CELL AND TISSUE ENGINEERING OF ARTICULAR CARTILAGE VIA REGULATION AND ALIGNMENT OF PRIMARY CHONDROCYTE USING MANIPULATED TRANSFORMING GROWTH FACTORS AND ECM PROTEINS

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PhD

2010

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Effect of transforming growth factor-beta (TGF-β1, 2 and 3) on the biological regulation and wound repair of chondrocyte monolayers with and without presence of ECM proteins

A thesis submitted for the degree of

Doctor of Philosophy

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2010

STATEMENT OF ORIGINALITY

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ACKNOWLEDGEMENTS

It is a pleasure to thank those who made this work and thesis possible. First and foremost I would like to thank my both project supervisors **Dr. Mansour Youseffi** and **Dr. Morgan C.T. Denyer** for their willingness to supervise this joint collaboration research project between School of Engineering, Design and Technology – Medical Engineering Department, and School of Life Sciences.

I greatly appreciate all their contributions including time, patience, motivation, ideas, and funding to make this postgraduate experience productive and precious. Through their support and encouragement it was possible to have 9 publications, so far.

I would also like to thank Dr. S. T. Britland and Professor D. J. Tobin for their kind attitude allowing me to use facilities of their laboratories. Without this contribution, the completion of this thesis was not possible.

Thanks to the technical and secretarial Staff of the School of Life Sciences and School of Engineering for their assistance during this work.

My thanks also goes to the Royal Academy of Engineering for their financial support to attend the 15th International biomedical science and technology symposium-BIOMED 2009. METU/Northern Cyprus.

My special thanks also goes to my wife and my daughter, for their continued patience, understanding, motivation and support for completion of this thesis. I would like to thank all those people who have contributed directly and indirectly to this research program, and thesis.

May God bless you all

“I dedicate this thesis to my wife, Mahin Joushak, and daughter, Nila, for their patience, support, encouragement, and unconditional love.

Their continuous moral support and enormous sacrifice has enabled to accomplish this thesis.”

LIST OF ASSOCIATED PUBLICATIONS

Published Conference Papers:

1- Jamil, M. M. Abdul, Sefat, F., Khaghani, S. A., Batista Lobo, S., Javid, F. A., Youseffi, M., Britland, S. T., Liu, S. G., See, C. W., Somekh, M. G and Denyer, (2008),"Cell Imaging With The Widefield Surface Plasmon Microscope,"IFMBE Proceedings, 21 Issue:1-2 (2008), Springer, Berlin, pp. 528-531, ISBN 1680-0737.

2- F. Sefat, R.F. Berends, M.C.T Denyer, S.A. Khaghani and M. Youseffi, "The anti-scarring (wound closure) properties of TGF-β3, BSA/HCl and HCl in cultured human bone cell monolayer," World congress on engineering (WCE) 2009, ISBN: 978-988-18210-1-0 pp 1866-1871.

3- F. Sefat, R.F. Berends, M.C.T. Denyer, S.A. Khaghani and M. Youseffi, "The effect of growth factor beta (TGF-β3) and BSA/HCl on Trypsinisation of bone cell monolayer," Proceeding of WCE 2009, ISBN: 978-988-18210-1-0, pp 1859-1862.

Abstracts:

1- Khaghani, Seyedali; Sefat, Farshid; Denyer, Morgan; Youseffi, Mansour, "Alignment of rat primary chondrocyte cells to collagen type-I, fibronectin and laminin," JOURNAL OF ANATOMY, Volume: 213, Issue: 3 (2008), Pages: 351-351 Published: 2008.

2- Seyedali Khaghani, Morgan Denyer, Mansour Youseffi, Samira Lobo, and Farideh A. Javid, "Effect of transforming growth Factor-β1 in biological regulation of primary Chondrocyte," 15th International Biomedical Science and

Technology Symposium, 16-19, August 2009, METU-Northern Cyprus (Güzelyurt), ISBN: 978-975-01656-5-8, p. 68-68.

3- S. A. Khaghani, M.C.T. Denyer, M. Youseffi, R.F. Berends, F. Sefat, S. Lobo, and F.A. Javid' "Purification of Primary Chondrocyte Cells extracted from knee joint of Sprague-Dawley rats," JOURNAL OF ANATOMY, Volume:215, Issue: 6 (2009), Pages: 711-712.

4- R.F. Berends, M. Youseffi, F. Sefat, S.A. Khaghani and M. Denyer. "Investigating Keratinocyte cell responses to ECM proteins using Microcontact printing," JOURNAL OF ANATOMY, Volume: 215, Issue: 6 (2009), Pages: 711-711.

5- Seyedali Khaghani, Morgan Denyer, Farshid Sefat and Mansour Youseffi, "Effect of Transforming Growth Factor-Beta2 on Cartilage Wound Repair," SSBII-11, Surface Science of Biologically Important Interfaces, 2009, Keele University, From fundamental surface science to clinical application.

6- Sefat, F.; Denyer, M.; Khaghani, S. A.; Youseffi, M.; "The role of ECM protein (collagen, fibronectin and laminin) in cellular adhesion (cell alignment) and cell engineering of bone," JOURNAL OF ANATOMY, Volume: 213, Issue: 3, pages: 349-349.

Grants:

1. The Royal Academy of Engineering conference grant for the 15th International biomedical science and technology symposium (BIOMED-2009/METU), N. Cyprus.

Abstract: Articular cartilage is an avascular and flexible connective tissue found in joints. It produces a cushioning effect at the joints and provides low friction to protect the ends of the bones from wear and tear/damage. It has poor repair capacity and any injury can result pain and loss of mobility. One of the common forms of articular cartilage disease which has a huge impact on patient's life is arthritis. Research on cartilage cell/tissue engineering will help patients to improve their physical activity by replacing or treating the diseased/damaged cartilage tissue.

Cartilage cell, called chondrocyte is embedded in the matrix (Lacunae) and has round shape *in vivo*. The *in vitro* monolayer culture of primary chondrocyte causes morphological change characterized as dedifferentiation. Transforming growth factor-beta (TGF-β), a cytokine superfamily, regulates cell function, including differentiation and proliferation. The effect of TGF-β1, 2, 3, and their manipulated forms in biological regulation of primary chondrocyte was investigated in this work. A novel method was developed to isolate and purify the primary chondrocytes from knee joint of neonate Sprague-Dawley rat, and the effect of some supplementations such as hyaluronic acid and antibiotics were also investigated to provide the most appropriate condition for *in vitro* culture of chondrocyte cells.

Addition of 0.1mg/ml hyaluronic acid in chondrocyte culture media resulted an increase in primary chondrocyte proliferation and helped the cells to maintain chondrocytic morphology.

TGF-β1, 2 and 3 caused chondrocytes to obtain fibroblastic phenotype, alongside an increase in apoptosis. The healing process of the wound closure assay of chondrocyte monolayers were slowed down by all three isoforms of TGF-β. All three types of TGF-β negatively affected the strength of chondrocyte adhesion. TGF-β1, 2 and 3 up regulated the expression of collagen type-II, but decreased synthesis of collagen type-I, Chondroitin sulfate glycoprotein, and laminin. They did not show any significant change in production of S-100 protein and fibronectin. TGF-β2, and 3 did not change expression of integrin-β1 (CD29), but TGF-β1 decreased the secretion of this adhesion protein.

Manipulated TGF-β showed huge impact on formation of fibroblast like morphology of chondrocytes with chondrocytic phenotype. These isoforms also decreased the expression of laminin, chondroitin sulfate glycoprotein, and collagen type-I, but they increased production of collagen type-II and did not induce synthesis of fibronectin and S-100 protein. In addition, the strength of cell adhesion on solid surface was reduced by manipulated TGF-β. Only manipulated form of TGF-β1 and 2 could increase the proliferation rate. Manipulation of TGF-β did not up regulate the expression of integrin-β1in planar culture system.

The implications of this R&D work are that the manipulation of TGF-β by combination of TGF-β1, 2, and 3 can be utilized in production of superficial zone of cartilage and perichondrium. The collagen, fibronectin and hyaluronic acid could be recruited for the fabrication of a biodegradable scaffold that promotes chondrocyte growth for autologous chondrocyte implantation or for formation of cartilage.

Keywords: Primary chondrocyte cell; Cell/Tissue engineering; TGF-β1, 2 and 3; Monolayer cell culture, Pellet culture; Cell marker; Wound closure assay; Integrin-β1 (CD29)

LIST OF ABBREVIATIONS

µl	micro litre
2D	two dimensional
3D	three dimensional
ACI	autologous chondrocyte implantation
ADTC5	ataxia telangiestasia group D-complementing
ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
BSA	bovine serum albomin
CAMs	cell adhesion molecules
CHC	chondrocyte cell
CIA	chondrocyte induced arthritis
COMP	cartilage oligomeric matrix protein
CSPG	chondroitin sulfate proteoglycon
D	diameter
DAH	differential adhesion hypothesis
df	degree of freedom
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DVT	deep vein thrombosis
ECM	extracellular matrix
ECN	engineered cartilage generated by nasal chondrocyte
EDTA	ethylenediaminetetraacetic acid
ePPi	extracellular inorganic pyrophosphate
F	F-statistics (Fisher) - value
FAs	focal adhesions
Fcrit	critical F - value
FCS	fetal calf serum
FGF	fibroblast growth factor
GAG	glycosamin glycon
H_0	null hypothesis
HA	hyaluronic acid
HAC	Human articular chondrocyte
HBSS	hank’s balanced salt solution
HCl	hydrochloric acid
HEPES	(Hydroxyethyl)-1-piperazine]ethanesulfonic acid
ICC	immunocytochemistry
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
ILK	integrin linked kinase
JIA	juvenile idiopathic arthritis
M	mole
MACT	matrix associated autologous chondrocyte transplantation
MCP	microcontact printing
Min.	Minute
ml	millilitre

mM	milli mole
MPa	mega Pascal
mRNA	messenger ribonucleic acid
MS	mean of squares
mTOR	mammalian target of rapamycin
ng	nano gramm
NIH	national institute of health
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
OP	osteogenic protein
PCM	pericellular matrix
PDMS	Polydimethylsiloxane
Pen/Strep	penicillin/streptomycin
PGA	polyglycolic acid
pH	pressure (presence) of hydrogen
PK	protein kinas
RA	rheumatoid arthritis
RAC	Rat articular chondrocyte
rIL	recombined interleukin
rpm	revolution per minute
RPMI	Roswell park memorial institute
SD	standard deviation
SE	standard error
Sec.	Second
SFB	synovial fibroblast
SRp40	serine rich protein
SS	sum of squares
TC	tissue culture
TGF	transforming growth factor
TMJ	temporomandibular join
TNF	tumour necrosis factor
U/ml	unit per millilitre
w/v	weight per volume
WSPR	wide field surface Plasmon resonance

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CHAPTER I

1 Introduction to Cartilage Tissue Engineering

1.1 Introduction

The future of medicine, drug therapy and treatment of diseases requires the knowledge and ability of developing methods which have fewer side effects and more mimic natural tissue.

Body takes drugs through several routs. Most of the treatment methods and application routs have disadvantages along with their advantages (Kopacek, 2007). Side effects are major causes of limitation of the drugs (Wallace, 2001). Also surgery has its own difficulty (Wakefield et al., 2008) such as immobilization, obesity, imperfect results, slow healing and in some cases danger of death.

Cell and tissue engineering is a rapidly growing field and number of reports showing successful progression in fabrication of engineered tissues such as *in vitro* engineered heart valve tissue (Mendelson and Schoen, 2006), (Schleicher et al., 2009), (Metcalfe and Ferguson, 2006) and *in vivo* engineered skin (Carlson et al., 2008) have indicated that regenerated tissues may be extremely useful in therapeutic procedures. Although there are advantages in tissue engineering such as elimination of need for immunosuppression (Chuang et al., 2009), reductions of transplantation complications

and a decreased need for donor tissue, there are still many challenges before the use of this technology becomes routine.

In cartilage repair, cartilage extraction, i.e., the digestion and isolation of cartilage cells (chondrocytes) are just a few of the challenges of the long process of cartilage tissue engineering. Chondrocyte cells express genes (Sniekers et al., 2009) involved in the formation of cartilage (Ibold et al., 2009) which are not yet fully studied and the extracellular matrix proteins produced by chondrocytes *in vitro* are different to those produced by chondrocytes *in vivo.* These different characteristics of chondrocyte make research in the area more challenging. Another challenge is that the effect of some cytokines in different forms on formation of cartilage and on behaviour of chondrocyte is not sufficiently investigated yet. Transforming growth factor-beta superfamily is a type of cytokine which is secreted by cells in the form (Koli et al., 2001).

This research is focuses on a novel protocol for the isolation, purification, culture and study of primary chondrocyte cells *in vitro*, with the aim of examining the effect of known cytokines (TGF-β1, TGF-β2 and TGF-β3) on chondrocyte behaviour.

1.2 Anatomy and Histology of Cartilage

Cartilage is a flexible connective tissue found in many regions in the body, such as the ear, trachea, nose, ribs, joints, bronchial tube and intervertebral discs. Cartilage maintains the shape of some tissues i.e. ear and provides a cushioning effect in the joints and supports other tissues within the body. Articular cartilage lines bones in joints to reduce wear and produce almost a frictionless environment for bones to slide over each

others. It also reduces the pressure arising from our weight by absorbing the shock at the ends of the bones. Cartilage is a type of connective tissue which does not have blood vessels and nerves or lymphatics (Desjardins and Hurtig, 1990), and its cells are enclosed in small cavities called lacunae. Consequently cartilage has limited repair capacity for damages and injuries. Even surgery is not promising healing of damaged cartilage particularly in adults (Bos et al., 2007).

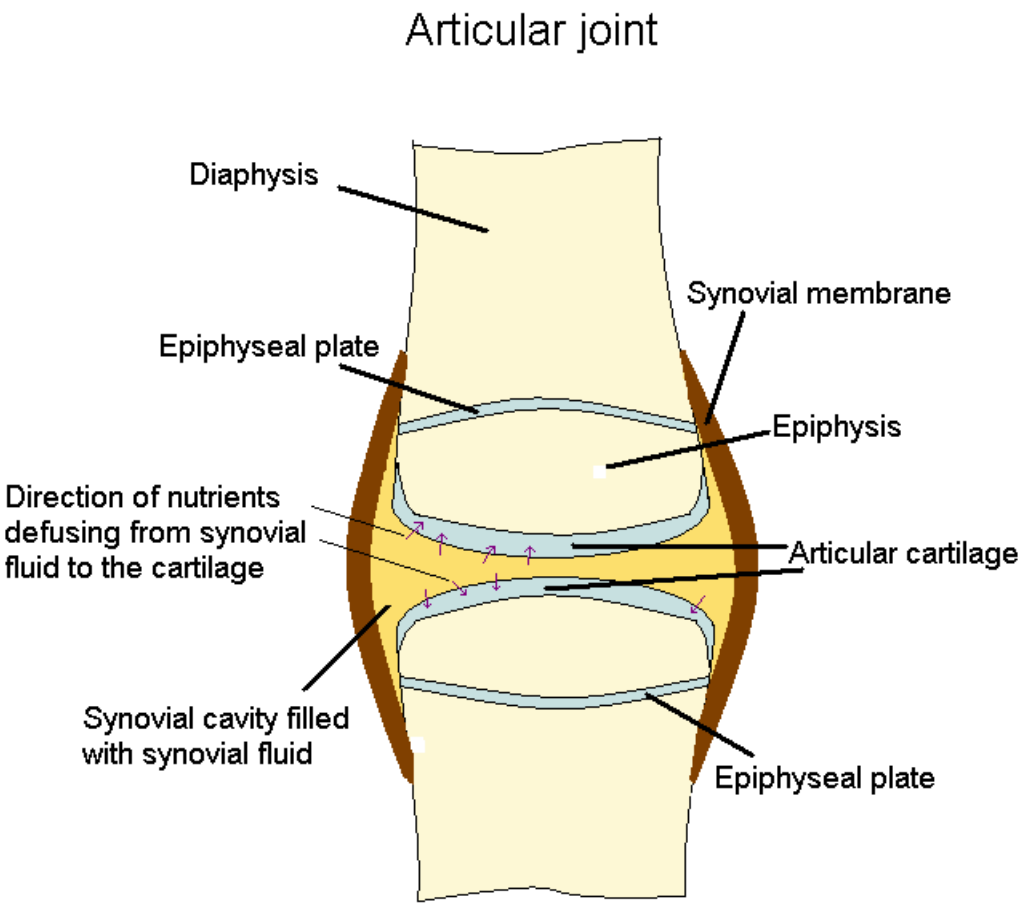


Figure 1.1 Schematic diagram of an articular joint, cartilage, synovial cavity, membrane, and epiphyseal plate.

Three types of cartilage can be distinguished: Hyaline cartilage, elastic cartilage and Fibrocartilage (Guilak, 2003).

Hyaline cartilage is white-bluish in colour, semitransparent, thin and extremely strong but flexible tissue which is most abundant type of cartilage found in the human body (Guilak, 2003). This type of cartilage exists in articular surface of bones, epiphysal plate of the bones, larynx, trachea, nasal septum and in the bronchia tree. Although hyaline cartilage is soft tissue and contains ca. 80% water (James and Uhl, 2001) (Balassa, 1987), it exposes highly resistant to pressure and shear stress on the synovial joints and maintains the form of the tissue.

Elastic cartilage is very similar to hyaline cartilage but with large amount of elastic fibres. This type of cartilage is found in the auricle of the external ear, in the wall of external auditory meatus, Eustachian tube, epiglottis and part of larynx. Elastic cartilage is found where the supportive tissue need possess elasticity and insures the opening of tubes which are surrounded by cartilage (Gillogly et al., 1998).

Fibrocartilage is a mixture of fibrous tissue and cartilaginous tissue. It is flexible, tough tissue with fibrous bundles and white appearance. Fibrocartilage is a special type of cartilage that contains type-I collagen in addition to type-II collagen. It exists in the annulus fibrosis of intervertebral disks, temporomandibular joints (TMJ) and in the junction between large tendons and articular cartilage in large joints (meniscus) (McIlwraith, 2007).

Longitudinal bone growth is also the results of chondrogenesis (formation of cartilage) and following calcification of cartilage cells within the epiphyseal growth plate, a

process called endochondral ossification and osteogenesis (formation of bone). Epiphyseal plate is a thin layer of cartilage found between epiphysis and diaphysis of growing bone plate (See Figure 1.1 and 1.2).

