and thus provides a foundation for future studies.

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| | |
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| **ABTS** | 2,2'azino-bis(3'ethylbenzthiazoline-6 sufonic acid) |
| **ADAMT** | A disintegrin and metalloproteinase with thrombospondin motifs |
| **BMP** | Bone morphogenic protein |
| **DAF** | Decay accelerating factor |
| **ECM** | Extracellular matrix |
| **FGF** | Fibroblast growth factor |
| **FN** | Fibronectin |
| **GAG** | Glycosaminoglycan |
| **GDF** | Growth and differentiation factor |
| **HA** | Hyaluronan |
| **HAS** | Hyaluronan synthase |
| **HABP** | Hyaluronan binding protein |
| **IAP** | Intra-articular fluid pressure |
| **IGF** | Insulin-like growth factor |
| **IL** | Interleukin |
| **LN** | Laminin |
| **με** | Microstrain |
| **MMP** | Matrix metalloproteinase |
| **OA** | Osteoarthritis |
| **P** | Passage |
| **PBS** | Phosphate buffered saline |
| **PD** | Population doublings |
| **PG** | Proteoglycan |
| **PGE2** | Prostaglandin E2 |
| **PMA** | Phorbol myristate acetate |
| **SF** | Synovial fluid |
| **SFB** | Synovial fibroblast |
| **SMP** | Synovial macrophage |
| **sIL-R** | Soluble interleukin receptor |
| **RA** | Rheumatoid arthritis |
| **RT** | Room temperature |
| **TBE** | Tris-borate EDTA |
| **TGFβ** | Transforming growth factor beta |
| **TIMP** | Tissue inhibitor of metalloproteases |
| **TMB** | Tetramethyl benzidine |
| **TNFα** | Tumour necrosis factor alpha |
| **TNF-R** | Tumour necrosis factor-receptor |
| **UDPGD** | Urindine-diphospho-glucose-dehydrogenase |
| **VCAM-1** | Vascular cell adhesion molecule-1 |

# Acknowledgements

I wish to thank Professor Charles Archer for allowing me the opportunity of studying for this Ph.D. I fully appreciate the supervision, support and encouragement he has provided throughout my time at Cardiff University.

I wish to thank the following for their help during my Ph.D. Dr Gary Dowthwaite, for his guidance and supervision. Dr Samuel Webster for help in all areas of molecular biology and for his patience. Dr Anwen Williams for her valuable advice and support and also for providing the synovium samples, without which this study could not have been completed. Dr Nick Amos for his help and assistance in all aspects of cytokine biology. Professor David Kipling and all the staff in the Department of Pathology, UWCM for their assistance during my time spent in their laboratory.

In addition, I would like to thank all the members of the Connective Tissue Research Laboratories. In particular my support network, Samantha Redman, Joanna Bishop, Rhiannon Fish, Dr Ilyas Khan, Kirsty Richardson and Dr Sarah Oldfield whom have all provided help, advice and a happy working environment.

Finally, I wish to give special thanks to my family. To my sister Sarah, her friendship is truly appreciated, and to my mum and dad for their tremendous support in all I aim to achieve.

# Chapter 1

# General Introduction

# 1.1 General Introduction

## 1.1.1 The Synovial Joint

An extensive range of friction free movement is provided by the synovial joint and joint capsule structure is vital to joint function. The fluid-filled cavity is positioned between the opposing articular cartilages of the long bones and is surrounded by a thick fibrous sheath, lined by the much thinner synovium. The capsule is supported by ligaments and tendons providing protection from loading yet allowing a wide range of movement. Nerves and blood vessels pass through the capsule and are important in the active protection of the capsule and its ligaments.

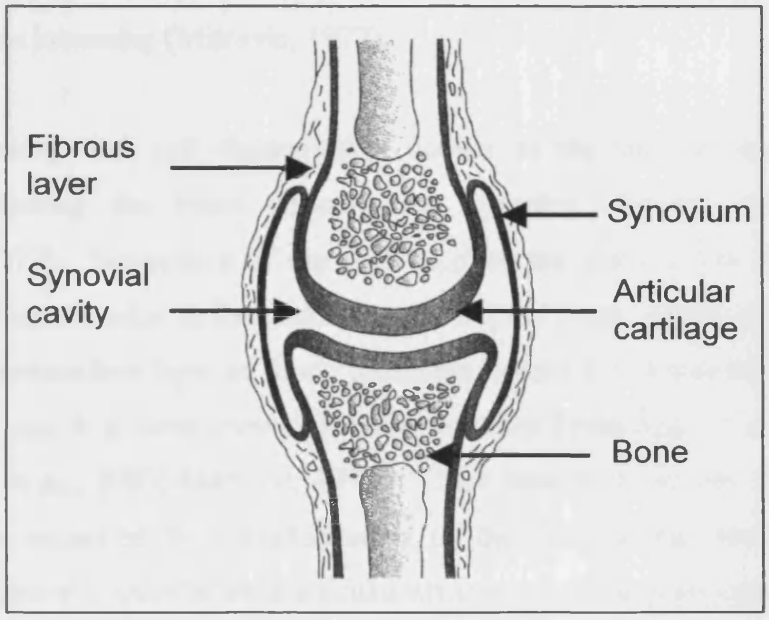


**Figure 1.1.1 Schematic diagram of the diarthrodial joint (adapted from Colour Textbook of Histology, 1997).**

## 1.1.2 Development of the Joint

The factors controlling joint development are not well understood. It is thought that the joint develops from a distinct population of cells but the lack of specific biological or molecular markers for these cells only acts to impede research into joint development. Joint development can be divided into two stages; an initial condensation stage and a second separation phase (Bernays, 1878; Mitrovic, 1978).

Intially, the homogeneous, avascular mesenchymal core of the limb bud condenses to form the cartilaginous anlagen. The cartilage anlagen are then separated by a narrow, dense blastemal mesenchyme region known as the interzone. The interzone is comprised of three layers – the two outer most layers eventually form the articular cartilage and the intermediate layer allows the cavity to form.

During the separation phase, the periphery of the cartilage blastema forms the synovial mesenchyme. The mesenchymal cells here are arranged in parallel with the boundary of the synovial cavity and are flattened in appearance (Whillis, 1940). Within the synovial mesenchyme distinct cell condensations can be observed and give rise to the menisci, ligaments and tendons (McDermott, 1943; Mitrovic, 1978; Whillis, 1940). Blood vessels are present at the periphery of the joint at sites of maximal tissue loosening (Mitrovic, 1977).

Tissue loosening and cell degeneration occurs in the intermediate layer of the interzone allowing the space between the opposing cartilage anlagen to form (Mitrovic, 1977). Separation of the skeletal elements starts in the periphery of the joint with discontinuous clefts coalescing to form a single cavity (Mitrovic, 1978). Within the intermediate layer as tissue loosening occurs an accumulation of interstitial fluid is seen and it is now known that this contains hyaluronan (Craig et al., 1990; Dowthwaite et al., 2003; Mitrovic, 1978). Once separation occurs, the cartilaginous elements are expanded by chondrification of the surrounding blastemal tissue by appositional growth and the intra-articular structures of the joint remain in the same position relative to the adult joint (Haines, 1947; Mitrovic, 1977).

At the early stages of joint development, gene expression is uniform between the skeletal elements and joint interzone whereas during the later stages, differential gene expression between the skeletal elements and interzonal cells are reflected in the morphological differences of these regions (Craig et al., 1987; Koyama et al., 1995; Nalin et al., 1995). Gene expression by cells of the developing joint allows the synthesis of families of signalling molecules, which can control cellular communication and influence joint development (Francis-West et al., 1999; Nalin et al., 1995). One of the largest families is the bone morphogenic proteins (BMP), a

subgroup of the $TGF_{\beta}$ family. The BMPs were initially associated with a role in inducing bone differentiation but further research has shown BMPs are involved in a diverse range of biological processes including cell proliferation, cell differentiation and cell-fate determination (Chang et al., 1994; Hogan, 1996; Kingsley, 1994; Rosen and Thies, 1992). A close relative of the BMPs are the growth and differentiation factors (GDF). Indeed, GDF-5 has been shown to signal through the same receptors as the BMP family (Nishitoh et al., 1996). The expression of four members of the BMP/GDF family during joint development especially in the chondrogenic regions in the later stages of development has lead to the belief that BMPs/GDFs do play a role in joint development (Hogan, 1996; Lyons et al., 1989; Lyons et al., 1990; Rosen et al., 1996; Storm et al., 1994; Zou et al., 1997). It has been shown that these signalling molecules can either work alone or synergistically to modulate chondrogenesis by controlling chondrocyte proliferation and differentiation and in recruiting non-chondrogenic cells (Brunet et al., 1998; Chen et al., 1991; Duprez et al., 1996; Storm et al., 1994; Storm and Kingsley, 1996). The activity of BMP-3 and 4 have also been shown to enhance cartilage matrix synthesis *in vitro*, contributing to maintenance of the cartilage phenotype (Luyten et al., 1994; Luyten et al., 1992; Vukicevic et al., 1989). Misexpression of either BMP or GDF-5 resulted in skeletal limb defects due to alterations in cell proliferation or condensation emphasising the importance of these genes in controlling cartilage development and skeletal morphogenesis (Duboule, 1995; Duprez et al., 1996; Gruneberg and Lee, 1973).

From this research it can be seen that the sequence of events allowing for joint formation are specific and controlled. A combination of a number of factors such as cell necrosis, apoptosis, vascular influences, cellular migration and differential matrix synthesis at the presumptive joint line have all been suggested to be involved in separation of the cartilage anlagen (Bernays, 1878; Craig et al., 1987; Edwards et al., 1994; Milaire, 1947; Mitrovic, 1978; Mitrovic, 1977). The exact stimuli that are responsible for instigating cavity formation and maintaining the joint cavity are at present unknown, although mechanical factors are thought to play a major regulatory role in this process (Anderson and Brorasmussen, 1961; Dowthwaite et al., 1998; Fell and Canti, 1934; Mitrovic, 1977; Murray and Drachman, 1969; Osborne et al., 2002). The removal of mechanical stimuli by neuromuscular blockers *in ovo* or in limb organ

culture prevents cavitation in uncavitated joints (primary fusion) (Drachmann and Sokoloff, 1966; Osborne et al., 2002). Additionally, removal of mechanical stimuli after cavitation has occurred results in a secondary fusion of the joint cavity (Drachmann and Sokoloff, 1966; Fell and Canti, 1934; Hamburger and Waugh, 1940; Murray and Drachman, 1969).

As mentioned previously, tissue loosening in the interzone allows the cartilage anlagen to separate. Apoptosis and cells necrosis have been implicated in joint formation as a reason for cell loss within the interzone. Cells within the intermediate layer of the interzone showed degeneration and characteristics of cell necrosis and it has been suggested that this occurs to eliminate cells that would otherwise undergo chondrogenesis (Mitrovic, 1978). However, apoptosis is unlikely to act alone in producing the fully formed joint space, as a very high proportion of cells would have to undergo cell death in order to clear the cavity of cells (Craig et al., 1990; Mitrovic, 1978).

It is clear that the separation of the cartilaginous elements along the potential joint line requires a local change in extracellular matrix to allow a joint space to form. This alteration in ECM properties could be due to enzymatic or mechanical breakdown of adherent ECM molecules, the synthesis of non-adherent components or indeed could involve both synthesis and catabolism of the extracellular matrix (Edwards, 1994a; Mitrovic, 1977).

There is little evidence for enzymatic degradation occurring along the presumptive joint line. Indeed, Mitrovic (1978) found no evidence of protease or lipase activity in the developing joint however, presence of acid phosphatase activity along the joint line was detected, but disappeared when cavitation began. This suggests that lysosomal enzymes are involved in the modification of the extracellular matrix (ECM), yet if these enzymes are to degrade components then they need to be endocytosed which would involve the action of matrix metalloproteinases (MMPs) (Milaire, 1947; Mitrovic, 1977). At present evidence is currently lacking for increased local levels of MMP activity at the developing joint line (Edwards et al., 1996).

### 1.1.3 Mechanical Stimuli & Joint development

As mentioned above, movement has long been regarded as essential for joint cavity formation. Disruption of cell-cell cohesion at the joint line arising from mechanical forces generated by muscle activity could allow separation to occur (Drachmann and Sokoloff, 1966; Mitrovic, 1982). Conversly, Hasty *et al.* (1993) have shown that in myogenin deficient mice, the joint forms even though there is no contracting skeletal muscle to generate mechanical forces. Passive motion within the uterus as a driving factor in the process of joint cavitation cannot therefore be ruled out as a factor. Interestingly, histological evidence of tissue loosening and accumulation of synovial fluid within the cavity are first seen when fetal movements are recognized by the mother (McDermott, 1943; Whillis, 1940). Studies in chick and human limbs have shown that there is very little, if any, growth in interzone cells but those of the cartilaginous anlagen show division and growth (Lewis, 1977). Thus, the local mechanical forces from the differential growth rate between the cartilaginous and synovial components may be involved in cavity formation. As paralysis has only a small effect on the growth rates (Edwards and Wilkinson, 1996) mechanical stimuli might not be the only factor responsible for formation of the joint line, as cavities are still able to form. Therefore, it is likely that the disruptive mechanical forces involved do not act alone in producing the well-defined joint line.

Movement induced mechanical stimuli could alter the synthesis and degradation of ECM during development of the joint cavity (Dowthwaite et al., 1998; Dowthwaite et al., 2003; Pitsillides et al., 1999). This biochemical change in ECM components is a certain probability as a loss of tensile properties in the interzone leads to tissue loosening and eventual cell separation. Although working on chick joints, which show analogy to mammals except they contain fibrocartilage as opposed to hyaline articular cartilage, Craig *et al.* (1987) showed less type II collagen concomitant with an increase in type I collagen during cavitation. Work by Nalin *et al.* (1995) showed evidence for type I collagen and type II collagen expression in the interzonal cells prior to cavitation yet at cavitation only type I collagen was observed in the interzone cells. These studies suggest differential matrix turnover does occur during joint development.

As well as the removal of adherent molecules within the joint line an increase in the synthesis of non-adherent components such as hyaluronan (HA) could also be involved in joint cavitation. Indeed, evidence for differential hyaluronan synthesis at the joint line is becoming more apparent and mechanical stimuli could be responsible for hyaluronan synthesis. As already discussed, immobilised limbs fail to form cavities and this failure to cavitate is associated with alterations in HA synthesis at the developing joint line (Pitsillides, 1999).

### 1.1.4 Hyaluronan & Joint Development

Hyaluronan was first isolated in 1934 by Meyer & Palmer and its extraction from the umbilical cord, vitreous humour of the eye, synovial fluid and streptococci all led to the discovery of its physiochemical characteristics (Blumberg and Ogston, 1958; Comper and Laurent, 1978; Kendall et al., 1937; Meyer, 1947). HA is classed as a glycosaminoglycan (GAG) due to its repeating disaccharide units of D-glucuronic acid linked to N-acetyl-D-glucosamine that forms a negatively charged, high molecular weight polysaccharide. Although belonging to the GAG family, it lacks a covalently linked polypeptide and is unsulphated.

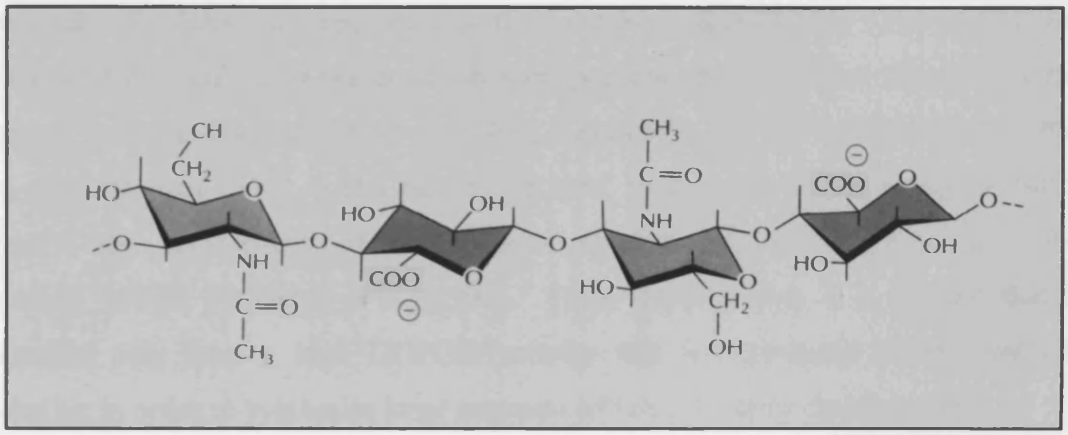


**Figure 1.1.2 The repeating glucuronic acid and *N*-acetylglucosamine subunits of hyaluronan (reproduced from Biochemistry, 1995).**

Previously, HA was considered to act simply as a space-filling molecule secreted into a cavity which had developed by another mechanism. Now, evidence suggests a

much more critical role, with local synthesis of HA occurring at the first signs of cavitation and continuing after development to help maintain a fully functional joint (Craig et al., 1990; Pitsillides et al., 1995).

As stated earlier, a change in ECM components appears to be involved in cavity formation. Anderson & Brorasmussen (1961) demonstrated the accumulation of a metachromatic substance at the joint line and following this work, Anderson (1961) showed that this could contain HA. Indeed, it was later shown that this metachromatic substance was HA and that HA was present at the joint line concominant with the first signs of cavitation (Craig et al., 1990). This work also showed that labelling for HA was more intense in the developing synovial tissue than in the tissue surrounding the cartilage anlagen and that the HA binding sites remained localized to the cavity and were maintained as the joint enlarges. The development of cytochemical assays and immunolabelling studies allowed the synthesis of HA by local cells to be assessed. Work by Pitsillides *et al.*(1995) concentrated on the activity of uridine-diphosphoglucose-dehydrogenase (UDPGD), the enzyme involved in producing precursors for HA. An increase in UDPGD activity occurred in the interzonal cells suggesting an increase in HA synthesis. The precursors produced by UDPGD could enter glycosaminoglycan (GAG) synthesis and not HA synthesis but low levels of sulphate incorporation at the joint line suggested the UDP-glucuronate synthesized by UDPGD is preferentially incorporated into HA rather than chondriotin sulphate synthesis (Hascall VC et al., 1991; Hinchcliffe, 1977; Pitsillides et al., 1995). The expression of CD44, a HA binding protein, by the cells of the interzone further supports the involvement of HA in joint formation (Dowthwaite et al., 1998; Edwards, 1994b; Pitsillides et al., 1995). From these studies, it is evident that the interzonal cells have a high UDPGD activity that is maintained during and after cavitation in order to synthesise large amounts of HA for cavity development.

Toole (1981) demonstrated that low concentrations of HA promoted cell aggregation whereas high concentrations of HA promote cell separation. The high levels of HA produced along the joint line would, therefore, promote cell separation as hyaluronan binding proteins (HABPs) become saturated accounting for the loss of tensile properties in the ECM (Toole, 1981). Dowthwaite *et al.* (1998) used low molecular

weight oligosaccharides of HA to disrupt HA:HABP interactions in developing chick joints. This distruption in HA:HABP interactions resulted in fused joints and these fusions were associated with decreases in UDPGD activity and HABP expression.

These results show that HA is essential in cavity formation, yet the signals which promote HA synthesis remain unclear. Recent work by Dowthwaite *et al.* (2003) involved subjecting embryonic chick fibrocartilage cells to mechanical strain and then assessing levels of HA synthesized, the UDPGD activity and expression of HABP. This work showed that mechanically induced stimuli are probably a major regulatory factor in joint cavity formation as UDPGD activity increased when cells were subjected to strain producing higher amounts of HA. An increase in the number of occupied HABP sites was also observed.

### 1.1.5 Synovium

The synovium develops from the peripheral, vascularised tissue surrounding the presumptive joint cavity. As the joint flexes, the synovium needs to be able to move with the joint yet still surround the cavity. Thus, it needs to increase its surface area as the joint develops. There are three mechanisms that could facilitate this increase in synovium area. The spontaneous growth of new tissue matrix is the first possibility but little evidence exists to support this mechanism (Henderson, 1987b). The second mechanism involves irreversible stretching and reorganisation of the cellular and matrix components, with movement playing a key factor (Drachmann and Sokoloff, 1966). The final mechanism of increasing the surface area involves separation or splitting of the lining tissue. Lamellar projections on the synovial surface may perforate and then separate to give further projections, or splitting of collagen bundles deep within the tissue followed by remodelling of the matrix may produce a larger continuous surface (Henderson, 1987a).

The synovium is 50-60μm in thickness in the human knee joint and 10-20μm in the rabbit knee (Levick and McDonald, 1989; Steven et al., 1991) and is split into two layers; the intima and subintima. Previously, it was difficult to distinguish between these two layers but now their different morphologies have been established. The

intima consists almost solely of synovial macrophages and synovial fibroblasts, the main cell types of the synovium. The subintima, which can be absent in some species is less cellular but synovial macrophages, synovial fibroblasts, mast cells and adipocytes are present. The proportion of collagen bundles is higher in the subintima than the intima and throughout the subintima there runs a rich, specialised capillary network (Mason et al., 1999).

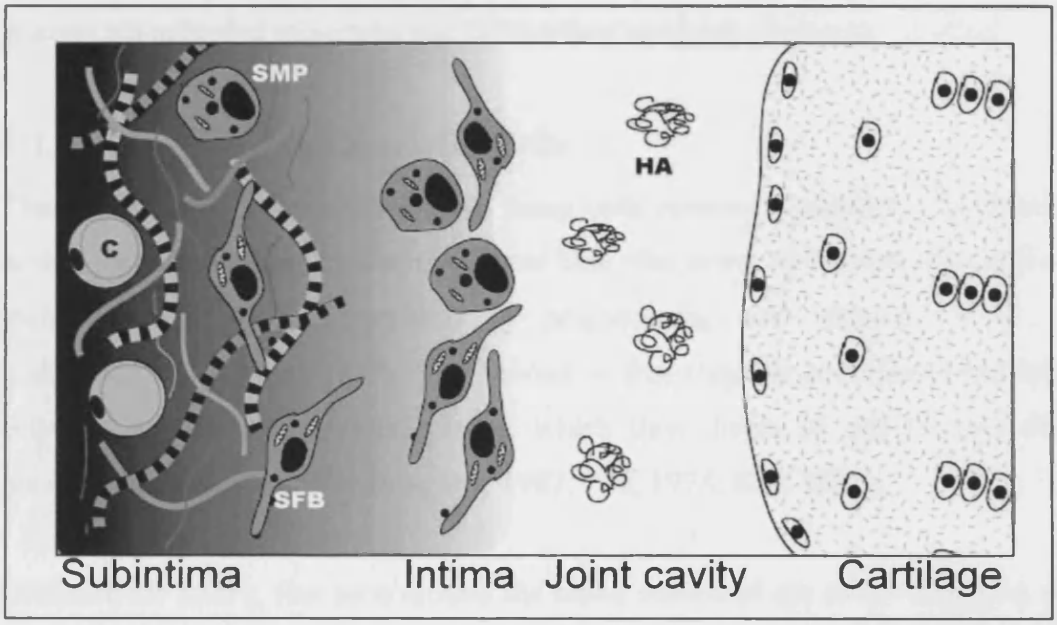


**Figure 1.1.3 Schematic diagram of the synovial joint. SFB=synovial fibroblast; SMP=synovial macrophage; C=capillary; HA=hyaluronan molecule.**

The synovium lies on a layer of either areolar, fibrous or adipose tissue and this layer influences the cells on the synovial surface and the degree of vascularisation of the subintima. Areolar synovium consists of a thick, cellular intima and a subintima that is formed from loose connective tissue rich in blood vessels. The fibrous type has a thin cellular lining that contains relatively few cells lying on a collagenous subintima (Key, 1932). There is no basal lamina, so the cells are in direct contact with the synovial fluid thus allowing the passage of plasma components and macromolecules synthesised by the synovial cells, such as hylauronan, to pass into the synovial fluid (Ghadially, 1983; Mason et al., 1999).

### 1.1.5.1 Cells of the Synovium

There are two main cell populations within the synovium. The macrophage-like type A cells and the fibroblast-like type B cells. The type A cells are similar to mature macrophages in other tissues and so are referred to as synovial macrophages (SMP). The type B cells show a wide range of properties, some of which are similar to fibroblasts, and are often referred to as synovial fibroblasts (SFB). Both cell types are distinct from each other, showing structural and functional differences yet are able to interact via adhesion molecules and cell surface receptors (Edwards, 1999b).

### 1.1.5.2 Origin of the Synovial Cells

The origins of both types of synovial lining cells remain in question. The first theory is that they derive from connective tissue cells that arrive at the synovial surface with their behaviour then determined by neighbouring cells (Barratt et al., 1977; Luckenbill and Cohen, 1967). The second is that they are a distinct synovial lining cell line defined during development, which then divide *in situ* to give the final phenotype (Edwards and Willoughby, 1982; Fell, 1978; Key, 1932).

Evidence for SMPs, first seen around the blood vessels of the developing joint (Okada et al., 1981), suggest that they are derived from a bone marrow cell line and once in the synovium show functional specialisation by interacting with SFBs (Edwards and Willoughby, 1982; Hogg et al., 1985). Further support for the macrophage lineage comes from macrophage deficient mice which contain no SMPs in the synovium (Naito et al., 1991).

Division of SFBs occurs at a very low rate, indicated by the low DNA content within the cells (Henderson, 1987a; Mohr et al., 1975). If this is the case, then slow turnover between cell death and cell division suggests that the synovial cells are rarely damaged in healthy synovium. This low rate of cell division may be attributed to cell migration to the synovium from the underlying connective tissue, with the type B cell phenotype being determined by local signals once migratory cells are within the tissue. Wilkinson *et al.* (1993) have shown that injections of air into subcutaneous tissue led to the formation of synovial type membranes, but the fibroblasts here lack

some of the typical SFB characteristics such as high UDPGD activity. Other signals, either chemical or mechanical, could be required to induce the SFB phenotype.

Recent work by De Bari *et al.*, (2001) supports the theory of a distinct synovial lining cell line predetermined in a restricted population during embryogenesis. This, and work by Nishimura *et al.*, (1999) and Fickert *et al.*, (2003) shows that the cells of synovial tissue can undergo chondrogenesis, myogenesis and osteogenesis highlighting the possibility that a mesenchymal stem cell is present within the adult synovial membrane.

## Synovial Fibroblasts

SFBs contain a large nucleus, and a small amount of cytoplasm occupied by an extensive network of rough endoplasmic reticulum (Iwanaga et al., 2000). One of the main properties of the SFBs that differs from other fibroblasts is their ability to synthesise HA. The enzyme UDPGD, which is responsible for converting UDP-glucose to UDP-glucuronate, is considered the rate limiting step in HA synthesis as it provides the precursor sugars for HA synthesis (McGarry and Gahan, 1985; Molz and Danishefsky, 1971). Elevated UDPGD activity is associated with the intimal layer of the synovium and it has been shown that the resident SFBs are responsible for this activity (Pitsillides and Blake, 1992; Wilkinson et al., 1992). Further work by Pitsillides *et al.* (1993) has shown the UDPGD activity in intimal fibroblasts is six or more times higher than in the cells of the subintima. It is not know what controls UDPGD activity, though in diseased synovium this activity is down-regulated, leading to a decrease in HA synthesis (Pitsillides et al., 1994). One possibility could be the continued surface shearing forces occurring during movement of the joint, since surface cells in contact with the synovial fluid show the highest UDPGD activity (Edwards, 1994b).

The second enzyme involved in HA synthesis, and present in high quantities within the SFBs, is hyaluronan synthase (HAS) (Edwards, 1999a). This enzyme consists of seven putative membrane-spanning domains, two at the N-terminal end and the remainder at the C-terminal end. The catalytic site and binding sites for the HA precursors are thought to exist between these two regions (Weigel et al., 1997). HA

chains are elongated by the alternate transfer of the precursors UDP-glucuronic acid and UDP-N-acetylglucosamine. This process takes place on the inner aspect of the plasma membrane allowing the HA macromolecule to be secreted from the cell surface where it can be shed into the synovial fluid or be retained by the cell in the form of a pericellular coat (Prehm, 1984; Spicer et al., 1997).

There are three HAS enzymes encoded by three separate but related *HAS* genes forming part of the mammalian *HAS* gene family (Spicer and McDonald, 1998). Although these genes show homology to each other, the HAS enzymes that they code for exhibit distinct properties, at least in *in vitro*. The size of the HA chain can vary depending on the HAS enzyme. *In vitro*, HAS1 and HAS2 yield products of $2 \times 10^5$ to $2 \times 10^6$ Da whereas, HAS3 synthesises a smaller HA chain with a Mr of $1 \times 10^5$ to $1 \times 10^6$ Da (Itano et al., 1999). The rate at which HAS is able to elongate the HA chain also differs such that HAS1 and HAS2 synthesise HA almost ten times faster than HAS3 (Itano et al., 1999), yet what governs this difference in elongation rate remains unknown.

The factors controlling *has* expression remain unknown although Jacobson (2000) and Recklies (2001) have both shown that various cytokines and growth factors such as IGF, FGF, TGFβ and IL-1, can differentially induce expression of the three *HAS* genes, and this corresponds to observed increases in HAS protein levels. It has also been demonstrated that *HAS* gene expression can be modulated by mechanical stimuli (Dowthwaite et al., 1999). This study demonstrated that after application of mechanical strain to chick fibrocartilage cells, *HAS3* expression was induced.

Thus, *HAS* gene expression, HAS enzyme synthesis and subsequent HA synthesis rely on a number of factors including cell type and external stimuli (Dowthwaite et al., 1998; Jacobson et al., 2000; Recklies et al., 2001; Spicer et al., 1997). The advantage of three HAS isoforms, able to respond to different stimuli, could provide the cell with the flexibility it needs to control HA synthesis and in respond to differing externally generated cues.

The vascular cell adhesion molecule (VCAM-1) is constitutively expressed by SFBs (Morales-Ducret et al., 1992). Its expression is thought to be predetermined, although it could require a signal from the SMPs to be expressed (Edwards, 1994c). VCAM-1 is able to bind to VLA-4, the α4β1 integrin on SMPs. It is conceivable, therefore, that this interaction between SFBs and SMPs anchors the SMPs in the matrix of the synovium. This allows granulocytes to enter the fluid-filled cavity and remove the waste components, which can then be reabsorbed by the SMPs thus keeping the synovial cavity free of waste or cell debris that would otherwise accumulate and interfere with joint function (Edwards, 1994c; Fairburn et al., 1993).

VCAM-1 is co-expressed with decay accelerating factor (DAF; CD55). High expression of DAF by SFBs, even before cavity formation has begun, is observed (Edwards and Wilkinson, 1996). This early expression is thought to be the result of positional information but may also be dependent on mechanical stimuli produced by shear stresses along the presumptive joint line (Edwards, 1999b).

## Synovial Macrophages

The cytoplasm of the SMPs is filled with loosely arranged ER and a prominent Golgi complex with a scattering of vacuoles and vesicles often in close proximity to the cavity (Shannon and Graham, 1971; Southwick and Bensch, 1971; Wyllie et al., 1964). SMPs show strong non-specific esterase activity and this property is often used as a marker to distinguish the SMPs from SFBs (Broker et al., 1990). Like all other macrophages, those in the synovium are phagocytic and covered by microvilli and microplicae. It has been demonstrated that the SMPs are active in the uptake of foreign substances that have been injected into the joint cavity (Iwanaga et al., 2000). Thus, a role of adsorption and degradation of cell debris, antigens and extracellular components is suggested for the synovial SMPs (Okada et al., 1981; Senda et al., 1999; Shannon and Graham, 1971).

The well-developed vesicular and lysosomal system of SMPs suits the critical role of keeping the synovial cavity free of waste so allowing full movement of the joint. The position of the SMPs within the synovium is also significant as they absorb debris that needs to be excreted from the synovial cavity and deposit it in the lymphatic system

where it can be removed. While some reports state that SMPs are unevenly distributed in the synovium, others report that they congregate at the tips of the synovial villi (Iwanaga et al., 2000; Stikichi et al., 1999). SMPs are also present at high density at the synovium-cartilage junction. Here, SMPs are likely to be involved in disposing of waste from the cartilage (Senda et al., 1999).

### 1.1.5.3 Extracellular Matrix

The composition of synovial extracellular matrix (ECM) is essential in maintaining healthy joint function and comprises sixty six percent of the synovial volume (Price et al., 1996). The movement, positioning and content of macromolecules within the synovium provides sufficient outflow resistance to retain the correct volume and composition of synovial fluid within the synovial cavity (Price et al., 1995).

The cells of the synovium are separated by 2μm gaps with the interstitial spaces subdivided with components of the ECM including collagens, glycoproteins and proteoglycans (Levick, 1996). There is no defined basement membrane so the cell layer's discontinuous interstitial matrix is exposed directly to the synovial fluid allowing for exchange of nutrients and waste products.

### Collagen

Collagen is responsible for resisting the tensile forces and shear stresses that synovium can experience (Kempson, 1974). The collagens are usually defined by the presence of a distinctive triple helical domain containing peptide chains with the repeating triplet of Gly-X-Y, where X and Y are often proline and hydroxyproline (Sandell et al., 1999). These chains combine to form collagen fibers that are protected from protease attack as the amide bonds between the chains are within the molecule. A rich diversity of collagens has now been discovered and although they are unique in structure their role as structural proteins is essential (Ayad and Sandell, 1996). Collagens within the synovium account for 20-50% of tissue dry weight. This high content of collagens in the synovium reduces the cross-sectional area available for fluid flow enhancing the synovium's ability to retain synovial fluid within the cavity (Eyre and Muir, 1975; Price et al., 1996).

Collagens I, III, IV, V and VI have all been detected within the synovium (Ashhurst, 1991; Levick and McDonald, 1990; Linck et al., 1983; Pollock et al., 1990; Worrall et al., 1991). Collagen type VI is rich in the cell binding motif RGD and has been implicated in the binding of matrix molecules such as HA and laminin. Thus, this collagen could anchor synoviocytes to the matrix so ordering matrix architecture (Kielty et al., 1992; Pollock et al., 1990; Wolf and Carsons, 1991). The network forming collagen type IV is a component of basement membranes but in the synovium it forms fine striated microfibrils that surround the SFBs. Non-striated microfibrils of collagen type VI are confined to the intima whilst collagen type III, the major striated fibrillar collagen extends into the subintima (Mason et al., 1999). Type I collagen interacts with collagen type III helping maintain fibril formation and growth (Sandell et al., 1999). Collagens type I, type III and type V extend into the subintima organising fibrils into parallel bundles that produce a criss-cross arrangement that could provide some of the elastic properties of the synovium (Levick et al., 1999).

## Glycoprotein

Glycoproteins are another important group of molecules within the synovial ECM. A role in cytoskeletal organisation, cell migration, growth and differentiation has been suggested for synovial glycoproteins due to the presence of specific binding domains (Henderson, 1987b). Fibronectin is one such glycoprotein consisting of two large subunits, which are joined by disulphide bonds near their carboxy termini with each folded subunit linked by polypeptide chains. Fibronectin shows a pericellular distribution (Revell et al., 1995) and Linck *et al.* (1983) have provided evidence that fibronectin bound HA may function to control protein exchange between the synovium and synovial fluid.

Laminin is a flexible complex that is formed from three long polypeptide chains. These, like fibronectin, contain functional binding regions that are able to bind to collagens and laminin receptors. Laminin is another component of basement membranes but although LN-1, LN-10 and LN-11 have all been detected in the synovial ECM there is no distinct basement membrane in the synovial cell layer (Ghadially, 1983; Konttinen et al., 1999). However, both fibronectin and laminin are

located in the basal part of the cell layer along with collagen type IV another component of basement membranes (Pollock et al., 1990).

Integrins are specific cell surface receptors (Hynes, 1992), influencing cell adhesion and migration. These molecules are the principle receptors in binding cells to their ECM and although this interaction is weak the integrins are at a high density on the cell surface. The main laminin receptor in the synovium is the integrin α6β1, but the collagen receptors α1β1 and α2β1 also bind laminin in the synovium (Sorokin et al., 1990). Pirilä *et al.* (2001) have shown that α6β1 is specific to the SFBs. In the synovium, integrins are thought to attach the SFBs to the matrix but their involvement in cellular signalling suggests they could also regulate the release of MMPs (Konttinen et al., 1999).

## Proteoglycan

Proteoglycan (PG) monomers consist of glycosaminoglycans bound to a protein core. The PG properties are then determined by the GAGs bound to the protein (Hardingham and Fosang, 1992; Jackson et al., 1991; Paulsson et al., 1987). PGs are able to interact non-covalently and form proteoglycan aggregates such as aggrecan or versican (fig.1.1.4). Aggrecan is found in many connective tissues including that of the cartilage matrix. Aggrecan allows the tissues to withstand compressive loads (Hascall, 1988; Mason et al., 1982; McDevitt, 1988) and is formed by the covalent attachment of keratan-sulphate and chondroitin-sulphate chains to a core protein. The monomers then become non-covalently bound to a HA molecule with link proteins stabilizing the interaction between HA molecule and PG monomer (Hardingham, 1979). Within the synovium the highly negatively charged PGs are able to occupy very large hydrophobic volumes and along with the collagen network contribute to the strength and stiffness of the synovium (Hascall and Hascall, 1981; Jackson et al., 2001). The PGs, decorin and biglycan have been found in the synovium (Bianco et al., 1990; Vogel and Trotter, 1987; Worrall et al., 1992). These two PGs have both been found carrying either chondrotin sulphate or dermatan sulphate chains yet little else is known about which PGs carry particular GAGs within the synovium. The levels of GAGs however, have been calculated and from this data the total GAG concentration within the synovial ECM has been estimated at 4.1mg $ml^{-1}$ (Price et al.,

contrast to cartilage, Price *et al.* (1995) showed that keratan sulphate was present in higher concentrations than the chondroitin sulphates within the synovium and there was a higher ratio of chondroitin-4-sulphate to chondroitin-6-sulphate. These studies would suggest that the difference in GAG distribution is a reflection of the different roles of synovium and cartilage within the joint.

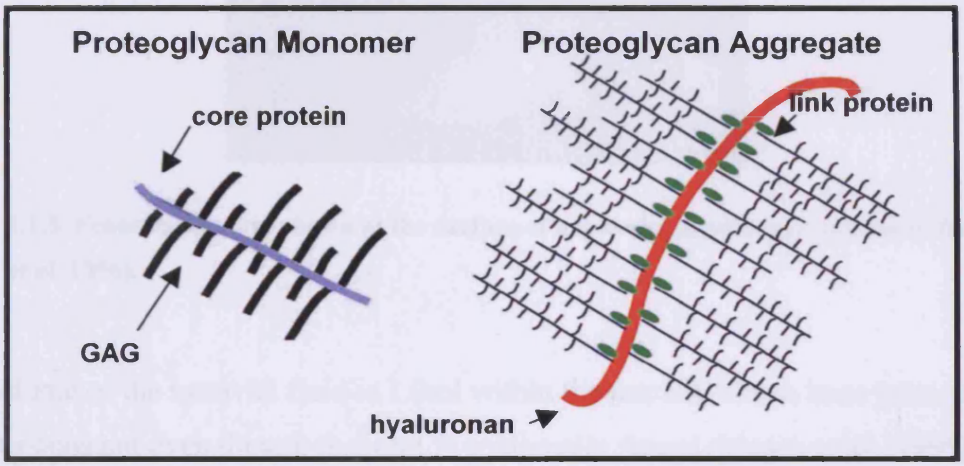


**Figure 1.1.4 Diagrammatic representation of proteoglycan monomer and aggregate.**

## 1.1.6 Synovial Fluid

The synovial fluid (SF) is an ultrafiltrate of plasma, and as a result its composition is very similar to plasma, with the addition of molecules synthesised and secreted by the SFBs (Levick, 1996). The concentration of electroclytes and small solutes, like urea, are almost identical as these can pass easily through the membranes. Glucose concentration is lower in the SF than the plasma as chondrocytes utilise this sugar as an energy source. Plasma proteins are also seen at lower levels as they are sieved by the capillary walls. The addition of HA and lubricin secreted by the SFBs completes SF composition (Jay et al., 2000; Mason et al., 1999; Pitsillides and Blake, 1992).

The capillaries of the synovium play an important role in synovial fluid production. Capillaries are abundant within the synovium, concentrated 35μm below the surface with the lymphatics lying deeper in the subsynovium (Knight and Levick, 1983). The capillaries show polarized distribution towards the joint cavity and the presence of polarized fenestrations on the capillaries reduces the distance solutes need to travel

facilitating fluid production (Levick, 1995; Levick, 1996; Yamashita and Ohkubo, 1993)(fig.1.1.5).

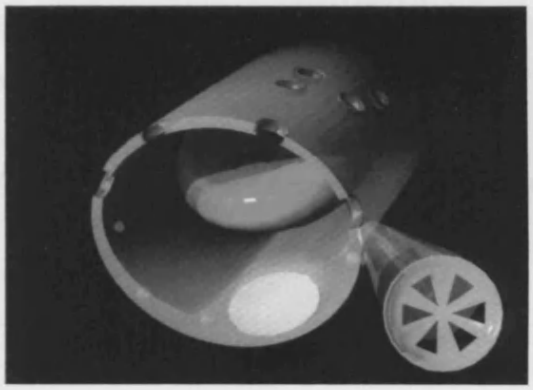

**Figure 1.1.5 Fenestrations are shown at the surface of a synovial capillary (reproduced from Levick *et al*, 1996).**

The volume of the synovial fluid is 1.0ml within the normal human knee joint and this remains constant even though the joint is continually flexed (Mason et al., 1999). The composition and volume of fluid within the cavity is essential, reducing friction between the articular cartilage surfaces so allowing free movement of the joint. The high concentration of HA contributes to the SF's functionality. HA is a linear polymer with a molecular mass of several million kDa and intermolecular and intramolecular interactions allow for the formation of a random coil figuration in solution, even at very low HA concentrations (Laurent, 1970). This secondary structure of HA, discovered by x-ray diffraction and spectroscopy, shows the occurrence of intramolecular reactions between the polar groups of the HA molecule. These H-bonds between the sugar residues and water molecules act to stabilise and stiffen the HA molecule, forming a helical arrangement (Scott, 1998). The conformation the HA molecule adopts allows it to form a flexible molecular network that will enable shear stresses to be applied, as the HA coils can deform and elongate absorbing the strain (Wik and Wik, 1998).

The synovial fluid reduces friction and wear by providing a smooth and low-friction coating for the articular cartilage surfaces. The SF is suited to the role with a low friction coefficient resulting in part from one of its components called lubricin (McCutcheon, 1967). Lubricin was first isolated and characterised over 30 years ago

and shows similar structural properties to proteoglycans with oligosaccharides attached to a core protein (Swann et al., 1985; Swann et al., 1981; Swann et al., 1977). Lubricin, like HA, is synthesised by the SFBs and secreted into the SF (Jay et al., 2000). This places lubricin in the ideal position to act as a boundary lubricant within the SF (Swann et al., 1981). More recently, work by Jay *et al.* (2000) showed proteolytic removal of lubricin from SF dramatically increased friction within the joint.

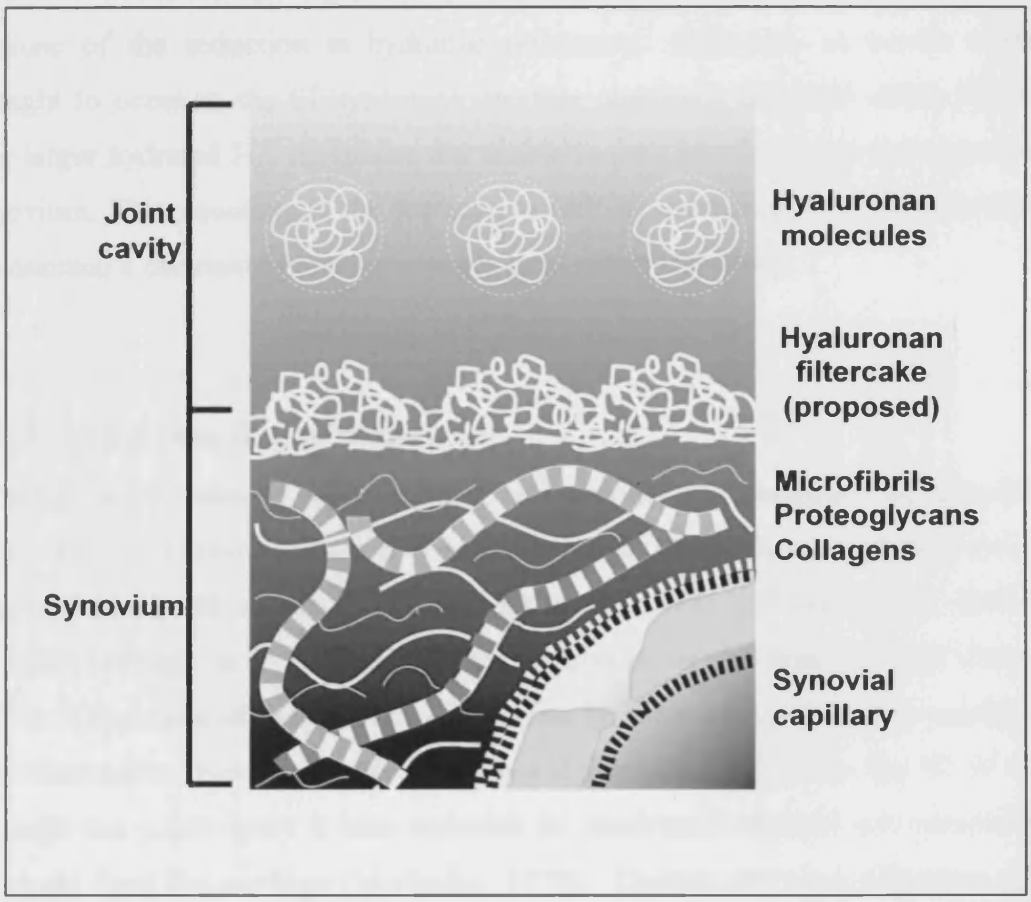


**Figure 1.1.6 Schematic diagram of proposed 'filtercake' hypothesis.**

The architecture of the synovium is very important in maintaining the synovial fluid. The continued trans-synovial fluid flow between synovium and synovial fluid allows the transport of nutrients and the removal of waste products during joint movement, while maintaining a constant volume of SF. Observations from previous work have suggested that flow is able to occur simultaneously both into and out of the synovial cavity at different points over the synovial surface (Levick and McDonald, 1994). The

rate of the fluid flow into the cavity has been calculated at approximately 2-4μl.h.cm$^2$ synovium in the human synovial joint (Levick, 1987).

When the joint is flexed an increase in the intra-articular fluid pressure (IAP) is observed (Levick, 1991). This pressure results in an increase in synovial surface area, an increase in interstitial spaces within the synovium and an increase in macromolecule concentration (Price et al., 1996). Normally, if IAP were to increase then this would be accompanied by an increase of fluid into the synovial cavity because of the reduction in hydraulic resistance. However, molecular sieving is thought to occur at the SF/synovium interface creating a filtercake effect (fig.1.1.6). The larger hydrated HA molecules are unable to pass into the interstitial spaces of the synovium. This counteracts the increase in IAP by producing outflow resistance, so maintaining a constant volume of viscous fluid within the cavity.

### 1.1.7 Synovium & Cartilage

Cartilage is an avascular tissue utilizing both aerobic and anaerobic respiration (Lane et al., 1977). However, its sparse cell distribution results in a metabolic rate of less than one hundredth of other cells (Barratt et al., 1977). In most joints, half of the articular cartilage is in contact with the synovium or the synovial fluid (Forrester, 1978). Movement of the joint ensures a route for molecular exchange as cartilage and synovium move over each other (Simkin and Pizzorno, 1974). As the SF is washed through the joint cavity it can replenish its nutritional content and remove waste products from the cartilage (Maroudas, 1970). During joint immobilization, glucose diffusion is slow resulting in lowered synthesis leading to overall matrix loss. This process supports the role of joint movement being necessary for the supply of oxygen and nutrients to the cartilage and also for the removal of its waste products via the synovium.

### 1.1.8 Osteoarthritis

Osteoarthritis (OA) is a painful and disabling disease that shows variable distribution throughout the joints of the body. In a healthy joint, the cartilage is organised with a low cell to matrix ratio. The chondrocytes maintain tissue homeostasis allowing dissipation of compressive loads during movement of the joint. Cells of the synovium also contribute to joint mobility by synthesising components of the synovial fluid filling the joint cavity. As OA progresses, the organised cartilage matrix, including proteoglycans and collagens breaks down and the synovium extends further into the cavity. Neighbouring chondrocytes try to rescue the damaged matrix but the process is disordered (Sandell and Aigner, 2001). The constituents of the synovial fluid alter and this adds to the reduction in joint mobility. Eventually subchondral bone is exposed, and the increased stress causes microfractures and cysts. The eventual outcome is an inflamed, painful joint with restricted movement.

Previous research into OA has suggested injury to the joint, genetic factors or an as yet unknown stimulus maybe responsible for disease onset (Fukui et al., 2001; Hedbom and Hauselmann, 2002; Smith et al., 1997; Terchek et al., 2002). Regardless of aetiology, it is clear that the relatively steady state of anabolic and catabolic processes that exist in normal healthy cartilage becomes uncoupled. In early OA, structural changes occur in the OA cartilage ECM. Type II collagen and aggrecan are two of the major components of cartilage matrix providing the mechanical properties of compressive resistance and elasticity. As synovial cells and chondrocytes respond to inflammatory stimuli, aggrecan is one of the first matrix components to be lost (Mankin and Lippiello, 1970). ADAMTs (a disingtigrin and metalloproteinase with thrombospondin motifs) particularly ADAMTS-4 and ADAMTS-5 have been implicated in aggrecan degradation in arthritis (Tortorella et al., 2001). This proteoglycan loss is thought to be reversible whereas, the later stages of matrix degradation, involving loss of collagens is irreversible (Mort and Billington, 2001). Another family member of the metalloproteases found within OA cartilage are the MMPs and these are responsible for collagen degradation (Billinghurst et al., 1997; Chubinskaya et al., 1999; Knauper et al., 1996; Vincenti et al., 1994). Recently it has been suggested that aggrecan protects the collagen from enzymatic cleavage but once aggrecan has been broken down then the collagen is exposed to MMP proteolysis

(Dahlberg et al., 2000; Kozaci et al., 1997; Pratta et al., 2003). Ultimately the structural integrity of the matrix is lost.

The chondrocytes respond to the initial stimuli by trying to rescue the damaged matrix. Enhanced expression and activity of anabolic growth factors such as TGF-β and IGF-I have been observed in OA cartilage and synovial fluid, presumably to try and increase collagen and PG content (Hedbom and Hauselmann, 2002; McQuillan et al., 1986; Middleton and Tyler, 1992; Morales and Hascall, 1988). The release of several pro-inflammatory cytokines eg. IL-1β, IL-1α and $TNF_{\alpha}$, believed to be the principle mediators of OA, also occurs in OA cartilage (Fukui et al., 2001; Tetlow et al., 2001). These pro-inflammatory cytokines stimulate catabolic processes inhibiting biosynthesis of essential components of cartilage ECM (Sandell and Aigner, 2001) while also increasing activity of degenerative enzymes such as MMPs and ADAMTs (Goldring et al., 1988; Tetlow et al., 2001; Tortorella et al., 2001). The effect each cytokine has on matrix degradation is not only dependent on cytokine concentration but the effect of modulating cytokines (IL-6, IL-8) and also anti-inflammatory cytokines (IL-4, IL-10) must be taken into consideration (Chomarat et al., 1995; Feldmann and Maini, 2001; Firestein, 2003; Fukui et al., 2001; Lubberts et al., 2000).

Clusters of chondrocytes are observed within OA cartilage. This proliferation prevents synthesis of new matrix yet proliferation would increase the numbers of cells able to synthesis matrix components (Rothwell and Bentley, 1973; Schumacher et al., 2002). Although the chondrocytes show an increase in synthetic rate, they are unable to produce 'normal' cartilage matrix (Lippiello et al., 1977; Matyas et al., 1995);. Again, this loss of ordered tissue adds to the loss in the mechanical properties of the cartilage.

Increased synthesis of nitric oxide (NO), a highly reactive free radical, has been shown to occur *in vitro* upon stimulation with IL-1β and $TNF_{\alpha}$ (Palmer et al., 1993). If the same occurs within the OA joint then increased concentrations of ILβ and $TNF_{\alpha}$ during OA could add to the degradation of the cartilage ECM as NO can inhibit collagen and PG synthesis as well as increasing MMP activity (Hauselmann et al., 1994).

Although the major pathogenic processes are located within the cartilage, components released from the cartilage into the synovial fluid act to modulate synovial cells into a response that might contribute to disease progression as the synovial cells are also able to release soluble cytokines and be a source of proteases (Hedbom and Hauselmann, 2002; Pelletier et al., 1995; Saito et al., 2002). Smith *et al.* (1997) demonstrated an increased synthesis of proinflammatory cytokines within the synovium correlating with an increase in articular cartilage damage, potentiating disease progression.

OA has a high prevalence within the population with between 1.3-1.75 million people affected in England and Wales alone (Reginster, 2002). Due to the ageing population, the incidence of OA will only increase unless beneficial treatments are found. Along the pathway from the first signs of cartilage matrix degradation to the loss of areas of cartilage within the joint, there are several possible points for intervention. Inhibition of pro-inflammatory cytokines, induction of anabolic enzymes, inhibitors of MMPs and ADAMTs are routes many believe will help in preventing or at least slowing down the progression of this disease (Malfait et al., 2002; Yao et al., 2001). Some protease inhibitors, however, have proved unsuccessful due to the complexity of cartilage metabolism and also because of the inability to determine severity of OA (Mort and Billington, 2001). Combining other strategies, including detection of biochemical disease markers within the synovial fluid, cartilage, or synovium to indicate the stage of OA, with biologic agents might prove a more beneficial approach. Cell and tissue transplantation is also a possibility and already in use in the form of autologous cartilage implantation (ACI). However, this is not recommended for treatment of late stage OA (Jackson et al., 2001). More recently the discovery of a cartilage progenitor cell has lead to the viable possibility that chondroprogenitor cells can be expanded *in vitro* and transplanted back into the defect where they will be able to generate new cartilage and replace areas of damage (Archer et al., 2000; Dowthwaite et al., 2004). As these methods remain in their infancy at present, then treatment of OA is temporary at first using analgesics, NSAIDs and corticosteroids to reduce pain and inflammation. A recommendation of weight loss and gentle exercise can also help alleviate pain and improve joint mobility. If these treatments fail then disease progression ultimately results in joint replacement surgery.

### 1.1.9 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is the most common inflammatory arthritis affecting almost one percent of the world's population (Robbins et al., 2003). In the mid-20th century it became clear that this debilitating disease could be the result of autoimmune attack and since then research has accumulated to support this hypothesis (Firestein, 2003; Zvaifler, 1973).

During RA, the synovium's usually flat surface is thrown into villi, the core of which contain aggregates of inflammatory cells including polymorphs, lymphocytes, plasma cells, mast cells and macrophages (Collins, 1949; Davies, 2003; Fassbender, 1985). T-cells are also present in this infiltrate but their response *in vitro* when in the presence of RA synovial cells alters. A lower proliferative rate was observed, especially in comparison to other antigen-mediated processes (Smeets et al., 1998; Stastny, 1976). Presence of T-cell cytokines/chemokines were in low abundance in the RA synovium compared with cytokines synthesised by the SFBs and SMPs. This cytokine imbalance allows induction of MMP synthesis and the inhibition of collagen synthesis leading to tissue degeneration (Burger et al., 1998; Hyka et al., 2001).

Hyperplasia of the synovium is one of the main factors associated with RA and although SFBs have been shown to undergo rapid proliferation *in vivo* in the presence of immune and inflammatory cells, it is still not known whether this hyperplasia is due to cell proliferation or whether it is a breakdown in the apoptosis signal (Davies, 2003; Leung et al., 2002; Tanaka et al., 2002). Chronic inflammation is thought to lead to DNA damage and experiments inhibiting p53, a gene involved in the cell cycle and apoptosis, results in fibroblasts with characteristics similar to SFBs from RA tissue.

As the synovium proliferates, it encroaches upon the cartilage at the periphery of the joint and eventually overgrows the surface of the cartilage forming a pannus. Here the SFBs show an activated phenotype expressing adhesion molecules and secreting factors such as matrix degrading enzymes including aggrecanases and MMPs (Deleuran et al., 1992; Foey et al., 2000; Woolley et al., 1977). There are a broad array of cytokines and chemokines also synthesised by both types of synovial cells,

which potentiate the inflammatory response (Cheon et al., 2002; Scott et al., 1997). The result of this response is the eventual degradation of the cartilage matrix (Smeets et al., 2001; van der Laan et al., 2003).

Increased vascularisation is evident in RA synovium. The lymphatic vessels become more numerous and prominent within both layers of the synovium (Davies, 2003; Xu et al., 2003). Another effect of the disease is the increase in volume of the synovial fluid resulting in swollen joints, one of the main symptoms of RA (Davies, 2003; Freemont et al., 1983). Eventually focal joint erosions can be seen. These usually occur at the joint margins but focal bone resorption is also observed in the subchondral bone and these subchondral lesions can penetrate the overlying cartilage. Evidence suggests that osteoclasts mediate this process via signals from cytokines and inflammatory mediators (Goldring, 2002).

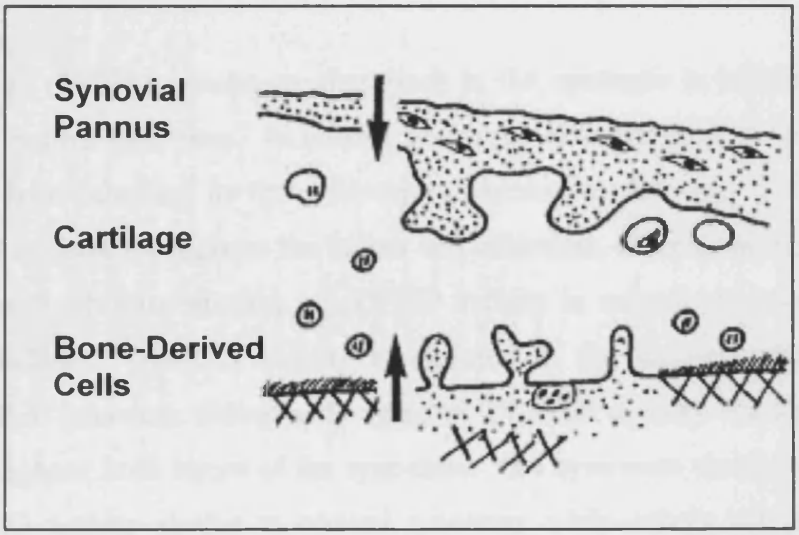


**Figure 1.1.7 Diagram showing the bi-directional erosion of cartilage. The predominant cell types observed in the synovial pannus are SMPs, SFBs, mast cells and neutrophils (adapted from Molecular Mechanisms in Rheumatoid Arthritis, 1995).**

Proinflammatory cytokines produced locally in RA synovial joints contribute directly or indirectly to RA pathogenesis. Biological agents which modulate these cytokines are one of the main treatments in targeting the disease. As the cytokine network is so complex, then blocking proinflammatory pathways while leaving anti-inflammatory pathways unaffected is very difficult. This has lead to novel gene therapies where *in*

*vitro* cells are manipulated and injected into specific sites within the joint so targeting the exact area of inflammation. Due to their involvement with inflammation, the transcription factor NF-κβ and cell cycle gene p53 have been targeted as ideal candidates for this research (Makarov et al., 1997; Taniguchi et al., 1999).

### 1.1.10 Diseased Synovium

There are a number of changes within the synovium associated with both RA and OA and one of these is the modification of the synovial architecture. Instead of an even distribution of SFBs and SMPs within the synovium, the SMPs migrate to occupy the outer most layer of the intima (Worrall et al., 1991). This strategic positioning of SMPs nearer to the cavity allows the removal of waste products and cell debris that accumulates in the SF. There is also an increase in cell numbers due to leucocyte infiltration and the proliferation of SFBs (Henderson, 1987a) (fig.1.1.9A).

Pitsillides *et al.* (1994) have shown alterations in HA synthesis in inflamed synovium compared to normal synovium. In normal synovium, HA was present throughout the intima with faint labelling in the subintima, whereas in RA and OA tissue, HA labelling was uniform throughout the intima and subintima, corresponding to a change in cell position with inflammation. UDPGD activity in normal synovium correlates with HA labelling. UDPGD activity is elevated in the intima compared to the subintima. RA synovium shows a decrease in UDPGD activity but this activity is uniform throughout both layers of the synovium. OA synovium varies, some samples show UDPGD activity similar to normal synovium while others are similar to RA tissue depending on the degree of infiltration present (Pitsillides and Blake, 1992; Wilkinson et al., 1992).

It has already been established that the maintenance of a high HA concentration in SF and synthesis of the correct sized HA polymer is important for joint function. HA concentration in the SF of RA and OA patients is lower than that of normal SF leading to reduced viscosity. This decrease in HA concentration results from an alteration in UDPGD activity and subsequently decreased HA synthesis during

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**Figure 1.1.8** The synovium of a 71 year old male. Note the thin intimal layer (arrowed).

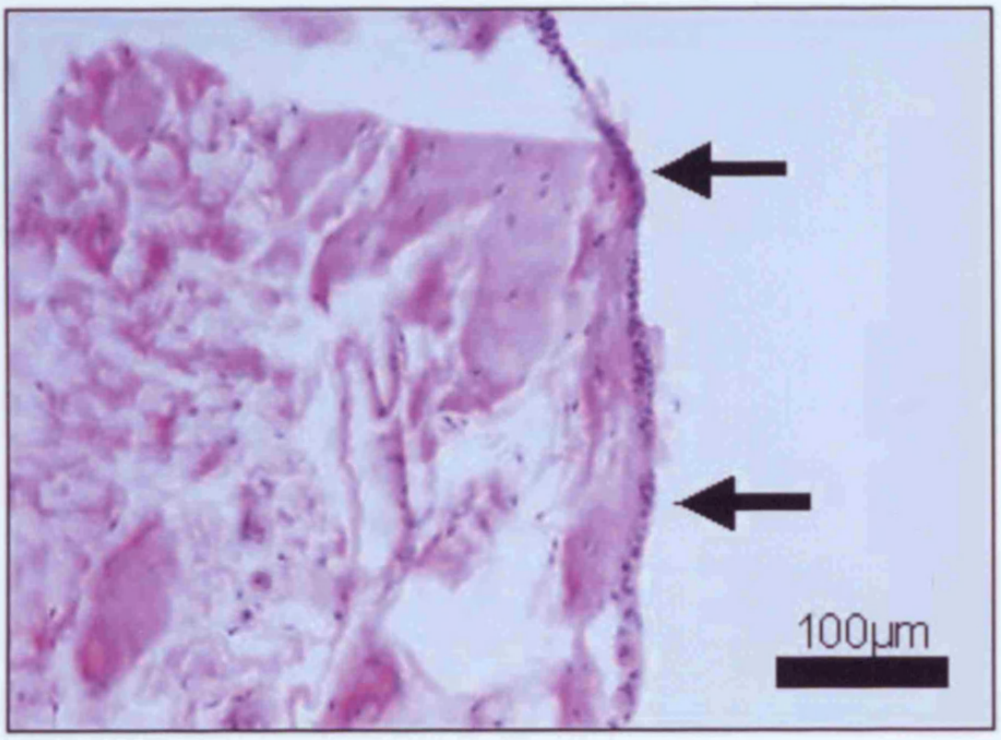
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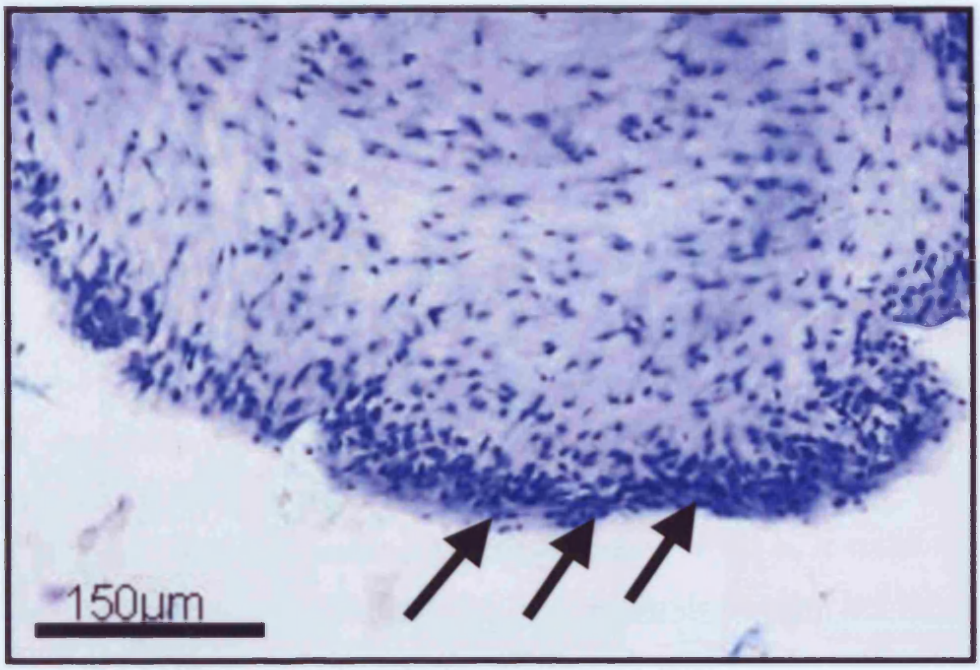
**Figure 1.1.9** Synovium from 70 year old male diagnosed with osteoarthritis. A) Note the high cell density of the synovial surface. B) Note the leucocyte infiltration (arrowed, MagX125).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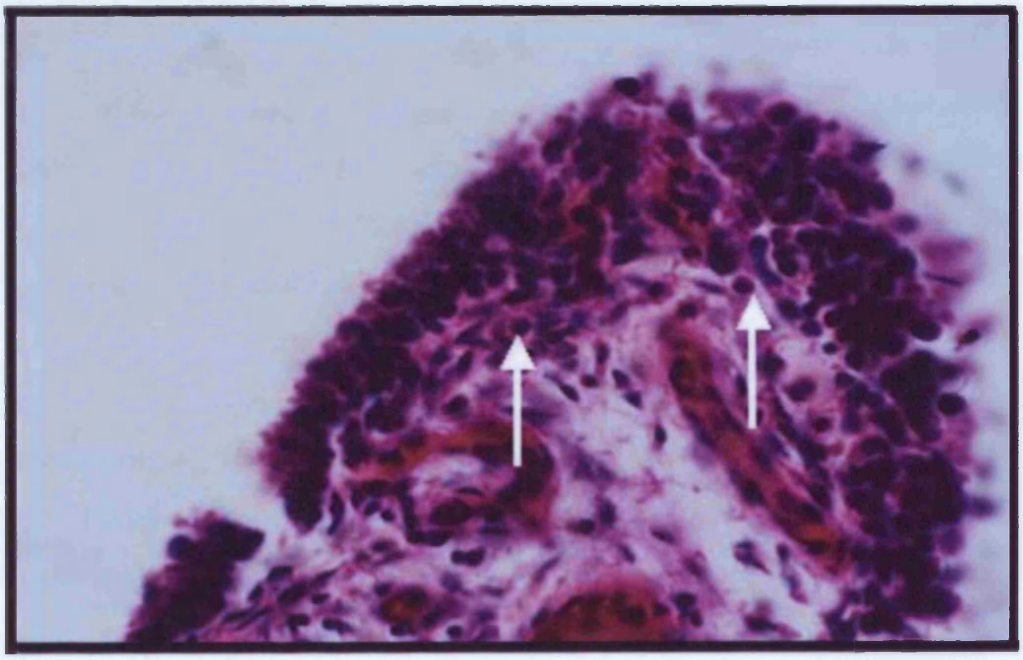


disease (Miyaguchi et al., 2001; Wik and Wik, 1998; Yamada et al., 2000). Whether this change is a result of HA dilution, HA degradation or changes in HA synthesis is still not clearly understood. The ratio of free:bound HA within RA synovium is higher than that of normal synovium yet *in vitro* lower molecular weight HA is synthesized (Pitsillides et al., 1994). The smaller the HA molecule the more mobile it is and so it can pass more easily through the synovium and into the capillaries. As a result, high plasma HA serum levels are seen in patients suffering from RA (Balazs et al., 1967; Sundblad, 1953). These levels of serum HA are often assessed as a sign of the severity of the disease. OA tissue can show a response similar to either normal or RA tissue depending on the degree of infiltration and recently, Miyugachi *et al.* (2001) have shown the presence of a low molecular weight HA in the synovial fluid from OA patients. This low molecular weight product could be a result of HAS3 expression as this produces the smallest HA polysaccharide *in vitro* and has a higher $V_{max}$ than HAS2 (Spicer and McDonald, 1998). A smaller HA molecule would explain the increased mobility of HA into the circulation and therefore the decrease in synovial fluid viscosity during disease. Synovial fibroblasts from OA tissues show HAS1 message levels that are more abundant than HAS2 and HAS3 message levels *in vitro* yet *in vivo* HAS1 and HAS2 message levels are similar, with HAS3 showing the least abundance (Recklies et al., 2001). The expression of the three *HAS* genes are higher *in vivo* than *in vitro* which suggests an increased level of HA biosynthesis in tissue. These three enzymes are essential for HA synthesis, yet little is known about their regulatory factors, especially in diseased tissues. Dowthwaite *et al* (1999) have demonstrated an increase in *HAS3* expression in fibrocartilage cells subjected to mechanical strain while *HAS2* expression remained constitutive. If the same was to occur in the synovium, then movement of the joint could regulate *HAS* gene expression in the synovial cells, maintaining HA concentrations within the synovial fluid and so allowing friction free articulation of the joint. Thus, the reintroduction of mechanical stimuli to immobile OA joints could be beneficial in increasing HA synthesis and increasing mobility.

### 1.1.11 Aims of the Project

Mechanical stimuli play a critical role in joint cavitation and the alterations in HA synthesis which occur along the presumptive joint line during joint development have been linked to mechanical stimuli. Continued flexion of the joint and hence HA synthesis is essential in maintaining a fully functioning adult joint. During osteoarthritis however, there is a reduction in the concentration of HA within the synovium and synovial fluid contributing to joint pain and immobility. This could be due to the cells reducing their HA synthesis as a facet of the disease. The loss of mechanical stimuli to the cells however, may lead to a further reduction in HA synthesis.

This study aims to determine whether the synovial cells derived from OA samples are still capable of modulating HA synthesis in response to mechanical strain by using a four-point bending mechanical loading jig. This study should provide us with a greater understanding of HAS regulation and subsequent HA synthesis within the synovial joint and the role mechanical stimuli plays in maintaining this synthesis.

# Chapter 2

# Effects of Long Term Culture on Synovial Cells

## 2.1 Introduction

The synovium consists mainly of two cell types, the synovial fibroblasts (SFB) and the synovial macrophages (SMP). These two different cell populations interact to maintain the healthy synovium (Chomarat et al., 1995; Edwards, 1999). Mast cells, T-cells, B-cells, endothelial cells, leukocytes and neutrophils are also present within the synovium and these cell types increase in number during during OA and RA contributing to the inflammatory response (Collins, 1949; Davies, 2003; Fassbender, 1985).

Experiments conducted with cells isolated from the synovium requires the phenotypic features to be as close as possible to that observed *in vivo* to produce reliable and reproducible results. Isolation of synovial cells can involve the digestion of the synovium using collagenase/trypsin or by explant outgrowth. However, with both these techniques a mixed population of cells is obtained and alters with time in culture. Alterations in growth rates and an increase in the frequency of genotype changes can be seen to occur (Clarris and Fraser, 1968; Ermis et al., 1995; Tsai et al., 1996). The data suggest the synovial cells deviate from their *in vivo* phenotype during *in vitro* growth. It is often the case that researchers use synovial cells from different passages to conduct their experiments. If the change in synovial cell cultures is significant between passages then variability in results could occur.

Generation of cell lines for future experimentation requires the *in vitro* culture of cells isolated from normal, OA and RA synovium. The aim of this work was to investigate the type of synovial cells obtained from the synovium and the effects of repeated-passage have on SFBs and SMPs and their respective markers, allowing the determination of whether to use low or high passage cells for future experiments.

## 2.2 Materials and Methods

### 2.2.1 Synovium Samples

Synovium was obtained at the time of surgical knee replacement for degenerative osteoarthritis with the relevant ethical approval. Samples were obtained from both female (n=4) and male (n=3) patients with an age range 63-82 years (table.2.2.1).

Excised tissue was placed into sterile phosphate buffered saline (PBS) and stored at 4°C until collection. The samples were cultured on the same day as they were excised.

| Sample | Sex | Age (years) |
|---|---|---|
| OA9 | Female | 72 |
| OA10 | Female | 73 |
| OA11 | Male | 65 |
| OA12 | Female | 63 |
| OA13 | Female | 82 |
| OA14 | Male | 66 |
| OA15 | Male | 66 |

**Table 2.2.1 Details of synovium samples obtained at knee joint replacement surgery.**

### 2.2.2 Cell Isolation & Culture

Synovial cells were isolated from diced tissue by digestion in 295IU mg $ml^{-1}$ type I collagenase (Sigma, Poole, UK) in Dulbecco's 50% Minimal Essential Medium/ 50% Nutrient Mix F12 (DMEM/F12; Life Technologies Ltd, Glasgow, UK) and placed on a roller for 3 hours at 37°C with vigorous shaking every 30 minutes. Following digestion, DMEM/F12 was removed into a sterile tube and an equal amount of DMEM/F12 added to the remaining synovium. The sample was vigorously shaken, media removed and placed into another sterile tube. Both fractions were centrifuged at 1000 x g for 5 minutes at 4°C and pelleted cells were suspended in DMEM/F12 containing 1% penicillin-streptamycin ($10^4$IU $ml^{-1}$/$10^4$ μg $ml^{-1}$; Sigma), L-glutamine (200mM; Life Technologies, Paisley, UK), hydrocortisone (4mg $ml^{-1}$; Sigma)[40mg hydrocortisone powder dissolved in 5ml 100% ethanol and 5ml sterile PBS], Insulin-Transferrin-Selenium (200mM; Life Technologies) and 20% foetal calf serum

(Sigma) [DMEM/F12+]. Following centrifugation at 1000 x g for 5 minutes at 4°C, cells were resuspended in DMEM/F12+ and the fractions combined. Cells were counted, seeded as required and incubated at 37°C in a 5% $CO_2$/air atmosphere.

**Cell Passage**

Once isolated, cells were plated into T75 flasks and allowed to reach confluence with a media change of DMEM/F12+ every four days. Primary cultures were grown to confluence, trypsinised (Appendix 2.1) and re-seeded at a 1:2 ratio in T75 flasks.

### 2.2.3 Immunocytochemistry

Cells seeded in 35 mm dishes were grown to confluence, the media removed and cells washed in PBS. The cells were fixed in 95% ethanol (Sigma) for 10 minutes and the cells washed in PBS.

Cells were incubated with blocking serum diluted in PBS-T for 20 minutes at room temperature to prevent unspecific binding of primary antibody. Excess serum was removed and the primary antibody diluted (table 2.2.2) in PBS-T, applied to the cells and incubated overnight at 4°C. The cells were washed 3 times in PBS-T (5 minutes each) then incubated with appropriate secondary antibody. The alexa fluor 488 (Molecular Probes Europe, The Netherlands) used depended on the primary antibody (table 2.2.2). The secondary antibody was diluted to 10μg $ml^{-1}$ in 1% blocking serum for 60 minutes at room temperature. Cells were then washed in PBS (3 X 5 minutes) and one drop of Vectashield plus propidium iodide (Vector Labs, UK) was applied and the cells cover slipped.

Negative controls consisted of PBS-T instead of primary antibody or relevant mouse/rabbit IgGs. Positive controls used were frozen sections of synovium due to the lack of human cell cultures available.

**Cell Counts**

One hundred cells or more were counted per 35mm dish from three random areas on the dish. Labelled and non-labelled cells were noted and one-way ANOVA tests were

applied to the cell counts with Tukey post-hoc tests. This statistical test was used to allow the comparison of data from more than two samples.

| Name of Clone | Antigen Specificity | Subclass | Dilution | Source | Reference |
|---|---|---|---|---|---|
| BRIC 128 | CD55 | IgM | 1:10 | IBGRL Research Products, Bristol. | Mushens R *et al.*, (1990) J Immunol Meth 131:83-89 |
| N-19 | Integrin α6 | IgG | 1:500 | Chemicon International Inc, CA, USA | Hynes RO (1992) Cell 69:11 |
| UCHM-1 | CD14 | IgG | 1:50 | Chemicon International Inc, CA, USA | Linch DC *et al.*, Blood (1984) 63:566 |
| KP1 | CD68 | IgG | 1:50 | Dako, Cambridgshire , UK | Warnke, RA *et al.*, Am J Pathol (1989) 135:1089 |
| P3C4 | VCAM-1 | IgM | 1:100 | Chemicon International Inc, CA, USA | Blood (1993) 81:2272 |

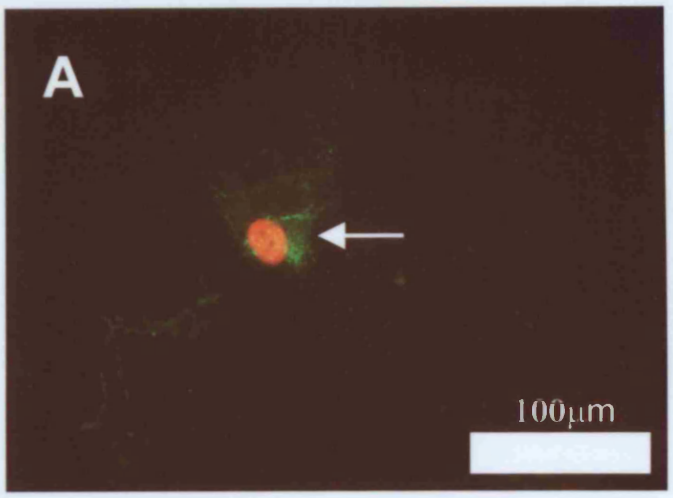
**Table 2.2.2 List of synovial cell markers.**

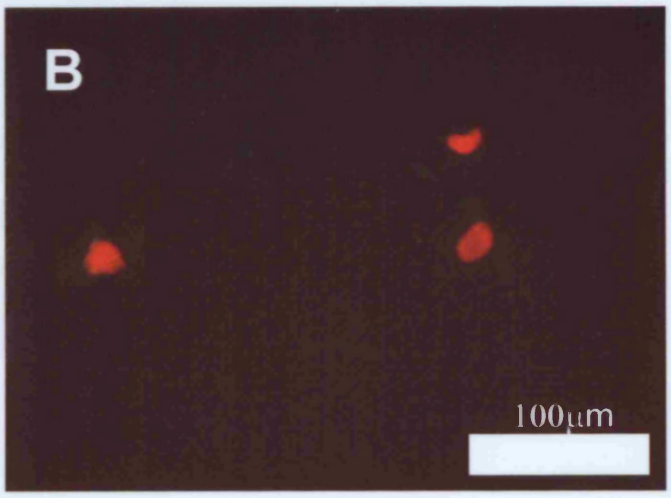
## 2.3 Results

The expression of the macrophage marker CD14 in synovial cell cultures can be seen in fig.2.3.1A. The expression of CD14 was seen to significantly decrease with repeated passage (fig.2.3.1C). The expression of the macrophage marker CD68 was present in the synovial membrane and was maintained in synovial cell cultures (fig.2.3.2A & B). The expression of this macrophage marker was seen to significantly decrease with repeated passage (fig.2.3.2E).

Expression of the fibroblast marker VCAM-1 observed in the synovial membrane is maintained in synovial cell cultures (fig.2.3.3A & B) yet there was no significant difference in the number of labelled cells in passage 1 cultures compared to repeated-passage cells (fig.2.3.3E). Expression of integrin α6 was seen in the synovial membrane and in synovial cultures. Significantly more cells expressed integrin α6 at passage 2 cells compared with passage 1 cells (fig.2.3.4A & B). The expression of this integrin showed significantly decreased expression in passage 5 and passage 6 cultures compared with passage 2 cells (fig.2.3.4D). Expression of CD55 was observed in the synovial membrane and in cultured cells at each passage (fig.2.3.5A & B). No significant differences in CD55 expression was found between passage 1 and the repeated passage cells (fig.2.3.5F).

**Figure 2.3.1** Immunolabelling for CD14. A representative synovial cell culture (A) labelled for CD14. Negative controls of no primary antibody showed no labelling in cultured cells (B). Percentage expression of CD14 in repeated passage OA synovial cell cultures (C). *$<P<0.05$ compared to passage 1 cultures.


A
100μm


B
100μm

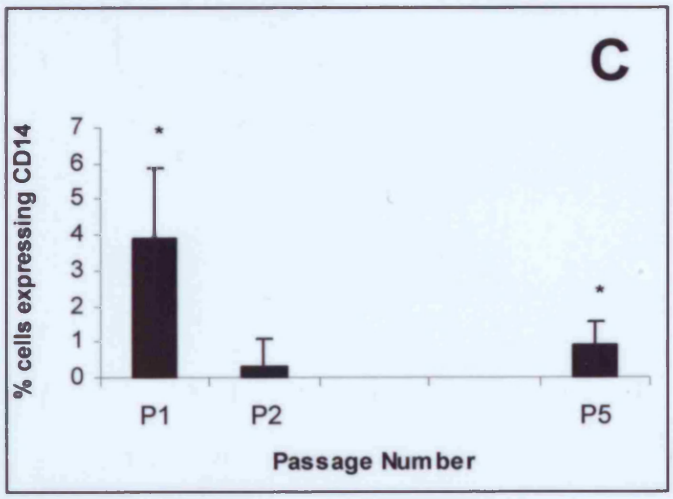

C
% cells expressing CD14
7
6
5
4
3
2
1
0
*
*
P1
P2
P5
Passage Number

**Figure 2.3.2** Immunolabelling for CD68. Immunolabelling of a representative synovial membrane (A) and synovial cell culture (B) labelled for CD68. Negative controls of no primary antibody (A & C) and mouse IGGs (D) showed no labelling in synovial membrane or cultured cells. Percentage expression of CD68 in repeated passage OA synovial cell cultures (E). *$<P<0.05$ compared to passage 1 cultures.

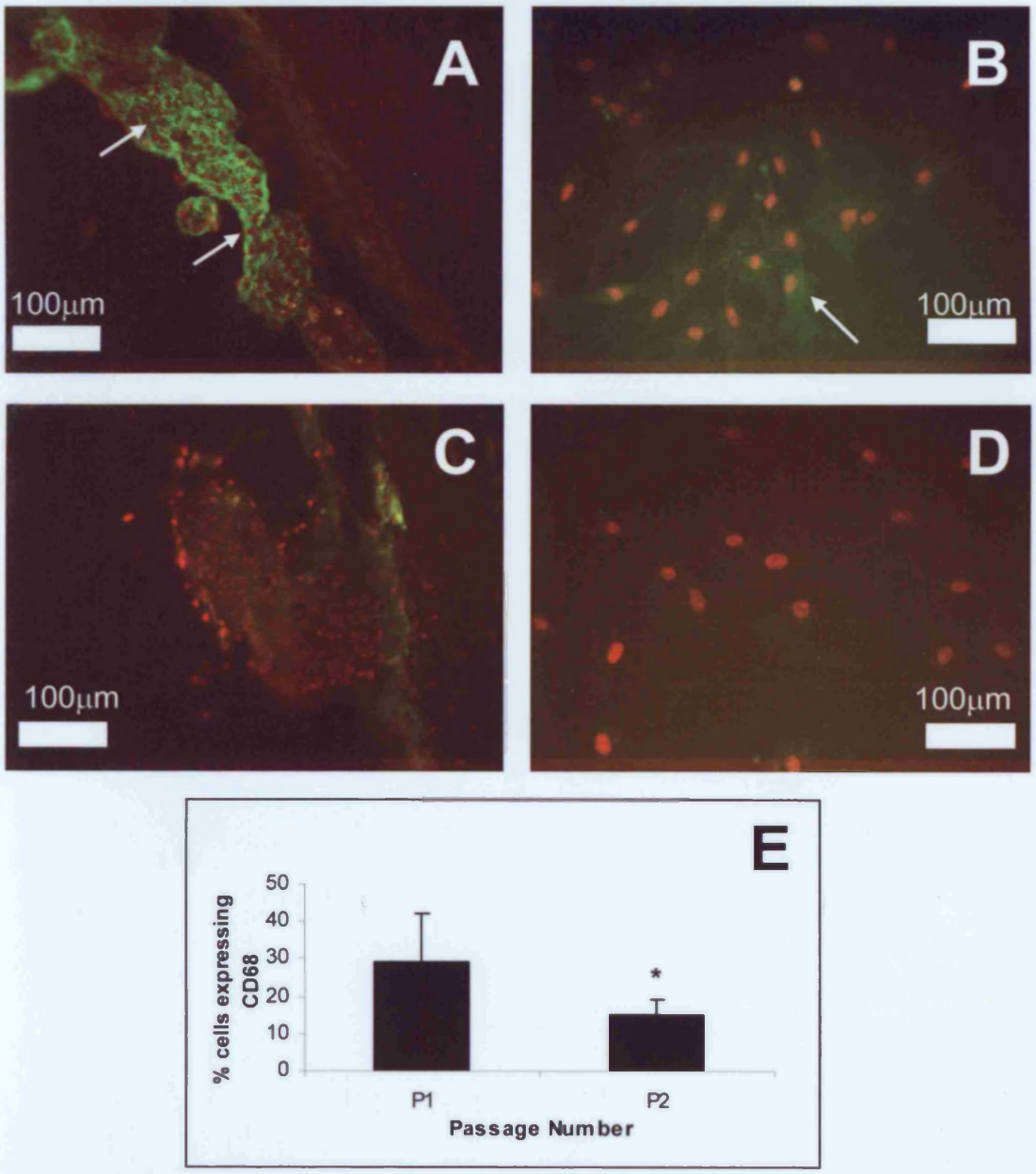

A
100μm
B
100μm
C
100μm
D
100μm
E
% cells expressing CD68
50
40
30
20
10
0
*
P1
P2
Passage Number

**Figure 2.3.3** Immunolabelling for VCAM-1. Immunolabelling of a representative synovial membrane (A) and synovial cell culture (B) labelled for VCAM-1. Negative controls of no primary antibody showed no labelling in synovium (C) or cultured cells (D). Percentage expression of VCAM-1 in repeated passage OA synovial cell cultures (E).

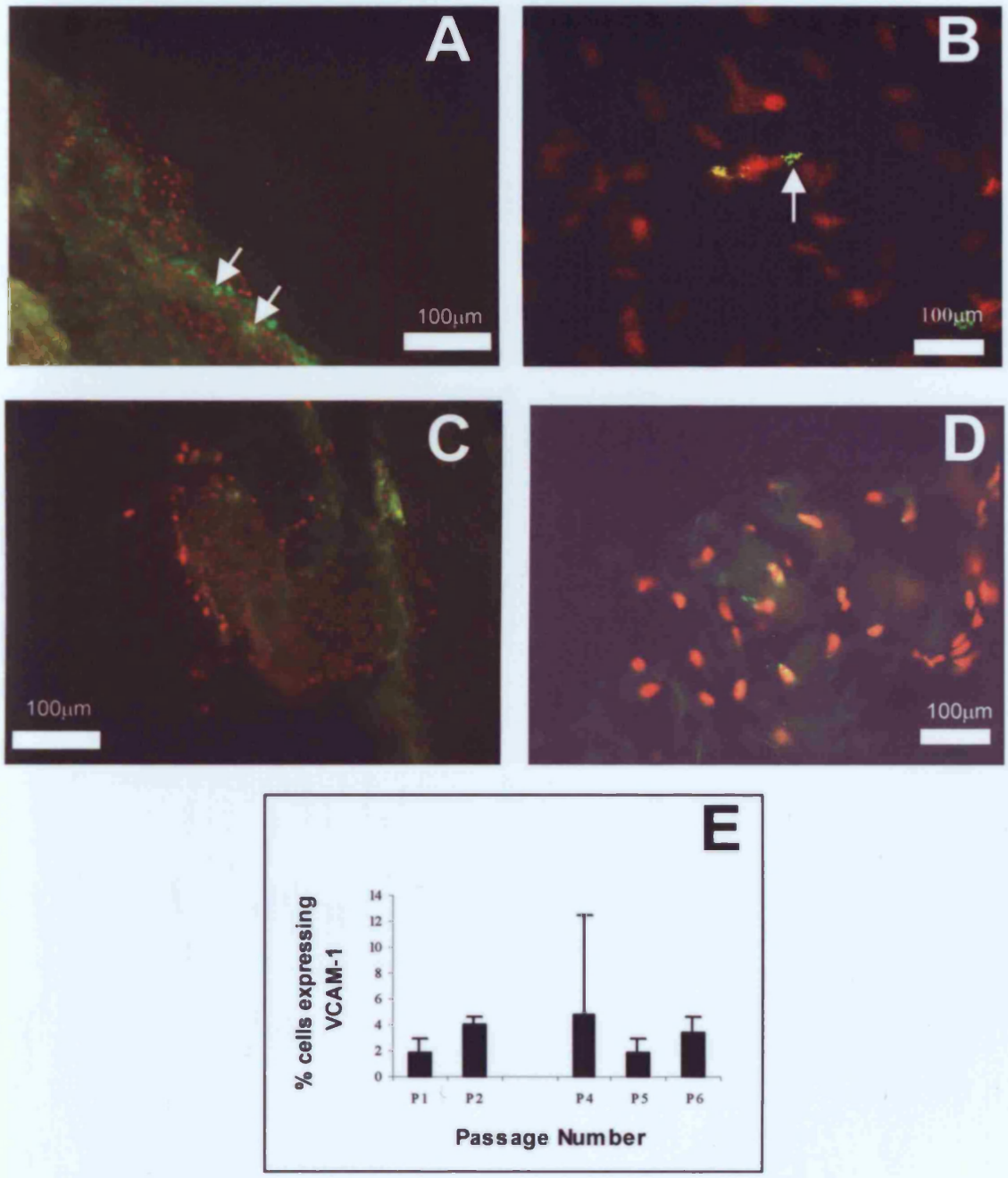

A
100µm
B
100µm
C
100µm
D
100µm
E
% cells expressing VCAM-1
14
12
10
8
6
4
2
0
P1
P2
P4
P5
P6
Passage Number

**Figure 2.3.4** Immunolabelling for integrin α6. Immunolabelling of a representative synovial membrane (A) and synovial cell culture (B) labelled for integrin α6. Negative controls of no primary antibody showed no labelling in synovium (C). Percentage expression of integrin α6 in repeated passage OA synovial cell cultures (D). *$<P<0.05$ compared to passage 1 cultures.

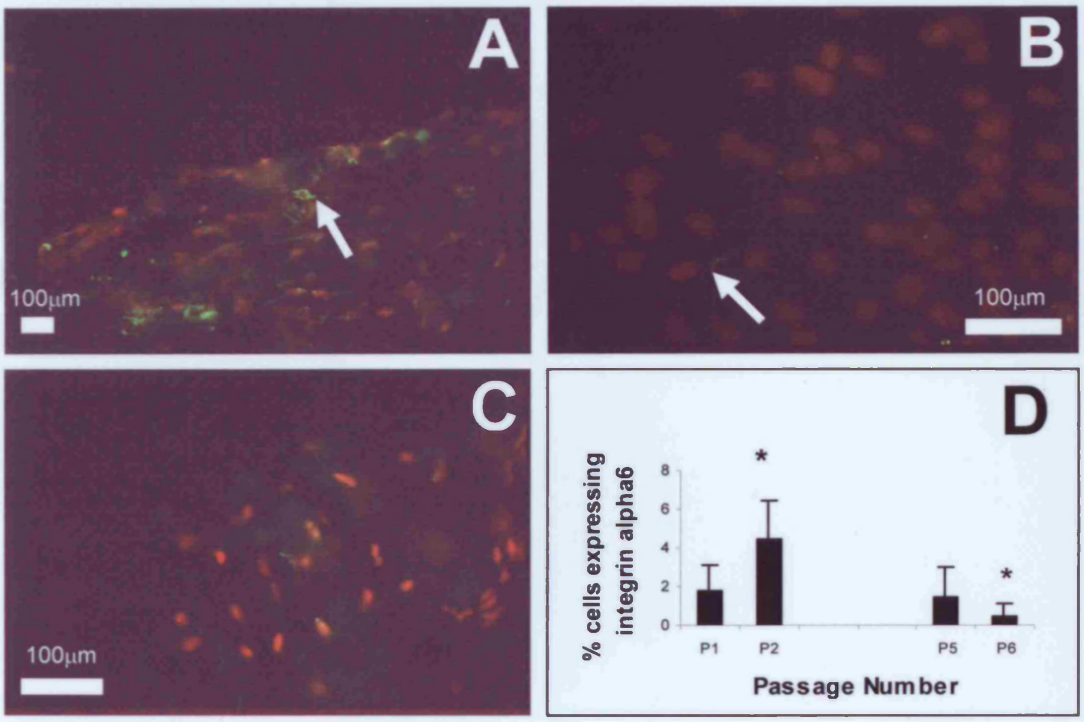
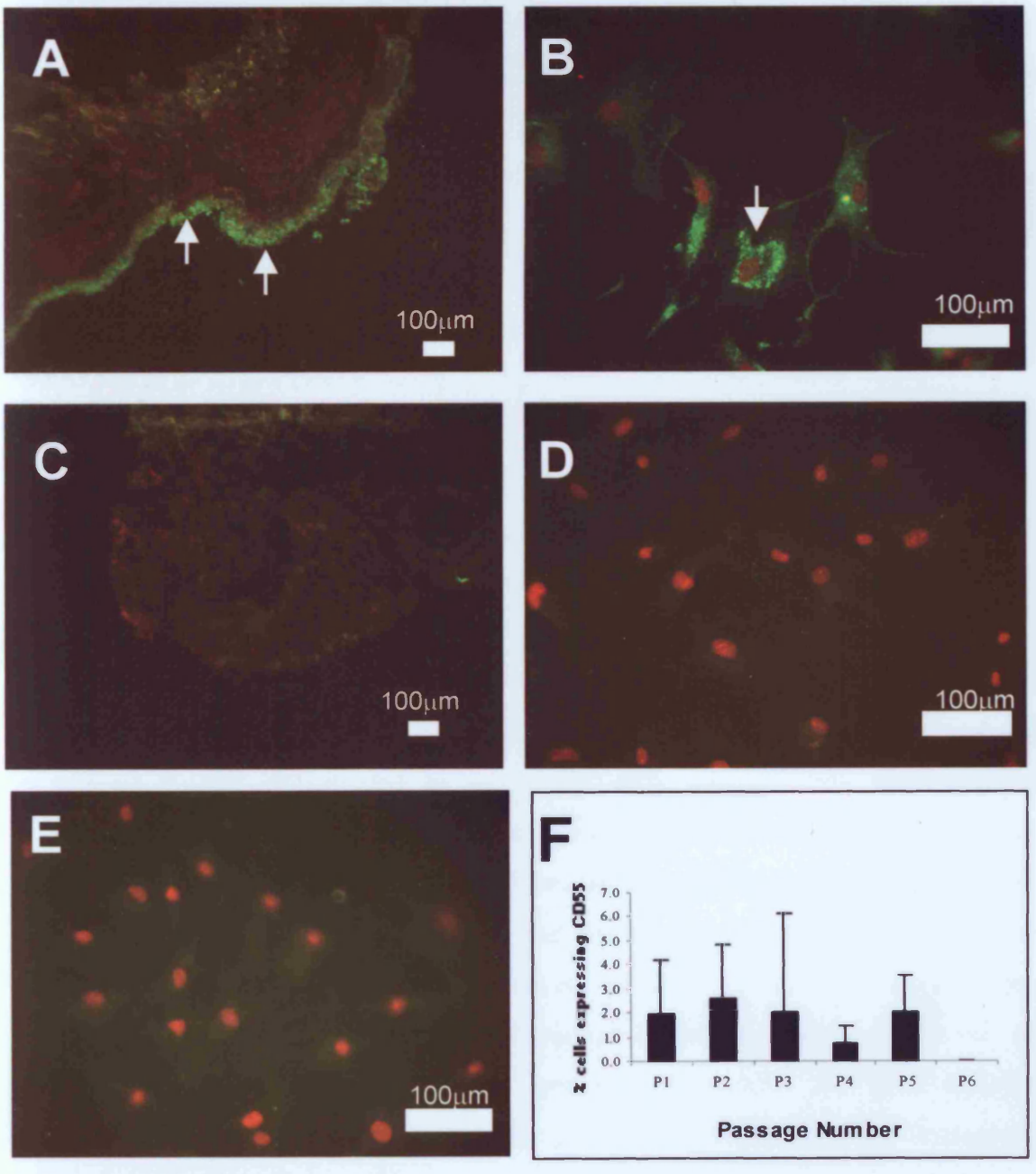

A
100μm
B
100μm
C
100μm
D
% cells expressing integrin alpha6
8
6
4
2
0
*
*
P1
P2
P5
P6
Passage Number

**Figure 2.3.5** Immunolabelling for CD55. Immunolabelling of a representative synovial membrane (A) and synovial cell culture (B) labelled for CD55. Negative controls of no primary antibody showed no labelling in synovium (C) or cultured cells (D). Percentage expression of CD55 in repeated passage OA synovial cell cultures (E).


A
100μm
B
100μm
C
100μm
D
100μm
E
100μm
F
% cells expressing CD55
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
P1
P2
P3
P4
P5
P6
Passage Number

## 2.4 Discussion

The synovial membrane is a relatively acellular structure which shows hyperplasia and cell infiltration during OA and RA (Davies, 2003; Henderson, 1987). The main cell types of the synovial membrane are the SFBs and SMPs and these two cell types can be distinguished from each other by a series of cell markers. Immunofluorescent labelling for CD14 positive macrophages and VCAM-1 and integrin alpha6 positive fibroblasts was observed in the OA synovial membrane and relates to previous published studies (Morales-Ducret et al., 1992; Pirila et al., 2001; Smith et al., 2003; Wilkinson et al., 1992; Wilkinson et al., 1993). Labelling for CD68 and CD55 is seen in the synovial membrane but these cell markers can label for fibroblasts and macrophages so do not clearly define the cell types (Falini et al., 1993).

Removing cells from their *in vivo* environment will alter their phenotype to some extent but the isolation of both SFBs and SMPs were observed in cell cultures from synovial tissue with the maintenance of cell phenotype. However, it seems that the long-term *in vitro* culture of synovial cells results in a cell type and cell phenotype change (Clarris and Fraser, 1968; Ermis et al., 1995; Marsh et al., 1978; Tsai et al., 1996; Zimmerman et al., 2001). A loss of SMPs with repeated-passage is a common occurrence in cell culture and in this study early passage cells labelled more extensively for SMP markers than late passage cells (Zimmerman et al., 2001). The loss of SMPs could lead to the conclusion that the late passage cultures consist solely of SFBs. This might indeed be the case but the loss of CD55 and integrin alpha6 labelling suggests that although the cultures are labelling for fibroblast markers they are losing the stimuli to maintain their phenotype in culture, leading to the loss of synovial fibroblast characteristics. The loss of cell markers has been reported previously so is not uncommon to this study (Edwards et al., 1997). The decrease in expression of cell markers could be a result of the loss of the SMPs. The SFBs and SMPs have been shown to interact within the synovium so removing the SMPs from cell cultures could prove detrimental to the SFBs (Chomarat et al., 1995; Edwards, 1999).

In conclusion, cell culture inevitably results in a modification of the cell as it is removed from it's *in vivo* environment. Fortunately cell culture techniques have progressed to such an extent that the *in vitro* growth conditions are as representative of the *in vivo* growth conditions as possible. Within long term cultures however, the synovial cell cultures show alterations. The loss of macrophages and other infiltrating cells with repeated-passage cultures and the loss of fibroblast marker expression on the synovial fibroblasts suggests that the lower passage cells are more representative of the true synovial cell. As a result future experiments in this study will use synovial cell cultures at passage 1, passage 2 and passage 3.

# Chapter 3

# Effects of Mechanical Strain on Hyaluronan Synthesis in Synovial Cells

## 3.1 Introduction

The sequence of events involved in joint cavity formation are complex and controlled. A full explanation for the separation of the cartilage anlagen allowing formation of the fluid-filled cavity is yet to be provided. However, it has been established that a process of cell loss combined with the turnover of matrix molecules occurs allowing the cells of the interzone to separate and form a joint cavity (Archer et al., 1994; Edwards, 1994; Fell and Canti, 1934; Mitrovic, 1971; Mitrovic, 1974).

Hyaluronan, the unsulphated glycosaminoglycan, was originally thought of as a space-filling molecule but it is now clear that HA plays a major role in joint cavitation. Hyaluronan is one of the matrix molecules detected at the first signs of cavitation and the large quantities of HA synthesised along the presumptive joint line are thought necessary for cell separation by a process of receptor saturation (Craig et al., 1990; Toole, 1981). UDPGD, the enzyme responsible for providing HA precursors for HA synthesis, can also be detected along the presumptive joint line and it has been determined that the UDP-glucoronate synthesized by UDPGD enters HA synthesis and not GAG synthesis (Hinchcliffe, 1977; Pitsillides et al., 1995). The presence of the HA binding protein, CD44, on the interzonal cells emphasizes the importance of HA in joint cavitation. Indeed, the disruption of HA:HABP interactions using low molecular weight HA oligosaccharides prevents joint cavitation, further highlighting the importance of HA in the cavitation process (Dowthwaite et al., 1998; Pitsillides et al., 1995).

Mechanical stimuli have been suggested as a mechanism influencing joint formation and HA synthesis. Removal of mechanical stimuli in uncavitated joints, either *in ovo* or in limb organ cultures, has been shown to prevent cavitation (Drachmann and Sokoloff, 1966; Fell and Canti, 1934; Osborne et al., 2002). More recently, the effects of mechanical stimuli on developing chick fibrocartilage cells have been demonstrated. The cells responded to brief periods of mechanical strain with an increase in media HA detected twenty minutes post strain. At twenty four hours, HA concentrations were maintained and increased CD44 expression was also observed

(Dowthwaite et al., 1998; Dowthwaite et al., 2003). As a result mechanical stimuli have been suggested to influence HA metabolism within the developing joint and movement of the adult joint is the likely stimulus for the continued synthesis of HA.

In OA joints, the synovium shows alterations in HA synthesis and subsequently the concentration of HA within the synovial fluid decreases. As the disease progresses, friction-free articulation of the joint is reduced, leading to loss of mechanical stimuli as the joint becomes immobile. The advantages of being able to increase HA synthesis and so increase HA within the joint cavity using precise mechanical stimuli would prove a less invasive and practical treatment for OA. Indeed, a number of studies involving controlled exercise programs combining walking, aerobic and muscle strengthening exercises have shown an increase in flexibility of the joint and significant decreases in pain within a short period of time (Fisher et al., 1993; Minor et al., 1989; Penninx et al., 2001; Rejeski et al., 1998; Sullivan et al., 1998). It has also been shown that non-weight bearing or dynamic aerobic exercises did not aggravate the OA joints as no indication of joint inflammation was found (Beals et al., 1985). Furthermore, the participants reported a decrease in knee pain. Cessation of these exercises resulted in the loss of the functional benefits obtained, demonstrating movement of the joint results in improvements in mobility. This work demonstrates that although the exact mechanisms of HA synthesis are still unknown it is likely that mechanical stimuli are an important regulatory factor in maintaining a fully functioning joint.

The aim of this work was to investigate the effects of mechanical strain on HA metabolism of synovial cells from OA knees. Establishing whether the synovial cells from OA knees are still able to respond to short periods of mechanical strain or whether due to age or disease they have become non-responsive to mechanical stimuli would result in a better understanding of mechanical stimuli and the regulation of HA synthesis in the functioning of the adult joint.

## 3.2 Materials and Methods

### 3.2.1 Synovium Samples

Synovium samples were obtained as in Chapter 2 (2.2.1). Normal synovial samples were obtained within 24 hours post-mortem, again with the relevant ethical approval. Details of the samples are listed below.

| Sample | Type | Sex | Age (years) |
|---|---|---|---|
| OA1 | OA | Female | 57 |
| OA2 | OA | Male | 64 |
| OA3 | OA | Female | 74 |
| OA4 | OA | Female | 79 |
| OA5 | OA | Male | 77 |
| OA8 | OA | Male | 79 |
| N1 | normal | Female | 67 |
| N2 | normal | Unknown | 71 |

**Table 3.2.1 Details of synovium samples obtained at knee joint replacement surgery.**

### 3.2.2 Cell Isolation & Culture

Cells were isolated from the synovium as described in Chapter 2 (2.2.2). Briefly, diced tissue was digested in 295IU mg $ml^{-1}$ type I collagenase (Sigma, Poole, UK) for 3 hours at 37°C. Cells were centrifuged at 1000 x g for 5 minutes, resuspended then centrifuged at 1000 x g for 5 minutes. Cells were counted and seeded as required in DMEM/F12 containing 1% penicillin-streptomycin ($10^4$IU $ml^{-1}$/$10^4$μg $ml^{-1}$; Sigma), L-glutamine (200mM; Life Technologies, Paisley, UK), hydrocortisone (4mg $ml^{-1}$; Sigma)[40mg hydrocortisone powder dissolved in 5ml 100% ethanol and 5ml sterile PBS], insulin-transferrin-selenium (200mM; Life Technologies) and 20% foetal calf serum (FCS;Sigma) and incubated at 37°C.

### 3.2.3 Mechanical Strain

Cells were used at passage 1, 2 and 3. The cells were trypsinised (Appendix 2.1), counted and plated at a density of 0.25 x $10^6$ cells $ml^{-1}$. One ml of cell suspension was placed into a well of the four-well strain plate (fig.3.2.1).

The confluent cells were serum deprived for 18 hours, and fresh serum-free media (DMEM/F12 containing 1% penicillin-streptamycin ($10^4$IU $ml^{-1}$/$10^4$μg $ml^{-1}$; Sigma), L-glutamine (200mM; Life Technologies), hydrocortisone (4mg$ml^{-1}$; Sigma)[40mg hydrocortisone powder dissolved in 5ml 100% ethanol and 5ml sterile PBS], and 1% Insulin-Transferrin-Selenium (200MM; Life Technologies) [DMEM/F12-] added 30 minutes prior to strain.

The four-point bending mechanical loading jig (fig.3.2.2) was calibrated accordingly (Appendix.3.1) so reproducible uniaxial strains could be applied to the cells. Cells were strained at 4000με, 6000με or 10000με at a frequency of 1 Hz for 10 minutes (figure.3.2.3.). Controls comprised cells that did not undergo strain, static controls and cells that did not undergo strain but were subjected to media perturbation using a platform shaker set at 60 rev/min for 10 minutes, flow controls.

### 3.2.4 Cell Viability

To determine the effect of strain on the viability of the synovial cells a trypan blue assay was used. Cells were strained as described then at 1 hour, 6 hours and 24 hours media was removed and 1ml trypsin (Sigma) added to each well for 5 minutes at 37°C. The cell/trypsin solution was removed then 1ml DMEM/F12+ was added to wash the wells of remaining cells and the contents centrifuged at 1000 x g for 3 minutes. The supernatant was removed and 1ml fresh DMEM/F12+ added. A 20μl aliquot of cell suspension was added to 20μl of trypan blue (Sigma), mixed and left for a few minutes before counting both live and dead cells with a haemocytometer.

Images of cells were also taken prior to trypsinisation to assess gross morphology of strained and static control cells.

### 3.2.5 Hyaluronan ELISA

Duplicate samples of media collected from each of the strain, static control and flow control plates at 1 hour, 6 hours and 24 hours were assessed for HA concentration using a competitive ELISA plate-based assay (Fosang, Hey et al. 1990).

A Ninety six well plate was coated with 100μl/well of 100μg $ml^{-1}$ HA (Calbiochem, San Diego, CA, USA) in 20mM sodium carbonate (Sigma-Aldrich), pH9.6 and left overnight on a shaker. The plate was washed 3 times in PBS-Tween 0.1% (PBS-T) and the plate blocked with 1% bovine serum albumin (Sigma-Aldrich)/PBS for 1 hour at 37°C.

The plate was washed 3 times in PBS-T. Fifty μl of sample (run in duplicate) or standard was added to the plate. A standard series of 10-0.00976μg $ml^{-1}$ of HA was run in triplicate and diluted in PBS-T. A max well contained 50μl of PBS-T. Next 50μl of biotinylated HA binding protein (Seikagaku Coorporation, Chuo-ku, Japan) was added at 1:1000 (0.22μg $ml^{-1}$) to all wells except the blank which consisted of 100μl PBS-T. The plate was incubated overnight on a shaker at room temperature.

The plate was washed 3 times in PBS-T then 100μl of 1:500 streptavidin-biotinylated HRP complex (Amersham Pharmacia Biotech, Buckinghamshire, UK) diluted in PBS-T was applied. The plate was incubated at 37°C for 40 minutes on a shaker. The plate was then washed 4 times in PBS-T and 100μl ABTS substrate (2,2'Azino-bis (3-ethylbenzthiazoline-6 sulfonic acid; Sigma) was added for 30-45 minutes and the absorbance read at 405nm.

**Statistical Analysis**

A set of three plates were subjected to strain, static control and flow control giving an n number of 3. Each plate consisted of 4 wells and statistical analysis showed HA media concentrations from 4 wells from the same plate did not show statistical difference. Data from the 3 plates subjected to either strain, control or flow control were combined for statistical analysis. All statistical analysis for changes in HA media concentrations between normal and OA patients, control and flow, control and strained cells were analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc tests.

**Sample Number**

The human tissue samples obtained for these experiments often varied in size and type. As a result, mechanical strain experiments could not be performed from each patient at all passages and at the 3 magnitudes of strain.

### 3.2.6 UDPGD Activity

UDPGD activity was assessed using a previously described cytochemical method (Mehdizadeh *et al*, 1991). Briefly, twenty four hours after strain media was removed and the cells were washed in PBS. Cells were then incubated for 3 hours at 37°C in nitrogen saturated medium containing 5.5mM UDP-glucose (Sigma), 1.5mM NAD (BDH Lab Supplies, Poole, UK) and 3.7mM nitrotetrazolium blue chloride (NBT) (Sigma-Aldrich) in 30% solution polyvinyl alcohol (Sigma) in 0.05%M glycylglycein buffer, pH7.8 (BDH Lab Supplies). Controls comprised cells incubated in medium without the UDP-glucose substrate. Once colour development occurred the cells were washed in distilled water and mounted in aquamount (BDH Lab Supplies).

**Figure 3.2.1** Strain plate consisting of four separate silicon wells on a polycarbonate base.

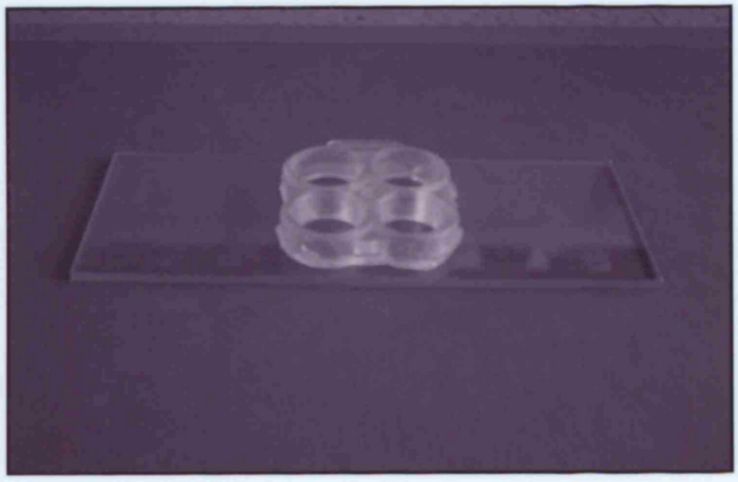


**Figure 3.3.2** Mechanical loading jig.

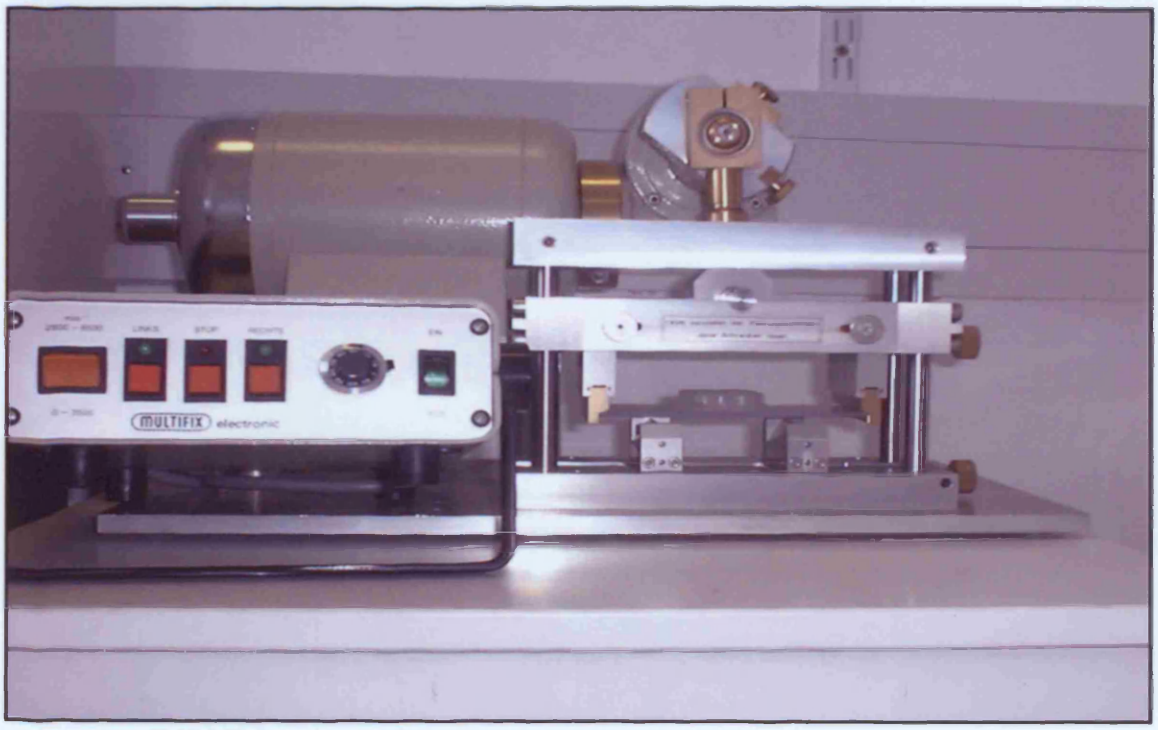
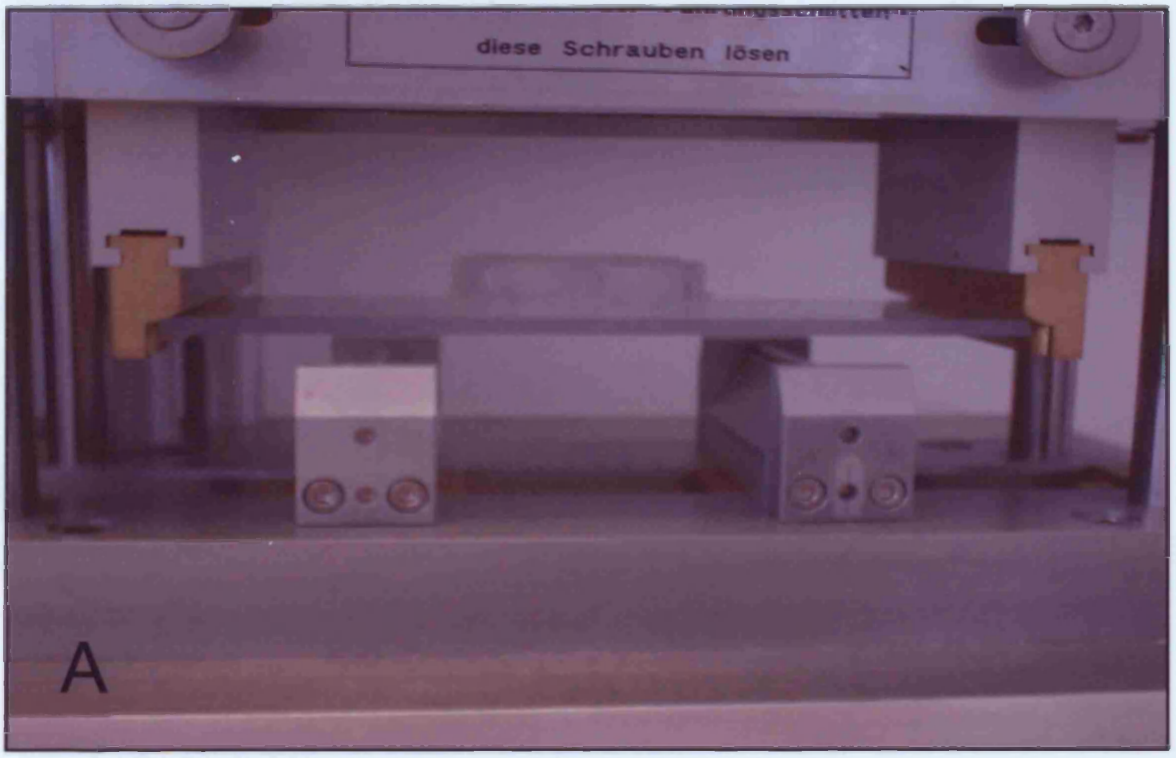
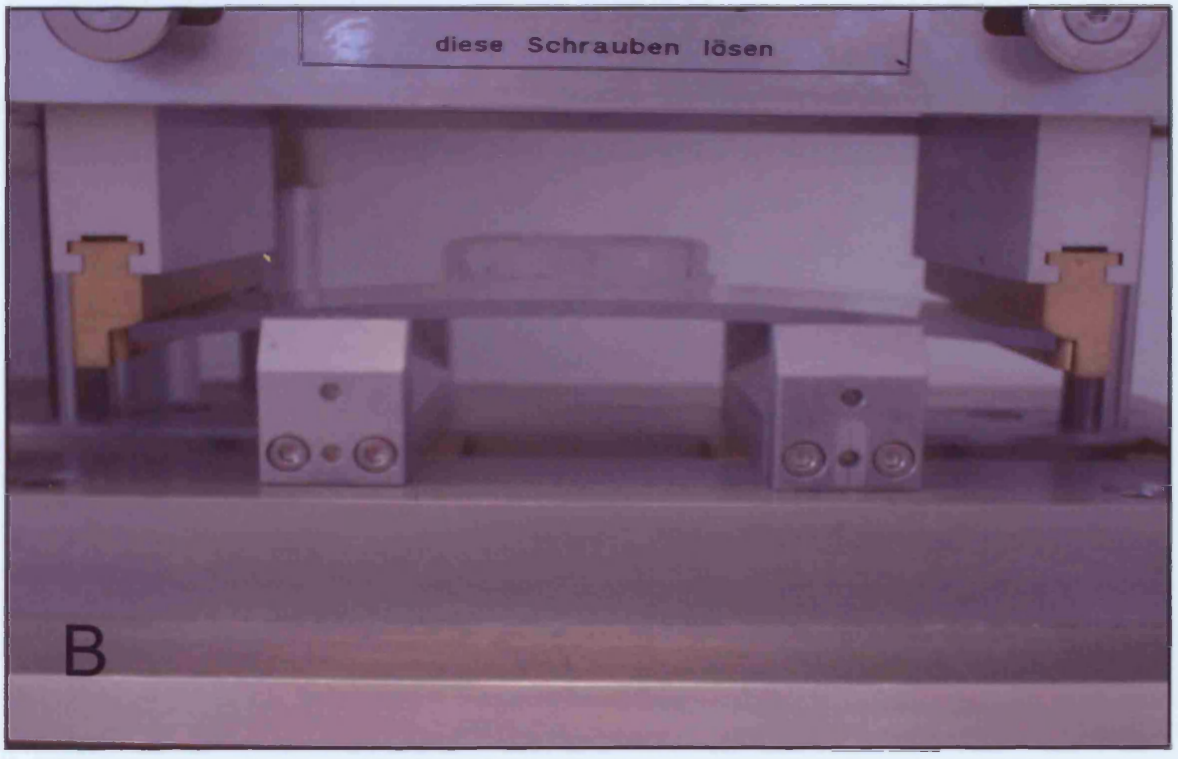

LINKS
STOP
RECHTS
EIN
MULTIFIX electronic

**Figure 3.2.3** Plates under mechanical loading go from a straight (A) to flexed (B) position.


diese Schrauben lösen
A


diese Schrauben lösen
B

## 3.3 Results

### 3.3.1 Effects of Mechanical Strain on Synovial Cells

No differences in the gross morphology between the control cells and the cells subjected to strain were evident at 24 hours post treatment (fig.3.3.1). Cell viability assays on the control cells and cells subjected to the 3 magnitudes of strain showed over 90% viability in both the control and strained cells 24 hours post-treatment.

### 3.3.2 Static and Flow Controls

The HA concentration of the media from static and flow control normal synovial cells displayed no significant differences in media HA concentration at 1 hour, 6 hours or 24 hours (fig.3.3.2A).

During the initial hour post-treatment media obtained from OA synovial cells subjected to flow alone and from static controls showed increases in HA concentrations. However, no significant difference between flow control and static control media HA concentrations were observed at the 1 hour time point (fig.3.3.2B). Although the Media HA concentration decreased in both the flow and static controls at 6 hours, no significant differences in HA concentrations between flow and static controls were evident. At 24 hours an increase in media HA concentration was observed in both the flow and static controls, compared to the 6 hour time point. However, there was no significant difference between flow and static control media HA concentration at 24 hours.

### 3.3.3 Patient Variability

**Variability with passage**

The media from control cells from patient OA2 showed increasing media HA concentration with time at each passage. A significant difference ($P<0.05$) in media HA concentration between passages occurred at the 6 hour time point (fig.3.3.3A). OA3 control cells exhibited an increase in media HA concentration at passage 1 and passage 3 but at passage 2, media HA concentration decreased with time. Media HA

concentration from the passage 1 cells at 6 and 24 hours were significantly higher ($P<0.05$) than the media HA concentrations obtained from passage 2 and passage 3 cells (fig3.3.3B). Control media from patient OA4 showed an overall increase in HA concentration at 24 hours with each passage with both passage 1 and passage 2 cells containing significantly less HA in their media compared to passage 3 cells (fig.3.3.3C). Overall, significant differences in media HA concentration between each passage and at each time point were observed. As a result, the data from each patient at the 3 passages could not be accumulated for statistical analysis.

**Cross patient variability**

Synovial cell media HA concentration from normal patients ranged from 0.32μg $ml^{-1}$ to 3.8μg $ml^{-1}$ at the 1 hour time point, 0.26μg $ml^{-1}$ to 6.23μg $ml^{-1}$ at the 6 hour time point and 0.99μg $ml^{-1}$ to 26μg $ml^{-1}$ at the 24 hour time point (Appendix.3.4). The HA concentration of media from synovial cells of OA patients ranged from 0.74μg $ml^{-1}$ to 5.11μg $ml^{-1}$ at the 1 hour time point, 0.14μg $ml^{-1}$ to 16.55μg $ml^{-1}$ at the 6 hour time point and 0.20μg $ml^{-1}$ to 9.88μg $ml^{-1}$ at the 24 hour time point (Appendix.3.5). The concentration of media HA from each of the individual patients at the same time point and passage showed significant differences. As a result media HA concentrations, from separate patients at each time point and at each passage, could not be combined for statistical analysis (fig.3.3.4).

## 3.3.4 Normal Patient Response to Mechanical Strain

**Patient N1**

Normal control cells at passage 1 and 2 exhibited an initial increase in media HA concentration which was followed by a decrease at 6 hours and an increase at 24 hours. The control cells at passage 3 had an increase in their media HA concentration at 1 hour that continued to increase up to 6 hours but decreased at 24 hours (fig.3.3.5 A-C).

Subjecting normal cells to 4000με increased media HA concentration at 1 hour followed by a decrease at 6 hours, then an increase at 24 hours in media from all 3

passages. The strained cells followed the same trend in media HA concentration with time as the control cells. However, significantly less HA was seen in the passage 1 cells at 1 hour after 4000με ($P<0.0001$; fig.3.3.5A). In the passage 2 cells significantly less HA was seen at all time points ($P<0.05$; fig.3.3.5B).

The effect of applying strain at 6000με was to increase media HA at 1 hour followed by a decrease at 6 hours then an increase at 24 hours in both passage 2 and passage 3 cells. When compared to control cell media HA concentration the passage 2 strained cells showed significantly less media HA at the 6 hour time point ($P<0.05$; fig.3.3.5B).

At 10000με an initial increase at 1 hour in media HA continued to increase up to 6 hours in the passage 1 and 3 cells. In the passage 2 cells, a decrease in media HA concentration was apparent at 6 hours post strain compared to 1 hour (f.ig.3.3.5A). Elevated media HA concentration was seen at 24 hours compared to 6 hours in strained cells from the 3 passages. The effect of straining the cells was reduced media HA concentrations in the strained cells with a significant decrease observed at the 6 hour time point in the passage 2 cells (fig.3.3.5B). However, passage 3 cells showed a significant increase ($P<0.01$) in media HA concentration at 24 hours compared to the control cells media HA concentration (fig.3.3.5C).

**Patient N2**

The control cells at passage 1 exhibited an initial increase in media HA at 1 hour, which then decreased at 6 hours, but increased at 24 hours (fig.3.3.6).

The cells subjected to 6000με showed an initial increase at 1 hour in their media HA concentration which was followed by a decrease at 6 hours and then an increase at the 24 hour time point. The strained cells showed a similar trend in media HA synthesis with time as the control cells, with a significant increase ($P<0.05$) observed at the 6 hour time point.

### 3.3.5 Summary: Changes in Media HA Concentrations from Normal Synovial Cells

| | 4000με | | | 6000με | | | 10000με | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 hr | 6 hrs | 24 hrs | 1 Hr | 6 Hrs | 24 hrs | 1 hr | 6 hrs | 24 hrs |
| Passage1 | ↓ | – | – | – | ↑ | – | – | – | – |
| Passage2 | ↓ | ↓ | ↓ | – | ↓ | – | ↓ | – | – |
| Passage3 | – | – | – | – | – | – | – | – | ↑ |

**Table 3.3.1 Changes in media HA concentrations from normal synovial cells.**

At 4000με the cells at passage 1 responded with a significant decrease in media HA at the one hour time point. The passage 2 cells responded with significant increases in media HA at all time points. Passage 3 cells showed no significant response at 4000με.

In response to 6000με the passage 1 cells showed a significant increase at 6 hours whereas the passage 2 cells showed a significant decrease in media HA concentrations compared to the control cells. Passage 3 cells showed no significant response at 6000με.

The effect of straining passage 2 cells at 10000με revealed a significant decrease in media HA at the 1 hour compared with control cell media HA concentration. At passage 3, cells responded with significant increases in media HA concentration at both 1 hour and 24 hours when subjected to 10000με.

### 3.3.6 OA Response to Mechanical Strain

**Patient OA1**

One hour after strain, passage 1 control cells gave an initial increase in their media concentration, which decreased at 6 hours but then increased at 24 hours (fig3.3.7).

The cells strained at 6000με showed an initial increase in media HA concentration which continued to increase up to 24 hours. When compared to control cells media HA concentration the strained cells showed no significant differences ($P<0.05$) in media HA concentration at any time point after strain.

**Patient OA2**

Control cells at all passages showed an initial increase in media HA concentration and media HA concentration continued to increase up to the 24 hour time point (fig.3.3.8).

The effect of 6000με on the cells resulted in an increase in media HA up to the 6 hour time point in media from all passages. Media HA continued to increase up to the 24 hour time point in passage 2 cells whereas passage 3 cells showed a decrease in media HA concentration at 24 hours. Passage 1 cells showed significantly higher ($P<0.05$) levels of HA within the media of strained cells at the 1 hour time point whereas passage 3 cells showed a significant decrease in media HA concentration 1 hour after being subjected to 6000με.

**Patient OA3**

Passage 1 and passage 3 control cells had increasing media HA concentration with time post strain (fig.3.3.9A & C). At passage 2 the control cells, after an initial increase at 1 hour showed a decrease in their media HA concentrations at 6 hours and a further decrease was observed at 24 hours (fig.3.3.9B).

Cells strained at 4000με showed increasing media HA concentration with time at both passage 1 and passage 3. The effect of strain on the cells resulted in a significant increase ($P<0.01$) in HA concentration within the media compared to the control cells in passage 1 cells at 24 hours (fig.3.3.9A).

Applying 10000με to passage 1 cells resulted in an increase in media HA concentration at 1 hour which fell at the 6 hour time point yet continued to increase in the passage 2 and 3 cells. At 24 hours, media HA concentration increased in the cells from all passages. When compared with control cells, passage 1 cells showed significantly less ($P<0.1$) HA within the media at the 6 hour time point whereas

passage 3 cells showed a significant increase ($P<0.1$) in media HA at 1 hour and 24 hours compared with control cells (fig.3.3.9C).

**Patient OA4**

Media from control passage 1 and passage 2 cells showed an initial increase in HA concentration which then decreased at 6 hours (fig.3.3.10A & B). At 24 hours, media HA concentration increased. Passage 3 control cells showed an increase in media HA up to 6 hours which was then decreased at 24 hours (fig.3.3.10C).

The cells strained at 4000με gave an initial increase in media HA concentration which decreased at the 6 hour time point in passage 1 and 2 cells but continued to increase in passage 3 cells. At the 24 hour time point media HA concentration decreased in passage 1 cells yet increased in passage 2 and 3 cells. Passage 3 cells showed significantly higher ($P<0.05$) media HA concentration compared to the control cells at 24 hour time point (fig.3.3.10C).

Application of 6000με to the cells gave an increase in media HA concentration up to 6 hours in passage 1 and 3 cells. This was followed by a decrease in media HA concentration at the 24 hours time point in passage 1 cells whereas an increase in media HA in passage 3 cells was seen. Passage 2 cells showed increased media HA at 1 hour which then decreased at 6 hours but increased at the 24 hour time point. Passage 1 cells showed significantly higher media HA compared to the control cells at the 1 hour time point whereas passage 2 cells showed a significant decrease ($P<0.05$) in media HA at the 24 hour time point (fig.3.3.10A & B).

HA accumulated in the media of cells subjected to 10000με at 1 hour at all passages. At 6 hours, passage 1 and 3 cells' media HA concentration continued to increase whereas passage 2 cells showed a decrease. At 24 hours, passage 1 and 2 cells showed an increase in media HA concentration yet passage 3 cells showed a decrease. Passage 1 cells showed significant increases ($P<0.1$) in media HA at the 6 hour and 24 hour time point compared to the control cells whereas the passage 3 cells showed significantly reduced ($P<0.05$) media HA concentration compared with control cells (fig.3.3.10A & C).

**Patient OA5**

Control passage 3 cells exhibited an initial increase in their media HA concentration at 1 hour which increased up to 6 hours but a decrease in media HA concentration was observed at 24 hours (fig.3.3.11).

The effect of straining the cells at 6000με resulted in an increase in media HA concentration at 1 hour which continued to increase with time. The cells response to 6000με however, was significantly less ($P<0.05$) media HA at all time points when compared to the control cells.

## 3.3.7 Summary: Changes in Media HA Concentrations from OA Synovial Cells

| | 4000με | | | 6000με | | | 10000με | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 hr | 6 hrs | 24 hrs | 1 hr | 6 hrs | 24 hrs | 1 hr | 6 hrs | 24 hrs |
| Passage1 | ↓ | — | ↑ | ↑ | — | — | — | ↑ ↓ | — |
| Passage2 | — | — | — | — | — | ↓ | — | — | — |
| Passage3 | — | — | ↑ | ↓ | ↓ | ↓ | ↓ | — | ↑ |

**Table 3.3.2 Changes in media HA concentration from OA synovial cells.**

At 4000με the cells responded in passage 1 and 3, with an increase in HA concentration within the media of the strained cells compared to the control cells. A significant decrease in media HA concentration was observed in OA3 passage 1 cells at 1 hour although by 24 hours media HA concentration was significantly higher in the strained cells compared with the control cells (fig.3.3.9A).

In response to 6000με, passage 1 cells exhibited an increase in media HA concentration, with significant increases observed in OA2 and OA4 at the 1 hour time point (fig.3.3.8A, 3.3.8C & 3.3.9A). The effect of straining passage 2 cells was a general reduction in HA accumulated in the media from the strained cells compared to the control cells and a significant decrease ($P<0.05$) was observed in OA4 at the 24 hour time point (fig.3.3.8B & 3.3.10B). Passage 3 cells when subjected to strain

showed significant decreases ($P<0.05$) in media HA compared to the control cells (fig.3.3.8C & 3.3.10C & 3.3.11).

The effect of straining passage 1 at 10000με was to either significantly increase ($P<0.1$) media HA concentration as in patient OA4 (fig.3.3.10A) or to significantly decrease ($P<0.05$) media HA as in patient OA3 (fig.3.3.9A). Passage 3 cells post-strain showed significantly reduced ($P<0.05$) HA in the media of cells from patient OA4 at the 1 hour time point whereas patient OA3 showed significantly increased ($P<0.1$) HA in the media compared to the control cells (fig.3.3.10C & 3.3.9C).

### 3.3.8 Effects of Mechanical Strain on Normal and OA Synovial Cells

Normal cells produced the highest concentration of media HA at passage 3 whilst patient OA4 showed the same trend, the other OA patients showed higher HA concentrations in media produced by passage 1 cells. Subjecting OA and normal cells to strain resulted in higher concentrations of media HA at the 24 hour time point compared with the 1 hour time point. An increase in strain magnitude resulted in a lower Media HA concentration at each passage in the normal cells. This was not observed in OA synovial cells.

Subjecting normal cells to 4000με and 6000με resulted in the same trend in media HA concentration at all 3 passages, ie. increase at 1 hour followed by a decrease at 6 hours and then an increase at 24 hours. The effect of strain on OA cells produced a different response to that of normal cells. An increase in media HA concentration was observed with increasing time when the cells were strained at 4000με. At 6000με, OA cells either showed continued increase in media HA concentration with time or an increase up to 6 hours followed by a decrease at 24 hours. Patient OA4, passage 2 cells were the only sample to show the same response as the normal cells. The response of normal cells to 10000με was different depending on the passage, and the same was evident in the OA cells. Each patient at the different passages showed differing trends in media HA concentration with time.

### 3.3.9 Comparison of Media HA Concentration from a Normal and an OA Patient

To allow a comparison between strain magnitude and passage number, data for 1 normal patient (N1) and 1 OA patient (OA4) were converted to percentage change in media HA concentration relative to unstrained controls. Here media HA concentrations from strained cells at each time point were converted to percentage increase or decrease over the static control cells media HA concentrations (Appendix 3.6).

**Changes in media HA concentration in passage 1 cells**

The effect of mechanical strain on passage 1 normal synovial cells was a decrease in percentage HA release in the strained synovial cells compared to the control cells at all magnitudes of strain and at each time point except for the 10000με cells at 24 hours where a percentage increase was observed (fig.3.3.12A). In contrast, the effect of strain on the OA cells was a percentage increase in HA release at all strain magnitudes and at all time points (fig.3.3.12B).

**Changes in media HA concentration in passage 2 cells**

Normal passage 2 cells showed a percentage decrease in HA release when the cells were subjected to 4000με. The effect of 10000με on normal cells was a percentage decrease in HA release at 1 hour and 6 hours and at 24 hours (fig.3.3.12C). Application of strain to the OA synovial cells showed the opposite effect, with a percentage increase in HA release observed at 4000με, 6000με and 10000με (fig.3.3.12D). However, as in the normal cells, the OA cells followed the same trend with time at each of the 3 magnitudes of strain.

**Changes in media HA concentration in passage 3 cells**

Normal cells subjected to 4000με and 6000με showed greater percentage increases in Media HA concentrations than the strained OA cells at passage 3 (fig.3.3.12E & F). OA cells also showed the same trend as normal cells, with an increase in media HA concentration at the 1 hour time point, which decreased at 6 hours then increased at 24 hours. The effect of 10000με on normal cells was to reduce percentage HA release compared with control cells at 1 hour and 6 hours. At the 24 hour time point an

increase was seen. The OA cells when strained gave an initial percentage increase at 1 hour which then decreased. As a result less HA was seen in the media of strained cells compared to control cells at 6 hours and 24 hours at 10000 με.

**Summary**

In summary, mechanical strain of 4000με, 6000με and 10000με produced significant changes in media HA concentration in normal synovial cells. The increases or decreases observed depended on strain and passage number of the cells. For example at 4000με, decreases in media HA concentration were observed at all passages whereas 6000με and 10000με produced both increases and decreases.

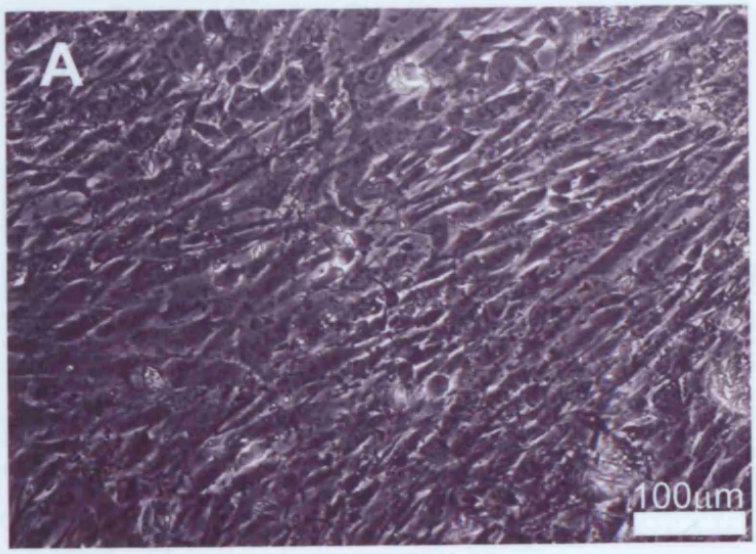
In OA synovial cells when mechanical strain was applied to the cells a significant change in media HA concentration occurred at all magnitudes of strain. Applying 4000με and 10000με to the cells resulted in both increases and decreases in media HA but this depended on cell passage number. Application of 6000με resulted in a decrease in media HA concentration at all passages.

The response to mechanical strain between normal and OA cells differed. At 4000με normal synovial cells showed significant decreases in media HA concentration whereas OA cells showed both increases and decreases. At 6000με and 10000με both increases and decreases were observed in both OA and normal cells. It was however, the 10000με at 24 hours which produced an increase in both normal and OA patients.

### 3.3.10 UDPGD Activity in OA Synovial Cells

OA synovial cells were assayed for UDPGD activity 24 hours after the application of strain. In strained samples, intense activity (as indicated by dark blue reaction product) was observed relative to unstrained controls (fig.3.3.13B & fig.3.3.14B). Additionally, background UDPGD activity was observed in control cells by comparison with assay controls (fig.3.3.13A & D & fig.3.3.14A & D). This increase in UDPGD activity was apparent in all samples subjected to strain.

**Figure 3.3.1** Gross morphology of a representative OA synovial cell culture 24 hours post-treatment. Patient OA3 cells were subjected to 10 minutes of mechanical strain (6000$\mu\varepsilon$) or remained as static controls.


A
100µm

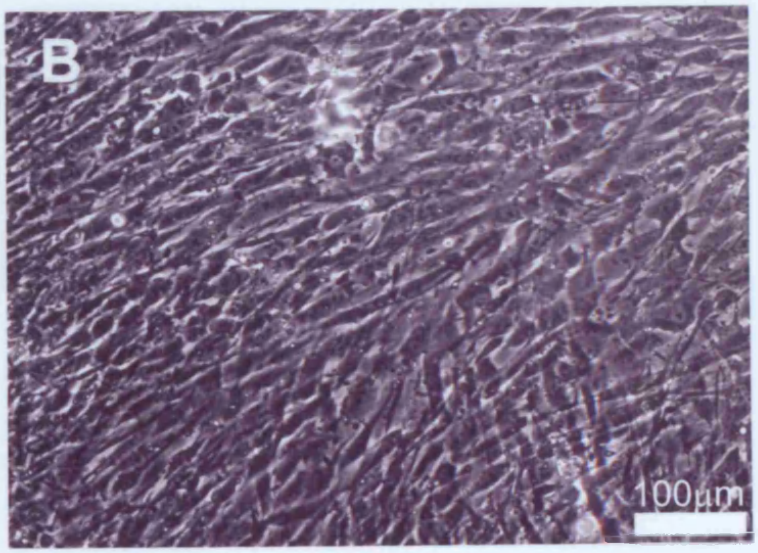
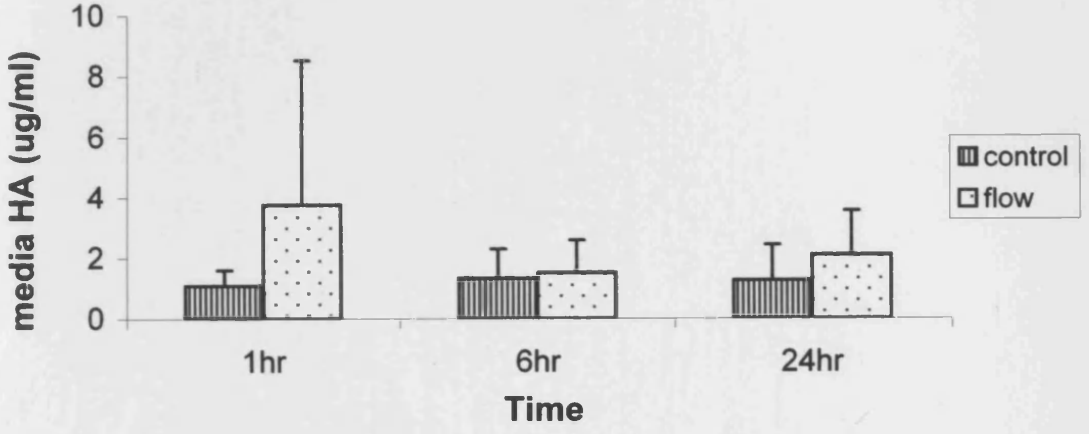

B
100µm

**Figure 3.3.2** Media HA concentrations from A) normal synovial cells and B) OA synovial cells. Cells were serum deprived for 24 hours, then fresh serum free media added. The cells remained static (control) or were subjected to media perturbation (flow) for 10 minutes and the media sampled at 1 hour, 6 hours and 24 hours. Error bars represent standard deviations.

A


10
8
6
4
2
0
media HA (ug/ml)
control
flow
1hr
6hr
24hr
Time

B

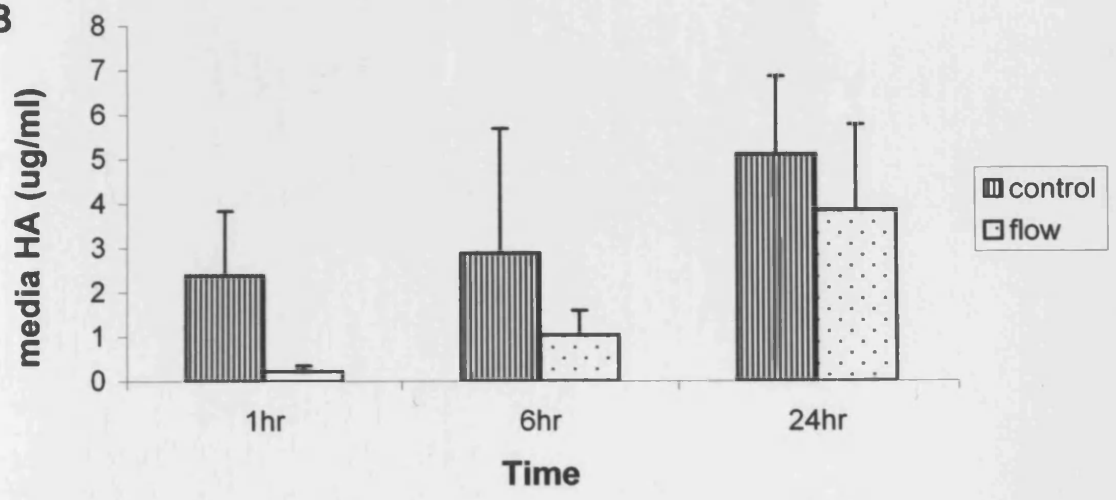
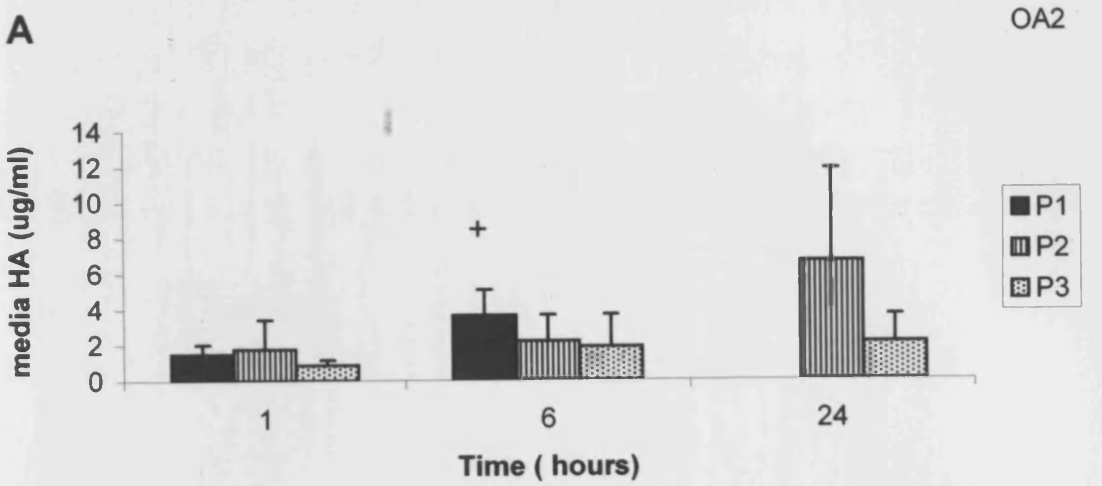
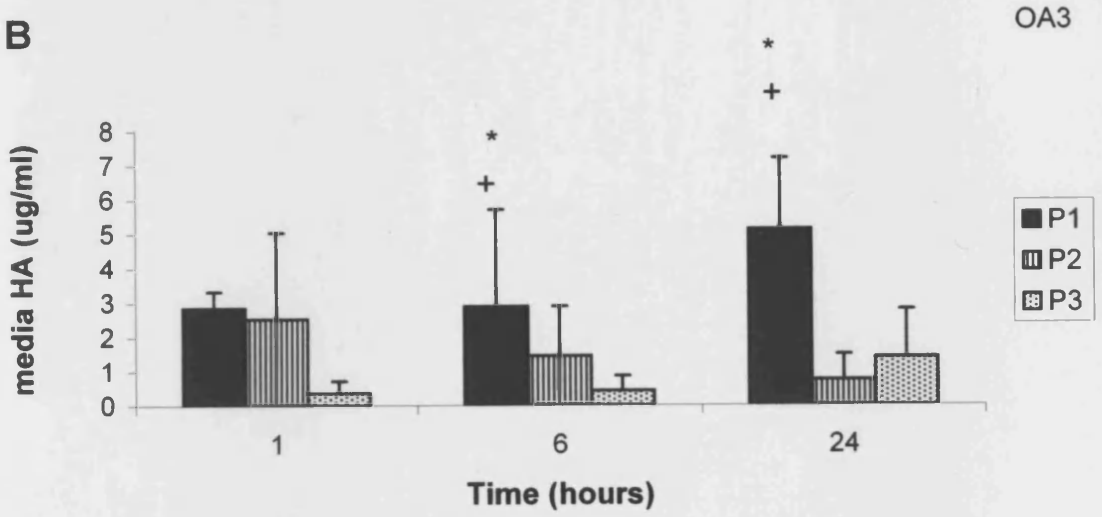

8
7
6
5
4
3
2
1
0
media HA (ug/ml)
control
flow
1hr
6hr
24hr
Time

**Figure 3.3.3** Media HA concentrations from synovial cells at three passages for three separate samples. Cells were serum deprived for 24 hours, then fresh serum free media added and the media sampled at 1 hour, 6 hours and 24 hours. Error bars represent standard deviations. A) patient OA2 B) patient OA3 C) patient OA4. *$P<0.05$ compared with passage 2. +$P<0.05$ compared with passage 3.


A
OA2
media HA (ug/ml)
14
12
10
8
6
4
2
0
+
1
6
24
Time ( hours)
P1
P2
P3


B
OA3
media HA (ug/ml)
8
7
6
5
4
3
2
1
0
*
+
*
+
1
6
24
Time (hours)
P1
P2
P3

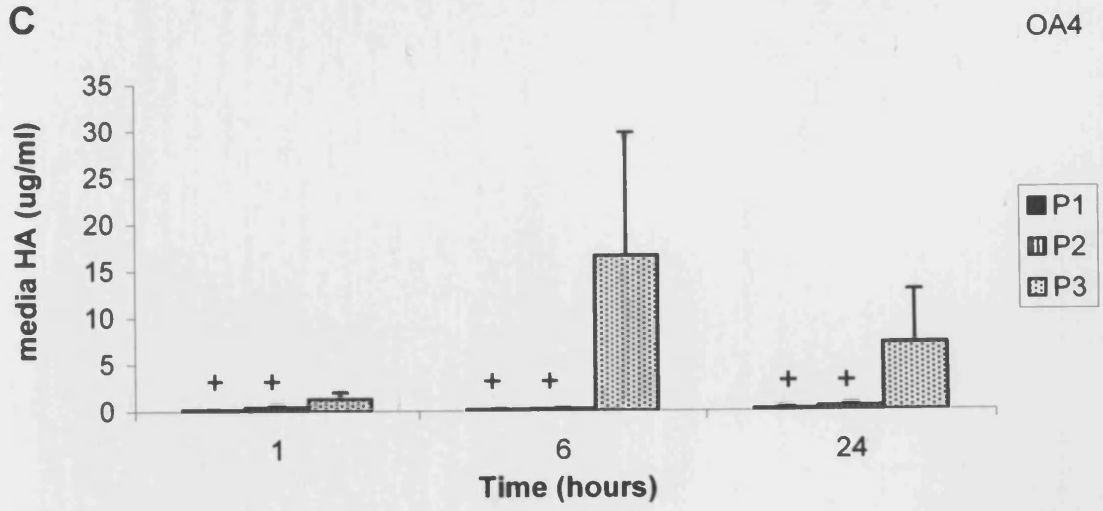
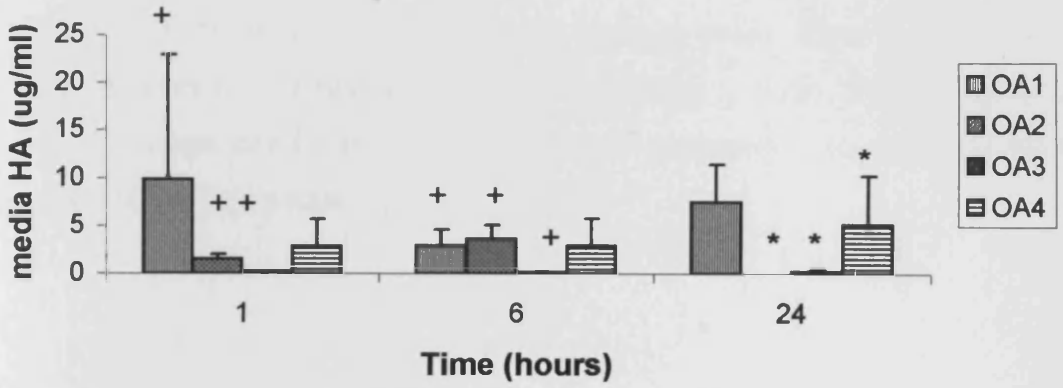
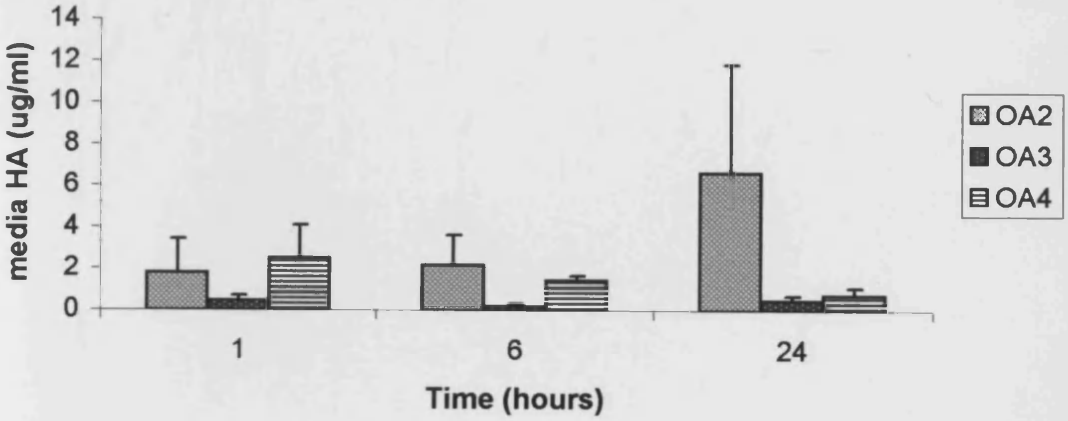

C
OA4
media HA (ug/ml)
35
30
25
20
15
10
5
0
+ +
+ +
+ +
1
6
24
Time (hours)
P1
P2
P3

**Figure 3.3.4** Media HA concentrations from synovial cells from five OA patients. Cells were serum deprived for 24 hours, then fresh serum free media added and the media sampled at 1 hour, 6 hours and 24 hours. Error bars represent standard deviations. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.05$ compared with OA1; #$P<0.05$ compared with OA3; +$P<0.05$ compared with OA4.

A


media HA (ug/ml)
25
20
15
10
5
0
+
+ +
+ +
+
* *
*
1
6
24
Time (hours)
OA1
OA2
OA3
OA4

B


media HA (ug/ml)
14
12
10
8
6
4
2
0
1
6
24
Time (hours)
OA2
OA3
OA4

C

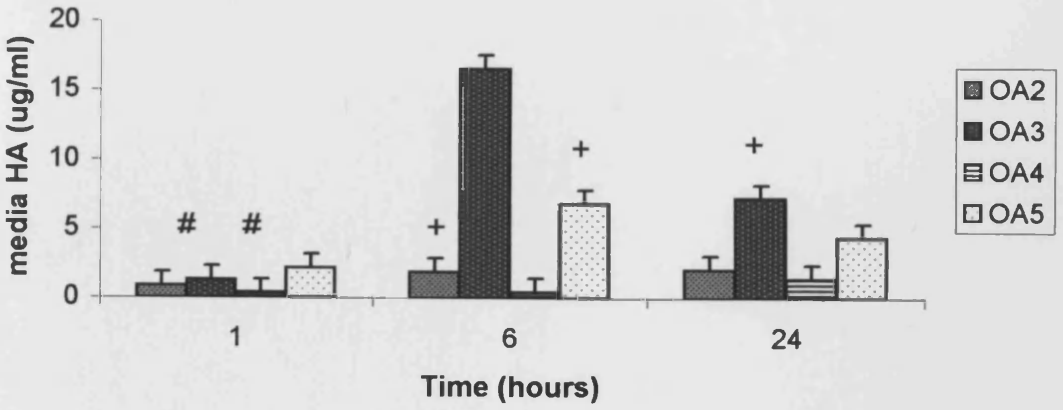
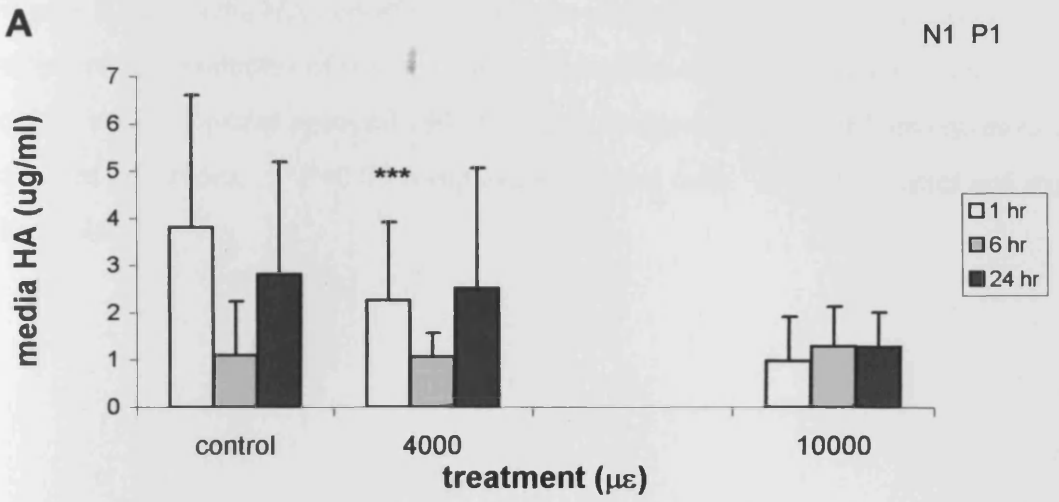
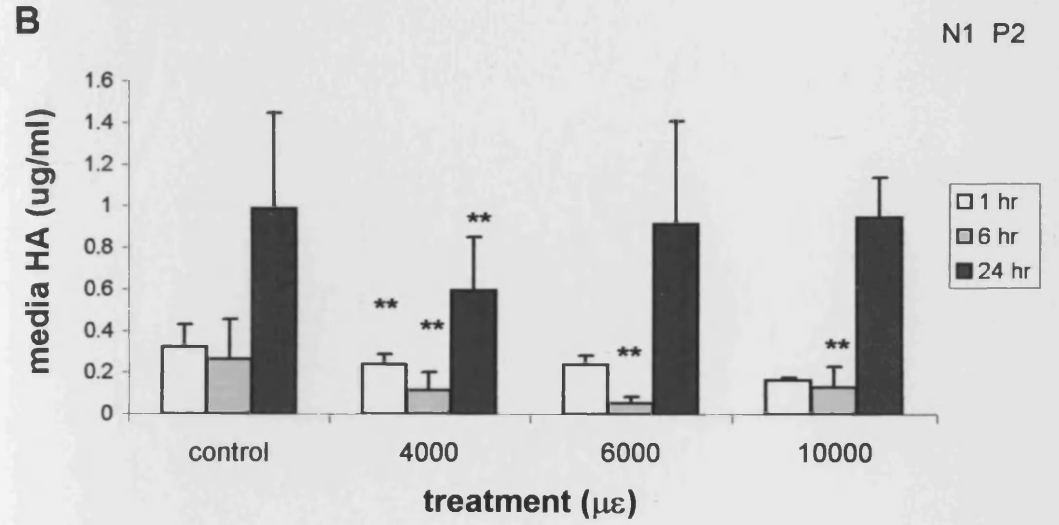

media HA (ug/ml)
20
15
10
5
0
# #
+
+
+
1
6
24
Time (hours)
OA2
OA3
OA4
OA5

**Figure 3.3.5** Media HA concentrations from patient N1. Normal synovial cells were subjected to 10 minutes of mechanical strain and the media sampled at 1 hour, 6 hours and 24 hours. Control synovial cells did not undergo strain. Error bars represent the standard deviations. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. ***$P<0.001$ compared to control cells. **$P<0.05$ compared to control cells. N=3 for control and strain data points.


A
N1 P1
media HA (ug/ml)
7
6
5
4
3
2
1
0
***
1 hr
6 hr
24 hr
control
4000
10000
treatment (με)


B
N1 P2
media HA (ug/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
1 hr
6 hr
24 hr
control
4000
6000
10000
treatment (με)

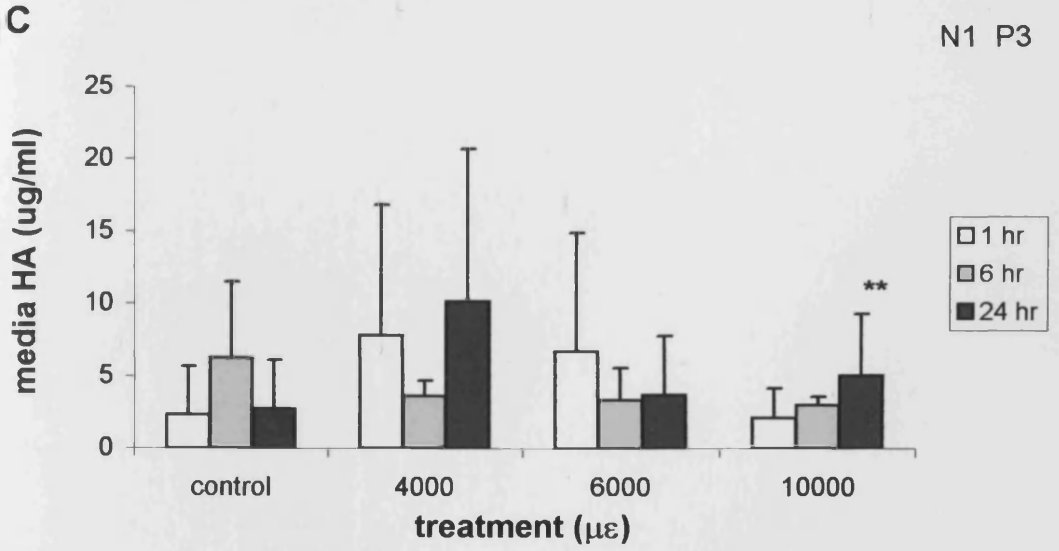

C
N1 P3
media HA (ug/ml)
25
20
15
10
5
0
**
1 hr
6 hr
24 hr
control
4000
6000
10000
treatment (με)

**Figure 3.3.6** Media HA concentrations from patient N2. Normal synovial cells were subjected to 10 minutes of mechanical strain and the media sampled at 1 hour, 6 hours and 24 hours. Control synovial cells did not undergo strain. Error bars represent the standard deviations. $^{**}P<0.05$ compared to control cells. N=3 for control and strain data points.