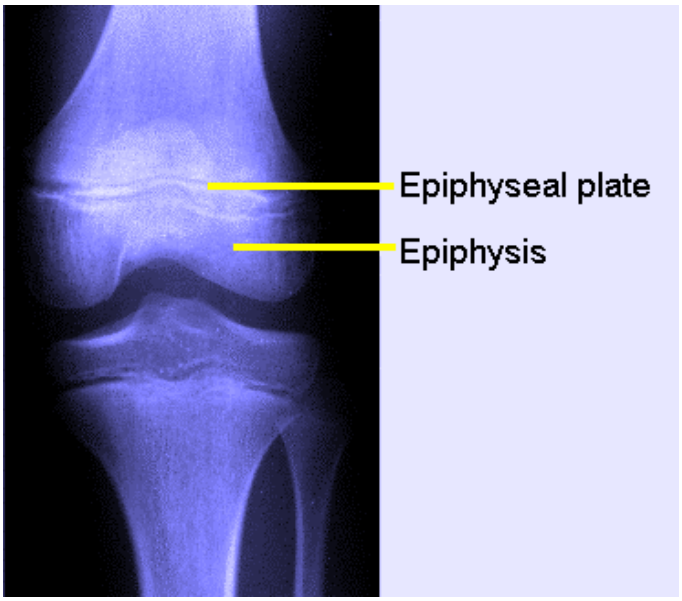


Figure 1.2 Anterior radiograph of epiphysis and epiphyseal plate (Bell et al., 2009)

Although cartilage is poorly innovated and vascularised cartilage growth is achieved by appositional growth and/or by interstitial growth. Appositional growth describes the formation of new cartilage by cells derived from undifferentiated cells at the surface of the cartilage or perichondrium, whereas, interstitial growth describes the proliferation of chondrocyte cells themselves and the associated synthesis of cartilage extracellular matrix proteins and the consequent formation of new cartilage (See Figure 1.3) (Henrikson et al., 1997, Rafique, 2010).

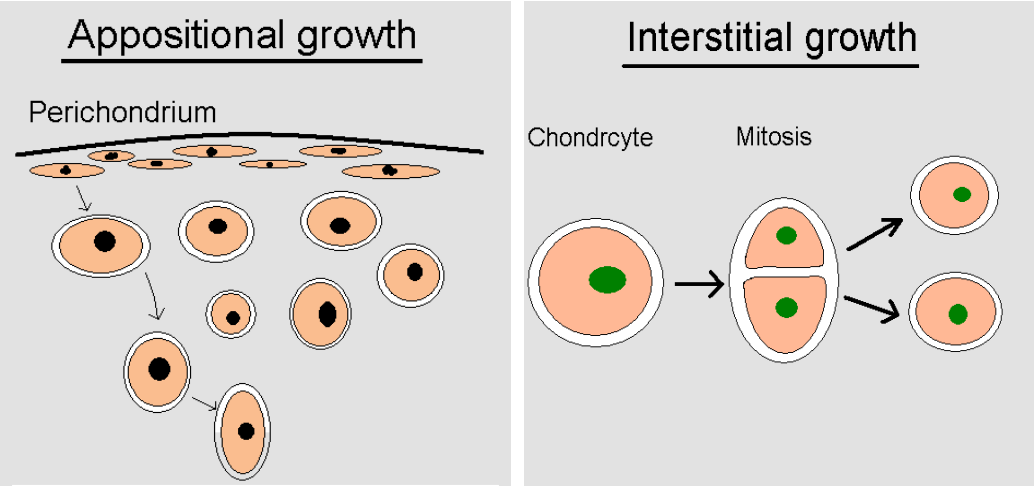


Figure 1.3 Two types of cartilage growth: (left) Appositional growth from perichonrium, and (right) Interstitial growth within cartilage (Rafique, 2010).

Cartilage in the movable joints is surrounded with a membrane called synovial membrane which seals the joint and prevents penetration of any particles separated from solid tissues in the joints in the joint. Synovial membrane is filled with non-Newtonian fluid with high rate of viscosity (Hou et al., 1989). A non-Newtonian fluid is a fluid whose viscosity is not constant and the relation between shear stress and strain rate is non-linear (Irvine-Jr. and Park, 1987).

Nutritions diffuse through synovial fluid to reach cartilage (Smith and Wood, 1991).

Articular cartilage consists of six zones (See Figure 1.4) in which chondrocytes make up approximately 10% of total cartilage volume in human adults (Guilak, 2003), the remaining 90% is formed from extracellular matrix molecules. However, during fetal life, the cell volume is significantly higher, but the number of cells decreases with age (Weiss, 1998).

Cartilage is completely surrounded by a narrow region called pericellular matrix (PCM). The structure and properties of the PCM may significantly influence the mechanical environment of the chondrocyte (Choi et al., 2008), and also the permeability of PCM has a significant effect on convective transport to and from chondrocytes (Leonidas G. Alexopoulosa et al., 2005).

At the surface of cartilage which is in contact with the synovial fluid, the chondrocytes are flattened and parallel to the surface (Mow and Huiskes, 2004). This region of cartilage is called the superficial zone (See Figure 1.4). In the layers below the superficial zone, the chondrocytes have a more rounded shape and their density decreases. The chondrocytes in the midzone synthesize more extracellular matrix and reveal a morphological phenotype closer to that of hyaline cartilage (See Figure 1.4). The layer below the midzone contains chondrocytes that are slightly elongated in vertical axis, this region is referred to as the deep zone (Silberberg et al., 2005). However, in the deep zone the collagen content is minimal and instead the concentration of aggrecan increases and fibril diameter becomes maximal. Below the deep zone the chondrocytes become calcified where they develop a hypertrophic phenotype and reach a differentiation stage similar to fracture repair. Cell density in this zone is the lowest when compared to the other cartilage zones (See Figure 1.4). The unique feature of hypertrophic cells is their ability to calcify the ECM and synthesize type-X collagen which enables excellent structural integration between the calcified zone and the underlying subchondral bone plate (Meyer and Wiesmann, 2006).

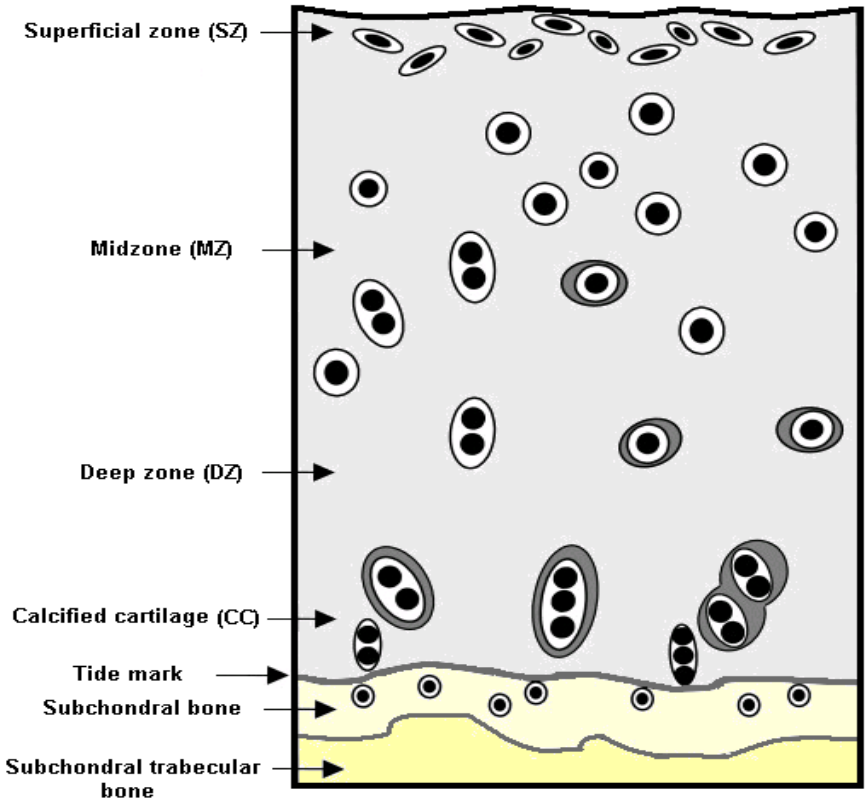


Figure 1.4 Schematic zonal structure of articular cartilage (Gardner et al., 1987).

The extracellular matrix of cartilage consists of differentially distributed collagen fibrils and non collagenous proteins (See Figure 1.5). Collagenous proteins (fibres), and water are the main components of extracellular matrix and structure of cartilage whereas the non-collagenous proteins play major role in the function and signalling of chondrocyte (Meyer and Wiesmann, 2006).

The extracellular matrix of cartilage consists of a number of different proteins including collagen-type II, type IX, type XI, aggrecan, link protein, hyaluronan, S-100 protein and the cartilage oligomeric matrix proteins; matrilin-1 and matrilin-2. Figure 1.5

schematically shows the binding of collagen type-II and type-IX together, where collagen type-IX bridge collagen fibrils with other macromolecules (Pei et al., 2008). Glycosaminoglycans are bound to each other to form proteoglycan aggregates, and these entire molecules bind to hyaluronic acid which itself is bound to collagen meshwork.

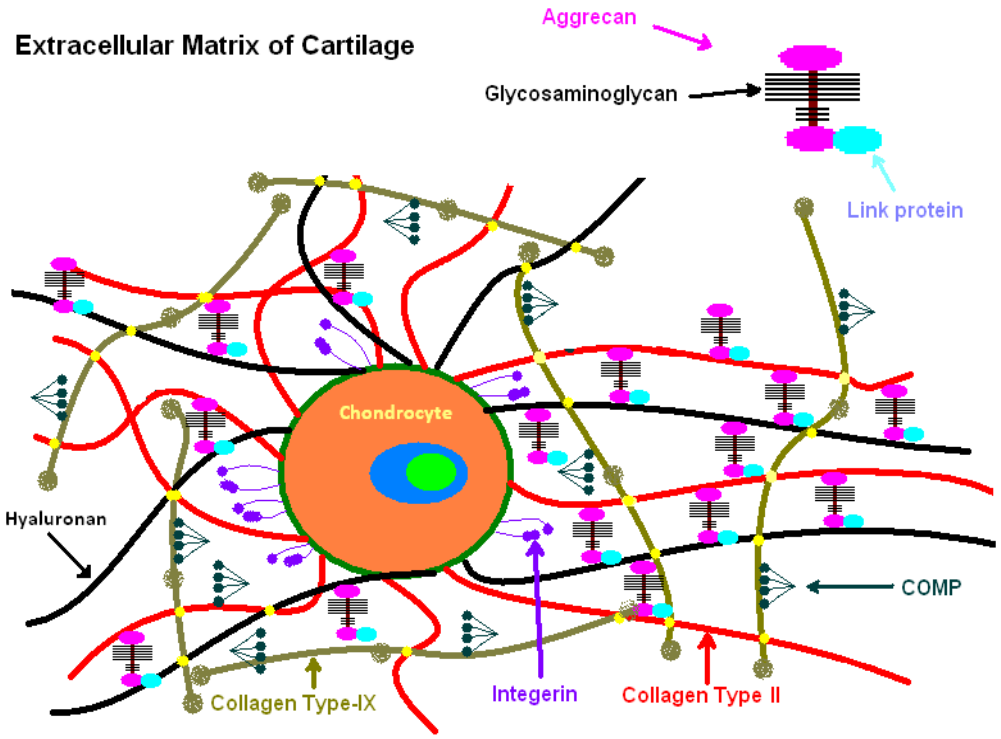


Figure 1.5 Schematic structure of cartilage with various proteins involved in the formation of ECM of cartilage (Chen et al., 2006).

Cartilage also has mechanical properties that allow it to withstand and distribute compressive loads of the body and provides friction-free condition in the joints (McIlwraith, 2007).

The extracellular matrix making up the bulk of cartilage is secreted by chondrocytes. Chondcocytes are enclosed in spaces called lacunae within the cartilage. The encapsulation of chondrocytes in lacunae and the avascular nature of cartilage decreases the inflammatory process which in turn is thought to play a role in decreasing the reparative capacity of cartilage (Cohen et al., 2006).

Cartilage degeneration caused by injuries and congenital abnormalities is a great clinical challenge (Gillogly et al., 1998). Damage to cartilage results an incomplete wound healing and can be followed by progressive and chronic lesions (Bhosale and Richardson, 2008). Cartilage can be injured by tears, general wear or injury resulting from genetic factors or overuse of tissue during sport (McIlwraith, 2007).

Damage to the cartilage does initiate a reparative response (Meyer and Wiesmann, 2006). However, deep cartilage defects result in the loss of non-collagenous matrix (Meyer and Wiesmann, 2006) which requires complete tissue repair and in more severe cases when the lesion is in the fibrillar network and involves significant cell death the cartilage does not heal (Chubinskaya et al., 2008). In contrast if a lesion occurs in the collagenous matrix, there is a degree of regeneration. In this case treatment of cartilage by a cell therapy method and engineered tissue could enhance tissue repair (Cohen et al., 1992).

1.3 Cartilage Repair Strategy

Articular cartilage provides an almost frictionless surface, which none of the artificial constructs have been able to successfully reproduce. There is a rapidly growing interest in cartilage repair with particular focus on tissue engineering and cell therapy (Horas et al., 2003). Bhosale and Richardson (2008) investigated the structure of cartilage, its injuries and management. The results of their investigation revealed that some of the cartilage lesion treatments such as knee arthroscopies underestimated many of the complications associated with treatment (Bhosale and Richardson, 2008). Cartilage injuries in the joint if left untreated lead to premature early arthritis and affect daily activities. They reported that various different treatment methods of cartilage regeneration have shown encouraging results, but unfortunately none have proved to be the ultimate solution.

Cartilage lesion and disease can be treated in two ways; non-surgical drug/physiotherapy related treatment and surgical treatment (Gillogly et al., 1998). Drugs for cartilage disease treatment are generally divided into painkillers (Analgesics), non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs such as cortisone.

Analgesics or pain killers are drugs which relieve the pain. They function by acting either on peripheral nervous system and either blocking the conduction of pain signals to the brain or by acting on the central nervous system through interfering with the brain's analysis and recognition of these signals. Some examples of analgesics are aspirin, paracetamol, iboprophin, morphine and NSAIDs (NSAIDs are anti-inflammatory drugs) (Cleeland et al., 1994).

Steroidal anti-inflammatory drugs can be considered as being the cortisone contained drugs (Levin, 2008). These types of drugs deliver a relatively high dose of steroid to the joint and diminish the pain by reduction of local inflammation but they do not cure the diseases. Steroids have side effect such as stress, increase in blood pressure and can be very harmful to liver (Bagrov et al., 2009). Thus steroidal treatments tend to be avoided in favour of NSAIDs.

NSAIDs are drugs which do not contain cortisone (corticosteroid) (Ong et al., 2007) and decreases glucose uptake by cells and increases glucose release by liver. Unfortunately some ibuprofen like NSAIDs have inhibitory effects on the synthesis of glycosaminoglycan (GAGs) by chondrocyte (Dingle, 2007) and thus may be harmful in the treatment of cartilage disease. Interestingly the magnitude of pain relief in osteoarthritis with NSAIDs is not greater than achieved result with paracetamol (acetaminophen) (Zhang et al., 2004). Also in some cases drug therapy of cartilage disease (corticosteroid therapy) causes immunosuppression (Zhang et al., 2004).

Physiotherapy or physical therapy is recognized as increasing joint mobility (Jamtvedt et al., 2008) and it is well known that cartilage repair without regular movement cannot take place. Joint movement provides appropriate condition to promote secretion of proteins such as glucosaminoglycan (GAG) and chondroitin sulfate by chondrocytes which assist regeneration of cartilage (Dutton, 2004).

Although physiotherapy and rehabilitation applications also benefit pain management of Rheumatoid Arthritis (RA) (Kavuncu and Evcik, 2004) but a complete cure is not feasible.

Additionally, Lowe et al (2007) reported that the interventions including physiotherapy functional exercises after discharge result in short term benefit after total knee arthroplasty. Their results show that the effect sizes were small to moderate, with no long term benefit (Lowe et al., 2007).

There are five surgical treatment methods used in the treatment of damaged cartilage;

- *Arthroplasty* describes a process of total or partial joint replacement. However, replacement joints tend to undergo a process of loosening with time and are likely to fractures during operation (Pour et al., 2003).

-*Arthroscopy* which describes the removal of loose bits of separated cartilage. Arthroscopy cannot heal the lesion but it reduces pain and increases mobility (Brody, 1986). The disadvantages of arthroscopy include *Deep Vein Thrombosis (DVT)* caused by formation of blood clot in the veins (Michot et al., 2002), loosening of the joint (Kingsley and Mehta, 2007), infection and consequently increased risk of dislocation (Widjaja et al., 2006).

- *Osteochondral grafting* describes a process in which a fragment (plug) is removed from a donor site (healthy bone and cartilage) and implanted in a recipient site (injured area) (Lo and Chang, 2003). The disadvantages and complications of this treatment method include stiffness in recipient site and degenerative changes in both donor and recipient sites (Yoshizumi et al., 2002), excessive postoperative bleeding and pain at the donor site.

- *Autologous chondrocytes implantation* (ACI) in which healthy chondrocytes are harvested from healthy and minimal functional tissue and implanted over the lesion

after *in vitro* cultivation (Candrian et al., 2008), (Horas et al., 2003). The aim of this technique is the production of a graft or chondrocyte rich suspension to cover the injured area and facilitate regeneration of cartilage (Yongzhong et al., 2006). Autologous chondrocyte implantation involves the collection of approximately 200- 300 mg of healthy cartilage harvested by arthroscopy (Minas and Peterson, 2005, Minas and Peterson, 1999). Following this the chondrocytes are cultured for about six weeks until the cell numbers have reached between 5-10 million cells (Giannetti et al., 2005). At this stage the expanded cell population can be reintroduced into the cartilage wound site (Minas and Peterson, 2005, Minas and Peterson, 1999). Complications of ACI include; infection and formation of scar tissue around the edges of periosteal patch which may in turn require arthroscopy after ACI (Surgeryinformation, 2008).

- *Mesenchymal stem cell* (MSC) based therapy in which stem cells derived from bone marrow are cultured *in vitro* and implanted in the injured area (Majumdar et al., 2000). It is believed that the bone marrow stromal cells may be recruiting in the region of the lesion and that localised environmental factors such as regional chemistry, cytokines and growth factors may promote chondrogenesis (Erickson et al., 2002).

- *Creating bleeding microfractures within the subchondral bone* is another cartilage treatment method (Miller et al., 2004). Theoretically penetration in subchondral bone causes bleeding and consequently blood clotting in any cartilaginous defects. A process which is then followed by the formation of fibrocartilage (Edwards, 2002). Microfracture treatment has disadvantages such as risk of a deep hole which could damage the bone (Edwards, 2002).

Of the methods so far described only those associated with the use of autologous chondrocyte or stem cell derived chondrocyte can be truly described as using cell and tissue engineering techniques.

1.4 Statement of the Problem

There are several cartilage diseases and injuries. Some of these diseases are inheritable and are related to the genetic factor/s whereas some causes are due to aging and injuries.

One of the common forms of articular cartilage damage is arthritis, an inflammation of joints. Arthritis has more than a hundred different types causing incredible pain and can result in the loss of mobility (Neugebauer et al., 2007).

Arthritis is divided in two categories; inflammatory arthritis and degenerative arthritis (Yamashita et al., 2002). Inflammatory arthritis causes pain and stiffness on the joints after periods of immobility such as sitting or sleeping, in comparison degenerative arthritis is characterized by pain after an activity (Dearborn and Jergesen, 2005).