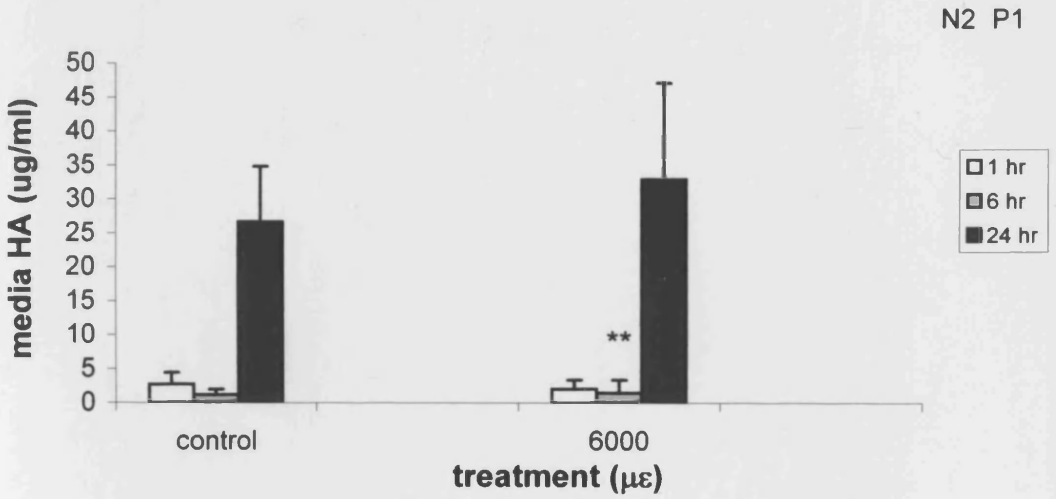

N2 P1
media HA (ug/ml)
50
45
40
35
30
25
20
15
10
5
0
**
control
6000
treatment (με)
1 hr
6 hr
24 hr

**Figure 3.3.7** Media HA concentrations from patient OA1. Passage 1 OA synovial cells were subjected to 10 minutes of mechanical strain and the media sampled at 1 hour, 6 hours and 24 hours. Control synovial cells did not undergo strain. Error bars represent the standard deviations. N=3 for control and strain data points.

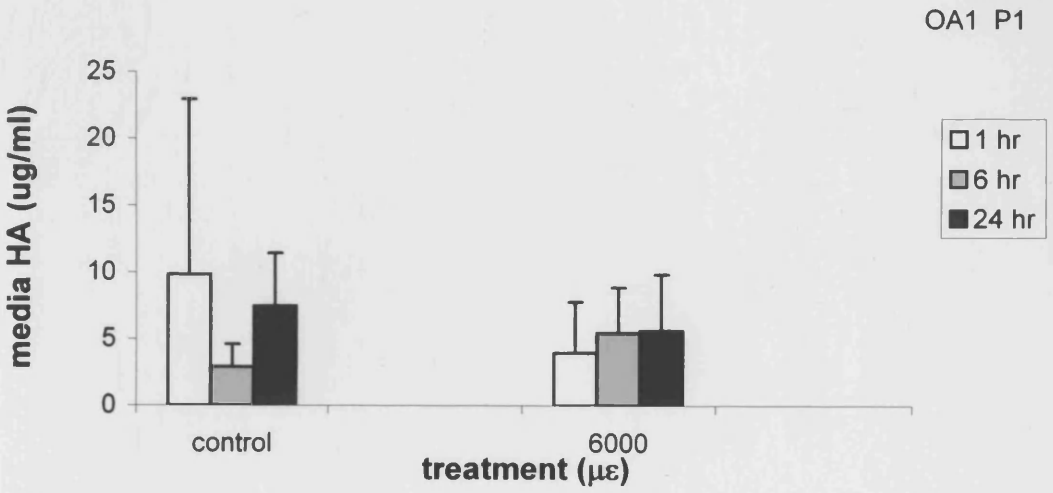
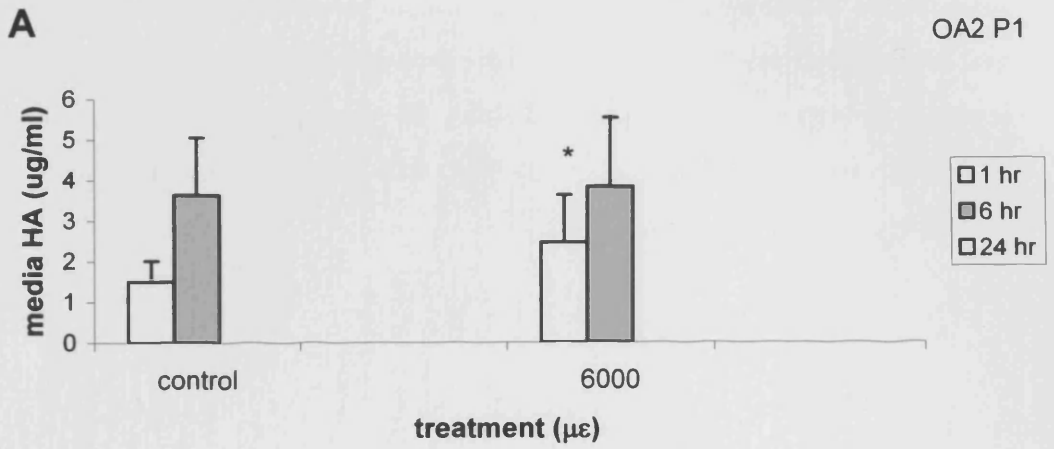
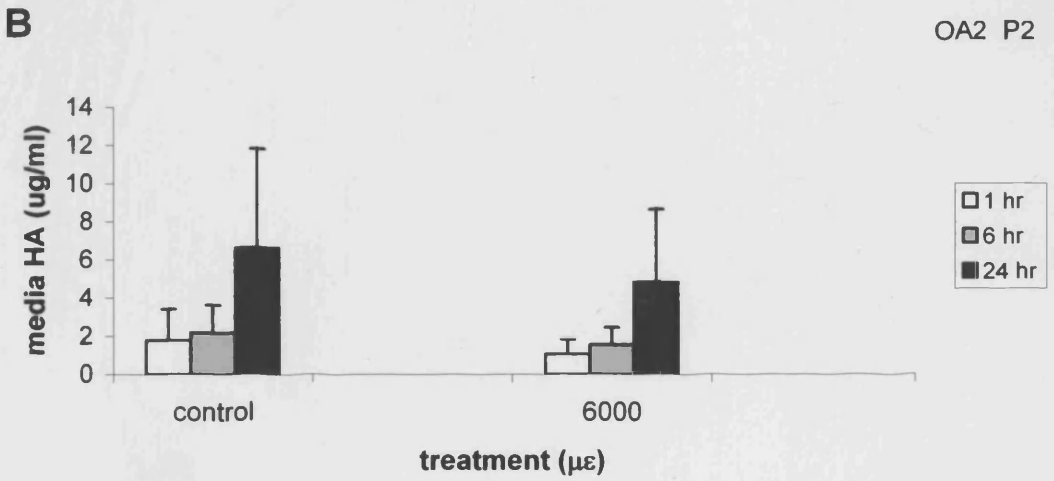

OA1 P1
media HA (ug/ml)
25
20
15
10
5
0
control
6000
treatment (με)
1 hr
6 hr
24 hr

**Figure 3.3.8** Media HA concentrations from patient OA2. OA synovial cells were subjected to 10 minutes of mechanical strain and the media sampled at 1 hour, 6 hours and 24 hours. Control synovial cells did not undergo strain. Error bars represent the standard deviations. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.05$ compared to control cells. N=3 for control and strain data points.


A
OA2 P1
media HA (ug/ml)
6
5
4
3
2
1
0
*
control
6000
treatment (με)
1 hr
6 hr
24 hr


B
OA2 P2
media HA (ug/ml)
14
12
10
8
6
4
2
0
control
6000
treatment (με)
1 hr
6 hr
24 hr

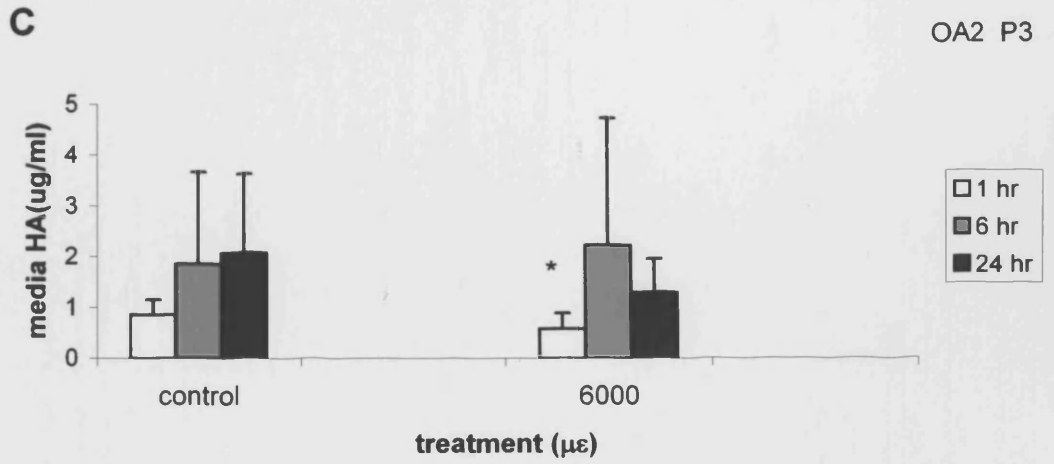
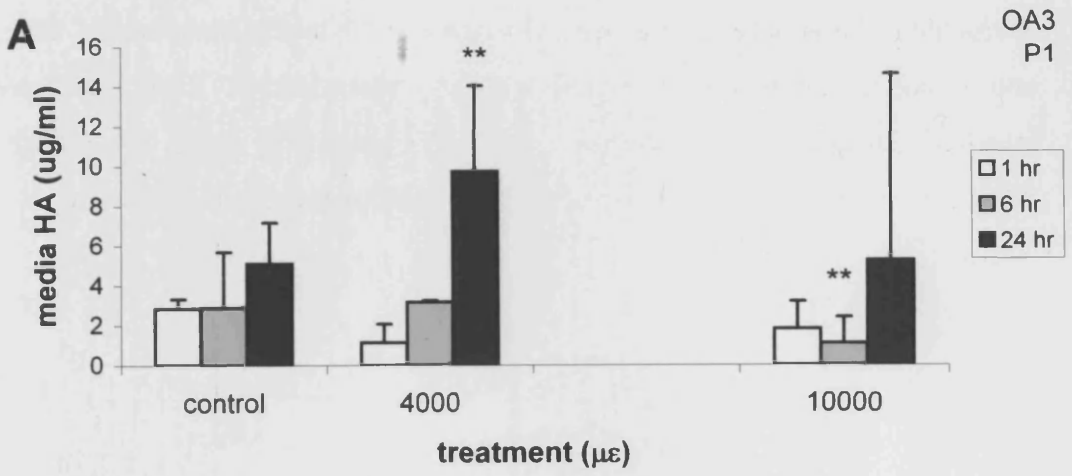
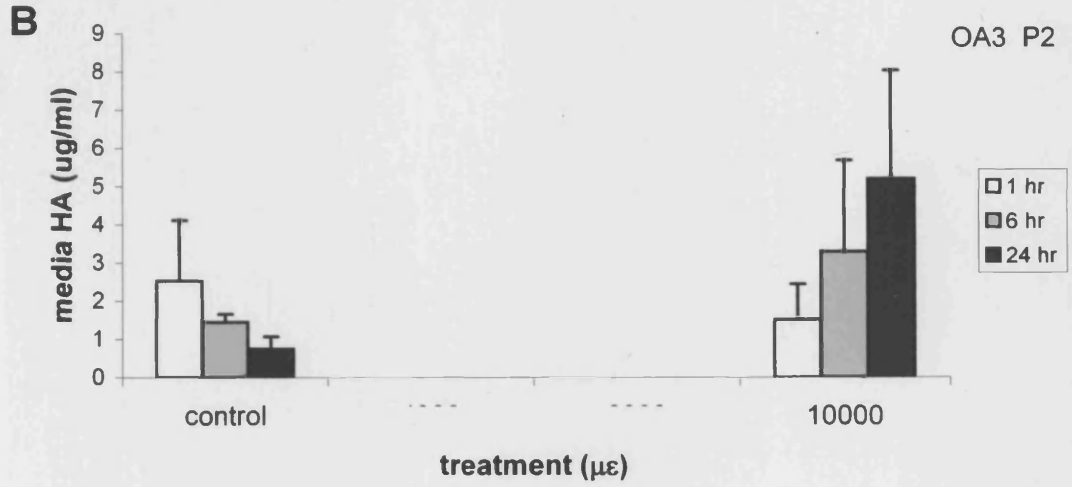

C
OA2 P3
media HA(ug/ml)
5
4
3
2
1
0
*
control
6000
treatment (με)
1 hr
6 hr
24 hr

**Figure 3.3.9** Media HA concentrations from patient OA3. OA synovial cells were subjected to 10 minutes of mechanical strain and the media sampled at 1 hour, 6 hours and 24 hours. Control synovial cells did not undergo strain. Error bars represent the standard deviations. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. $^{**}P<0.01$ compared to control cells. $^{**}P<0.05$ compared to control cells. N=3 for control and strain data points.


A
OA3
P1
media HA (ug/ml)
16
14
12
10
8
6
4
2
0
**
**
1 hr
6 hr
24 hr
control
4000
10000
treatment (με)


B
OA3 P2
media HA (ug/ml)
9
8
7
6
5
4
3
2
1
0
1 hr
6 hr
24 hr
control
10000
treatment (με)

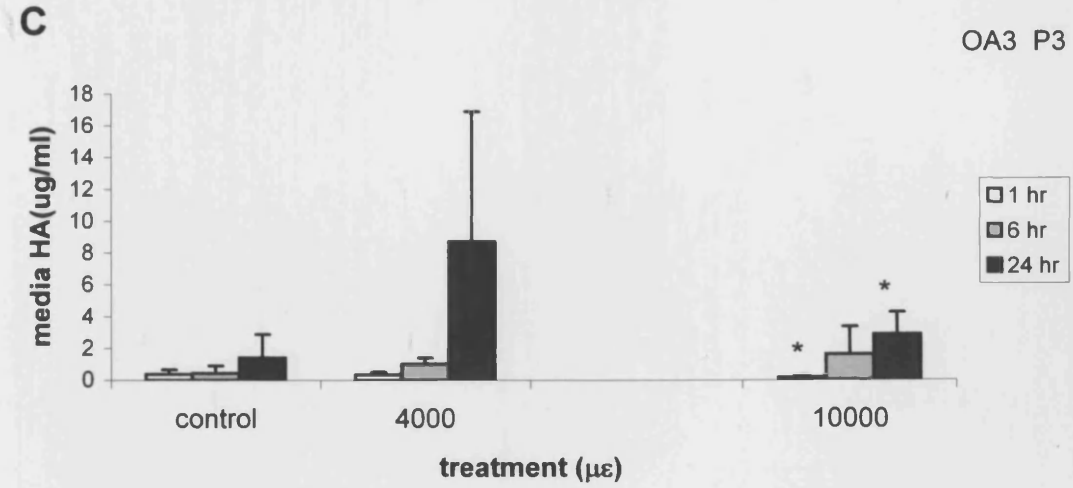
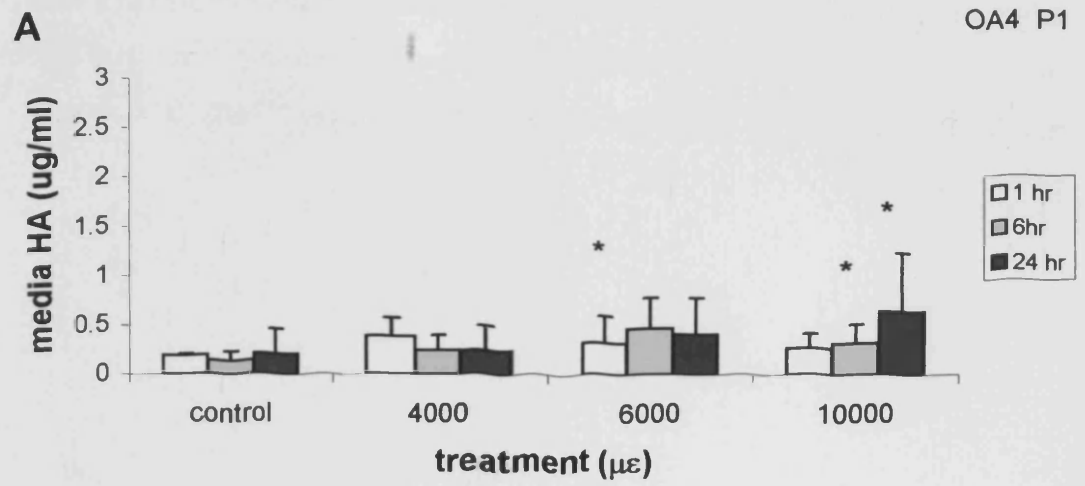
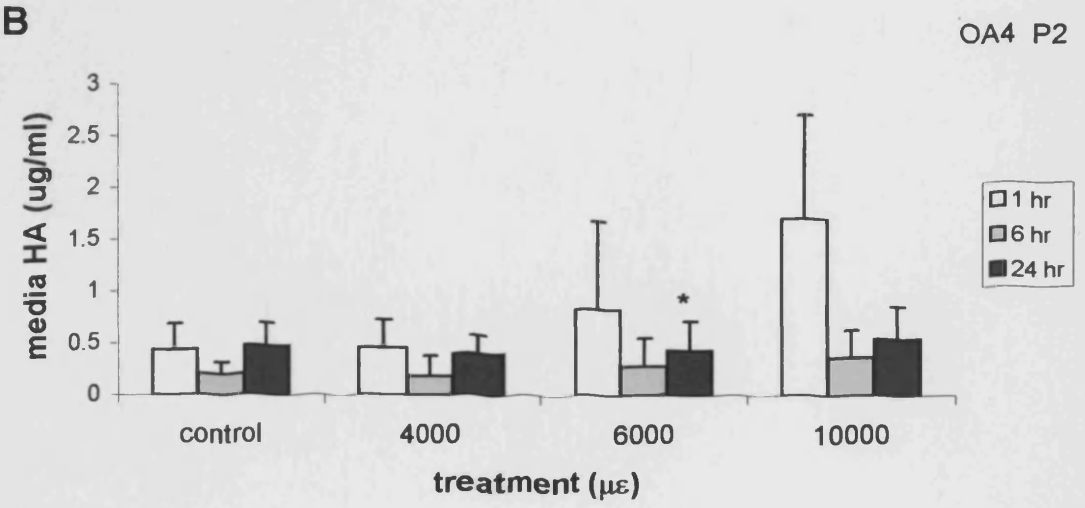

C
OA3 P3
media HA(ug/ml)
18
16
14
12
10
8
6
4
2
0
*
*
1 hr
6 hr
24 hr
control
4000
10000
treatment (με)

**Figure 3.3.10** Media HA concentrations from patient OA4. OA synovial cells were subjected to 10 minutes of mechanical strain and the media sampled at 1 hour, 6 hours and 24 hours. Control synovial cells did not undergo strain. Error bars represent the standard deviations. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.05$ compared to control cells. N=3 for control and strain data points.


A
OA4 P1
media HA (ug/ml)
3
2.5
2
1.5
1
0.5
0
1 hr
6hr
24 hr
control
4000
6000
10000
treatment (με)


B
OA4 P2
media HA (ug/ml)
3
2.5
2
1.5
1
0.5
0
1 hr
6 hr
24 hr
control
4000
6000
10000
treatment (με)

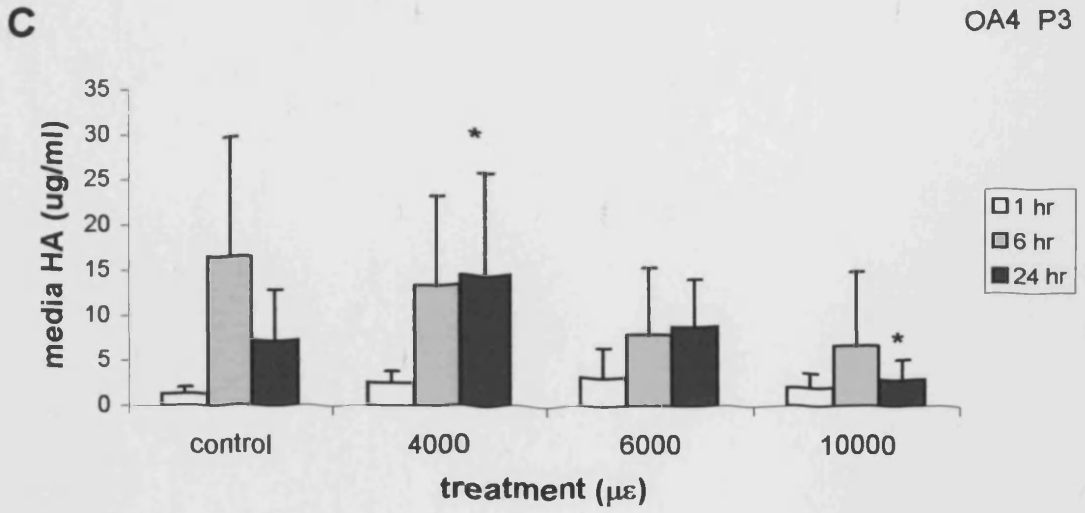

C
OA4 P3
media HA (ug/ml)
35
30
25
20
15
10
5
0
1 hr
6 hr
24 hr
control
4000
6000
10000
treatment (με)

**Figure 3.3.11** Media HA concentrations from patient OA5. OA synovial cells were subjected to 10 minutes of mechanical strain and the media sampled at 1 hour, 6 hours and 24 hours. Control synovial cells did not undergo strain. Error bars represent the standard deviations. *$P<0.05$ compared to control cells. N=3 for control and strain data points.

OA5 P3

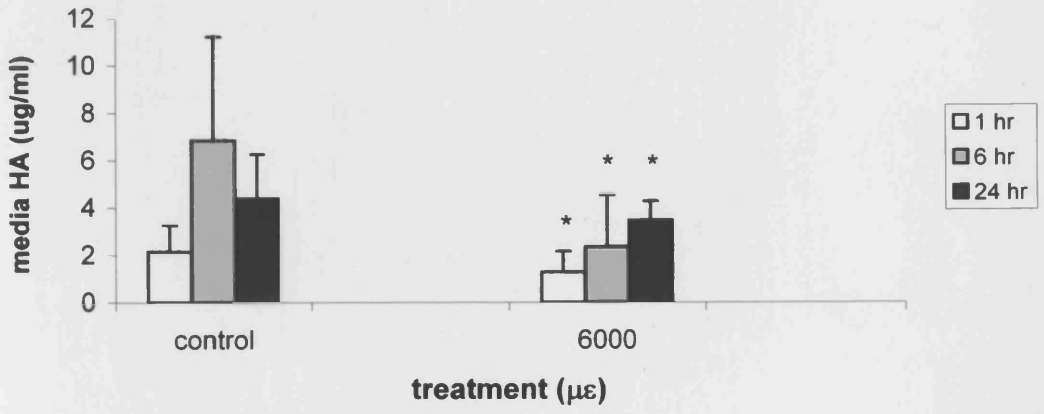
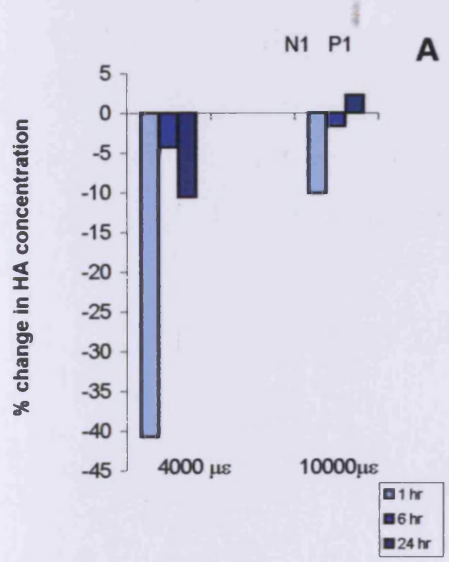
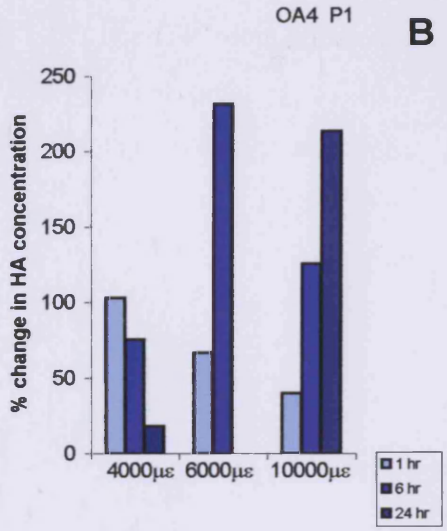
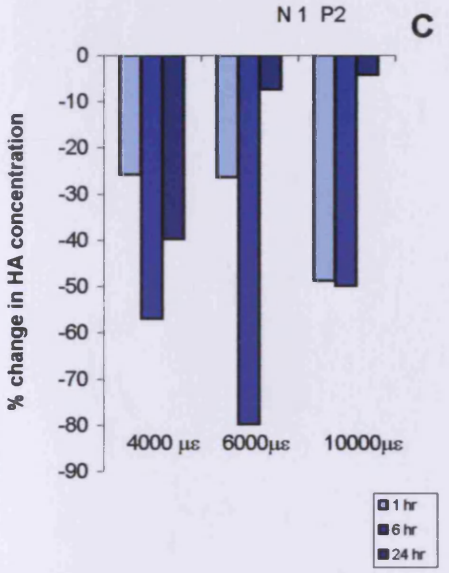
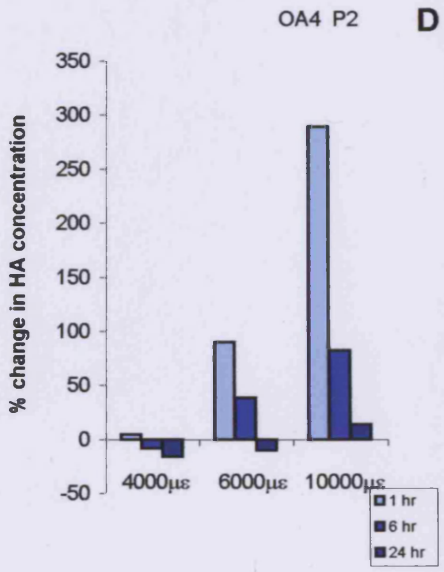

media HA (ug/ml)
12
10
8
6
4
2
0
control
6000
treatment (με)
1 hr
6 hr
24 hr
*
*
*

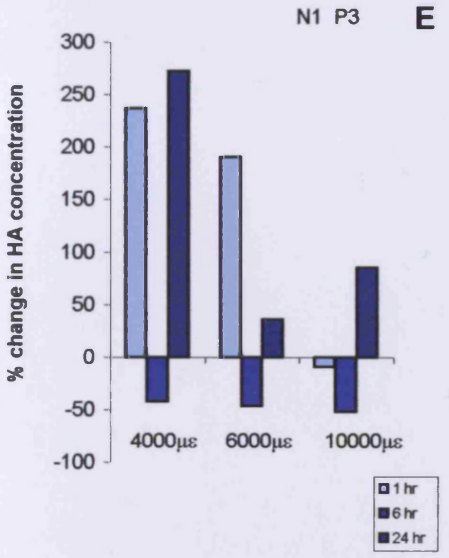
**Figure 3.3.12** The effect of mechanical strain on media HA concentration from normal (sample N1) and OA (sample OA4) synovial cells. Media was sampled at 1 hour, 6 hours and 24 hours post strain. Concentrations of HA in the media have been converted to percentage of HA in the media above or under the static control media HA concentration. A) normal passage 1 cells; B) OA passage 1 cells; C) normal passage 2 cells; D) OA passage 2 cells; E) normal passage 3 cells; F) OA passage 3 cells.


N1 P1
A
% change in HA concentration
5
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
4000 µε
10000µε
1 hr
6 hr
24 hr


OA4 P1
B
% change in HA concentration
250
200
150
100
50
0
4000µε
6000µε
10000µε
1 hr
6 hr
24 hr


N 1 P2
C
% change in HA concentration
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
4000 µε
6000µε
10000µε
1 hr
6 hr
24 hr


OA4 P2
D
% change in HA concentration
350
300
250
200
150
100
50
0
-50
4000µε
6000µε
10000µε
1 hr
6 hr
24 hr


N1 P3
E
% change in HA concentration
300
250
200
150
100
50
0
-50
-100
4000µε
6000µε
10000µε
1 hr
6 hr
24 hr

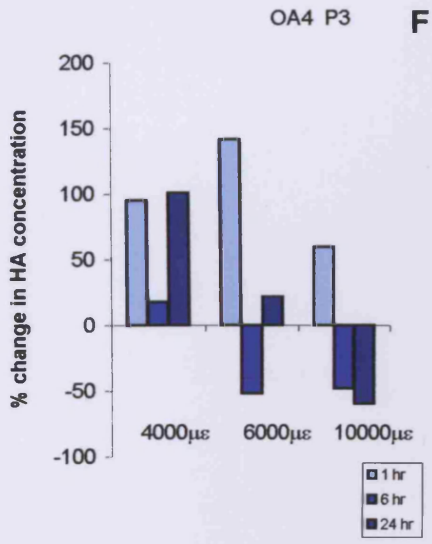

OA4 P3
F
% change in HA concentration
200
150
100
50
0
-50
-100
4000µε
6000µε
10000µε
1 hr
6 hr
24 hr

**Figure 3.3.13** UDPGD activity of cultured synovial cells from patient OA8. A&C shows static cells while B&D shows strained cells. C&D show the control reactions. Note the deeper blue reaction product in strained cells (B) when compared to static cells (A).

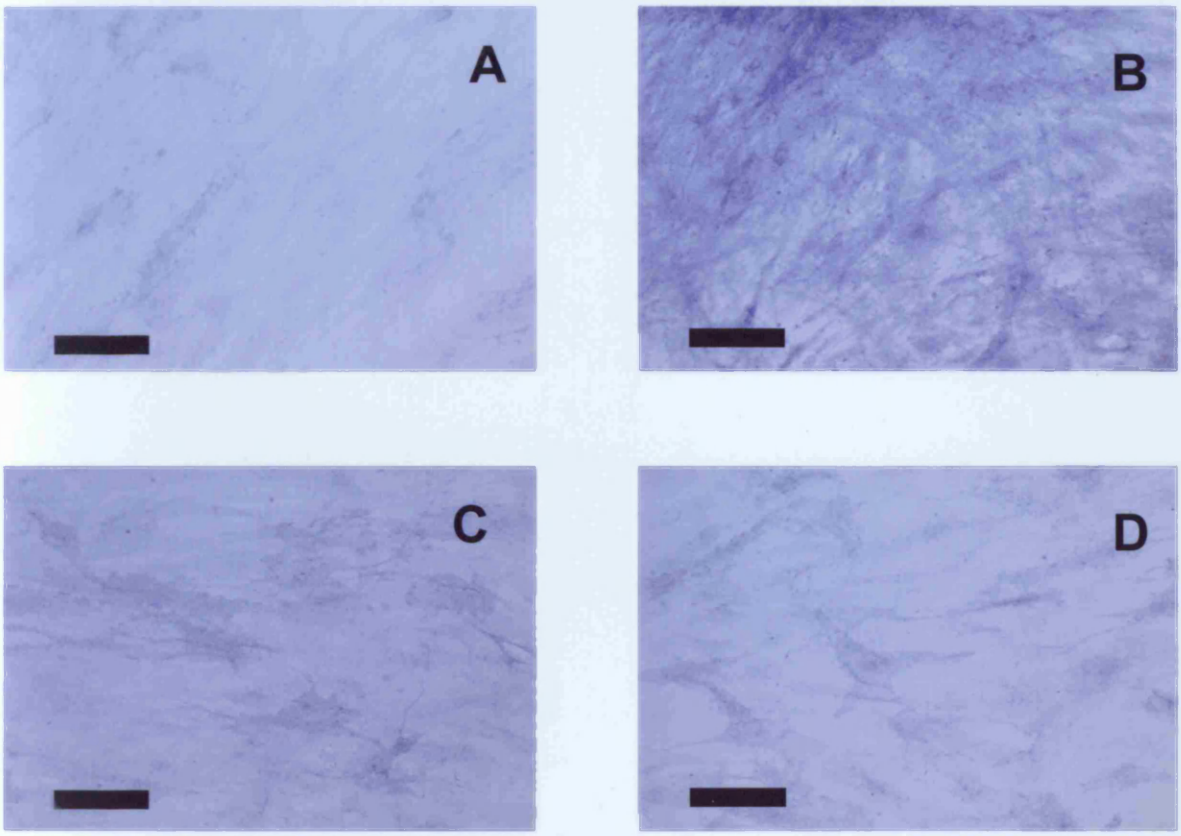
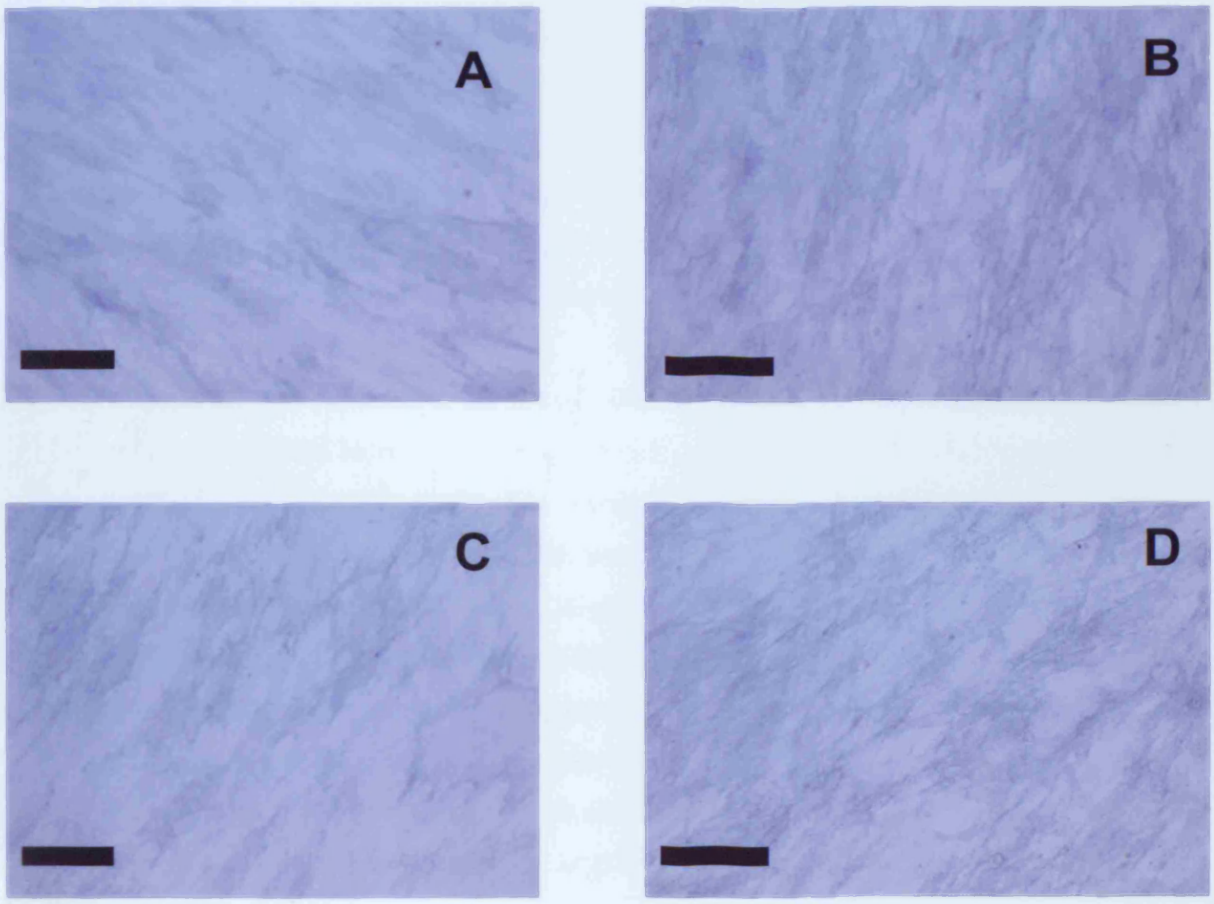

A
B
C
D

**Figure 3.3.14** UDPGD activity of cultured synovial cells from patient OA3. A&C shows static cells while B&D shows strained cells. C&D show the control reactions. Note control cells are lighter blue.


A
B
C
D

## 3.4 Discussion

The data presented here demonstrates that cells derived from both normal and OA synovium respond to mechanical strain by altering HA release into the surrounding media. Hyaluronan synthesis during joint development has been attributed in part to mechanical stimuli and, studies showing increased HA synthesis *in vitro* when cells from the developing joint were subjected to mechanical stimuli support this hypothesis (Dowthwaite et al., 1998; Dowthwaite et al., 2003; Dowthwaite et al., 1999; Edwards et al., 1994; Pitsillides et al., 1995). The continued synthesis of HA is thought a necessary requirement for maintaining a fully functioning adult joint and this current work supports the data that mechanical stimuli are vital for maintaining joint function. Synovial cells responded to a brief period of mechanical strain and maintained their response in altered HA synthesis. It is clear from this work however, that normal and OA cells respond differently to mechanical strain.

The general trend observed in normal synovial cell cultures in response to mechanical strain was an initial increase in media HA followed by a decrease at 6 hours then an increase at 24 hours post-strain. The increase in media HA at the 1 hour time point could be due to newly synthesised HA secreted into the media or from HA released from the pericellular coat either as a result of mechanical stimuli or due to the decreased levels of HA in the media at the start of the experiment. Previous work has shown that HA can signal through negative and positive feedback mechanisms which could explain the increase in HA (Larnier et al., 1989; Philipson and Schwartz, 1984; Philipson et al., 1985). These feedback mechanisms could also be responsible for the decrease in media HA concentration at the six hour time point, yet this decrease in media HA could occur in different ways. Firstly, the cessation of HA synthesis, explained by a reduction in HAS mRNA expression, HAS synthesis or in HAS activity. Internalisation of HA could occur via HABPs, reducing HA concentration within the media. Indeed, CD44 has been established as the principle cell surface HA receptor and is present on synovial cells (Aruffo et al., 1990; Edwards et al., 1993; Henderson et al., 1994; Henderson et al., 1993). Internalisation of HA via its CD44 receptor has previously been demonstrated and the addition of exogenous HA to the

cell cultures decreases HA synthesis, the mechanisms of which are likely to be saturation of CD44 (Collis et al., 1998; Dowthwaite et al., 1998; Edwards et al., 1994). The decrease in HA secretion into the media could also be attributed to the loss of nascent HA within the pericellular coat. As a result, the cell would continue to synthesise HA yet it would be retained within the pericellular coat so would not be detected. Previous work has shown the effect of digestion of the pericellular coat is to increase levels of HA synthesis and CD44 expression (Dowthwaite et al., 2003).

Lastly, the loss of HA within the media could also be a result of hyaluronidase digestion of the HA molecules. Hyaluronidases are present in mammalian cells and are responsible for cleaving the HA molecule (Afify et al., 1993; Flannery et al., 1998; Laurent and Fraser, 1992; Meyer, 1971). In different tissues, HA has different turnover rates and this is, in part, due to hyaluronidases (Stern, 2003). Treatment of cell cultures with hyaluronidases stimulates HA synthesis and high levels of hyaluronidase can modulate CD44 expression (Larnier et al., 1989; Philipson and Schwartz, 1984; Stern, 2003; Tanabe et al., 1993). The hyaluronidases are able to generate different sized disaccharides from the HA chain and these molecules are able to interact within the environment producing different effects (Afify et al., 1993; Lepperdinger et al., 2001; Noble, 2002).

The increased concentrations of HA at 24 hours suggests the HA levels within the surrounding media have reached a minimum level or that the levels of HA within the pericellular coat are at a maximum and so HA is secreted into the surrounding media. HA synthesis could be switched on again via increased expression of HAS mRNA or increased activity of HAS. If the pericellular coat has retained enough HA, then HA intended for the pericellular coat could simply be secreted into the media from the pericellular coat. Again, these data suggest a feedback mechanism exists that enables the synovial cells to sense the levels of HA that have been synthesised. More intriguingly, despite the presence of a HA pericellular coat, the cells seem to sense concentrations of HA distant and beyond this coat.

The response of the OA cells with time either showed a gradual increase in HA synthesis over the 24 hour period or an increase up to 6 hours then a decrease at 24 hours. Both these trends could be due to a breakdown in the HA synthesis feedback

pathway. The cells response to HA concentrations within the surrounding media could be hampered due to the disease and so the cells could take longer to respond to the stimuli. If the HA media concentrations could have been monitored over a longer period of time, then this might give a better insight into the response to mechanical stimuli. The fact that the OA control cells and OA strained cells respond differently with time suggests that the response to mechanical stimuli is altered perhaps as part of the disease process, but it can be seen that the OA synovial cell cultures are capable of synthesising HA.

Although the cells were subjected to 10 minutes of mechanical strain, the alterations in HA synthesis are evident 24 hours later. The results, however, indicate a difference in the synovial cell cultures from normal and OA tissues, in response to the three magnitudes of strain. Normal synovial cell cultures at 24 hours showed a decrease in media HA synthesis when subjected to 4000με whereas at 10000με an increase in media HA concentration was observed. In contrast, the OA synovial cell cultures showed an increase in media HA concentration at 4000με and a decrease at both 6000με and 10000με. Whilst the exact mechanical strain to which cells in the synovium are subjected to are at present unknown we do know that the lowest strain magnitude of 4000με applied to the cells in this investigation is physiological in a wide range of bones from a number of different species (Lanyon, 1996). Thus, it is the lowest strain of 4000με that is able to increase and maintain HA synthesis in OA synovial cell cultures. The normal cells, however, showed a decrease in media HA concentration when subjected to 4000με. Previous work has shown that HA synthesis can be controlled by the amount of HA present in the surrounding milieu and could explain the reduction in HA synthesis in the normal cell cultures post strain (Dowthwaite et al., 2003; Larnier et al., 1989; Philipson et al., 1985). The responses to 10000με by normal and OA synovial cell cultures is different and the increase in HA synthesis by the normal cells might be considered more advantageous. An increase in media HA concentration would lead to a more viscous synovial fluid allowing greater loads to be applied and preventing synovial fluid leaking from the cavity (Coleman et al., 1999; Coleman et al., 2000).

The differences in media HA concentrations between normal and OA synovial cells seen in response to strain was highlighted in the different passages. At passage 1 and

2, normal cells showed decreases in media HA concentration when subjected to 4000με whereas passage 3 cells showed no changes in media HA concentration in response to strain. When the cells were subjected to 10000με an increase in media HA concentration was observed only in the passage 3 cells. The OA synovial passage 1 cells gave an increase in media HA concentration at the three magnitudes of strain whereas passage 3 cells showed an increase only when strained at 4000με and 10000με. These results might suggest that the cells become less mechano-sensitive with passage as they do not receive mechanical cues *in vitro*. Further explanation of the response to mechanical strain by the cells at the different passages could be the result of alterations in cell cultures due to *in vitro* growth. It has previously been shown that synovial cell cultures alter in both cell population and cell characteristics with the length of time in culture (Clarris and Fraser, 1968; Marsh et al., 1978; Smith and Hamerman, 1969; Zimmerman et al., 2001). As a result, a loss of macrophage and other infiltrating cells with repeated passage can be observed. This prevents the cell-cell interactions between the SFBs and SMPs which occur *in vivo* and which might be needed for transduction of mechanical stimuli *in vitro* (Edwards, 1999). The development of a more fibroblastic cell line has also been suggested to occur with repeated passage and could result in a different response to mechanical stimuli *in vitro* (Marsh et al., 1978; Zimmerman et al., 2001).

The loss of mobility within the OA joint is one of the main symptoms associated with OA and this has often been attributed in part to the loss of hyaluronan from the synovial fluid and synovium. Whether the synovial cells are unable to synthesise HA due to the disease or whether the loss of HA synthesis observed in the synovium is due to the reduction in mechanical stimuli or is a combination of both of these factors is not clear. From this work, however, it can be established that OA synovial cell cultures are able to respond to mechanical stimuli. In particular, the lowest magnitude of strain increased HA synthesis in OA synovial cell cultures. Whether this would transfer to an *in vivo* situation remains to be researched. Previous work of controlled exercise based studies as a treatment for OA have been shown to reduce joint pain and increase joint mobility and this could be due to the increase in HA synthesis as a result of mechanical stimuli (Fisher et al., 1993; Minor et al., 1989; Penninx et al., 2001; Rejeski et al., 1998; Sullivan et al., 1998). It seems that HA does indeed play an important role in joint mobility and mechanical stimuli are necessary to maintain

the HA synthesis within the synovial joint. Thus, development of exercise based therapies could prove beneficial as a treatment for OA.

# Chapter 4

# Effects of Mechanical Strain on Hyaluronan Synthase mRNA Expression in Synovial Cells

## 4.1 Introduction

Since the discovery of hyaluronan (HA) in 1934 (Meyer and Palmer, 1934), this polysaccharide has generated a great amount of interest, due to the variety of physical and biochemical properties it exhibits that are able to influence cell behaviour. HA has been shown to modulate cellular migration and differentiation, regulate the organisation and metabolism of the ECM and be an important factor in metastasis, wound healing and inflammation (Hall and Turley, 1995; Ichikawa et al., 1999; Kozaci et al., 1997; Laurent and Fraser, 1992; Lee et al., 2000; Lesley et al., 1993; Toole, 1990). The variety of functions HA is able to perform is partly due to the nature of its synthesis and the sizes of the polymer chain and the concentrations at which it is synthesised leads to differing biological and physical characteristics.

HA synthesis begins with conversion of UDP-glucose to UDP-glucuronate by the enzyme uridine-diphosphoglucose dehydrogenase (UDPGD) and this has been shown to be the rate-limiting step of HA synthesis (McGarry and Gahan, 1985; Molz and Danishefsky, 1971). UDPGD activity provides the precursors for the next step in HA synthesis that involves the enzyme hyaluronan synthase (HAS). HAS is responsible for extending the HA polymer at the reducing end of the HA chain (Prehm, 1984). Here UDP-glucuronate (UDP-glc) or UDP-N-acetylglucosamine (UDP-glcNAc) are transferred onto the reducing end sugar of the HA chain, with displacement of the terminal UDP (fig.1.1). This is in contrast to the synthesis of GAGs, which are synthesised in processes involving elongation of the sugar chains bound to core proteins (Mason et al., 1982). Simultaneously, the HA chain is extruded in to the ECM while still being extended in the cell. This process of synthesis allows the formation of large chains of HA as they are not restricted in size by modifications in the Golgi compartment, or vesicular transport to the cell surface, as in the synthesis of other macromolecules (Kjellen and Lindahl, 1991).

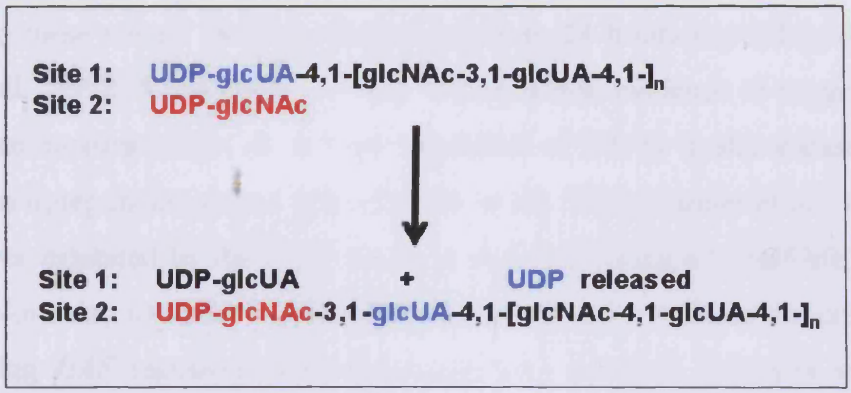



**Figure 4.4.1 Example of proposed HA chain polymerisation**

HAS resides on the plasma membrane and consists of seven putative membrane-spanning domains, two of which are located at the N-terminal end of the molecule while the other five are located at the C-terminal end (Prehm, 1984). HAS is unusual because of the large number of functions that are required for the overall polymerisation of the HA chain. It has been suggested that vertebrate HAS is part of a multicomponent enzyme complex comprising of 2 binding sites for the 2 different sugar nucleotides, 2 different glycosyltransferase activities, 1 or more binding sites that can anchor the HA polymer to the enzyme and a ratchet-like transfer reaction site that moves the growing polymer one sugar at a time (Mian, 1986; Weigel et al., 1997).

Research into the bacterial *HAS* genes lead to the discovery of a multigene family encoding distinct isoenzymes (DeAngelis, 1996; Itano et al., 1999; Watanabe and Yamaguchi, 1996). As research progressed to eukaryotic organisms three mammalian enzymes, HAS1, HAS2 and HAS3, encoded by three *HAS* genes on different chromosomes were discovered and have contributed to a greater understanding of HA synthesis (Spicer and McDonald, 1998). The 3 *HAS* genes show similar homology yet *in vitro* have been shown to be responsible for the synthesis of three distinct HAS enzymes capable of synthesising HA of different chain lengths and at different rates (Itano et al., 1999). If the same were to occur *in vivo*, then this would provide some answers to the many biological and physical properties of HA. The regulation of *HAS* gene expression remains obscure. Levels of media HA concentrations have been shown to correlate with HAS mRNA levels in some studies whilst in others this is not the case (Jacobson et al., 2000; Recklies et al., 2001). Growth factors and cytokines have been shown to act as stimuli in HA synthesis but alterations in HA detected in

response to these stimuli can vary from 4 hours to 24 hours depending on cell type (Heldin et al., 1992; Suzuki et al., 1995). There is also evidence to suggest negative feedback mechanisms occur, as *in vitro* depletion of HA by hyaluronidase treatment results in an upregulation of HA (Dowthwaite et al., 2003; Larnier et al., 1989). The low turnover exhibited by the HAS enzymes and the variation in half-life, which can range from one day to three weeks depending on cell type, adds to the complexity of understanding *HAS* regulation and subsequent HA synthesis (Morales and Hascall, 1988; Tammi et al., 1991).

Synovial fibroblasts within the adult joint are responsible for synthesising HA (Pitsillides and Blake, 1992; Wilkinson et al., 1992). The HA is either retained in the synovium or secreted into the synovial fluid, maintaining a constant volume and level of viscosity necessary for frictionless movement of the joint (Levick, 1991; Levick, 1996). Low abundance of *HAS* gene expression in cultured synovial cells has caused some difficulties in detecting the three *HAS* genes but Recklies *et al.*, (2001) demonstrated that *HAS1* and *HAS2* are in similar abundance whereas *HAS3* is the least abundant. The differential expression of the three *HAS* genes suggest each plays an important role in maintaining joint function. As the genes are responsible for HAS expression and, as the three HAS proteins are each able to synthesise different sized HA polymers at different rates, this could allow the cells the flexibility to synthesise the necessary HA polymers required to maintain synovial fluid viscosity and to prevent synovial fluid leaking out from the cavity when the joint is flexed.

In OA synovium, there is evidence of a decrease in HA synthesis by the synovial fibroblasts and a consequent decrease in HA concentration of the synovial fluid, leading to a reduction in joint mobility (Pitsillides et al., 1994; Yamada et al., 2000). At present, little research exists on differential *HAS* gene expression within OA joints but alterations in HA synthesis occurring in OA joints would suggest regulation of *HAS* gene expression is involved. It is interesting to note that OA patients show high HA serum levels and this could be due to increased HAS3 message levels (Miyaguchi et al., 2003). HAS3 synthesises the smallest HA molecule *in vitro*, if the same were to occur *in vivo* then the HA molecule could more easily be lost to the lymphatic system than larger HA molecules (Miyaguchi et al., 2001). If HA is not retained within the synovial fluid then this will affect synovial fluid viscosity and volume.

As OA progresses, the OA joint becomes painful to move and the observed decrease in HA synthesis could be attributed in part, to the reduction in mechanical stimuli. HA synthesis within the developing joint cavity is involved in cell separation of the cartilage anlagen and the stimuli for HA synthesis here could well be mechanical since cavitation only occurs after the musculature becomes functional (Craig et al., 1990). Indeed, mechanical stimuli has been shown to induce *HAS3* expression in developing chick fibrocartilage cells whereas *HAS2* expression remained constitutive (Dowthwaite et al., 2003; Dowthwaite et al., 1999). Thus, reduction in HA synthesis in the OA joint could be due partly, to the reduction or loss of mechanical stimuli on the three *HAS* genes. The ability to regulate *HAS* expression through mechanical stimuli could prove beneficial in treating OA patients by increasing HA concentration within the synovial fluid and so improving joint mobility.

The aim of this work was to try and establish a greater understanding of HAS regulation in the synovial cells from OA knees. The previous chapter has demonstrated the effect of mechanical strain on HA synthesis. An increase in media HA concentration was observed when the cells were subjected to 4000με whereas 6000με and 10000με gave variable results depending on the patient. Investigation of the differential expression of the three *HAS* genes in synovial cells and subsequent alterations in gene expression when synovial cells are subjected to mechanical stimuli should provide a more indepth understanding of the regulation of HA synthesis.

## 4.2 Materials and Methods

### 4.2.1 Synovium Samples

Synovium samples were obtained as in Chapter 2 (2.2.1). Details of the samples are listed below.

| Sample | Type | Sex | Age (years) |
|---|---|---|---|
| OA1 | OA | Female | 57 |
| OA2 | OA | Male | 64 |
| OA3 | OA | Female | 74 |
| OA4 | OA | Female | 79 |
| OA5 | OA | Male | 77 |
| OA6 | OA | Male | 79 |
| OA7 | OA | Male | 79 |
| N1 | normal | Female | 67 |
| N2 | normal | Unknown | 71 |

**Table 4.2.1 Details of synovium samples obtained at knee joint replacement surgery.**

### 4.2.2 Cell Isolation & Culture

Cells were isolated from the synovium as described in Chapter 2 (2.2.2). Briefly, diced tissue was digested in 295IU mg $ml^{-1}$ type I collagenase (Sigma, Poole, UK) for 3 hours at 37°C. Cells were centrifuged at 1000 x g for 5 minutes, resuspended then centrifuged at 1000 x g for 5 minutes. Cells were counted and seeded as required in DMEM/F12 containing 1% penicillin-streptamycin ($10^4$IU $ml^{-1}$/$10^4$μg $ml^{-1}$; Sigma), L-glutamine (200mM; Life Technologies, Paisley, UK), hydrocortisone (4mg $ml^{-1}$; Sigma)[40mg hydrocortisone powder dissolved in 5ml 100% ethanol and 5ml sterile PBS], Insulin-Transferrin-Selenium (200mM; Life Technologies) and 20% foetal calf serum (Sigma) and incubated at 37°C.

### 4.2.3 Mechanical Strain

The cells were subjected to mechanical strain as described in Chapter 3 (3.2.3). Briefly, cells were serum deprived for 18 hours then fresh serum free media was added. The cells were strained at 4000με, 6000με and 10000με for 10 minutes using

a four-point bending loading jig. Control cells remained static for 10 minutes and flow control cells remained static but were subjected to media perturbation for 10 minutes.

### 4.2.4 RNA Isolation

RNA from the strained, static control and flow control cells were isolated 24 hours post-strain using the Qiagen RNeasy Mini kit (Qiagen Ltd, West Sussex, UK) following manufacturers instructions. RNA was stored in solution at -80°C until required.

The quality of extraction was verified by 1% (w/v) agarose (Helena Biosciences, Europe)/ Tris-Borate EDTA buffer (TBE, Sigma-Aldrich) gel and by measuring light absorbance at 260nm and 280nm. The concentration of RNA in the extraction was calculated using the equation:

$$[RNA] = A_{260} \times D \times 40ug/ml$$

where D = the final dilution factor, and $A_{260}$ = the absorbance of the solution at 260nm.

### 4.2.5 Real-Time PCR

Reverse transcription polymerase chain reaction was used to establish HAS expression by the strained, static control and flow control cells. A reverse transcription reaction was performed to transcribe the RNA to cDNA using the following reaction mix.

| Product | Volume |
|---|---|
| RNA (1µg/ml) | 10µl |
| 5X MMLV RT buffer (Promega, Southampton, UK) | 10µl |
| MMLV Reverse Transcriptase (200U/ul, Promega) | 0.5µl |
| dNTP (25mM each, Promega) | 1µl |
| Random Hexamer (500µg/ml, Promega) | 1µl |
| Rnasin (400U/µl, Promega) | 1.25µl |
| Distilled Rnase & Dnase free $H_2O$ | 26.25µl |
| Total | **50µl** |

**Table 4.2.2 Reagents for cDNA synthesis.**

The reaction mix was incubated at 25°C for 10 minutes, followed by 48°C for 60 minutes then a 95°C step for 10 minutes to denature the reverse transcriptase enzyme. All cDNA was synthesised using a Perkin Elmer cDNA thermal cycler. The RT products were used immediately or stored at -20°C.

The PCR primers in table 4.2.3 were designed using Bio/Oligo Primer Design software and supplied by Invitrogen (Paisley, UK).

| Sequence (5'-3') | Primer | Annealing Temperature °C | Product (bp) |
|---|---|---|---|
| CAT CGC TGC CTA TCA AGA AGA | Forward human HAS2 | 65 | 454 |
| CCC AAC ACC TCC AAC CAT | Reverse human HAS2 | | |
| TCA TCT CTG CCC CCT CTG CTG | Forward human GAPDH | 53 | 444 |
| CCT CCG ACG CCT GCT TCA C | Reverse human GAPDH | | |
| CGG CCT GTT CCC CTT CTT CGT | Forward human HAS1 | 62 | 347 |
| CGT GTG CTA CGC TGC GGA CCA | Reverse human HAS1 | | |
| CAG CCT CCT CCA GCA GTT CC | Forward human HAS3 | 55 | 317 |
| TAA CCG TGG CAA TGA GGA G | Reverse human HAS3 | | |

**Table 4.2.3 Oligonucleotide primers for RT-PCR analysis.**

PCR for HAS2 was then performed on a GeneAMP PCR system 9700 (PE Applied Biosystems) using the above primers and the following reaction products. The PCR mix consisted of the reagents described in table 4.2.4.

The PCR reaction started at 95°C for 30 seconds, then cycled at 95°C for 30 seconds, the annealing temperature (listed in table.4.2.3) for 60 seconds, then 72°C for 60 seconds for 35 cycles, followed by an elongation step of 72°C for 6 minutes. The product was stored at 4°C until required.

| Product | Volume |
|---|---|
| Forward primer (50µm, Invitrogen) | 1µl |
| Reverse primer (50µm, Invitrogen) | 1µl |
| RT product | 1µl |
| dNTPs (10mM each, Promega) | 1µl |
| Taq DNA polymerase (95U/µl, Promega) | 0.25µl |
| 10X buffer (Promega) | 5µl |
| $MgCl_2$ (25mM, Promega) | 3µl |
| $dH_2O$ | 37.75µl |
| **Total** | **50µl** |

**Table 4.2.4 Reagents for HAS2 PCR.**

PCR for HAS1 and HAS 3 was performed using the published primer sequences (Recklies *et al.*, 2001) shown in table 4.2.3. The PCR mix was as follows.

| Product | Volume |
|---|---|
| Forward primer (50µm, Invitrogen) | 1µl |
| Reverse primer (50µm, Invitrogen) | 1µl |
| RT product | 1µl |
| dNTPs (10mM each, Promega) | 1µl |
| Amplitaq Gold (5U/µl, Applied Biosciences, CA, USA) | 0.3µl |
| 10X PCR X10 Gold buffer (Applied Biosciences) | 5µl |
| $MgCl_2$ (25mM, Applied Biosciences) | 3µl |
| $dH_2O$ | 37.75µl |
| **Total** | **50µl** |

**Table 4.2.5 Reagents for HAS1 and HAS3 PCR.**

The PCR reaction was the same as above with the cycle number increased to 45.

Positive controls of human tendon fibroblast cDNA were run with each reaction to ensure the PCR was effective.

**Gel Electrophoresis**

Eighteen µl of the PCR product was run on a 2.0% (w/v) agarose/ TBE gel at 70V for 35 minutes along with phiX DNA/*Hae* III marker (Promega). The gel was stained with ethidium bromide (Sigma) at 1µg/ml for 20 minutes and the excess washed off

with tap water. The bands were visualised on an ultraviolet light box and the gel documented using Quantitiy One software (BioRads Labs, Hertfordshire, UK).

**Analysis of PCR products**

To determine that the bands visualised on the gel were the gene of interest, each PCR product was purified, using the QIAquick PCR purification kit (Qiagen) following the manufacturers instructions. The purification products were then sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosciences) following manufacturers instructions.

### 4.2.6 Quantitative Real-Time PCR

The relative expression levels of HAS1, HAS2 and HAS3 in strained and unstrained synovial cells from OA patients and normal patients were determined using the ABI Prism 7700 TaqMan quantitative polymerase chain reaction system (Applied Biosystems, CA, USA).

Primers and dual-labelled probes were designed using Oligo Primer Design software to detect HAS1, HAS2 and HAS3 and purchased from Invitrogen and Sigma respectively. The relative expression levels of each HAS gene from control and strained cells were all normalised to 18S rRNA (Applied Biosystems, CA, USA).

| | Forward Primer | Reverse Primer | TaqMan Probe |
|---|---|---|---|
| **HAS 1** Acc. NM_001523 | 911-GCG GGC TTG TCA GAG CTA-931 | 995-AAC TGC TGC AAG AGG TTA TTC CTA TAT-968 | ACTGTGTATCCTG CATCAGCGGTCCT CTA |
| **HAS 2** Acc. U54804 | 45-GCA GCT CAT TGA ACC AGA GA-65 | 130-AAG ACT CAG CAG AAC CCA GGA A-108 | CCCCAGCCAAAGA CTTTTCTCCCAATT CT |
| **HAS 3** Acc.Af232772 | 3445-GGT AAG ACT GCT GGT TGA CAT CAG-3469 | 3547-CAG AAT GAT TGC CTG GAT CA-3527 | CCCAACCCATTGA AGGCTGGAAGG |

**Table 4.2.6 Quantitative PCR primer and probe sequences.**

The probe reporter dye used for detection of the HAS amplicons was 6-carboxyfluorescein (FAM), quenched by 6-carboxy-tetramethyl-rhodamine

(TAMRA). The rRNA reagents were purchased as a kit (Applied Biosystems). The probe reporter dye was VIC (proprietary dye, Applied Biosystems), quenched by TAMRA.

Amplification of both HAS and 18S rRNA was performed in a 96 well plate prepared as per the manufacturers instructions (Eurogentec Ltd, Hampshire, UK) with a total reaction volume of 25µl. Primer and probe concentrations were optimised and are indicated in table.4.2.7. Each reaction used 0.5µl of cDNA template. All reactions were performed in triplicate and to certify reactions were not contaminated negative controls replacing cDNA with water were run in parallel.

| Target | Primer | Probe |
|---|---|---|
| 18S rRNA (Applied Biosciences) | 50nM | 200nM |
| HAS 1 | 50nM | 100nM |
| HAS 2 | 300nM | 100nM |
| HAS 3 | 50nM | 100nM |

**Table 4.2.7 Quantitative PCR primer and probe reaction concentrations.**

Reactions were run on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with cycling conditions set at 95°C, 10 minutes, then 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute.

Before conducting the experiment on the samples a validation plate was run. This was to establish the efficiencies of the gene interest and the 18S rRNA to determine whether the standard curve or comparative Ct method was needed.

For HAS1 and HAS2, the comparative Ct method could be applied. The efficiencies of the HAS and 18S rRNA amplicons were shown to be equal, so eliminating the need for a standard curve (Appendix 4.2).

For HAS3, a dilution series of control synovial cDNA was run in triplicate for both the gene of interest (HAS3) and the standard (18S rRNA) on the same 96 well plate as the samples (Appendix 4.4). Thus, Ct (cycle threshold; AppliedBiosystems, 1997)

values and total RNA quantities were used to generate a relative standard curve (Appendix 4.4).

**Statistical Analysis**

The mean Ct values and standard deviation values were determined as per Applied Biosystems instructions (Appendix 4.2 & 4.4). Thus data are presented as relative levels of mRNA for each gene of interest normalised to either control normal or control OA synovial cells. Changes in mRNA quantities were analysed using one-way analysis of variance (ANOVA) of normalised Ct values for pairwise contrasts with Tukey error protection at a 95% confidence interval.

**Sample Number**

As a result of low cell number, cells from some patients could not be subjected to the three magnitudes of strain at each passage. Therefore, subsequent QPCR analysis of HAS expression could not be performed for each patient at all passages and at the three magnitudes of strain.

## 4.3 Results

### 4.3.1 HAS1, HAS2 and HAS3 Gene Expression

Sequence analysis of PCR products generated using HAS1, HAS2 and HAS3 gene-specific primers each produced a positive match to the published gene sequences for HAS1, HAS2 and HAS3 in mRNA extracted from cultured synovial fibroblasts.

**HAS Gene Expression in Normal Synovial Cells**

Subjecting the cells to 4000με HAS1 revealed mRNA expression in strained cells whereas HAS1 mRNA expression was not detected in control cells at passage 1 (fig.4.3.1). At passage 3, HAS1 expression was detected in both strained and control cells. HAS2 mRNA expression was detected in strain and control cells at all passages. The expression of HAS3 mRNA was not detected in control or strain cells at any passage.

Analysis of HAS1 mRNA expression post-6000με revealed variable results. Strained and control cells from patient N2 at passage 1 and 2 did not express HAS1 whereas HAS1 expression was detected in strained and control cells at passage 3. HAS2 expression was detectable in strained and control cells from both normal patients and in cells from all passages. HAS3 mRNA expression was not detected in cells from patient N1 subjected to strain or in control cells but was detected in patient N2 in both strained and control cells.

HAS1 mRNA expression was detected in both strained and control cells when the cells were strained at 10000με. Strained and control cells were both seen to express HAS2 mRNA in cells from the 3 passages. HAS3 mRNA was expressed in both strained and control cells at passage 1 but was not detected at passage 3 when cells were subjected to 10000με.

**HAS Gene Expression in OA Synovial Cells**

Analysis of synovial fibroblast HAS1 mRNA expression by PCR at 24 hours post-4000με revealed no differences in expression patterns between the strained and control cells, with both sets of cells expressing HAS1 mRNA, except for Patient OA3 at passage 3 which did not express HAS1 mRNA (fig.4.3.2). HAS2 mRNA was expressed in both strained and control cells in all patients and at all passages regardless of strain. HAS3 mRNA was absent in both strained and control synovial fibroblasts except Patient OA3 passage 3 cells which expressed HAS3 mRNA in both strain and control cells.

When cells were subjected to 6000με, HAS1 mRNA expression was detected in both strained and control cells at all passages except in Patient OA7, passage 1 cells. HAS2 mRNA expression was expressed in both strained and control cells in all patients and at all passages regardless of strain. HAS3 mRNA expression could not be detected in over half of patients. Patients OA1 and OA7 passage 1 cells however, expressed HAS3 mRNA expression in control cells but not in strained cells.

Analysis of HAS1 mRNA expression in the cells subjected to 10000με showed expression in strained and control cells, except for patient OA3 passage 3 cells and OA4 passage 1 cells which showed no expression in both strain and control cells. HAS2 mRNA expression was detectable in both strained and control cells in all patients and at all passages. HAS3 mRNA expression was detected in patient OA3 in all but the control passage 3 cells, in the strained passage 3 cells from patient OA5 and in the strained passage 1 cells from patient OA4. HAS3 mRNA expression was not detected in the remainder of the patients sampled, either in control or strained cells.

## 4.3.2 QPCR data: Normal Patient Response to Mechanical Strain

**Patient N1**

<u>HAS1 mRNA expression.</u> At passage 1, HAS1 mRNA expression was significantly decreased ($P<0.01$) relative to the control cells when the cells were strained at 4000με (fig.4.3.3A). The effect of 10000με on HAS1 mRNA expression was not

significantly different in the strained cells relative to the control cells (fig.4.3.3A). At passage 2 and 3, no significant differences in HAS1 expression were observed in the cells subjected to 4000με, 6000με and 10000με relative to the control cells (fig.4.3.3B & C).

HAS2 mRNA expression. At passage 1, a significant decrease ($P<0.001$) in HAS2 mRNA expression was seen at 4000με relative to the control cells (fig.4.3.4A). At passage 2, a significant increase ($P<0.0001$) in HAS2 mRNA expression was detected at 6000 and 10000με, with the greatest increase in HAS2 mRNA expression at 6000με (fig.4.3.4B). At passage 3, no significant differences in HAS2 mRNA expression were observed at the 3 magnitudes of strain relative to the control cells (fig.4.3.4C).

HAS3 mRNA expression. At passage 1, no significant differences in HAS3 mRNA expression in the strained cells relative to the control cells were apparent when the cells were subjected to 4000με and 10000με (fig.4.3.5A). At passage 2 and 3, significant increases in HAS3 mRNA expression relative to the control cells resulted when the cells were subjected to 4000με (fig.4.3.5B & C). At 6000με no significant differences were observed between the strained and control cells. Significant decreases ($P<0.001$) in HAS3 mRNA expression were observed when the passage 2 and 3 cells were strained at 10000με, relative to the control cells (fig.4.3.5B & C).

**Patient N2**

HAS2 mRNA expression. At passage 1, no significant differences in HAS2 mRNA expression relative to the control cells was observed when the cells were strained at 6000με (fig.4.3.6A).

HAS3 mRNA expression. At passage 1, no significant differences in HAS3 mRNA expression in the strained cells relative to the control cells was observed when the cells were subjected to 6000με (fig.4.3.6B).

### 4.3.3 OA Patient Response to Mechanical Strain

**Patient OA2**

HAS1 mRNA expression. At passage 1 and 3 no significant differences in HAS1 mRNA expression were observed when the cells were subjected to 6000με, relative to HAS1 mRNA expression in the control cells (fig.4.3.7A & B).

HAS2 mRNA expression. At passage 1, a significant decrease ($P<0.001$) in HAS2 mRNA expression was observed in the cells that were strained at 6000με relative to the control cells (fig.4.3.7C).

**Patient OA3**

HAS1 mRNA expression. At passage 1 and 2, no significant differences in HAS1 mRNA expression relative to the control cell HAS1 mRNA expression were detected when the cells were subjected to 4000με, 6000με and 10000με (fig.4.3.8A & B). At passage 3, a significant increase ($P<0.001$) in HAS1 mRNA expression relative to control cells could be seen at 4000με (fig.4.3.8C). At 10000με, a significant increase ($P<0.001$) in HAS1 mRNA expression was seen in the passage 3 strained cells relative to control cells.

HAS2 mRNA expression. At passage 2, no significant differences in HAS2 mRNA expression in the strained cells relative to the control cells were observed when the cells were subjected to 4000με (fig.4.3.9A). At 10000με, a significant increase ($P<0.01$) in HAS2 mRNA expression in the strained cells relative to the control cells was detected. At passage 3 significant increases ($P<0.01$) in HAS2 mRNA expression was observed at 4000με and 10000με relative to control cells (fig.4.3.9B).

**Patient OA4**

HAS1 mRNA expression. At passage 1, no significant differences were observed in HAS1 mRNA expression relative to the control cells HAS1 mRNA expression when the cells were subjected to 4000με. The effect of 6000με resulted in a significant increase ($P<0.001$) in HAS1 mRNA expression in the strained cells relative to the control cells whereas 10000με resulted in a significant decrease ($P<0.001$) in HAS1

mRNA expression (fig.4.3.10A). At passage 2, a significant increase ($P<0.01$) in HAS1 mRNA expression in strained cells relative to control cells was observed in the cells subjected to 4000με and 6000με (fig.4.3.10B & C). The effect of 6000με on passage 3 cells HAS1 mRNA expression resulted in a significant increase ($P<0.01$) relative to the control cells. At 4000με and 10000με no changes in HAS1 expression were detected.

HAS2 mRNA expression At passage 1, cells subjected to 6000με showed a significant increase ($P<0.001$) in HAS2 mRNA expression (fig.4.11A). There was no significant difference between HAS2 mRNA expression in the cells subjected to 4000με and 10000με relative to the control cells. At passage 2, no significant differences in HAS2 mRNA expression were observed in the strained cells relative to the control cells when the cells were subjected to 4000με and 6000με (fig.4.3.11B). At 10000με, a significant decrease ($P<0.001$) in HAS2 mRNA expression in the strained cells was apparent (fig.4.3.11B). At passage 3, no significant effects in HAS2 mRNA expression were observed when the cells were subjected to strain regardless of magnitude (fig.4.3.11C).

HAS3 mRNA expression At passage 1, no significant effects in HAS3 mRNA expression were observed in the cells subjected to 4000με and 10000με (fig.4.3.12A). At 6000με a significant increase ($P<0.001$) in HAS3 mRNA expression in the strained cells relative to the control cells HAS3 mRNA expression was detected (fig.4.3.12A). Passage 2 cells showed significant increases ($P<0.001$) in HAS3 mRNA expression in the cells subjected to 4000με and 6000με relative to the control cells (fig.4.3.12B). No significant difference in HAS3 mRNA expression was seen when the cells were subjected to 10000με. The effect of 4000με on HAS3 mRNA expression in passage 3 cells was a significant increase ($P<0.001$) relative to the control cells (fig.4.3.12C). Straining the cells at 6000με and 10000με resulted in no significant differences in HAS3 mRNA expression relative to the control cells (fig.4.3.12B & C).

**Patient OA6**

HAS1 mRNA expression At passage 1, no significant differences in HAS1 mRNA expression in the strained cells relative to the control cells were seen at 4000με and

10000με (fig.4.3.13A). Cells at passage 2 showed a significant increase ($P<0.01$) in HAS1 mRNA expression relative to control cells when subjected to 4000με (fig.4.3.13B). The cells subjected to 6000 and 10000με showed no significant differences in HAS1 mRNA expression relative to control cells. At passage 3, HAS1 mRNA expression in strained cells was significantly increased ($P<0.01$) relative to control cells at 6000με (fig.4.3.13C). At 4000με and 10000με, no significant differences between HAS1 expression in the strained cells relative to the control cells was observed (fig.4.3.13C).

HAS2 mRNA expression At passage 1, no significant effects of strain on HAS2 mRNA expression was observed when the cells were subjected to 4000με or 10000με relative to control cells (fig.4.3.14A). At passage 2, significant increases ($P<0.0001$) in relative HAS2 mRNA expression were seen when the cells were strained at the 4000με, 6000με and 10000με (fig.4.3.14B). Strain resulted in a significant increase ($P<0.0001$) in HAS2 mRNA expression in the passage 3 cells subjected to 6000με and 10000με relative to the control cells (fig.4.3.13C). When 4000με was applied to the cells no significant difference was observed in HAS2 mRNA expression between the strained and control cells (fig.4.3.13C).

### 4.3.3 Summary: Normal Patient Response to Mechanical Strain

| | 4000με | | | 6000με | | | 10000με | | |
|---|---|---|---|---|---|---|---|---|---|
| | HAS 1 | HAS 2 | HAS 3 | HAS 1 | HAS 2 | HAS 3 | HAS 1 | HAS 2 | HAS 3 |
| Passage1 | ↓ | ↓ | – | – | – | ↑ | – | – | – |
| Passage2 | – | – | – | – | ↑ | – | – | ↑ | ↓ |
| Passage3 | – | – | ↑ | – | – | – | – | – | ↓ |

**Table.4.3.1 Expression of HAS mRNA in normal synovial cells.**

When passage 1 cells were subjected to 4000με HAS1 and HAS2 mRNA expression could be seen to significantly decrease ($P<0.01$) relative to the control cells. At

passage 3, the effect of 4000με on HAS3 mRNA expression resulted in a significant increase in expression ($P$<0.001) relative to the control cells.

Passage 1 cells showed a significant increase in HAS3 mRNA expression relative to control cells when 6000με was applied. The effect of strain on passage 2 cells was a significant increase ($P$<0.0001) in HAS2 mRNA expression in the strained cells relative to the control cells.

The cells at passage 2, when subjected to 10000με showed a significant increase ($P$<0.001) in HAS2 mRNA expression as a result of strain whereas a significant decrease ($P$<0.001) was observed in HAS3 mRNA expression relative to control cells. The effect of strain on HAS3 mRNA expression in the passage 3 cells was a significant decrease ($P$<0.001) in expression relative to control cells.

### 4.3.4 Summary: OA Patient Response to Mechanical Strain

| | 4000με | | | 6000με | | | 10000με | | |
|---|---|---|---|---|---|---|---|---|---|
| | HAS 1 | HAS 2 | HAS 3 | HAS 1 | HAS 2 | HAS 3 | HAS 1 | HAS 2 | HAS 3 |
| Passage1 | – | – | – | ↑ | ↓ ↑ | ↑ | ↓ | – | – |
| Passage2 | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | – | ↑ ↓ | – |
| Passage3 | ↑ | ↑ | ↑ | ↑ | ↑ | – | ↑ | ↑ | – |

**Table 4.3.2 Expression of HAS mRNA in OA synovial cells.**

Cells at passage 2 and passage 3 showed significant increases ($P$<0.001) in HAS1, HAS2 and HAS3 mRNA expression in strained cells relative to control cells.

Passage 1 cells when subjected to 6000με showed a significant increase ($P$<0.001) in HAS1 mRNA expression relative to the control cells. The effect of strain on HAS2 and HAS3 mRNA expression was a significant increase ($P$<0.001) relative to the control cells, yet one patient showed a significant decrease in HAS2 mRNA expression. Passage 2 cells showed significant increases in HAS1, HAS2 and HAS3

mRNA expression in cells that underwent strain relative to control cells. Passage 3 cells showed a significant increase ($P<0.001$) in both HAS1 and HAS2 expression when they were subjected to strain relative to the control cells HAS1 and HAS2 expression and there was no detectable change in HAS3 expression.

Passage 1 cells showed a significant decrease ($P<0.001$) in HAS1 mRNA expression relative to control cells when they were subjected to 10000$\mu\varepsilon$ whilst HAS2 and HAS3 remained unchanged. The effect of strain on passage 2 cells was a significant increase in HAS2 mRNA expression in the strained cells relative to the control cells in one patient whereas another patient showed a significant decrease ($P<0.0001$). Passage 3 cells, when strained, showed a significant increase ($P<0.001$) in HAS1 mRNA expression whereas HAS2 mRNA expression showed both a significant increase and a significant decrease in two different patients relative to the control cells.

### 4.3.4 Changes in HAS mRNA Expression in OA Synovial Cells Relative to Normal Synovial Cells

**HAS mRNA expression in unstrained normal and OA synovial cells**

HAS1 mRNA expression in passage 1 OA cells showed significant decreases ($P<0.05$) relative to normal cell HAS1 mRNA expression (fig.4.3.15A & C). At passage 2 and passage 3, the OA cells showed significant increases ($P<0.1$) in their HAS1 mRNA expression relative to the normal control cells (fig.4.3.15B & D).

At passage 1, OA cells HAS2 expression was variable compared with normal samples (fig.4.3.16). Patient OA2 exhibited increased ($P<0.01$) HAS2 expression (fig.4.3.16A) whereas Patients OA3, OA4 and OA6 had decreased ($P<0.05$) HAS2 mRNA expression (fig.16.3.16B, C & D). All OA samples at passage 2 and 3 exhibited significantly higher ($P<0.001$) HAS2 mRNA expression compared to controls (fig.4.3.15B, C & D).

HAS3 mRNA expression in passage 1 OA cells was significantly lower ($P<0.05$) than the normal cells (fig.4.3.17). In passage 2 and 3 cells, HAS3 mRNA expression was not significantly different in the OA cells relative to the normal cells (fig.4.3.17).

**HAS mRNA expression in normal and OA synovial cells at 4000με**

Passage 2 cells showed significant increases ($P<0.01$) in HAS1 mRNA expression in the OA cells relative to the normal cells (fig.4.3.18B & C.). However, passage 1 and passage 3 cells showed no significant differences in HAS1 mRNA expression when the cells were subjected to strain.

A significant decrease ($P<0.1$) in HAS2 mRNA expression in OA cells relative to normal cells was apparent in passage 1 cells (fig.4.3.19B). Passage 2 OA cells showed a significant decrease ($P<0.01$) in HAS2 mRNA expression relative to the normal cells in one patient yet a significant increase ($P<0.01$) could be seen in another patient (fig.4.3.19B & C). All three OA patients at passage 3 showed significant increases ($P<0.001$) in HAS2 expression relative to normal cells (fig.4.3.19).

HAS3 mRNA expression in OA cells was not significantly different to the normal cells HAS3 mRNA expression in passage 1, 2 or 3 cells when they were subjected to the three magnitudes of strain (fig.4.3.20).

**HAS mRNA expression in normal and OA synovial cells at 6000με**

In passage 1 OA cells there was a significant increase in HAS1 mRNA expression relative to the normal cells HAS1 mRNA expression (fig.4.3.21D.). In passage 2 OA cells, no differences in HAS1 mRNA expression were observed relative to the normal cells (fig.4.3.21). At passage 3, the cells showed a significant increase ($P<0.01$) in HAS1 mRNA expression relative to the normal cells (fig.4.3.21D).

HAS2 mRNA expression in passage 1 OA cells showed significant increases ($P<0.001$) in two of the patients relative to the normal cells (fig.4.3.22A & C). Significant decreases ($P<0.0001$) in HAS2 mRNA expression were seen in OA passage 2 strained cells relative to the normal strained cells whereas significant increases ($P<0.0001$) were observed in passage 3 OA cells (fig.4.3.22B, C & D).

A significant increase ($P<0.05$) in HAS3 mRNA expression in strained OA cells relative to strained normal cells was observed in passage 1 and passage 3 cells whereas significant decrease ($P<0.01$) in HAS3 mRNA expression could be seen in the passage 2 OA cells relative to the normal cells (fig.4.3.23).

**HAS mRNA expression in normal and OA synovial cells at 10000με**

HAS1 mRNA expression in passage 1 and passage 3 OA cells decreased significantly ($P<0.0001$) relative to normal strained cells in all patients sampled (fig.4.3.24.). At passage 2, OA cells showed no significant differences in HAS1 mRNA expression relative to normal cells (fig.4.3.24).

There was no differences in HAS2 mRNA expression in strained OA cells relative to strained normal cells at passage 1 (fig.4.3.25). At passage 2 and passage 3, HAS2 mRNA expression in OA cells was significantly increased ($P<0.01$) relative to normal cells HAS2 mRNA expression (fig.4.3.25B & C).

The effect of strain on passage 1 OA cells was a significant decrease ($P<0.05$) in HAS3 mRNA expression relative to normal cells at passage 1 (fig.4.3.26). In passage 2 cells, OA cells showed significantly increased ($P<0.01$) HAS3 mRNA expression in the strained cells relative to the normal cells (fig.4.3.26). HAS3 mRNA expression was no different in the OA strained cells relative to the normal strained cells.

**Summary**

In summary, the majority of OA synovial cells subjected to the 3 magnitudes of strain showed an increase in HAS1, HAS2 and HAS3 mRNA expression. It was 10000με which produced the most variable results in HAS mRNA expression between passage number and magnitude of strain. In normal synovial cells, mechanical strain both induced and decreased HAS mRNA expression at 4000με and 10000με while increases in HAS mRNA expression were observed at 6000με. The expression of HAS mRNA was different between OA and normal synovial cells at each of the 3 strains with observed changes in expression of HAS1, HAS2 and HAS3 mRNA occurring more frequently in the OA synovial cells compared to the normal synovial cells.

**Figure 4.3.1** Changes in HAS mRNA expression from a representativ passage 3 synovial fibroblast cell culture. Cells were subjected to 10 minute and RT-PCR performed 24 hours post-strain as described in materials and HAS1 and HAS2 mRNA is expressed in strained and control cells at 4000με and 10000με. HAS3 mRNA was not expressed in the strained or contro 4000με, 6000με and 10000με.

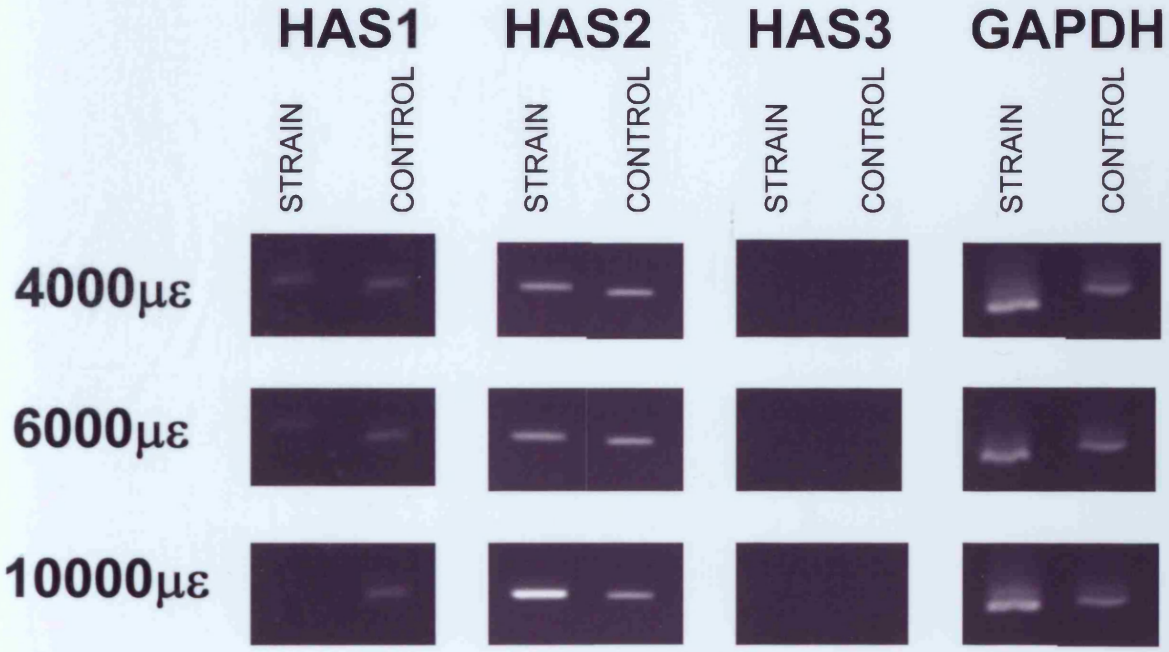

HAS1
HAS2
HAS3
GAPDH
STRAIN
CONTROL
STRAIN
CONTROL
STRAIN
CONTROL
STRAIN
CONTROL
4000με
6000με
10000με

**Figure 4.3.2** Changes in HAS mRNA expression from a representative passage 3 OA synovial fibroblast cell culture. Cells were subjected to 10 minutes of strain and RT-PCR performed 24 hours post-strain as described in materials and methods. HAS1 and HAS2 mRNA is expressed in strained and control cells at 4000με, 6000με and 10000με. Control and strained cells were positive for HAS3 mRNA at 4000με and 6000με whereas control and strained cells at 10000με were negative for HAS3 expression. Typical results from one of the patients sampled.

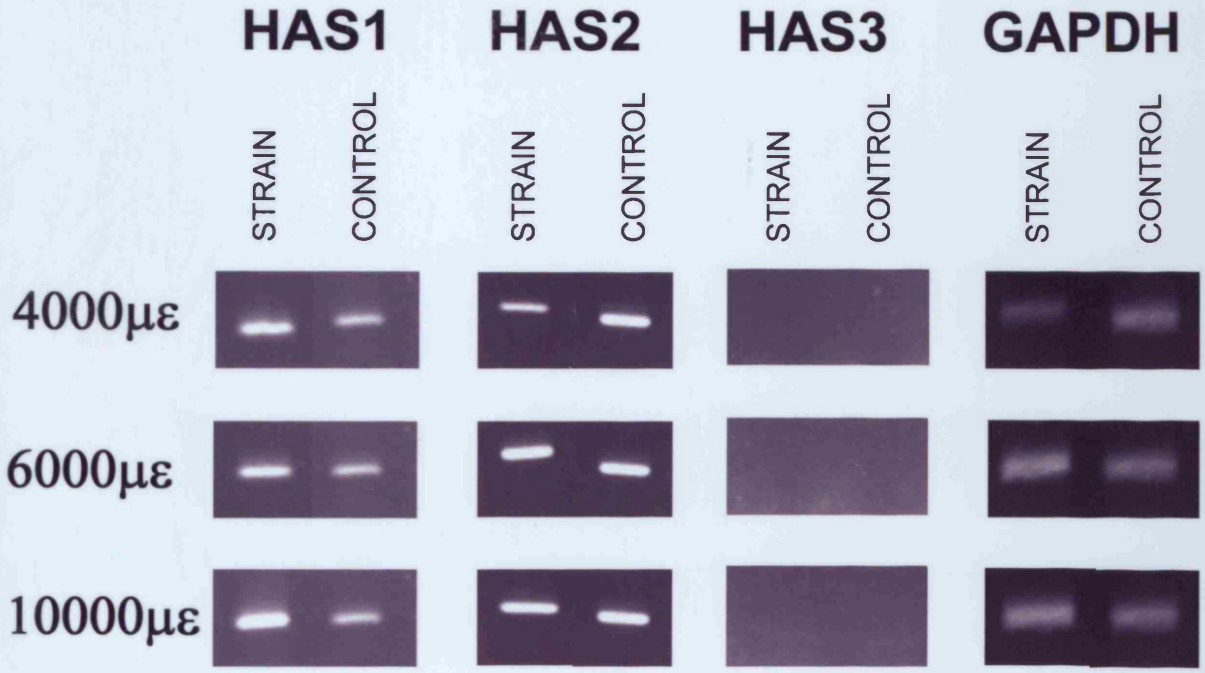

HAS1
HAS2
HAS3
GAPDH
STRAIN
CONTROL
STRAIN
CONTROL
STRAIN
CONTROL
STRAIN
CONTROL
4000με
6000με
10000με

**Figure 4.3.3** HAS1 mRNA expression in normal synovial cells from Patient
RNA was purified from cells that had been subjected to mechanical strain fo
minutes and from control cells, 24 hours post-treatment. Strained cell HAS1 m
expression has been normalised to control cell HAS1 mRNA expression as desc
in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage 3
*$P<0.01$ relative to control cells.

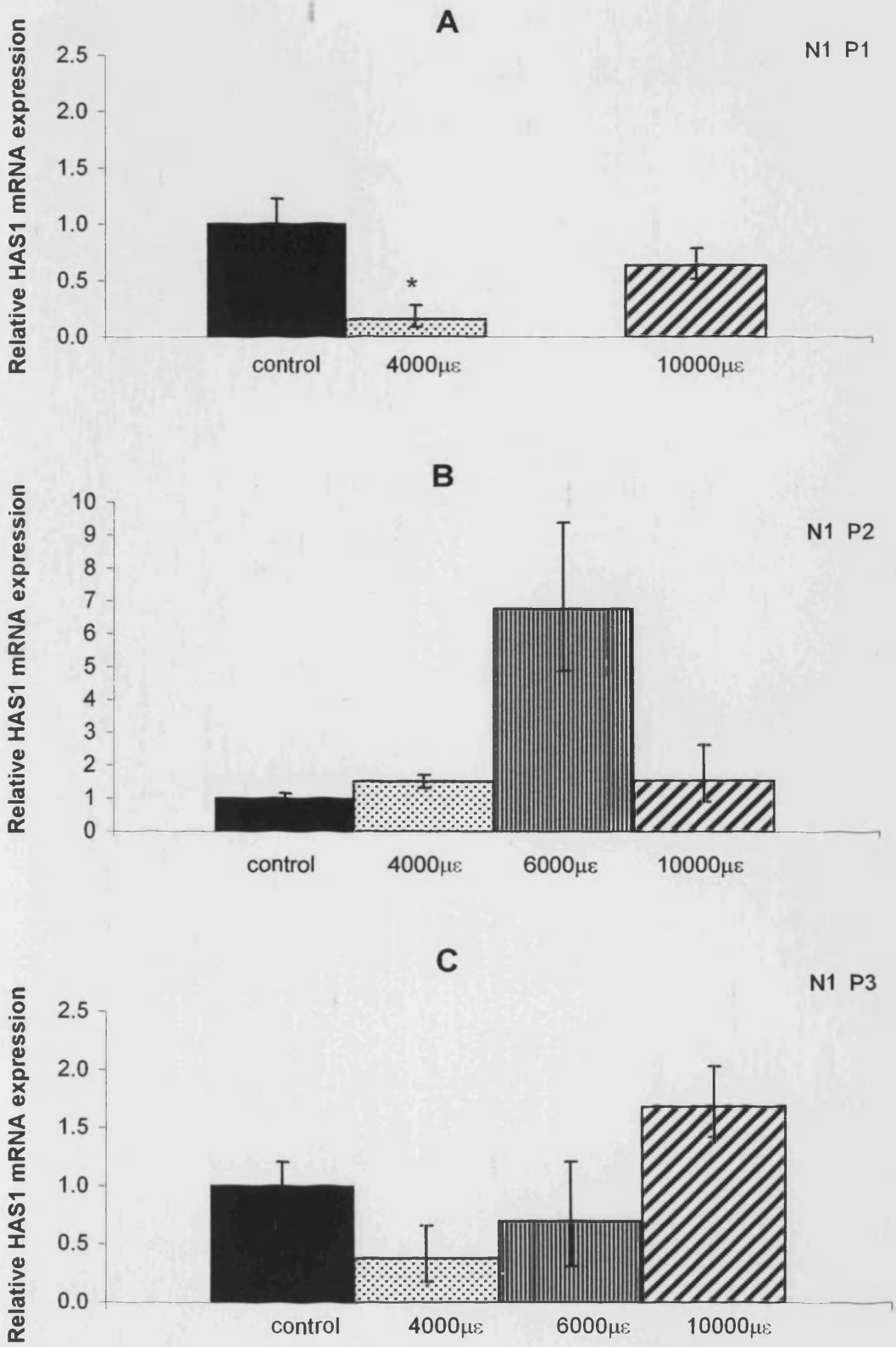
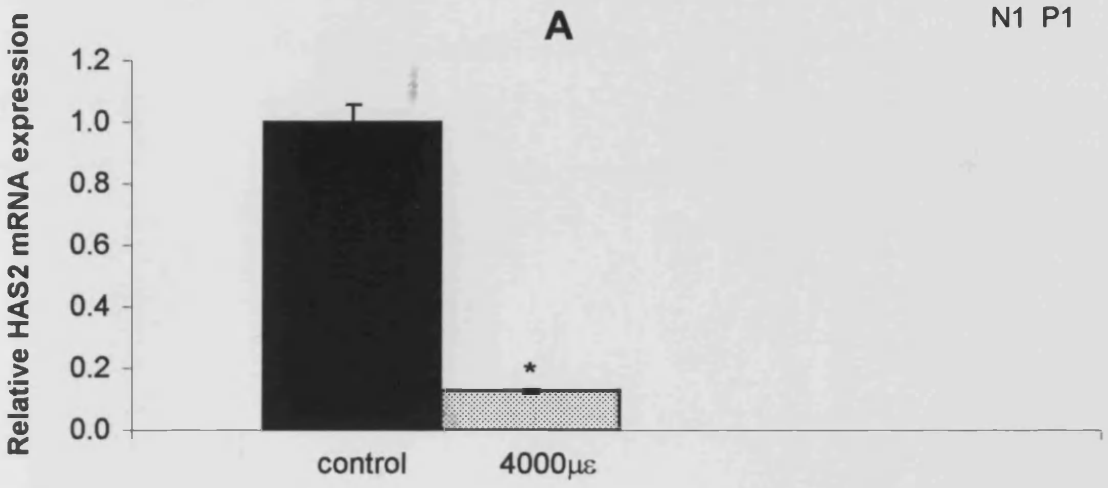
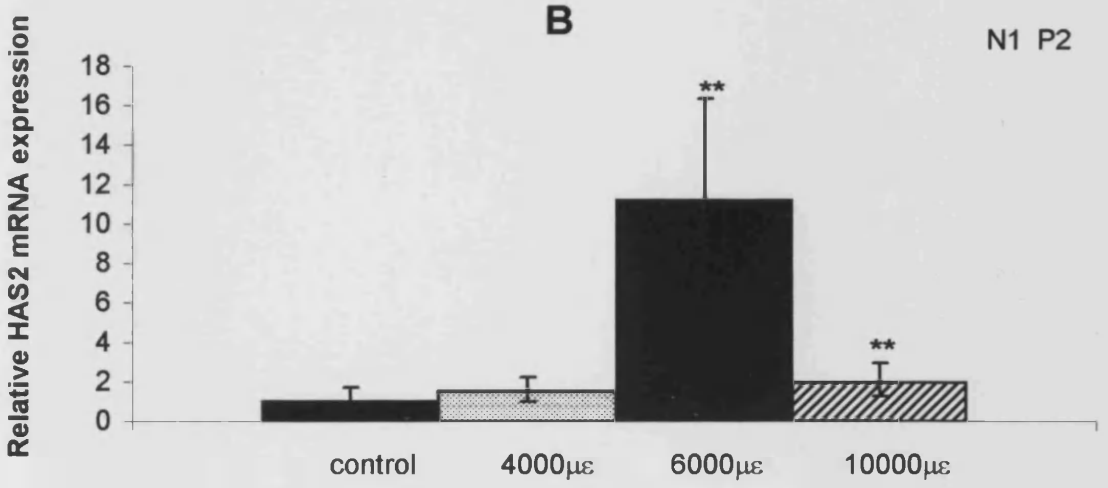

A
N1 P1
Relative HAS1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
control
4000με
10000με
B
N1 P2
Relative HAS1 mRNA expression
10
9
8
7
6
5
4
3
2
1
0
control
4000με
6000με
10000με
C
N1 P3
Relative HAS1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
control
4000με
6000με
10000με

**Figure 4.3.4** HAS2 mRNA expression in normal synovial cells from Patien
RNA was purified from cells that had been subjected to mechanical strain f
minutes and from control cells, 24 hours post-treatment. Strained cell HAS2 m
expression has been normalised to control cell HAS2 mRNA expression as dese
in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage 3
*$P$<0.001 relative to control cells. **$P$<0.0001 relative to control cells.


A
N1 P1
Relative HAS2 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
control
4000με


B
N1 P2
Relative HAS2 mRNA expression
18
16
14
12
10
8
6
4
2
0
**
**
control
4000με
6000με
10000με

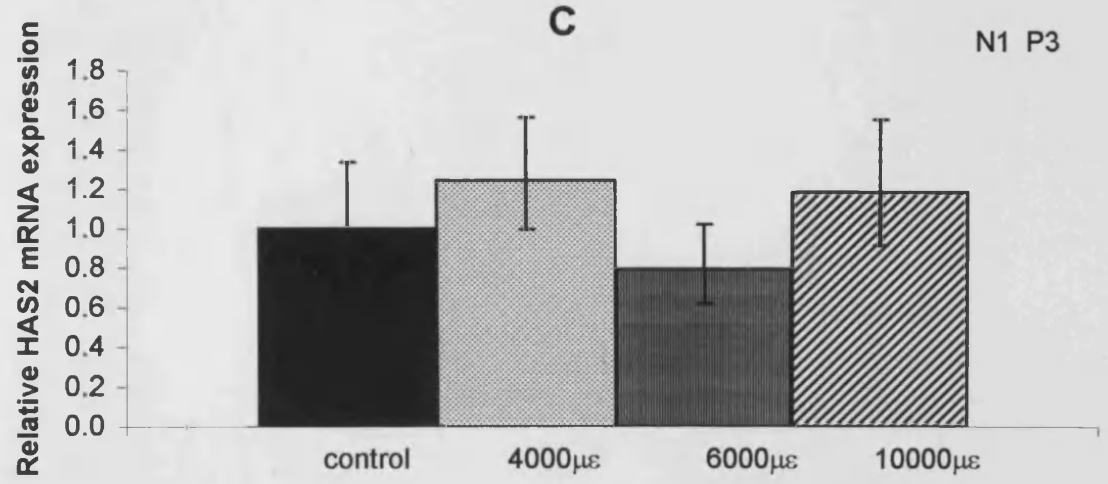
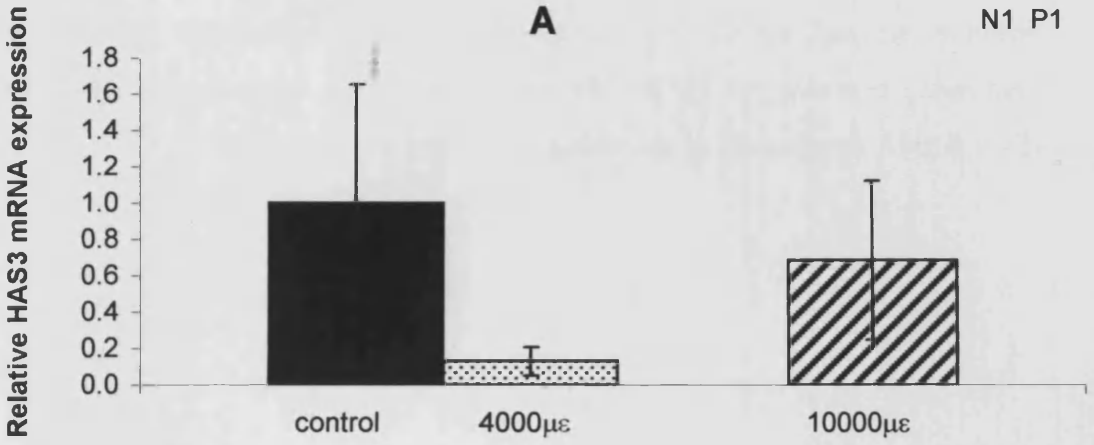
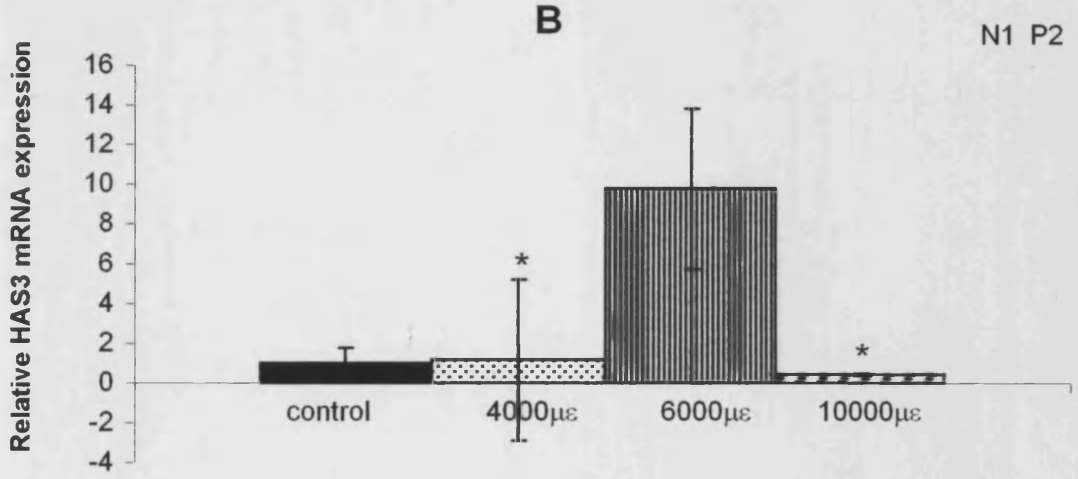

C
N1 P3
Relative HAS2 mRNA expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control
4000με
6000με
10000με

**Figure 4.3.5** HAS3 mRNA expression in normal synovial cells from Pati
RNA was purified from cells that had been subjected to mechanical strain
minutes and from control cells, 24 hours post-treatment. Strained cell HAS3
expression has been normalised to control cell HAS3 mRNA expression as de
in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage
*$P$<0.01 relative to control cells. **$P$<0.001 relative to control cells.


A
N1 P1
Relative HAS3 mRNA expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control
4000με
10000με


B
N1 P2
Relative HAS3 mRNA expression
16
14
12
10
8
6
4
2
0
-2
-4
*
*
control
4000με
6000με
10000με

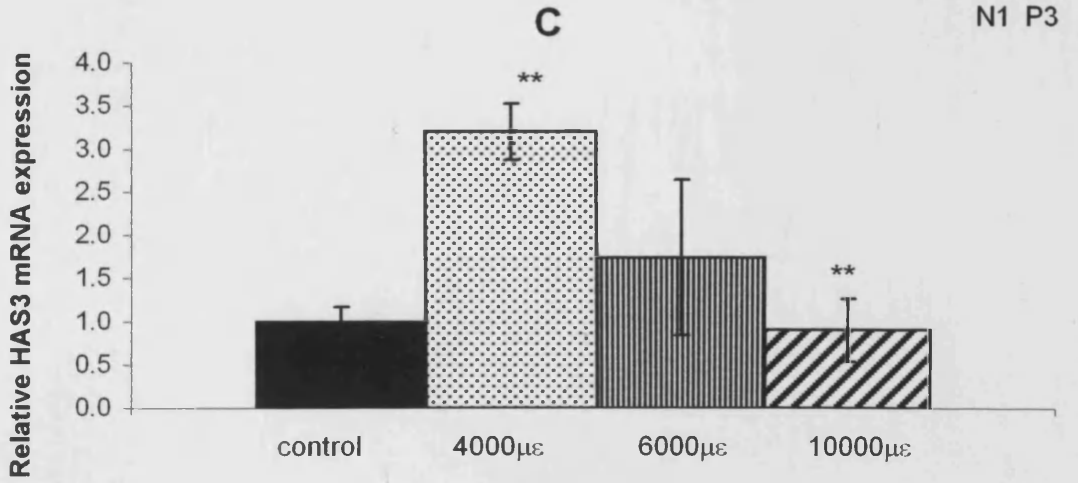
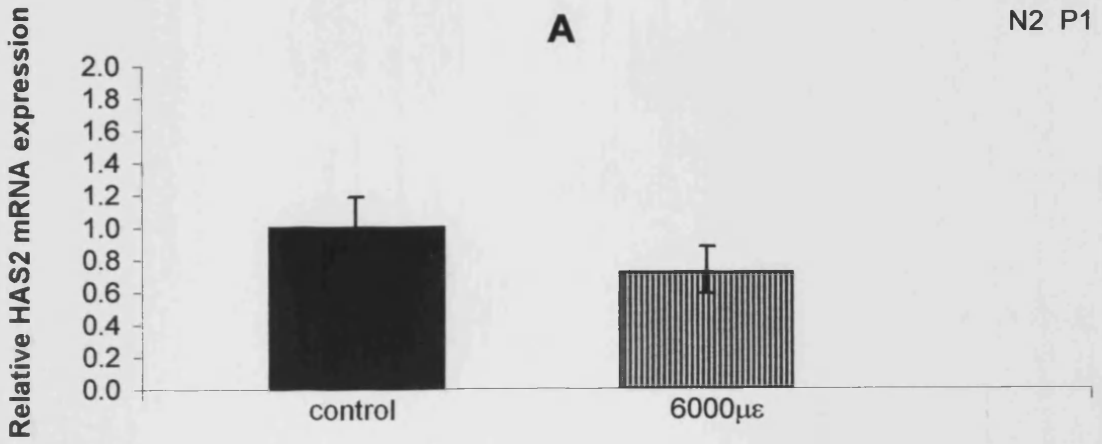

C
N1 P3
Relative HAS3 mRNA expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
control
4000με
6000με
10000με

**Figure 4.3.6** A) HAS2 mRNA expression and B) HAS3 expression in normal pa 1 synovial cells from Patient N2. RNA was purified from cells that had subjected to mechanical strain for 10 minutes and from control cells, 24 hours treatment. Strained cell HAS mRNA expression has been normalised to control HAS mRNA expression as described in materials and methods.


A
N2 P1
Relative HAS2 mRNA expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control
6000με

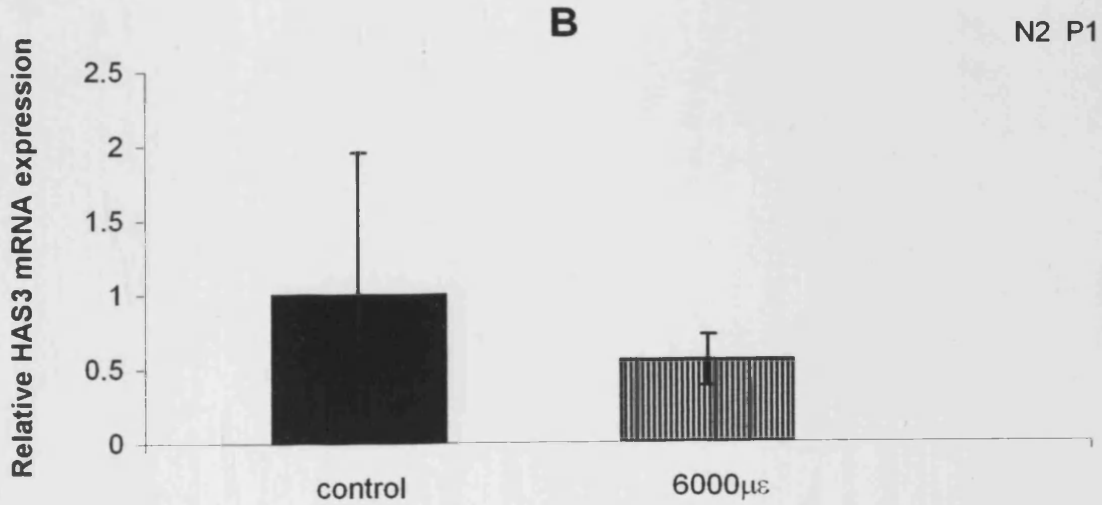
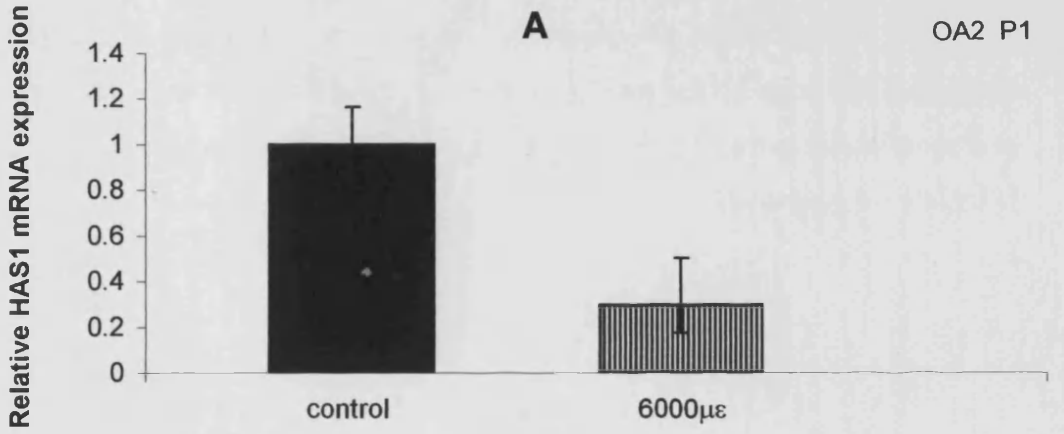
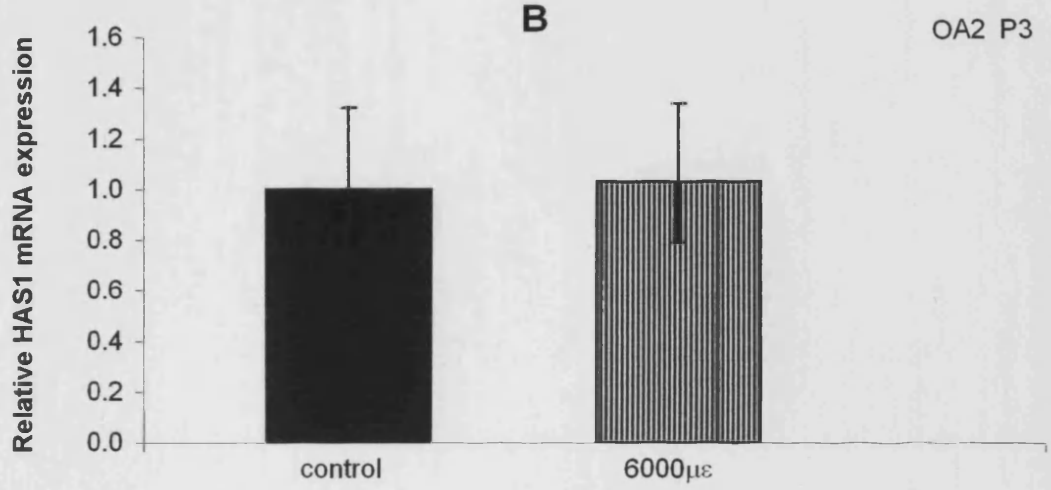

B
N2 P1
Relative HAS3 mRNA expression
2.5
2
1.5
1
0.5
0
control
6000με

**Figure 4.3.7** A) HAS1 mRNA expression in passage 1 in OA synovial cells from Patient OA2; B) HAS1 expression in passage 3 cells from Patient OA2; C) HAS2 expression in passage 1 cells from OA2. RNA was purified from cells that had been subjected to mechanical strain for 10 minutes and from control cells, 24 hours post-treatment. Strained cell HAS mRNA expression has been normalised to control cell HAS mRNA expression as described in materials and methods. *$P<0.001$ relative to control cells.


A
OA2 P1
Relative HAS1 mRNA expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
6000με


B
OA2 P3
Relative HAS1 mRNA expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control
6000με

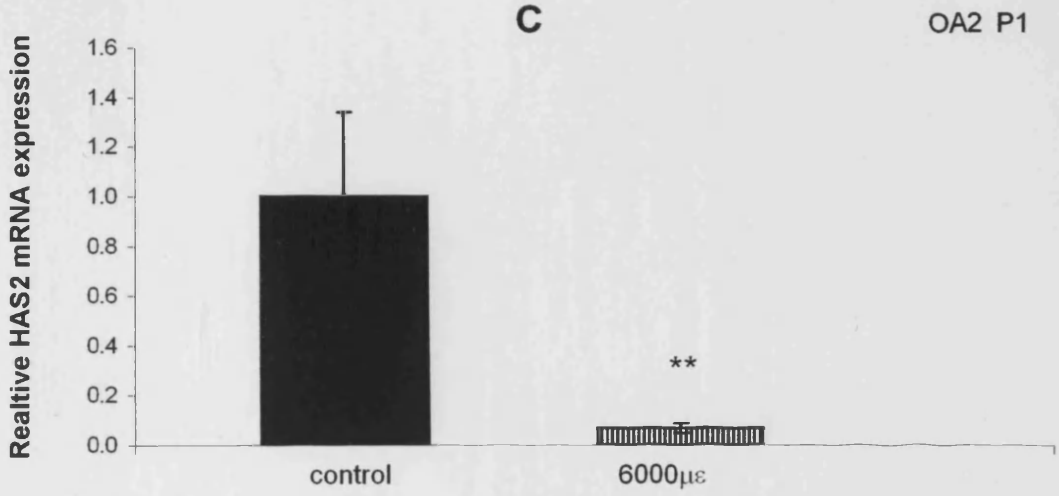
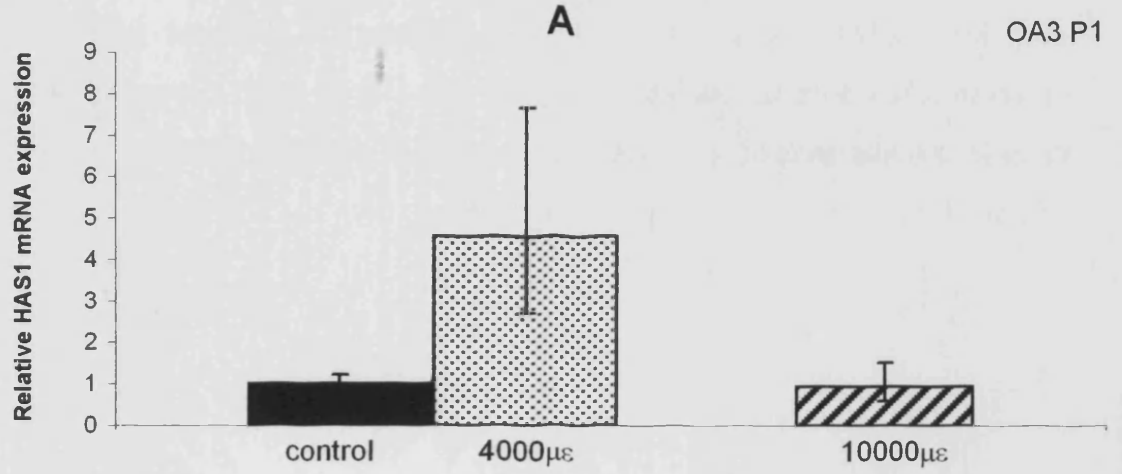
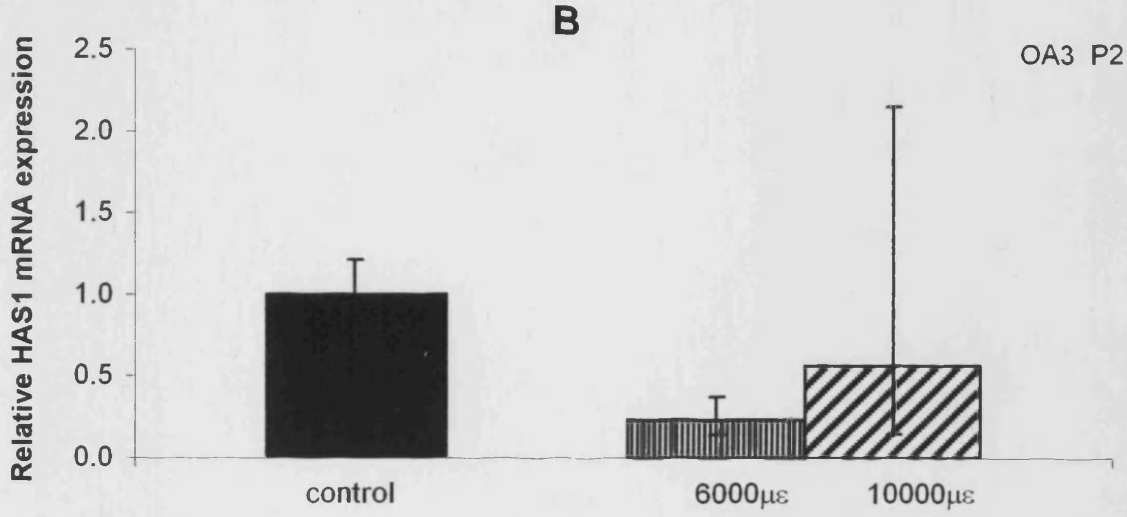

C
OA2 P1
Realtive HAS2 mRNA expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
control
6000με

**Figure 4.3.8** HAS1 mRNA expression in OA synovial cells from Patient OA3. RNA was purified from cells that had been subjected to mechanical strain for 10 minutes and from control cells, 24 hours post-treatment. Strained cell HAS1 mRNA expression has been normalised to control cell HAS1 mRNA expression as described in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.001$ relative to control cells.


A
OA3 P1
Relative HAS1 mRNA expression
9
8
7
6
5
4
3
2
1
0
control
4000με
10000με


B
OA3 P2
Relative HAS1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
control
6000με
10000με

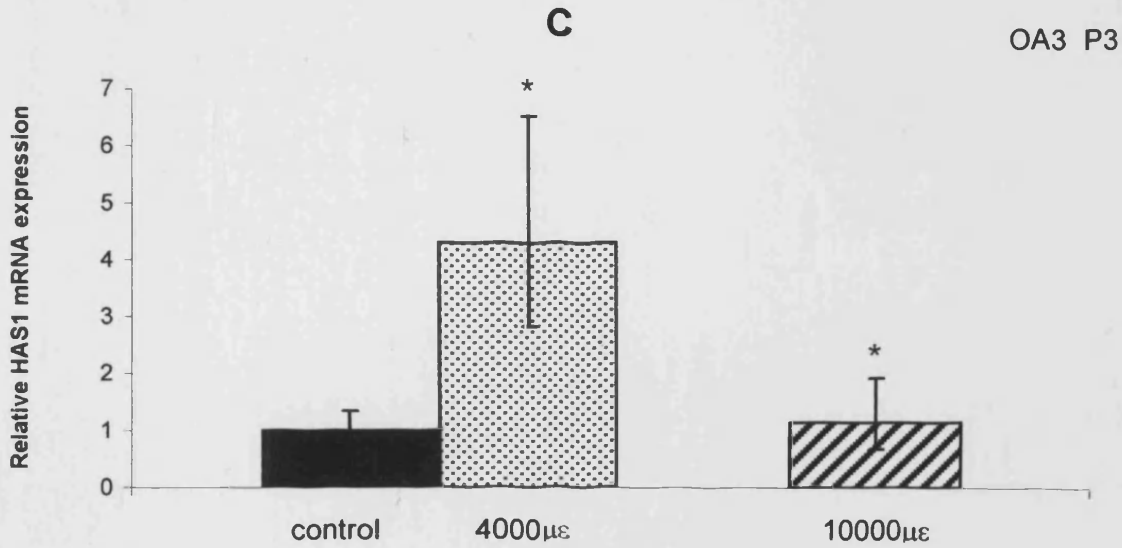
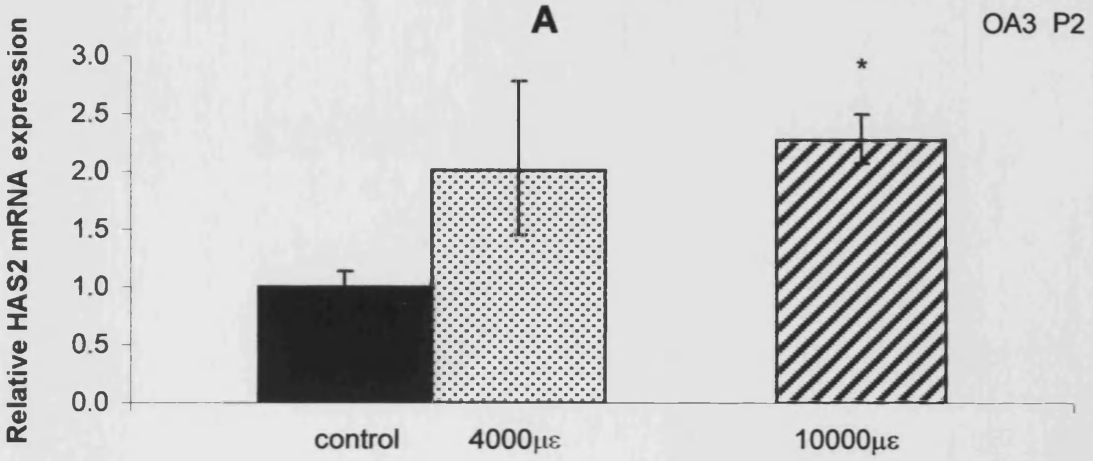

C
OA3 P3
Relative HAS1 mRNA expression
7
6
5
4
3
2
1
0
*
*
control
4000με
10000με

**Figure 4.3.9** HAS2 mRNA expression in OA synovial cells from Patient OA3 was purified from cells that had been subjected to mechanical strain for 10 and from control cells, 24 hours post-treatment. Strained cell HAS2 expression has been normalised to control cell HAS2 mRNA expression as d in materials and methods. A) passage 2 cells; B) passage 3 cells. *$P<0.01$ re control cells.


A
OA3 P2
Relative HAS2 mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
control
4000με
10000με

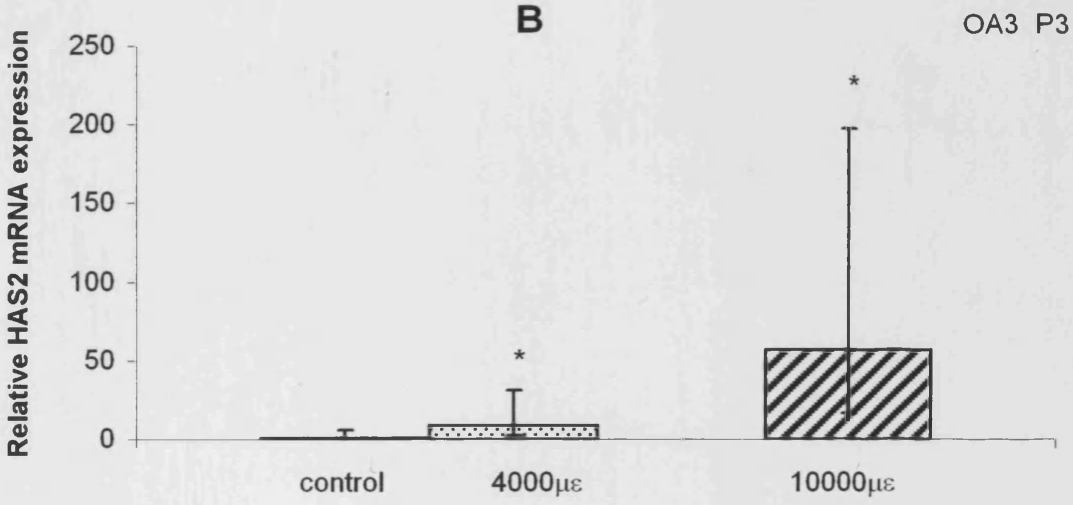
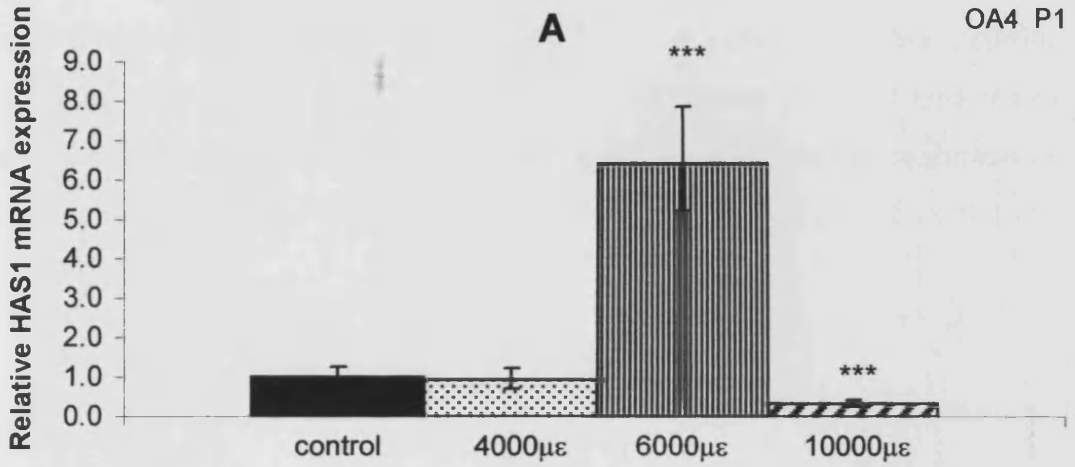
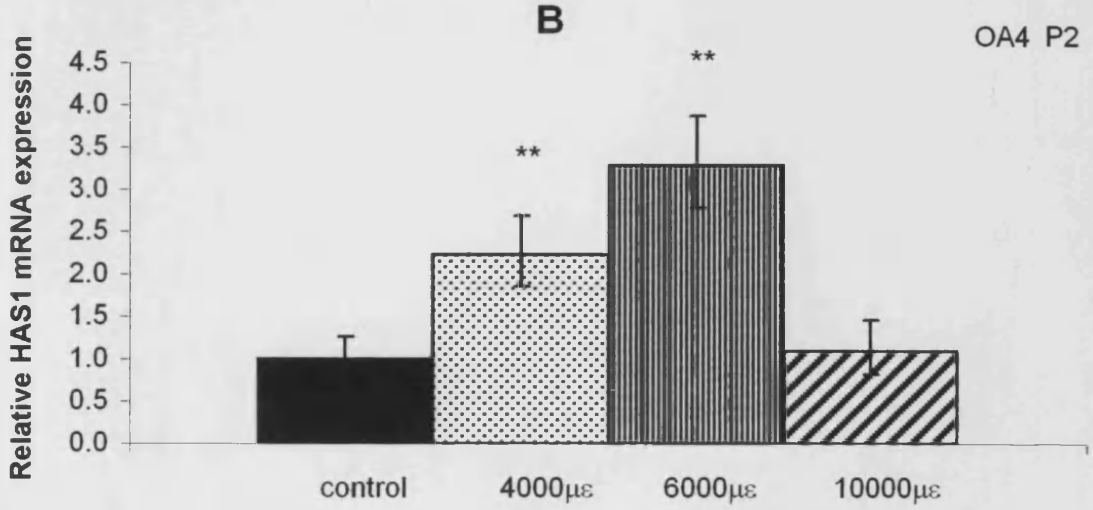

B
OA3 P3
Relative HAS2 mRNA expression
250
200
150
100
50
0
*
*
control
4000με
10000με

**Figure 4.3.10** HAS1 mRNA expression in OA synovial cells from Patient C. RNA was purified from cells that had been subjected to mechanical strain for minutes and from control cells, 24 hours post-treatment. Strained cell HAS1 mRNA expression has been normalised to control cell HAS1 mRNA expression as described in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.01$ relative to control cells. **$P<0.001$ relative to control cells.


A
OA4 P1
Relative HAS1 mRNA expression
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***
***
control
4000με
6000με
10000με


B
OA4 P2
Relative HAS1 mRNA expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
control
4000με
6000με
10000με

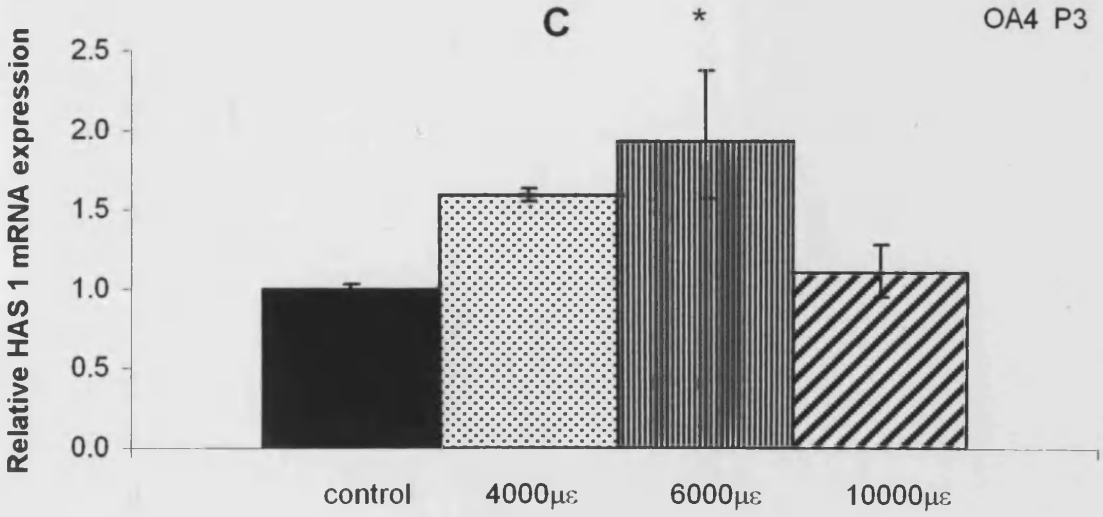
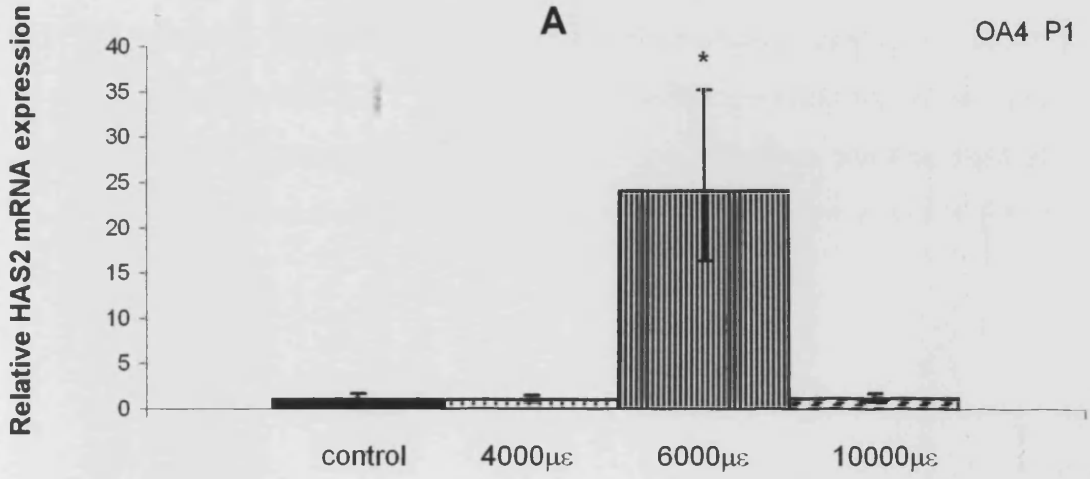
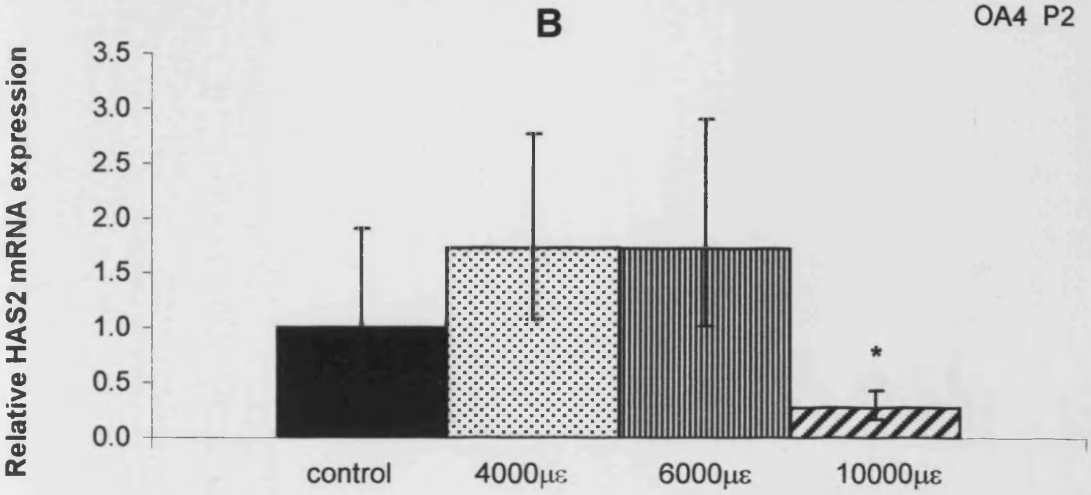

C
OA4 P3
Relative HAS 1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
control
4000με
6000με
10000με

**Figure 4.3.11** HAS2 mRNA expression in OA synovial cells from Patient OA4. RNA was purified from cells that had been subjected to mechanical strain for 10 minutes and from control cells, 24 hours post-treatment. Strained cell HAS2 mRNA expression has been normalised to control cell HAS2 mRNA expression as described in materials and methods. A) passage1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.001$ relative to control cells. **$P<0.0001$ relative to control cells.


A
OA4 P1
Relative HAS2 mRNA expression
40
35
30
25
20
15
10
5
0
*
control
4000με
6000με
10000με


B
OA4 P2
Relative HAS2 mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
control
4000με
6000με
10000με

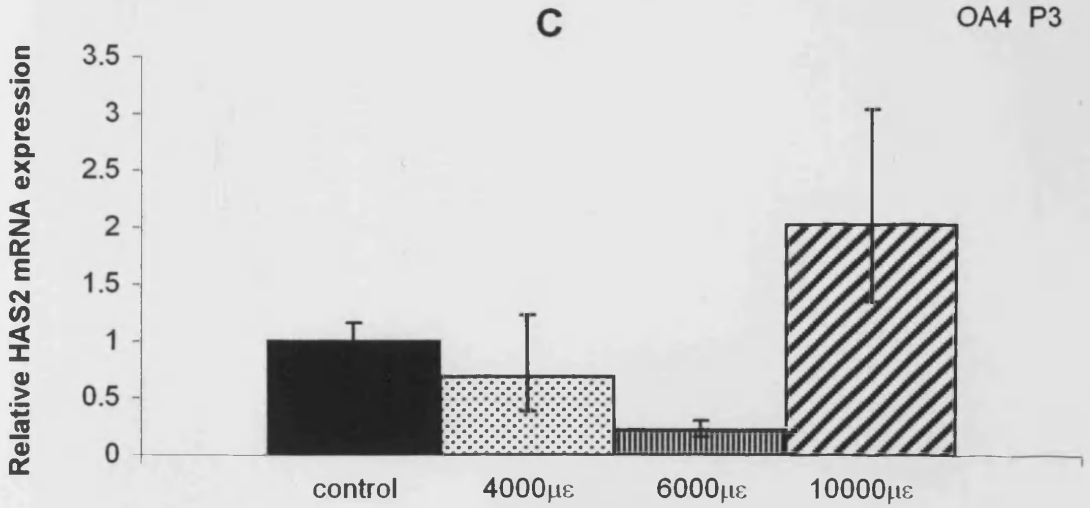
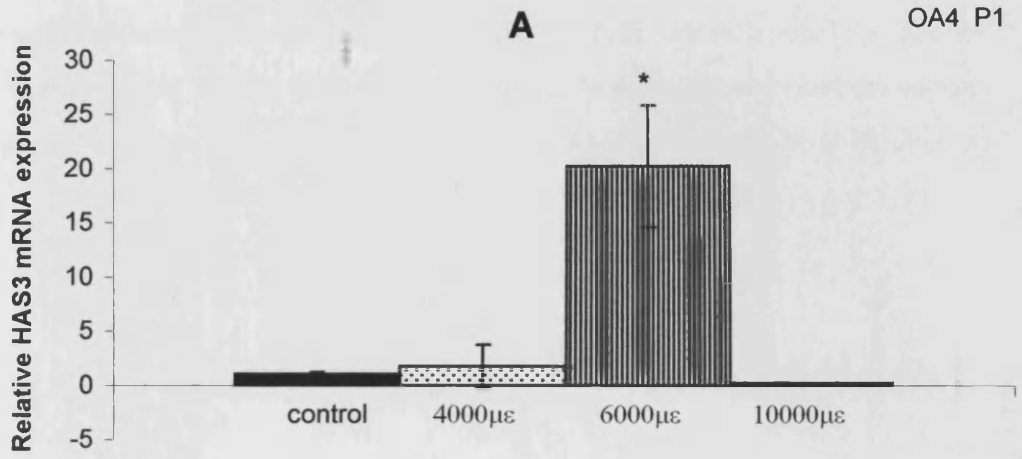
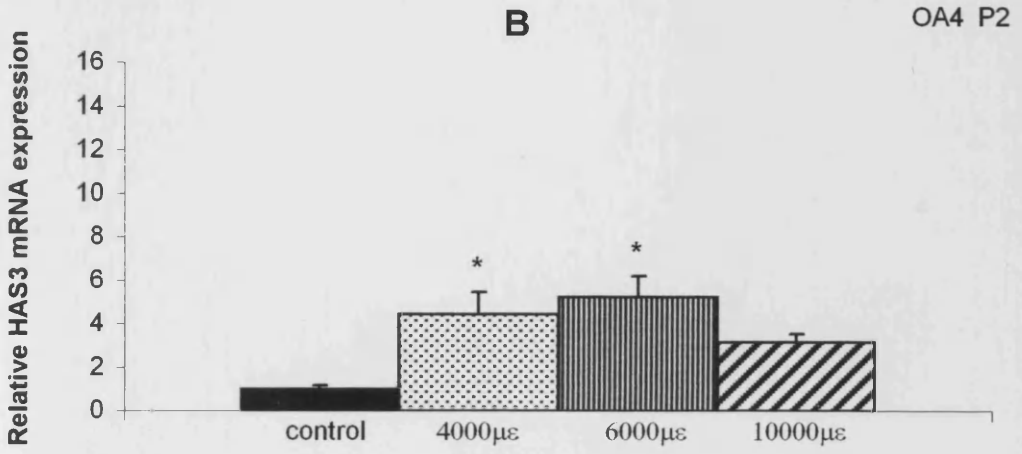

C
OA4 P3
Relative HAS2 mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
control
4000με
6000με
10000με

**Figure 4.3.12** HAS3 mRNA expression in OA synovial cells from Patient OA4. RNA was purified from cells that had been subjected to mechanical strain for 10 minutes and from control cells, 24 hours post-treatment. Strained cell HAS3 mRNA expression has been normalised to control cell HAS3 mRNA expression as described in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.001$ relative to control cells. **$P<0.0001$ relative to control cell.


A
OA4 P1
Relative HAS3 mRNA expression
30
25
20
15
10
5
0
-5
*
control
4000με
6000με
10000με


B
OA4 P2
Relative HAS3 mRNA expression
16
14
12
10
8
6
4
2
0
*
*
control
4000με
6000με
10000με

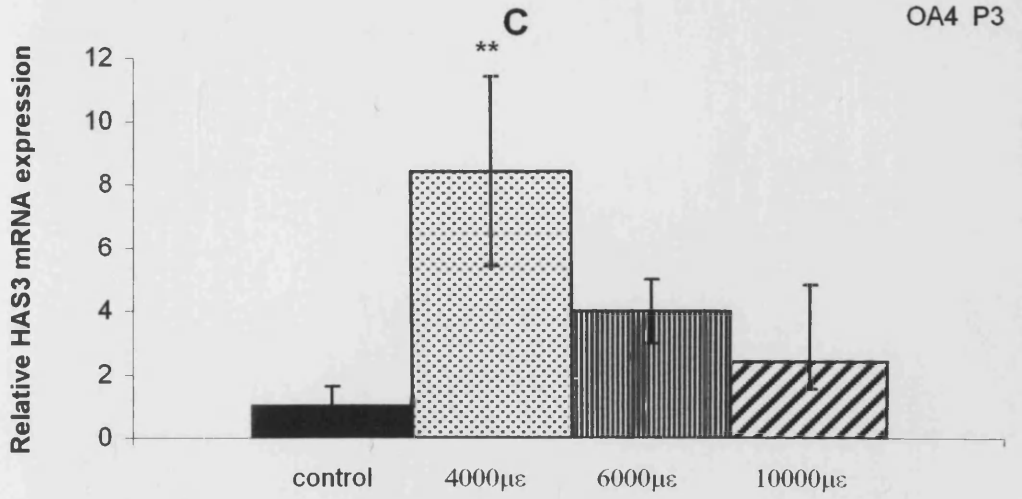
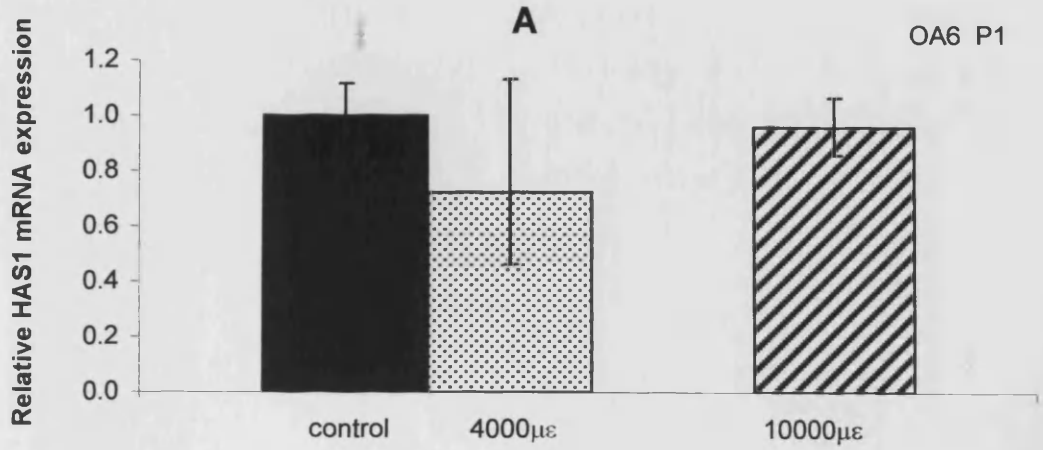
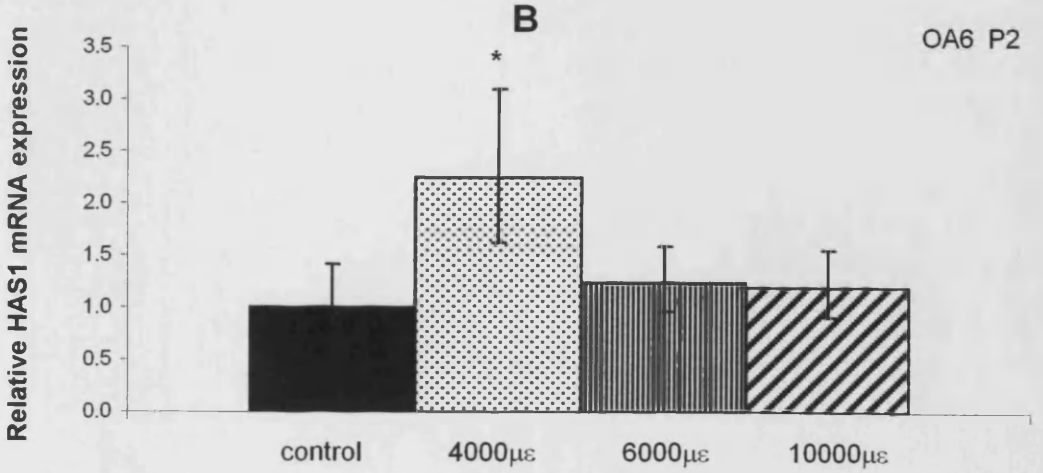

C
OA4 P3
Relative HAS3 mRNA expression
12
10
8
6
4
2
0
**
control
4000με
6000με
10000με

**Figure 4.3.13** HAS1 mRNA expression in OA synovial cells from Patient OA6. RNA was purified from cells that had been subjected to mechanical strain for 10 minutes or from control cells, 24 hours post-treatment. Strained cell HAS1 mRNA expression has been normalised to control cell HAS1 mRNA expression as described in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.01$ relative to control cells. **$P<0.0001$ relative to control cell.


A
OA6 P1
Relative HAS1 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control
4000με
10000με


B
OA6 P2
Relative HAS1 mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
control
4000με
6000με
10000με

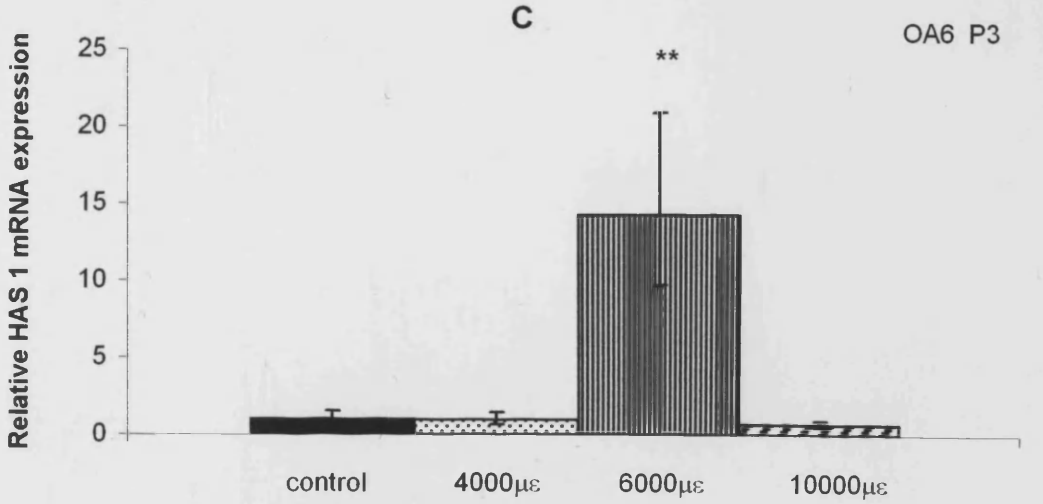
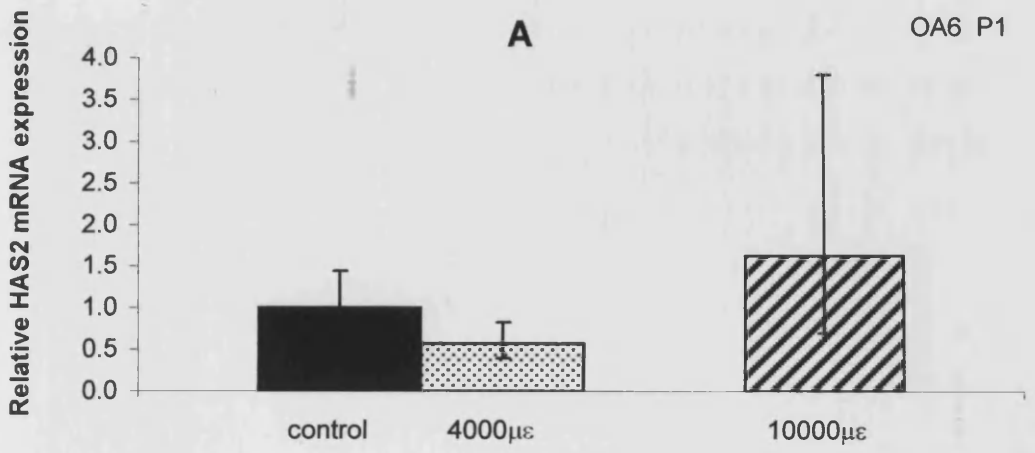
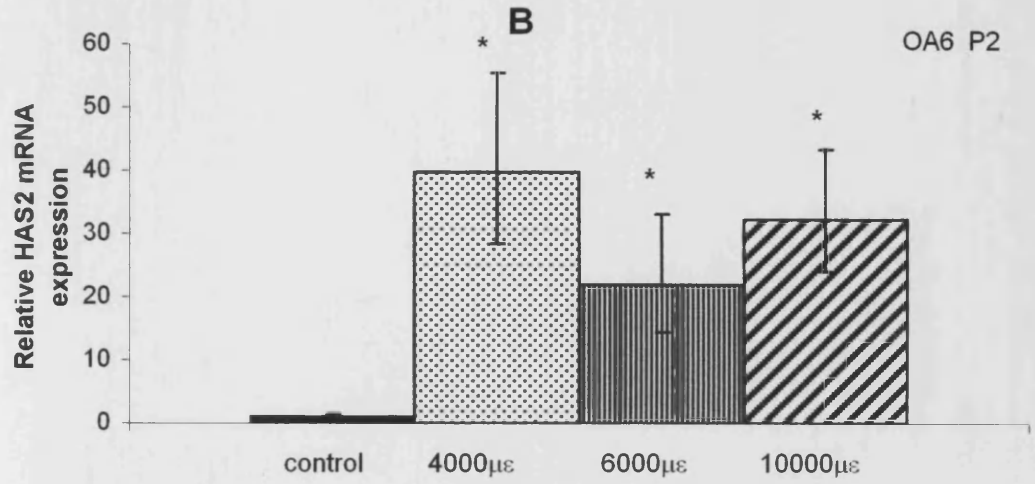

C
OA6 P3
Relative HAS 1 mRNA expression
25
20
15
10
5
0
**
control
4000με
6000με
10000με

**Figure 4.3.14** HAS2 mRNA expression in OA synovial cells from Patient OA6. RNA was purified from cells that had been subjected to mechanical strain for 10 minutes and from control cells, 24 hours post-treatment. Strained cell HAS2 mRNA expression has been normalised to control cell HAS2 mRNA expression as described in materials and methods. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.0001$ relative to control cells.


A
OA6 P1
Relative HAS2 mRNA expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
4000με
10000με


B
OA6 P2
Relative HAS2 mRNA expression
60
50
40
30
20
10
0
*
*
*
control
4000με
6000με
10000με

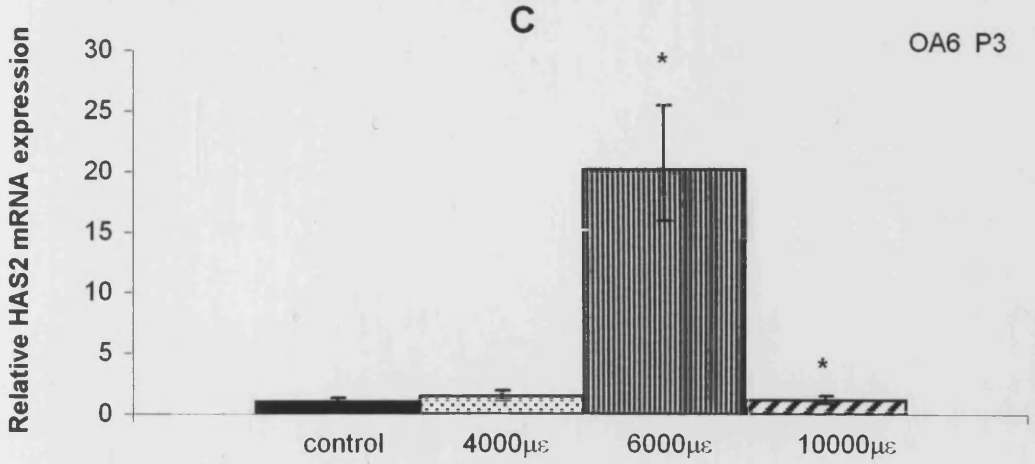

C
OA6 P3
Relative HAS2 mRNA expression
30
25
20
15
10
5
0
*
*
control
4000με
6000με
10000με

**Figure 4.3.15** HAS1 mRNA expression from A) Patient OA2; B) Patient OA3; C) Patient OA4; D) Patient OA6. RNA was purified from control cells 24 hours post-treatment. OA control cell HAS1 mRNA expression was normalised to normal control cell HAS1 mRNA expression as described in materials and methods. *$P<0.05$ relative to normal strained cells.

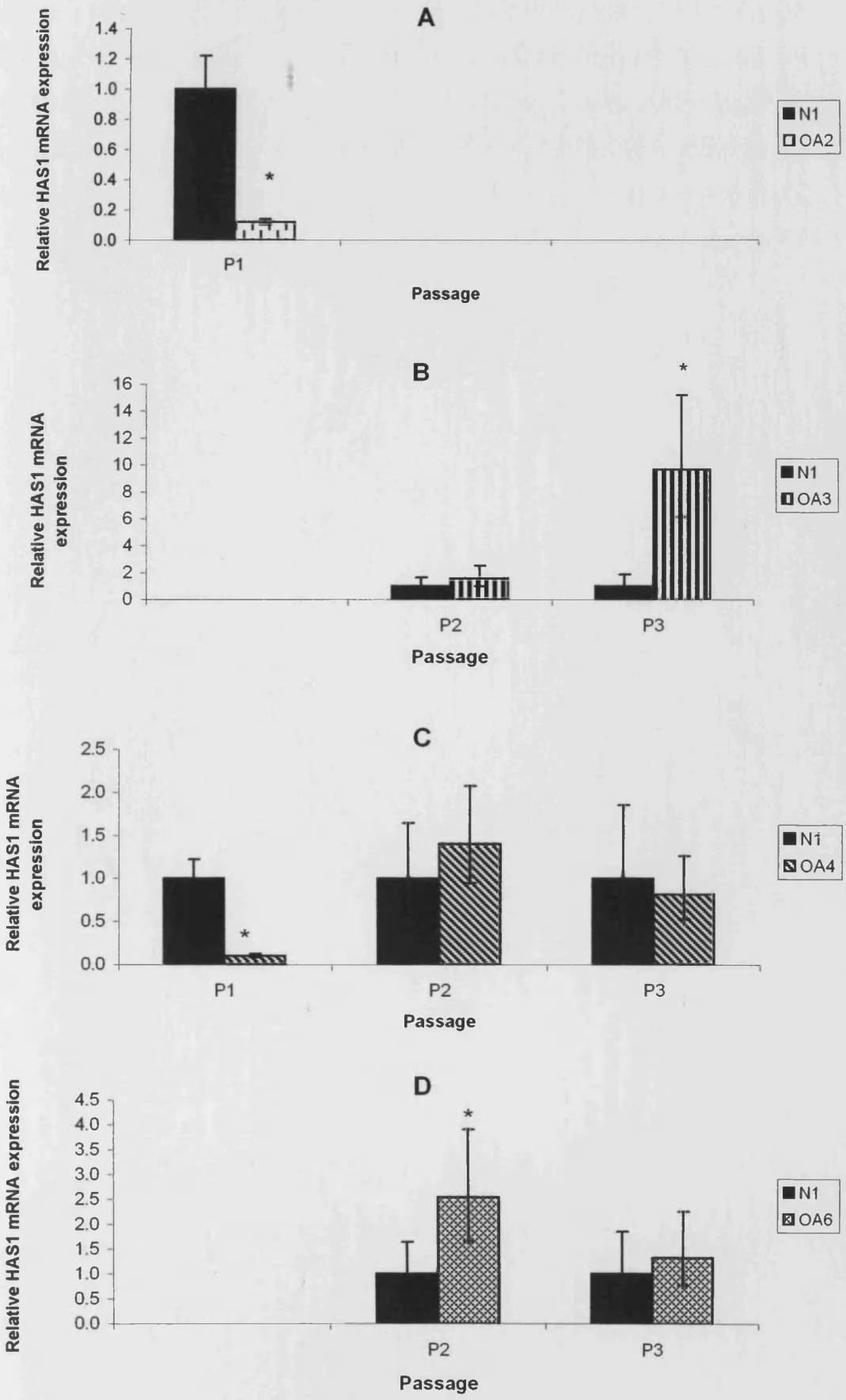
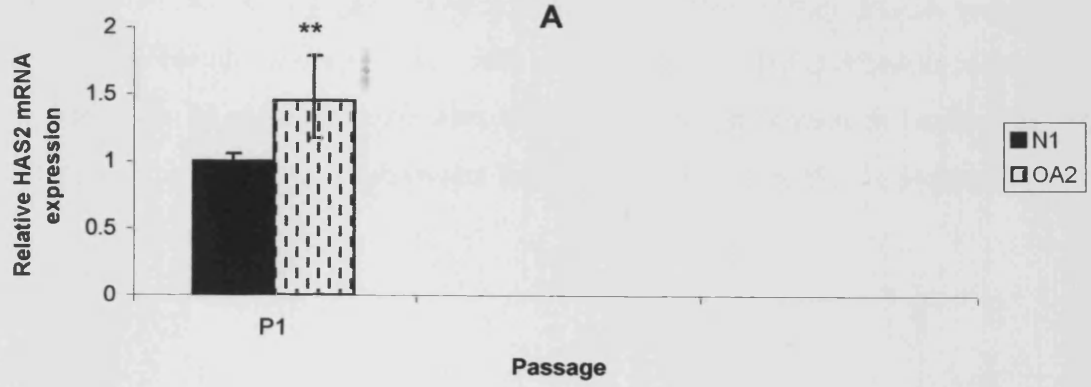
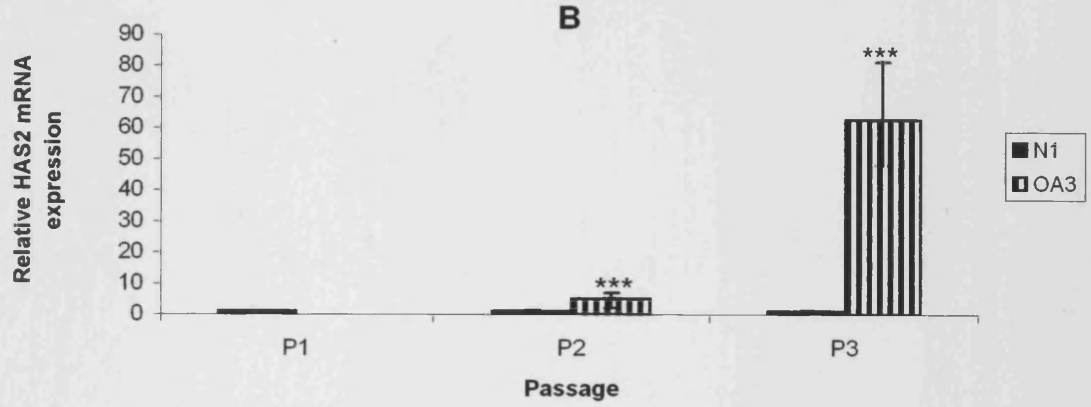
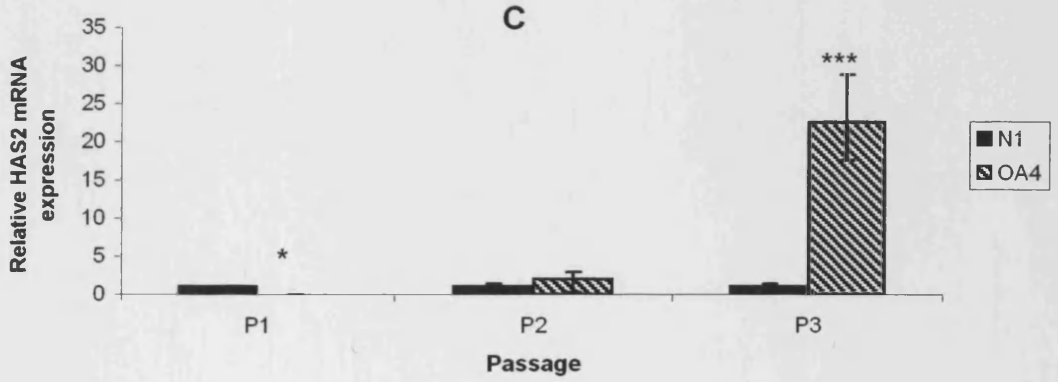

A
Relative HAS1 mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
P1
Passage
N1
OA2
B
Relative HAS1 mRNA expression
16
14
12
10
8
6
4
2
0
*
P2
P3
Passage
N1
OA3
C
Relative HAS1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
P1
P2
P3
Passage
N1
OA4
D
Relative HAS1 mRNA expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
P2
P3
Passage
N1
OA6

**Figure 4.3.16** HAS2 mRNA expression from A) Patient OA2; B) Patient OA3 Patient OA4; D) Patient OA6. RNA was purified from control cells 24 hours treatment. OA control cell HAS2 mRNA expression was normalised to n control cell HAS2 mRNA expression as described in materials and methods. *P relative to normal strained cells; **$P<0.01$ relative to normal strained ***$P<0.001$ relative to normal strained cells.