Some major forms of arthritis are; *Osteoarthritis, Rheumatoid arthritis and Juvenile idiopathic arthritis*

- *Osteoarthritis*: Apart from sudden motion, wear, tear and dislocation of a fragment of cartilage another possible cause of cartilage lesion is gradually wear of cartilage tissue (Pritzker et al., 2006) (Peterson and Renstroem, 2004). Osteoarthritis may arise because of aging, obesity or problems related to the structure of bone and joint (Karvonen et al., 1994). Immobility is another cause of osteoarthritis, as the cartilage requires movement

(Karvonen et al., 1994). Osteoarthritis is the most common type of arthritis and can occur in all articular joints particularly in knees, hips and feet. In sever cases osteoarthritis can require treatment with complete joint replacement (Harms et al., 2007).

- *Rheumatoid arthritis* is chronic types of arthritis that progresses in three periods. The inflammation and swelling of the synovial lining and around the joint is the first period which causes warmth, pain, stiffness, redness. The second stage is associated with rapid cell division and cell growth which causes the synovium to thicken. This is followed by digestion of bone and cartilage by released enzymes from inflamed cells in the third stage. This can often cause the involved joint to lose its shape and alignment. Associated with this there is more pain resulting and a loss of movement (Banning, 2005).

- *Juvenile idiopathic arthritis (JIA)* is another types of arthritis which occurs in children under sixteen. The cause of juvenile arthritis is not well known yet. However, it is known that immune system of the body attacks the healthy cartilage tissue and synovium (Murray et al., 1998). It is not a heredity disease but heredity can increase its development (Petty et al., 1998). JIA may cause fever, rash, lymph node swelling and may affect the child's heart.

Imbalanced Cytokines: The role of imbalanced cytokines in joint disease is well documented. Inteleukin-1 and tumour necrosis factor-α are the main proinflammatory and catabolic cytokines involved in disease initiation and progression. Interleukin-1 suppresses joint repair by inhibiting collagen synthesis (Goldring, 2007). Other proinflammatory cytokines may amplify or modulate this process, whereas anti-

inflammatory cytokines, which are often detected in osteoarthritis tissues, may counteract the tissue destruction and inflammation (Goldring, 2007). The effect of imbalances of tumour necrosis factor (TNF)-α and members of the interleukin (IL)-1 and the TGF superfamily have been found in association with osteoarthritis (Müller, 2002). These advances in knowledge have led to the development of novel anticytokine therapies for joint diseases such as rheumatoid arthritis (Arend, 2001) (Khoury et al., 2008). However, it is not yet clear whether anti-cytokine therapy controls the arthritis, as it is not effective in all patients (Berg, 2001).

1.5 Cartilage Tissue Engineering

Drug and surgical methods are mainly used to treat the cartilage lesions, however, degeneration of cartilage and consequently pain is still a serious medical issue (Nesic et al., 2006).

When diseases cause the loss of tissues and functionality, and this loss cannot be treated using drug based treatments, the generation and re-implantation of engineered tissues or organs become therapeutically attractive (Lalan et al., 2001).

The history of cell and tissue engineering dates back to the 19th century when an English physiologist, Sydney Ringer, developed a salt solution to maintaining isolated animal hearts (Inoue, 1997). Ross Granville Harrison an American biologist published some results from his experiments about methodology of tissue culture (Harrison et al., 2004, Nicholas, 1960).

Whilst tissue engineering of skin is the most successful in the field of regeneration medicine (MacNeil, 2008), most other cell and tissue engineered products are still in the development stage. To date there are no engineered tissues that have taken on all of the characteristics of the tissue they have been designed to replace (Metcalfe and Ferguson, 2006).

In the case of cartilage, cell and tissue engineering generally involves a process in which patient's tissue is harvested from autologous, healthy tissue from a non load bearing part of the body. Another cell source is the bone marrow derived stem cells which have the potential to develop into other cell types (Ikada, 2006a). In both cases cells are isolated and then cultured in such a way as to allow the generation of a replacement tissue (Ossendorf et al., 2007) (Figure 1.6). This involves a process where harvested cells are grown in or on artificially generated biocompatible/biodegradable 3D matrices (Ikada, 2006b).

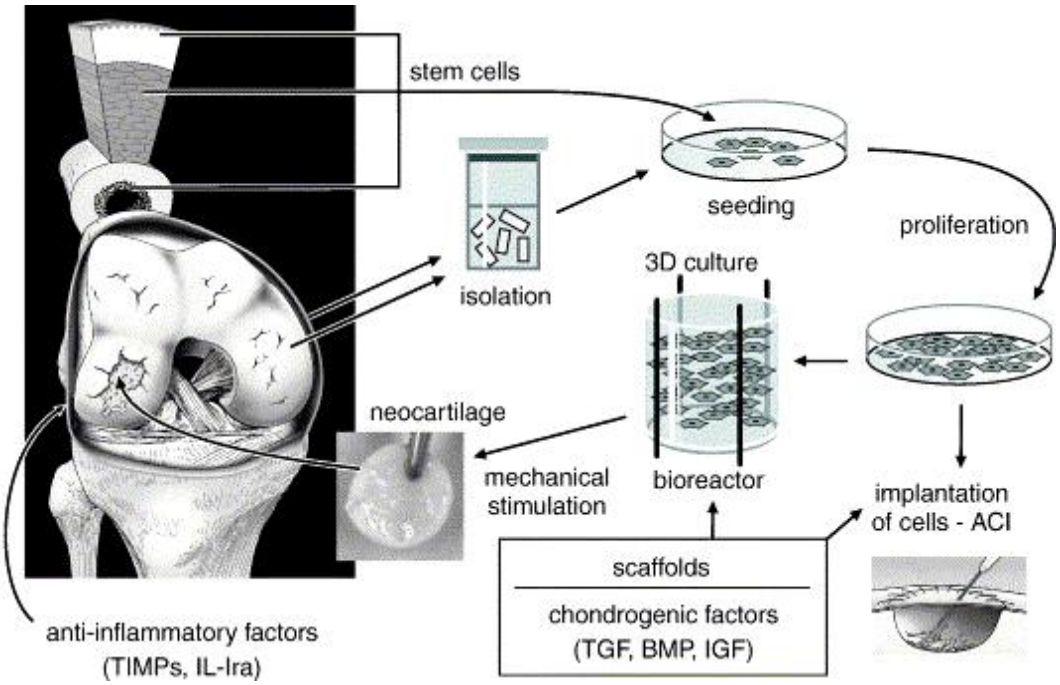


Figure 1.6 Basic schematic cell and tissue engineering of cartilage from biopsy until implantation (Nesic et al., 2006).

Biocompatibility or biodegradability of a material is the ability to perform an application within the body whilst causing minimum damage to the blood and surrounded tissues (Zhang, 2004). Development of biodegradable scaffolds for tissue engineering which can perform and support complete functionality of engineered tissue is still a big challenge. The aim of using biodegradable materials is to design porous three-dimensional scaffolds in which autologous cells are seeded. For these systems to function they have to provide appropriate conditions for cell attachment and growth, whilst allowing the cells to keep their *in vivo* phenotype until transplantation into the defect site (Woods et al., 2007). Injectable materials such as naturally derived alginate, aggaros and polysaccharide gels are often used for cartilage tissue engineering to avoid the need of invasive surgery (Tuli et al., 2003).

The process of tissue engineering is carried out with production of an implantable tissue within a bioreactor. Bioreactors in cartilage engineering provide an environment in which the cells are subjected to the appropriate hydrostatic pressures gas-exchange regimen and cyclic mechanical stresses required to mimic the internal environment of joints (Bilodeau and Mantovani, Nov. 2004, Bilodeau and Mantovani, 2004). These allow the cells seeded in scaffold to acquire a suitable functionality and scaffold deformational loading at physiological levels (Ateshian et al., 2001). Additionally, by automating and standardizing tissue manufacture in controlled, *in vivo* mimicked environments, bioreactors could reduce production and materials costs thus enabling a wider use of engineered tissues (Martin et al., 2004). Carrier et al (2000) reported that a rotating bioreactor improves cell quantity, distribution and metabolism in comparison to the fixed or agitated culture vessels. In rotating bioreactor gases exchange such as oxygen and carbondyoxide and nutrient transport is thought to be similar to the *in vivo* conditions. Their results show that agitating the culture vessels creates a turbulent zone, therefore a rotating bioreactor is more appropriate for tissue cultures especially for the culture of fragile tissues (Carrier et al., 2000).

Gooch et al (2001) investigated the effect of mechanical stress on engineered tissue. Their results indicate that the mechanical environment can significantly influence the development of engineered tissues cultured *in vitro* (Gooch et al., 2001). Lagana et al (2008), Gooch et al (2001) demonstrated that mechanical stimuli enhance chondrogenesis of chondrocytes cultured *in vitro*. Different mechanical stimuli act simultaneously *in vivo* in cartilage tissue and their effects have been extensively studied *in vitro* by providing mechanical stimuli in bioreactor (Darling and Athanasiou, 2003). Wang and Mao (2002) used a bioreactor to demonstrate how different mechanical

stimuli, i.e. shear stress and hydrostatic pressure, can be combined in different ways to study the mechanobiology and increase in proliferation of tissue engineered cartilage (Wang and Mao, 2002). Shear stress was imposed on cells by forcing the culture medium through the scaffolds, whereas a high hydrostatic pressure up to 15 MPa was generated by pressurizing the culture medium. Lagana, Moretti et al (2008) performed a fluid-dynamic experimental tests and reported successful validation of the bioreactor which has been carried out by dynamic culture of tissue-engineered cartilage constructs (Laganà et al., 2008). Their bioreactor system allowed the investigation of the combined effects of different mechanical stimuli on the development of engineered cartilage, as well as other possible three-dimensional tissue-engineered constructs. In 2005 Stevens et al reported successful tissue growth using a bioreactor. They showed that large volumes of bone can be engineered in a predictable manner, without the need for cell transplantation and growth factor administration. Their report concluded that harvested bone could be transplanted and integrated into contralateral tibial defects after 6 weeks with no apparent morbidity at the donor site. Furthermore, in a proof-of-principle study, they showed that by inhibiting angiogenesis and promoting a more hypoxic environment within the "*in vivo* bioreactor space," cartilage formation could be exclusively promoted (Stevens et al., 2005).

Most challenges related to cartilage tissue engineering focus around developing methods to stimulate chondrocyte proliferation, and incorporation into the lesion area (Tuan, 2007) and fabrication of three dimensional porous scaffolding appropriate for chondrocyte culture (Vasita and Katti, 2006). As cartilage tissue contains less than 10% cells (chondrocytes) the major issue in cartilage tissue repair is the lack of enough cells to cover the injured area. (Ikada, 2006a). Stimulation of chondrocyte causes

proliferation and secretion of cartilage extracellular matrix is the most challengeable aspect of cartilage tissue repair. Kermani (2001) reported that the cytokines play important roles in regulating cell function such as proliferation, migration, and matrix synthesis. His experiment showed that cytokines and anti-cytokines are critical in clinical use for accelerating wound healing. Also Murray et al (1998) examined the effect of interleukin-4 in the different forms of juvenile rheumatoid arthritis and spondyloarthropathy and found that interleukin-4 could restrict some joint diseases such as rheumatoid arthritis. However, there is evidence that other cytokines may promote cartilage damage. For example Tetlow and Wolley (1995) indicated that cartilage erosions are often associated with the micro-environmental expression of TNF alpha, IL-1 beta, stromelysin-1, and collagenase (Tetlow and Woolley, 1995).

According to Banning (Banning, 2005), one of the main causes of joint diseases is the blockage of inflammation process and in consequence a poor cartilage repair. Glycosaminoglycan (GAG) is one of most prominent extracellular matrix components found in cartilage (Stephens and Seegmiller, 2005). Dingle (2007) investigated the effect of non-steroidal anti inflammatory drugs (NSAIDs) on synthesis of GAG in cartilage. He divided NSAIDs into 3 categories, with respect to their *in vitro* function on human osteoarthritis cartilage: Those such as aceclofenac, tenidap and tolmetin, which can stimulate GAG synthesis and those such as piroxicam, tiaprofenic acid and aspirin, which have no significant effect on GAG synthesis; and the third group like naproxen, ibuprofen and indomethacin, which significantly inhibit GAG synthesis. Dingle's reported that some highly effective anti-inflammatory agents may have adverse effects on cartilage integrity when employed during long term treatment (Dingle, 2007).

Yamashita et al (2002) used fibroblast growth factor-2 (FGF-2) to determine severity of joint disease in Adjuvant-Induced arthritis in rats. Their results suggested that FGF-2 modulated disease progression, but did not affect initiation of the arthritis (Yamashita et al., 2002).

As shown above there are many reports regarding using of various cytokines, growth factors and developed chondrocyte culture methods to stimulate the chondrocyte proliferation *in vivo* and *in vitro*. However, until now there are very few reports looking at the effect of the transforming growth factor beta family of cytokines (TGF-β1, TGF-β2 and TGF-β3) on chondrocyte cell-cell and cell-ECM adhesion, cell proliferation, migration and wound healing.

Transforming growth factor-β (TGF-β) superfamily includes a large group of soluble extracellular proteins which regulate the development in both vertebrates and invertebrates (Raftery and Sutherland, 2002). TGF-β family regulate the cell functions such as migration, apoptosis, proliferation and differentiation (Krauss, 2006). Davidson and Scharstuhl et al (2005) investigated the change of transforming growth factor-beta signalling in cartilage of old mice and found that the TGF-β2 and 3 were reduced in 2 year old mice in comparison to 5 months old animals. The conclusion of their reports shows that the TGF-beta appears to play an important role in repair of cartilage and a lack of TGF-beta responsiveness in old mice might be at the root of osteoarthritis (Davidson et al., 2005).

In 2007 Oka et al used TGF-β2 to examine the role of TGF-β signalling in regulation of chondrogenesis and osteogenesis during mandibular development. Their data suggested that there are differential signal flows in response to TGF-β control of chondrogenesis

and osteogenesis during mandibular development (Oka et al., 2007). Henson and Vincent (2007) identified whether chondrocyte growth into a 3D scaffold could be observed following single impact load and culture. They investigated the effect of bone morphogenic protein-2 (BMP-2) on chondrocyte behaviour in 3D culture system. They cultured the extracted cartilage into 3D scaffolds for 20 days and reported that the addition of BMP-2 to the culture medium quantitatively reduced the repair response. This suggests that BMP-2 may have inhibitory effects which prevent articular cartilage from healing itself and consequently causes the appearance of chronic damage (Henson and Vincent, 2007).

Pohlers et al (2007) published a paper about up-regulation of the transforming growth factor-β pathway in rheumatoid arthritis (RA). They reported that the pathogenetic role of TGF-β-induced effects on Synovial Fibroblast (SFBs) in RA (Pohlers et al., 2007).

The importance of transforming growth factors in wound healing has caused significantly increase in research on these types of cytokines. Rolfe et al (2007) investigated the role for TGF-β1 induced cellular responses during wound healing. They compared the response of fibroblasts derived from early human fetal skin (non-scarring) and their mature (scarring) postnatal counterparts to the TGF-β1. This revealed that fetal fibroblasts differentiate into myofibroblasts (Rolfe et al., 2007).

Shah et al (1999) investigated the effect of manipulation of TGF-β on the wound healing process and found that the effects of TGF-β3 is inhibited by the high levels of TGF-β1and 2 from the inflammatory cells in adult tissue. Thus increasing the levels of TGF-β3 caused scar-free wound healing (Shah et al., 1999).

Related work was also carried out by Mark Ferguson and O'Kane (2004) who identified some TGF-β1 related therapeutic targets. They found that the growth factor profiles differ significantly in embryonic and adult tissue. For example, levels of TGF-β1 and TGF-β2 in embryonic wounds are very low, however the levels of TGF-β3 is much higher in comparison to adult wounds (Ferguson and O'Kane, 2004). Similarly, Gorvey et al (2005) found that by inhibiting TGF-β1 and TGF-β2, whilst simultaneously increasing TGF-β3 concentrations in adult wounds, near embryonic repair could be achieved in adults (Gorvy et al., 2005).

In 2006 Davidson et al investigated TGF-β induced cartilage repair. They found that the cartilage was maintained by TGF-β1 but fibrosis was blocked in the presence of Smad7 which is an inhibitor of transforming growth factor-beta superfamily signalling (Davidson et al., 2006) (Benchabane and Wrana, 2003).

Again in 2007 Bos et al (2007) examined the effect of TGF-β1 in early wound healing reactions of immature and mature articular cartilage along with the effects of TGF-β1 on chondrocyte proliferation and glycosaminoglycan synthesis in a three dimensional chondrocyte culture. They reported that TGF-β1 induced repair in immature cartilage but induced little or no repair in mature articular cartilage defects (Bos et al., 2007). Bos et al suggested that the addition of TGF-β1 may induce cartilage repair responses in mature cartilage as they observed in immature, developing cartilage (Bos et al., 2007).

It is well established that the highest reparative capacity of tissue occurs during embryonic development. However, to date there is nothing in the literature to suggest that cartilage can be fully repaired in embryos. However, Walker et al (2005) created experimental wound within embryonic chick sternum and studied synthesis of some

extracellular matrix proteins such as the collagens and chondroitin-4. He found that creation of experimental lesion in chick embryonic cartilage caused a rapid chondrocyte migration into the lesion area and a delay in the apoptosis would normally expect in adult wounds (Walker et al., 2005). These experiments suggest that manipulating transforming growth factors within cartilage may lead to improved cartilage repair capacity.

Another great challenge in relation to cartilage tissue engineering is the 3D culture method and utilization of biomaterials and various ECM proteins such as Hyaluronan, different types of collagens. Filová et al (2008) used a composition of collagen type-I, hyaluronic acid and fibrin to culture the chondrocyte 3-dimensionally (Filová et al., 2008) to enhance chondrocyte proliferation.

Hyalograft C is an novel tissue-engineering approach for the treatment of articular cartilage lesions involving the implantation of *in vitro* expanded autologous chondrocytes grown on a three-dimensional hyaluronan-based scaffold (Pavesio et al., 2003) (Tognana et al., 2007).

Although Pavesio et al (2003) and Tognana et al (2007) reported the success of Hyalograft C for cartilage tissue engineering, Weidenbecher et al (2007) utilized Hyalograft C scaffold and concluded that in rabbits, Hyalograft C initiated a foreign body reaction if implanted intra- or paralaryngeally, leading to cartilage degradation and possible graft failure. These findings suggested limitations on the environment in which Hyalograft C can be applied (Weidenbecher et al., 2007).

Providing an appropriate condition such as 3D culture system and stimulation of cartilage cell by cytokines may induce chondrocytes to secrete specific proteins which lead to expression of cartilage extracellular matrix. Libera et al (2004) researched on isolated human disc chondrocytes and reported that CHCs have a strong capability for the synthesis and secretion of disc specific proteins as well as for a regulated disc specific matrix maturation (Libera et al., 2004).

Martin et al (2001) cultured bovine chondrocyte in two dimensional and three dimensional culture systems using polyglycolic acid scaffold (PGA) in the presence of fibroblast growth factor and bone morphogenetic protein. They reported that application of fibroblast growth factor-2 (FGF-2) during 2D culture system caused a reduction in expression of fibroblastic molecules and induced reactions to BMP-2 during 3D cultivation on polyglycolic acid (PGA) scaffolds (Martin et al., 2001).