A
Relative HAS2 mRNA expression
2
1.5
1
0.5
0
**
P1
Passage
N1
OA2


B
Relative HAS2 mRNA expression
90
80
70
60
50
40
30
20
10
0
***
***
P1
P2
P3
Passage
N1
OA3


C
Relative HAS2 mRNA expression
35
30
25
20
15
10
5
0
*
***
P1
P2
P3
Passage
N1
OA4

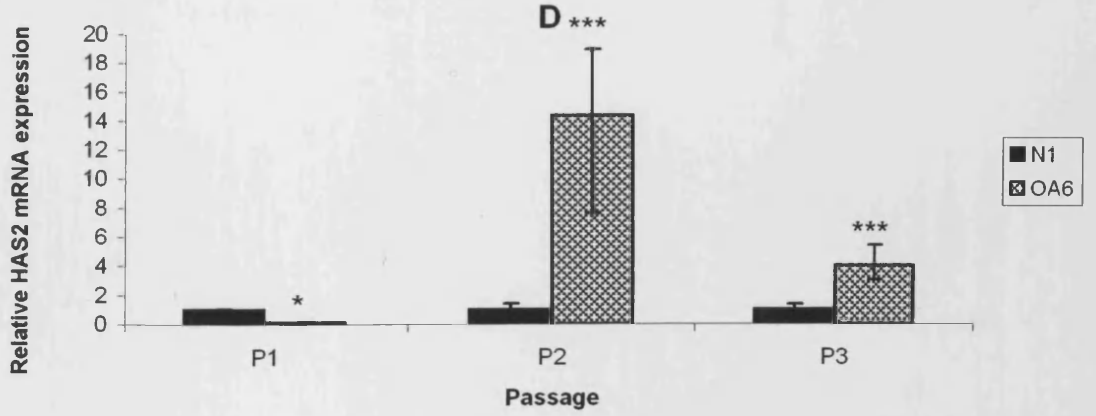

D
Relative HAS2 mRNA expression
20
18
16
14
12
10
8
6
4
2
0
*
***
***
P1
P2
P3
Passage
N1
OA6

**Figure 4.3.17** HAS3 mRNA expression from Patient OA4. RNA was purified from control cells 24 hours post-treatment. OA control cell HAS3 mRNA expression was normalised to normal control cell HAS3 mRNA expression as described in materials and methods. *$P<0.05$ relative to normal strained cells.

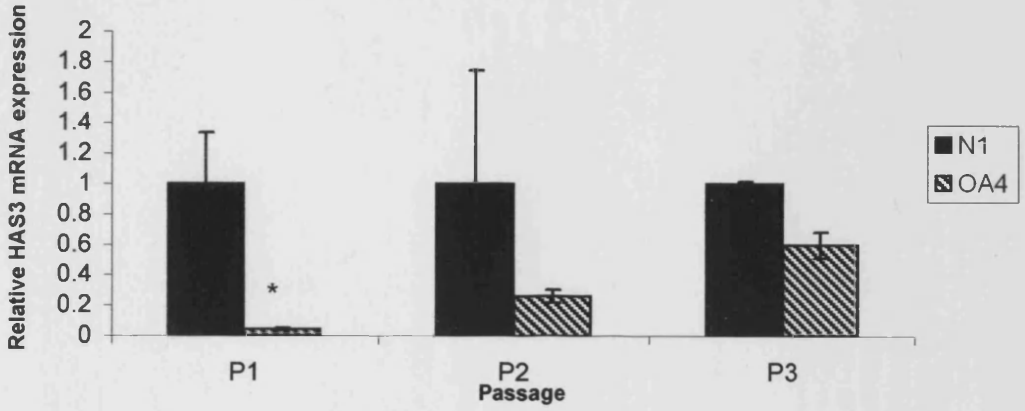

Relative HAS3 mRNA expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
P1
P2
P3
Passage
N1
OA4

**Figure 4.3.18** HAS1 mRNA expression from A) Patient OA3; B) Patient OA4; C) Patient OA6. RNA was purified from cells subjected to 4000με for 10 minutes, 24 hours post treatment. OA and strained cell HAS1 mRNA expression was normalised to normal strained cell HAS1 mRNA expression as described in materials and methods section. *$P<0.01$ relative to normal strained cells.

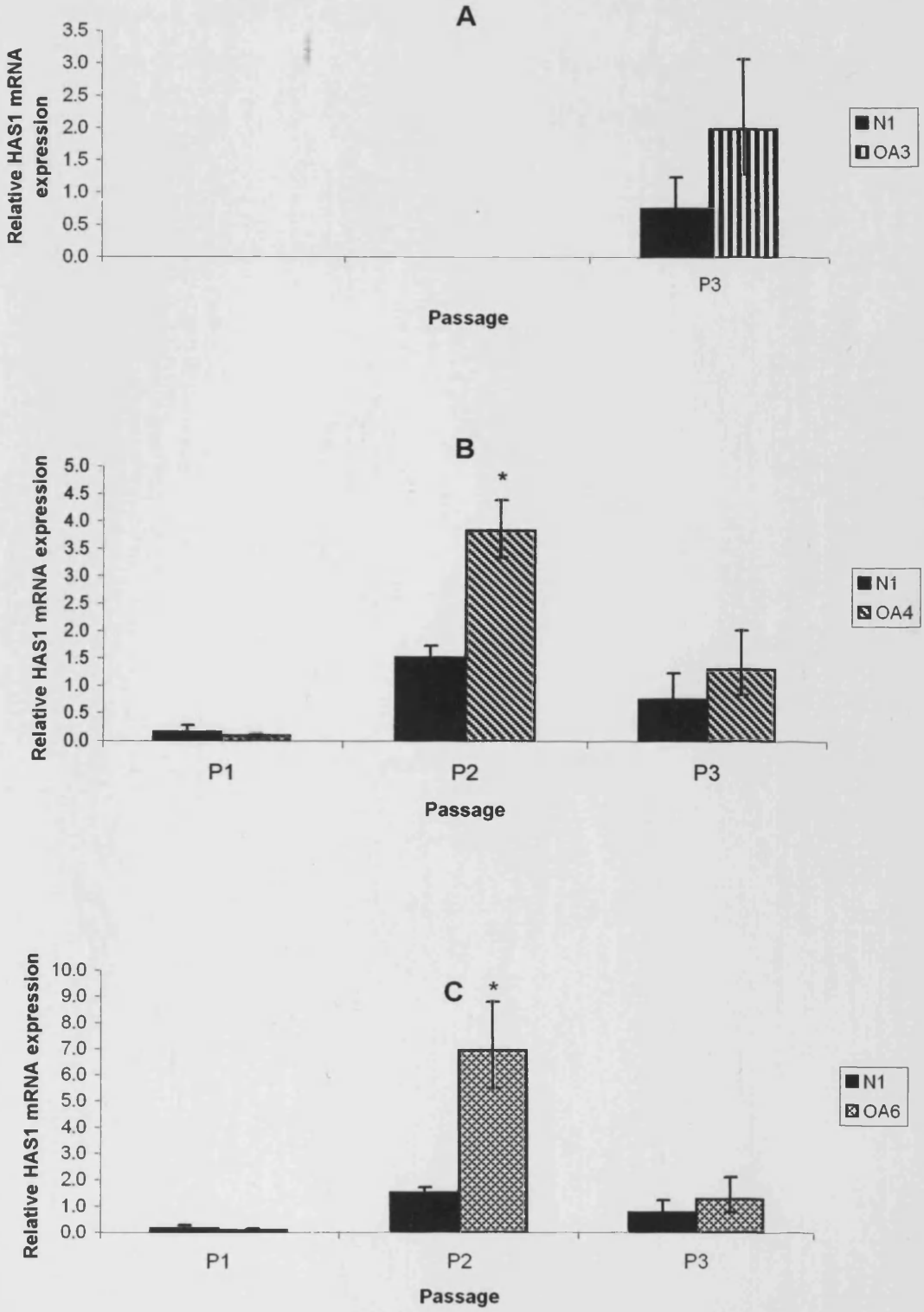

A
Relative HAS1 mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P3
Passage
N1
OA3
B
Relative HAS1 mRNA expression
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
P1
P2
P3
Passage
N1
OA4
C
Relative HAS1 mRNA expression
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
P1
P2
P3
Passage
N1
OA6

**Figure 4.3.19** HAS2 mRNA expression from A) Patient OA3; B) Patient OA4; C) Patient OA6. RNA was purified from cells subjected to 4000με for 10 minutes, 24 hours post treatment. OA strained cell HAS2 mRNA expression was normalised to normal strained cell HAS2 mRNA expression as described in materials and methods. *$P<0.1$ relative to normal strained cells. **$P<0.01$ relative to normal strained cells. ***$P<0.001$ relative to normal strained cells.

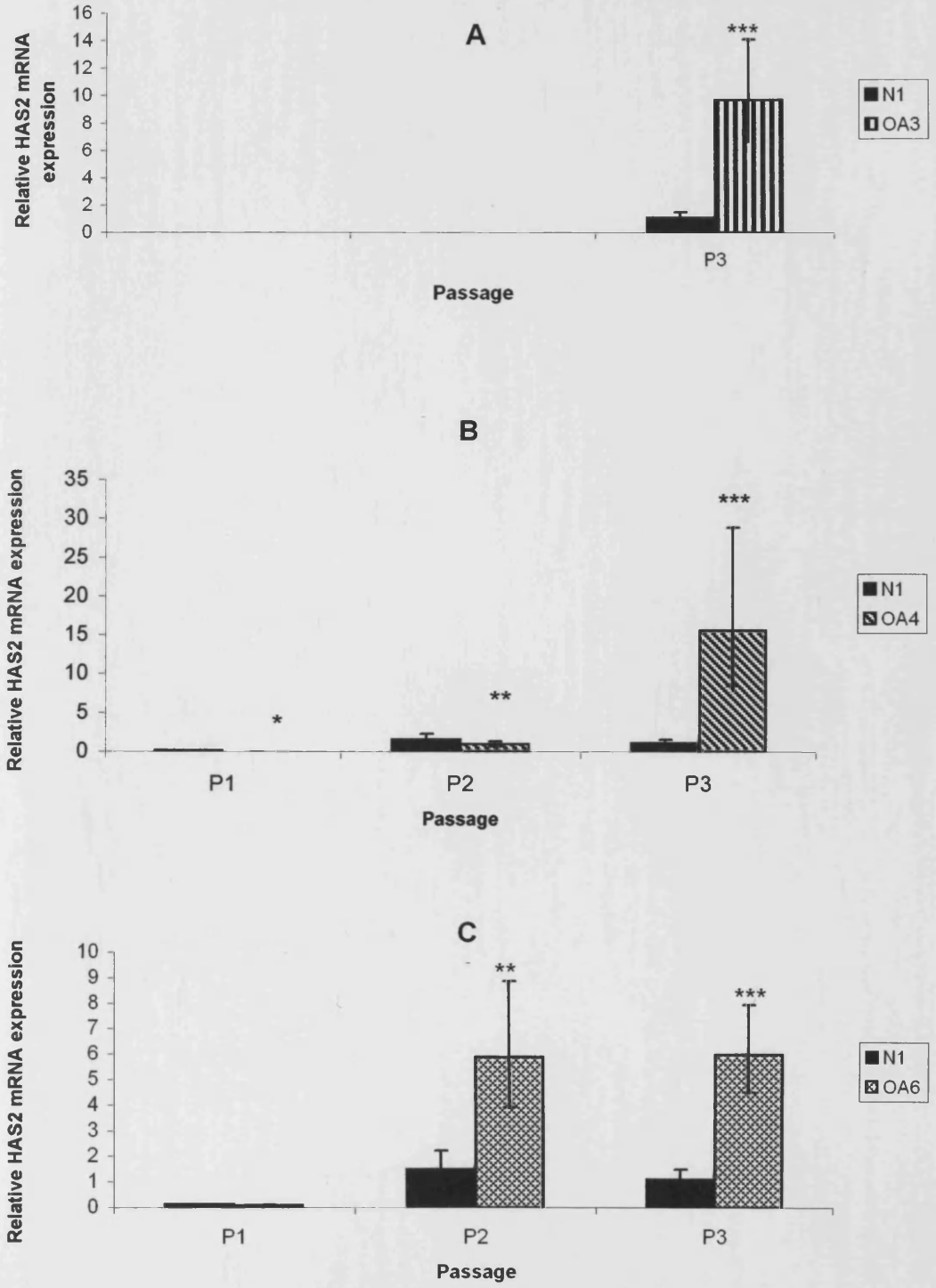

A
Relative HAS2 mRNA expression
16
14
12
10
8
6
4
2
0
***
N1
OA3
P3
Passage
B
Relative HAS2 mRNA expression
35
30
25
20
15
10
5
0
*
**
***
N1
OA4
P1
P2
P3
Passage
C
Relative HAS2 mRNA expression
10
9
8
7
6
5
4
3
2
1
0
**
***
N1
OA6
P1
P2
P3
Passage

**Figure 4.3.20** HAS3 mRNA expression from Patient OA4. RNA was purified from cells subjected to 4000$\mu\varepsilon$ for 10 minutes 24 hours post-treatment. OA strained cell HAS3 mRNA expression was normalised to normal strained cell HAS3 mRNA expression as described in materials and methods section.

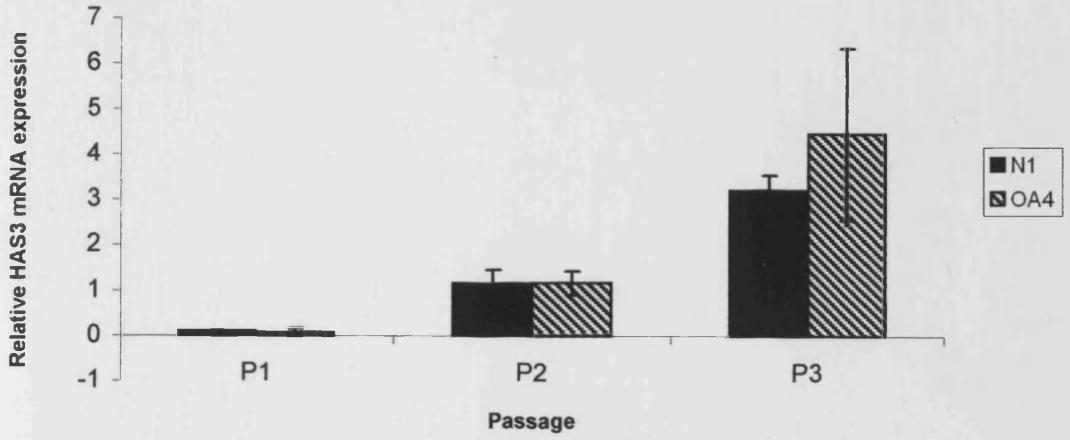

Relative HAS3 mRNA expression
7
6
5
4
3
2
1
0
-1
P1
P2
P3
Passage
N1
OA4

**Figure 4.3.21** HAS1 mRNA expression from A) Patient OA2; B) Patient OA3; C) Patient OA4; D) Patient OA6. RNA was purified from cells subjected to 6000$\mu\varepsilon$ for 10 minutes 24 hours post-treatment. OA strained cell HAS1 mRNA expression was normalised to normal strained cell HAS1 mRNA expression as described in materials and methods. *$P<0.1$ relative to normal strained cells. **$P<0.01$ relative to normal strained cells.

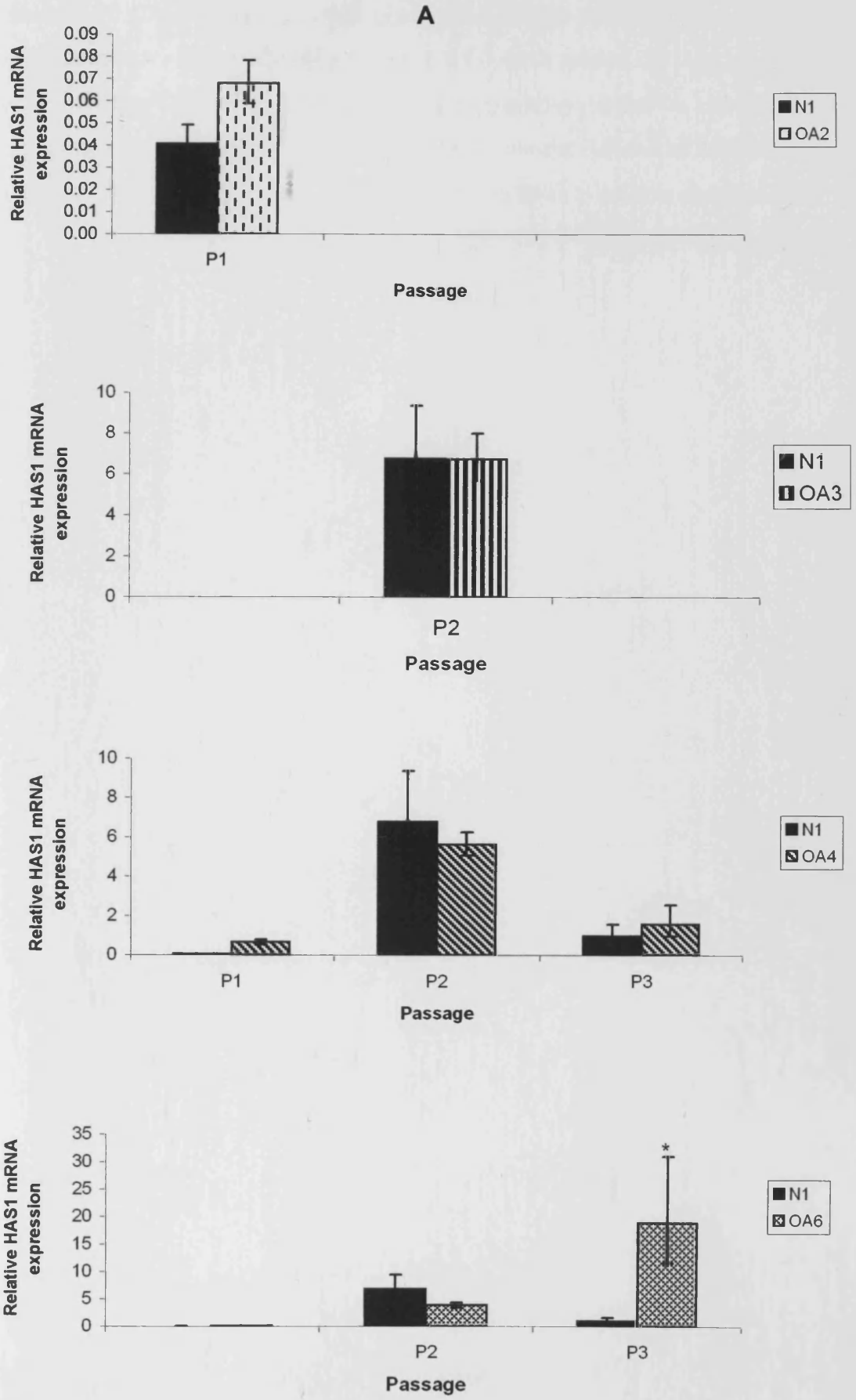

A
Relative HAS1 mRNA expression
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
P1
Passage
N1
OA2
Relative HAS1 mRNA expression
10
8
6
4
2
0
P2
Passage
N1
OA3
Relative HAS1 mRNA expression
10
8
6
4
2
0
P1
P2
P3
Passage
N1
OA4
Relative HAS1 mRNA expression
35
30
25
20
15
10
5
0
*
P2
P3
Passage
N1
OA6

**Figure 4.3.22** HAS2 mRNA expression from A) Patient OA2; B) Patient OA3; C) Patient OA4; D) Patient OA6. RNA was purified from cells subjected to 6000με for 10 minutes 24 hours post-treatment. OA strained cell HAS2 mRNA expression was normalised to normal strained cell HAS2 mRNA expression as described in materials and methods section. *$P<0.01$ relative to normal strained cells. **$P<0.001$ relative to normal strained cells. ***$P<0.0001$ relative to normal strained cells.

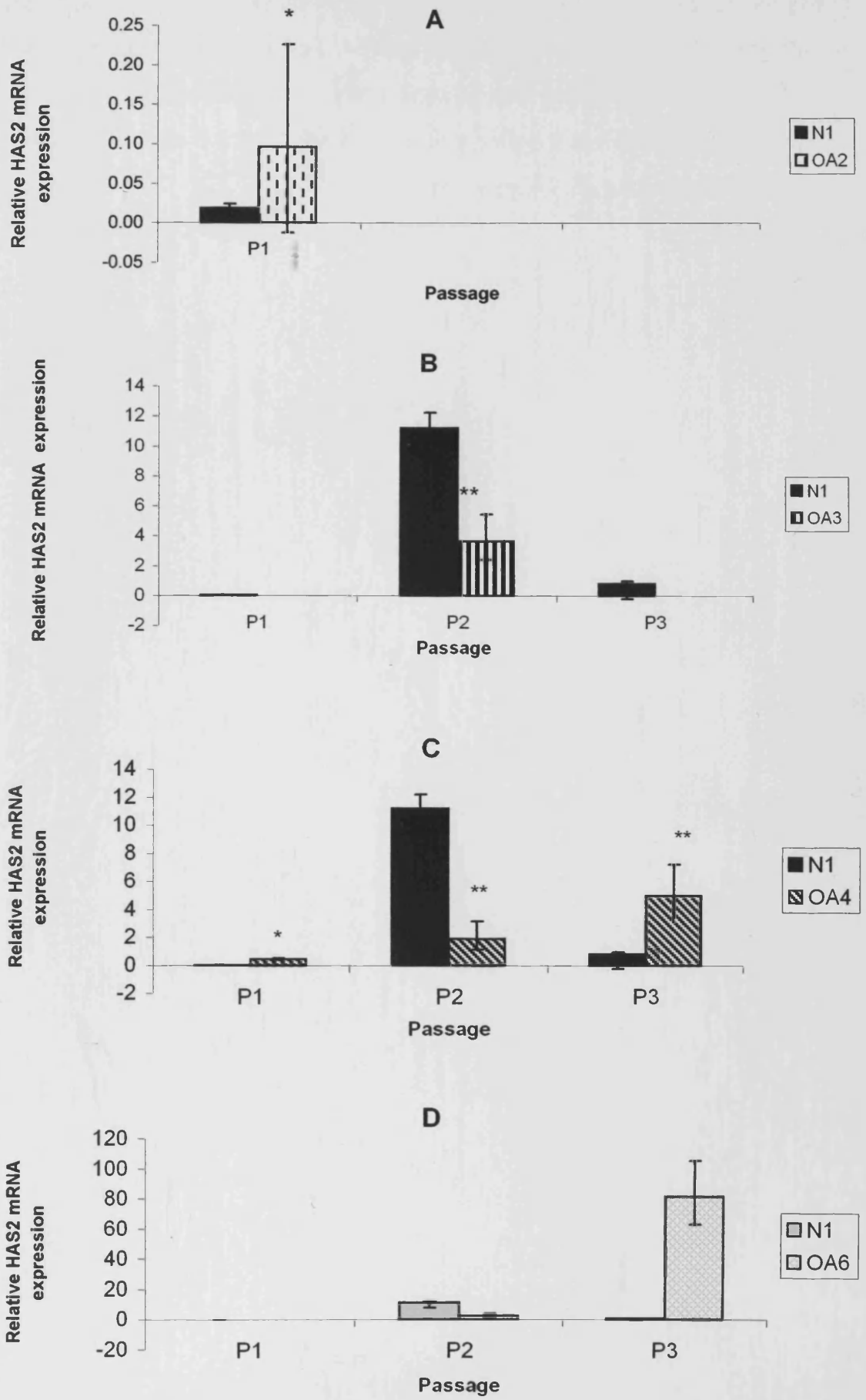

A
Relative HAS2 mRNA expression
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
*
P1
Passage
N1
OA2
B
Relative HAS2 mRNA expression
14
12
10
8
6
4
2
0
-2
**
P1
P2
P3
Passage
N1
OA3
C
Relative HAS2 mRNA expression
14
12
10
8
6
4
2
0
-2
*
**
**
P1
P2
P3
Passage
N1
OA4
D
Relative HAS2 mRNA expression
120
100
80
60
40
20
0
-20
P1
P2
P3
Passage
N1
OA6

**Figure 4.3.23** HAS3 mRNA expression from Patient OA4. RNA was purified from cells subjected to 6000$\mu\varepsilon$ for 10 minutes 24 hours post-treatment. OA strained cell HAS3 mRNA expression was normalised to normal strained cell HAS3 mRNA expression as described in materials and methods. *$P$<0.1 relative to normal strained cells. **$P$<0.01 relative to normal strained cells.

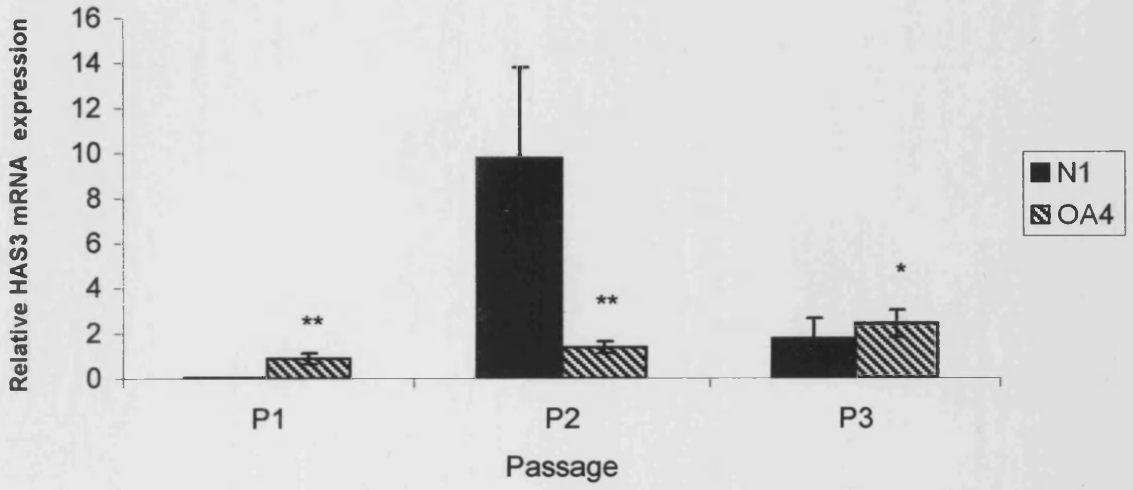
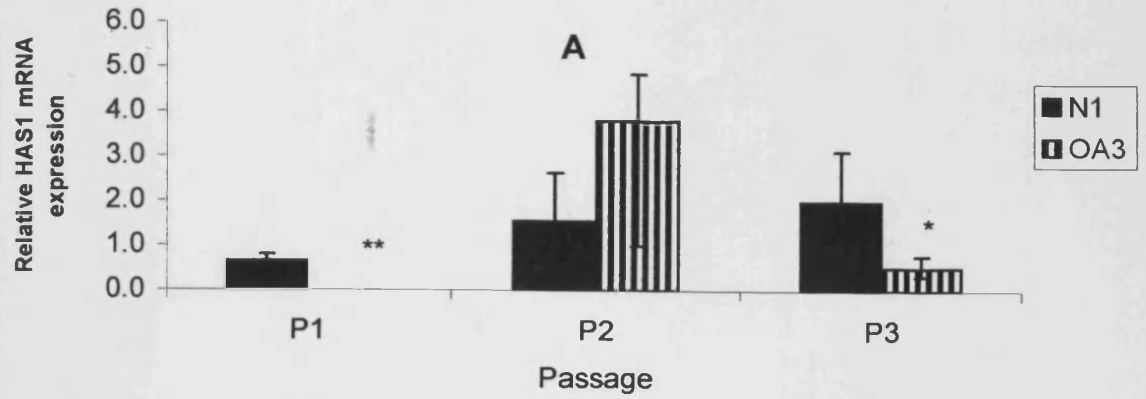
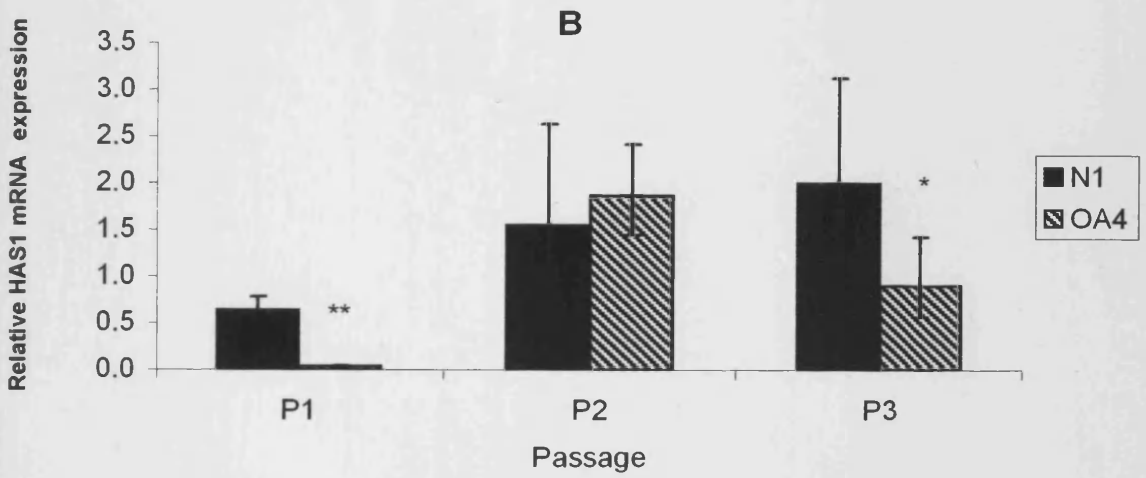

Relative HAS3 mRNA expression
16
14
12
10
8
6
4
2
0
**
**
*
P1
P2
P3
Passage
N1
OA4

**Figure 4.3.24** HAS1 mRNA expression from A) Patient OA3; B) Patient OA4; C) Patient OA6. RNA was purified from cells subjected to 10000με for 10 minutes 24 hours post-treatment. OA strained cell HAS1 mRNA expression was normalised to normal strained cell HAS1 mRNA expression as described in materials and methods. *$P<0.001$ relative to normal strained cells. **$P<0.0001$ relative to normal strained cells.


A
Relative HAS1 mRNA expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
*
P1
P2
P3
Passage
N1
OA3


B
Relative HAS1 mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
P1
P2
P3
Passage
N1
OA4

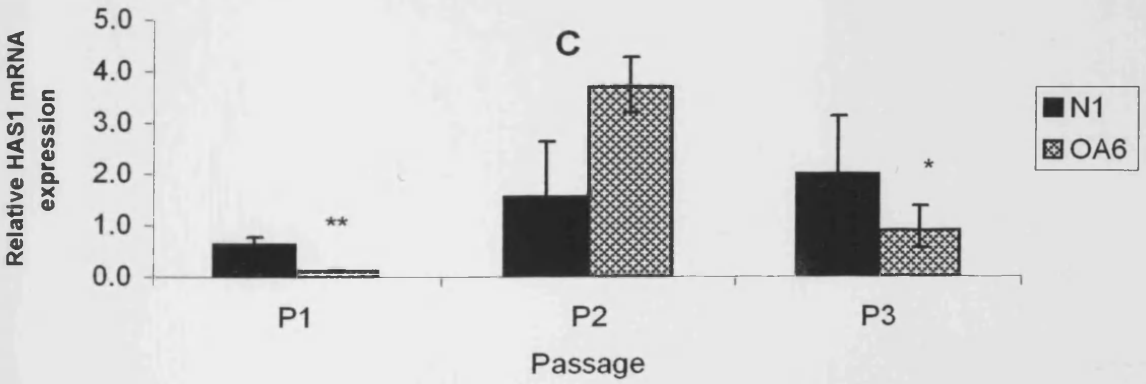

C
Relative HAS1 mRNA expression
5.0
4.0
3.0
2.0
1.0
0.0
**
*
P1
P2
P3
Passage
N1
OA6

**Figure 4.3.25** HAS2 mRNA expression from A) Patient OA3; B) Patient OA4; C) Patient OA6. RNA was purified from cells subjected to 10000$\mu\varepsilon$ for 10 minutes 24 hours post-treatment. OA strained cell HAS2 mRNA expression was normalised to normal strained cell HAS2 mRNA expression as described in materials and methods. *$P<0.01$ relative to normal strained cells.

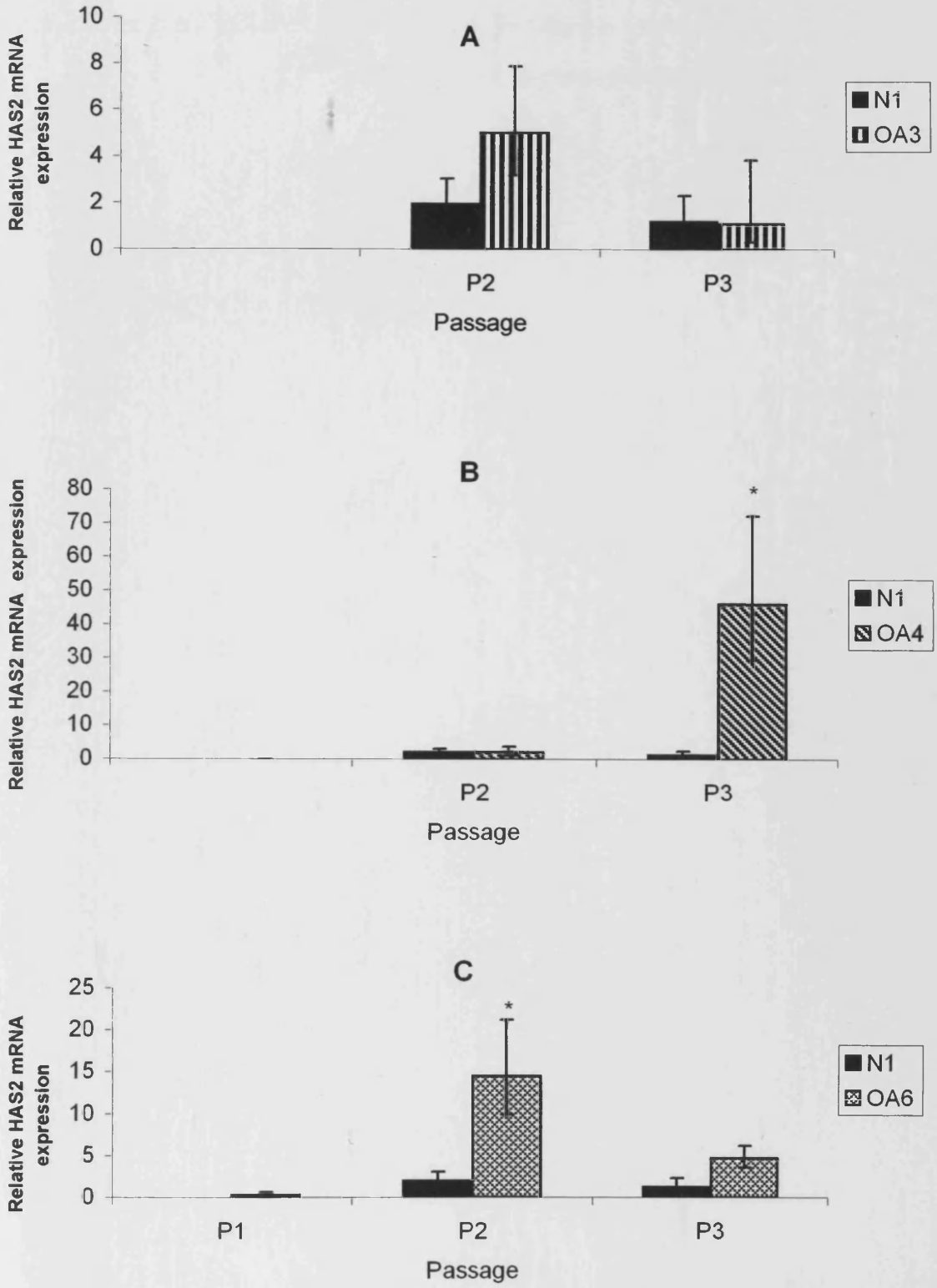
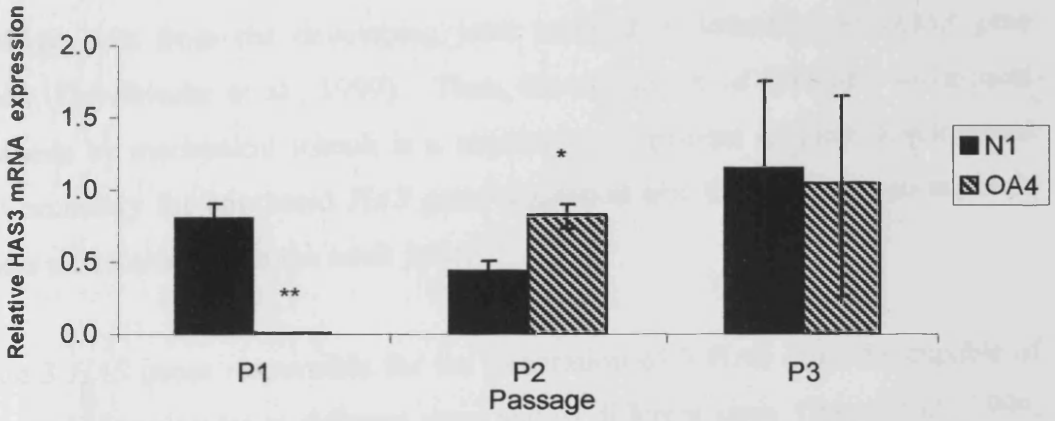

A
Relative HAS2 mRNA expression
10
8
6
4
2
0
P2
P3
Passage
N1
OA3
B
Relative HAS2 mRNA expression
80
70
60
50
40
30
20
10
0
*
P2
P3
Passage
N1
OA4
C
Relative HAS2 mRNA expression
25
20
15
10
5
0
*
P1
P2
P3
Passage
N1
OA6

**Figure 4.3.26** HAS3 mRNA expression from Patient OA4. RNA was purified from cells subjected to 10000$\mu\varepsilon$ for 10 minutes 24 hours post-treatment. OA strained cell HAS3 mRNA expression was normalised to normal strained cell HAS3 mRNA expression as described in materials and methods. *$P<0.05$ relative to normal strained cells. **$P<0.01$ relative to normal strained cells.


Relative HAS3 mRNA expression
2.0
1.5
1.0
0.5
0.0
**
*
N1
OA4
P1
P2
P3
Passage

## 4.4 Discussion

Results from this work show that synovial cells cultured from normal and OA synovium differentially express 3 *HAS* genes *in vitro*. Previous work has shown the regulation of the *HAS* genes to be controlled by physiological stimuli including cytokines and growth factors (Jacobson et al., 2000; Recklies et al., 2001). Data from this current study demonstrate that mechanical stimuli can also regulate HAS expression in synovial cells derived from normal and OA donors. The importance of HA and mechanical stimuli during joint development has been detailed. The removal of mechanical stimuli during joint development prevents the separation of the cartilage anlagen and this is linked to alterations in HA synthesis (Drachmann and Sokoloff, 1966; Edwards et al., 1994; Fell and Canti, 1934; Osborne et al., 2002; Pitsillides, 1999; Pitsillides et al., 1995). Indeed, mechanical strain applied to fibrocartilage cells from the developing joint resulted in induction of *HAS3* gene expression (Dowthwaite et al., 1999). Thus, the regulation of *HAS* and subsequent HA synthesis by mechanical stimuli is a necessary component of joint development and also necessary for continued *HAS* gene regulation and HA synthesis to maintain frictionless movement within the adult joint.

There are 3 *HAS* genes responsible for the generation of 3 HAS enzymes capable of synthesising HA molecules at different rates and of different sizes (Itano et al., 1999; Spicer and McDonald, 1998). From this current work it can be seen that all 3 *HAS* genes show differential expression when synovial cells were subjected to mechanical stimuli. If the same were to occur *in vivo*, then this would provide the cell with some control over its biological functions. The HA molecules provide different functional roles within the cell and surrounding environment depending on size (Rooney et al., 1995; West and Kumar, 1989). For example, within the synovial joint, the larger HA molecules are able to prevent synovial fluid loss from the synovial cavity more so than the smaller HA molecules (Coleman et al., 1999).

The response to the three magnitudes of strain (4000με, 6000με and 10000με) was different between the normal and OA cells. 4000με is the physiological strain applied

to cells *in vivo* based on measurements from bone surfaces during normal activity and *in vitro* 4000με resulted in an increase in all three of the *HAS* genes in the OA synovial cell cultures (Lanyon, 1996). Conversely, a decrease in HAS1 and HAS2 mRNA expression was seen in normal cells. Although the normal synovial cell cultures showed an increase in HAS2 and HAS3 mRNA expression at 6000με this did not occur at each passage. The OA synovial cell cultures however, showed increases in all three HAS mRNA expression relative to the control cells at each passage when subjected to 6000με. Application of 10000με to the normal synovial cell cultures resulted in alterations in HAS2 and HAS3 mRNA expression whereas HAS1 and HAS2 expression could be seen to alter in the OA synovial cells. Thus, the normal cells showed fewer alterations in *HAS* gene expression in response to strain compared to the OA cells. Whether this is because the normal synovial cells are able to synthesise the necessary amounts of HA without mechanical stimuli cannot be confirmed from this study. It has been shown that feedback mechanisms are involved in HA synthesis, so, if there is an adequate concentration of HA being synthesised, further synthesis could be switched off via a reduction in HAS mRNA expression (Philipson and Schwartz, 1984; Philipson et al., 1985; Stern, 2003).

HA concentrations decrease within the synovial fluid of OA patients and this could be attributed to the loss of response to mechanical stimuli by the cells due to the disease. These results however, demonstrate that OA synovial cells constitutively express the three *HAS* genes and differential expression occurs as a result of mechanical stimuli. The observed increase in mRNA expression of the 3 *HAS* genes in response to 4000με and 6000με suggests the cell needs to increase it's HA synthesis and also needs to synthesise the 3 differently sized HA molecules. The synthesis of the larger HA chains in the synovial joint as a result of HAS1 and HAS2 synthesis, would allow buffering outflow preventing the synovial fluid decreasing in volume and viscosity as a result of joint flexion. The larger HA molecules interact forming a network above the synovium controlling interstitial fluid flow into and out of the synovial cavity (Coleman et al., 1999). The role of the smaller HA molecule, synthesised by HAS3 *in vitro*, is less clear. High levels of this smaller HA molecule can be seen in the synovial fluid and plasma of OA patients (Miyaguchi et al., 2001). The smaller HA molecule is able to buffer synovial outflow but to a lesser extent than the larger HA molecules (Coleman et al., 2000). The role of this smaller HA molecule could be in

cell signalling as a variety of genes have been shown to be expressed in response to lower molecular mass HA allowing the regulation of biological properties within the cell (Oertli et al., 1998; Slevin et al., 1998). The low molecular weight HA chains might also stimulate macrophage responses during inflammatory situations (McKee et al., 1996; Termeer et al., 2000). Thus, the expression of HAS3 mRNA upon the application of mechanical strain could be to generate HA molecules of smaller molecular weights that could be involved in the inflammatory process. The generation of this smaller HA might, in contrast, simply act to increase the levels of HA within the synovial fluid in a shorter time sequence.

Response at 10000με in *HAS* expression resulted in an increase in HAS1 and HAS2 mRNA expression in the OA synovial cultures. The requirement to synthesise HA molecules with a high molecular weight could be a reflection of the exceedingly high load applied to the cells. A greater viscosity in synovial fluid would be required compared to the lower magnitudes of strain and greater fluid outflow buffering would also be needed.

The effect of passage number on the synovial cell cultures needs to be taken into consideration when assessing the results. As this study and others have shown there is a difference between low and high passage cell cultures (Clarris and Fraser, 1968; Marsh et al., 1978; Zimmerman et al., 2001). Here, it can be seen that normal synovial cell cultures each showed a different response to the three magnitudes of strain depending on the passage number. A significant difference between control and strained cell HAS mRNA expression was not observed in the three genes simultaneously at any passage. In contrast, the OA synovial cell cultures' HAS mRNA response at each passage at the low magnitudes of strain was an increase in each of the 3 *HAS* genes. At 10000με the response at each passage was different.

Although it must be taken into consideration that changes in mRNA expression do not always correspond to protein synthesis and subsequent HA synthesis, the observations obtained from this study suggest that mechanical stimuli do influence the regulation of the three *HAS* genes allowing the cell to synthesise the required size of HA molecule, with the response dependent on disease state and passage number.

# Chapter 5

# Effects of Mechanical Strain on Cytokine Synthesis by Synovial Cells

## 5.1 Introduction

The cytokine family compromises a diverse group of soluble proteins and peptides that modulate the functional activities of individual cells and tissues and are involved in inflammatory processes (Firestein, 2003; Fortunato and Menon, 2003; Grooten et al., 1993; Kaden et al., 2003; Kawano et al., 1988; Tsuyama et al., 2003; Warner et al., 1987). Almost all cytokines detected are pleiotropic effectors showing multiple biological activities depending on the cell signalling pathway stimulated by the cytokine. The circumstance of the cell – its growth state, type of neighbouring cells, cytokine concentration and presence of other cytokines will influence the effect of a single cytokine thus allowing the transmission of diverse signals to different subsets of cells. Response to cytokine stimulation is rapid. Once the cytokine has bound to its receptor, the receptor undergoes oligomerisation. Kinases are activated, phosphorylating cytoplasmic transcription factors which are able to translocate to the nucleus where they bind to unique DNA sequences in the promoter regions of cytokine responsive genes. The translation of these genes leads to protein synthesis and an appropriate response. Once released, the cytokine concentrations are controlled through soluble receptors and specific or unspecific binding proteins. Further release is controlled by positive and negative feedback mechanisms or by other cytokines (Feldmann et al., 1996b; Nishimoto et al., 2000; Silacci et al., 1998).

Regulation of matrix synthesis and degradation is an integral requirement for maintenance of a healthy joint. During RA and OA however, impaired matrix turnover occurs involving an imbalance between synthesis of pro-inflammatory and anti-inflammatory cytokines (Feldmann et al., 1996a; Firestein, 2003). The synovium contributes to disease progression releasing pro-inflammatory cytokines yet also attempting to restore the damaged matrix with the release of anti-inflammatory cytokines. The cytokine network in OA and RA has been extensively studied at both the mRNA and protein level in an attempt to understand the complex interactions occurring during disease progression and to develop treatments that block pro-inflammatory processes (Feldmann et al., 1996a; Moreland, 1999).

Tumour necrosis factor alpha (TNFα) and interleukin-1 (IL-1) have been considered the main pro-inflammatory cytokines involved in arthritis (Firestein, 2003; Firestein et al., 1992; Fukui et al., 2001; Hedbom and Hauselmann, 2002). Both of these cytokines are synthesised and secreted by synovial cells and chondrocytes and have been shown to inhibit or induce their own synthesis and are controlled by complex feedback mechanisms depending on cell type (Alsalameh et al., 1999). Enhanced synthesis of both TNFα and IL-1 have been observed in RA and OA synovium and cartilage, inducing pro-inflammatory processes such as proteinase synthesis, inhibition of new matrix synthesis and increasing matrix catabolism (Goldring et al., 1988; Goldring et al., 1990; Hauptmann et al., 1991; Hollander et al., 1994; Hutchinson et al., 1992; Ismaiel et al., 1992; Miyasaka et al., 1988; Westacott et al., 1990; Yoshida et al., 1992). Intact and degraded matrix molecules within the synovial fluid also contribute to proteinase synthesis and cytokine release, as well as synoviocyte activation, potentiating disease progression (Biswas and Dayer, 1979; Chu et al., 1991; Deleuran et al., 1994; Gurr et al., 1990; Saito et al., 2002; Wurster and Lust, 1984). In response to these catabolic signals, the joints response is an attempt to diminish disease activity. Increased levels of soluble cytokine receptors such as p55 tumor necrosis factor-receptor (TNF-R), p75 TNF-R and soluble interleukin-1-receptor (sIL-1R) are present in the synovial fluid of RA and OA patients and within the chondrocytes themselves (Butler et al., 1994; Firestein et al., 1992; Malyak et al., 1993; Martel-Pelletier et al., 1992; Pelletier and Martel-Pelletier, 2002; Roux-Lombard and Steiner, 1992; Smith et al., 1997; Symons et al., 1991). The TNF-Rs are able to bind to TNFα and inactivate TNFα whereas sIL-1-R prevents processing of the IL-1 protein so acting as inhibitors (Symons et al., 1995). Although these inhibitors can be detected at elevated concentrations, (in the case of TNF-R four fold), this is not enough to inactivate the enhanced levels of TNFα and IL-1 synthesised during disease (Brennan et al., 1989; Cope et al., 1992; Roux-Lombard et al., 1993).

The cytokine interleukin-6 (IL-6) is involved in the inflammatory process in RA and OA and is synthesised by the both synovial cells and chondrocytes (Guerne et al., 1989; Rosenbaum et al., 1992). The physiological stimuli for IL-6 includes both IL-1 and TNFα and as with both of these pro-inflammatory cytokines, IL-6 concentration is elevated in the synovial fluid of patients with RA and OA and in media from synovial

cell cultures from inflamed tissues (Bucala et al., 1991; Hirano et al., 1988; Mihara et al., 1995; Nishimoto et al., 2000; Okamoto et al., 1997; Ritchlin, 2000). Stimulation of synovial fibroblasts with IL-6 does not induce collagenase or prostaglandin $E_2$ (PGE2) synthesis, suggesting an anti-inflammatory or mediating role (Hauptmann et al., 1991; Lotz and Guerne, 1991). IL-6 has a glycosylated membrane protein receptor as well as soluble receptor that act as physiological regulators of IL-6 activity (Mihara et al., 1995; Nishimoto et al., 2000). The concentration of the soluble IL-6 receptor (sIL-6R) has been shown to be elevated 2.5 fold in the synovial fluid of inflamed joints and in combination with IL-6, the soluble receptor is capable of increasing TIMP expression 3 fold. Since tissue inhibitors of metalloproteases (TIMP) are responsible for blocking MMP activity, this adds credence to the role of IL-6 in mediating anti-inflammatory responses (Silacci et al., 1998).

Synovial cells are one of the many cell types that synthesise the chemokine interleukin-8 (IL-8) and both OA and RA tissues exhibit increased synthesis of this chemokine (Brennan et al., 1990; Koch et al., 1991; Koch et al., 1992b; Loetscher et al., 1994; Rampart et al., 1992; Seitz et al., 1991). IL-8 is a low molecular weight pro-inflammatory chemokine frequently found at high concentrations in diseases associated with neutrophil influx such as cystic fibrosis, gastroduodenal disease, psoriasis and respiratory disease (Alaaeddine et al., 1999; Brennan et al., 1990; Crabtree and Lindley, 1994; Dean et al., 1993; Kulke et al., 1996; Mahida et al., 1992; Oppenheim et al., 1991). As IL-8 is a potent chemoattractant for neutrophils, it plays a role in inducing neutrophil activation and recruitment involving the regulation of adhesion molecules on the neutrophil cell surface (Koch et al., 1992a; Nanki et al., 2001). In OA and RA, IL-8 has been shown to enhance inflammatory cell migration and is often found at very high concentrations within the cells of the cartilage-pannus junction (Deleuran et al., 1994). The pro-inflammatory cytokines TNFα and IL-1 have both been shown to induce mRNA expression and subsequent protein synthesis of IL-8, which during RA and OA could be associated with the increase in inflammatory cells seen in the synovial membrane (Bedard and Golds, 1993; Koch et al., 1991; Rathanaswami et al., 1993; Tobe et al., 2002; Wang et al., 1997).

The therapeutic effects of physiotherapy as a treatment for OA has already been discussed in terms of HA synthesis and the beneficial effects this has on joint function

(Chapter 3). Nevertheless, it is clear that during OA and RA, the equilibrium that exists between the cytokines and their inhibitors is disrupted and this needs to be taken into consideration if mechanical strain is to be used as a treatment for OA.

The effects of mechanical strain on chondrocytes has been more widely studied than the effects of strain on synovial fibroblasts, the results of these studies however, are contradictory. Mechanical strain has been shown to reduce IL-1 and TNFα induced expression of pro-inflammatory mediators *in vitro,* down-regulate nitric oxide expression and synthesis and increase proteoglycan and collagen synthesis augmenting the reparative process of the cartilage matrix (Deschner et al., 2003; Long et al., 2001) (Gassner et al., 2000; Xu et al., 2000). The induction of interleukin-4 synthesis is also observed leading to anti-inflammatory signals (Lee et al., 2000). Conversly, mechanical strain has also been shown to increase mRNA expression of the pro-inflammatory cytokines and matrix metalloproteinases (MMP) (Deschner et al., 2003; Honda et al., 2000). It must be born in mind that these pro-inflammatory responses were detected when chondrocytes were subjected to high levels of cyclic strain.

The effect of mechanical strain on RA synovial cells resulted in a reduction of mRNA expression, protein synthesis and activity of MMP-1 and MMP-13 even when the cells had been subjected to IL-1 and TNFα stimulation (Sun and Yokota, 2001a; Sun and Yokota, 2001b). The anti-inflammatory effect of strain is further supported by data showing that TIMP-1 and TIMP-2 are up regulated after synovial cells have been subjected to strain (Sun et al., 2003). An exercise based study also showed the beneficial effects of strain, with observed reductions in IL-1 concentration in the synovial fluid (Messier et al., 2000). IL-8 synthesis has been shown to increase in bronchial epithelial cells that were subjected to mechanical stimuli and if the same were to occur in synovial cells then this would facilitate disease progression (Oudin and Pugin, 2002).

The aim of this work was to determine the effects of mechanical strain on IL-1, IL-6, IL-8 and TNFα synthesis from synovial cells derived from the synovium of normal and OA donors.

## 5.2 Materials and Methods

### 5.2.1 Synovium Samples

Synovium samples were obtained as in Chapter 2 (2.2.1). Details of the samples are listed below.

| Sample | Type | Sex | Age (years) |
|---|---|---|---|
| OA3 | OA | Female | 74 |
| OA4 | OA | Female | 79 |
| OA6 | OA | Male | 79 |
| N1 | normal | Female | 67 |

**Table 5.2.1 Details of synovium samples obtained at knee joint replacement surgery.**

### 5.2.2 Cell Isolation & Culture

Cells were isolated from the synovium as described in Chapter 2 (2.2.2). Briefly, diced tissue was digested in 295IU mg $ml^{-1}$ type I collagenase (Sigma, Poole, UK) for 3 hours at 37°C. Cells were centrifuged at 1000 x g for 5 minutes, resuspended and centrifuged at 1000 x g for 5 minutes. Cells were counted and seeded as required in DMEM/F12 containing 1% penicillin-streptomycin ($10^4$IU $ml^{-1}$/$10^4$μg $ml^{-1}$; Sigma), L-glutamine (200mM; Life Technologies, Paisley, UK), hydrocortisone (4mg $ml^{-1}$; Sigma)[40mg hydrocortisone powder dissolved in 5ml 100% ethanol and 5ml sterile PBS], insulin-transferrin-selenium (200mM; Life Technologies) and 20% foetal calf serum (FCS) (Sigma) and incubated at 37°C.

### 5.2.3 Mechanical Strain

The cells were subjected to mechanical strain as described in Chapter 3 (3.2.3). Briefly, cells were serum deprived for 18 hours and fresh serum free media was added. The cells were strained at 4000με, 6000με and 10000με for 10 minutes using a four-point bending loading jig. Control cells remained static for 10 minutes and flow control cells remained static but were subjected to media perturbation for 10 minutes.

### 5.2.4 Cytokine ELISA

Samples of media collected from the strain and control plates at 24 hours were assayed for interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNFα) concentration using an ELISA plate-based assay developed by Biosource Int. Inc. (Ca, USA).

Plates were coated with 100μl per well of 10μl coating antibody (IL-1, IL-6, IL-8 or TNFα) diluted in 10.5ml phosphate buffered saline (PBS, Sigma, Poole, UK). These were incubated overnight at 4°C. The coating was removed and plates washed in PBS then blocked with 300μl 1% bovine serum albumin (BSA; Sigma) in PBS. The plates were incubated for 2 hours at room temperature and then washed in PBS-Tween (PBS-T) three times. One hundred μl of sample or standard was added. A standard series ranging from 15 to 1000pg $ml^{-1}$ was run on each plate in triplicate. Samples and standards were diluted in DMEM/F12 when necessary and duplicate samples were assayed. Fifty μl biotin-detection antibody diluted 1:1375 in PBS was added to the plates and incubated for 2 hours at room temperature. The plates were washed thoroughly in PBS-T and 100μl strepavidin-HRP conjugate (Biosource Int Inc, CA, USA) added at a dilution of 1:1000. This was incubated for 30 minutes at RT. Plates were washed in PBS-T and 100μl developing buffer added. The developing buffer consisted of 10ml citrate buffer (Sigma), 10μl hydrogen peroxide (Sigma), and 100μl tetramethyl benzidine (TMB; Sigma) and was prepared immediately before use. Plates were gently shaken for 20-30 minutes and once colour had developed the reaction was quenched with 50μl $H_2SO_4$ (12.5%). The absorbencies were read at 405nm.

**Statistical Analysis**

All statistical analysis for changes in cytokine concentrations between normal and OA patients and control and strained cells were analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc tests. This statistical test was applied in order to compare more than 2 sets of data.

**Sample Number**

As a result of low cell number, cells from some patients could not be subjected to the three magnitudes of strain at each passage. Media was retained for HA analysis and this also reduced the sample number available for cytokine analysis.

# 5.3 Results

## 5.3.1 Patient Variability

**Variability with passage**

Media IL-6 and IL-8 concentrations from the three passages of the normal synovial cells showed significant differences ($P<0.05$) and as a result, the data from each passage could not be combined for statistical analysis (fig.5.3.1). Media cytokine concentrations from the three passages of OA synovial cells showed no significant differences, except for IL-8 concentrations, so the data from each passage could be combined for statistical analysis (fig.5.3.2).

**Cross patient variability**

Media cytokine concentrations from the separate patients showed significant differences ($P<0.05$) so the data from separate patients could not be combined for statistical analysis (fig.5.3.3).

## 5.3.2 Normal Patient Response to Strain

**TNFα**

No significant differences between the cells subjected to the three magnitudes of strain and the control cells were observed in media TNFα concentrations (fig.5.3.4A).

**Interleukin-1**

No significant differences in media IL-1 concentrations in the media from cells subjected to the three magnitudes of strain compared to the control cells were seen (fig.5.3.4B).

**Interleukin-6**

The cells subjected to 4000με and 6000με at passage 1, 2 and 3 showed no significant differences in their media IL-6 concentrations compared to the control cells (fig.5.3.5). When cells were subjected to 10000με, a significant increase ($P<0.05$) in

media IL-6 concentration was observed in the passage 1 cells (fig.5.3.5A) but no significant differences were observed in the passage 2 and passage 3 cells (fig.5.3.5B & C).

**Interleukin-8**

Cells at passage 1, 2 and 3 showed no significant differences in media IL-8 concentration compared with the control cells when they were subjected to 4000με and 6000με (fig.5.3.6). The passage 1 cells, when subjected to 10000με showed a significant increase ($P<0.05$) in media IL-8 concentration (fig.5.3.6A). Passage 2 and 3 cells showed no significant differences in media IL-8 concentration when subjected to 10000με.

**Summary: Cytokine Media Concentrations from Normal Synovial Cells**

| Passage | 4000με | 6000με | 10000με |
|---|---|---|---|
| P1 | | | IL-6 increase<br>IL-8 increase |
| P2 | | | |
| P3 | | | |

**Table 5.3.1 Changes in cytokine media concentration from normal cells.**

## 5.3.3 OA Patient Response to Strain

**TNFα**

The passage 3 cells showed no significant differences in media TNFα concentration compared to the control cells, when subjected to the three magnitudes of strain (fig.5.3.7A).

**Interleukin-1**

The passage 3 cells showed no significant differences in media IL-1 concentrations compared to the control cells, when subjected to the three magnitudes of strain (fig.5.3.7B).

**Interleukin-6**

Passage 3 cells from three different patients, when subjected to the three magnitudes of strain, showed no significant differences in media IL-6 concentrations compared to the control cells (fig.5.3.8).

**Interleukin-8**

Passage 1 cells showed a significant increase ($P<0.05$) in media IL-8 concentration when subjected to 10000με whereas subjecting the cells to 4000με and 6000με resulted in no significant differences in media IL-8 concentrations compared to the control cells (fig.5.3.9). Passage 2 and 3 cells showed no significant differences in media IL-8 concentrations compared to the control cells when they were subjected to 4000με, 6000με and 10000με (fig.5.3.10).

**Summary: Cytokine Media Concentrations from OA Synovial Cells**

| Passage | 4000με | 6000με | 10000με |
|---|---|---|---|
| P1 | | | IL-8 increase |
| P2 | | | |
| P3 | | | |

**Table 5.3.2 Changes in cytokine media concentration from OA cells**

## 5.3.4 Effects of Mechanical Strain on Normal and OA Cells

**TNFα**

No significant differences in TNFα media concentrations from normal control cells compared to the OA control cells were observed. When the cells were subjected to the three magnitudes of strain no significant differences in media TNFα concentrations were seen in the normal strained cells compared to the OA strained cells (fig.5.3.11).

**Interleukin-1**

No significant differences in the IL-1 media concentrations from normal control cells compared to the OA control cells were observed. When the cells were subjected to the three magnitudes of strain, 4000με resulted in an increase in the media IL-1

concentrations in the normal cells whereas the OA cells showed a decrease in IL-1 concentration which was significantly less ($P<0.0001$) than the response of the normal cells (fig.5.3.12). No significant differences in the IL-1 media concentrations were observed when the normal and OA cells were subjected to 6000με and 10000με (fig.5.3.12).

**Interleukin-6**

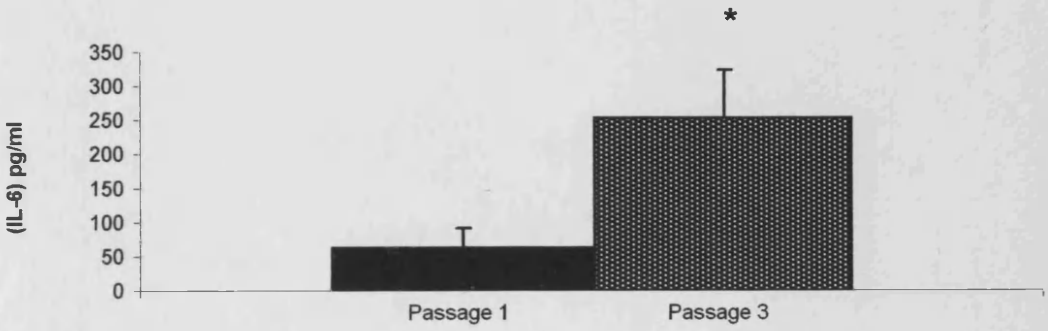
Passage 1 and 3 control cells from normal and OA patients showed significant differences in media IL-6 concentrations whereas passage 2 cells showed no significant differences. When the cells were subjected to 4000με, the passage 1 normal cells responded with significantly higher ($P<0.05$) media IL-6 concentration compared to the OA cells (fig.5.3.13A). Passage 2 OA cells showed significantly less ($P<0.05$) media IL-6 compared to the normal cells IL-6 response when normal and OA cells were strained at 6000με (fig.5.3.13B). Passage 3 cells showed no significant differences in IL-6 media concentration between normal and OA cells when subjected to the three magnitudes of strain (fig.5.3.13C).

**Interleukin-8**

Control cells from the three passages from control normal and OA patients showed significant differences in media IL-6 concentrations ($P<0.05$). When the cells were subjected to 10000με, the passage 1 cells responded with media IL-8 concentrations significantly less than media IL-8 concentrations synthesised by normal cells in response to strain (fig.5.3.14A). Passage 2 cells showed significantly less media IL-8 compared to the normal cells when the cells were strained at 6000με (fig.5.3.14B). No significant differences between OA and normal cells IL-8 media concentration were observed in cells response to 4000με and 10000με at passage 2 (fig.5.3.14B). Passage 3 cells showed no significant differences between normal and OA cells when subjected to the three magnitudes of strain (fig.5.3.14C).

**Figure 5.3.1** A) Media interleukin-6 and B) media interleukin-8 concentrations from synovial cells at passage 1 and passage 3 from Patient N1. Cells were serum deprived for 24 hours, then fresh serum free media added and the media sampled at 24 hours. Error bars represent standard deviations. $*P<0.05$.

A


*
350
300
250
200
150
100
50
0
(IL-6) pg/ml
Passage 1
Passage 3

B

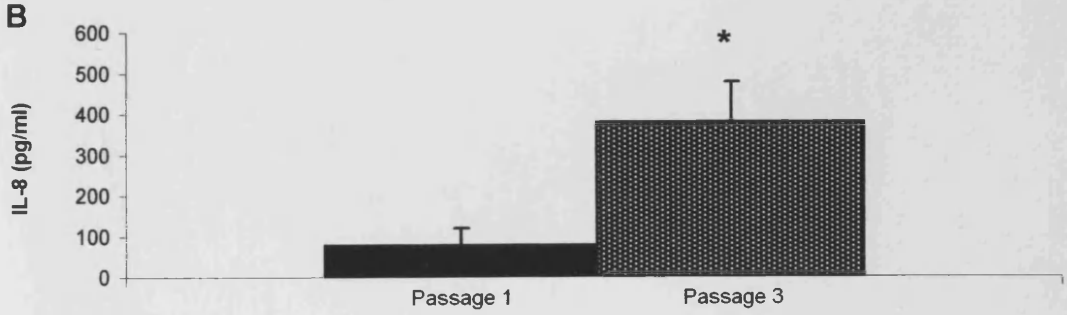
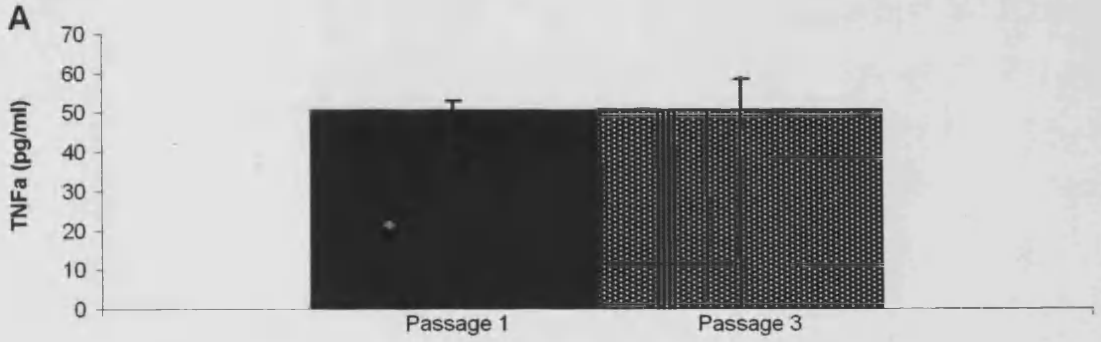
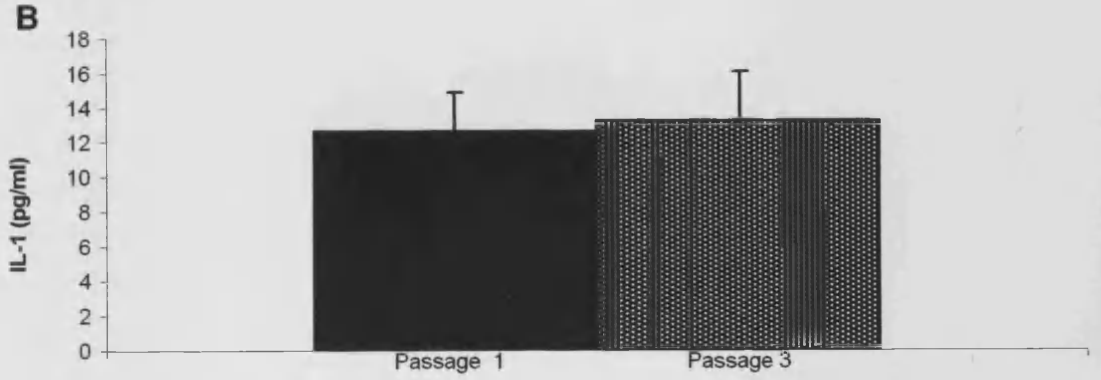
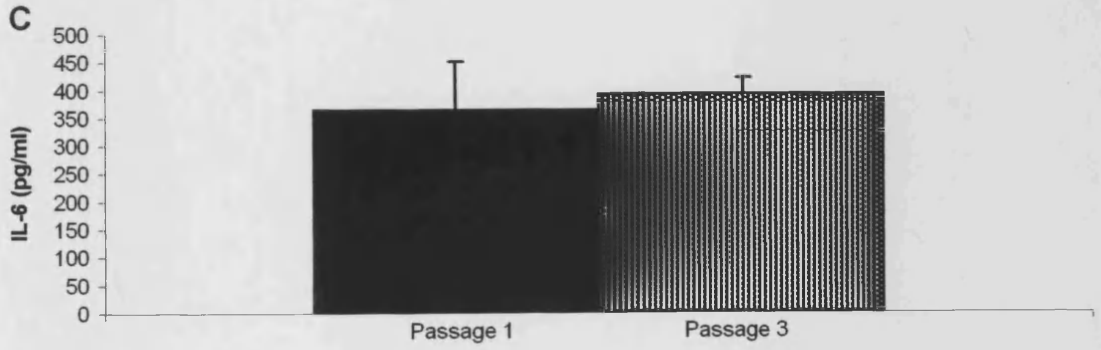

*
600
500
400
300
200
100
0
IL-8 (pg/ml)
Passage 1
Passage 3

**Figure 5.3.2** A) Media tumour-necrosis factor α; B) media interleukin-1; C) media interleukin-6 and D) media interleukin-8 concentrations from synovial cells at passage 1 and passage 3 from an OA patient. Cells were serum deprived for 24 hours, then fresh serum free media added and the media sampled at 24 hours. Error bars represent standard deviations. $^{*}P<0.05$.


A
TNFa (pg/ml)
70
60
50
40
30
20
10
0
Passage 1
Passage 3


B
IL-1 (pg/ml)
18
16
14
12
10
8
6
4
2
0
Passage 1
Passage 3


C
IL-6 (pg/ml)
500
450
400
350
300
250
200
150
100
50
0
Passage 1
Passage 3

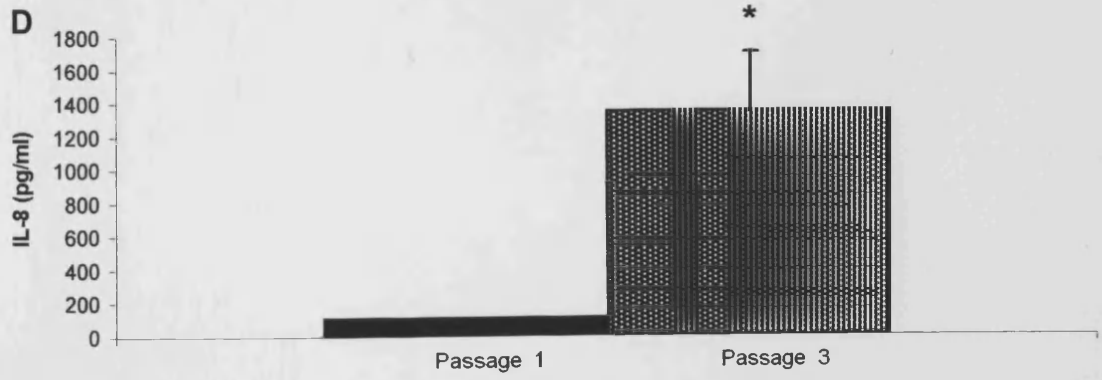
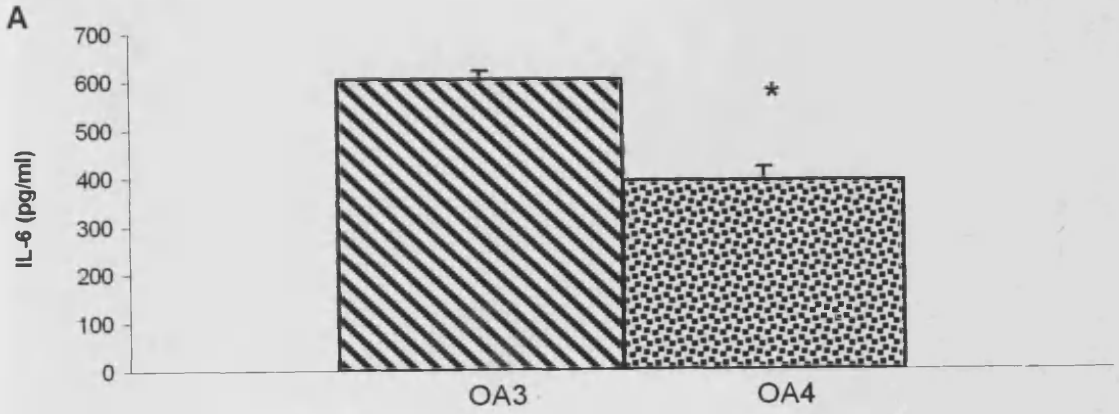

D
*
IL-8 (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
Passage 1
Passage 3

**Figure 5.3.3** A) Media interleukin-6 and B) media interleukin-8 concentrations from synovial cells from two different OA patients. Cells were serum deprived for 24 hours, then fresh serum free media added and the media sampled at 24 hours. Error bars represent standard deviations. $^{*}P<0.05$.


A
IL-6 (pg/ml)
700
600
500
400
300
200
100
0
*
OA3
OA4

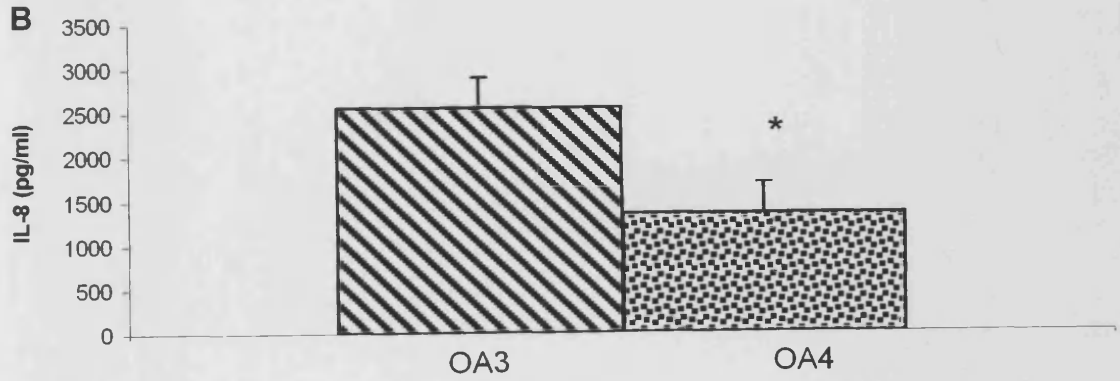
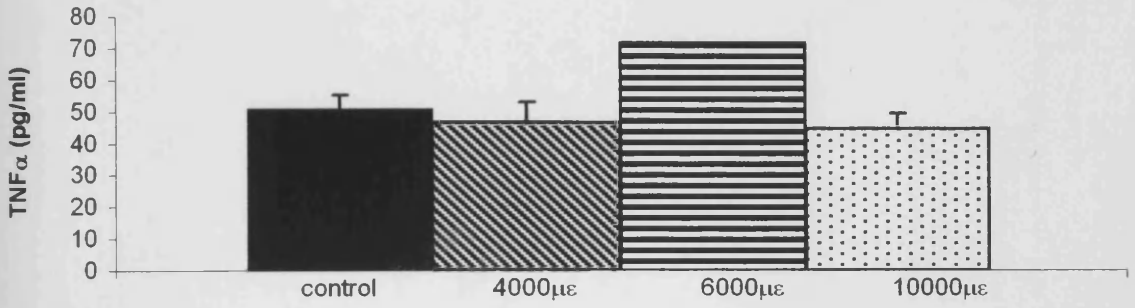

B
IL-8 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
*
OA3
OA4

**Figure 5.3.4** A) Media tumour-necrosis factor α and B) media interleukin-1 concentrations. Normal passage 3 synovial cells were subjected to mechanical strain and the media sampled at 24 hours. Control synovial cells did not undergo strain. Error bars represent standard deviations.


TNFα (pg/ml)
80
70
60
50
40
30
20
10
0
control
4000με
6000με
10000με

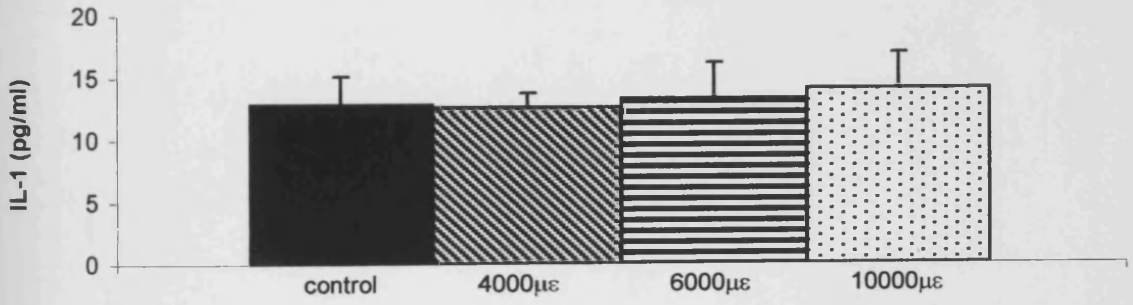
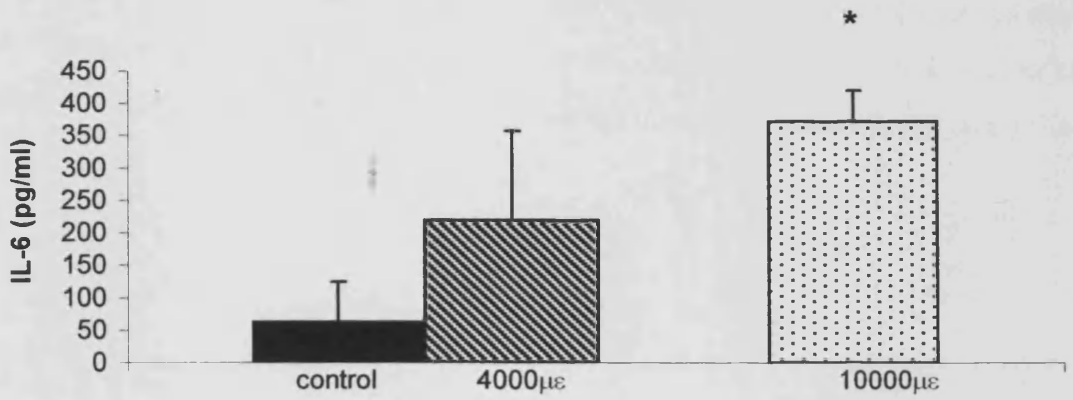
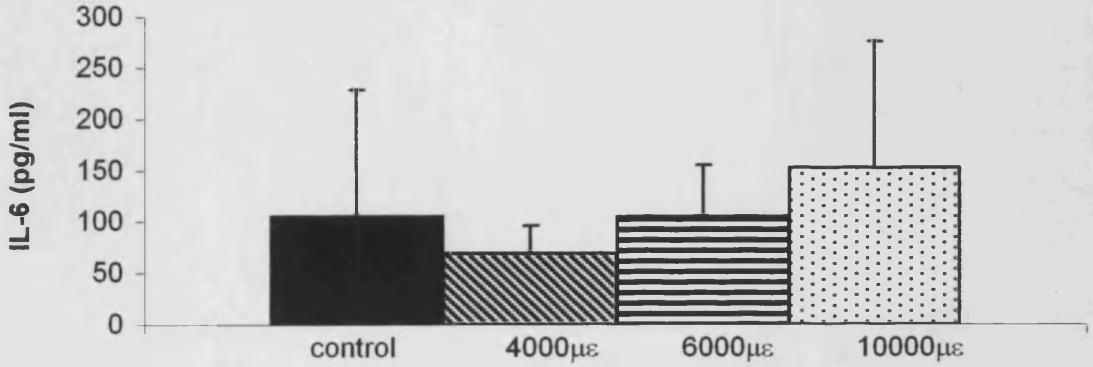

IL-1 (pg/ml)
20
15
10
5
0
control
4000με
6000με
10000με

**Figure 5.3.5** Media interleukin-6 concentrations from Patient N1. Normal synovial cells were subjected to mechanical strain and the media sampled at 24 hours. Control synovial cells did not undergo strain. Error bars represent standard deviations. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. $^{*}P<0.05$ compared to control cells.

A


*
450
400
350
300
250
200
150
100
50
0
IL-6 (pg/ml)
control
4000με
10000με

B


300
250
200
150
100
50
0
IL-6 (pg/ml)
control
4000με
6000με
10000με

C

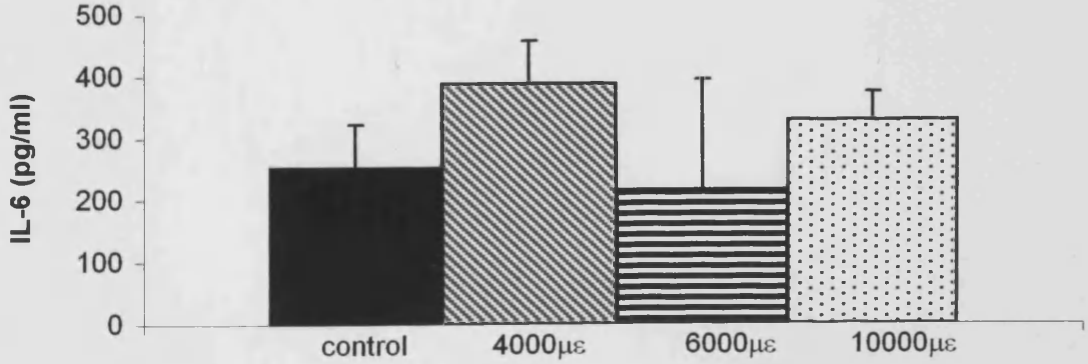
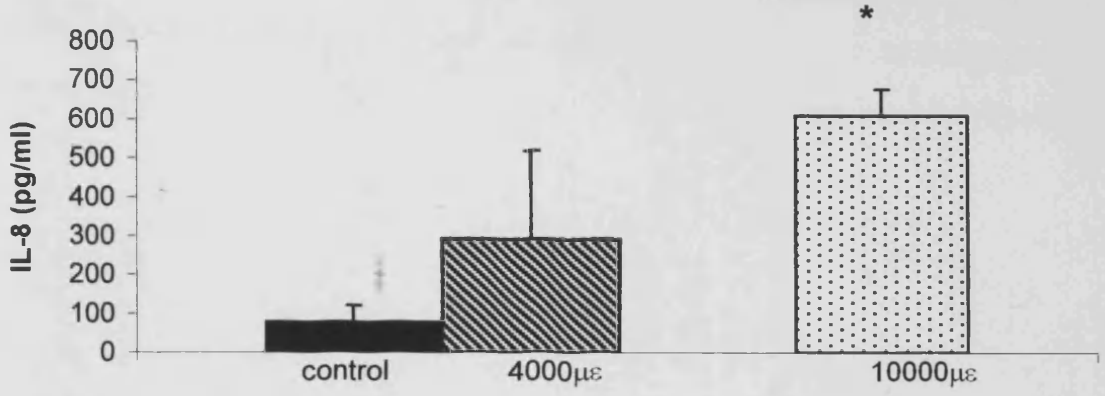
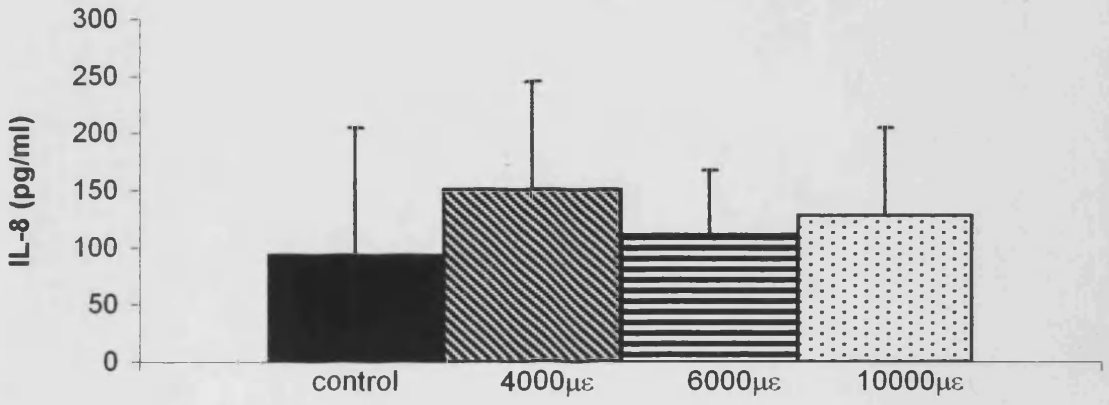

500
400
300
200
100
0
IL-6 (pg/ml)
control
4000με
6000με
10000με

**Figure 5.3.6** Media interleukin-8 concentrations from Patient N1. Normal synovial cells were subjected to mechanical strain and the media sampled at 24 hours. Control synovial cells did not undergo strain. Error bars represent standard deviations. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. *$P<0.05$ compared to control cells.

A


*
800
700
600
500
400
300
200
100
0
IL-8 (pg/ml)
control
4000με
10000με

B


300
250
200
150
100
50
0
IL-8 (pg/ml)
control
4000με
6000με
10000με

C

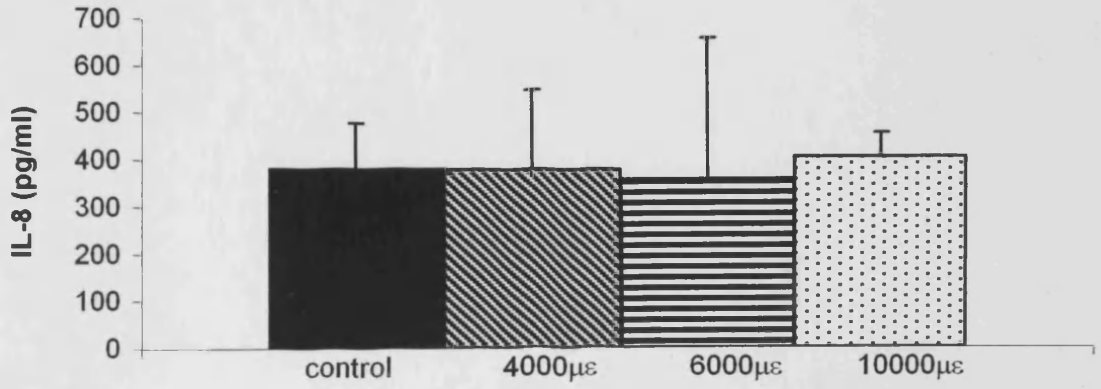
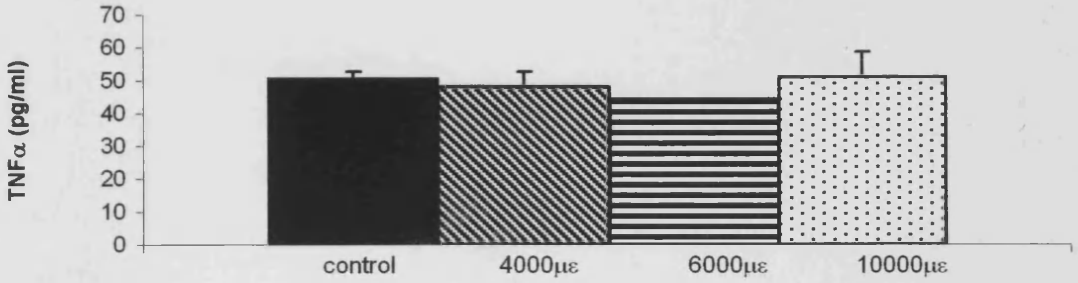

700
600
500
400
300
200
100
0
IL-8 (pg/ml)
control
4000με
6000με
10000με

**Figure 5.3.7** A) Media tumour-necrosis factor α and B) media interleukin-1 concentrations. OA passage 3 synovial cells were subjected to mechanical strain and the media sampled at 24 hours. Control synovial cells did not undergo strain. Error bars represent standard deviations.

A


TNFα (pg/ml)
70
60
50
40
30
20
10
0
control
4000με
6000με
10000με

B

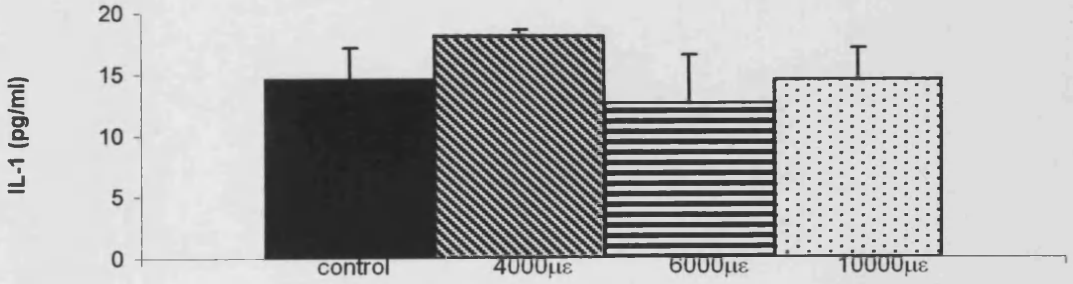
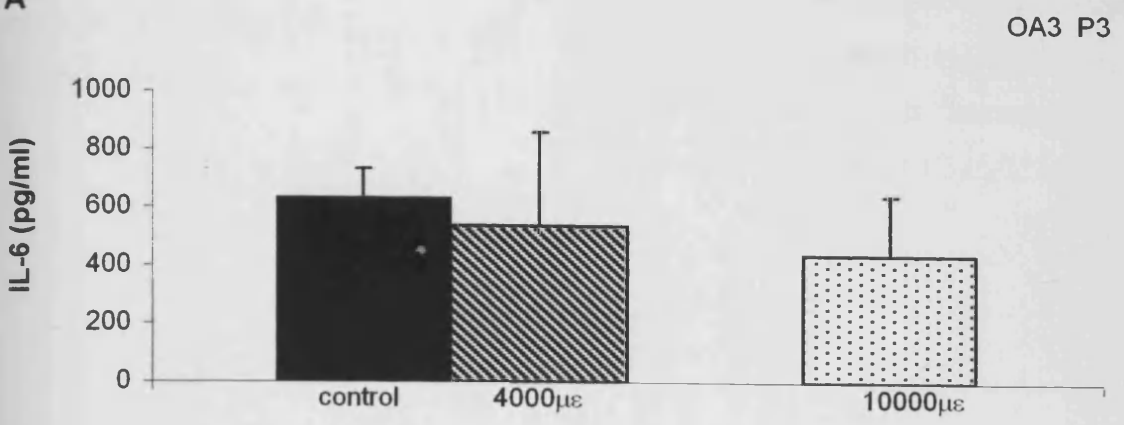
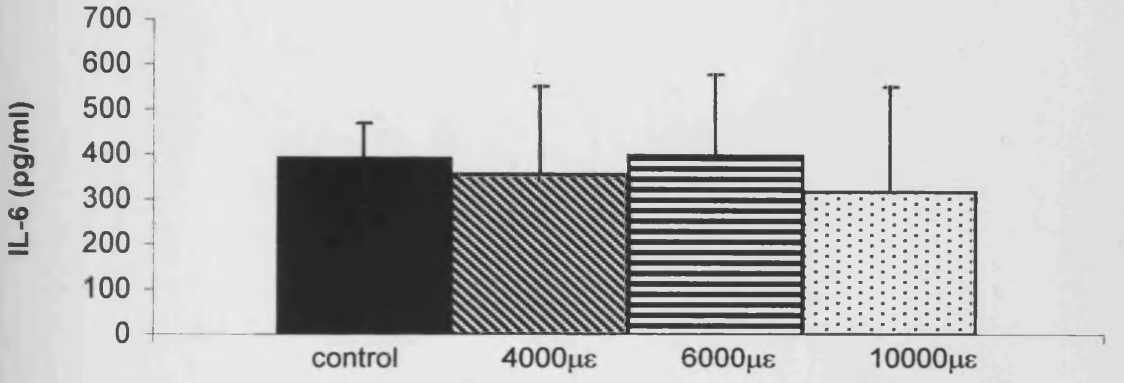

IL-1 (pg/ml)
20
15
10
5
0
control
4000με
6000με
10000με

**Figure 5.3.8** Media interleukin-6 concentrations from A) patient OA3; B) patient OA4 and C) patient OA6. OA synovial cells were subjected to mechanical strain and the media sampled at 24 hours. Control synovial cells did not undergo strain. Error bars represent standard deviations.

A


OA3 P3
IL-6 (pg/ml)
1000
800
600
400
200
0
control
4000με
10000με

B


OA4 P3
IL-6 (pg/ml)
700
600
500
400
300
200
100
0
control
4000με
6000με
10000με

C

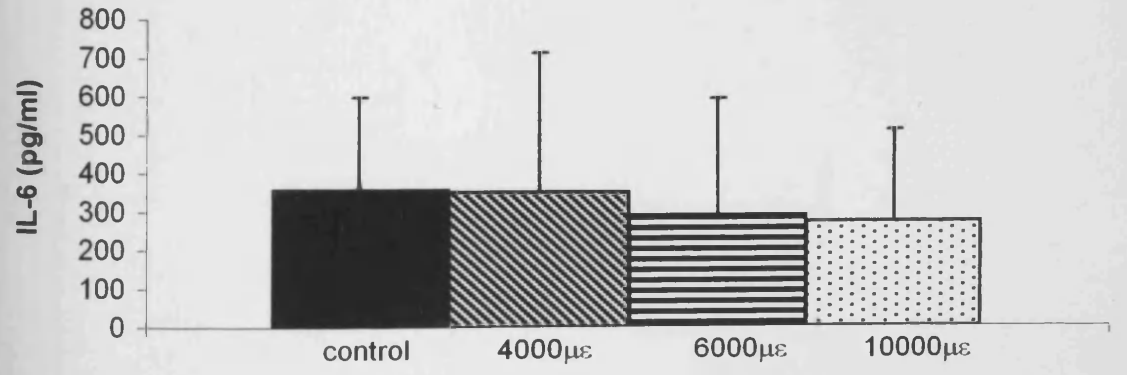

OA6 P3
IL-6 (pg/ml)
800
700
600
500
400
300
200
100
0
control
4000με
6000με
10000με

**Figure 5.3.9** Media interleukin-8 concentrations from Patient OA4. Passage 1 OA synovial cells were subjected to mechanical strain and the media sampled at 24 hours. Control synovial cells did not undergo strain. Error bars represent standard deviations. *$P<0.05$ compared to control cells.

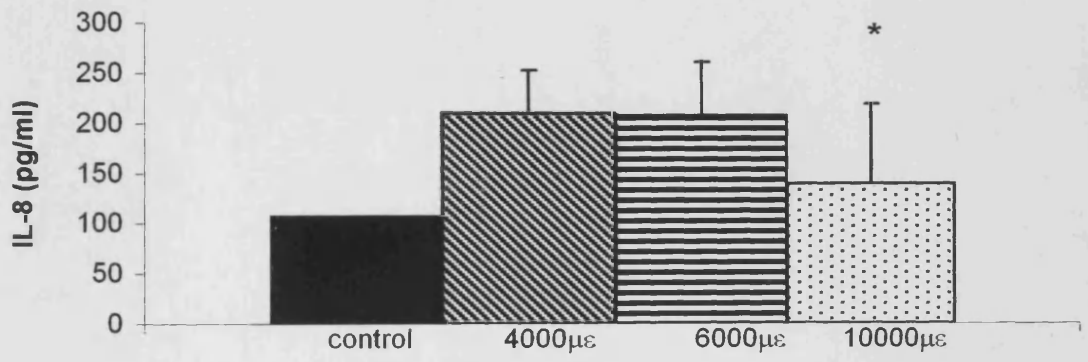
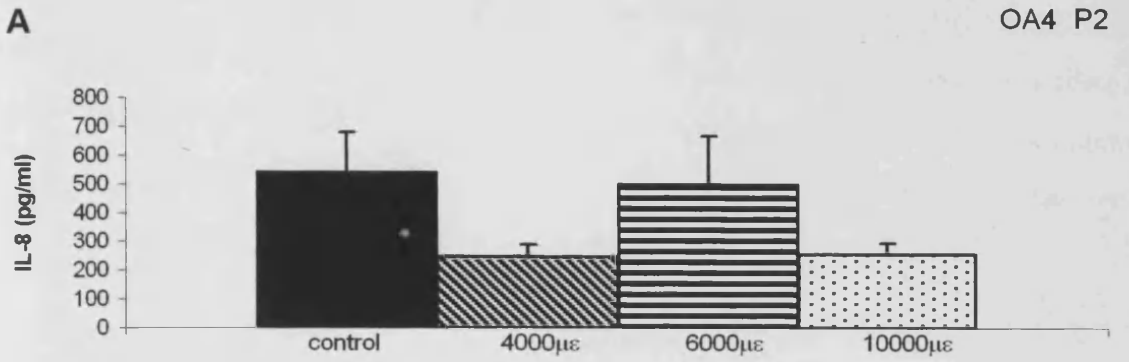
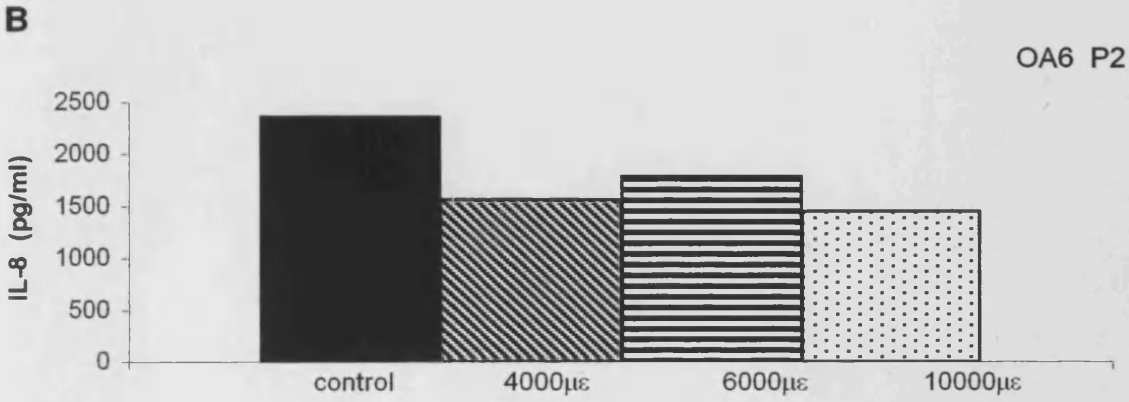
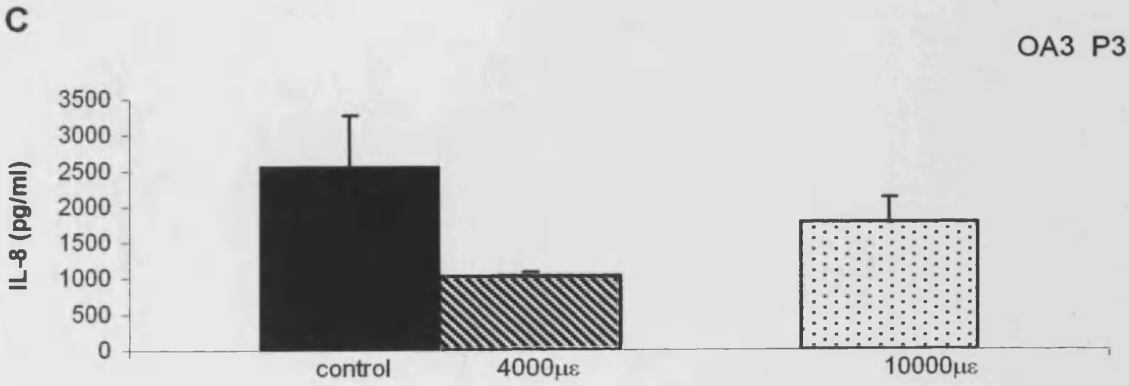

IL-8 (pg/ml)
300
250
200
150
100
50
0
*
control
4000με
6000με
10000με

**Figure 5.3.10** Media interleukin-8 concentrations from A) patient OA4 and B) patient OA6 at passage 2; C) patient OA3 and D) patient OA6 at passage 3. OA synovial cells were subjected to mechanical strain and the media sampled at 24 hours. Control synovial cells did not undergo strain. Error bars represent standard deviations.


A
OA4 P2
IL-8 (pg/ml)
800
700
600
500
400
300
200
100
0
control
4000με
6000με
10000με


B
OA6 P2
IL-8 (pg/ml)
2500
2000
1500
1000
500
0
control
4000με
6000με
10000με


C
OA3 P3
IL-8 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
control
4000με
10000με

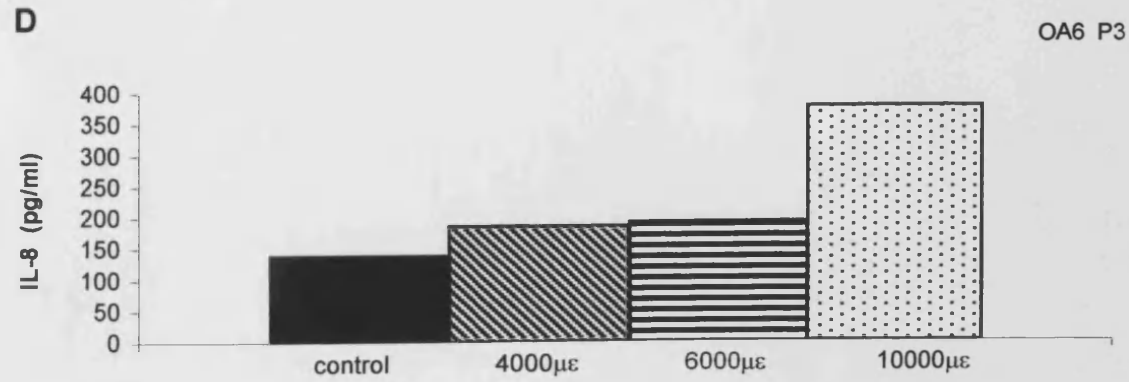

D
OA6 P3
IL-8 (pg/ml)
400
350
300
250
200
150
100
50
0
control
4000με
6000με
10000με

**Figure 5.3.11** Media TNFα concentrations from normal and OA synovial cells. Cells were subjected to mechanical strain, the media sampled at 24 hours and the percentage differences between the control and strained cells calculated. Error bars represent standard deviations.

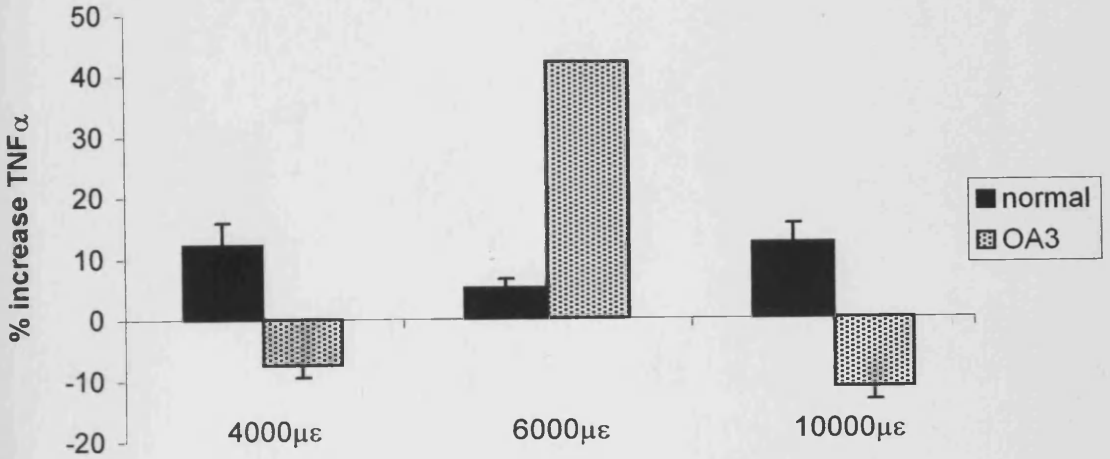

50
40
30
20
10
0
-10
-20
% increase TNFα
4000με
6000με
10000με
normal
OA3

**Figure 5.3.12** Media interleukin-1 concentrations from normal and OA synovial cells. Cells were subjected to mechanical strain, the media sampled at 24 hours and the percentage differences between the control and strained cells calculated. Error bars represent standard deviations. *$P<0.0001$ compared to normal cells.

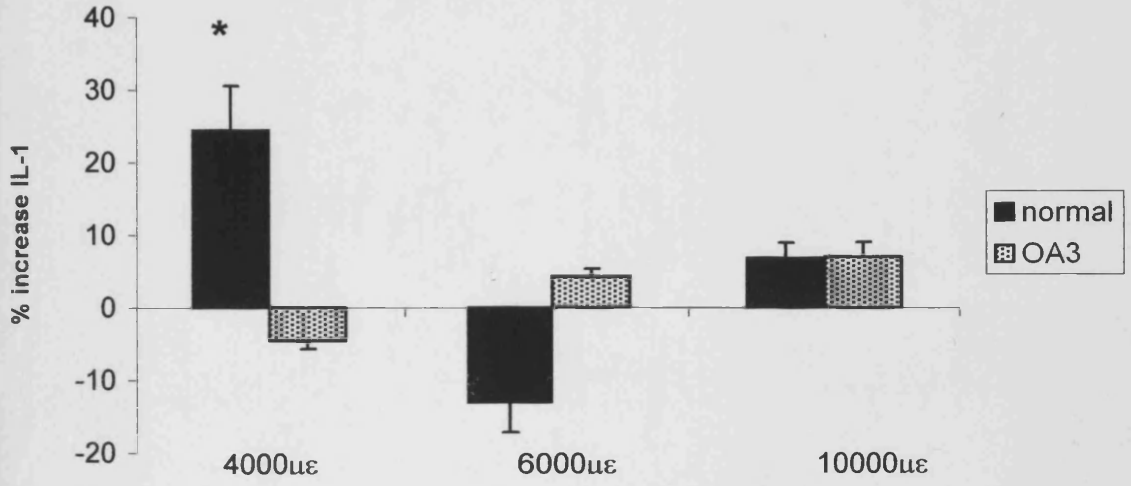
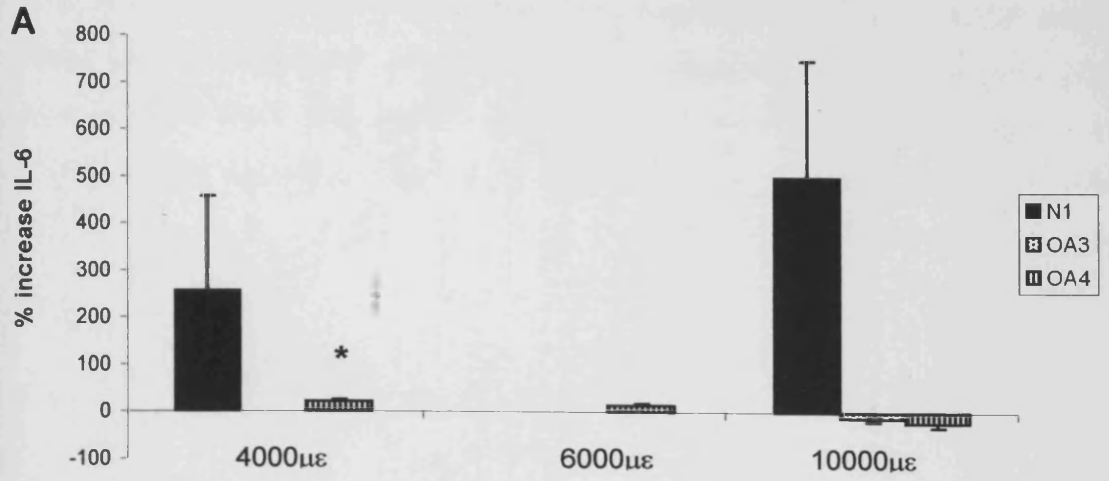
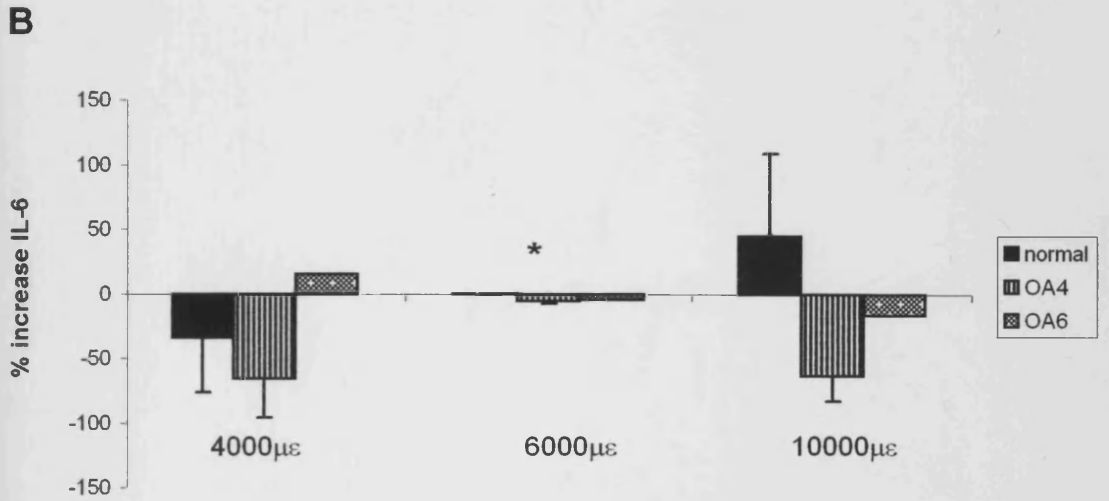

40
30
20
10
0
-10
-20
% increase IL-1
*
4000με
6000με
10000με
normal
OA3

**Figure 5.3.13** Media interleukin-6 concentrations from normal and OA synovial. Cells were subjected to mechanical strain, the media sampled at 24 hours and the percentage differences between the control and strained cells calculated. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. Error bars represent standard deviations. *$P<0.05$ compared to normal cells.


A
% increase IL-6
800
700
600
500
400
300
200
100
0
-100
*
4000με
6000με
10000με
N1
OA3
OA4


B
% increase IL-6
150
100
50
0
-50
-100
-150
*
4000με
6000με
10000με
normal
OA4
OA6

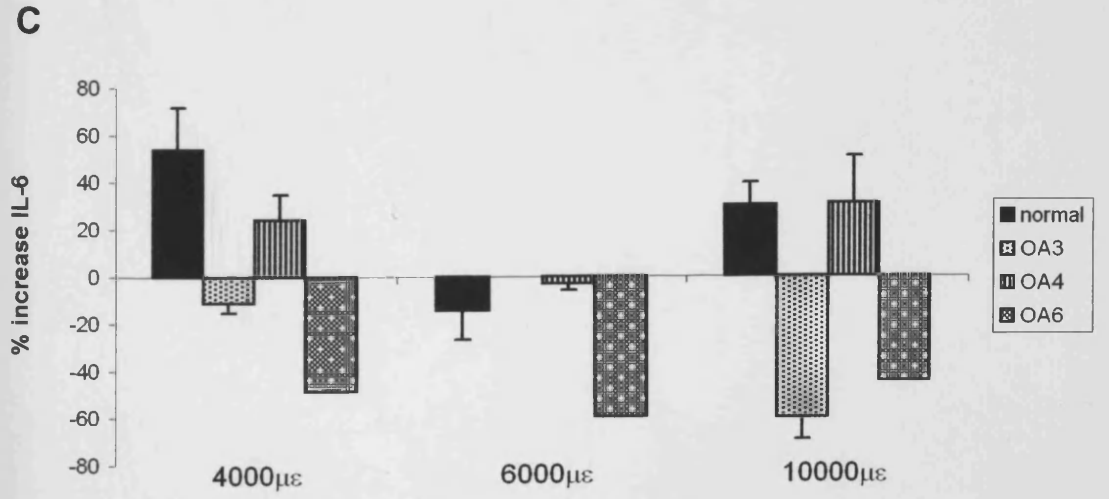
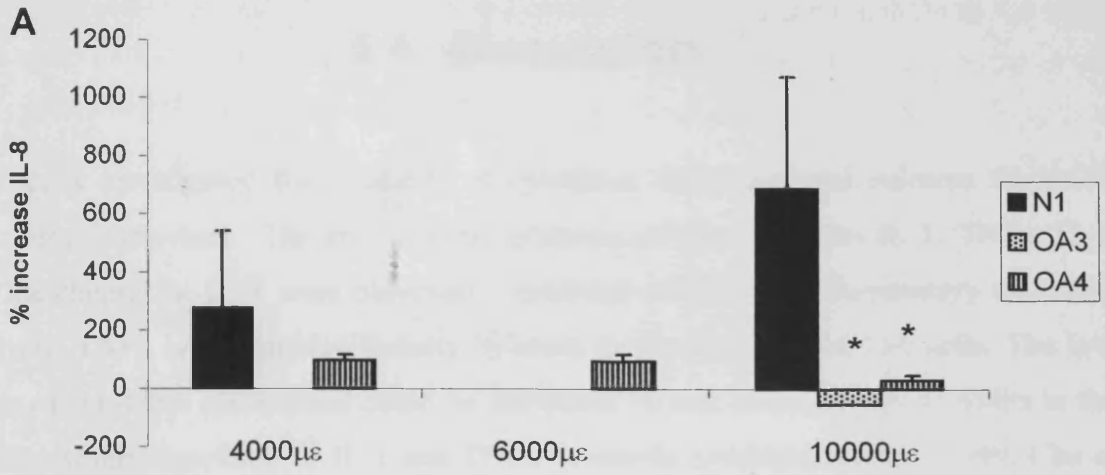
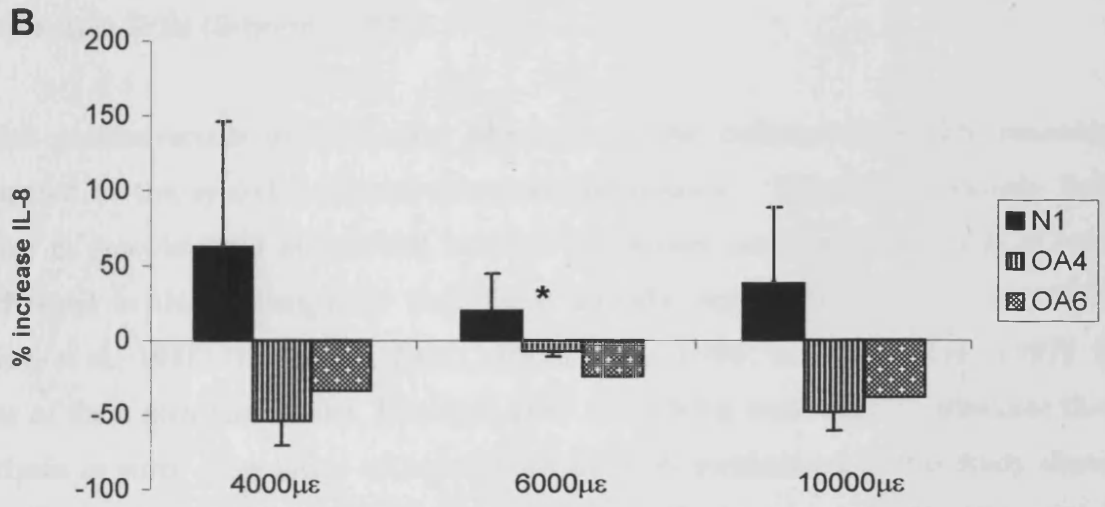
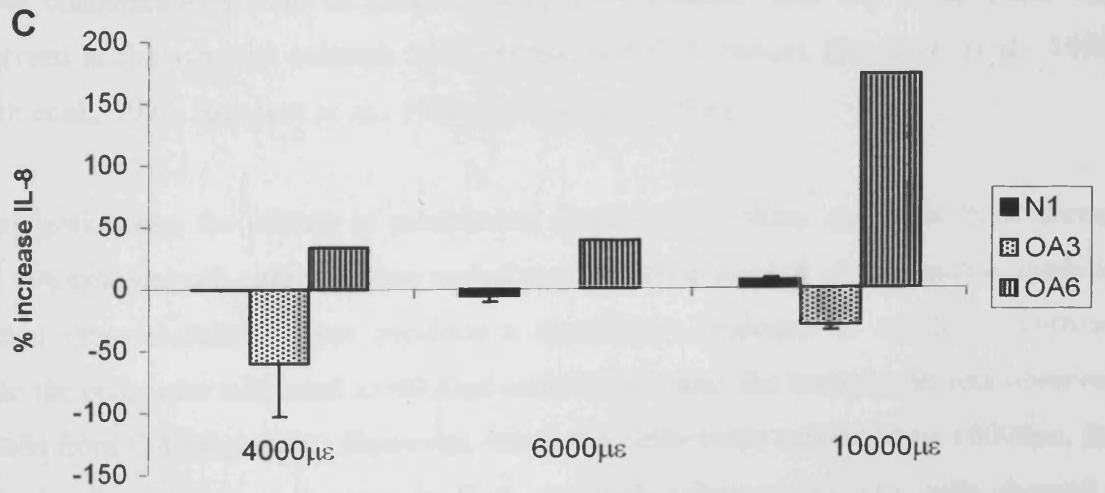

C
% increase IL-6
80
60
40
20
0
-20
-40
-60
-80
4000με
6000με
10000με
normal
OA3
OA4
OA6

**Figure 5.3.14** Media interleukin-8 concentrations from normal and OA synovial. Cells were subjected to mechanical strain, the media sampled at 24 hours and the percentage differences between the control and strained cells calculated. A) passage 1 cells; B) passage 2 cells; C) passage 3 cells. Error bars represent standard deviations. *$P<0.05$ compared to normal cells.


A
% increase IL-8
1200
1000
800
600
400
200
0
-200
4000με
6000με
10000με
N1
OA3
OA4
*
*


B
% increase IL-8
200
150
100
50
0
-50
-100
4000με
6000με
10000με
N1
OA4
OA6
*


C
% increase IL-8
200
150
100
50
0
-50
-100
-150
4000με
6000με
10000με
N1
OA3
OA6

## 5.4 Discussion

This study investigated the synthesis of cytokines from synovial cultures from OA and normal synovium. The spontaneous synthesis of the cytokines IL-1, TNFα, IL-6 and the chemokine IL-8 were observed. Synthesis of the pro-inflammatory cytokines IL-1 and TNFα were not significantly different in the normal and OA cells. The low levels of cytokines synthesised could be attributed to the low numbers of SMPs in the cultures which contribute to IL-1 and TNFα synthesis (Alsalameh et al., 1999; Chu et al., 1991; Okamoto et al., 1997). The SMPs interact with SFBs and loss of this interaction could lead to a withdrawal of cytokine stimulation *in vitro* and subsequent alterations in SFBs (Edwards, 1999).

Higher concentrations of IL-6 were observed in the cultures from OA synovium compared to the synovial cultures from normal tissues. This has previously been shown in synovial fluid and in cell cultures yet, lower concentrations of IL-6 were synthesised in this investigation than those already reported.(Bucala et al., 1991; Hirano et al., 1988; Hirth et al., 2002; Mihara et al., 1995; Okamoto et al., 1997) In some of these previous studies, however, IL-1 and TNFα were used to stimulate IL-6 synthesis *in vitro*. The lower concentrations of IL-6 synthesised in this study shows the ability of the cells to constitutively secrete IL-6 (Guerne et al., 1989; Okamoto et al., 1997). As with IL-6, IL-8 is detected in the synovial fluid of normal patients at lower concentrations than in patients with joint disease and the same trend was observed in the synovial cultures from normal and OA tissues (Brennan et al., 1990; Koch et al., 1992; Rampart et al., 1992; Seitz et al., 1991).

Investigation into the effects of mechanical strain on cytokine synthesis from normal and OA synovial cell cultures gave varied results. Application of mechanical strain to normal synovial cells did not produce a significant response in cytokine synthesis when the cells were subjected to 4000με and 6000με and the same trend was observed in cells from OA synovium. However, when the cells were subjected to 10000με, the normal cells showed an increase in IL-8 synthesis whereas the OA cells showed a decrease. IL-8 is considered a potent chemoattractant involved in the process of

inflammation, so a decrease in this chemokine could reduce disease progression, although it must be taken into consideration that it is unlikely that one cytokine alone will be able to halt the disease (Deleuran et al., 1994; Koch et al., 1992). Mechanical strain of 10000με did not alter the synthesis of IL-1, TNFα or IL-6 in either the normal or the OA synovial cells.

A recent exercise based study has shown a reduction in IL-1 concentration within the synovial fluid, with reported decrease in joint pain (Messier et al., 2000). Indeed, IL-1 concentration was significantly decreased in OA cells compared to the normal cells when cells were subjected to 4000με. The higher magnitudes of strain showed no differences between IL-1 concentrations from normal or OA cells in response to strain. Lower concentrations of IL-6 were synthesised by the OA cells whereas normal cells showed higher IL-6 concentrations when subjected to the lower magnitudes of strain (4000με, 6000με). It is also interesting to note that it was the passage 1 and 2 cells that showed a response to strain, indicating that higher passage cells may not be able to respond to strain due to the length of time in culture or the loss of cytokine stimuli provided by the SMPs which reduce in number with passage (Hirth et al., 2002; Zimmerman et al., 2001). A difference between IL-8 synthesis from normal and OA cells was also observed in the lower passage cells. Here, higher levels of IL-8 were seen in the normal cells compared to the OA cells in response to both 6000με and 10000με. A decrease in IL-8 synthesis within the synovium and a loss of subsequent secretion into the OA joint could reduce the inflammatory effects associated with disease. Overall, the OA cells have responded to mechanical strain synthesising lower concentrations of cytokine compared to the normal cells.

Studies have shown the effects of strain on chondrocytes and tendon cells and these have shown strain does not induce or abrogate cytokine synthesis unless cells are stimulated with cytokines (Archambault et al., 2002; Gassner et al., 2000; Long et al., 2002). The low levels of cytokines synthesised by the synovial cells and the lack of response to strain may indicate that cytokine stimulation is needed for synovial cells to respond to mechanical strain. Previous work used cells from RA tissue which shows more of an inflammatory response than OA tissues. Consequently, synthesis of higher levels of cytokines are observed *in vivo* in RA tissues compared to OA tissues

which might explain the low levels of cytokines synthesised by the OA cells in this study.

The effect of passage number on cytokine synthesis also needs to be taken into consideration. Previous work has concentrated on the effects of MMP activity/synthesis in synovial cells and has shown these increase with strain (Sun and Yokota, 2002). These studies, however, used cells at passages 8 and greater whereas this investigation used cells at passage three or lower and could explain the differences in response to strain. Alterations in *in vitro* features of the synovial cells could be responsible for the differences in cytokine response to mechanical strain. It has been shown that infiltrating cells such as macrophages decrease with increasing passage number and the loss of the SFB and SMP interaction and subsequent loss of cytokine stimulation may be reflected in synovial cell response (Chomarat et al., 1995; Hirth et al., 2002; Marsh et al., 1978; Zimmerman et al., 2001).

Current treatments for OA and RA involve the blockade of the cytokine pathways however, the aim of this work was to investigate the effects of strain on cytokine synthesis in order to develop exercise based therapies as an alternative treatment for OA. This investigation was hampered by the low number of synovial samples used and needs to be taken into consideration when assessing the results. Overall, it can be seen that the synovial cells from both OA and normal synovium respond to the greatest magnitude of strain, which is higher than the physiological strains the joint undergoes during flexion (Lanyon, 1996). Mechanical strain does not however, affect all of the cytokines and chemokines investigated. At present, this work does not provide enough evidence necessary to show that cytokine production can be modulated by mechanical strain but does show OA and normal cells are responsive to mechanical strain and both respond differently. Whether mechanical strain can be used to modulate cytokine synthesis will require further studies on a greater number of synovial samples.

# Chapter 6

# Effects of hTERT Infection on Synovial Cells

# 6.0 Introduction

A two stage model for the control of cellular growth has been proposed by Shay et al., (1991). The first, or M1 (mortality stage 1) represents the onset of senescence in normal cells. Replicative cell senescence is characterised by continued cell viability without further cell division yet alterations in gene expression and phenotype are observed and can be overridden in certain cell populations leading to an extended life span (Allsopp et al., 1995; Allsopp and Harley, 1995; Bodnar et al., 1998; Martin et al., 2002; Wei and Sedivy, 1999). Eventually, however, cell proliferation will be stopped in a stage initially defined as crisis or M2 (mortality stage 2). Here, karyotypic instability and widespread apoptosis occurs (Maser and DePinho, 2002; Wei and Sedivy, 1999). Cells that survive M2 are considered immortal in that they can divide infinitely.

Replication of linear eukaryotic chromosomes during cell proliferation poses a problem as DNA polymerase is unable to synthesise DNA in 3'- 5' direction or start DNA chain synthesis *de novo*. The presence of telomerase, a functional ribonucleoprotein complex consisting of an integral RNA telomeric template (hTERC) and a reverse transcriptase subunit (hTERT) however, allows chromosome replication (Feng et al., 1995; Nakamura and Cech, 1998). Telomerase exists at the ends of chromosomes and in humans telomeres consist of hundreds and thousands of tandem repeats, which are simple, repetitive DNA sequences. The hexanucleotide telomeric sequence in humans is TTAGGG and it is this sequence that acts as an RNA template for telomerase. The RNA template of the telomerase complex binds to the telomere and the reverse transcriptase subunit allows elongation of the DNA, beginning with the telomere. This mechanism of chromosome elongation during cell proliferation ensures the maintenance of chromosome length. The loss of telomeres leads to end-to-end chromosomal fusions, facilitates increased genetic recombination and triggers cell death through apoptosis, as can be seen to occur in the M1 stage of growth. (Blasco et al., 1997; Counter et al., 1992; Knauper et al., 1996). The ability of the cells to escape M2 is thought due to the restoration and/or stabilisation of telomere length.

Loss of between 50-200 nucleotides of the telomeric sequence at each cell division results in the progressive shortening of telomere length and it is this shortening of the telomere that is thought to control entry into senescence leading to genetic instability. (Chang and Harley, 1995; Harley, 1997; Hastie et al., 1990; Lee et al., 1998). Once the telomere reaches a critical length, the cell will no longer be able to undergo replicative proliferation and so becomes senescent (Kipling and Faragher, 1999; Reddel, 1998). The disruption of telomerase activity has been shown to cause telomere shortening and cell senescence (Yu et al., 1990) whereas the activation of telomerase can result in telomere lengthening and cell immortilisation (Bodnar et al., 1998; Counter, 1998). These data suggest that there is a critical telomere length necessary to prevent senescence and that telomerase activity is involved in controlling cell senescence.

Normal human somatic tissues have been shown to lack telomerase activity and in these cell types, telomere shortening is thought to act as a molecular counting device, indicating the number of times a cell has divided, triggering proliferative arrest when the telomeres become too short (Harley et al., 1990; Kim et al., 1994; Wright and Shay, 2002). Telomere length can be seen to decrease with age and is considered a mechanism controlling cellular aging by limiting cell proliferation (Harley et al., 1990; Prowse and Greider, 1995). Hayflick *et al.*, (1961) showed cell growth arrest was entered to *in vitro* after a characteristic number of cell doublings and this depended on cell type. For example, human fibroblasts undergo on average 60 population doublings whereas chondrocytes undergo between 30-35 population doublings before becoming senescent (Allsopp and Harley, 1995; Kolettas et al., 1995). In addition to time in culture, the age of the donor seems to affect cellular senescence. Hayflick (1965) and Campsi (2000) both demonstrated that cells cultured from older individuals exhibited a lower proliferative lifespan than those from younger individuals and a relationship between number of population doublings and the life span of the organism in chondrocyte cultures was demonstrated by Adolphe (1983). Indeed, *in vivo* age related changes in phenotype such as decreased response to growth factors, decreased production of extracellular matrix components and increased apoptosis occurs in relation to accumulating senescent cells (Adams and Horton, 1998; Guerne et al., 1995; Price et al., 2002). A decrease in telomere length *in vitro* occurs in human fibroblasts and chondrocytes with increasing passage number

and if the same occurs *in vivo*, this could be the mechanism controlling cellular ageing by limiting cell proliferation (Harley et al., 1990; Parsch et al., 2003; Prowse and Greider, 1995). Further research however, disputes the relationship of donor age and cellular senescence. Cells from old and young donors were shown to senesce at the same point *in vitro* suggesting cell proliferation *in vitro* is not simply related to age (Cristofalo et al., 1998). The influence of many genetic and epigenetic factors involved in cell proliferation are thought to affect telomerase activity and, as a result, telomeres have been shown to be longer in an older donor compared to that of a younger donor (Harley, 1997; Reddel, 1998). Additional work demonstrating that although humans have a longer life span than rodents, it is rodents that have the longest telomeres (Kakuo et al., 1999). Thus, the length of the telomere might not be the critical factor in cell senescence. As long as telomerase maintains a stable telomere length during cell proliferation then cell senescence is prevented (Allsopp et al., 1992; Greider, 1990; Harley et al., 1990; Prescott and Blackburn, 1997; Roy et al., 1998). Furthermore, telomerase length may have a protective effect on short telomeres by preventing further shortening once a critical telomere size has been achieved, above this critical size telomerase activity is not required (Yang et al., 1999; Zhu et al., 1999).

The ability to maintain cellular life span while preserving the diploid status of growth characteristics, gene expression patterns and phenotype of primary cells has important implications for biological research. Access to cells usually limited by life span *in vitro* or availability would be a useful tool to the researcher. As a result, immortalisation of cell types is a rapidly expanding area of research. Extension of the life spans of human somatic cell types including endothelial cells, bone marrow stromal cells, retinal pigment cells and skin fibroblasts has been increased by the transfection of telomerase cDNA (hTERT) (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999; Simonsen et al., 2002; Steinert et al., 2000; Yang et al., 1999). Normal cells showed a limited proliferative potential *in vitro* with characteristics of a senescent phenotype. The ectopic expression of telomerase reverse transcriptase hTERT however, inhibited replicative senescence and the cells maintained a normal differentiation potential *in vitro* (Simonsen et al., 2002). Phenotypic markers were maintained and often resembled those of a younger phenotype and the cells did not acquire transformed or malignant characteristics (Jiang et al., 1999; Morales et al.,

1999; Simonsen et al., 2002; Young et al., 2003). The increase in population doubling (PD) number varied between cell type. An increase of 10 PD was observed in chondrocytes whereas a 6 fold increase was seen in human mesenchymal stem cells from bone (Simonsen et al., 2002). The PD rate also equalled that of young cells. The reason for this increase in replicative capacity is thought to be because of the stabilisation in telomere length or an actual increase in telomere length by the introduction of ectopic hTERT (Bodnar et al., 1998; Wood, 2001).

Conclusions drawn for previous work on synovial cells suggest that cultures from the same passage should be used for experimentation as synovial cell cultures in long-term *in vitro* studies showed alterations in cell type, cell morphology and growth rates (Lories et al., 2003; Marsh et al., 1978; Zimmerman et al., 2001). Rapid proliferation of cells occurs in lower passage cells yet this is seen to decrease in cells from higher passage cultures (beyond passage 15) (Clarris and Fraser, 1968; Saksela, 1962). The change in morphological features and growth rates is associated with chromosomal deviations and also the loss of individual cell populations (Castor, 1960; Clarris and Fraser, 1968; Saksela, 1962). The synovial cultures have a finite lifespan *in vitro* and long-term cultures show detrimental morphological alterations which result in cell senescence and eventual cell death (Bartfeld, 1965; Hayflick, 1965; Martin et al., 1970). Establishment of an immortalised cell line providing a limitless supply of heterogeneous synovial cells would aid greatly in overcoming the problems associated with primary synovial cell culture.

A rabbit synovial fibroblast cell line, HIG-82 has been established (Georgescu et al., 1988). This cell line however, was produced by spontaneous transformation of an ageing late-passage population of primary cells. The HIG-82 cell line can be activated by a number of stimuli, such as phorbol myristate acetate (PMA) and can express similar SFB cell markers as primary synovial cells, for example CD44 (Galea-Lauri et al., 1993; Georgescu et al., 1988). The HIG-82 cells do not respond to IL-1 in exactly the same manner as SFBs, the responsiveness to IL-1 is lost probably as a consequence of repeated-passage (Gouze et al., 2003; Lin et al., 1996). The HIG-82 cells do however, synthesise factors capable of inducing synthesis of $PGE_2$ and collagenase by chondrocytes (Watanabe et al., 1986). Synovial fibroblasts (SFBs) usually synthesise no or little nitric oxide (NO) and HIG-82 cells show the

same trend *in vitro* (Stefanovic-Racic et al., 1994). When both SFBs and HIG-82 cells are exposed to IL-1 and TNFα, SFBs increase NO synthesis whereas HIG-82 cells show no change in NO synthesis. Thus, synovial cells have the potential to be immortalised but there exists a difference between these cells and the SFBs that needs to be considered during experimentation.

The aim of this work was to immortalise human synovial fibroblasts from normal, OA and RA donors. At present, limited availability and the variability between donors is disadvantageous when using SFBs for research. Generation of an immortalised SFB cell line with an extended life span and that maintains characteristics of an early culture would benefit research, allowing study of the disease at the cellular level.

## 6.2 Materials and Methods

### 6.2.1 Synovium Samples

Synovium samples were obtained as in Chapter 2 (2.2.1). Details of the samples are listed below.

| Sample | Type | Sex | Age (years) |
|---|---|---|---|
| OA1 | OA | Female | 57 |
| OA3 | OA | Female | 74 |
| OA2 | OA | Male | 64 |
| OA16 | OA | Male | 55 |
| OA17 | OA | Female | 75 |
| RA1 | RA | Male | 56 |
| N2 | normal | Unknown | 71 |
| N3 | normal | Female | 89 |
| N4 | normal | Female | 55 |
| N5 | normal | Unknown | Unknown |

**Table 6.2.1 Details of synovium samples obtained at knee joint replacement surgery.**

### 6.2.2 Cell Isolation & Culture

Cells were isolated from the synovium as described in Chapter 2 (2.2.2). Briefly, diced tissue was digested in 295IU mg $ml^{-1}$ type I collagenase (Sigma, Poole, UK) for 3 hours at 37°C. Cells were centrifuged at 1000 x g for 5 minutes, resuspended in DMEM/F12+, then centrifuged at 1000 x g for 5 minutes. Cells were counted and seeded as required in DMEM/F12 containing 1% penicillin-streptamycin ($10^4$IU $ml^{-1}$ /$10^4$μg $ml^{-1}$; Sigma), L-glutamine (200mM; Life Technologies, Paisley, UK), hydrocortisone (4mg $ml^{-1}$; Sigma)[40mg hydrocortisone powder dissolved in 5ml 100% ethanol and 5ml sterile PBS], Insulin-Transferrin-Selenium (200mM; Life Technologies) and 20% foetal calf serum (Sigma) and incubated at 37°C.

### 6.2.3 Construction of Vector

The human telomerase cDNA was provided by Geron Corporation (Menlo Park, Ca, USA). The telomerase retroviral expression plasmid was constructed by inserting the human telomerase cDNA into the *pBABEpuro* vector from Clontech (Palo Alto, Ca, USA) (appendix 6.1). A control plasmid consisting of the *puro* resistant gene with no telomerase cDNA was also constructed. Briefly, 5μg of either control vector plasmid or telomerase construct was transfected into the *ψCRIP* packaging cell line using the Profection Calcium phosphate kit as per manufacturers instructions (Promega UK, Southamton, UK). Cells that had taken up the vector were then selected by culturing for 10 days in the presence of 350μg $ml^{-1}$ of G418. Retrovirus was pooled from the supernatant of stably transfected *ψCRIP* producing cells.

### 6.2.4 Infection & Selection of Synovial Cells

Infection was carried out by incubating monolayer synovial cells with 0.45μm-filtered supernatants of retroviral producing cells in the presence of 8μg $ml^{-1}$ of polybrene (Sigma). The infection medium was in contact with the synovial cells for 48 hours then cells were expanded with non-selective medium (DMEM/F12+). Cells were re-fed 48 hours later. Infected cells were submitted to selection for 14 days in the presence of 1.0μg $ml^{-1}$ puromycin (Sigma) before reverting to non-selective media.

Cells from three samples, (normal (N2), OA (OA1) and RA (RA1)) were infected at passage 9. In each case the cell lines were infected with *pBABEpuro*-telomerase retrovirus or with *pBABEpuro* retrovirus as a control. Cells were labelled as shown in table 6.2.2.

| | Retroviral Infection | | |
|---|---|---|---|
| **Parent Cell Sample** | None | *pBABEpuro*-telomerase | *pBABE-puro* |
| **N2** | Normal | N-hTERT | N-chTERT |
| **OA1** | OA | OA-hTERT | OA-chTERT |
| **RA1** | RA | RA-hTERT | RA-chTERT |

**Table 6.2.2 Labelling of retroviral cell lines and non-infected cell lines.**

### 6.2.5 Telomeric Repeat Amplification Protocol (TRAP Assay)

Cells at selected passages were allowed to reach confluence, trypsinised (appendix 2.1) and then washed in PBS before being frozen at 100,000 cells per pellet in liquid nitrogen. These cell pellets were stored at -80°C until needed.

Cell pellets were defrosted at room temperature and lysis buffer (appendix 6.2) added proportional to the cell count (in most cases 5000 cells per μl). The samples were incubated at 4°C for 30 minutes then centrifuged at 100000 x g at 4°C. The supernatant was removed, divided into 2 separate samples and frozen at -80°C prior to the TRAP assay.

The first step of the TRAP assay uses nascent telomerase to extend the TS primer sequence (5'-AATCCGTCGAGCAGAGTT-3'). Cells were diluted 1:5 in lysis buffer (appendix 6.2) and each extract was split into 2 aliquots, one aliquot was incubated at 85°C for 10 minutes to destroy nascent telomerase activity (negative control). Human 293 kidney cells (CACCS) at 5000 cells $\mu l^{-1}$ were used as positive controls. Fifty μl of premix (appendix 6.1) consisting of reaction buffer (appendix 6.2), 100ng TS primer (MWG biotech Ltd, Milton Keynes, UK), and 1μg T4 gene 32 protein (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to 1μl of prepared cell extract. After capping with mineral oil, this mixture was incubated for 30 minutes at 30°C, followed by a soak at 92°C using a DNA Thermal cycler (Perkin Elmer).

The second step of the TRAP assay involves extension of the nascent telomerase products created in the above step using the polymerase chain reaction (PCR). Two μl of final mix (appendix 6.2) containing 100ng CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') (MWG biotech Ltd), 2.5U Taq Polymerase (Promega, Southampton, UK) and 0.5 x $10^{-18}$ of internal standard was added to each extract. The PCR was run for 31 cycles of 92°C for 30s; 50°C for 30s and 72°C for 90s with a final soak at 4°C.

Detection of the TRAP assay products involved loading 30µl of the samples onto a 10% polyacrylamide (19:1) 20cm Protean II gel containing 1.5 X tris borate EDTA (TBE) that was run at 300V for 3 hours. The gel was stained with Sybr Gold (Sigma) at a 1:10,000 dilution in $H_2O$ for 10 minutes. Excess stain was washed off and the gel destained for 3 minutes in $dH_2O$. Bands were visualized on a STORM ultraviolet light box (Amersham Biosciences) and the gel documented using Image Quant software (Molecular Dynamics).

### 6.2.6 Immunohistochemistry

Cells were fixed in cold 95% ethanol (Sigma) for 10 minutes or 0.4% paraformaldehyde (Sigma) depending on the antibody used and the cells washed in PBS. Labelling for collagen IV required enzymatic pre-treatment. The cells were equilibrated in 0.1M tris-acetate (Sigma) for 5 minutes, and then incubated in chondroitinase (0.25U/ml in tris-acetate pH7.8; Sigma) and hyaluronidase (1U/ml in tris-acetate pH7.8; Sigma) for 2 hours at 37°C. Cells were washed for 3 times (5 minutes each) in PBS-T and primary antibody applied.

| Name of clone | Antigen Specificity | Subclass | Source | Reference |
|---|---|---|---|---|
| BRIC 128 | CD55 | IgM | IBGRL Research Products, Bristol. | Mushens R & Scott M (1990) J.Immunol.Meth. 131:83-89 |
| COL-94 | Collagen Type IV | IgM | Sigma, Poole, UK | Sanes JR *et al* (1990) Journal Cell Biology 111:1685 |
| HM-2 | MAP-2 | IgG | Sigma, Poole, UK | Huber G & Matus A (1984) J.Neuroscience 4:151-160 |

**Table 6.2.3 List of synovial fibroblast markers.**

Cells were labelled with a variety of synovial fibroblast markers (table 6.2.3) and the immunohistochemistry was performed as described in Chapter 2 (2.2.3). Briefly, cells were incubated with blocking serum to prevent unspecific binding of primary antibody. Excess serum was removed and the primary antibody diluted (table 6.2.4) in PBS-T, applied to the cells and incubated overnight at 4°C. The cells were washed and incubated with secondary antibody. Alexa fluor 488 (Molecular Probes Europe,

The Netherlands) used depended on the primary antibody (table 6.2.4). The secondary antibody was diluted to 10μg $ml^{-1}$ in 1% blocking serum in PBS for 60 minutes at room temperature. Cells were washed in PBS and mounted in Vectashield containing 1μg $ml^{-1}$ propidium iodide (Vector Labs, UK).

Negative controls consisted of PBS-T instead of primary antibody or Mouse/Rabbit IgGs. Positive controls used were frozen sections of synovium due to the lack of human cell cultures available.

| Antigen Specificity | Fix | Blocking Serum | Dilution of Antibody | Alexa Fluor 488 |
|---|---|---|---|---|
| CD55 | 95% EtOH | Goat | 1:10 | Goat α mouse |
| Collagen TypeIV | 95% EtOH | Goat | 1:500 | Goat α mouse |
| MAP-2 | 4% paraformaldehyde | Goat | 1:800 | Goat α mouse |
| PGP9.5 | 95% EtOH | Goat | 1:400 | Goat α rabbit |

**Table 6.2.4 Protocol for antibody labelling.**

## 6.2.7 Population Doublings

Confluent cultures were subjected to enzymatic dissociation with trypsin (appendix 2.1) and cell numbers determined by counting 2 aliquots of the resulting suspension in a haemocytometer. Cells were re-seeded at 50,000 cells per T25 flask. The population doublings were calculated using the following equation:

$$PD = Log_{10} (N/N_0) \times 3.33$$

where N is the number of cells at trypsinisation and $N_0$ is the number of cells initially seeded (Cristofalo et al., 1998).

Remaining cells were seeded into 35mm dishes for cell senescence assays, immunohisochemistry or RNA isolation, or were frozen and stored at -80°C.

### 6.2.8 Senescence Associated β-Galactosidase Staining

Cells were washed in PBS and fixed in 0.4% formaldehyde for 3 minutes at room temperature then washed in PBS. Fresh senescence associated β-Gal stain solution (SA-β-Gal) was made immediately before use and consisted of 1mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (β-Gal;Sigma) dissolved in 1ml dimethylformamide. B-gal was then added to 100ml 40mM citric acid (Sigma) / 40mM sodium phosphate (Sigma), pH6.0 / 5 mM potassium ferrocyanide (Sigma) / 5 mM potassium ferricyanide (Sigma) / 150 mM sodium chloride (Sigma)/ 2 mM magnesium chloride (Sigma). Cells were incubated with the SA-β-Gal solution at 37°C for 16 hours. Cells were washed with $dH_2O$, then counterstained in haematoxylin for 1 minute. Cells were thoroughly washed in PBS and mounted with Vectashield (Vectorlabs). Young bovine chondrocytes were used as a negative control and late passage bovine chondrocytes were used as a positive control.

Three dishes from each sample were counted for total cell number and number of cells labelled for SA-β-gal senescent activity. A total of one hundred cells were counted in three random areas of each dish using a x20 objective lens. One-way ANOVA, wih Tukey post hoc analysis was used to calculate differences between infected and control synovial cells. This particular statistical test was used as it allowed the comparasion of more than 2 sets of data.

### 6.2.9 Gene Expression

RT-PCR for hyaluronan synthase 2 (HAS2) mRNA expression was performed as described in Chapter 4 (4.2.4 & 5). Briefly, 10,000 cells were seeded in 35mm dishes and allowed to reach confluence, and RNA isolated using an RNeasy minikit (Qiagen Ltd, UK). The RNA was reverse transcribed to cDNA and the cDNA generated was amplified using the reverse transcriptase polymerase chain reaction and HAS2 specific primers as described in Chapter 4 (table 4.2.3). PCR products were run on a 1.5% (w/v) agarose/TBE gel and visualised using Quantity One software (Bio-Rad Labs).

## 6.3 Results

### 6.3.1 TRAP Assay

The TRAP assay demonstrated that there was no telomerase activity in synovial cells from normal, OA and RA cell samples (fig.6.3.1). The same normal, OA and RA cell samples when infected with the hTERT construct were positive for telomerase activity at passage 11 (fig.6.3.2).

### 6.3.2 Cell Morphology

The morphologies of N-hTERT and N-chTERT infected cells resembled that of the uninfected parent cell sample (N) (fig.6.3.3A-D). All samples contained thin, spindle-shaped cells during growth whist spindle-shaped and stellate cells were present in confluent cultures (fig.6.3.3B). In addition, no obvious differences in cell morphology between early and late passage normal cells were apparent regardless of infection with of hTERT (fig.6.3.F & G).

The morphologies of OA-hTERT and OA-chTERT synovial cells were similar in appearance to that of the OA synovial cells at passage 11 (fig.6.3.4A-C). As passage number increased in both the infected and parent OA synovial cell cultures, a reduced proliferative capacity was accompanied by an increase in the larger stellate shaped cells (fig.6.3.4D & E).

Cells from RA-hTERT and RA-chTERT cultures showed similar morphology to each other at passage 11 (fig.6.3.5A & B). With an increase in passage number, an increase in cell size was noted (fig.6.3.5C).

### 6.3.3 Immunohistochemistry

Collagen type IV was used as a marker of synovial fibroblast phenotype. Normal synovial cell cultures showed collagen type IV labelling in both early and late passage

cultures (fig.6.3.6A & B). N-hTERT cells exhibited collagen type IV labelling in late passage cultures (fig.6.3.6E).

Positive labelling for collagen type IV was seen in OA cultures at both early and late passages (fig.6.3.7A & B). The infected OA and RA synovial cells, (OA-hTERT and RA-hTERT) showed collagen type IV expression in late passage cultures (fig.6.3.7C & D).

CD55 was expressed in early OA synovial cell cultures (fig.6.3.8A) but was not observed in late passage cultures (fig.6.3.8B). OA-hTERT cells did not express CD55 at late passages (fig.6.3.8E). Expression of CD55 was seen in cultures from the RA-hTERT cells in late passage cultures (fig.6.3.8G).

With time in culture cells morphology became neuronal-like. As a result MAP-2 labelling was performed. Cultured synovial cells from both normal and OA cultures at early and late passage exhibited MAP-2 labelling (fig.6.3.9A-C). The infected N cells, N-hTERT showed MAP-2 labelling in late passage cells (fig.6.3.9E & F).

### 6.3.3 Population Doublings

Synovial cells were grown in monolayer culture over a period of a year to determine the effects of hTERT infection on population doublings (PD). PD was calculated each time the cultures were trypsinised and passed to new flasks. Accumulated PDs were plotted as a function of time to show changes in population growth rates that occurred with successive passage.

Normal synovial cells exhibited the greatest PD of 18 at 270 days in culture (fig.6.3.10A). The normal synovial cells infected with hTERT and chTERT showed lower population doublings at this time point. N-hTERT cells had reached a PD of 13 whereas the cells infected with chTERT reached a PD of 8 when the experiment was terminated (fig.6.3.10A). The parental normal cells, (N) were infected with chTERT and hTERT during its growth phase. The growth rate of parental normal cells was similar to the growth rate of synovial cells from other normal donors (fig.6.3.10B).

The OA synovial cells exhibited 10 PD in 249 days of culture and similar results were obtained with the hTERT infected OA cells, which exhibited 11 PD at the same number of days in culture (fig.6.3.10C). OA-chTERT cells exhibited a much lower number of PDs, with only 4 PD observed (fig.6.3.10C). The OA parental cells (OA) were infected with chTERT and hTERT during the growth phase (fig.6.3.10D). The growth rate of parental OA cells was similar to the growth rate of other OA synovial cells (fig.6.3.10D).

The RA synovial cells showed similar population doubling times to the hTERT infected RA cells (fig.6.3.10E). The RA-hTERT cells exhibited a higher PD of 11 in 200 days in culture whereas the chTERT cells reached a PD of 7 in the same number of days (fig.6.3.10E).

### 6.3.4 Cell Senescence

In normal synovial cell cultures, the percentage of cells labelled for β-Gal activity increased with passage number (fig.6.3.11E). Normal cells infected with hTERT and ch-TERT showed β-gal activity (fig.6.3.11C & D). A significant increase ($P<0.05$) in percentage of senescent cells compared to the normal parental cells (N) at the same number late passage culture was observed in the N-hTERT cells (fig.6.3.11E). A significant increase ($P<0.05$) in the number of senescent cells in N-chTERT cultures at passage 23 was observed compared to the N cells (fig.6.3.11E). N-chTERT cells showed no significant differences in the number of senescent cells compared to the N-hTERT cells in late passage cultures (fig.6.3.11E).

OA synovial cells showed β-Gal activity in both early and late passage cultures (fig.6.3.12A). No significant differences in the numbers of senescent cells in the early passage compared to the late passage cells were observed (fig.6.3.12E). The OA-hTERT cells showed β-Gal activity (fig.6.3.12B) and did not show a significant difference in the number of senescent cells compared to parental OA cells, at the same passage (fig.6.3.12E).

### 6.3.5 Gene Expression

Hyaluronan synthase 2 expression was seen in cultures from the synovial cells and infected cells from normal, OA and RA donors (fig.6.3.13). HAS 2 expression was maintained in all late passage cultures until each cell culture was terminated (fig.6.3.13).

**Figure.6.3.1** TRAP assay for telomerase activity in synovial cells from normal (N), OA and RA donors. Cell extracts from parental cell samples were subjected to the TRAP assay. Telomerase activity is demonstrated by multiple bands as telomerase extension products have been amplified by PCR. Telomerase positive human 293 kidney cells were used as a positive control (293 lane). The absence of bands in the negative control lanes (-ve) are a result of heated-treated cell extracts in which nascent telomerase activity has been destroyed.

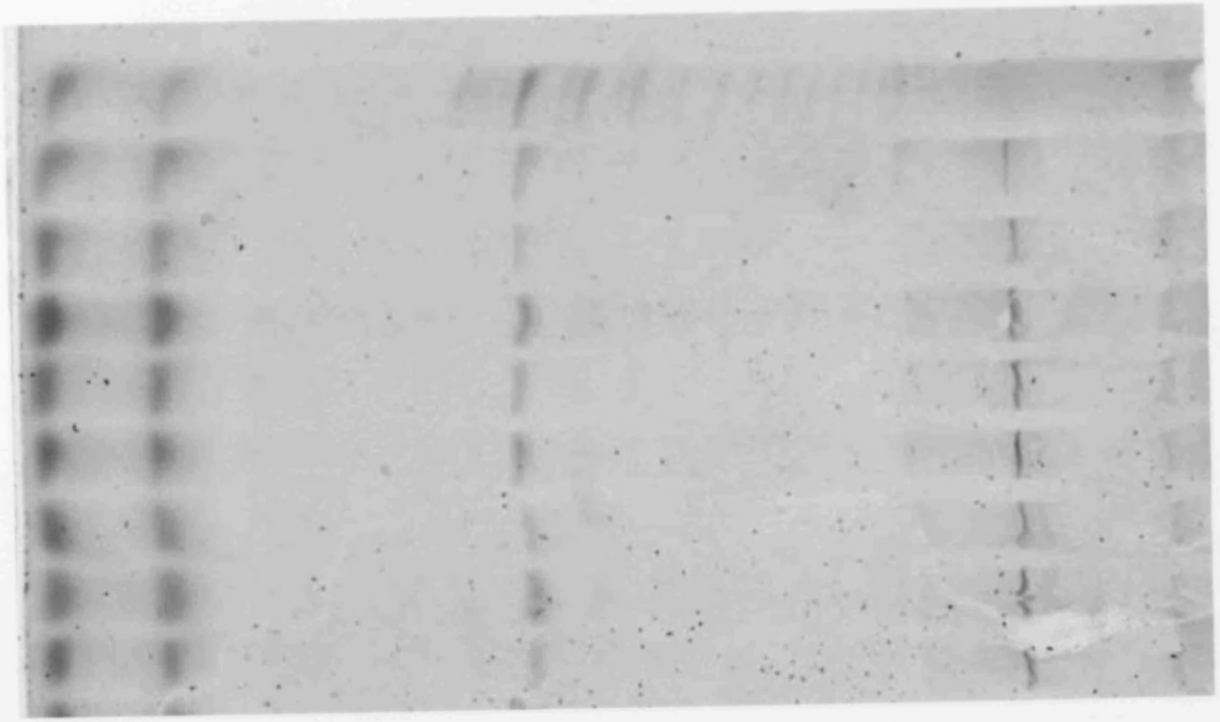

293
293-ve
N
N-ve
OA
OA-ve
RA
RA--ve
Blank

**Figure.6.3.2** TRAP assay for telomerase activity in telomerase infected (hTERT) and telomerase control infected (chTERT) normal (N), OA and RA synovial cells. Cell extracts from infected parental cells were subjected to the TRAP assay. Telomerase activity is demonstrated by multiple bands as telomerase extension products have been amplified by PCR. Telomerase positive human 293 kidney cells were used as a positive control (293 lane). The absence of bands in the negative control lanes (-ve) are a result of heated-treated cell extracts in which nascent telomerase activity has been destroyed.

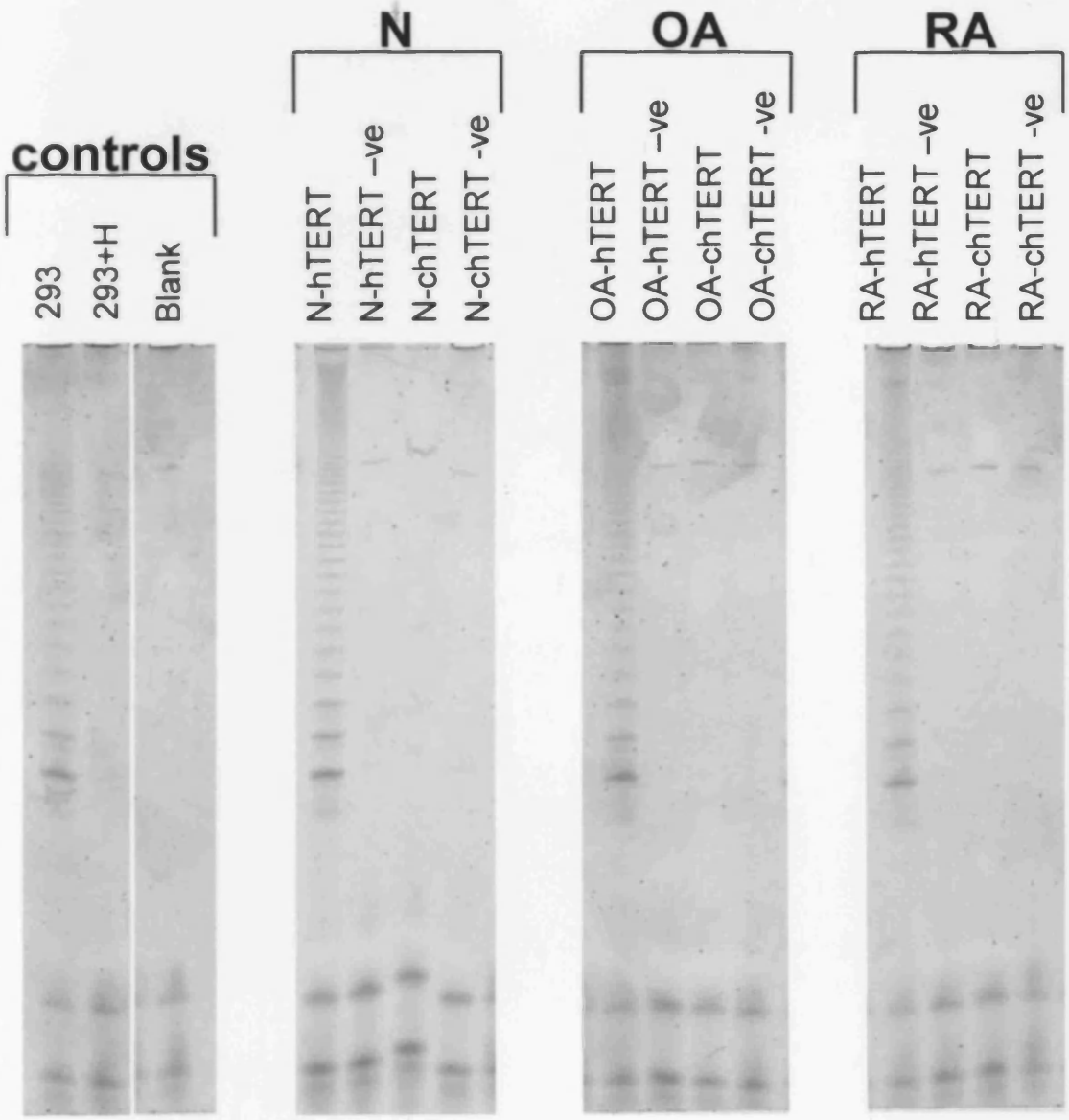

controls
293
293+H
Blank
N
N-hTERT
N-hTERT –ve
N-chTERT
N-chTERT -ve
OA
OA-hTERT
OA-hTERT –ve
OA-chTERT
OA-chTERT -ve
RA
RA-hTERT
RA-hTERT –ve
RA-chTERT
RA-chTERT -ve

**Figure.6.3.3** Morphology of synovial cell cultures from normal tissue. Parental cells from a normal donor and infected normal cells were monitored from early to late passage. The similarities between infected and non-infected cells were noted. Scale bars, 100μm.

A) N-hTERT non-confluent synovial cell culture at passage 14.

B) N-hTERT synovial cell culture at passage 14. A stellate-shaped cell (st) and spindle-shaped cells (sp) are present within the culture.

C) N-chTERT synovial cell culture at passage 13.

D) Normal synovial cell culture at passage 14.

E) Normal non-confluent synovial cell culture at passage 20.

F) Normal synovial cell culture at passage 20. Spindle-shaped cells are present within the culture (sp).