Yate et al (2005) and recently Freyria et al (2009) isolated chondrocytes and cultured them in a 3D porous collagen sponge and analysed for expression of chondrocyte specific proteins such as collagen type-II and aggrecan. The experiment of Yates et al (2005) suggested that the 3D culture system provided a favourable environment for chondrogenesis (Yates et al., 2005), whereas Freyria et al (2009) showed that the collagen type-I sponge provided most appropriate condition for 3 dimensional chondrocyte culture (Freyria et al., 2009).

One of the challenges related to the 3D chondrocyte is the cell migration within the scaffold. Henson and Vincent (2007) cultivated chondrocyte in a gelatine scaffold and studied cell migration. They also looked at the effect of bone morphogenic protein (BMP-2) in the culture medium and found that this cytokine has negative effects on cell

viability and behaviour. Although cell migration was observed, the tissue repair capacity in the presence of BMP-2 was significantly reduced.

A study of zonal changes in 3D culture of chondrocyte can be useful to research the relation among cellular, perichondrium and extracellular deformation in articular cartilage. Choi et al (2008) reported that the pericellular matrix PCM can serve as either a protective layer for the chondrocyte or a transducer that increases strain until cellular-level.

In the superficial zone, cellular-level strains are always lower than tissue-level strains. In the middle and deep zones, however, tissue strains (tissue deformations) below 25% are amplified at the cellular level, while tissue strains above 25% are decreased at the cellular level. Their finding suggested that the cellular-level strain is homogenous throughout the cartilage (Choi et al., 2008).

Engineered cartilage generated by different type of cartilage cells such as nasal chondrocytes (ECN) could be responsive to different kinematic loadings which are applied to the chondrocytes of articular cartilage. Candrian et al (2008) harvested chondrocytes from five individuals and cultured them in 3D porous polymeric scaffolds. The chondrocytes were exposed to cyclic deformation and surface motion during various time periods. This revealed that the chondrocytes isolated from nasal cartilage were as responsive to physical forces as cells derived from articular cartilage (Candrian et al., 2008). Candrian et al (2008) suggested that the nasal chondrocytes could be used as a cell source for articular cartilage repair.

Two different types of substrates coated with chondrocytes were implanted subcutaneously in a swine model by Monroy et al (2007). After 10 weeks post-implantation they evaluated the neocartilage using Hematoxylin-Eosin and Safranin-O staining. Their report indicated a similarity between neocartilage and native cartilage and that both contained 60% GAG. They also confirmed the production of an elastic neocartilage coating, elicited a low inflammatory reaction, and an associated 30% increase in the rate of wound healing (Monroy et al., 2007).

In 2008 Moroni et al (2008) designed a 3D hybrid scaffold based on biomaterial assembly. They obtained bone mimicking constructs that displayed the mechanical toughness of ceramics and the flexibility of polymers. Their report demonstrated the regeneration capacity of stem cell seeded scaffold for both cartilage and bone *in vivo* (Moroni et al., 2008).

Parallel to the researches related to cartilage tissue therapies, autologous chondrocyte implantation (Candrian et al., 2008) or matrix associated autologous chondrocyte transplantation (MACT) are becoming more common (Trattnig et al., 2007).

Chubinskaya et al (2008) investigated the metabolism of autologous chondrocytes after initial expansion immediately before implantation. They cultured the chondrocytes in monolayers and in alginate beads and looked at the effect of insulin-like growth factor-1 (IGF-1), osteogenic protein-1 (OP-1), and a combination of both. They tested the synthesis of proteoglycan and the content of DNA. Their report indicated that in monolayer culture the morphology of chondrocytes changed to fibroblastic cell. In alginate, they maintained chondrocytic phenotype. Growth factors, especially combined growth factors, induced chondrocyte proliferation. OP-1 stimulated the cartilage-

specific matrix and induced the greatest accumulation of collagen type II and fibronectin. Unfortunately, by cell counting, they found that matrix synthesised by autologous chondrocyte implantation cells was reduced when compared with that synthesised by normal chondrocytes (Chubinskaya et al., 2008).

Bentley et al (2008) compared the autologous chondrocyte implantation (Candrian et al., 2008) with mosaicplasty for osteochondral defects in the knee and reported that both were successfully used in the repair of defects of the articular cartilage of the knee. A functional assessment after both ACI and mosaicplasty on 100 patients showed that 88% had excellent or good results after ACI compared with 69% after mosaicplasty. Arthroscopy at one year demonstrated excellent or good repairs in 82% after ACI and in 34% after mosaicplasty (Bentley et al., 2008). In comparison Madry and Pape (2008) studied the result of autologous chondrocyte implantation and concluded that long-term studies are required to determine the effect of ACI in the treatment of osteoarthritis (Madry and Pape, 2008).

Noticeably significant research on chondrocyte adhesion is lacking. In this thesis cell response in terms of cell phenotype, adhesion, alignment, wound healing capacity and secretion of extracellular matrix molecules will be examined in relation to exposure to TGF-β1, β2 and β3 in 2 dimensional culture systems. This work will involve aspects of cell isolation, purification, cell culture, immunocytochemistry and also the use of the recently developed widefield surface plasmon microscope (WSPR) to examine the cell surface interface. The WSPR system is almost unique in that it enables the visualisation of interfacial interactions at high submicron lateral resolutions and sub nanometric Z-axis resolutions (Jamil et al., 2008).

1.6 Cytokines Therapy and Wound Healing of Cartilage

Cytokines are group of macromolecules synthesized by cells in response to immune stimuli to regulate the cell's function (Galvani and Cowley, 1992) . They exert their effects on the same cells (autocrine activity) or on the neighbouring cells (paracrine activity) by interacting with specific receptors (Balkwill, 2000).

Cytokines act as signalling proteins, and like hormones play an important role in pathophysiological and homeostatic processes such as fever, wound healing, inflammation, tissue repair and fibrosis. Cytokines are very active in regulation of cell function such as proliferation, migration, and matrix synthesis (Kermani and Pham, 2001). Some diseases such as degeneration of intervertebral disks could be caused by altering the expression of cytokines such as Interleukin-1 (IL-1) (Maitre et al., 2007). Investigation the role of cytokines in the development of diseases could be helpful in the therapy of some disorders.

Interleukin (IL)-18 is also a member of the IL-1 family that exerts proinflammatory effects. Joosten et al (2004) used Interleukin (IL)-18 to investigate whether it induces joint inflammation and joint destruction directly or via induction of other cytokines such as IL-1 and tumour necrosis factor (TNF). They reported that IL-18 induces joint inflammation independently of IL-1 (Joosten et al., 2004). Ye and Tang et al (2004) also used the IL-18 and investigated the actions of varying doses of recombinant rat IL-18 (rIL-18) on the course of type II collagen-induced arthritis (CIA) in BB (BioBreeding) rats, including clinical and immune events. They reported that small doses of rIL-18 (10 and 50 μg/rat) increased arthritis incidence and severity when a low-potency collagen

type-II preparation was used for immunization (YE et al., 2004). Their results indicated that treatment of inflammatory arthritis can be supported by using IL-18 antagonists.

Transforming growth factor-β (TGF-β1, 2, and 3) has been implicated in the ontogenetic transition from scarless fetal repair to adult skin repair with scaring. Fibromodulin which is a member of the small leucine-rich proteoglycan family, has been suggested as a biologically significant mediator of fetal scarless repair (Stoff et al., 2007). There is evidence that fibromodulin may be a biologically relevant modulator of TGF-β activity during scar formation (Soo et al., 2000). Spagnoly et al (2007) studied the role of transforming growth factor β (TGF-β) signalling in mice lacking the TGF-β type-II receptor gene in their limbs. They found that TGF-β receptor II signalling regulates growth and differentiation factor-5 (GDF-5), joint morphogenic gene expression (Spagnoli et al., 2007). Han et al (2007) studied the effect of transforming growth factor-β1 on regulation of fibronectin isoform expression and splicing factor SRp40 expression during pro-chondrogenic cell line (ATDC5) chondrogenic maturation. Their report showed that the effects of TGF-β1 on fibronectin isoform splicing during chondrogenesis may be largely dependent on its effect on SRp40 isoform expression (Han et al., 2007).

Cailotto et al (2007) examined the production of extracellular inorganic pyrophosphate (ePPi) in chondrocytes and the signalling pathways involved in the regulation of Ank gene expression by TGF-β1. Their report shows that TGF-β1 increases ePPi levels, mainly by the induction of the Ank gene, which requires activation of Ca2+-dependent Protein Kinase C (PKC) pathways in chondrocytes (Cailotto et al., 2007).

In 2006 Tchetina et al examined the effect of TGF- β2 on collagen cleavage on human osteoarthritic cartilage (Tchetina et al., 2006). Before Tchetina et al, Okazaki et al (1996) studied the effects of transforming growth factor beta-1, beta-2 and basic fibroblast growth factor (bFGF) on articular chondrocytes obtained from immobilised rabbit knees. However, they did not use TGF-beta 3. They reported that the TGF beta-1 or TGF beta-2 in combination with bFGF exerted synergistic effects on cell proliferation in articular chondrocytes obtained from the rabbit. Their results suggest a critical role of cytokine combinations in the development of articular cartilage degeneration after immobilisation (Okazaki et al., 1996). There are very few reported research articles related to use of TGF-β1, TGF-β2 and TGF-β3 in chondrocyte regulation. Thorp et al (1992) reported on the effects of transforming growth factor-beta 1, -beta 2 and -beta 3 in cartilage and bone cells during endochondral ossification in the chick. They discovered that TGF-beta localization controlled an increase in type II collagen and mRNA expression in transitional chondrocytes, suggesting a role for TGF-beta in the induction of synthesis of extracellular matrix (Thorp et al., 1992).

1.7 Research Objectives

This thesis will concentrate on examining the effect of transforming growth factor beta isomers such as TGF-β1, TGF-β2 and TGF-β3 and their combination on biological regulation of primary chondrocyte cells isolated from articular cartilage of Sprague-Dawley rat and development of new methods to induce proliferation of chondrocyte in planar and 3D culture system. Key to understanding of behaviour of chondrocyte and how growth factors modify cellular response is being able to examine how cells interact with their environment. In this thesis rat chondrocyte behaviour will be examined via cytokine induced changes in cell phenotype, cytokine induced cell alignment to patterned extracellular matrix molecules (proteins), and cytokine induced modification in cell surface attachment by studying integrin expression and by examining cytokine induced changes in chondrocyte secretion of extracellular matrix molecules.

This may allow the development of methods that promote cell proliferation *in vitro* whilst maintaining *in vivo* characteristics, which in turn may allow the generation of implantable autologous chondrocyte.

CHAPTER II

2 Isolation and Purification of Primary Chondrocyte Cells Extracted from the Knee Joint of Neonate Rats

2.1 Introduction

Articular cartilage is a special type of tissue that covers the ends of bones at the joints and is surrounded by other tissue types such as bone, synovial tissue, tendon, ligament and nerve tissue (Davies et al., 2008, Bernstein et al., 2009).

Depending on the research objects, the segregation of cartilage could be very difficult. Cartilage is a tissue with very low repair capacity (Selmi et al., 2008, Bos et al., 2007), and obtaining a sample of cartilage tissue from a healthy donor source is very difficult. There are various animals suitable for research on cartilage tissue such as bovine, rabbit, calf, rats. The Sprague-Dawley rat is considered to be one of the most suitable tissue sources for biopsied cartilage (Appleton et al., 2007).

A primary cell is a non-immortalized cell harvested directly from living organism (Stoop et al., 2007), and experiments on primary cells are different to those carried out on cell lines (Mi et al., 2005). Harvesting pure cells during tissue isolation is almost impossible as isolated tissue includes various cell types and requires separation of the desired cell type from unwanted cells mixed in the culture during the isolation process.

The isolation of cartilage from the knee joint of neonate rats is just the beginning of the most challenging process of chondrocyte cell culture and cartilage tissue engineering (Dan-ning and McCormick, 2005).

Even with good experience, the isolation of pure cartilage from surrounding tissues, such as bone, tendons and blood vessels, is almost impossible.

In spite of the attention given to the process of extraction, such as removal of tendon and muscle tissues, some part of the bone was mixed with the cartilage, thus requiring purification (Koplov, 1994).

Removing the Epiphyseal plates from the knee joint and the enzymatic digestion of cartilage without scratching of bone was the method used in this work for isolation of cartilage in neonate rats.

Many members of the integrin family with β1 subunit are involved in chondrocyte adhesion (Loeser, 2000), and any blockage of these proteins will cause the loss of chondrocyte attachment ability or will detach this cell type from the culture surfaces, other cells or extracellular matrix (Gigout et al., 2008).

Integrins with β1 subunit are widely expressed by variety of cell types of connective tissue (Hirai et al., 2007, Miyata et al., 2000), and thus this appropriate purification of chondrocyte cells, are considered essential for cartilage tissue engineering.

Differential adhesion hypothesis (DAH) is another technique used to score out unwanted cells from co-cultured cells in the monolayer (Foty and Steinberg, 2005).

In this research a method similar to DAH was used to separate chondrocyte cells from other cell types, i.e. bone, ligament, and fibroblast cells.

By seeding the cells and replacing the culture flasks at different time periods and counting the non-attached cells using a haemocytometer, we were able to study the attachment and detachment abilities of chondrocytes and other cell types such as bone cells on the solid surface.

A good success rate of chondrocyte purification was, therefore, achieved by using differential adhesion technique.

2.2 Materials and Methods

A series of experiments were required for the isolation of chondrocyte cells including enzymatic digestion of the extracted tissue, isolation of cells, purification of chondrocyte, and recording of data using some sterile and non-sterile materials, as discussed below.

2.2.1 Sterilised Equipment

Isolation of cartilage from knee joint of a neonate rats required separation of the skin from the leg and separation of the leg by cutting the femur between knee and hip with a sterilised scissor, forceps and a scalpel. Other tools and materials required for isolation of cartilage until culture of cells were pipette controller, sterile pipette, 5 ml non

pyrogenic pipette, 15 ml, 50 ml centrifuge tubes, 25 cm^2 culture flask, 100 mm^2 Petri dish, cutting board, laboratory stirrer, laminar flow hood and cell culture incubator

2.2.2 Sterile Materials and Reagents

Reagents used during isolation and purification process were cell culture medium (DMEM), Hank's Balanced Salt Solution (HBSS), Trypsin and Collagenase.

High glucose (4500mg/l glucose), (Li et al., 2008) Dulbecco's modified eagle medium (DMEM) is a sterile-filtered cell culture medium which needed to be supplemented with 20% v/v fetal calf serum (FCS), 2.5 U/ml L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1µg amphoterysin B (Fungizone) (Vunjak-Novakovic and Freshney, 2006) (Sigma Aldrich, UK).

Hank's Balanced Salt Solution was used to wash the tissue and was buffered with phosphate to maintain its physiological pH in atmospheric conditions.

Trypsin is widely used in cell culture research and bioproduction to remove adherent cells from surfaces, via trypsinisation, depending on the type of cells (Moody et al., 2006). Trypsin is delivered at 500 ml and needed to be aliquoted into 2 or 4 ml within a few hours of thawing.

Collagenase was used as a crude enzyme for tissue dissociation since it contains the enzyme required to attack native collagen and reticular fibres. Other enzymes hydrolyse

other proteins, polysaccharides and lipids in the extra-cellular matrix of connective and epithelial tissues.

Also 70% ethanol was prepared and utilized for sterilization of tools, tubes and flasks.

2.2.3 Protocol of Isolation of Chondrocyte Cells

The cartilage tissues were extracted and enzymatically digested and then the chondrocyte cells were acquired from the knee joints of neonate rats using the following method:

According to schedule one, methods of euthanasia, a procedure under the Animals Act 1986, six three-day-old rats were euthanized by disconnection of spinal cord. The specimen was laid on its back and the leg was stretched using forceps. Skin was opened at the hip and the leg was cut at a point between knee and hip joint. The cutting of the femur was sufficient to get the leg separated from the body.

The widest dimension of the knee joint of the neonate Sprague-Dawley rat was roughly about two millimetres, as shown in Figure 2.1. Hence, isolation of the cartilage from a ca. 2 mm-diameter joint surface under laboratory conditions required great accuracy and effort.

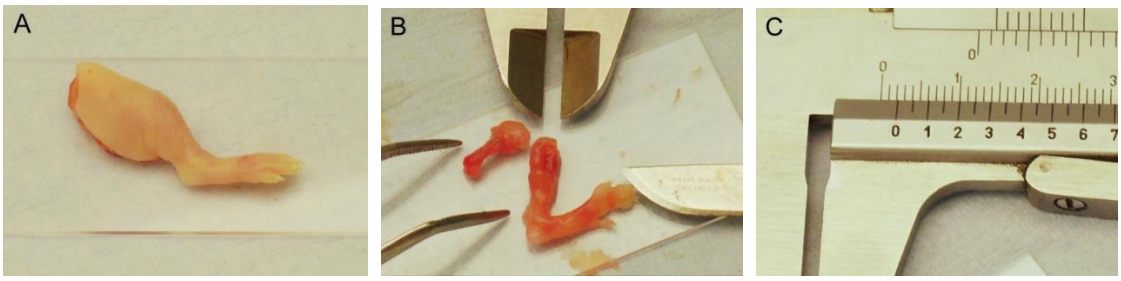


Figure 2.1 (a) Leg of a neonate rat; (b) Separated knee joint; and (c) Measured thickness of the knee joint.

Extra attention was required during the separation of the leg from the body because the object was small and the knee of the neonate rat was difficult to distinguish from its ankle as it was hidden by skin.

After separation of the leg the skin was removed using scalpel and forceps and the leg was washed with HBSS. This eliminated the possibility of having adipose, keratinocyte and blood cells in the culture. Also, the removal of the patella with patellar tendon reduces the possibility of having tenocytes in the culture.

The next step was disconnecting the tibia and fibula from the femur, which made it possible to have access to the cartilage. Exact separation of joint was required in this section because the harvestable cartilage was not well recognisable.

The patella, the anterior and posterior ligaments were removed in the first stage and the femur and tibia were separated carefully without cutting or removing any bone fragments.

The explanted cartilage and unfamiliar tissue(s) from each joint was nearly 8mg (See Figure 2.2), and thus there was a need for the isolation of more joints and, consequently, this increased the risk of death in the extracted tissues.

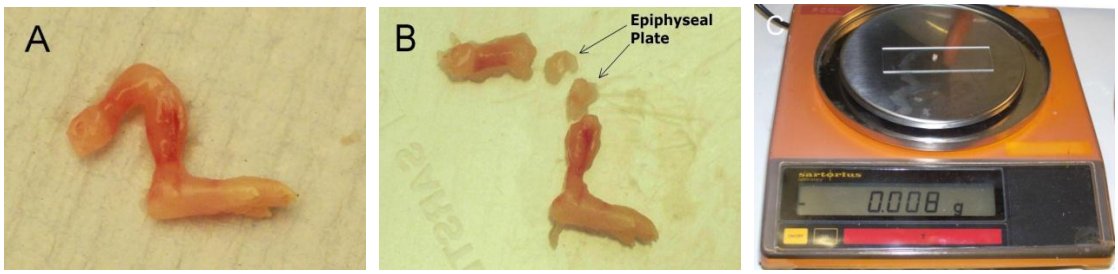


Figure 2.2 (a) knee joint of a Sprague-Dawley rat, (b) isolated joint of object; and (c) the weight of an epiphyseal plate isolated from knee joint.