G) N-hTERT synovial cell culture at passage 24. Spindle-shaped cells are present within the culture (sp).

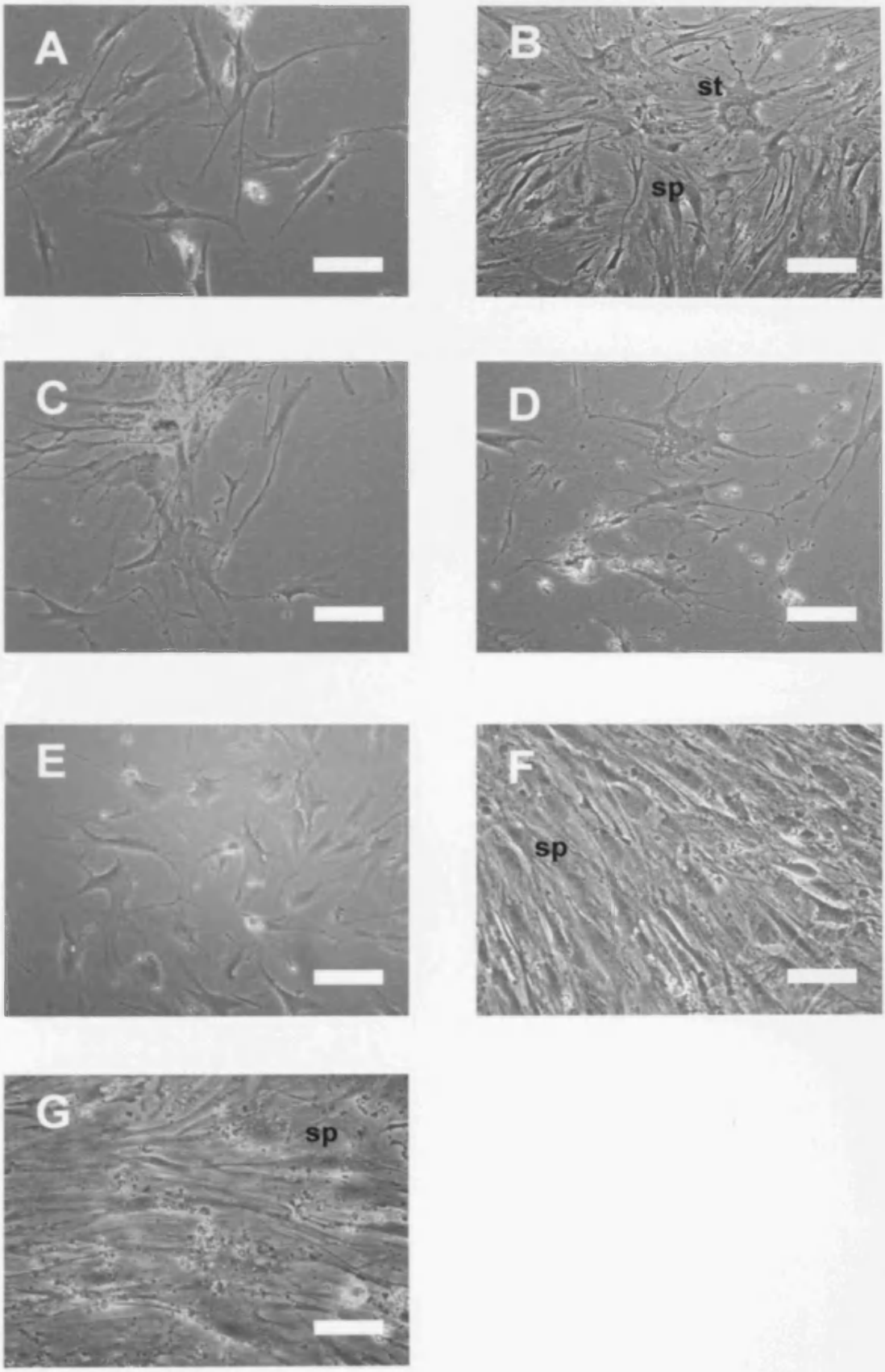

A
B
st
sp
C
D
E
F
sp
G
sp

**Figure.6.3.4** Morphology of synovial cell cultures from osteoarthritic synovium. Parental cells from an OA donor and infected OA cells were monitored from early to late passage. The similarities between infected and non-infected cells were noted. Scale bars, 100μm.

A) OA-hTERT synovial cell culture at passage 11.

B) OA-chTERT synovial cell culture at passage 11.

C) OA synovial cell culture at passage 11.

D) OA synovial cell culture at passage 19. Stellate-shaped cells are present within the culture (st).

E) OA-hTERT synovial cell culture at passage 20. Stellate-shaped cells are present within the culture (st).

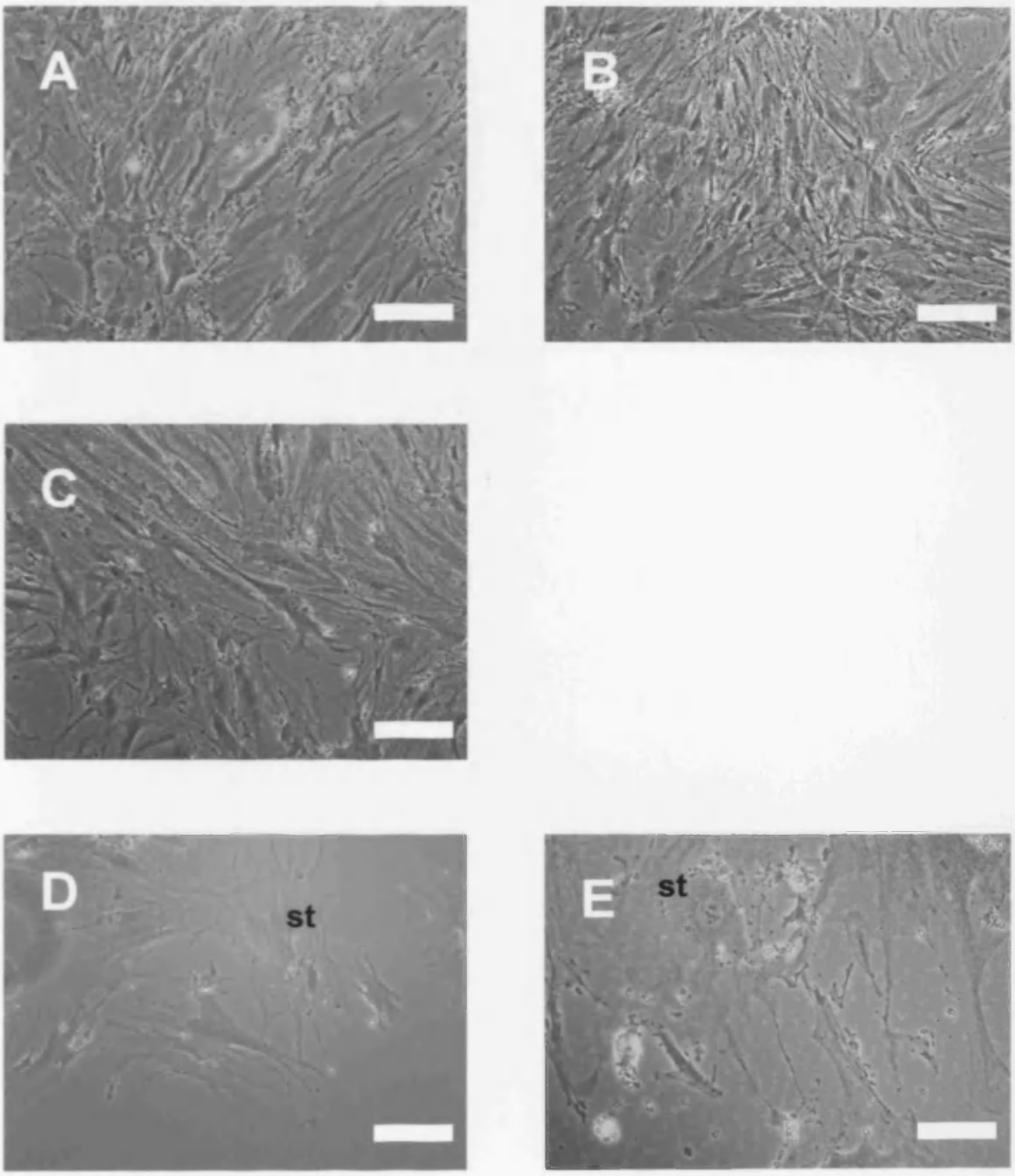

A
B
C
D
st
E
st

**Figure.6.3.5** Morphology of synovial cell cultures from rheumatoid tissue. Infected RA parental cells were monitored in culture. The similarities between infected and non-infected cells were noted. Scale bars, 100μm.

A) RA-hTERT synovial cell culture at passage 11.

B) RA-chTERT synovial cell culture at passage 11.

C) RA-hTERT synovial cell culture at passage 20.

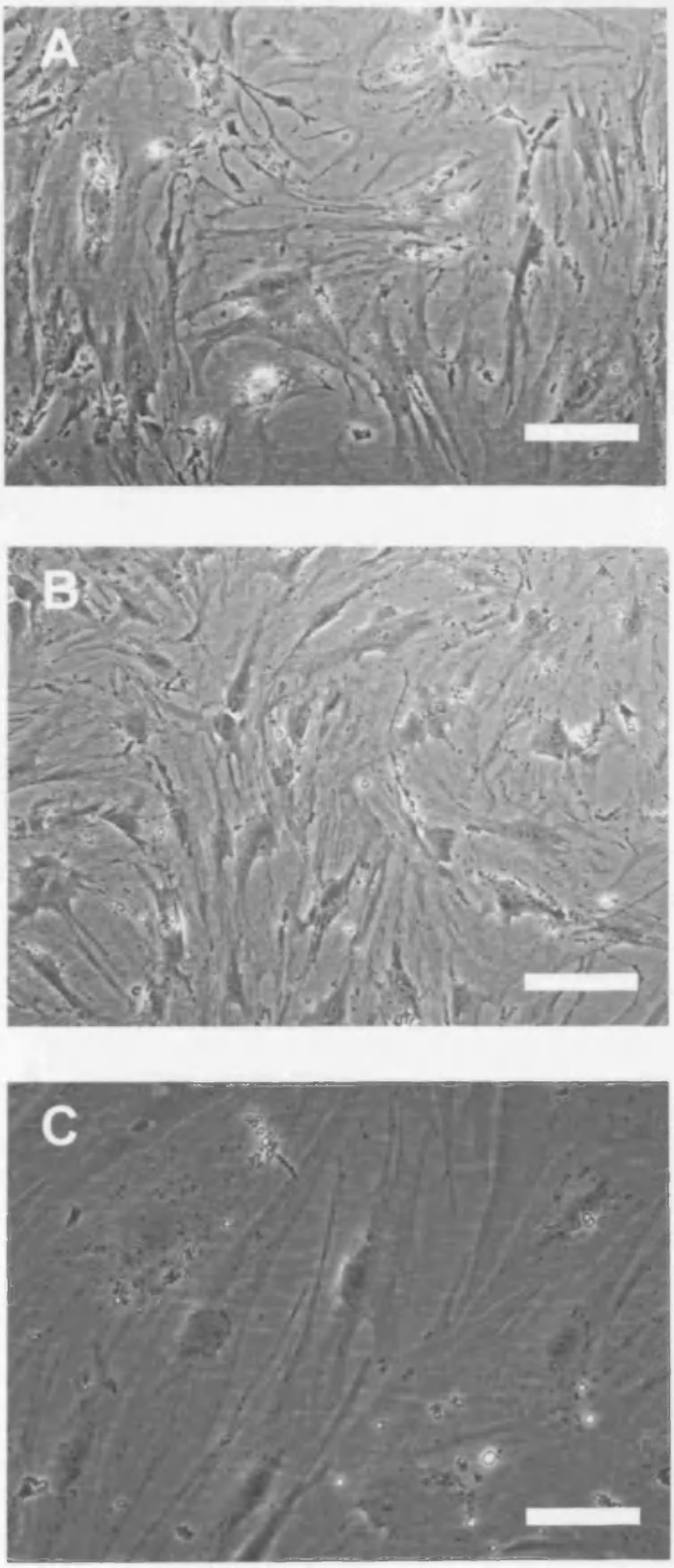

A
B
C

**Figure.6.3.6** Immunolabelling of normal synovial cell cultures for collagen type IV. Cells from parental or hTERT infected normal cells were labelled for collagen type IV in early and late passage cultures. Scale bars, 100μm.

A) Representative early passage normal parental cells (N4, P4). Cells that show labelling for collagen IV are indicated (arrow).

B) Late passage normal parental cells (N, P20). Cells that show labelling for collagen IV are indicated (arrow).

C) Negative control of early passage normal parental cells (without anti-collagen type IV) (N4, P4).

D) Negative control of early passage normal parental cells (without anti-collagen type IV antibody) (N4, P4).

E) N-hTERT cells at P20. Note the positive label for collagen IV within the cell cluster.

F) Negative control of N-hTERT cells at P20 (antibody replaced with IgGs) (OA, P20).

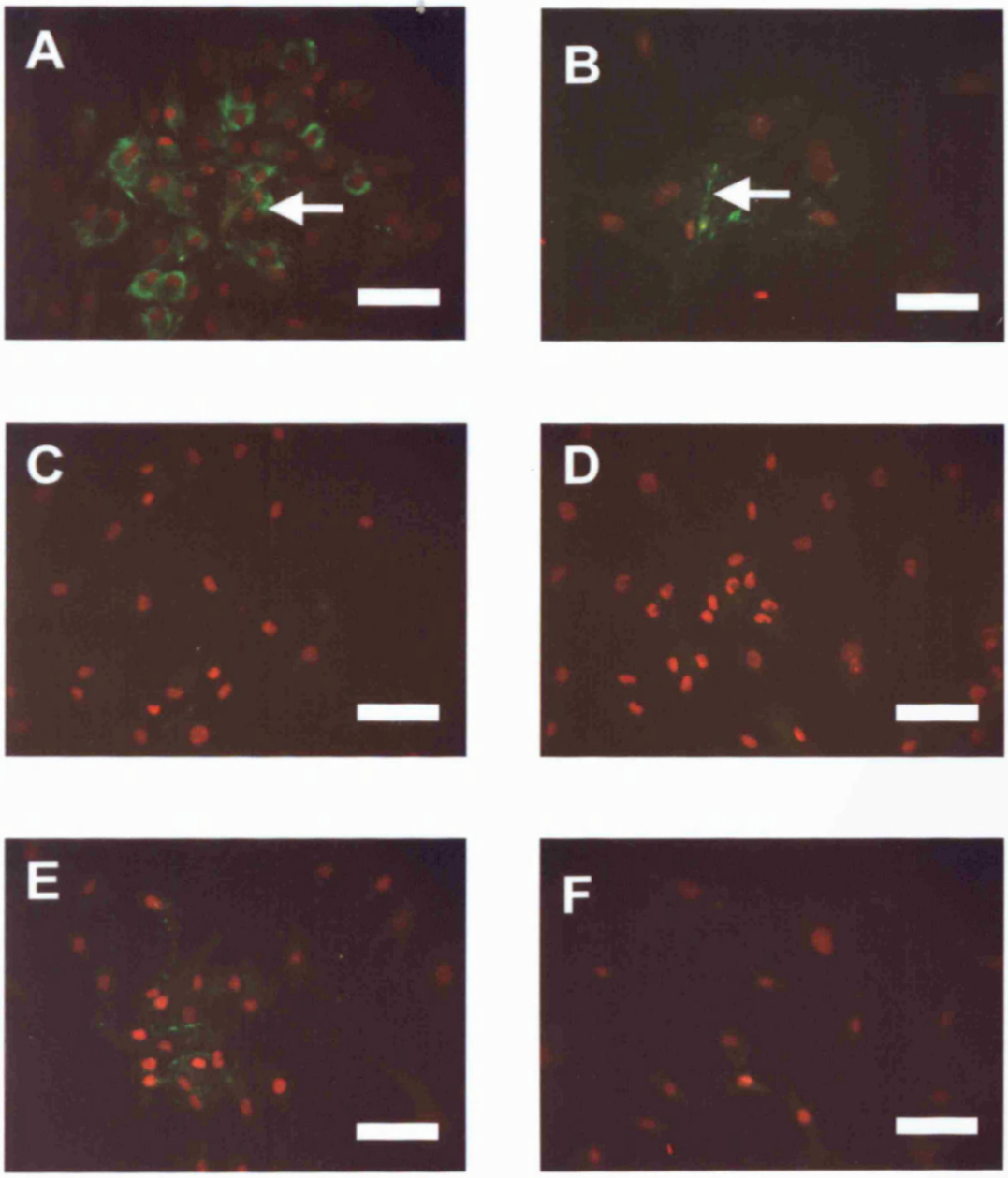

A
B
C
D
E
F

**Figure.6.3.7** Immunolabelling of OA and RA synovial cell cultures for collagen type IV. Scale bars, 100μm.

A) Representative early passage parental OA cells (OA3, P4). Cells labelled for collagen type IV are indicated (arrow).

B) Late passage parental OA cells (OA, P20). Cells labelled for collagen type IV are indicated (arrow).

C) OA-hTERT cells at P20. The cell at the centre of the image displays collagen type IV expression.

D) RA-hTERT cells at P16. Cells labelled for collagen IV are indicated (arrow).

E) Negative control of early passage OA cells (without anti-collagen type IV)(OA3, P4).

F) Negative control of early passage parental OA cells (antibody replaced with IgGs) (OA3, P4).

G) Negative control of late passage parental OA cells (without anti-collagen type IV)(OA, P20).

H) Negative control of early passage parental OA cells (antibody replaced with IgGs) (OA, P20).

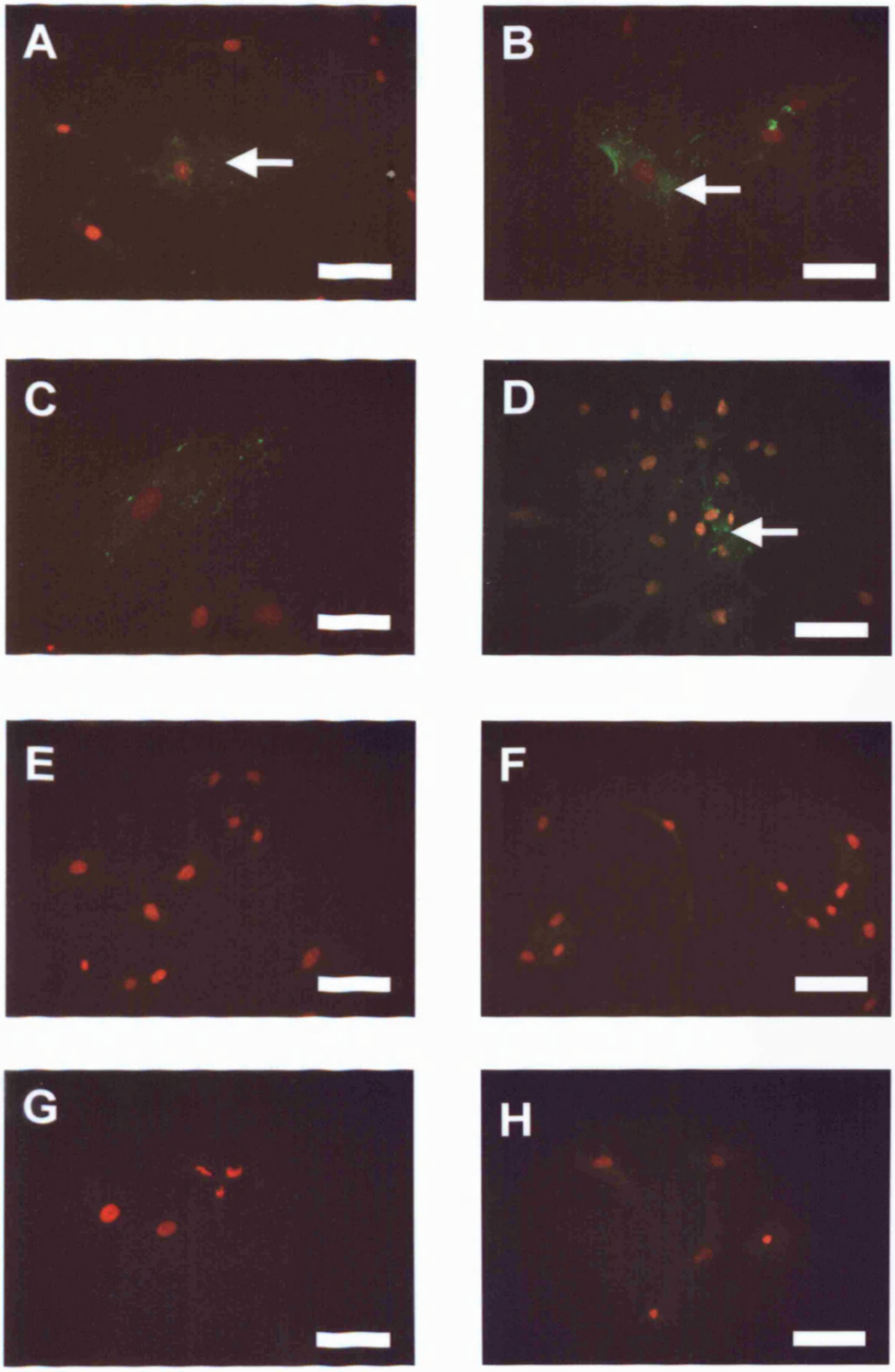

A
B
C
D
E
F
G
H

**Figure 6.3.8** Immunolabelling of OA and RA synovial cell cultures for CD55. Scale bars, 100μm.

A) Representative early passage parental OA cells (OA17, P2). Cells labelled for CD55 expression are indicated (arrow).

B) Representative late passage parental OA cells (OA, P20). No positive CD55 label was observed.

C) Negative control of early passage OA cells (without anti-collagen type IV)(OA17, P2).

D) Positive control; synovial section from an OA sample. The intense immunolabel along the edge of the section indicates CD55 expression (arrow).

E) OA-hTERT cells at P20.

F) Negative control OA-hTERT cells at P20 (without anti-collagen type IV).

G) RA-hTERT cells at P16. Cells labelled for CD55 expression are indicated (arrow).

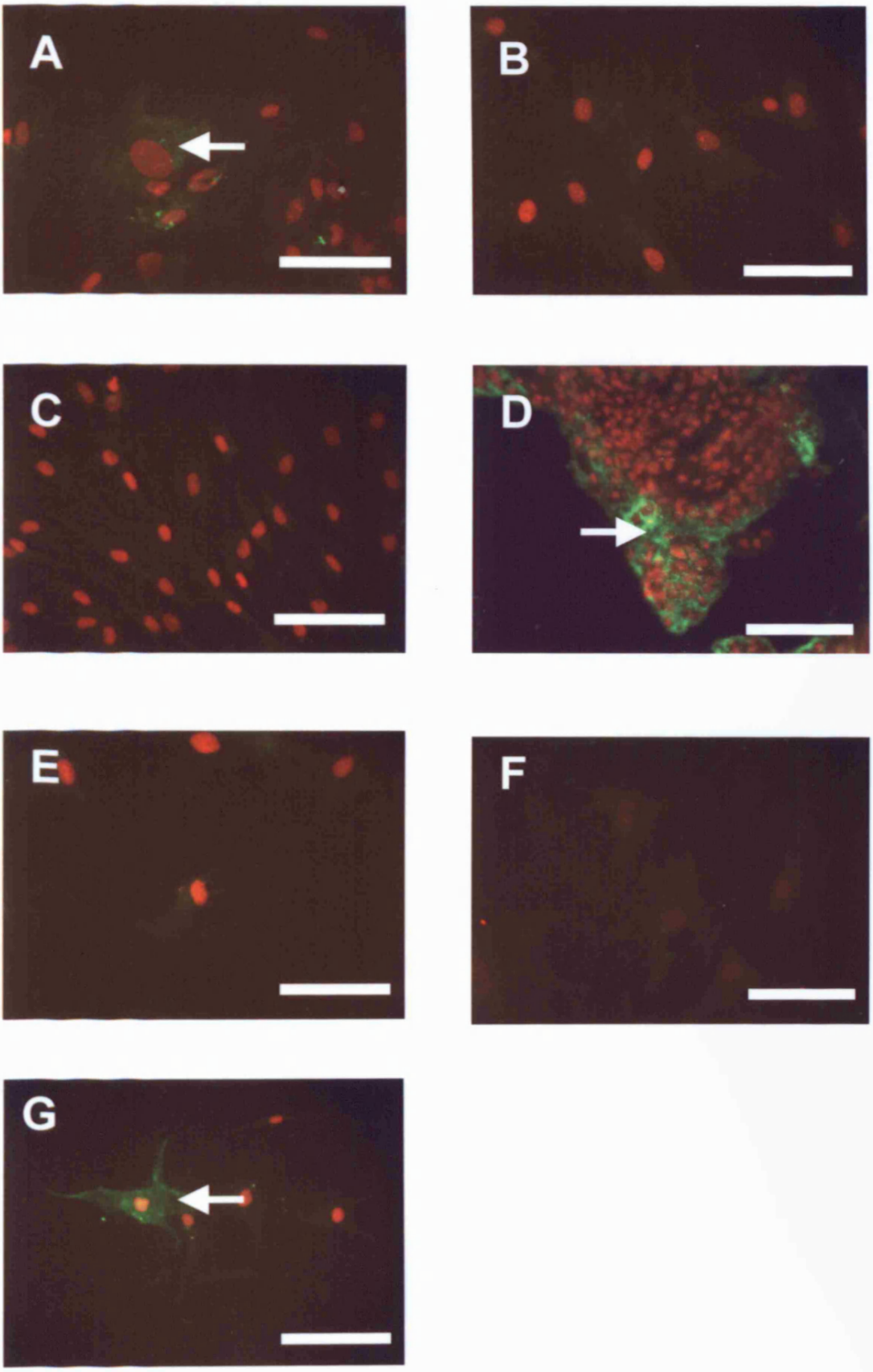

A
B
C
D
E
F
G

**Figure.6.3.9** Immunolabelling of OA and normal synovial cell cultures for MAP-2. Cells from parental cells and infected hTERT normal and OA cells were labelled for MAP-2 in early and late passage cultures. Scale bars, 100μm.

A) Representative early passage parental OA cells (OA2, P1). Note the positive labelling for MAP-2 (arrow).

B) Representative late passage parental normal cells (N5, P8). Note the positive labelling for MAP-2 (arrow).

C) Representative late passage parental OA cells (OA16, P10). Note the positive labelling for MAP-2 (arrow).

D) Negative control of late passage parental OA cells (without anti-collagen type IV)(OA16, P10).

E) N-hTERT cells at P17. Note the positive labelling for MAP-2 (arrow).

F) N-hTERT cells at P24. Note the positive labelling for MAP-2 (arrow).

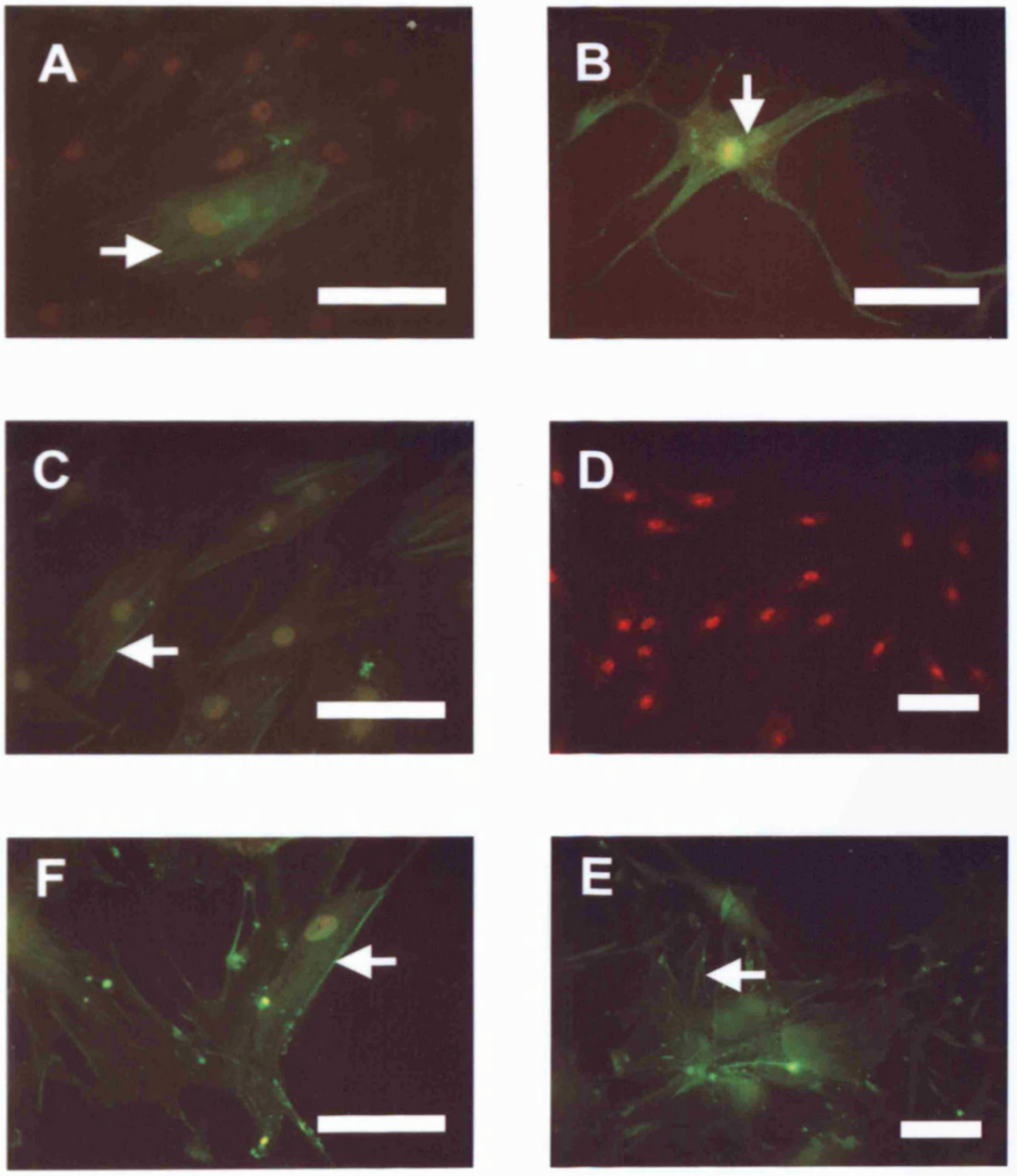

A
B
C
D
F
E

**Figure.6.3.10** Growth curves of normal synovial cells. Cells were grown in monolayer and subcultured when confluent. Cell number was counted at each passage and used to calculate population doublings.

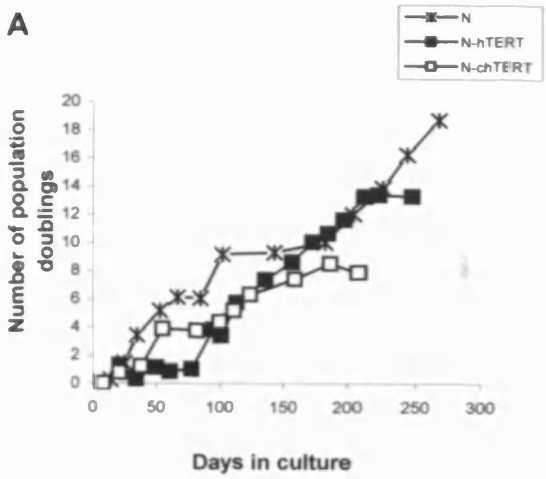
A) Growth curves of hTERT infected, chTERT infected cells and the corresponding parental normal synovial cells from a 71year old donor.

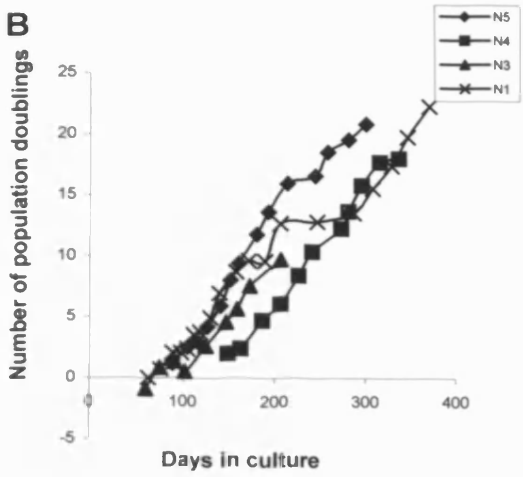
B) Growth curves of normal synovial cell cultures derived from four normal donors.

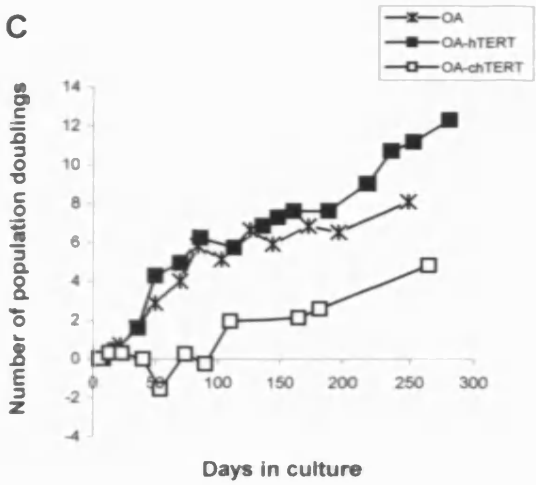
C) Growth curves of the hTERT infected, chTERT infected OA cells and the corresponding parental OA synovial cells from a 57 year old donor.

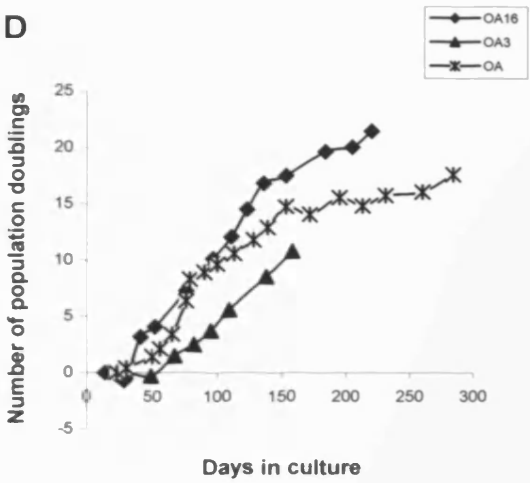
D) Growth curves of OA synovial cell cultures derived from three OA donors.

E) Growth curves of the hTERT infected, chTERT infected RA cells and the corresponding parental RA synovial cells from a 56 year old donor.


A
N
N-hTERT
N-chTERT
Number of population doublings
Days in culture


B
N5
N4
N3
N1
Number of population doublings
Days in culture


C
OA
OA-hTERT
OA-chTERT
Number of population doublings
Days in culture


D
OA16
OA3
OA
Number of population doublings
Days in culture

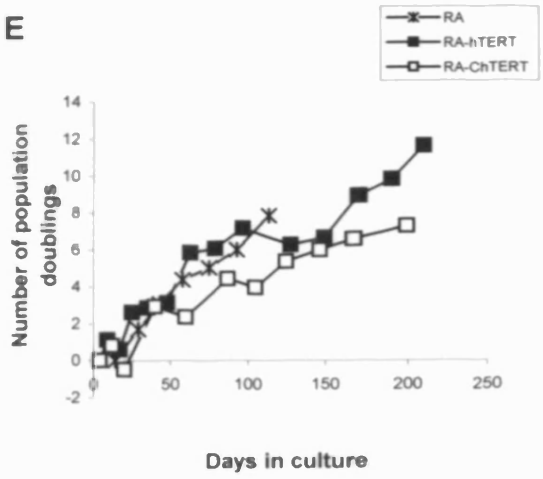

E
RA
RA-hTERT
RA-ChTERT
Number of population doublings
Days in culture

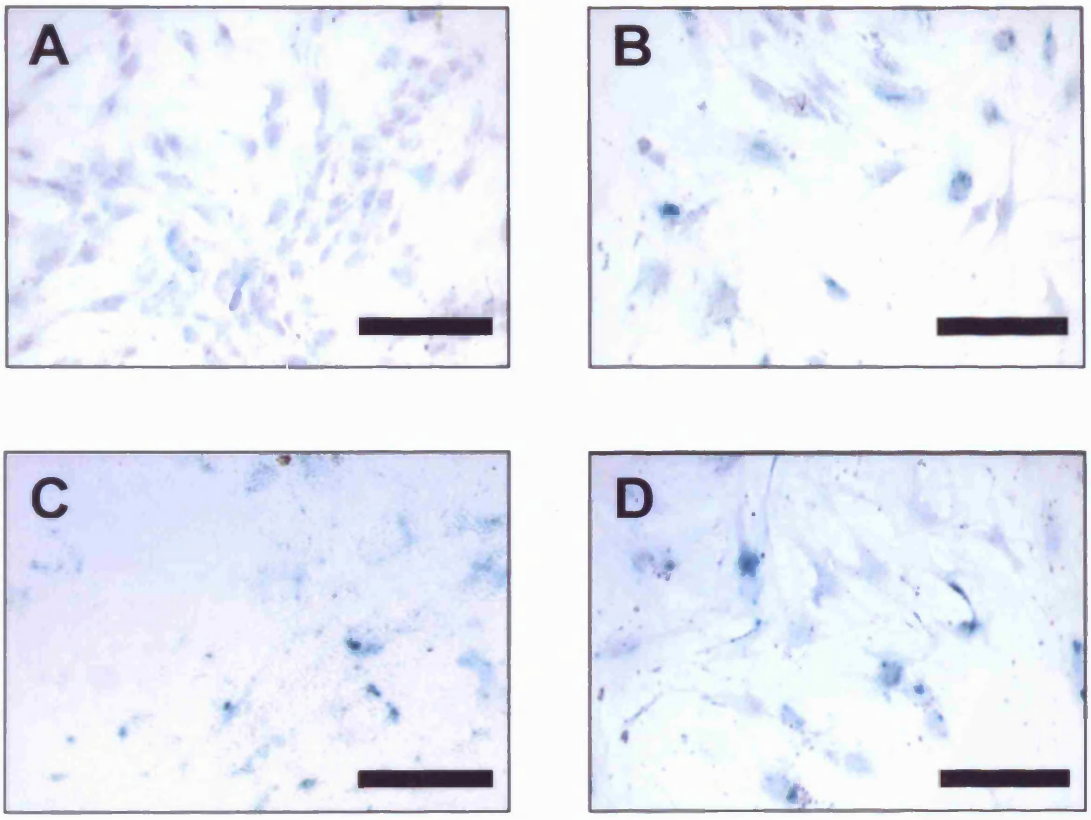
**Figure 6.3.11** Cell senescence associated β-gal activity in synovial cell cultures.

A) and B) β-gal staining of a representative normal synovial cell culture.

C) N-hTERT synovial cells. Scale bars, 100μm.

D) N-chTERT synovial cells. Scale bars, 100μm.

E) Graph to show percentage of cells labelled for β-gal activity in parental normal early and late passage cultures and in infected normal cells at late passage. *$P<0.05$ compared to normal late passage cultures.


A
B
C
D

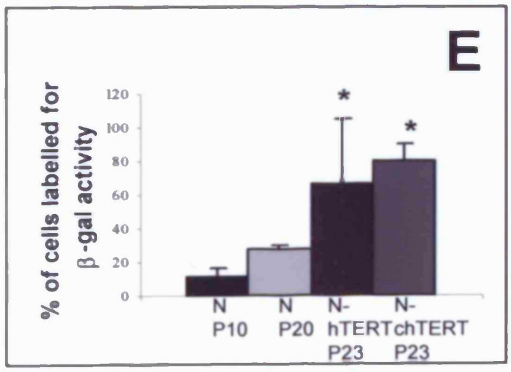

E
% of cells labelled for β-gal activity
120
100
80
60
40
20
0
*
*
N P10
N P20
N-hTERT P23
N-chTERT P23

**Figure.6.3.12** Cell senescence associated β-gal activity in OA synovial cell cultures.

A) β-gal staining of a representative OA synovial cell culture. Note the intense blue reaction product (arrow). Scale bars, 50μm.

B) OA-hTERT synovial cells. Scale bars, 100μm.

C) Positive controls consisted of senescent chondrocytes. Scale bars, 50μm.

D) Negative controls were young chondrocytes. Scale bars, 50μm.

E) Graph to show percentage of cells labelled for β-gal activity in parental OA and in infected OA cells at late passage.

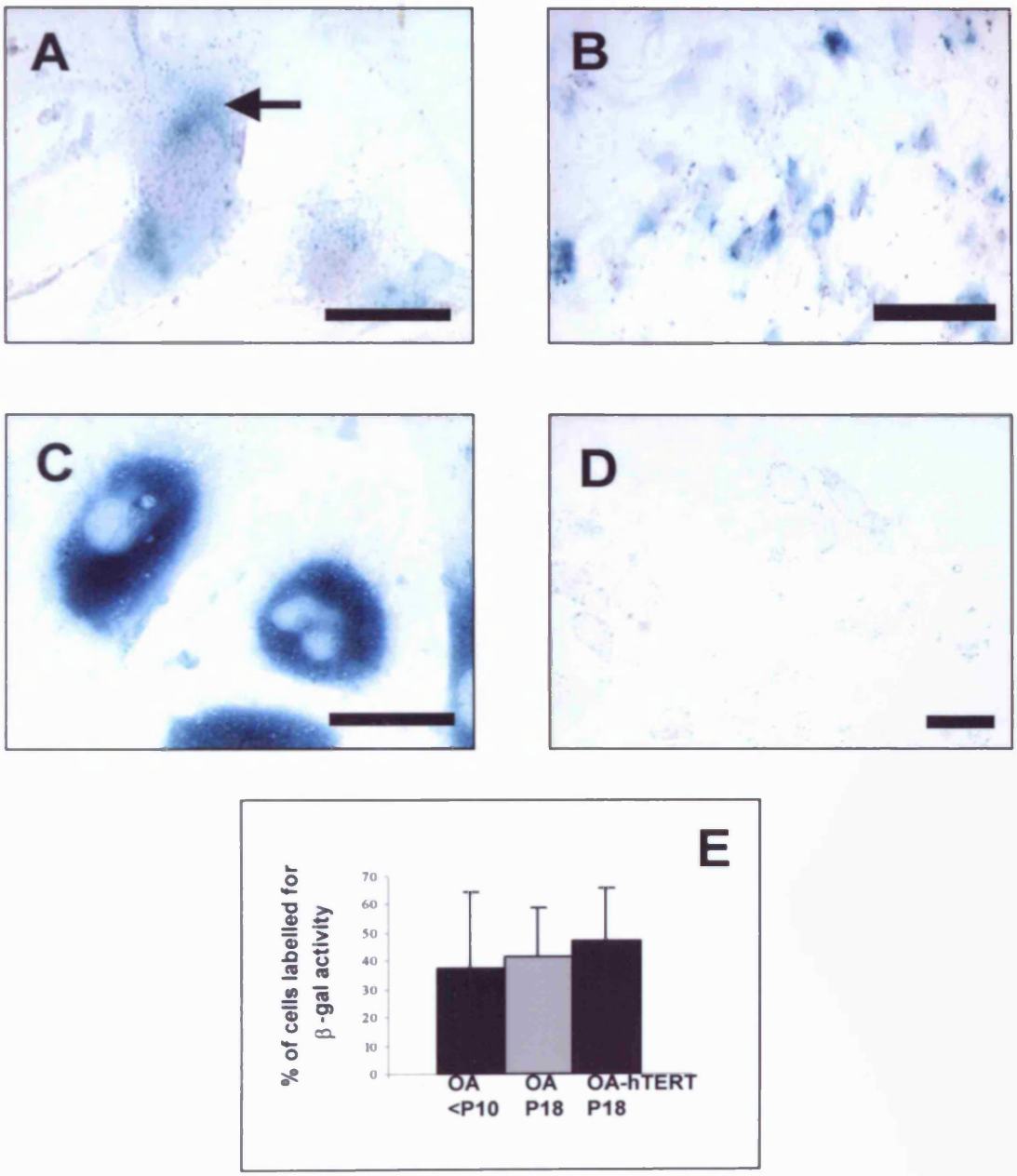
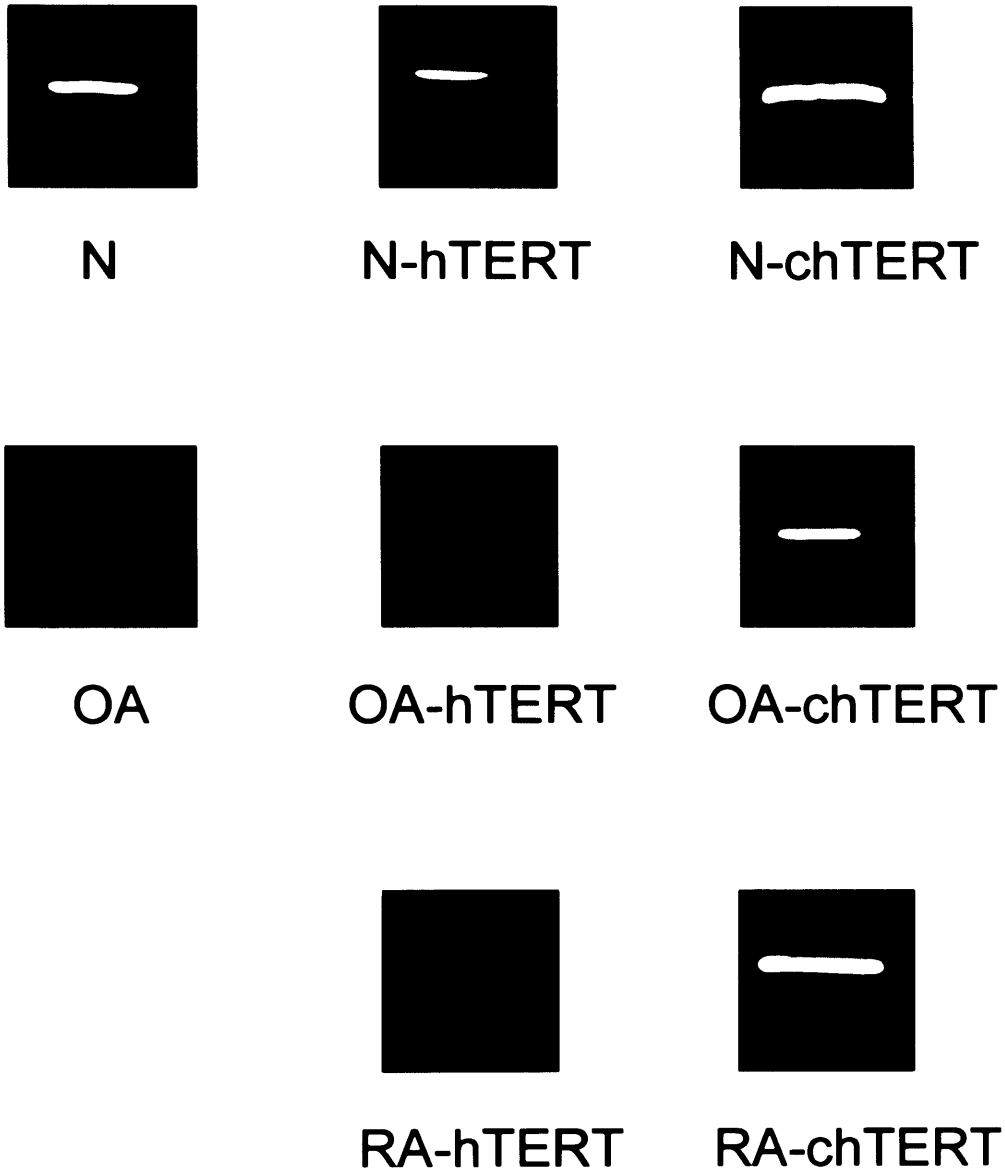

A
B
C
D
E
% of cells labelled for β-gal activity
70
60
50
40
30
20
10
0
OA <P10
OA P18
OA-hTERT P18

**Figure.6.3.13** Synovial cell hyaluronan synthase 2 mRNA expression from infected late passage cultures of synovial cells and the corresponding parental cell cultures. Nore that all samples expressed HAS2 mRNA expression. N=normal; OA=osteorthritic; RA=rheumatoid.


N
N-hTERT
N-chTERT
OA
OA-hTERT
OA-chTERT
RA-hTERT
RA-chTERT

## 6.1 Discussion

This study showed that normal, OA and RA synovial cells expressed telomerase activity when they received the hTERT construct as evaluated by the TRAP assay. Previous studies have shown the lack of telomerase activity in human somatic cells and synovial cells from normal and OA donors are no different (Tsumuki et al., 2000; Yamanishi et al., 1999; Yamanishi et al., 1998; Yudoh et al., 1999). Synovial cells from RA donors however, showed no telomerase activity in this study but previous work both supports and contradicts this result (Yamanishi et al., 1999; Yudoh et al., 1999). It has been suggested that it is the infiltrating mononuclear cells and not the SFBs which are responsible for the detected telomerase activity (Yamanishi et al., 1998). The stage at which the cells were subjected to the TRAP assay in this study was at passage 9, by which point infiltrating cells would not be present.

The normal synovial cells reached a higher population doubling than the normal cells infected with the hTERT expression vector at the end of the experiment. Interestingly, the hTERT cells reached confluence between passages in a fewer number of days than the non-infected (N) cells. The OA cells infected with the hTERT expression vector increased the population doubling number by 4 compared to the OA cells. As with the N-hTERT cells, the OA-hTERT cells reached confluence in less time than the non-infected cells. The RA cells, at termination of the experiment showed a population doubling of 8 and if one looks at the RA-hTERT cells at this point, the PD is 7 yet the cells have been passed twice more than the RA cells. Again, this suggests that the RA-hTERT cells are able to reach confluence quicker than the cells from the parent RA cultures. Normal, OA and RA cells infected with the ch-TERT expression vector showed the least number of population doublings. This result suggests that the infection of the cell with the vector plasmid is inhibiting the growth rates and might explain why the hTERT expressing cells do not show a greater proliferative capacity than the non-infected cells.

Cell senescence is classified as the loss of replicative proliferation by the cells yet the cells remain viable (Allsopp et al., 1995; Bodnar et al., 1998; Wei and Sedivy, 1999). Others have defined cell senescence as the cells taking longer than three weeks to

reach confluence (Gire and Wynford-Thomas, 1998). The growth rates of the N-hTERT cells initially showed 2 weeks or less to reach confluence after passaging throughout the majority of the experiment. At the last 2 passages however, cultures took 3 or more weeks to reach confluence. Significantly, more senescent cells were observed in the N-hTERT cell cultures compared to the N cell cultures which suggest the hTERT construct cannot prevent cell senescence. Whether hTERT expression would eventually allow hTERT infected cells to overcome this growth arrest stage was one of the aims of this study, but it seems the cells needed to be monitored for a greater period of time to determine this outcome. The OA hTERT cells showed a period of no growth that was over 3 weeks. The time required to reach confluence after this period decreased and this could indicate that a population of cells was able to overcome senescence. The decrease in time required to reach confluence could be the result of ectopic hTERT expression as the non-infected OA cells shows an increase in the number of days needed to reach confluence at this point which suggests cells might be entering growth arrest. Again the cell cultures needed to be maintained over a greater period of time to draw definite conclusions.

The immortalisation of a cell line not only requires an extended proliferative lifespan *in vitro* but the cells also need to maintain their phenotype. The expansion of synovial cells *in vitro* has been shown to lead to an increase in cell size (Marsh et al., 1978). This study shows similar results, the cells in late passage cultures although showing a spindle-like morphology characteristic of a fibroblast cell type, an increase in cell size was also observed. This increase in size may account for the increasing time required to reach cell confluence as cell passage number increases but as the cells increase in size it could be argued that they should reach confluence in a shorter period of time than smaller sized cells. The cells expressing the hTERT or chTERT vector plasmid showed similar gross morphology to the corresponding non-infected normal, OA and RA cells. The infected and non-infected cells from normal, OA and RA donors expressed typical phenotype markers in both early and late passage cultures. Previously, expression of fibroblast markers was lost with time in culture but this was not seen in cells cultured from these particular donors (Edwards et al., 1997; Zimmerman et al., 2001). The expression of hyaluronan synthase 2 mRNA was seen in infected and non-infected cultures from normal, OA and RA cells. As the enzyme

hyaluronan synthase is required for hyaluronan synthesis, this result indicates that the cell retains its synovial fibroblast characteristics (Edwards, 1999).

Overall, the results of this study showed that hTERT infection was not sufficient to extend the lifespan of a normal, OA or RA synovial cell population under typical monolayer culture conditions. Although ectopic hTERT expression has been shown sufficient to immortalise fibroblasts, other cell types require further intervention (Dickenson et al., 2000; Kiyono et al., 1998). Previous work suggests that viral oncogenes are required for by-passing M1 growth arrest while stabilisation of telomere length by ectopic hTERT expression is needed to by-pass M2 growth, thus generating an immortal cell line (Counter, 1998). Introduction of the high-risk human papilloma virus 16 (HPV16) oncogene E6 and E7 allows growth beyond M1 as E7 blocks the activity of the p16/pRb pathway that induces growth arrest and E6 expression also acts to overcome growth arrest by targeting the p53 pathway (Demers et al., 1994; Foster and Galloway, 1996; Scheffner et al., 1990). The requirements for immortalisation however, vary in different types of cells. It has been shown that chondrocytes require hTERT and both E6 and E7 expression to exceed the 'normal' number of population doublings, whilst E6 and E7 expression in endometrial cells is sufficient for immortalisation (Kyo et al., 2003; Martin et al., 2002). It seems therefore, that SFBs can not maintain an extended lifespan with ectopic hTERT expression alone and the expression of either one or both of the viral oncogenes is required. The expression of these viral oncogenes can lead to phenotypic disruption in some cell types during immortalisation and needs to be considered when planning future work (Bodnar et al., 1998; Jiang et al., 1999; Vaziri and Benchimol, 1998). Certain cells including the rabbit synovial cell line HIG-82 have become immortal through spontaneous transformation of late-passage cultures (Georgescu et al., 1988). The immortalised cell line showed similar morphology and properties to primary synovial cell cultures although responsiveness to some stimuli was lost (Lin et al., 1996; Stefanovic-Racic et al., 1994). The spontaneous transformation of human synovial fibroblasts could prove a possibility for the immortalisation of human synovial cells if they were monitored and maintained in culture.

Due to the nature of the synovium, cell cultures often retain a mixed population *in vitro*. As time in culture increases these infiltrating cells are lost and the remaining

cells show a more homogeneous phenotype (Marsh et al., 1978; Zimmerman et al., 2001). As a result, experiments on synovial cell cultures could produce varied results because of the differences in cell population and morphology at the different passages. If experiments are to be conducted using cells at the same passage then this would limit cell numbers. Synovial cells in culture show an increase in the time taken to reach confluence as passage number increases (Clarris and Fraser, 1968; Smith and Hamerman, 1969). The alterations associated with time *in vitro* and the fact that the cells eventually undergo replicative senescence also limits cell numbers (Hayflick, 1965; Hayflick and Moorhead, 1961). The immortalisation of a SFB cell line that maintains characteristics of a young phenotype with an increased proliferative capacity would allow a limitless and available supply of cells. At present, this study does not provide an immortalised cell line but does show that synovial cells retain some of their characteristics in long term culture yet whether this is due to ectopic hTERT expression requires further investigation.

# Chapter 7
# General Discussion

# 7.0 General Discussion

The importance of mechanical stimuli during joint cavitation and differential HA synthesis at the presumptive joint line has been established (Dowthwaite et al., 1998; Osborne et al., 2002; Pitsillides, 1999; Pitsillides et al., 1995). Furthermore, the reduction of HA concentration within the synovial fluid leads to painful and immobile joints, as in OA, suggesting that continued synthesis of HA, and hence continued mechanical stimuli is necessary for friction free articulation of the joint. At present, the decreases in HA concentration observed within the synovial fluid are attributed in part to the loss of mechanical stimuli as the joint becomes less mobile but could also be the result of a decrease in synovial cell responsiveness to mechanical stimuli (Pitsillides et al., 1999; Pitsillides et al., 1994). Treatments for OA include intra-articular HA therapies to try and increase HA within the synovial fluid, yet these have not always proved beneficial in reducing joint pain or immobility (Altman and Moskowitz, 1998; Dixon et al., 1988; Dougados et al., 1993; Petrella et al., 2002). Using exercise based therapies as a treatment for OA to increase the concentration of HA within the synovial joint could prove to be an appropriate and simple treatment measure. If exercise is to be considered as a method of increasing HA concentration within the synovial fluid to reduce pain and increase joint mobility, then the effects of mechanical stimuli *in vitro* first need to be investigated. Although mechanical stimuli has been shown to be important in inducing alterations in HA synthesis during joint development and in maintaining HA synthesis in the adult joint, it needs to be known whether the synovial cells from OA donors are still able to respond to mechanical stimuli. Observations from this study show that mechanical stimuli produces significant changes in HA media concentrations in synovial cell cultures.

Synovial cells derived from both normal and OA tissues were used in the study and it could be seen that response to mechanical strain was different between the two. Overall, it was the OA synovial cell cultures that gave the greatest changes in HA media concentration and HAS mRNA expression. These changes demonstrate that OA cells were able to respond to strain but this response was different to that of normal cells.

The response of the synovial cell cultures to the three magnitudes of strain in terms of HA media concentration was different with each strain. The OA cells however, showed greater increases in media HA concentration at the lowest magnitude of strain. This increase relates to the physiological magnitude of strain experienced during walking and so a simple walk would prove beneficial in reducing joint pain and increasing mobility (Lanyon, 1996).

| | HA | Passage 1 | | |
|---|---|---|---|---|
| | | HAS1 | HAS2 | HAS3 |
| 4000με | | | | |
| 6000με | | | | |
| 10000με | ↑ | ↓ | | |

| | HA | Passage 2 | | |
|---|---|---|---|---|
| | | HAS1 | HAS2 | HAS3 |
| 4000με | | | | |
| 6000με | ↓ | ↑ | | ↑ |
| 10000με | | | | |

| | HA | Passage 3 | | |
|---|---|---|---|---|
| | | HAS1 | HAS2 | HAS3 |
| 4000με | ↑ | | | ↑ |
| 6000με | | | | |
| 10000με | ↑ | ↑ | | |

**Table 7.1 Table to indicate media HA concentration from strained synovial OA cell cultures in relation to HAS mRNA expression.**

Expression of the three *HAS* genes was altered upon mechanical strain but this change was not as dependent on passage number as it was in alterations in media HA concentration. HAS mRNA expression does not always reflect protein and subsequent HA synthesis and this needs to be taken into consideration. If we correlate the data of HA media concentration and HAS mRNA expression in some patients, an increase in media HA concentration could be correlated to an increase in *HAS* mRNA expression (table 7.1). It is interesting to note that HAS1 mRNA expression increases at 10000με whereas HAS3 mRNA expression increases at 4000με, both correlating to an increase in media HA concentration. The *HAS* genes are responsible for the synthesis of differently sized HA molecules and the application

of the different magnitudes of strain could be reflected in the size of the HA molecule synthesised. An increase in the larger HA molecule has been shown to buffer outflow so retaining synovial fluid within the cavity whereas synthesis of the smaller HA molecule may facilitate cell signalling as low Mr weight HA has been shown to induce gene expression during inflammation (Coleman et al., 1999; Coleman et al., 2000; McKee et al., 1996; Termeer et al., 2000). The observed increase in HAS1 and HAS3 mRNA expression at 6000με accompanied by a decrease in media HA concentration may reflect post transcriptional regulation. The concentration of HA is reportedly more important than the size of the HA molecule in retaining a constant volume and viscosity of HA within the synovial fluid (Coleman et al., 2000; Scott et al., 2000) but for future work it would be interesting to determine the size of the HA molecules synthesised as a result of mechanical stimuli and investigate the correlation to HAS mRNA expression.

During OA, cytokine synthesis becomes unbalanced and although cells within the joint try and restore the equilibrium the disease progresses (Feldmann et al., 1996; Firestein, 2003). The use of exercise as a treatment would prove disadvantageous if pro-inflammatory cytokines were to be synthesised as a result of joint movement. Data from this study however, show that pro-inflammatory cytokine synthesis is not altered by mechanical stimuli although the synthesis of anti-inflammatory cytokines through mechanical strain would have proved an added advantage to exercise as a therapy for OA.

The use of human cells for this study was necessary but it has produced variability in the data. Whether these arise from age, sex, disease state or treatment program of patients can not be determined from the number of donors used in this study. The use of an increased number of patients might reduce the observed variability but it would be beneficial to develop a synovial cell line that provides a limitless supply of cells. This should also combat the problems of changes in cell characteristics during long-term *in vitro* growth. In primary cultures there exists a mixed population of cells which become more homogeneous with time in culture (Clarris et al., 1977; Marsh et al., 1978; Zimmerman et al., 2001). The loss of macrophages and other infiltrating cells and the increase in a more fibroblastic like cell type might indeed alter the response to strain and, thus, might not reflect the response *in vivo*. Changes in cell

characteristics such as cell markers and cell size were also observed and suggest cells are deviating from their *in vivo* phenotype. As a result, the use of synovial cells at the same passage is essential to produce reproducible and reliable results as this study has demonstrated. Thus, the immortalisation of synovial cells using ectopic hTERT expression to develop a cell line for future experiments was investigated with the aim of generating an available and limitless supply of synovial cells. Data obtained from this study however, show that hTERT alone was not sufficient to immortalise the cells, although the infected OA cells did show an increased population doubling compared to the non-infected cells. Cells retained characteristic synovial cell markers and gene expression but continued to increase in size and show increased senescence associated β-galactosidase activity. An immortal cell line would reduce some of the problems associated with the use of human tissue and so further investigation is definitely needed.

Further work into the effects of mechanical strain on OA synovial cells will require a greater number of donors to be sampled or an immortal cell line. This inturn might show definite trends in synovial cell response to mechanical strain. A similar argument pertains to both normal and RA cells alike. Using an animal cell line could have proved worthwhile for this study as it would have provided a baseline for our human data to be compared against. The established HIG-82 rabbit synovial cell line (CRL-1832: ATCC, USA) would have been an ideal candidate. Since finishing this study however, I have been made aware of a human synovial cell line (Hs 701.T: ATCC, USA) that could have proved even more beneficial to this study. It must be taken into consideration however that to date this cell line has not been characterised fully.

The mechanical strain that was applied to the cells reached a maximum of 1% due to the constraints of the mechanical loading jig. At present there are no measurements for the stretch the synovium undergoes within a joint due to the nature and situation of the tissue. Previous studies on tendon fibroblasts, periodontal ligament fibroblasts and dermal fibroblasts have all used stretches above 1%, in most cases starting at 5% and rising to 20% (Grymes and Sawyer, 1997; Neidlinger-Wilke et al., 2002; Sambajon et al., 2003; Skutek et al., 2003; van Griensven et al., 2003; Yamaoka et al., 2001; Yoshino et al., 2003). The application of mechanical strain at a higher

magnitude to that used in this study would therefore be an area of further work. The stretch applied to the cells was based on work by Lanyon who carried out his studies on bone (Lanyon, 1996). In retrospect, the stretch applied to synovial cells is likely to be higher than 1% especially in areas where the synovium covers a large surface area during joint flexion.

The ECM and cytoskeleton play significant roles in mechanotransduction with signalling through integrins and $Ca^{2+}$ channels resulting in changes in gene expression (Chiquet, 2003; Itano et al., 2003; Maniotis et al., 1997; Munevar et al., 2004). Coating the plates with various ECM proteins could have given us some insight into the role the cytoskeleton plays in mechanical transduction pathway and maybe would have provided a more physiological environment for the cells. Previous work has already shown different responses to mechanical strain when cells were adhered to different matrices providing evidence for the role of the ECM in mechanotransduction and a valid area for future study (Breen, 2000; Reusch et al., 1996) .

Regulation of HA synthesis could occur at three different levels, mRNA expression of the HAS genes, HAS protein expression and the activity of the HAS enzyme. Whether mechanical strain can directly affect HA synthesis at these three points remains to be seen. Evidence suggests the involvement of MAPKs (mitogen activated protein kinases) such as p38 in signalling to transcription factors within the cell nucleus controlling gene expression (Oudin and Pugin, 2002; Skutek et al., 2003). Indeed a study by Mascarenhas *et al* (2003) demonstrated the cessation of HAS3 mRNA expression on addition of a protein tyrosine kinase inhibitor. Work by Sun *et al* (2001) has shown alterations in transcription factors when mechanical strain was applied to the cells and more recently, Sun's work has shown mechanical strain induces post-transcriptional reduction in enzyme activity (Sun et al., 2003).

Evidence establishes that mechanical stimuli are able to signal to the cell to initiate a response yet it is also evident from this work and previous studies that the synovial cells are able to monitor HA levels within their surrounding environment and signal back to the cell. At present it is unclear how this process occurs but investigation into the HABPs, for example CD44 and RHAMM, should be considered.

Many area still exist which will require further research in order to improve our understanding of the regulation of HA metabolism via mechanical strain. Nevertheless, OA synovial cell cultures can be seen to increase HA synthesis upon mechanical stimulation.

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# Appendices

# Appendix 2.1

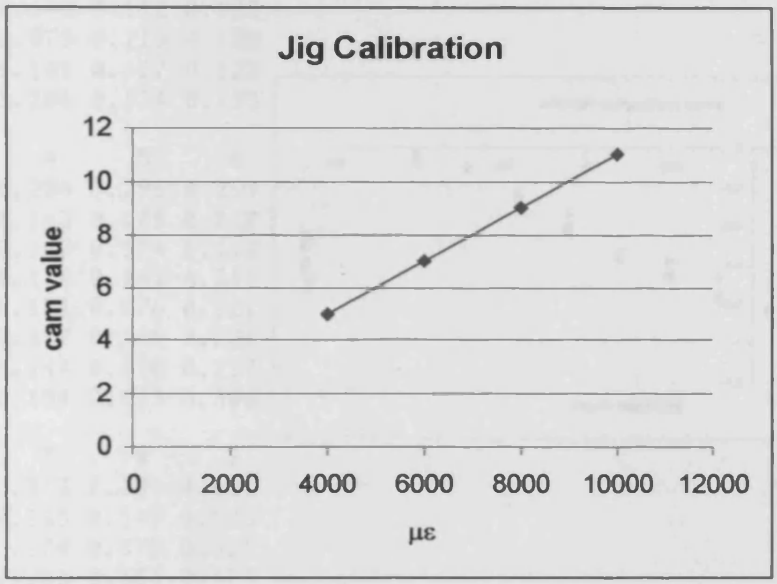
## Synovial Cell Passage

1) Remove media.
2) Wash cells in PBS.
3) Add 3ml trypsin (0.05%) per T25 flask.
4) Incubate at 37°C for 8 minutes.
5) Tap underneath of flask
6) Remove media.
7) Add 5ml DMEM/F12+ to flask. Use cell scrapper to remove remaining cells.
8) Combine with previous media.
9) Centrifuge at 1000 x g for 5 minutes.
10) Remove supernatant, flick bottom of tube to disrupt pellet.
11) Add 1ml DMEM/F12+, shake tube.
12) Count cells.

# Appendix 3.1

## Four point bending mechanical loading jig calibration

The jig was calibrated using an Omega strain gauge SG-3/350-LY13 with CIL 870 strain gauge amplifier (CIL Electronics, Worthing)




As strain is measured as a ratio between the change in length between 2 points;

$$\varepsilon = \frac{\Delta L}{L}$$

strains are often referred to as a percentage. Thus 10,000 με is equivalent to a 1% strain.

# Appendix 3.2

11/Mar/03
30 mins

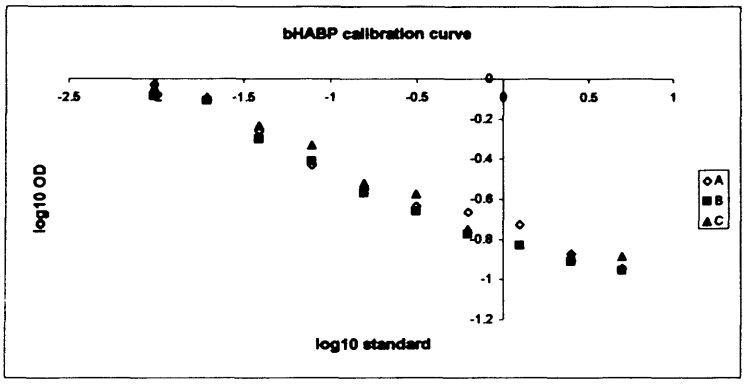
MULTISKAN MS

PRIMARY EIA
V. 1.4-0
ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 405

ABSORBANCES
30. JUL 1916 15:59:48

Max

| | 1 | 2 | 3 |
|---|---|---|---|
| A | 0.705 | 0.114 | 0.125 |
| B | 0.630 | 0.108 | 0.122 |
| C | 0.617 | 0.118 | 0.155 |
| D | 0.608 | 0.101 | 0.123 |
| E | 0.646 | 0.112 | 0.125 |
| F | 0.879 | 0.118 | 0.120 |
| G | 1.101 | 0.127 | 0.123 |
| H | 1.206 | 0.134 | 0.135 |




| | 4 | 5 | 6 |
|---|---|---|---|
| A | 0.254 | 0.296 | 0.269 |
| B | 0.143 | 0.178 | 0.217 |
| C | 0.167 | 0.174 | 0.210 |
| D | 0.134 | 0.162 | 0.211 |
| E | 0.151 | 0.176 | 0.221 |
| F | 0.157 | 0.165 | 0.221 |
| G | 0.144 | 0.178 | 0.227 |
| H | 0.154 | 0.179 | 0.306 |

| | 7 | 8 | 9 |
|---|---|---|---|
| A | 0.313 | 0.389 | 0.601 |
| B | 0.265 | 0.349 | 0.526 |
| C | 0.274 | 0.372 | 0.521 |
| D | 0.261 | 0.363 | 0.477 |
| E | 0.257 | 0.369 | 0.502 |
| F | 0.288 | 0.434 | 0.523 |
| G | 0.292 | 0.485 | 0.561 |
| H | 0.315 | 0.446 | 0.610 |

Blank

| | 10 | 11 | 12 |
|---|---|---|---|
| A | 0.834 | 0.963 | 0.090 |
| B | 0.706 | 0.925 | 0.129 |
| C | 0.810 | 0.927 | 0.103 |
| D | 0.692 | 0.745 | 0.160 |
| E | 0.818 | 0.859 | 0.135 |
| F | 0.819 | 0.845 | 0.139 |
| G | 0.799 | 0.942 | 0.102 |
| H | 0.820 | 0.856 | 0.088 |

# Appendix 3.2

**bHABP A**

| standard | OD1 | OD2 | OD3 |
|---|---|---|---|
| 5 | 0.114 | 0.108 | 0.118 |
| 2.5 | 0.125 | 0.122 | 0.156 |
| 1.25 | 0.254 | 0.143 | 0.167 |
| 0.625 | 0.296 | 0.178 | 0.174 |
| 0.3125 | 0.269 | 0.217 | 0.21 |
| 0.15625 | 0.313 | 0.265 | 0.274 |
| 0.078125 | 0.389 | 0.349 | 0.372 |
| 0.0390625 | 0.601 | 0.526 | 0.521 |
| 0.01953125 | 0.834 | 0.706 | 0.81 |
| 0.009765625 | 0.963 | 0.925 | 0.927 |

| avg |
|---|
| 0.113 |
| 0.134 |
| 0.188 |
| 0.216 |
| 0.232 |
| 0.284 |
| 0.370 |
| 0.549 |
| 0.783 |
| 0.938 |

| standard | avg.OD | log10 standard | log10 OD | | | HA | HA | HA | avg | std |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 0.113 | 0.698970004 | -0.945642338 | slope | -0.337173 | 3.928176 | 4.611392 | 3.546264 | 4.029 | 5 |
| 2.5 | 0.134 | 0.397940009 | -0.872895202 | intercept | -0.74275 | 2.989111 | 3.212419 | 1.579314 | 2.594 | 2.5 |
| 1.25 | 0.188 | 0.096910013 | -0.725842151 | rsq | 0.974546 | 0.364996 | 2.005672 | 1.265961 | 1.212 | 1.25 |
| 0.625 | 0.216 | -0.204119983 | -0.665546249 | | | 0.231839 | 1.047746 | 1.120608 | 0.800 | 0.625 |
| 0.3125 | 0.232 | -0.505149978 | -0.634512015 | | | 0.307883 | 0.582205 | 0.641669 | 0.511 | 0.3125 |
| 0.15625 | 0.284 | -0.806179974 | -0.54668166 | | | 0.196452 | 0.321871 | 0.291517 | 0.270 | 0.15625 |
| 0.078125 | 0.370 | -1.10720997 | -0.431796276 | | | 0.103102 | 0.142242 | 0.117713 | 0.121 | 0.078125 |
| 0.0390625 | 0.549 | -1.408239965 | -0.260164047 | | | 0.028376 | 0.042134 | 0.043345 | 0.038 | 0.039063 |
| 0.01953125 | 0.783 | -1.709269961 | -0.108053392 | | | 0.010738 | 0.017601 | 0.01171 | 0.013 | 0.019531 |
| 0.009765625 | 0.938 | -2.010299957 | -0.027642856 | | | 0.007009 | 0.007898 | 0.007848 | 0.008 | 0.009766 |

**bHABP B**

| standard | OD1 | OD2 | OD3 |
|---|---|---|---|
| 5 | 0.101 | 0.112 | 0.118 |
| 2.5 | 0.123 | 0.125 | 0.12 |
| 1.25 | 0.134 | 0.151 | 0.157 |
| 0.625 | 0.162 | 0.176 | 0.165 |
| 0.3125 | 0.211 | 0.221 | 0.221 |
| 0.15625 | 0.261 | 0.257 | 0.288 |
| 0.078125 | 0.363 | 0.369 | 0.434 |
| 0.0390625 | 0.477 | 0.502 | 0.523 |
| 0.01953125 | 0.692 | 0.818 | 0.819 |
| 0.009765625 | 0.745 | 0.859 | 0.845 |

| avg |
|---|
| 0.110 |
| 0.123 |
| 0.147 |
| 0.168 |
| 0.218 |
| 0.269 |
| 0.389 |
| 0.501 |
| 0.776 |
| 0.816 |

| standard | avg.OD | log10 standard | log10 OD | | | HA | HA | HA | avg | std |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 0.110 | 0.698970004 | -0.957293261 | slope | -0.347791 | 5.625291 | 4.13989 | 3.546264 | 4.437 | 5 |
| 2.5 | 0.123 | 0.397940009 | -0.911273436 | intercept | -0.789811 | 3.135577 | 2.989111 | 3.373826 | 3.166 | 2.5 |
| 1.25 | 0.147 | 0.096910013 | -0.831696965 | rsq | 0.975515 | 2.432143 | 1.708651 | 1.520389 | 1.886 | 1.25 |
| 0.625 | 0.168 | -0.204119983 | -0.77555327 | | | 1.385395 | 1.083454 | 1.312016 | 1.260 | 0.625 |
| 0.3125 | 0.218 | -0.505149978 | -0.662208073 | | | 0.632692 | 0.551505 | 0.551505 | 0.579 | 0.3125 |
| 0.15625 | 0.269 | -0.806179974 | -0.570786213 | | | 0.336723 | 0.352508 | 0.251465 | 0.314 | 0.15625 |
| 0.078125 | 0.389 | -1.10720997 | -0.410422704 | | | 0.126561 | 0.120574 | 0.074519 | 0.107 | 0.078125 |
| 0.0390625 | 0.501 | -1.408239965 | -0.300451322 | | | 0.05631 | 0.048394 | 0.042855 | 0.049 | 0.039063 |
| 0.01953125 | 0.776 | -1.709269961 | -0.109951766 | | | 0.018679 | 0.011373 | 0.011332 | 0.014 | 0.019531 |
| 0.009765625 | 0.816 | -2.010299957 | -0.08813247 | | | 0.015007 | 0.009838 | 0.010329 | 0.012 | 0.009766 |

**bHABP C**

| standards | OD1 | OD2 |
|---|---|---|
| 5 | 0.127 | 0.134 |
| 2.5 | 0.123 | 0.135 |
| 1.25 | 0.144 | 0.154 |
| 0.625 | 0.178 | 0.179 |
| 0.3125 | 0.227 | 0.306 |
| 0.15625 | 0.292 | 0.315 |
| 0.078125 | 0.485 | 0.446 |
| 0.0390625 | 0.561 | 0.61 |
| 0.01953125 | 0.799 | 0.82 |
| 0.009765625 | 0.942 | 0.856 |

| avg |
|---|
| 0.131 |
| 0.129 |
| 0.149 |
| 0.179 |
| 0.267 |
| 0.304 |
| 0.466 |
| 0.586 |
| 0.810 |
| 0.899 |

| standard | avg.OD | log10 standard | log10 OD | | | HA | HA | avg | std |
|---|---|---|---|---|---|---|---|---|---|
| 5 | 0.131 | 0.698970004 | -0.884389488 | slope | -0.350388 | 2.851651 | 2.432143 | 2.642 | 5 |
| 2.5 | 0.129 | 0.397940009 | -0.88941029 | intercept | -0.744107 | 3.135577 | 2.379099 | 2.757 | 2.5 |
| 1.25 | 0.149 | 0.096910013 | -0.826813732 | rsq | 0.972332 | 1.964644 | 1.609924 | 1.787 | 1.25 |
| 0.625 | 0.179 | -0.204119983 | -0.74836178 | | | 1.047746 | 1.030481 | 1.039 | 0.625 |
| 0.3125 | 0.267 | -0.505149978 | -0.574302787 | | | 0.509385 | 0.210063 | 0.360 | 0.3125 |
| 0.15625 | 0.304 | -0.806179974 | -0.517841305 | | | 0.241385 | 0.192776 | 0.217 | 0.15625 |
| 0.078125 | 0.466 | -1.10720997 | -0.332080315 | | | 0.0536 | 0.068729 | 0.061 | 0.078125 |
| 0.0390625 | 0.586 | -1.408239965 | -0.232473101 | | | 0.034806 | 0.027152 | 0.031 | 0.039063 |
| 0.01953125 | 0.810 | -1.709269961 | -0.091783147 | | | 0.012194 | 0.011291 | 0.012 | 0.019531 |
| 0.009765625 | 0.899 | -2.010299957 | -0.046240308 | | | 0.007483 | 0.00994 | 0.009 | 0.009766 |

# Appendix 3.3

| | HA ug/ml 1hr | HA ug/ml 6hr | HA ug/ml 24hr |
|---|---|---|---|
| | 0.417028 | 0.133926 | 0.077878 |
| | 0.463505 | 0.125835 | 0.106661 |
| | 0.295966 | 0.448709 | 0.177703 |
| | 0.25061 | 0.149627 | 0.047442 |
| | 0.554425 | 0.152044 | 0.004516 |
| | 0.234691 | 0.48574 | 0.076298 |
| | 0.773007 | 0.114759 | 0.376342 |
| | 0.463505 | 0.238947 | 0.514505 |
| | 0.141308 | 0.376971 | 0.21922 |
| | 0.295966 | | 0.798171 |
| **avg4000** | **0.389001** | **0.247395** | **0.239874** |
| **stdev4000** | **0.185095** | **0.149203** | **0.252367** |

effect of treatment

| treatment 1hr | |
|---|---|
| strain4000 | control |
| 0.41702818 | 0.175763 |
| 0.463505101 | 0.206136 |
| 0.295965511 | 0.187267 |
| 0.250609993 | 0.212892 |
| 0.554425156 | 0.175763 |
| 0.234691391 | |
| 0.773007495 | |
| 0.463505101 | |
| 0.141307516 | |
| 0.295965511 | |

effect of treatment

| treatment 6hr | |
|---|---|
| strain4000 | control |
| 0.133926283 | 0.157019 |
| 0.125834573 | 0.290629 |
| 0.448709324 | 0.230766 |
| 0.149626564 | 0.069068 |
| 0.152043899 | 0.078342 |
| 0.485739683 | 0.072006 |
| 0.114759162 | 0.089135 |
| 0.23894711 | |
| 0.376971009 | |

effect of treatment

| treatment 24hr | |
|---|---|
| strain4000 | control |
| 0.077877722 | 0.015533 |
| 0.106661131 | 0.224479 |
| 0.177702565 | 0.775947 |
| 0.047442259 | 0.209116 |
| 0.004516455 | 0.046543 |
| 0.076298239 | 0.018969 |
| 0.376342108 | 0.124119 |
| 0.514504727 | 0.047442 |
| 0.219219746 | 0.042328 |
| 0.798171174 | 0.528416 |

| | HA ug/ml 1hr | HA ug/ml 6hr | HA ug/ml 24hr |
|---|---|---|---|
| | 0.065809 | 0.362933 | 0.129689 |
| | 0.206136 | 0.152044 | 0.090037 |
| | 0.515856 | 0.113028 | 0.588509 |
| | 0.137033 | 0.260956 | 0.030375 |
| | 0.574912 | 0.583207 | 0.838701 |
| | 0.159961 | 0.981812 | 0.224479 |
| | 0.181413 | 0.838939 | 0.604723 |
| | 0.972041 | 0.230766 | 0.588509 |
| | 0.388947 | 1.004506 | 0.011265 |
| | 0.080299 | 0.33047 | 1.164226 |
| | 0.095599 | 0.343024 | |
| | 0.447398 | 0.407031 | |
| **avg6000** | **0.318784** | **0.467393** | **0.407051** |
| **stdev6000** | **0.271887** | **0.312901** | **0.371363** |

effect of treatment

| treatment 1hr | |
|---|---|
| strain6000 | control |
| 0.065809356 | 0.175763 |
| 0.206136139 | 0.206136 |
| 0.515855713 | 0.187267 |
| 0.137033432 | 0.212892 |
| 0.574912364 | 0.175763 |
| 0.159960701 | |
| 0.181412862 | |
| 0.972040926 | |
| 0.3889468 | |
| 0.080299187 | |
| 0.095599103 | |
| 0.447397871 | |

significant tukey

effect of treatment

| treatment 6hr | |
|---|---|
| strain6000 | control |
| 0.362933377 | 0.157019 |
| 0.152043899 | 0.290629 |
| 0.11302787 | 0.230766 |
| 0.260955792 | 0.069068 |
| 0.583206842 | 0.078342 |
| 0.981812092 | 0.072006 |
| 0.838938536 | 0.089135 |
| 0.230765912 | |
| 1.004505729 | |
| 0.330470043 | |
| 0.343023596 | |
| 0.407030978 | |

effect of treatment

| treatment 24hr | |
|---|---|
| strain6000 | control |
| 0.129689869 | 0.015533 |
| 0.090036641 | 0.224479 |
| 0.588508531 | 0.775947 |
| 0.030375463 | 0.209116 |
| 0.838701205 | 0.046543 |
| 0.224479434 | 0.018969 |
| 0.604723041 | 0.124119 |
| 0.588508531 | 0.047442 |
| 0.011265033 | 0.042328 |
| 1.164225865 | 0.528416 |

| | HA ug/ml 1hr | HA ug/ml 6hr | HA ug/ml 24hr |
|---|---|---|---|
| | 0.187267 | 0.48574 | 0.155003 |
| | 0.463505 | 0.466788 | 1.312953 |
| | 0.170309 | 0.399257 | 1.236047 |
| | 0.227159 | 0.607923 | 1.680146 |
| | 0.063989 | 0.280313 | 0.104403 |
| | 0.327554 | 0.336677 | 0.357691 |
| | 0.497704 | 0.075095 | 0.713386 |
| | 0.295966 | 0.093119 | 0.733579 |
| | 0.175763 | 0.118317 | 0.004274 |
| | | | 0.074755 |
| **avg10000** | **0.267891** | **0.318137** | **0.637224** |
| **stdev10000** | **0.142818** | **0.191099** | **0.599031** |

effect of treatment

| treatment 1hr | |
|---|---|
| strain10000 | control |
| 0.187268751 | 0.175763 |
| 0.463505101 | 0.206136 |
| 0.170309289 | 0.187267 |
| 0.227158825 | 0.212892 |
| 0.063989228 | 0.175763 |
| 0.327554484 | |
| 0.497703997 | |
| 0.295965511 | |
| 0.175762998 | |

effect of treatment

| treatment 6hr | |
|---|---|
| strain10000 | control |
| 0.485739683 | 0.157019 |
| 0.466788436 | 0.290629 |
| 0.399256844 | 0.230766 |
| 0.607923267 | 0.069068 |
| 0.280312615 | 0.078342 |
| 0.336677305 | 0.072006 |
| 0.07509492 | 0.089135 |
| 0.093118878 | |
| 0.11831722 | |

significant tukey

effect of treatment

| treatment 24hr | |
|---|---|
| strain10000 | control |
| 0.155002658 | 0.015533 |
| 1.312952759 | 0.224479 |
| 1.236046502 | 0.775947 |
| 1.680145872 | 0.209116 |
| 0.104403037 | 0.046543 |
| 0.357690708 | 0.018969 |
| 0.713385982 | 0.124119 |
| 0.733578732 | 0.047442 |
| 0.004273959 | 0.042328 |
| 0.074755111 | 0.528416 |

significant tukey

| | HA ug/ml 1hr | HA ug/ml 6hr | HA ug/ml 24hr |
|---|---|---|---|
| | 0.175763 | 0.157019 | 0.015533 |
| | 0.206136 | 0.290629 | 0.224479 |
| | 0.187267 | 0.230766 | 0.775947 |
| | 0.212892 | 0.069068 | 0.209116 |
| | 0.175763 | 0.078342 | 0.046543 |
| | | 0.072006 | 0.018969 |
| | | 0.089135 | 0.124119 |
| | | | 0.047442 |
| | | | 0.042328 |
| | | | 0.528416 |
| **avgcontrol** | **0.191564** | **0.140995** | **0.203289** |
| **stdevcontrol** | **0.017212** | **0.088745** | **0.254818** |

| n | 19 | | | |
|---|---|---|---|---|
| treatment 1hr | n | Mean | SD | SE |
| strain6000 | 0 | 0.467 | 0.313 | 0.0903 |
| control | 0 | 0.141 | 0.089 | 0.0335 |

| Source of variation | SSq | DF | MSq | F | p |
|---|---|---|---|---|---|
| treatment 6hr | 0.471 | 1 | 0.471 | 7.12 | 0.0162 |
| Within cells | 1.124 | 17 | 0.066 | | |
| Total | 1.595 | 18 | | | |

| Contrast | Difference | Tukey 95% CI | |
|---|---|---|---|
| strain6000 v control | 0.326 | 0.068 to 0.584 | (significant) |

| n | 16 | | | |
|---|---|---|---|---|
| treatment 6hr | n | Mean | SD | SE |
| strain10000 | 9 | 0.318 | 0.191 | 0.0637 |
| control | 7 | 0.141 | 0.089 | 0.0335 |

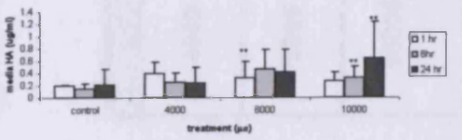
| Source of variation | SSq | DF | MSq | F | p |
|---|---|---|---|---|---|
| treatment 6hr | 0.124 | 1 | 0.124 | 5.10 | 0.0405 |
| Within cells | 0.339 | 14 | 0.024 | | |
| Total | 0.463 | 15 | | | |

| Contrast | Difference | Tukey 95% CI | |
|---|---|---|---|
| strain10000 v control | 0.177 | 0.009 to 0.345 | (significant) |

| n | 20 | | | |
|---|---|---|---|---|
| treatment 24hr | n | Mean | SD | SE |
| strain10000 | 10 | 0.637 | 0.599 | 0.1894 |
| control | 10 | 0.203 | 0.255 | 0.0806 |

| Source of variation | SSq | DF | MSq | F | p |
|---|---|---|---|---|---|
| treatment 24hr | 0.941 | 1 | 0.941 | 4.44 | 0.0493 |
| Within cells | 3.814 | 18 | 0.212 | | |
| Total | 4.755 | 19 | | | |

| Contrast | Difference | Tukey 95% CI | |
|---|---|---|---|
| strain10000 v control | 0.434 | 0.001 to 0.866 | (significant) |



# Appendix 3.4

## Normal Synovial Cell Cultures (HA media concentration µg/ml)

**N1**

| | | P1 | | P2 | | P3 | |
|---|---|---|---|---|---|---|---|
| µs | Time (hours) | control | strain | control | strain | control | strain |
| 4000 | 1 | 3.8 (±2.8) | 2.25 (±1.67) | 0.32 (±0.11) | 0.24 (±0.05) | 2.31 (±3.34) | 7.80 (±9.0) |
| | 6 | 1.10 (±1.14) | 1.06 (±0.51) | 0.26 (±0.19) | 0.11 (±0.09) | 6.23 (±5.23) | 3.61 (±1.07) |
| | 24 | 2.81 (±2.38) | 2.51 (±2.55) | 0.99 (±0.46) | 0.59 (±0.25) | 2.72 (±3.39) | 10.12 (±10.6) |
| 6000 | 1 | | | 0.32 (±0.11) | 0.24 (±0.04) | 2.31 (±3.34) | 6.72 (±8.16) |
| | 6 | | | 0.26 (±0.19) | 0.05 (±0.03) | 6.23 (±5.23) | 3.33 (±2.21) |
| | 24 | | | 0.99 (±0.46) | 0.91 (±0.49) | 2.72 (±3.39) | 3.69 (±4.11) |
| 10000 | 1 | 1.31 (±0.93) | 0.98 (±9.33) | 0.32 (±0.11) | 0.16 (±0.01) | 2.31 (±3.34) | 2.10 (±2.05) |
| | 6 | 1.2 (±0.99) | 1.3 (±0.83) | 0.26 (±0.19) | 0.13 (±0.1) | 6.23 (±5.23) | 2.99 (±0.56) |
| | 24 | 1.6 (1.77)± | 1.28 (±0.73) | 0.99 (±0.46) | 0.94 (±0.19) | 2.72 (±3.39) | 5.05 (±4.23) |

**N2**

| | | P1 | | P2 | | P3 | |
|---|---|---|---|---|---|---|---|
| µs | Time (hours) | control | strain | control | strain | control | strain |
| 4000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |
| 6000 | 1 | 2.67 (±1.72) | 1.99 (±1.36) | | | | |
| | 6 | 1.13 (±0.83) | 1.42 (±1.93) | | | | |
| | 24 | 26.62 (±8.17) | 32.81 (±14.12) | | | | |
| 10000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |

# Appendix 3.5

## OA Synovial Cell Cultures (HA media concentration µg/ml)

### OA1

| | | P1 | | P2 | | P3 | |
|---|---|---|---|---|---|---|---|
| µg | Time (hours) | control | strain | control | strain | control | strain |
| 4000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |
| 6000 | 1 | 9.83(±13.1) | 3.95(±3.82) | | | | |
| | 6 | 2.89(±1.69) | 5.42(±3.41) | | | | |
| | 24 | 7.45(±3.96) | 5.59(±4.12) | | | | |
| 10000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |

### OA2

| | | P1 | | P2 | | P3 | |
|---|---|---|---|---|---|---|---|
| µg | Time (hours) | control | strain | control | strain | control | strain |
| 4000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |
| 6000 | 1 | 1.49 (±0.53) | 2.46 (±1.16) | 1.77 (±1.65) | 1.05 (±0.77) | 0.86 (±0.30) | 0.58 (±0.31) |
| | 6 | 3.63 (±1.42) | 3.82 (±1.70) | 2.18 (±1.46) | 1.54 (±0.91) | 1.85 (±1.81) | 2.23 (±2.51) |
| | 24 | | | 6.62 (±5.18) | 4.81 (±3.79) | 2.07 (±1.55) | 1.30 (±0.66) |
| 10000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |

## OA3

| | | P1 | | P2 | | P3 | |
|---|---|---|---|---|---|---|---|
| µε | Time (hours) | control | strain | control | strain | control | strain |
| 4000 | 1 | 2.84 (±0.49) | 1.13 (±2.59) | | | 0.31 (±0.29) | 0.36 (±0.16) |
| | 6 | 2.89 (±2.81) | 3.17 (±0.09) | | | 0.51 (±0.48) | 1.00 (±0.40) |
| | 24 | 5.11 (±2.06) | 9.72 (±4.30) | | | 1.39 (±1.49) | 8.70 (±8.16) |
| 6000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |
| 10000 | 1 | 0.54 (±0.16) | 1.81 (±1.39) | 2.52 (±1.51) | 1.51 (±0.92) | 0.31 (±0.29) | 0.16 (±1.12) |
| | 6 | 2.89 (±2.81) | 1.08 (±1.32) | 1.44 (±0.21) | 3.28 (±2.34) | 0.51 (±0.48) | 1.60 (±1.74) |
| | 24 | 5.33 (±2.06) | 5.25 (±9.34) | 0.74 (±0.32) | 5.17 (±2.83) | 1.39 (±1.49) | 2.86 (±1.39) |

## OA4

| | | P1 | | P2 | | P3 | |
|---|---|---|---|---|---|---|---|
| µε | Time (hours) | control | strain | control | strain | control | strain |
| 4000 | 1 | 0.19(±0.02) | 0.39(±1.9) | 0.44(±0.25) | 0.46(±0.273) | 1.29(±0.74) | 2.52(±1.35) |
| | 6 | 0.14(±0.09) | 0.25(±0.15) | 0.20(±0.11) | 0.19(±0.193) | 16.55(±13.23) | 13.40(±9.92) |
| | 24 | 0.20(±0.25) | 0.24(±0.25) | 0.48(±0.22) | 0.40(±0.17) | 7.23(±5.67) | 14.5(±11.38) |
| 6000 | 1 | 0.19(±0.02) | 0.32(±0.27) | 0.44(±0.25) | 0.83(±0.85) | 1.29(±0.74) | 3.12(±3.36) |
| | 6 | 0.14(±0.09) | 0.47(±0.31) | 0.20(±0.11) | 0.28(±0.27) | 16.55(±13.23) | 7.95(±7.41) |
| | 24 | 0.20(±0.25) | 0.41(±0.37) | 0.48(±0.22) | 0.43(±0.29) | 7.23(±5.67) | 8.80(±5.33) |
| 10000 | 1 | 0.19(±0.02) | 0.27(±1.43) | 0.44(±0.25) | 1.7(±1.0) | 1.29(±0.74) | 2.06(±1.60) |
| | 6 | 0.14(±0.09) | 0.32(±0.19) | 0.20(±0.11) | 0.37(±0.26) | 16.55(±13.23) | 6.71(±8.21) |
| | 24 | 0.20(±0.25) | 0.64(±0.60) | 0.48(±0.22) | 0.54(±0.32) | 7.23(±5.67) | 2.93(±2.20) |

**OA5**

| | | P1 | | P2 | | P3 | |
|---|---|---|---|---|---|---|---|
| µg | Time (hours) | control | strain | control | strain | control | strain |
| 4000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |
| 6000 | 1 | | | | | 2.15 *(±1.12)* | 1.26 *(±0.88)* |
| | 6 | | | | | 6.83 *(±4.39)* | 2.33 *(±2.19)* |
| | 24 | | | | | 4.39 *(±1.88)* | 3.45 *(±0.82)* |
| 10000 | 1 | | | | | | |
| | 6 | | | | | | |
| | 24 | | | | | | |

# Appendix 3.6

## Percentage increases in media HA concentration

**normal cells (N1)**

| | | Passage | | |
|---|---|---|---|---|
| µs | Time (hours) | 1 | 2 | 3 |
| 4000 | 1 | -40.7 | -25.8 | 237.1 |
| | 6 | -4.3 | -56.9 | -42.1 |
| | 24 | -10.6 | -39.8 | 272.1 |
| 6000 | 1 | | -26.4 | 190.5 |
| | 6 | | -79.7 | -46.6 |
| | 24 | | -7.4 | 35.7 |
| 10000 | 1 | -10.1 | -48.8 | -9.1 |
| | 6 | -1.7 | -49.9 | -52.0 |
| | 24 | 2.2 | -4.2 | 85.5 |

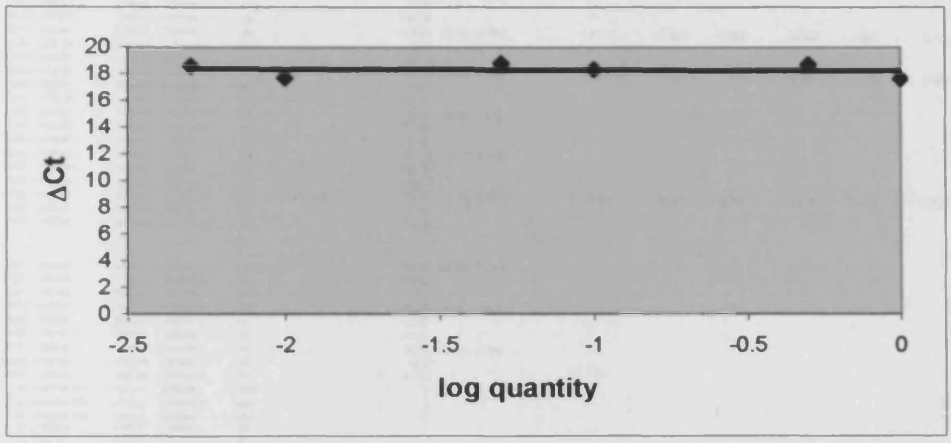
**OA cells (OA4)**

| | | Passage | | |
|---|---|---|---|---|
| µs | Time (hours) | 1 | 2 | 3 |
| 4000 | 1 | 103.1 | 4.4 | 95.4 |
| | 6 | 75.5 | -8.2 | 17.9 |
| | 24 | 18.0 | -16.0 | 100.9 |
| 6000 | 1 | 66.4 | 90.1 | 141.9 |
| | 6 | 231.5 | 38.2 | -52.0 |
| | 24 | | -10.3 | 21.9 |
| 10000 | 1 | 39.7 | 289.3 | 59.5 |
| | 6 | 125.6 | 82.6 | -48.3 |
| | 24 | 213.5 | 13.7 | -59.5 |

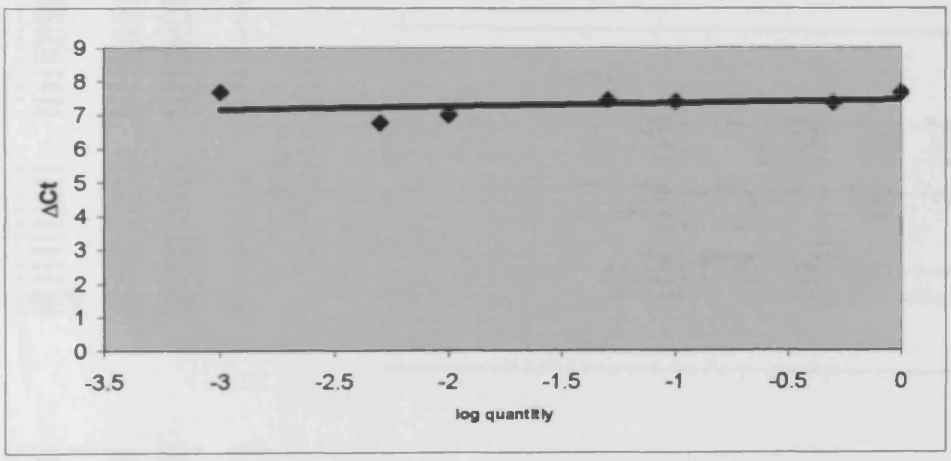
# Appendix 4.1

## ΔCt Validation Curves

HAS1




HAS2



# Appendix 4.2

| Well | Reporter | Type | Baseline Stdev | Amplitude | Ct | Quantity | Replicate | Ct Mean | Ct Stdev | RQ | Sample Name |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | FAM | UNKN | 3.010E-03 | 1.160E+00 | 34.2 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX1 |
| 2 | FAM | UNKN | 3.360E-03 | 1.090E+00 | 34.68 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX1 |
| 3 | FAM | UNKN | 3.010E-03 | 1.160E+00 | 35.15 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX1 |
| 4 | FAM | UNKN | 3.090E-03 | 1.200E+00 | 33.01 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 5 | FAM | UNKN | 3.090E-03 | 1.160E+00 | 33.68 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 6 | FAM | UNKN | 3.330E-03 | 1.280E+00 | 33.01 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 7 | FAM | UNKN | 2.370E-03 | 1.150E+00 | 33.73 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 8 | FAM | UNKN | 3.390E-03 | 1.280E+00 | 32.79 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 9 | FAM | UNKN | 3.090E-03 | 1.140E+00 | 34.09 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 10 | FAM | UNKN | 6.160E-03 | 1.210E+00 | 32.37 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 11 | FAM | UNKN | 1.710E-03 | 1.200E+00 | 32.4 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 12 | FAM | UNKN | 2.460E-03 | 1.280E+00 | 33.04 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 13 | FAM | UNKN | 3.380E-03 | 1.110E+00 | 34.0 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 14 | FAM | UNKN | 4.030E-03 | 9.190E-01 | 35.04 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 15 | FAM | UNKN | 2.830E-03 | 9.350E-01 | 35.04 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 16 | FAM | UNKN | 3.820E-03 | 1.040E+00 | 33.49 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 17 | FAM | UNKN | 2.390E-03 | 7.370E-01 | 35.01 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 18 | FAM | UNKN | 3.330E-03 | 1.080E+00 | 34.19 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 19 | FAM | UNKN | 2.770E-03 | 1.240E+00 | 31.49 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 20 | FAM | UNKN | 3.370E-03 | 1.280E+00 | 31.09 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 21 | FAM | UNKN | 3.230E-03 | 1.210E+00 | 31.08 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 22 | FAM | UNKN | 2.880E-03 | 1.360E+00 | 33.01 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 23 | FAM | UNKN | 3.470E-03 | 1.290E+00 | 33.03 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 24 | FAM | UNKN | 4.030E-03 | 1.260E+00 | 33.79 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 25 | FAM | UNKN | 2.750E-03 | 1.140E+00 | 33.03 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 26 | FAM | UNKN | 3.390E-03 | 1.030E+00 | 33.79 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 27 | FAM | UNKN | 3.390E-03 | 1.100E+00 | 33.01 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 28 | FAM | UNKN | 3.030E-03 | 9.460E-01 | 35.35 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 29 | FAM | UNKN | 3.480E-03 | 9.180E-01 | 34.44 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 30 | FAM | UNKN | 3.080E-03 | 8.880E-01 | 35.35 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 31 | FAM | UNKN | 3.310E-03 | 1.170E+00 | 32.35 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 32 | FAM | UNKN | 3.170E-03 | 1.160E+00 | 32.69 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 33 | FAM | UNKN | 1.850E-03 | 1.140E+00 | 33.03 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 34 | FAM | | | | | | | | | | |
| 35 | FAM | | | | | | | | | | |
| 36 | FAM | | | | | | | | | | |
| 37 | FAM | UNKN | 1.380E-03 | 1.010E+00 | 34.71 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX4 |
| 38 | FAM | UNKN | 2.380E-03 | 9.910E-01 | 36.2 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX4 |
| 39 | FAM | UNKN | 3.020E-03 | 0.000E+00 | 32.33 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX4 |
| 40 | FAM | UNKN | 2.050E-03 | 1.230E+00 | 30.73 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 41 | FAM | UNKN | 2.980E-03 | 1.280E+00 | 30.82 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 42 | FAM | UNKN | 2.170E-03 | 1.260E+00 | 30.86 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 43 | FAM | UNKN | 2.880E-03 | 1.190E+00 | 31.28 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 44 | FAM | UNKN | 3.070E-03 | 1.210E+00 | 30.07 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 45 | FAM | UNKN | 3.200E-03 | 1.270E+00 | 30.04 | 1.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 46 | FAM | STD | 3.070E-03 | 2.340E-03 | 40 | 0.000E+00 | STD | 0.000E+00 | 0.000E+00 | - | HAS1 |
| 47 | FAM | STD | 1.870E+00 | -6.000E+02 | 40 | 0.000E+00 | STD | 0.000E+00 | 0.000E+00 | * | HAS1 |
| 48 | FAM | STD | 1.000E+00 | -1.220E+01 | 40 | 0.000E+00 | STD | 0.000E+00 | 0.000E+00 | * | HAS1 |
| 49 | FAM | | 3.000E+00 | 1.010E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX1 |
| 50 | FAM | | 3.000E+00 | 0.000E+00 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX1 |
| 51 | FAM | | 3.000E+00 | 7.870E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX1 |
| 52 | FAM | | 3.000E+00 | 0.000E+00 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 53 | FAM | | 3.000E+00 | 1.270E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 54 | FAM | | 3.000E+00 | 1.220E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 55 | FAM | | 3.000E+00 | 1.490E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 56 | FAM | | 3.000E+00 | 7.870E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 57 | FAM | | 3.000E+00 | 0.000E+00 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 58 | FAM | | 3.000E+00 | -1.030E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY2 |
| 59 | FAM | | 3.000E+00 | 0.000E+00 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY2 |
| 60 | FAM | | 3.000E+00 | -1.000E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY2 |
| 61 | FAM | | 3.000E+00 | -1.400E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 62 | FAM | | 3.000E+00 | -1.130E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 63 | FAM | | 3.000E+00 | -1.070E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 64 | FAM | | 3.000E+00 | -6.380E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 65 | FAM | | 3.000E+00 | -1.000E+01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 66 | FAM | | 3.000E+00 | -9.910E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 67 | FAM | | 3.000E+00 | -6.100E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 68 | FAM | | 3.000E+00 | -1.200E-01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 69 | FAM | | 3.000E+00 | 7.870E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 70 | FAM | | 3.000E+00 | -4.380E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY2 |
| 71 | FAM | | 3.000E+00 | -1.100E-01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY2 |
| 72 | FAM | | 3.000E+00 | -1.040E-01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY2 |
| 73 | FAM | | 3.000E+00 | -3.180E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 74 | FAM | | 3.000E+00 | -9.760E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 75 | FAM | | 3.000E+00 | -6.330E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 76 | FAM | | 3.000E+00 | -6.280E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 77 | FAM | | 3.000E+00 | -6.950E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 78 | FAM | | 3.000E+00 | -1.370E-01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 79 | FAM | | 3.000E+00 | -4.680E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 80 | FAM | | 3.000E+00 | -6.100E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 81 | FAM | | 3.000E+00 | -2.710E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 82 | FAM | | | | | | | | | | |
| 83 | FAM | | | | | | | | | | |
| 84 | FAM | | | | | | | | | | |
| 85 | FAM | | 3.000E+00 | 3.040E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX4 |
| 86 | FAM | | 3.000E+00 | -1.330E-01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX4 |
| 87 | FAM | | 3.000E+00 | -1.040E-01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX4 |
| 88 | FAM | | 3.000E+00 | 2.380E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 89 | FAM | | 3.000E+00 | 7.870E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 90 | FAM | | 3.000E+00 | -1.020E-01 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TY1 |
| 91 | FAM | | 3.000E+00 | -3.020E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 92 | FAM | | 3.000E+00 | -6.100E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |
| 93 | FAM | | 3.000E+00 | -2.710E-02 | 0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | - | TX2 |

| Slope | 0 | optimizing threshold |
|---|---|---|
| Offset | 0 | optimizing threshold |
| R^2 | 0 | |
| FG Stdev | 0 | |

Threshold definition parameters

| | |
|---|---|
| Baseline s | 0 |
| Baseline e | 10 |
| Amp. dev. s | 0.0001100 |
| Multiplier | 10 |
| Threshold | 0.277 (Multiplier Manually) |

| Well | Reporter | Type | Baseline Stdev | Amplitude | Ct | Quantity | Replicate | Ct Mean | Ct Stdev | RQ | Sample Name |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 46 | VIC | UNKN | 6.000E-03 | 3.000E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX1 |
| 47 | VIC | UNKN | 4.000E-03 | 3.000E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX1 |
| 48 | VIC | UNKN | 2.710E-03 | 3.000E+00 | 18.78 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX1 |
| 49 | VIC | UNKN | 7.370E-03 | 3.000E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 50 | VIC | UNKN | 7.000E-03 | 3.000E+00 | 17.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 51 | VIC | UNKN | 3.010E-03 | 3.000E+00 | 18.04 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 52 | VIC | UNKN | 4.700E-03 | 3.000E+00 | 18.08 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 53 | VIC | UNKN | 4.460E-03 | 3.000E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 54 | VIC | UNKN | 6.000E-03 | 3.000E+00 | 18.77 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 55 | VIC | UNKN | 5.000E-03 | 3.000E+00 | 20.49 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 56 | VIC | UNKN | 4.070E-03 | 3.000E+00 | 20.89 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 57 | VIC | UNKN | 3.400E-03 | 3.370E+00 | 21.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 58 | VIC | UNKN | 3.400E-03 | 3.300E+00 | 23.25 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 59 | VIC | UNKN | 3.020E-03 | 3.320E+00 | 23.89 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 60 | VIC | UNKN | 2.900E-03 | 3.220E+00 | 24.08 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 61 | VIC | UNKN | 3.000E-03 | 3.420E+00 | 18.09 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 62 | VIC | UNKN | 3.010E-03 | 3.710E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 63 | VIC | UNKN | 4.350E-03 | 3.600E+00 | 18.45 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 64 | VIC | UNKN | 2.000E-03 | 3.300E+00 | 18.02 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 65 | VIC | UNKN | 1.840E-03 | 3.000E+00 | 18.09 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 66 | VIC | UNKN | 1.420E-03 | 3.000E+00 | 18.23 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 67 | VIC | UNKN | 4.760E-03 | 3.000E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 68 | VIC | UNKN | 5.070E-03 | 3.000E+00 | 18.49 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 69 | VIC | UNKN | 4.000E-03 | 3.000E+00 | 18.48 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY2 |
| 70 | VIC | UNKN | 6.070E-03 | 3.000E+00 | 18.7 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 71 | VIC | UNKN | 6.100E-03 | 3.000E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 72 | VIC | UNKN | 6.700E-03 | 3.310E+00 | 17.07 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 73 | VIC | UNKN | 2.000E-03 | 3.000E+00 | 20.48 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 74 | VIC | UNKN | 3.000E-03 | 3.400E+00 | 20.0 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 75 | VIC | UNKN | 3.200E-03 | 3.000E+00 | 21.48 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 76 | VIC | UNKN | 3.000E-03 | 3.400E+00 | 20.10 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 77 | VIC | UNKN | 6.000E-03 | 3.400E+00 | 20.70 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 78 | VIC | UNKN | 3.400E-03 | 3.400E+00 | 20.48 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 79 | VIC | | | | | | | | | | |
| 80 | VIC | | | | | | | | | | |
| 81 | VIC | | | | | | | | | | |
| 82 | VIC | | | | | | | | | | |
| 83 | VIC | | | | | | | | | | |
| 84 | VIC | | | | | | | | | | |
| 85 | VIC | UNKN | 3.700E-03 | 3.400E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX4 |
| 86 | VIC | UNKN | 3.740E-03 | 3.400E+00 | 18.00 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX4 |
| 87 | VIC | UNKN | 3.070E-03 | 3.000E+00 | 20.33 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX4 |
| 88 | VIC | UNKN | 2.070E-03 | 3.210E+00 | 18.11 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 89 | VIC | UNKN | 1.960E-03 | 3.400E+00 | 18.23 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 90 | VIC | UNKN | 2.000E-03 | 3.000E+00 | 18.21 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TY1 |
| 91 | VIC | UNKN | 3.100E-03 | 3.000E+00 | 14.02 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 92 | VIC | UNKN | 1.070E-03 | 3.000E+00 | 18.17 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 93 | VIC | UNKN | 1.000E-03 | 3.000E+00 | 18.24 | 0.000E+00 | | 0.000E+00 | 0.000E+00 | * | TX2 |
| 94 | VIC | STD | 3.300E-03 | 1.000E+00 | 40 | 0.000E+00 | STD | 0.000E+00 | 0.000E+00 | * | HAS1 |
| 95 | VIC | STD | 3.140E-03 | 1.070E+00 | 40 | 0.000E+00 | STD | 0.000E+00 | 0.000E+00 | * | HAS1 |
| 96 | VIC | STD | 7.070E-01 | -1.210E+01 | 40 | 0.000E+00 | STD | 0.000E+00 | 0.000E+00 | * | HAS1 |

| Slope | 0 | optimizing threshold |
|---|---|---|
| Offset | 0 | optimizing threshold |
| R^2 | 0 | |
| FG Stdev | 0 | |

Threshold definition parameters

| | |
|---|---|
| Baseline s | 3 |
| Baseline e | 12 |
| Amp. dev. s | 0.0001107 |

| | Ct | mean ± stdev | | ΔCt | (ΔCt-ΔCt) | St.Dev. ΔΔCt | $2^{-\Delta\Delta Ct}$ rel. to normalised sample | $2^{-\Delta\Delta Ct}$ + St. Dev | $2^{-\Delta\Delta Ct}$ - St. Dev | St. Dev (minus) | St. Dev. (plus) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14.55 | 14.95 | 0.411 | | | | | | | | |
| | 14.92 | | | | | | | | | | |
| | 15.37 | | | | | | | | | | |
| | 15.67 | 15.56 | 0.746 | | | | | | | | |
| | 16.25 | | | | | | | | | | |
| | 14.77 | | | | | | | | | | |
| N1 4000 | 15.28 | 14.41 | 0.817 | 2.673 | 0.842 | **0.157** | 0.087 | 0.281 | 0.069 | 0.124 | |
| | 13.66 | | | | | | | | | | |
| | 14.28 | | | | | | | | | | |
| N1 CONTROL | 11.78 | 11.73 | 0.204 | 0.000 | 0.289 | **1.000** | 0.819 | 1.221 | 0.181 | 0.221 | |
| | 11.51 | | | | | | | | | | |
| | 11.91 | | | | | | | | | | |
| | 11.55 | 12.1 | 0.488 | | | | | | | | |
| | 12.25 | | | | | | | | | | |
| | 12.49 | | | | | | | | | | |
| | 14.89 | 15.78 | 1.639 | | | | | | | | |
| | 17.65 | | | | | | | | | | |
| | 14.74 | | | | | | | | | | |
| | 16.41 | 16.35 | 0.182 | -0.477 | 0.503 | **1.392** | 0.982 | 1.972 | 0.410 | 0.580 | |
| | 16.5 | | | | | | | | | | |
| | 16.15 | | | | | | | | | | |
| | 16.76 | 16.83 | 0.469 | 0.000 | 0.663 | **1.000** | 0.631 | 1.584 | 0.369 | 0.584 | |
| | 16.4 | | | | | | | | | | |
| | 17.33 | | | | | | | | | | |
| | 16.82 | 16.83 | 0.39 | | | | | | | | |
| | 17.22 | | | | | | | | | | |
| | 16.44 | | | | | | | | | | |
| | 14.63 | 14.35 | 0.703 | | | | | | | | |
| | 13.54 | | | | | | | | | | |
| | 14.87 | | | | | | | | | | |
| N1 10000 | 12.44 | 12.39 | 0.229 | 0.657 | 0.307 | **0.634** | 0.513 | 0.785 | 0.122 | 0.150 | |
| | 12.14 | | | | | | | | | | |
| | 12.59 | | | | | | | | | | |
| | 14.88 | 14.07 | 1.712 | | | | | | | | |
| | 15.22 | | | | | | | | | | |
| | 12.1 | | | | | | | | | | |
| | 15.62 | 15.29 | 0.54 | | | | | | | | |
| | 15.59 | | | | | | | | | | |
| | 14.67 | | | | | | | | | | |
| | 16.73 | 15.45 | 1.109 | | | | | | | | |
| | 14.9 | | | | | | | | | | |
| | 14.73 | | | | | | | | | | |
| | 0 | | | | | | | | | | |
| | 0 | | | | | | | | | | |
| | 0 | | | | | | | | | | |

Data for ANOVA

N1
Effect of Treatment

| treatment | | |
|---|---|---|
| control | 4000 | 10000 |
| 11.78 | 15.28 | 12.44 |
| 11.51 | 13.66 | 12.14 |
| 11.91 | 14.28 | 12.59 |

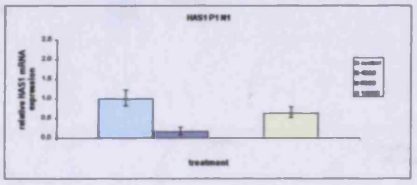
Results of ANOVA

n | 9

| treatment | n | Mean | SD | SE |
|---|---|---|---|---|
| control | 3 | 11.733 | 0.204 | 0.1178 |
| 4000 | 3 | 14.407 | 0.817 | 0.4719 |
| 10000 | 3 | 12.390 | 0.229 | 0.1323 |

| Source of variation | SSq | DF | MSq | F | p |
|---|---|---|---|---|---|
| treatment | 11.645 | 2 | 5.822 | 22.91 | 0.0016 |
| Within cells | 1.525 | 6 | 0.254 | | |
| Total | 13.169 | 8 | | | |

| Contrast | Difference | Tukey 95% CI | |
|---|---|---|---|
| control v 4000 | -2.673 | -3.936 to -1.411 | (significant) |
| control v 10000 | -0.657 | -1.919 to 0.606 | |
| 4000 v 10000 | 2.017 | 0.754 to 3.279 | (significant) |



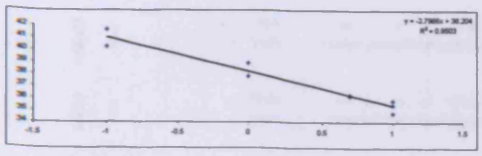
# Appendix 4.3

| Well | Sample Type | Starting Dilution | activity | Ct | Quantity | Residuals | Qty Mean | Qty StdDev | IPC | Sample Name |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Unknown | 2.338e-03 | 7.264e-01 | 34.23 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T21 |
| 2 | Unknown | 1.910e-03 | 6.482e-01 | 34.54 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 3 | Unknown | 2.483e-03 | 7.075e-01 | 34.05 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 4 | Unknown | 2.200e-03 | 7.010e-01 | 34.01 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha1 |
| 5 | Unknown | 1.800e-03 | 6.170e-01 | 34.68 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha1 |
| 6 | Unknown | 2.010e-03 | 6.446e-01 | 35.08 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha1 |
| 7 | Unknown | 2.080e-03 | 7.100e-01 | 34.5 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 1.0ul |
| 8 | Unknown | 1.680e-03 | 5.480e-01 | 35.3 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.6ul |
| 9 | Unknown | 1.580e-03 | 4.380e-01 | 36.09 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.1ul |
| 10 | Unknown | 1.700e-03 | 5.200e-01 | 37.58 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.06ul |
| 11 | Unknown | 2.110e-03 | 6.400e-01 | 40.22 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.01ul |
| 12 | NTC | 1.710e-03 | 1.010e-03 | 50 | 0.000e+00 | NTC | 0.000e+00 | 0.000e+00 | | NTC |
| 13 | Unknown | 2.100e-03 | 5.600e-01 | 34.35 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 14 | Unknown | 1.800e-03 | 5.000e-01 | 34.51 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 15 | Unknown | 1.880e-03 | 4.600e-01 | 34.69 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 16 | Unknown | 1.480e-03 | 4.710e-01 | 35.12 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha2 |
| 17 | Unknown | 2.080e-03 | 5.300e-01 | 37.38 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha2 |
| 18 | Unknown | 1.730e-03 | 4.660e-01 | 38.0 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha2 |
| 19 | Unknown | 2.020e-03 | 5.000e-01 | | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 1.0ul |
| 20 | Unknown | 1.800e-03 | 5.300e-01 | | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.6ul |
| 21 | Unknown | 1.870e-03 | 3.770e-01 | | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.1ul |
| 22 | Unknown | 1.480e-03 | 3.000e-01 | | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.06ul |
| 23 | Unknown | 2.080e-03 | 4.000e-01 | | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.01ul |
| 24 | Unknown | | | | | | | | | |
| 25 | Unknown | 2.185e-03 | 4.010e-01 | 36.12 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 26 | Unknown | 2.100e-03 | 3.860e-01 | 37.55 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 27 | Unknown | 1.880e-03 | 3.480e-01 | 37.67 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 28 | Unknown | 1.880e-03 | 4.310e-01 | 35.83 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 29 | Unknown | 1.200e-03 | 4.080e-01 | 36.69 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 30 | Unknown | 1.880e-03 | 4.110e-01 | 36.13 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 31 | Unknown | 1.840e-03 | 4.040e-01 | 35.50 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 1.0ul |
| 32 | Unknown | 1.760e-03 | 3.870e-01 | 37.1 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.6ul |
| 33 | Unknown | 2.210e-03 | 3.310e-01 | 37.51 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.1ul |
| 34 | Unknown | 1.010e-03 | 1.880e-01 | 50 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.06ul |
| 35 | Unknown | 1.010e-03 | 1.880e-01 | 50 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.01ul |
| 36 | Unknown | | | | | | | | | |
| 37 | Unknown | 2.360e-03 | 4.040e-01 | 36.03 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 38 | Unknown | 1.210e-03 | 3.230e-01 | 37 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 39 | Unknown | 1.840e-03 | 3.860e-01 | 37.25 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 40 | Unknown | 1.680e-03 | 3.870e-01 | 37.46 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 41 | Unknown | 1.760e-03 | 4.480e-01 | 36.01 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 42 | Unknown | 2.080e-03 | 4.030e-01 | 37.36 | 1.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 43 | Unknown | | | | | | | | | |
| 44 | Unknown | | | | | | | | | |
| 45 | Unknown | | | | | | | | | |
| 46 | Unknown | | | | | | | | | |
| 47 | Unknown | | | | | | | | | |
| 48 | Unknown | | | | | | | | | |
| 49 | Unknown | 0.000e+00 | 0.760e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T21 |
| 50 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T21 |
| 51 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 52 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha1 |
| 53 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha1 |
| 54 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha1 |
| 55 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 1.0ul |
| 56 | Unknown | 0.000e+00 | 1.760e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.6ul |
| 57 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.1ul |
| 58 | Unknown | 0.000e+00 | 4.380e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.06ul |
| 59 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.01ul |
| 60 | Unknown | 0.000e+00 | 4.380e-03 | 0 | 0.000e+00 | NTC | 0.000e+00 | 0.000e+00 | - | 0.1 |
| 61 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 62 | Unknown | 0.000e+00 | 7.480e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 63 | Unknown | 0.000e+00 | 4.380e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 64 | Unknown | 0.000e+00 | 4.670e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha2 |
| 65 | Unknown | 0.000e+00 | 7.310e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha2 |
| 66 | Unknown | 0.000e+00 | 4.370e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha2 |
| 67 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 1.0ul |
| 68 | Unknown | 0.000e+00 | 6.760e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.6ul |
| 69 | Unknown | 0.000e+00 | 4.020e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.1ul |
| 70 | Unknown | 0.000e+00 | 4.080e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.06ul |
| 71 | Unknown | 0.000e+00 | 6.760e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.01ul |
| 72 | Unknown | | | | | | | | | |
| 73 | Unknown | 0.000e+00 | 2.200e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 74 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 75 | Unknown | 0.000e+00 | 2.070e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 76 | Unknown | 0.000e+00 | 1.070e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha3 |
| 77 | Unknown | 0.000e+00 | 4.670e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha3 |
| 78 | Unknown | 0.000e+00 | 2.090e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha3 |
| 79 | Unknown | 0.000e+00 | 1.410e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 1.0ul |
| 80 | Unknown | 0.000e+00 | 4.380e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.6ul |
| 81 | Unknown | 0.000e+00 | 2.380e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.1ul |
| 82 | Unknown | 0.000e+00 | 1.070e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.06ul |
| 83 | Unknown | 0.000e+00 | 1.070e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.01ul |
| 84 | Unknown | | | | | | | | | |
| 85 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 86 | Unknown | 0.000e+00 | 0.000e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 87 | Unknown | 0.000e+00 | 0.100e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T23 |
| 88 | Unknown | 0.000e+00 | 4.020e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha4 |
| 89 | Unknown | 0.000e+00 | 4.020e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha4 |
| 90 | Unknown | 0.000e+00 | 0.070e-03 | 0 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | T alpha4 |
| 91 | Unknown | | | | | | | | | |
| 92 | Unknown | | | | | | | | | |
| 93 | Unknown | | | | | | | | | |
| 94 | Unknown | | | | | | | | | |
| 95 | Unknown | | | | | | | | | |
| 96 | Unknown | | | | | | | | | |

Slope 0 outlier/low density
intercept 0 outlier
RFU 0
FU StdDev 0

Threshold detection correction
Baseline s 0
Baseline e 50
Res. dbl. s 0.0774655
Modifiedbs 10
Threshold 0.049 (Calculated Manually)

| Concentration | Ct | log conc |
|---|---|---|
| 1.0ul | 34.2 | 1 |
| | 35.45 | 1 |
| | 35.63 | 1 |
| 0.6ul | 36.3 | 0.69897 |
| | 36.2 | 0.69897 |
| | | 0.69897 |
| 0.1ul | 36.88 | 0 |
| | | 0 |
| | 37.91 | 0 |
| 0.06ul | | -0.301029996 |
| | | -0.301029996 |
| | | -0.301029996 |
| 0.01ul | 40.22 | -1 |
| | 41.64 | -1 |
| | | -1 |



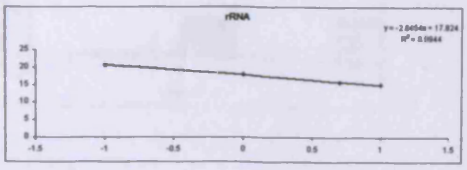

y = -3.8482x + 36.79 y=y-d/m Ct

| Well | Sample Type | Starting Dilution | activity | Ct | Quantity | Residuals | Qty Mean | Qty StdDev | IPC | Sample Name |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | VIC | | | | | | | | | |
| 2 | VIC | | | | | | | | | |
| 3 | VIC | | | | | | | | | |
| 4 | VIC | | | | | | | | | |
| 5 | VIC | | | | | | | | | |
| 6 | VIC | | | | | | | | | |
| 7 | VIC | | | | | | | | | |
| 8 | VIC | | | | | | | | | |
| 9 | VIC | | | | | | | | | |
| 10 | VIC | | | | | | | | | |
| 11 | VIC | | | | | | | | | |
| 12 | VIC | | | | | | | | | |
| 13 | VIC | | | | | | | | | |
| 14 | VIC | | | | | | | | | |
| 15 | VIC | | | | | | | | | |
| 16 | VIC | | | | | | | | | |
| 17 | VIC | | | | | | | | | |
| 18 | VIC | | | | | | | | | |
| 19 | VIC | | | | | | | | | |
| 20 | VIC | | | | | | | | | |
| 21 | VIC | | | | | | | | | |
| 22 | VIC | | | | | | | | | |
| 23 | VIC | | | | | | | | | |
| 24 | VIC | | | | | | | | | |
| 25 | VIC | | | | | | | | | |
| 26 | VIC | | | | | | | | | |
| 27 | VIC | | | | | | | | | |
| 28 | VIC | | | | | | | | | |
| 29 | VIC | | | | | | | | | |
| 30 | VIC | | | | | | | | | |
| 31 | VIC | | | | | | | | | |
| 32 | VIC | | | | | | | | | |
| 33 | VIC | | | | | | | | | |
| 34 | VIC | | | | | | | | | |
| 35 | VIC | | | | | | | | | |
| 36 | VIC | | | | | | | | | |
| 37 | VIC | | | | | | | | | |
| 38 | VIC | | | | | | | | | |
| 39 | VIC | | | | | | | | | |
| 40 | VIC | | | | | | | | | |
| 41 | VIC | | | | | | | | | |
| 42 | VIC | | | | | | | | | |
| 43 | VIC | | | | | | | | | |
| 44 | VIC | | | | | | | | | |
| 45 | VIC | | | | | | | | | |
| 46 | VIC | | | | | | | | | |
| 47 | VIC | | | | | | | | | |
| 48 | VIC | | | | | | | | | |
| 49 | VIC | dRNA | 1.480e-03 | 3.040e-01 | 13.87 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T21 |
| 50 | VIC | dRNA | 0.820e-03 | 3.000e-01 | 14.46 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T21 |
| 51 | VIC | dRNA | 0.710e-03 | 3.000e-01 | 14.37 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T21 |
| 52 | VIC | dRNA | 4.380e-03 | 4.200e-01 | 15.8 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha1 |
| 53 | VIC | dRNA | 5.480e-03 | 4.120e-01 | 16 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha1 |
| 54 | VIC | dRNA | 2.230e-03 | 4.000e-01 | 16.18 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha1 |
| 55 | VIC | dRNA | 6.000e-03 | 4.000e-01 | 16.18 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 1.0ul |
| 56 | VIC | dRNA | 3.760e-03 | 4.000e-01 | 16.38 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.6ul |
| 57 | VIC | dRNA | 3.010e-03 | 4.000e-01 | 17.46 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.1ul |
| 58 | VIC | dRNA | 2.200e-03 | 3.880e-01 | 17.31 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.06ul |
| 59 | VIC | dRNA | 2.010e-03 | 4.040e-01 | 20.87 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.01ul |
| 60 | VIC | NTC | 2.770e-03 | 3.000e-01 | 30.74 | 0.000e+00 | NTC | 0.000e+00 | 0.000e+00 | + | 0.1 |
| 61 | VIC | dRNA | 1.680e-03 | 3.760e-01 | 14.18 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 62 | VIC | dRNA | 1.230e-03 | 3.770e-01 | 14.23 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 63 | VIC | dRNA | 3.480e-03 | 3.760e-01 | 14.46 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 64 | VIC | dRNA | 2.200e-03 | 3.680e-01 | 16.04 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha2 |
| 65 | VIC | dRNA | 1.880e-03 | 4.040e-01 | 16.9 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha2 |
| 66 | VIC | dRNA | 2.100e-03 | 3.910e-01 | 20.32 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha2 |
| 67 | VIC | dRNA | 4.000e-03 | 4.100e-01 | 14.98 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 1.0ul |
| 68 | VIC | dRNA | 4.000e-03 | 4.100e-01 | 16.98 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.6ul |
| 69 | VIC | dRNA | 1.680e-03 | 3.910e-01 | 17.73 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.1ul |
| 70 | VIC | dRNA | 0.370e-03 | 4.140e-01 | 17.02 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.06ul |
| 71 | VIC | dRNA | 1.070e-03 | 3.870e-01 | 20.96 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.01ul |
| 72 | VIC | | | | | | | | | |
| 73 | VIC | dRNA | 2.880e-03 | 3.800e-01 | 17.01 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 74 | VIC | dRNA | 2.880e-03 | 3.800e-01 | 17.13 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 75 | VIC | dRNA | 1.760e-03 | 3.800e-01 | 17.3 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 76 | VIC | dRNA | 4.010e-03 | 4.000e-01 | 15.49 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha3 |
| 77 | VIC | dRNA | 5.000e-03 | 3.980e-01 | 15.01 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha3 |
| 78 | VIC | dRNA | 3.230e-03 | 3.820e-01 | 15.03 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha3 |
| 79 | VIC | dRNA | 4.010e-03 | 3.820e-01 | 15.07 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 1.0ul |
| 80 | VIC | dRNA | 7.000e-03 | 3.980e-01 | 15.00 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.6ul |
| 81 | VIC | dRNA | 1.000e-03 | 3.980e-01 | 15.03 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.1ul |
| 82 | VIC | dRNA | 1.000e-03 | 4.070e-01 | 15 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | 0.06ul |
| 83 | VIC | dRNA | 1.000e-03 | 3.800e-01 | 15 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | - | 0.01ul |
| 84 | VIC | | | | | | | | | |
| 85 | VIC | dRNA | 6.000e-03 | 3.980e-01 | 15.03 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 86 | VIC | dRNA | 7.000e-03 | 3.790e-01 | 14.98 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 87 | VIC | dRNA | 4.100e-03 | 3.790e-01 | 15 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T23 |
| 88 | VIC | dRNA | 4.100e-03 | 3.820e-01 | 15.01 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 89 | VIC | dRNA | 0.450e-03 | 3.870e-01 | 15.49 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 90 | VIC | dRNA | 2.010e-03 | 3.940e-01 | 15.07 | 0.000e+00 | | 0.000e+00 | 0.000e+00 | + | T alpha4 |
| 91 | VIC | | | | | | | | | |
| 92 | VIC | | | | | | | | | |
| 93 | VIC | | | | | | | | | |
| 94 | VIC | | | | | | | | | |
| 95 | VIC | | | | | | | | | |
| 96 | VIC | | | | | | | | | |

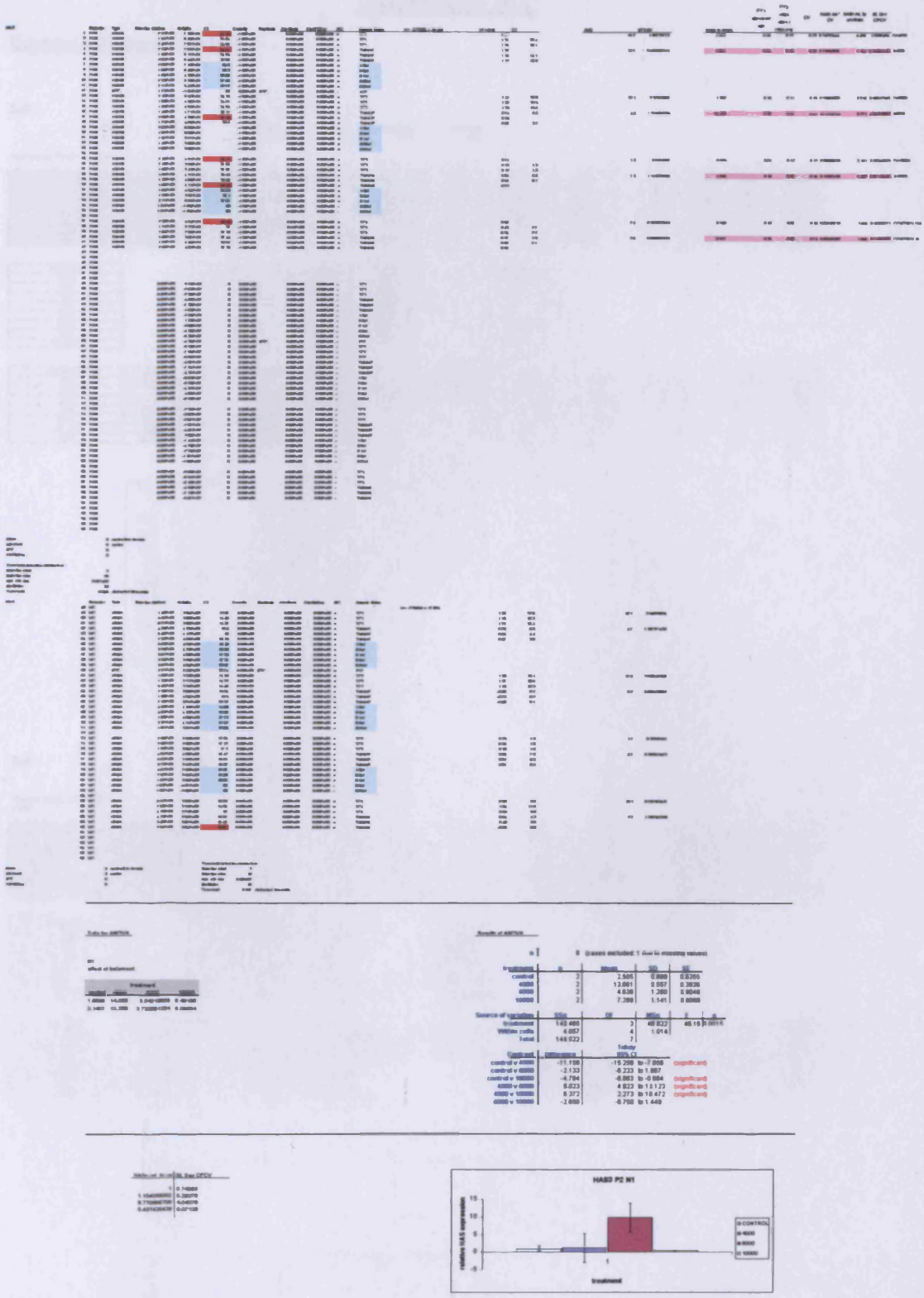
Slope 0 outlier/low density
intercept 0 outlier
RFU 0
FU StdDev 0

Threshold detection correction
Baseline s 0
Baseline e 50
Res. dbl. s 0.0394455
Modifiedbs 10
Threshold 0.207 (Calculated Manually)

| Concentration | Ct | log Conc |
|---|---|---|
| 1.0ul | 15.18 | 1 |
| | 14.98 | 1 |
| | 15.07 | 1 |
| 0.6ul | 15.00 | 0.69897 |
| | 15.00 | 0.69897 |
| | 15.00 | 0.69897 |
| 0.1ul | 17.46 | 0 |
| | 17.73 | 0 |
| | 18.00 | 0 |
| 0.06ul | | -0.301029996 |
| | | -0.301029996 |
| | | -0.301029996 |
| 0.01ul | 20.87 | -1 |
| | 20.96 | -1 |
| | | -1 |



# Appendix 4.4


HASD P2 N1
relative HAS expression
15
10
5
0
-5
CONTROL
4000
6000
10000
treatment

# Appendix 5.1

## Cytokine calibration curves

### IL-6

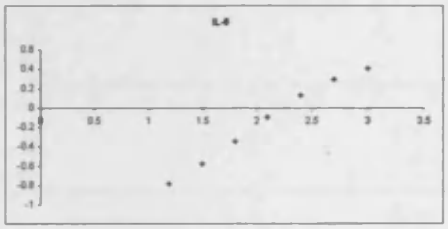
| Plate | Repeat | End time | Start temp. | End temp. | BarCode |
|---|---|---|---|---|---|
| 1 | 1 | 14:15:19 | 29.2 | 29.1 | N/A |

Absorbance (B 450 (0.1s) (A)

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.547 | 2.524 | 2.814 | 0.006 | 0.510 | 0.638 | 0.957 | 2.777 | 1.519 | 0.027 | 0.001 | 0.001 |
| 1.810 | 1.945 | 2.154 | 2.714 | 1.708 | 1.938 | 2.207 | 1.899 | 2.159 | 0.025 | 0.001 | 0.001 |
| 1.317 | 1.347 | 1.489 | 2.738 | 2.512 | 1.795 | 1.694 | 1.733 | 1.888 | 0.027 | 0.002 | 0.001 |
| 0.715 | 0.867 | 0.885 | 1.508 | 2.978 | 1.724 | 1.567 | 0.622 | 1.710 | 0.022 | 0.001 | 0.001 |
| 0.412 | 0.423 | 0.498 | 1.195 | 0.304 | 1.341 | 2.633 | 2.275 | 1.469 | 0.025 | 0.001 | 0.001 |
| 0.236 | 0.276 | 0.268 | 2.770 | 2.670 | 2.720 | 2.938 | 0.879 | 0.540 | 0.027 | 0.001 | 0.001 |
| 0.186 | 0.172 | 0.165 | 0.972 | 1.940 | 1.710 | 1.149 | 0.872 | 0.737 | 0.030 | 0.001 | 0.001 |
| 0.063 | 0.062 | 0.065 | 0.968 | 1.436 | 1.794 | 1.874 | 0.870 | 0.061 | 0.029 | 0.001 | 0.001 |

| Standard | OD1 | OD2 | OD3 | AVG OD |
|---|---|---|---|---|
| 1000 | 2.547 | 2.524 | 2.814 | 2.562 |
| 500 | 1.810 | 1.945 | 2.154 | 1.970 |
| 250 | 1.317 | 1.347 | 1.489 | 1.384 |
| 125 | 0.715 | 0.867 | 0.885 | 0.822 |
| 62.5 | 0.412 | 0.423 | 0.498 | 0.451 |
| 31.25 | 0.236 | 0.276 | 0.268 | 0.264 |
| 15.6 | 0.186 | 0.172 | 0.165 | 0.108 |
| BLANK | 0.063 | 0.062 | 0.065 | 0.063 |

| standard | avg OD | log10 standard | log10 OD | | | OD1 | OD2 | OD3 | avg OD | standard |
|---|---|---|---|---|---|---|---|---|---|---|
| 1000 | 2.561777812 | 3 | 0.408641426 | slope | 0.8963 | 774.0696 | 764.5103053 | 804.6604859 | 781.4 | 1000.0 |
| 500 | 1.969759372 | 2.698970004 | 0.294413032 | intercept | -1.5767 | 473.0077 | 522.9471078 | 607.0003 | 520.7 | 500.0 |
| 250 | 1.384448585 | 2.397940009 | 0.131781647 | rsq | 0.9996 | 284.2472 | 305.2222284 | 357.7409 | 316.4 | 250.0 |
| 125 | 0.822376181 | 2.096910013 | -0.08562522 | | | 171.8944 | 145.0998264 | 198.3864 | 144.3 | 125.0 |
| 62.5 | 0.451088044 | 1.795880017 | -0.345737272 | | | 58.3669 | 58.84103117 | 71.8870 | 62.3 | 62.5 |
| 31.25 | 0.263814721 | 1.494850022 | -0.578700875 | | | 34.1147 | 29.42948693 | 32.8186 | 28.5 | 31.3 |
| 15.6 | 0.185708294 | 1.193031996 | -0.790850755 | | | 13.7987 | 15.23330646 | 14.3702 | 14.5 | 15.6 |




### IL-8

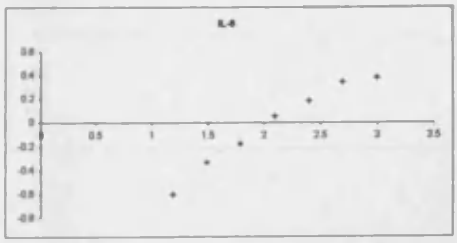
| Plate | Repeat | End time | Start temp. | End temp. | BarCode |
|---|---|---|---|---|---|
| 1 | 1 | 12:44:26 | 28.3 | 28.3 | N/A |

Absorbance (B 450 (0.1s) (A)
0.000

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.421 | 2.456 | 0.146 | 0.510 | 0.515 | 0.299 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 2.458 | 2.223 | 0.147 | 0.268 | 0.494 | 0.304 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 1.586 | 1.462 | 0.225 | 0.325 | 0.480 | 0.341 | 0.011 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.883 | 0.739 | 0.239 | 0.371 | 0.571 | 0.487 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.615 | 0.559 | 0.239 | 0.467 | 0.643 | 0.508 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.364 | 0.362 | 0.196 | 0.475 | 0.192 | 0.567 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.000 |
| 0.341 | 0.268 | 0.347 | 0.364 | 0.475 | 0.491 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.266 | 0.244 | 0.307 | 0.224 | 0.416 | 0.878 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.000 |

| standards | OD1 | OD2 | AVG OD |
|---|---|---|---|
| 1000 | 2.420578712 | 2.455815131 | 2.438196922 |
| 500 | 2.457887769 | 2.035.987 | 2.246394494 |
| 250 | 1.586011458 | 1.462543405 | 1.536178021 |
| 125 | 1.255104724 | 1.232451391 | 1.143778029 |
| 62.5 | 0.814894489 | 0.659968812 | 0.885293901 |
| 30.63 | 0.553851865 | 0.362973042 | 0.488367253 |
| 15.31 | 0.241198726 | 0.256391567 | 0.292295151 |
| 0 | 0.350471537 | 0.04434941 | 0.047395473 |

| standard | avg OD | log10 standard | log10 OD | | | OD1 | OD2 | avg | standard |
|---|---|---|---|---|---|---|---|---|---|
| 1000 | 2.438196922 | 3 | 0.387268018 | slope | 0.862037211 | 653.7239694 | 681.1252426 | 667.4 | 1000.0 |
| 500 | 2.246394494 | 2.698970004 | 0.349395487 | intercept | -1.173986419 | 582.6208693 | 473.7110143 | 578.2 | 500.0 |
| 250 | 1.536178021 | 2.397940009 | 0.186441855 | rsq | 0.99931436 | 309.8627671 | 273.0481468 | 291.5 | 250.0 |
| 125 | 1.143778029 | 2.096910013 | 0.058341147 | | | 147.4557339 | 195.3941762 | 171.4 | 125.0 |
| 61.25 | 0.885293901 | 1.791190063 | -0.176487111 | | | 85.5987340 | 82.3343764 | 84.0 | 61.3 |
| 30.63 | 0.488367253 | 1.486005198 | -0.329418021 | | | 45.98039943 | 23.50595327 | 34.7 | 30.6 |
| 15.31 | 0.292295151 | 1.184875191 | -0.601547564 | | | 10.17775798 | 11.81073908 | 10.9 | 15.3 |



**TNFa**

| Plate | Repeat | End time | Start temp. | End temp. | BarCode |
|---|---|---|---|---|---|
| 1 | 1 | 13:17:22 | 25.9 | 26.1 | N/A |

Absorbance db 450 (0.1s) (A)
0.000

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.778 | 3.272 | 0.398 | 0.065 | 0.050 | 0.790 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 2.583 | 1.500 | 0.364 | 0.067 | 0.080 | 0.667 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 1.485 | 0.798 | 0.259 | 0.067 | 0.064 | 0.055 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.679 | 0.345 | 0.258 | 0.058 | 0.059 | 0.073 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.192 | 0.187 | 0.173 | 0.061 | 0.058 | 0.065 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.346 | 0.342 | 0.169 | 0.100 | 0.053 | 0.067 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.236 | 0.222 | 0.158 | 0.067 | 0.067 | 0.268 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.075 | 0.071 | 0.079 | 0.061 | 0.061 | 0.062 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |

| standards |
|---|
| 1000 |
| 500 |
| 250 |
| 125 |
| 61.25 |
| 30.62 |
| 15.31 |
| 0 |

| OD1 | OD2 |
|---|---|
| 2.778 | 3.272 |
| 2.583 | 1.500 |
| 1.485 | 0.798 |
| 0.679 | 0.345 |
| 0.192 | 0.187 |
| 0.346 | 0.342 |
| 0.236 | 0.222 |
| 0.075 | 0.071 |

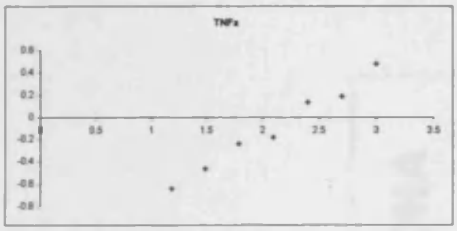
| AVG OD |
|---|
| 3.025 |
| 1.542 |
| 0.892 |
| 0.512 |
| 0.190 |
| 0.344 |
| 0.230 |
| 0.073 |

| standard | avg-OD | log10 standard | log10 OD |
|---|---|---|---|
| 1000 | 3.025 | 3 | 0.480788541 |
| 500 | 1.542 | 2.698970004 | 0.188148172 |
| 250 | 0.892 | 2.397940009 | 0.132288520 |
| 125 | 0.512 | 2.096910013 | -0.1179888 |
| 61.25 | 0.190 | 1.787106893 | -0.7208462727 |
| 30.62 | 0.344 | 1.486005196 | -0.4637553369 |
| 15.31 | 0.230 | 1.184975191 | -0.6389105453 |

| | |
|---|---|
| slope | 0.5021169026 |
| intercept | -1.342063907 |
| rse | 0.8787259604 |

| OD1 | OD2 |
|---|---|
| 1017.4972 | 1368.8728 |
| 304.9662 | 87.3024 |
| 576.5578 | 115.1725 |
| 178.3273 | 30.8090 |
| 76.7174 | 70.8063 |
| 30.6292 | 30.1419 |
| 18.3525 | 14.5477 |

| avg | standard |
|---|---|
| 1202.2 | 1000.000 |
| 481.2 | 500.000 |
| 345.8 | 250.000 |
| 104.5 | 125.000 |
| 73.5 | 61.250 |
| 30.5 | 30.620 |
| 15.5 | 15.310 |




**IL-1**

| Plate | Repeat | End time | Start temp. | End temp. | BarCode |
|---|---|---|---|---|---|
| 1 | 1 | 13:14:55 | 26.2 | 26 | N/A |

Absorbance db 450 (0.1s) (A)
0.000

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3.126 | 2.863 | 0.056 | 0.054 | 0.052 | 0.052 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 2.089 | 1.843 | 0.054 | 0.045 | 0.045 | 0.045 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 1.408 | 0.798 | 0.047 | 0.043 | 0.044 | 0.040 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.633 | 0.398 | 0.042 | 0.043 | 0.039 | 0.041 | 0.000 | 0.001 | 0.001 | 0.000 | 0.001 | 0.001 |
| 0.153 | 0.268 | 0.040 | 0.041 | 0.040 | 0.040 | 0.000 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.268 | 0.267 | 0.040 | 0.043 | 0.042 | 0.039 | 0.000 | 0.000 | 0.001 | 0.000 | 0.001 | 0.001 |
| 0.153 | 0.154 | 0.040 | 0.042 | 0.042 | 0.042 | 0.000 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 |
| 0.043 | 0.043 | 0.044 | 0.040 | 0.040 | 0.042 | 0.000 | 0.001 | 0.001 | 0.0001 | 0.001 | 0.002 |

| standards |
|---|
| 1000 |
| 500 |
| 250 |
| 125 |
| 61.25 |
| 30.62 |
| 15.31 |
| 0 |

| OD1 | OD2 |
|---|---|
| 3.126 | 2.863 |
| 2.089 | 1.843 |
| 1.408 | 0.798 |
| 0.633 | 0.398 |
| 0.268 | 0.268 |
| 0.153 | 0.154 |
| 0.043 | 0.043 |

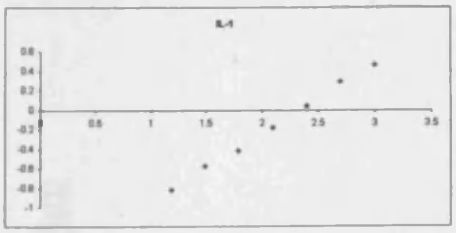
| AVG OD |
|---|
| 2.995 |
| 1.966 |
| 1.103 |
| 0.515 |
| 0.268 |
| 0.267 |
| 0.154 |
| 0.043 |

| standard | avg-OD | log10 standard | log10 OD |
|---|---|---|---|
| 1000 | 2.995 | 3 | 0.476985072 |
| 500 | 1.966 | 2.698970004 | 0.293577729 |
| 250 | 1.103 | 2.397940009 | 0.042588298 |
| 125 | 0.515 | 2.096910013 | -0.1803277708 |
| 61.25 | 0.268 | 1.787106893 | -0.4136747777 |
| 30.62 | 0.267 | 1.486005196 | -0.5729831153 |
| 15.31 | 0.154 | 1.184975191 | -0.8131741162 |

| | |
|---|---|
| slope | 0.7090011441 |
| intercept | -1.802906101 |
| rse | 0.0870[illegible] |

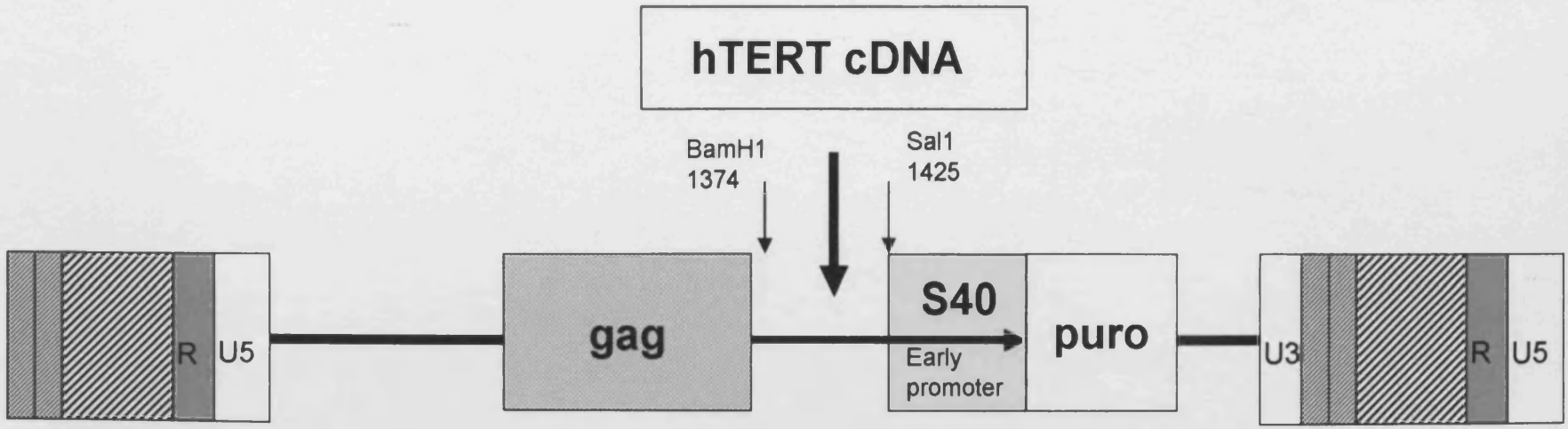
| OD1 | OD2 |
|---|---|
| 1071.1027 | 952.4781 |
| 603.1962 | 507.9627 |
| 247.8208 | 154.3610 |
| 112.4315 | 124.4142 |
| 33.2029 | 33.5487 |
| 15.2453 | 15.3085 |

| avg | standard |
|---|---|
| 962.2 | 1000 |
| 557.0 | 500 |
| 251.8 | 250 |
| 118.5 | 125 |
| 58.0 | 61.25 |
| 33.4 | 30.62 |
| 15.3 | 15.31 |



# Appendix 6.1

Vector Map



# Appendix 6.2

## TRAP assay reagents

**Lysis Buffer** (1ml)
100µl 0.1M Tris-HCL pH8.3
1.5µl 1M $MgCl_2$
10µl 0.1M EGTA
100µl Glycerol
100µl 5% CHAPS
688.5µl DEPC water

**Premix** (24 samples)
600µl 2x buffer (Rb)
4.8µl T4 gene 32 protein
4.8µl TS primer (100ng)
590µl DEPC water

**Reaction Buffer (Rb)**
20mM Tris-HCL pH8.3
1.5mM MgCl
63mM KCl
0.005% Tween
1mM EGTA
50µm dNTPs
0.1mg/ml BSA

**Final mix** (30 samples)
6µl CX primer (100ng)
15µl Taq polymerase (2.5U)
15µl ITAS
24µl DEPC $H_2O$

**Gel reagents**
25ml acrylamide
30ml 5 x TBE
45ml MilliQ water
500µl APS (100µg/ml)
100µl TEMED

# Publications

# EFFECTS OF MECHANICAL STRAIN ON HYALURONAN METABOLISM OF CELLS CULTURED FROM THE SYNOVIUM OF OSTEOARTHRITIC KNEES.

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**Introduction:** Hyaluronan (HA), the high molecular weight polysaccharide consisting of repeating glucuronic and N-acetylglucosamine units, is synthesized during joint cavitation (1). HA synthesis persists in the adult joint helping to sustain friction free articulation. Immobilization studies have shown that the removal of mechanical stimuli interferes with joint cavitation (2&3) and so mechanically induced factors are likely to play a major regulatory role in joint morphogenesis. This role of mechanical stimuli in maintaining hyaluronan synthesis in the adult joint is likely to be essential.

Previous work has shown that it is the synovial fibroblasts which synthesise HA within the synovium as high activity of the enzyme uridine diphosphoglucose dehydrogenase (UDPGD), required for precursor saccharides, has already been reported (4). Once synthesized, HA is secreted into the synovial cavity where it combines with the synovial fluid to help lubricate the joint. During osteoarthritis, along with a change in cellular architecture of the synovium, there is a decrease in the UDPGD activity of the synovial fibroblasts leading to a subsequent decrease in HA synthesis (5). This adds to the loss of joint mobility and knee pain associated with osteoartritis.

Although we understand how HA is synthesized, the stimuli controlling this synthesis remain elusive. We hypothesise that restoration of normal synovial HA concentrations is dependent upon mechanical stimuli and that OA synovial cells can respond to mechanical stimuli. Here we show that after a brief period of mechanical strain, cultured human OA synovial cells can increase and sustain media HA concentrations.

**Materials and Methods:** Synovium was obtained from patients (both male and female, age range 57-77 years) undergoing total knee joint replacement surgery with the relevant ethical approval and patient consent.

Synovial cells were isolated from diced tissue by digestion in 0.2mg $ml^{-1}$ type I collagenase in DMEM/F12 +5% foetal calf serum (FCS). Following digestion, cells were isolated then suspended in DMEM/F12 containing 1% penicillin($10^4 IUml^{-1}$)-streptamycin($10^4 \mu gml^{-1}$), L-glutamine (1g/L), hydrocortisone (4mg/ml), insulin-transferrin-selenium (200MM) and 20% FCS. Cells were counted and seeded at $1x10^6$ cells $ml^{-1}$ into T75 flasks and allowed to reach confluence with a media change of DMEM/F12+ every four days.

Once confluent, cells were trypsinised, counted and plated at a density of $0.015 \times 10^6$ cells $ml^{-1}$ with 1ml of cell suspension placed into a well of a 4-well strain plate. Confluent cells were serum deprived for 18 hours and fresh serum free media (DMEM/F12-) added 30 minutes prior to strain. Each experiment consisted of 6 plates, with every plate containing four wells. Plates were strained at 4000, 6000 or 10000με at a frequency of 1 Hz for 10 minutes using a mechanical loading jig. All strains were carried out at 37°C. Control plates consisted of cells that did not undergo strain acting as static controls and cells that were subjected to media flow without strain, flow controls. The media from each well was collected at 1 hour, 6 hours and 24 hours. The media HA levels were assayed using a HA ELISA as previously described by Fosang *et al* (1990)(6).

**Results:** First, results from strained, static control and flow control cells were analysed using a one-way ANOVA and Scheffe post hoc test. This showed there was no difference in hyaluronan levels between each of the individual strain plates, no difference between each of the static controls and no difference between the flow control plates. Comparisons of static control cells and flow control cells showed no significant differences in their media hyaluronan concentrations.

From the several cell lines subjected to the 3 different strains it can be seen that of the three strains tested it was the lowest strain of 0. 4% that gave the greatest significant increase between strained and static control cells. The highest strain (1%) produced a significant decrease in hyaluronan media levels at 24 hours.

Selected cell lines were then followed over a time course of 24 hours. The HA media from static cells could be seen to increase with time. However when cells were subjected to the 0.4% strain significantly higher hyaluronan media levels could be seen. For the 0.4% strain, this increase in HA media levels was significantly different between strained and static cells at each of the time points. All data were analysed using a one-way ANOVA followed by Tukey post hoc test.

Figure.1. Relative media hyaluronan levels 24 hours post strain. *p<0.05

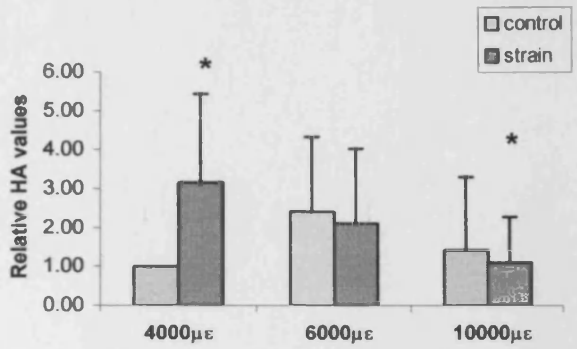
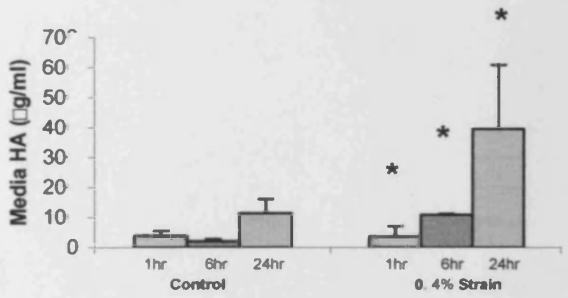



Figure.2. Hyaluronan media levels post 4000με. *p<0.05




**Discussion:** These results demonstrate that although the synovial cells have been cultured from the synovium of osteoarthritic patients which show reduced levels of hyaluronan, they are able to alter their hyaluronan synthesis when subjected to mechanical stimuli. The upregulation of HA levels was seen predominately in the cells subjected to the lowest strain while a decrease in HA levels was seen at a higher strain. This result suggests that the loss of mobility in osteoarthritic joints could be a contributory factor in the decrease of hyaluronan yet the synovial cells from OA synovium can be seen to increase hyaluronan synthesis on the reintroduction of mechanical stimuli. The determination of an appropriate strain and frequency required to restore hyaluronan levels to those of the normal synovial joint will be important in generating exercise based therapies for osteoarthritis.

**References:** 1) Craig et al 1990. J Anat. 171, 17-23. 2) Fell & Canti 1934. Proceedings of the Royal Society 116, 316-327. 3) Hamburger & Waugh 1940. Physiological Zoology 13, 367-384. 4) Wilkinson et al 1992. Arth & Rheum. 35(10), 1179-1184. 5) Pitsillides & Blake 1992. Ann.Rheum.Dis. 51, 992-995. 6) Fosang et al 1990. Matrix. 10, 306-13.

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***Abstract accepted for poster presentation at the 50th Annual Meeting of the Orthopaedic Research Society, March 2004.***