Epiphyseal plates from both the tibia and femur sides were carefully separated and immersed in 4 ml of trypsin, or alternatively 1% pronase F v/v (streptomyces griseus), and stirred for 15 minutes at 37ºC.

After 15 minutes, the supernatant was removed and transferred into a sterile 15ml centrifuge tube containing 6ml DMEM media (eventually RPMI-1640 media) supplemented with 10% FCS to stop activation of the trypsin. This step was repeated three times and finally all three suspensions were added together. The suspension was centrifuged for five minutes at 2000 rpm. The media were removed and discarded. The retained pellet was re-suspended with 1ml collagenase type-IA 10% v/v and stirred at 37ºC for 90 minutes. 4ml DMEM media (10% FCS; 5% L-glutamine; Penicillin/Streptomycin) was added to the suspension to stop collagenase action. The suspension was centrifuged at 2000 rpm for five minutes and the media was discarded.

The pellet was re-suspended in 6mL DMEM media supplemented with10% FCS and 0.1 mg/ml hyaluronic acid.

The media with cartilage cells and tissue fragments were put in flasks and incubated at 37ºC. After 24 hours non-attached cells and epiphyseal plates were removed and culture flasks incubated at 37°C until 70% confluence was achieved.

2.2.4 Purification of Chondrocyte Cells Isolated from Articular Cartilage

The purification of chondrocyte cells isolated from the knee joint of the rats was performed at subculture-II (passage-II). In the process of extraction and digestion of cartilage tissue it was suspected that some part of the bone was also removed (see Figure 2.3 for various cell morphologies before cell separation). The sequence of purification and relevant results are shown in Figures 2.3-2.10.

Chondrocyte cell staining was performed via immunocytochemical staining for identification of separated cells and specification of chondrocytes during the cell-purification progress. The isolated cells were separated seven times using their attachment abilities on a solid surface and detachment by digestion of their adhesion proteins. Also, early-detached cells were separated from non-detached/late-detached cells using collagenase.

During the processes of cell isolation, cell culture and purification, the cells were kept in a sterile environment, by spraying 70% ethanol in the laminar flow hood, and incubated in closed, sterile tissue-culture flasks and Petri dishes.

Labelling of the flasks and Petri dishes were carried out for the purpose of identification.

2.2.4.1 Protocol for Purification

After the second passage, the culture was left until 70-80% confluency. The cells were detached from the culture flask after ~ five minutes using trypsin, centrifuged at 2000 rpm and counted five times by haemocytometer. The numbers of counted cells were 109, 110, 106, 108 and 112, respectively. The sample mean (average) calculated from these cell numbers was 109. This number corresponds with the cell amount in the space with the dimensions of 1mm x 1mm x 0.1mm (0.1 ml) which is the volume of the chamber between coverslip and haemocytometer. The corresponding formula is 1mm x 1mm x 0.1mm = 0.1cm x 0.1cm x 0.01cm = 0.0001cm^3 = 0.0001ml. The amount of counted cells was multiplied by 10^4 which gave the cell number in one millilitre (109 x 10^4 /ml), and again by six to obtain the cell amount in 6ml. The final calculated cells were 654 x 10^4 .The cells were then plated into tissue-culture flasks and incubated for 20 minutes at 37ºC.

Due to cell counting there is approximately 125 µl reduction in the volume of cell suspension every time, which was taken into account.

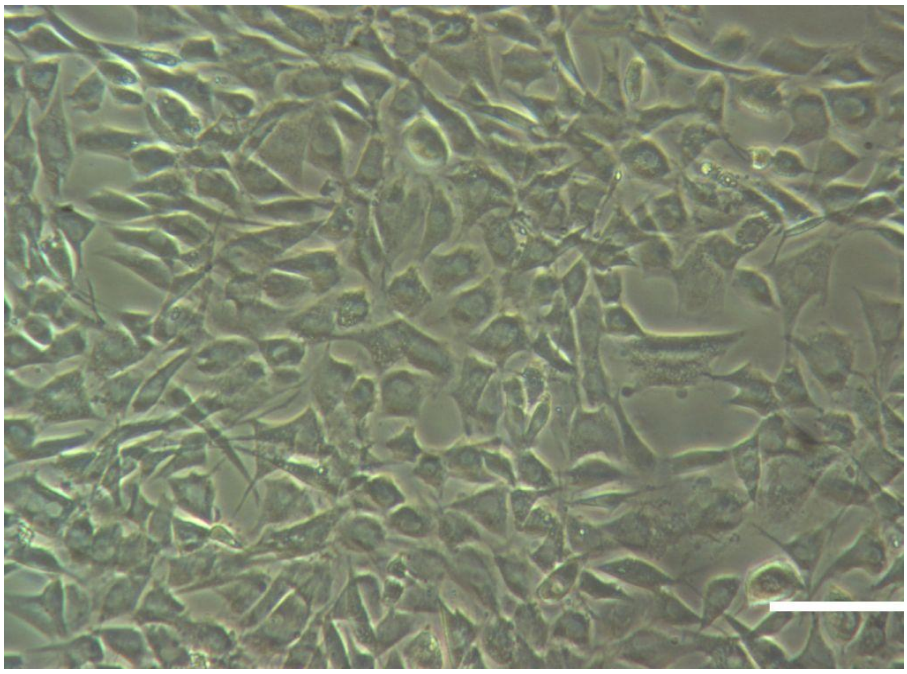


Figure 2.3 Various cell types with different morphologies such as chondrocytes, tenocytes, osteocytes and fibroblasts, before cell separation (Scale bar = 50 µm).

After 20 minutes, the media with non-attached cells were removed, re-suspended five-times, and seeded into a new tissue-culture flask. The numbers of cells in one chamber of haemocytometer were 67, 68, 63, 63 and 62, respectively. The sample mean (average) calculated from these numbers was 3,795,250 cells/6ml which is about 58% of the total amount of isolated cells. This meant that 42% of isolated cells had attached in 20 minutes. The cell suspension was transferred again into a new culture flask and incubated at 37ºC for another 20 minutes. Figures 2.4 to 2.11 show the process of purification for chondrocyte cell separation.

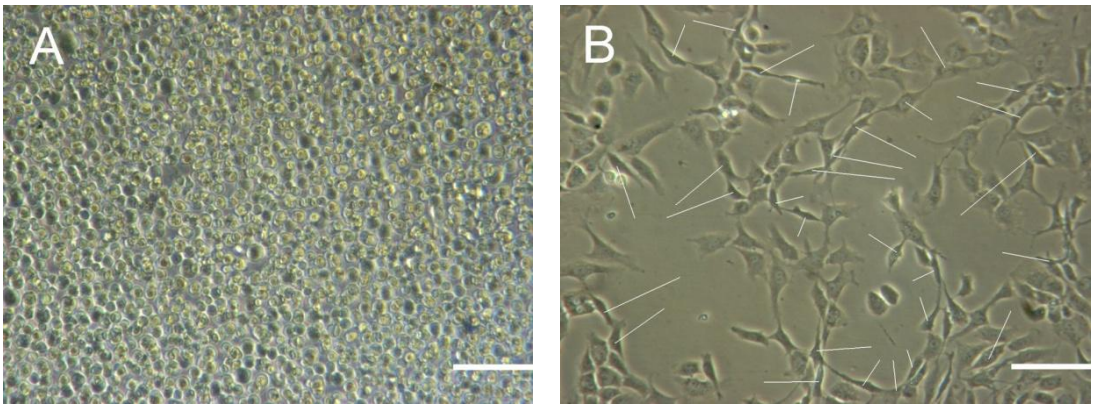


Figure 2.4 The purification process was repeated after 20 minutes of initial cell seeding (3,795,250/6ml), (A) and presence of chondrocytes and bone cells can be seen clearly, (B) (Scale bar = 100 µm).

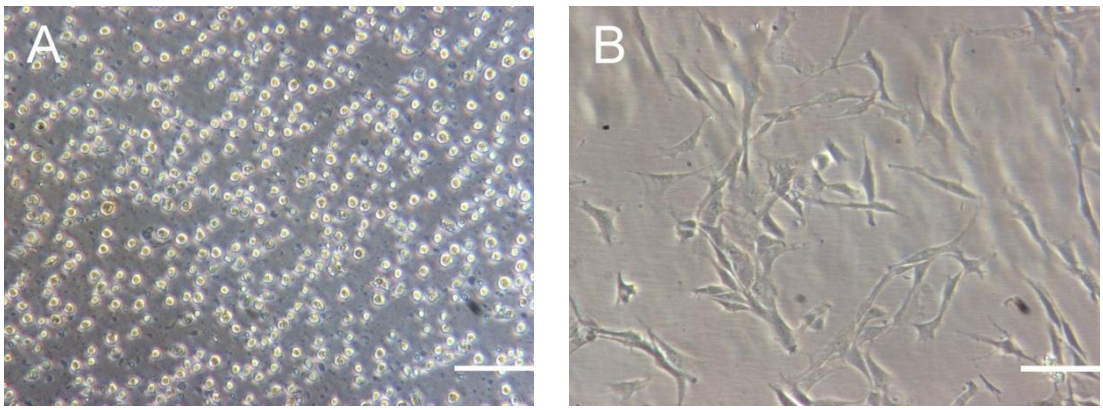


Figure 2.5 The removal of suspension with non-attached cells was repeated again after 40 minutes. The number of cells was 1,207,500 (Scale bar = 100 µm).

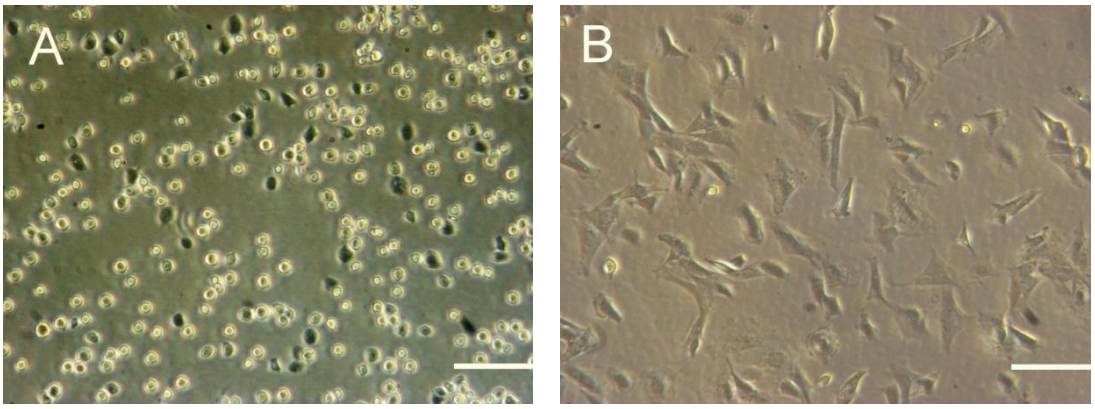


Figure 2.6 Morphologically, almost no chondrocyte cells was observed after 60 minutes of the purification process. The cell number at this stage reduced to 697,500 (Scale bar = 100 µm).

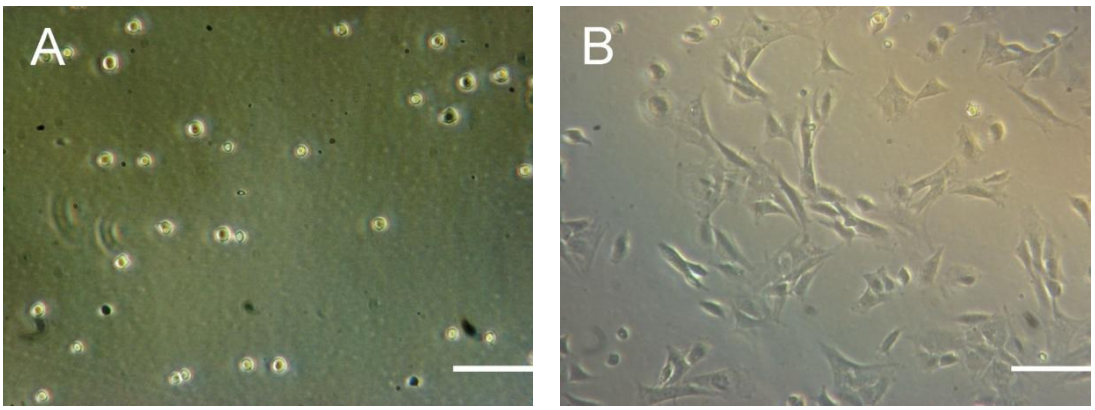


Figure 2.7 The purification process was repeated after 80 minutes with the number of cells at 550,000 (Scale bar = 100 µm).

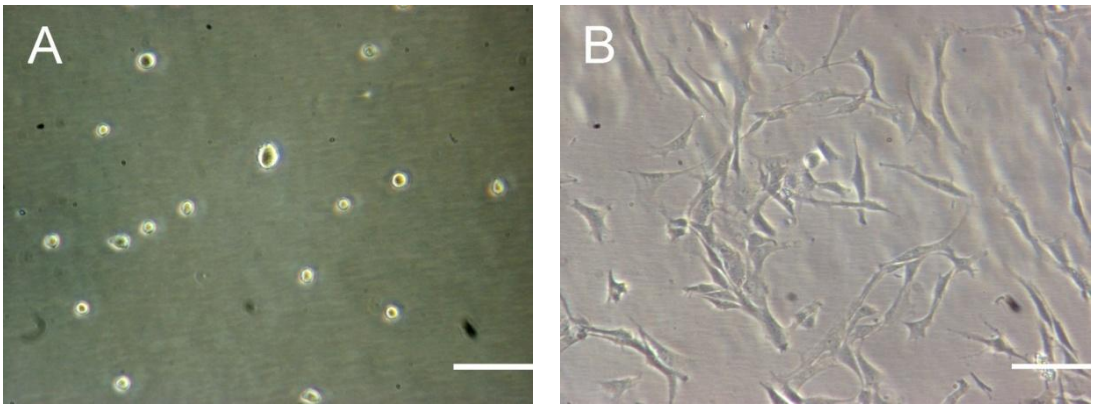


Figure 2.8 Non-attached cells after 100 minutes during chondrocyte purification. The number of cells was 365,500 (Scale bar = 100 µm).

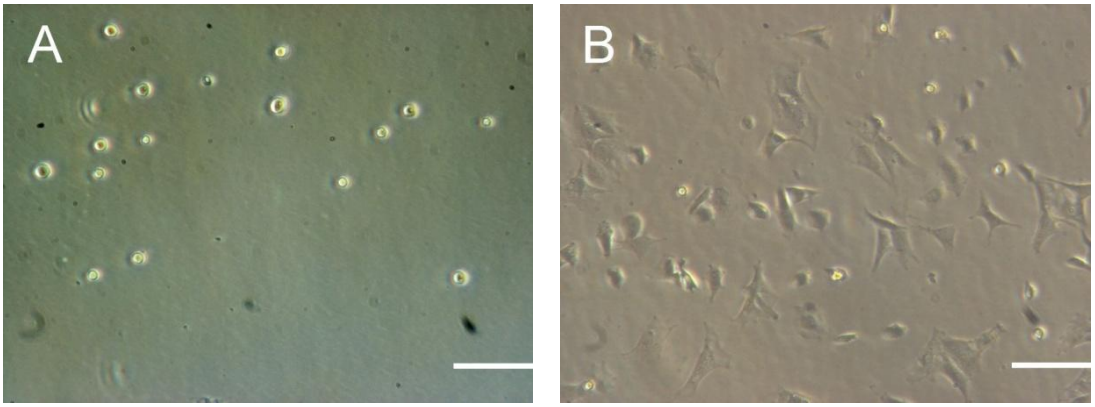


Figure 2.9 Little change in non-attached cells after 120 minutes. The number of cells was 336,000 (Scale bar = 100 µm).

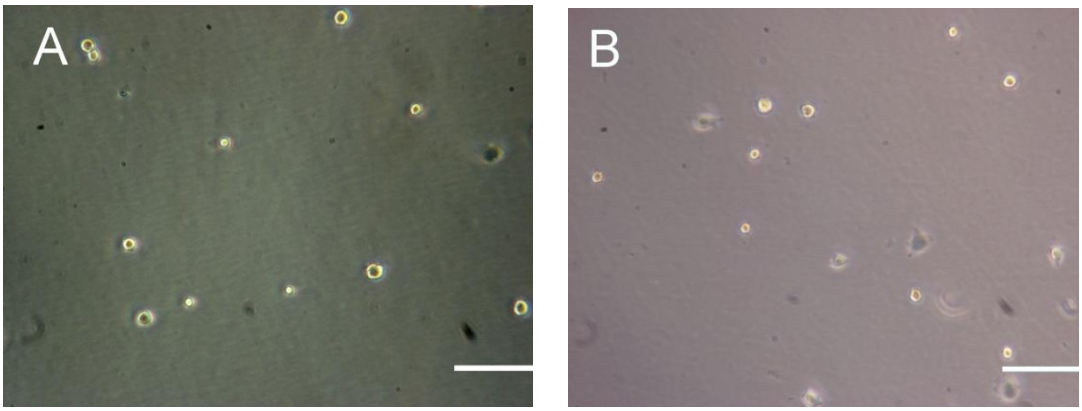


Figure 2.10 Very few cells were attached after 140 minutes. The number of cells was 312,625 (Scale bar = 100 µm).

After 140 minutes cell attachment was extremely slow, consequently, cell suspension was incubated for 240 minutes. The purification process was stopped as soon as the attachment of the majority of the cells was observed under the microscope, as can be seen in Figure 2.11.

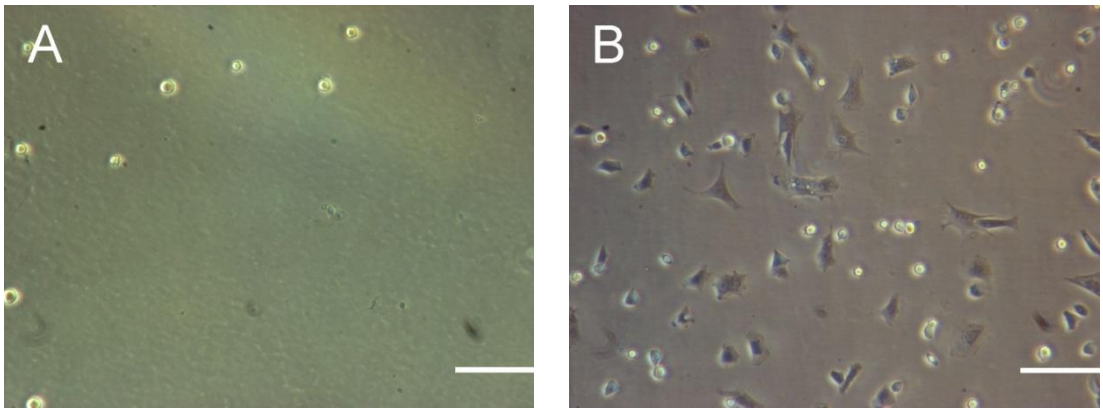


Figure 2.11 The remaining cells were attached during 240 minutes (Scale bar = 100 µm).

The statistical data obtained from cell separation were used to calculate the mean value, ± standard deviation, and by plotting a bar chart with error bars using Microsoft Office Excel, as shown in Figure 2.12.

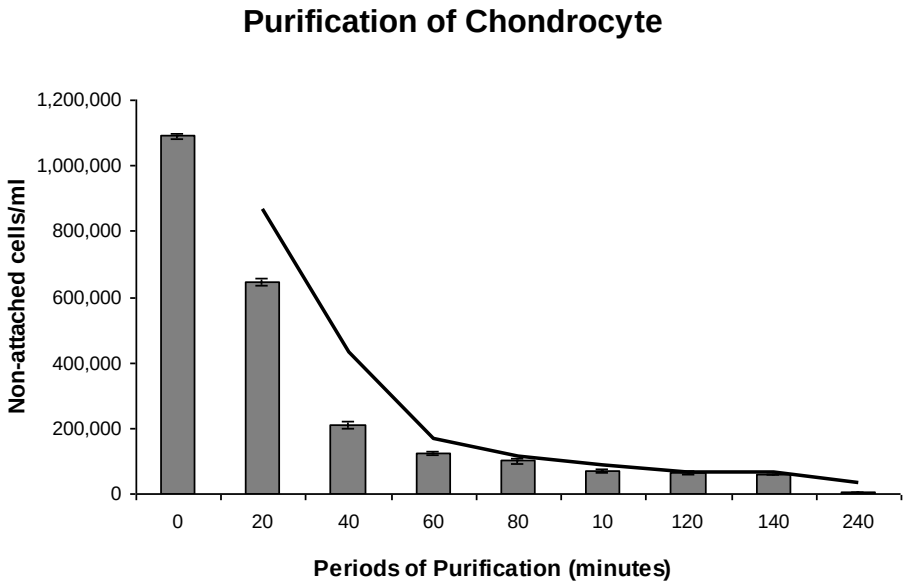


Figure 2.12 Bar chart for the purification of chondrocyte cells isolated from knee joints of neonate rats.

A total of 72 hours after the purification process, the cells were detached by trypsin, re-suspended in new cell-culture media, seeded on sterile glass coverslips and incubated at 37ºC. The non-attached cells were removed after 24 hours, and 48 hours after cell seeding, the coverslips were prepared for immunofluorescence imaging.

2.2.5 Immunocytochemistry

Immunocytochemistry (ICC) is a general method used to identify the antigens by binding of particular antibodies to these antigens. This binding can be detected using several methods such as applying a secondary antibody which binds to the primary antibody and causes a fluorescence effect (Meyer and Wiesmann, 2006) as shown schematically in Figure 2.13. This reaction, resulting from binding of secondary antibody to the primary antibody, is then imaged by fluorescence microscopy.

As described in Chapter One, cells synthesise several specific proteins. In the case of chondrocytes, these proteins include S-100, and collagen type-II which can be used as chondrocyte markers and also collagen type-I, an *in vitro* protein, secreted by chondrocytes. Identification of these molecules and confirmation of cell type was possible by using antibodies that rose against these markers.

After the isolation of the articular cartilage from the knee joint of the Sprague-Dawley rat and the enzymatic digestion of tissue and separation of cells, the cells were cultured in monolayer on the glass coverslips, fixed with 1% formaldehyde and immnocytochemical staining was performed.

Ten chondrocyte cultures were examined for the immuno-expression of two types of antibodies. Monoclonal anti S-100 (β-subunit, mouse IgG1 isotype) (Sigma Aldrich, UK) and monoclonal anti-collagen type-I (mouse IgG1 isotype) were used as primary antibodies and goat anti-mouse IgG, conjugated to Alexa Fluor 488 SFX Kits (Invitrogen, UK), was utilised as the secondary antibody.

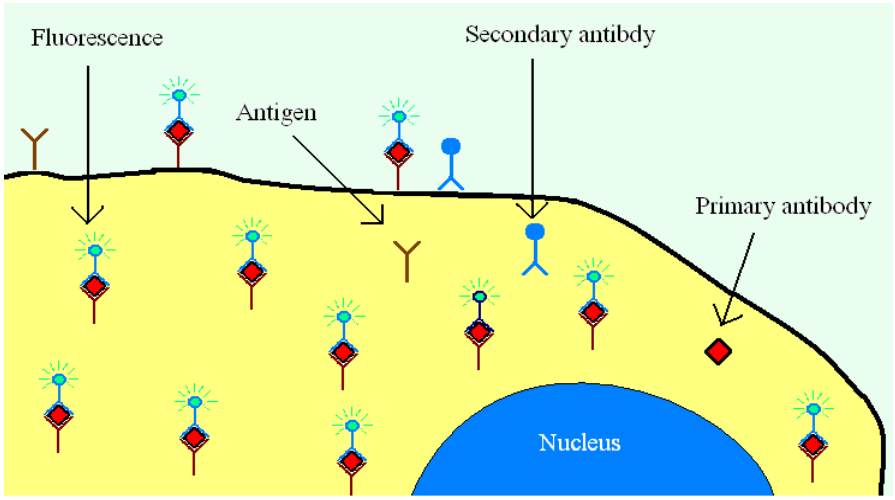


Figure 2.13 Schematic diagram of antigen, primary and secondary antibodies and immunofluorescence.

2.2.5.1 Preparation of Monoclonal Anti S-100 Antibody (β-Subunit)

The as-received S-100 antibody had to be thawed, aliquoted in 10 µL and immediately stored back at -20ºC, the temperature given by the supplier. A total of 50 mg BSA was dissolved in 5ml PBS or HBSS to produce 1% BSA w/v. 10 µL of primary antibody was required to be added into the 4,990 µL PBS or HBSS to make 5ml primary antibody with 1% BSA. Only 250-300 µL was adequate for each coverslip, and thus 5 ml was utilized to stain up to 20 coverslips.

2.2.5.2 Cell Fixation

Cells were fixed on the coverslips by using the following materials for fixation of cells: 2% formaldehyde solution made by adding 35.5 ml PBS, or alternatively 36.5% Hank's Balanced Salt Solution (HBSS) to 2 ml formaldehyde.

Old media were removed and the substrates (Petri dishes) were washed twice with HBSS. Cell-fixing solution (2% formaldehyde solution) was added to the substrates, left for five minutes and discarded. The substrates were washed three times with HBSS.

2.2.5.3 Protocol of Immunocytochemical Staining

Some of the *in vitro* antigens synthesised by chondrocyte are S-100 and type-I collagen (Meyer and Wiesmann, 2006). These antigens were labelled with appropriate antibodies and are demonstrated in immunocytochemical staining with the following protocols.

2.2.5.3.1 Triton X-100

Before immunostaining the cells, the permeability of the cell membrane was increased by utilising Triton X-100 which is a non-ionic surfactant that is used for biochemical applications to solubilise proteins (Sigma Aldrich, UK).

Preparation of 25ml of 0.1% Triton X-100 for five coverslips (5 ml for each coverslips) was as follows:

To make 10 x 5 ml = 50 ml of 0.1:100 Triton-X solution, 0.5 ml Triton X-100 (0.1%) was added to 49.50 ml HBSS by using the formula: $m_1 v_1 = m_2 v_2$

0.01 x 50 ml = 1 x v_2, v_2 = 0.5 ml, X ml + 0.5 ml = 50 ml X = 49.50 ml HBSS

2.2.5.3.2 Immunostaining Procedure

The first nine Petri dishes were assigned as positive (+), eight for the anti-S-100 antibody and one from the last purified cells for the anti-collagen type-I antibody and the 10th Petri dish was assigned as negative control (to see if there was any direct staining from the secondary antibody). After labelling the Petri dishes, 5 ml of prepared Triton X-100 (0.1%) (Sigma Aldrich, UK) was added to each Petri dish and incubated for 15 minutes at room temperature.

Triton X-100 was removed and the coverslips washed only once with PBS. Four drops of signal enhancer were applied to each coverslip and incubated at room temperature for 30 minutes. After 30 minutes the coverslips were washed carefully three times for five minutes each with 6ml HBSS on the shaking platform at room temperature. The HBSS was aspirated and only the third PBS was left in negative control. Diluted primary antibodies were added to appropriate (+) Petri dishes and incubated at room temperature for three hours in a humid environment.

To obtain the optimum results, a set of anti-S-100 primary antibody with two dilution ratios (1:500 and 1:1000) was prepared. For each respective ratio, 20 µL and 10 µL

primary antibody were added to 10 ml of 1% HBSS, as shown schematically in Figure 2.14.

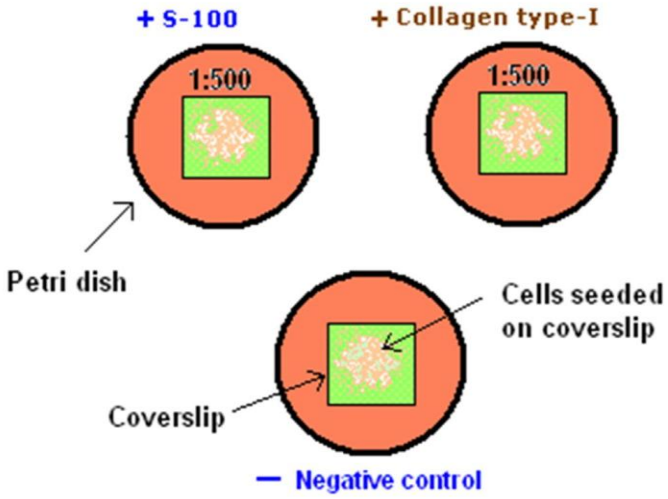


Figure 2.14 Schematic diagrams of cultures with two different dilution ratios prepared for immunostaining.

The primary antibody was removed from the positive (+) and HBSS from the negative (-) control and washed carefully three times for five minutes each with 6 ml HBSS on the shaking platform at room temperature. The third HBSS left for 30 minutes in the Petri dishes. HBSS was aspirated and the area around the coverslips in the Petri dishes was dried very carefully. The secondary antibody was then added to the coverslips and was prepared as follows. The common ratio of secondary antibody is 1:400. According to the supplier instruction, the final concentration of secondary antibody needed was 1-10 µg/ml where the delivered antibody had a concentration ratio of 2mg/ml. To make 4ml (4000 µl) antibody, 10 µl of secondary antibody was added to 3990 µl HBSS.

The Petri dishes were incubated with the secondary antibody for two hours at room temperature in a humid environment in the dark.

After two hours of incubation, the Petri dishes were washed three times, each time for 15 minutes with 6ml HBSS on the shaking platform at room temperature. The Petri dishes were mounted by leaving the last HBSS in them and incubated at four degrees in a dark and humid environment.

2.2.5.4 Immunofluorescence Microscopy

After seven days the bound antibodies were visualised by Alexa Fluor 488 goat anti-mouse SFX (Invitrogen, UK) as a secondary antibody. Chondrocytes cultured on coverslips without any added antibody were also prepared as negative control to compare and control the accuracy of immunocytochemistry.

Each Petri dish was imaged 10-times and the eight best images were used to obtain statistical and analytical data.

The stained S-100 was localised around the nuclei (also found in ECM) which confirmed the existence of chondrocytes, as shown in Figure 2.15.

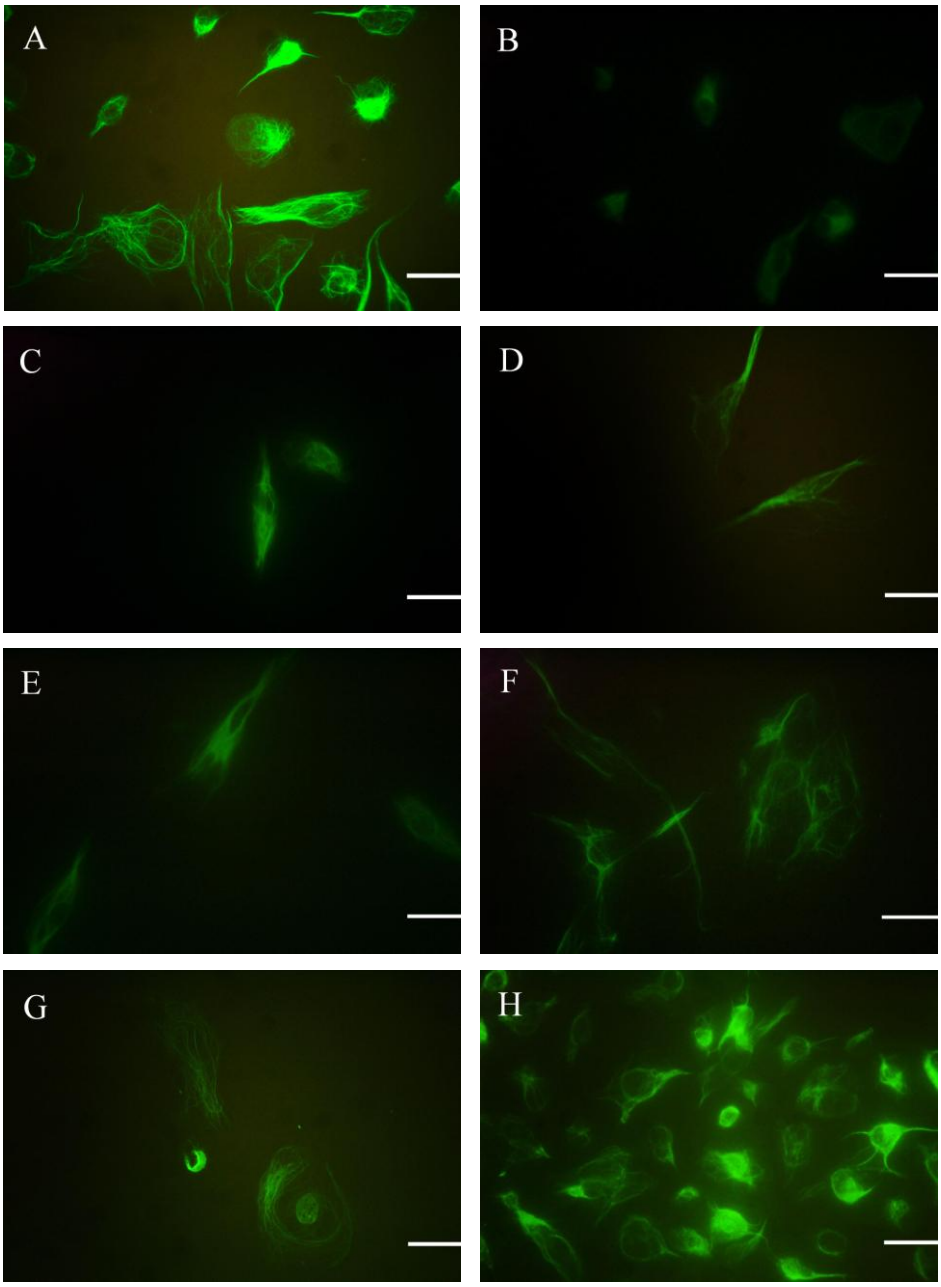


Figure 2.15 Fluorescence microscopy images of primary culture of cartilage isolated from the knee joints of Sprague-Dawley rats showing green stained S-100 antigen around the nuclei. The arrows show the interaction between the anti-S-100 antibody and the S-100 antigen, causing a fluorescence effect: a) before purification; b) - g) purification stages; h) final stage with purified chondrocytes (Scale bar = 50 μm).

The stained type-I collagen was localised around the cell membrane as part of the extra-cellular matrix (ECM) shown in Figure 2.16.

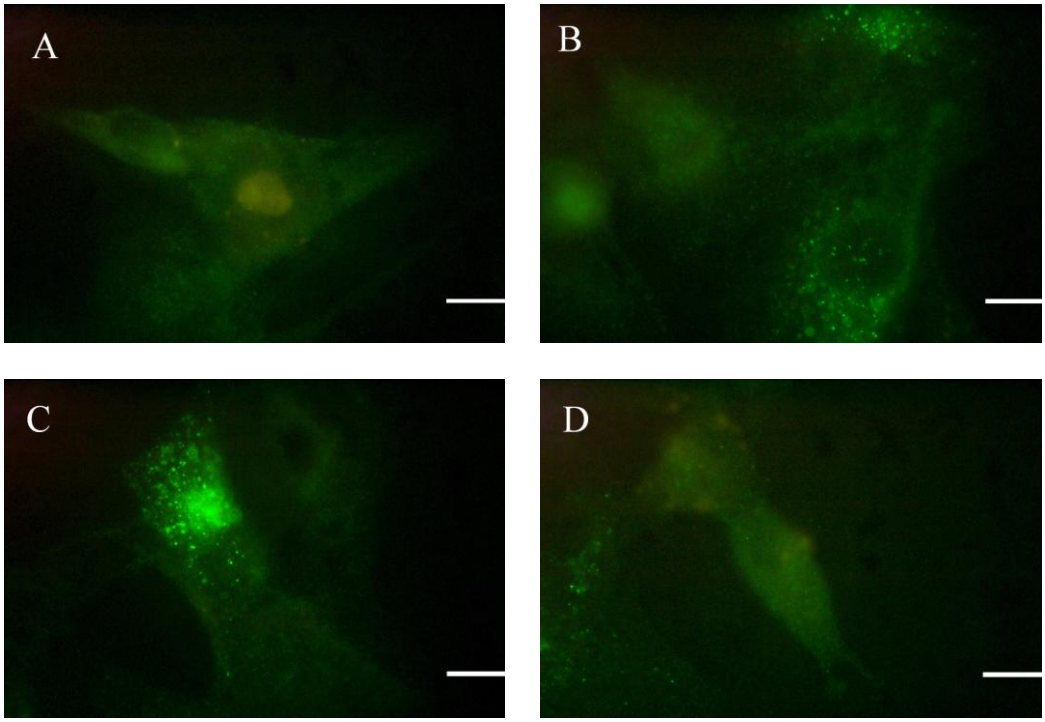


Figure 2.16 a) – d) Fluorescence micrographs of primary chondrocytes immunostained for type-I collagen. The arrows show the regions positively labelled by the anti-collagen-I antibodies (Scale bar = 50 µm).

Apart from some background staining caused by debris, there was almost no staining on the negative controls for S-100 and type-I collagen, as shown in Figure 2.17. This proved the chondrocyte separation from co-cultured cells, obtained in this work, after purification.

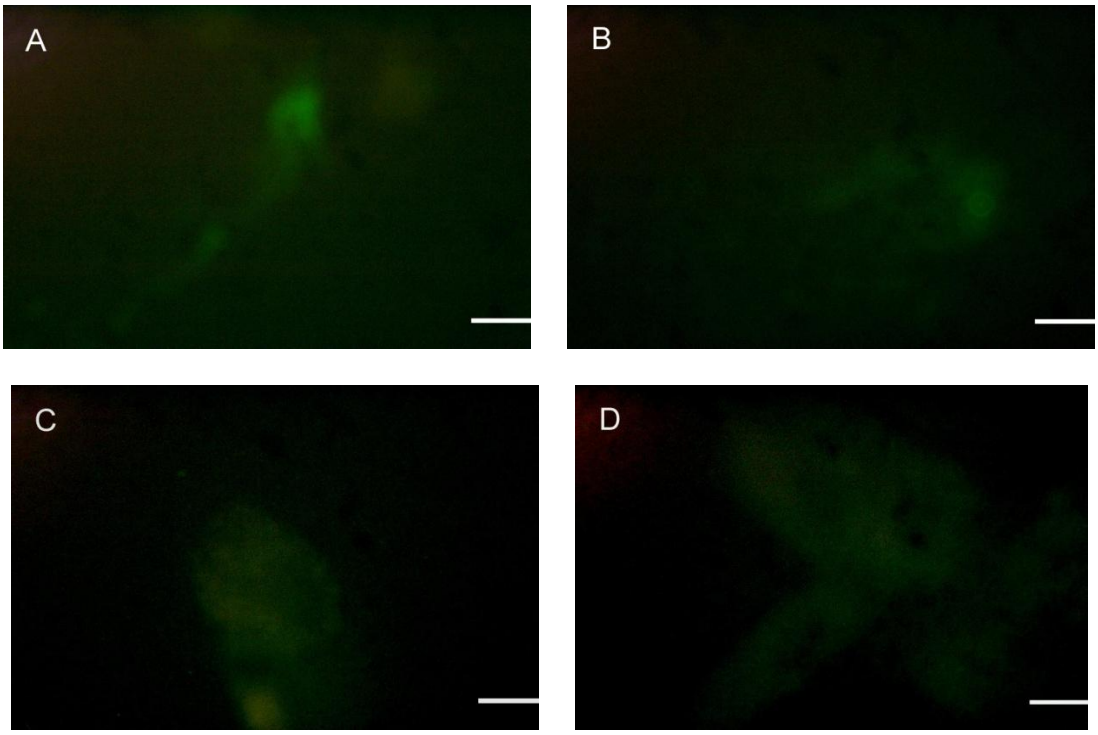


Figure 2.17 Fluorescent micographs of the negative controls for the S100 (a) and (b), and for type-I Collagen (c) and (d), (x10 Magnification), (Scale bar = 50 µm).

The stained cells with S-100 antibody on each group were counted and the average per image was plotted in the graph shown in Figure 2.18. The amount of the final purified cells shows the maximum staining, confirming the achievement of almost pure chondrocytes in culture, during this work.

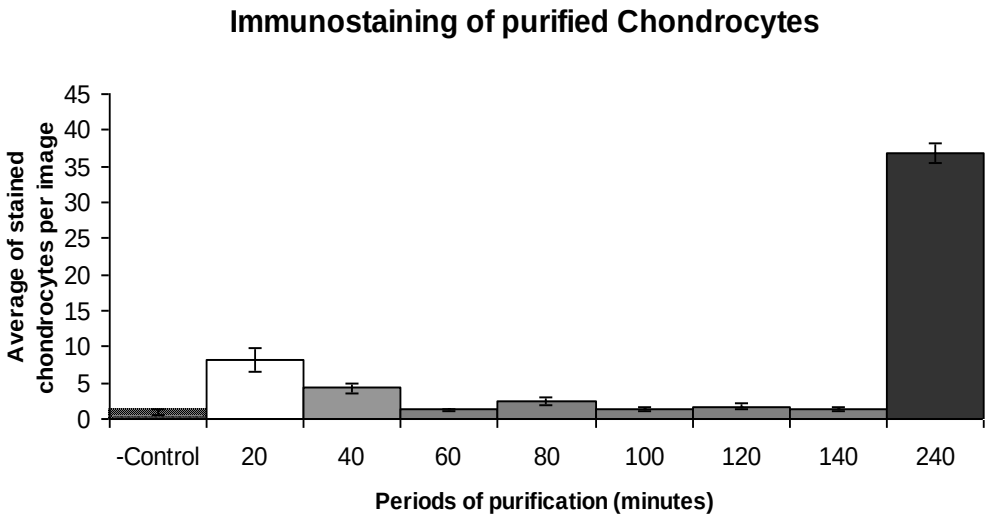


Figure 2.18 Graph of stained cells with S-100 antibody during the purification process.

2.3 Results and Discussion

At the stage of primary chondrocyte culture and cell separation, the results obtained from seeding of chondrocyte cells isolated from the articular cartilage of the Sprague-Dawley rat showed that they started attaching on a solid surface after about 80 minutes, but even after 120 minutes, most of the chondrocyte cells were not attached.

However, most of the chondrocytes attached after 180 minutes, and after four hours the non-attached cells were still visible in the culture. The bone cells and fibroblasts started attaching to the surface of the culture flask a few minutes after seeding. This difference in the attachment ability of different cell types was used to separate the chondrocytes from other co-cultured cell types.

The results obtained from the cell-purification process were put in Microsoft Excel to plot a graph shown in Figure 2.12. The straight line of the graph up to 50 minutes showed that the cells attach regularly on the substrate, which could be due to the presence of analogous cells in the culture system. In this case they are probably bone cells because they attached much faster than chondrocyte cells.

Between 40 and 120 minutes, the curve changed its shape and levelled off, which meant that the cell type was no longer analogous. These cells are probably nerve cells which are the offspring of bone nerves, tenocytes and also chondrocytes that started to attach on the surface after ~ 80 minutes.

The curve changed its shape again after 140 minutes and the line became level, almost constant, suggesting the attachment of analogous cells. The difference between these two recordings is about 2.9×10^{5} cells in 100 minutes, and as compared to the first purification stages, is very low.

The new method of purification of chondrocyte also evidenced the attachment of bone cells and fibroblasts before chondrocytes. Noticeably the bone cells adhere on the surface much faster than chondrocytes and fibroblasts also attached and detached faster than chondrocytes. Immunohydrochemistry staining with S-100 protein indicated that the isolated cells expressed S-100, thus confirming the successful isolation of chondrocytes. This was further confirmed by immunostaining for the collagen type-I and that the collagen type-I was localised around the cell membrane.

Also immunostaining proved that the chondrocyte exhibited different morphologies as seen in Figure 2.15-h. Depending on different zone of cartilage, chondrocytes exhibit

different morphologies (Hu and Athanasiou, 2006) (Gardner et al., 1987). The superficial zone and perichondrium contain fibroblast like chondrocytes, whereas cells from the middle zone demonstrate rounded shape. The chondrocytes from deeper zone have an elongated shape and hence different chondrocyte morphologies do exist within the in vivo cartilage in a natural state.

CHAPTER III

3 Culture of Chondrocyte

3.1 Introduction to Chondrocyte Cell Culture

The cell is the most basic unit of structure, function and reproduction of a living organism that consists of various organelles all of which work together. These organelles carry out processes such as respiration, migration, shape, digestion of nutrients, transportation of waste products out of the cell and many other functions. These materials and nutrients pass through the cell membrane by diffusion or protein channels (Alberts et al., 1983).

The cells, after being removed from the body, require maintenance and growth in an artificial environment supplemented with necessary nutrients under controlled conditions. The culture medium consists of Dulbecco's modified Eagle's medium (DMEM) containing 4.5mg/ml glucose, 4mM L-glutamine, 10% foetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml Fungizone (Vunjak-Novakovic and Freshney, 2006).

Normally, chondrocyte within the cartilage demonstrates a round-shape morphology and a phenotype by secretion of various extracellular matrix proteins, including type-II collagen, cartilage oligomeric matrix protein (COMP) and aggrecan, which are characteristically found in cartilage (Pei et al., 2008); (Schnabel et al., 2002); (Gigout et

al., 2008). Also, high-density chondrocyte cultured in monolayer keeps its rounded shape and shows characteristics similar to the *in vivo* condition (Brodkinb et al., 2004) as can be seen in Figure 3.1.

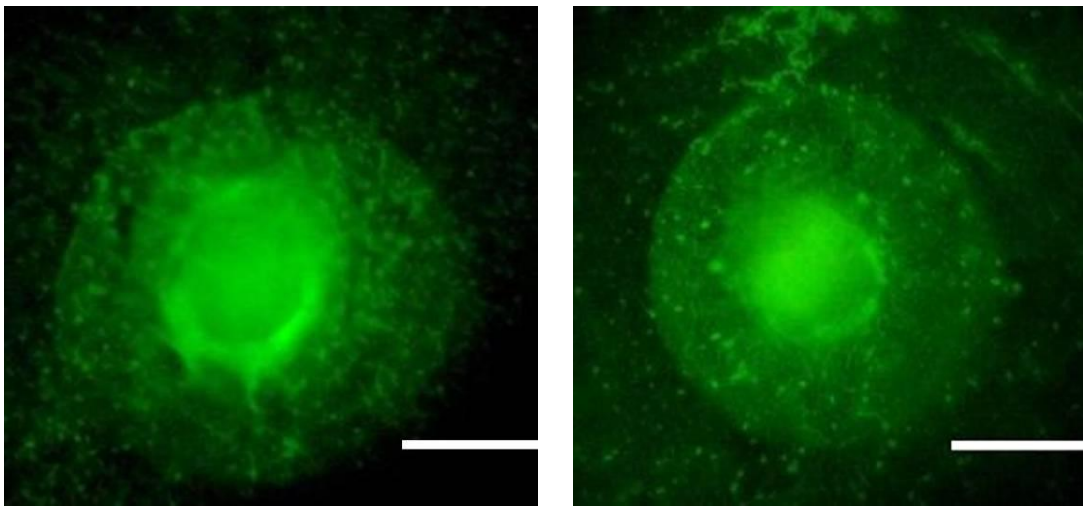


Figure 3.1 Photomicrograph of chondrocytes in monolayer with round morphology seeded at high density, obtained in this work (Scale bar = 50 μm).

However, once chondrocytes are cultured in a planar culture system, they lose their rounded shape and dedifferentiate into fibroblast-like morphology and produce collagen type-1 (Boubriak et al., 2009) as shown in Figure 3.2.

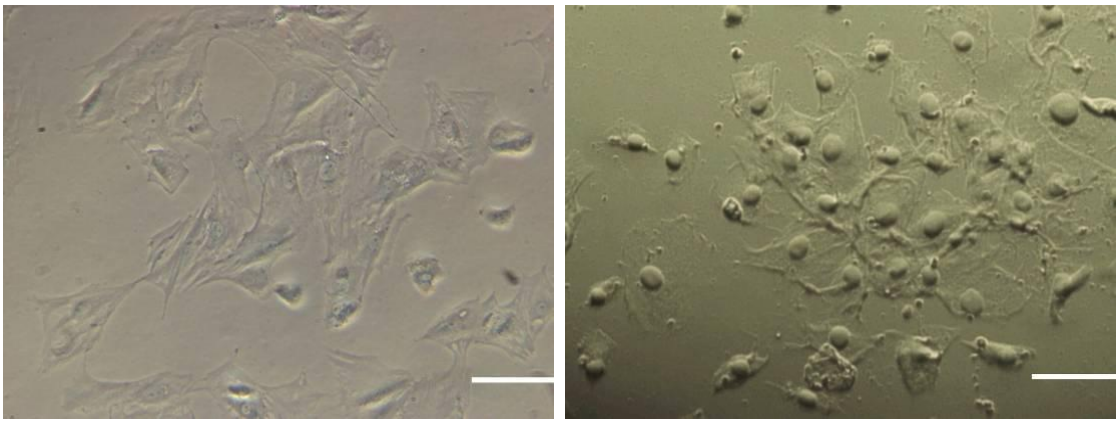


Figure 3.2 Photomicrographs of chondrocytes cultured in monolayer with fibroblast-like morphology (left) and under a light microscope (x10 Magnification), (right) fixed with 3% formaldehyde (x10 Magnification).

Chondrocyte in intact cartilage (*in vivo*) is mostly embedded within a space called lacunae. During *in vitro* preparation, because of cell shrinkage, a space between chondrocyte and ECM occurs, whereas, within the *in vivo* cartilage, the cell occupies whole lacunae (Steinert et al., 2009) as can be seen from Figure 3.3 (Tsai and Liua, 2002); (Underhill et al., 1998).

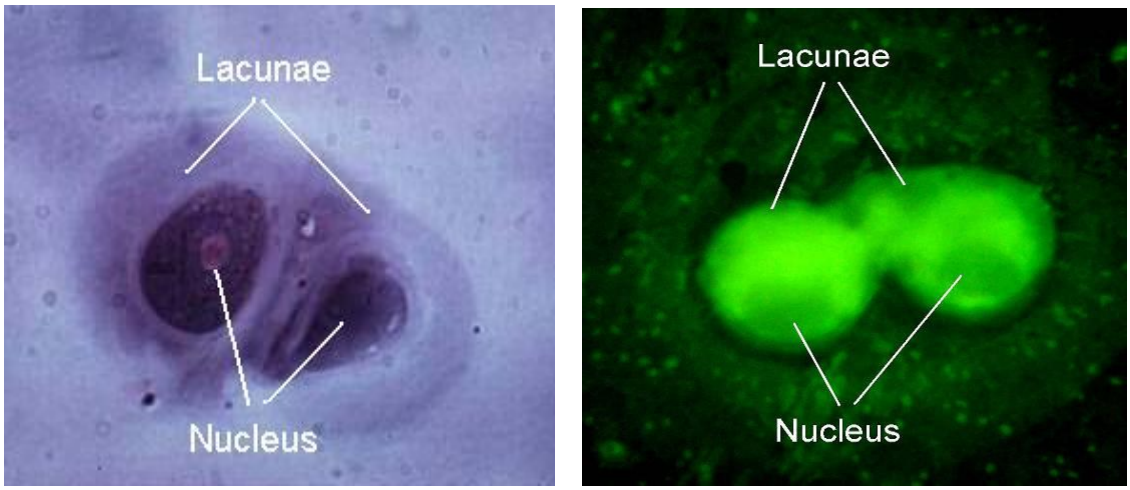


Figure 3.3 Chondrocytes in lacunae (left) and immunohistochemically stained chondrocyte (Petty, 2009) and (right) primary chondrocyte immunocytochemically stained for S-100.

There are various models of chondrocyte culture that facilitate the study of chondrocyte with the aim of cartilage repair so that the tissue has characteristics of normal articular cartilage.

3.2 Monolayer Culture

Chondrocyte culture is usually maintained at 37˚C and saturated with a mixture of 95% air and 5% CO_2. Another option to maintain the culture is the use of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) modified reagents (Sigma Aldrich, UK). The temperature could be set a degree below 37˚C to avoid accidental high-temperature fluctuation because the cells can survive at a lower temperature but not at a higher temperature.

Feeding of chondrocytes should be done at least twice a week via changing the whole media. The appropriate culture media for primary chondrocyte could be DMEM or RPMI-1640. Alternatively, a mixture of these media with Ham's F-12 (1:1) might induce chondrocyte proliferation (Dan-ning and McCormick, 2005). As in monolayer culture, chondrocyte undergoes dedifferentiation and secretion of collagen type-I instead of collagen type-II (Vinatier et al., 2009) (See Figure 3.4), as discussed in Chapter two.

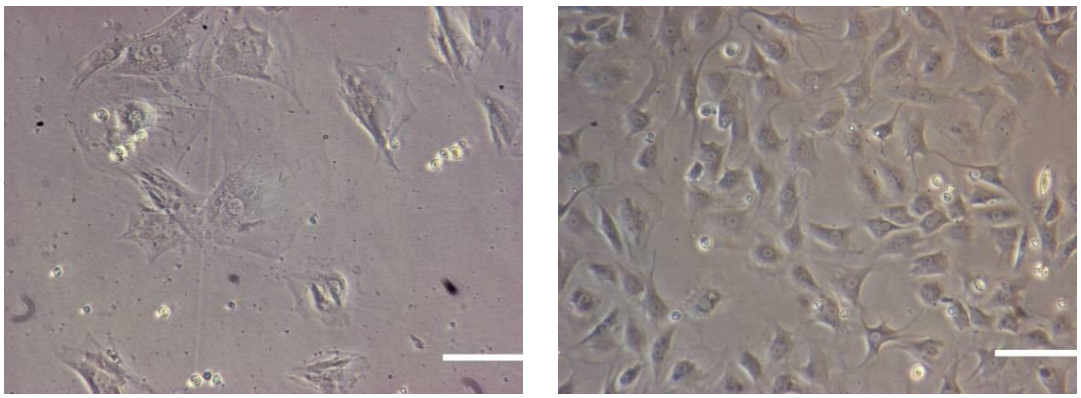


Figure 3.4 Primary chondrocyte in monolayer culture (left) of low density and (right) high-density chondrocyte culture under a phase-contrast microscope (x10 Magnification=10x; Scale bar = 50μm).

When the chondrocytes are cultured in monolayer at high density, they show a rounded shape, but at low density the cells show a fibroblast-like morphology (see Figure 3.4). The high density could preferably be achieved by cells seeding at 2×10^5 cell/ml (Bhatnagar and Nicoll, 2001).

It must be emphasised that any disturbance of cells in the form of mechanical stress, via washing with salt solution such as PBS or Hank's, change of media, aspiration of solution would affect cell proliferation and its behaviour. The major problem in cell culture might be contamination of the media and therefore, the use of antibiotics and Fungizone protects the cell culture from bacterial and yeast infection. Sterilisation by 70% ethanol is a basic strategy to combat contamination.

Before changing the media, the culture must be checked for its colour, as the colour of the culture media is a good indication of infection (Sigma Aldrich, UK).

Minimising contact between air and media and use of sterile material and equipment is the best way to prevent air-borne infections.

3.3 Three Dimensional (3D)-Pellet Culture

A key component of this study will be to examine the effects of a three-dimensional culture system on chondrocyte phenotype. One of the methods for studying the characteristics of chondrocytes *in vitro* in a 3D environment is via pellet culture (Kato et al., 1988).

Pellet culture is a type of 3D culture technique using an appropriate number of cells, centrifuged into a pellet and cultured in a floating environment without the possibility of cell attachment to the surface. The cell density has to be extremely high to be able to develop a pellet (Kwok-Sui Leung, 2008).

Examining chondrocyte in a 3D culture is particularly important because, in a monolayer culture, chondrocytes typically stop differentiation and develop a non-chondrocytic morphology and become more fibroblast-type. Dedifferentiated chondrocytes secrete type-1 collagen and lose the ability to produce chondrocyte-specific proteins, e.g. aggrecan and type-2 collagen, (Schnabel et al., 2002).

In contrast to other types of 3D culture system, such as aggarose, pure alginate or alginate-chitosan, chondrocyte cultured in a pellet expresses collagen type-2 (Ibold et al., 2009) and a significantly greater amount of DNA and glycosaminoglycan (Goldberg et al., 2005). Also, recently, Bernstein et al (2009) examined chondrogenic redifferentiation of chondrocyte in different 3D culture systems. Their report revealed that the chondrocytes cultured in pellets express a maximum amount of collagen type-2 when compared with other three-dimensional culture systems. This finding gives a new hypothesis, that chondrocyte from a pellet culture might be most appropriate for tissue engineering and autologous chondrocyte implementation (ACI).

3.3.1 Experimental Method: Cartilage Extraction, Isolation and Pellet Formation

Cartilage was extracted from the articular joint of a neonate Sprague-Dawley rat and the cartilage tissue covering epiphysis at the knee joint was predigested in 4ml of trypsin and further digested in 1ml of type-1 collagenase (See Chapter 2). The isolated chondrocytes were centrifuged and the pellet was re-suspended in 15ml of fresh media and cultured in monolayer in a 75cm² tissue-culture flask until 70-80% confluency was achieved. The cells were quantitatively expanded in monolayer and, after the third passage, chondrocytes were trypsinised, poured into six different 15ml centrifuge tubes and centrifuged at 700, 900, 1100, 1500, 1700 and 2000rpm individually. The supernatants were discarded and 10ml of fresh media was added on each pellet and incubated at 37˚C. All six pellet cultures were fed with fresh media by changing only 80% of the whole media every two days.

A light microscope was used for taking images after one week, 10 days, two and three weeks for further analysis (See Figure 3.5).

The longest dimension of the cells was measured six times and the mean value was obtained.

After three weeks, the pellets were minced and re-suspended in a high-glucose DMEM media supplemented with 10% FCS and 0.1mg/ml hyaluronic acid. 1 ml of cell suspension was centrifuged at 1400 rpm, media was discarded and the pellet was resuspended in 1 ml PBS. The viability of the cells obtained from the resuspended pellet in PBS was assessed by trypan blue (See Table 3.1).

The cells were seeded into a 25cm² tissue-culture flask with a cell density of 200,000 cell/ml and incubated at 37ºC until they were released from the pellet and attached to the surface of the culture flask.

3.3.2 Results and Discussion

Apart from the pellet centrifuged at 700rpm, other pellets failed to adhere. This suggested that these 3D pellet in an *in vitro* environment lacked key signals associated with the up-regulation of the integrins responsible for cell attachment.

The pellet after 700rpm centrifugation maintained the most viable cells when assessed with trypan blue. The 700rpm pellet maintained ~93% of their proliferation state (See Table 3.1), and hence the chondrocytes were able to survive in a prolonged culture that was probably in a relatively anaerobic environment (Figures 3.5-3.6).

Trypan blue assay	**Viable Cells**	**Non-viable cells**	**Viability (%)**
Count-1	18	1	94.47
Count-2	7	0	100
Count-3	18	2	90
Mean	14.33	1	93.47

Table 3.1 Evaluation of chondrocyte cell viability (cell/chamber) after transfer from pellet to monolayer culture system using trypan blue assay.

The viability of the chondrocyte cells cultured in pellet was statistically assessed by Single-factor ANOVA test with the hypothesis of equal amount of viable and non-viable cells (Null hypothesis). The results showed that the F crit = 7.7 < 12.9 = F, p = 0.029 < α = 0.05. α is the threshold of significance of the test and generally is set at α = 0.05, F is the probability of distribution of the samples under the null hypothesis, null hypothesis is the equality of the means, and F crit (Critical F) is a value that the test statistic must exceed to reject the hypothesis. In this case the results showed that F > Fcrit, and p < 0.05, which rejected the null hypothesis and proved that the amount of viable and non-viable cells were not equal.

In the first week of pellet culture, the extracellular matrix (ECM) was formed. It is well established that ECM acts as a scaffolding to hold the cells together. An increase in cell size within the ECM was visible in the first 12 days (See Figure 3.5-C). After 16 days, the pellet exhibited much fibrous formation (as shown in Figure 3.5-D). After about three weeks, pellet culture noticeably increased in fibril thickness and cell size (See Figure 3.5-E, and F).

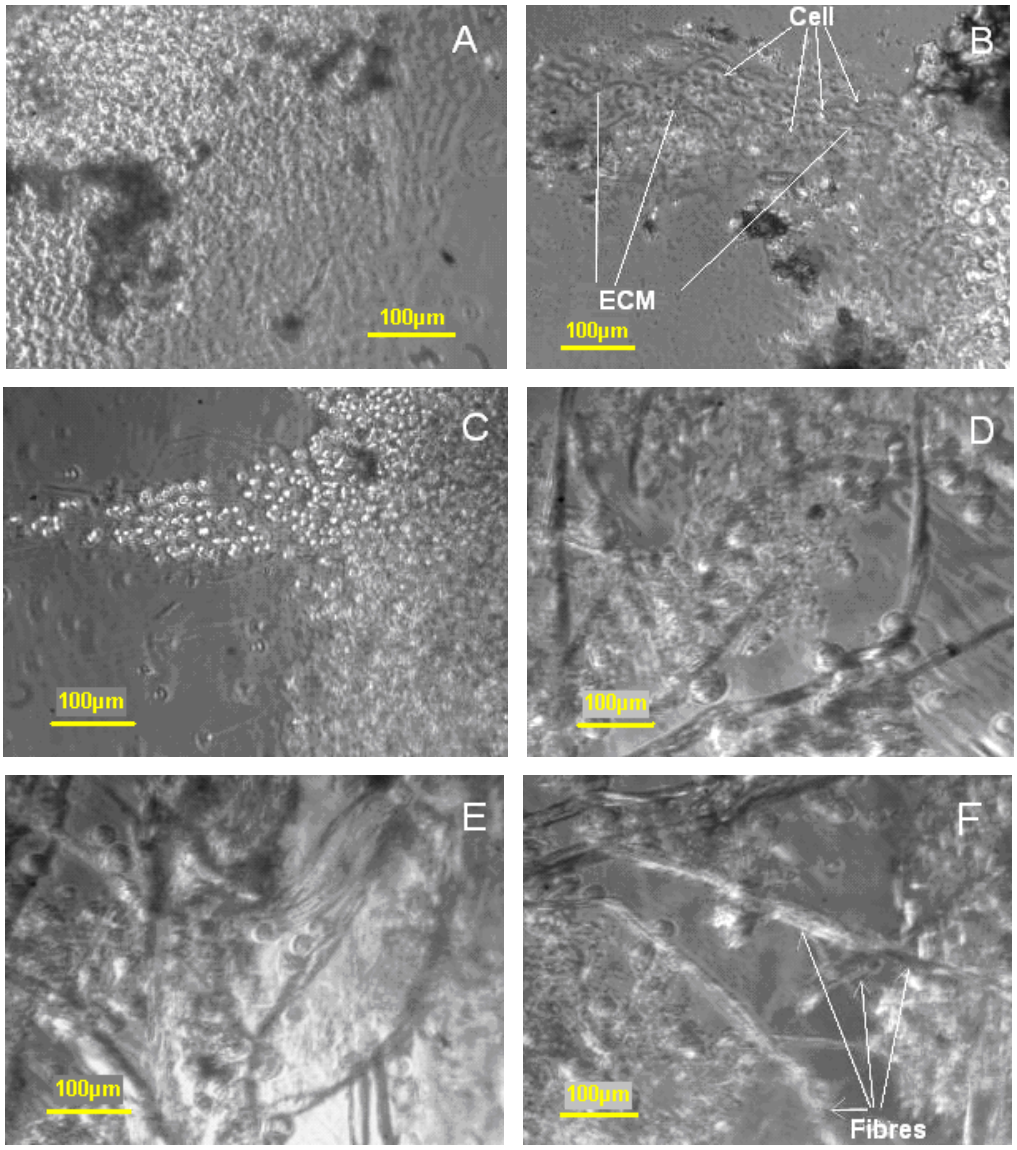


Figure 3.5 Micrographs of chondrocyte cells in a pellet after 700rpm centrifugation: A) after four days; B) formation of ECM after eight days; C) increased cell diameter after 12 days; D) increased cell size and synthesis of fibrils after 16 days; E) and F) cells and fibrils after three weeks.

Once the pellet was transferred to tissue culture flask, the cells migrated to the surface of the flask and attached with different morphology from those cells within the pellet (Figure 3.6).

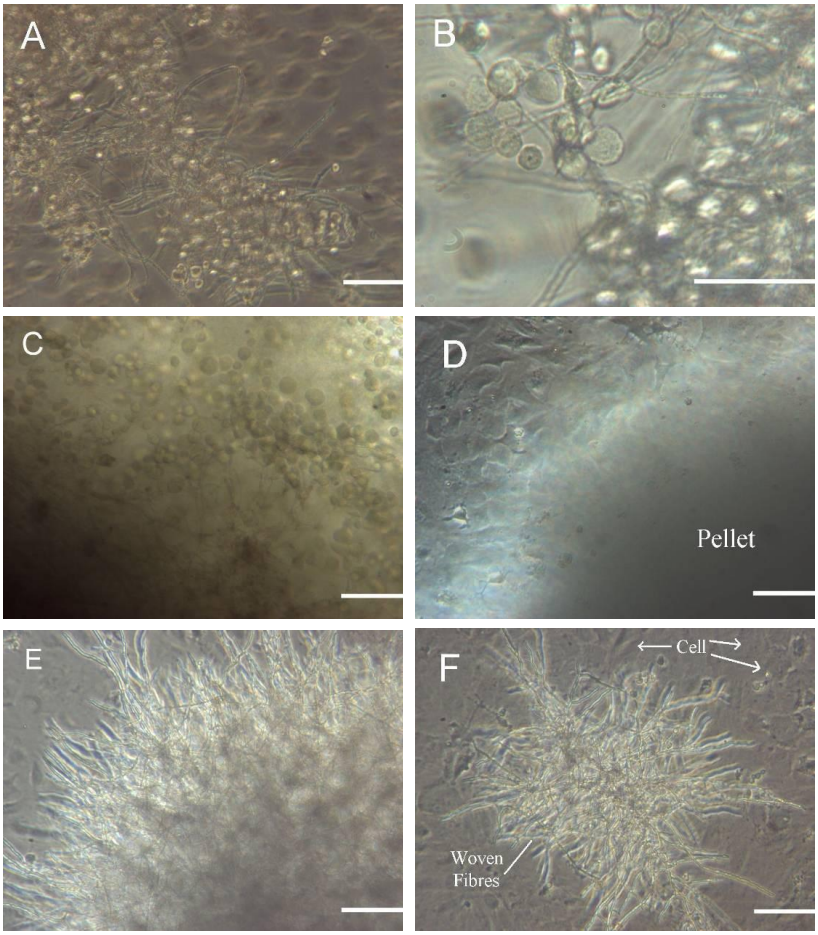


Figure 3.6 Chondrocyte cells attached to ECM molecules during pellet culture: A) Chondrocytes attached to fibres (10 x magnification), B) attached cells to ECM molecules (20 x magnification), C) Cells attached to the ECM inside the pellet, D) chondrocyte cells migrated from pellet (10 x magnification), E) and F) pellet without cells, (Scale bar = 100 µm).

The cells started to migrate out of the minced pellet and attached to the surface of the tissue culture flask about one hour after transfer from 3D-pellet to planar culture system (Figure 3.7). The cells obtained fibroblastic morphology as soon as they attached to the surface of the tissue culture flask (Figures 3.6-3.7). This cell migration continued until all cells were moved from pellet. The treatment of the surface of tissue culture flask was probably the reason for the cells relocation.

Typical shape of chondrocytes cultured at low density *in vitro*.

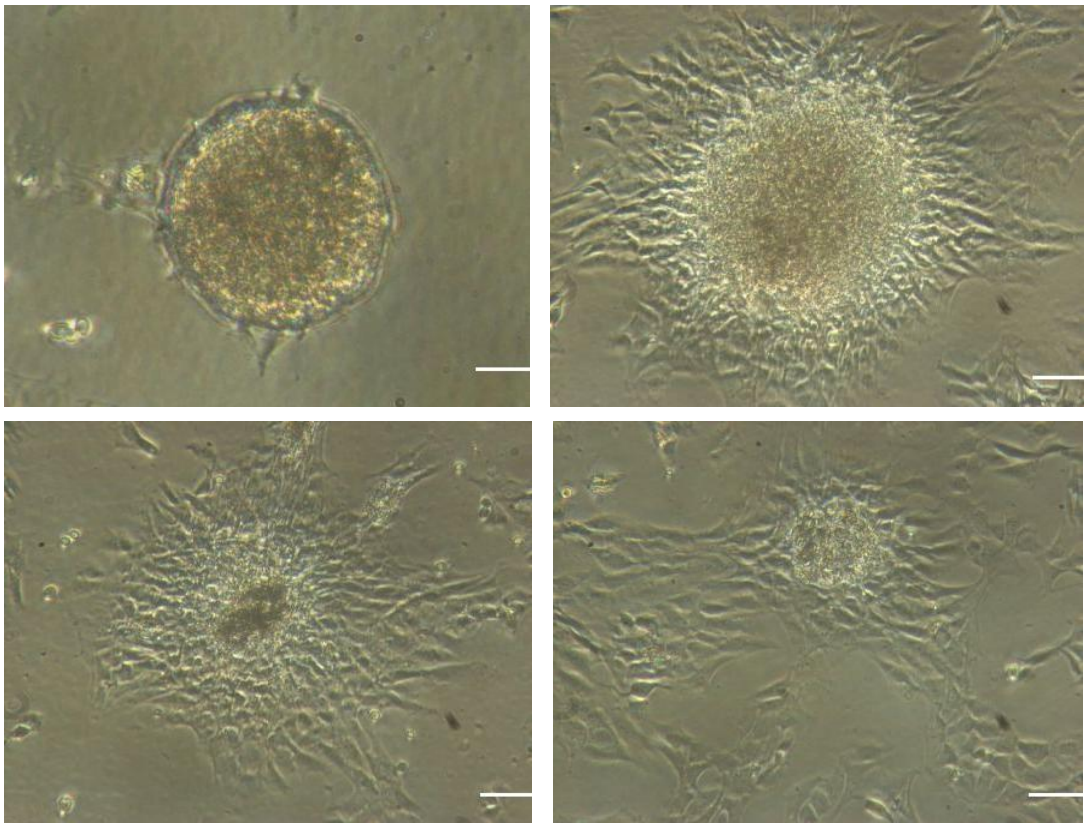


Figure 3.7 Gradual release and migration of chondrocytes (in 24 hours) from pellet centrifuged at 700 rpm (Scale bar = 50μm).

The size of the cells was measured 10 times every four days. The greatest increase in cell size (See Figure 3.8) was from 8.266 to 31.558µm in diameter after 16 days. The pellet size started to increase after eight days.

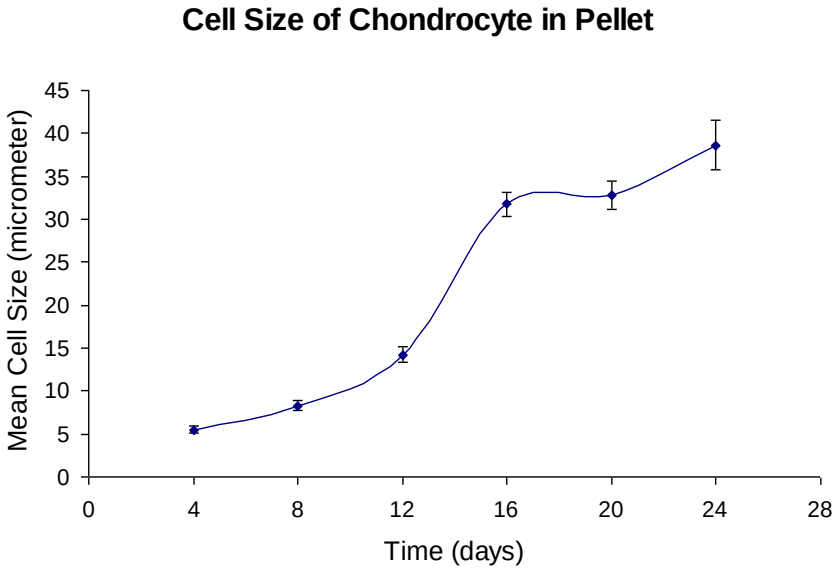


Figure 3.8 Diagram showing increase in primary chondrocyte cell size during three weeks in pellet culture.

Immunofluorscence staining with specific markers confirmed presence of Chondroitin Sulfate Proteoglycon-5 (CSPG-5) and Type-2 Collagen and that the chondrocytes in 3D-pellet culture synthesize these two proteins (Figure 3.9).

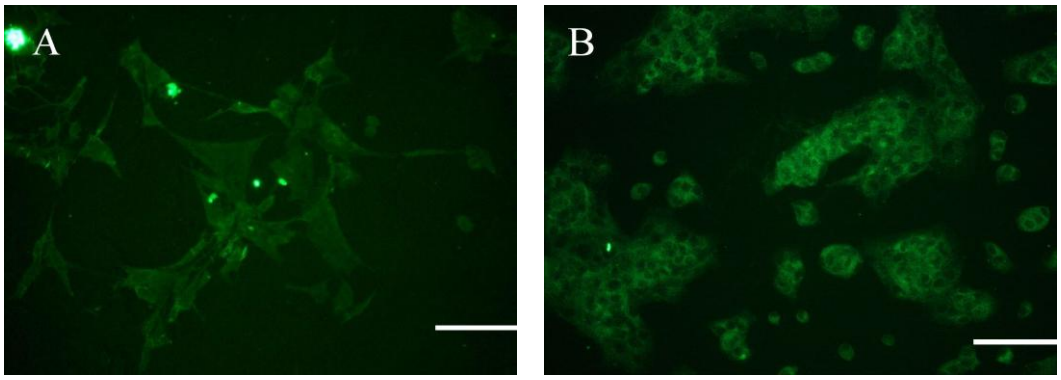


Figure 3.9 Chondrocyte from pellet stained for A) CSPG, and B) Collagen type-II. (Scale bar = 100 μm).

These results, therefore showed clearly that culture of chondrocytes in the form of pellet might be an alternative *in situ* cell culture method for storing cells. As the pellet maintained the cells in a viable and proliferative state without being stored in liquid nitrogen, for example, it might be biologically and financially even more appropriate for cell preservation.

3.4 Effect of Hyaluronic Acid (HA) on Regulation of Primary Chondrocyte

Hyaluronic acid (HA) is a natural glycosaminoglycan found in synovial fluid, cartilage and extracellular matrix and has an important structural and information carrier role in cartilage tissue (Tognana et al., 2007).

Several devices such as non-woven fibres, tubes, membranes and sponges can be produced by processing hyaluronic acid (Vindigni et al., 2009). These biocompatible scaffolds are biodegradable and resorbable by the host tissue.

Chondrocytes, human hepatocytes, mesencymal stem derived cells, and keratinocytes have been successfully cultured in hyaluronic acid meshes (Vindigni et al., 2009).

Hyaluronic acid by adhering to CD44 antigen (Ishida et al., 1997), induces a variety of signals, that regulate cells proliferation, synthesis of type-II collagen and formation of cartilage matrix protein (Akmal et al., 2005).

The purpose of this experiment was to study the effect of hyaluronic acid in the cellularity and ECM production of chondrocyte cultured in DMEM media supplemented with 10% FCS and 0.1 mg/ml HA.

3.4.1 Experimental Methods

A total of 50mg of hyaluronic acid sodium salt from a rooster comb (Sigma Aldrich, UK) was dissolved in DMEM media at a temperature below 0°C and in a sterile environment, as hyaluronic acid (HA) is not soluble at high temperatures. The solution was added to 500ml of media to make 0.1mg/ml and stored at 4°C for further use.

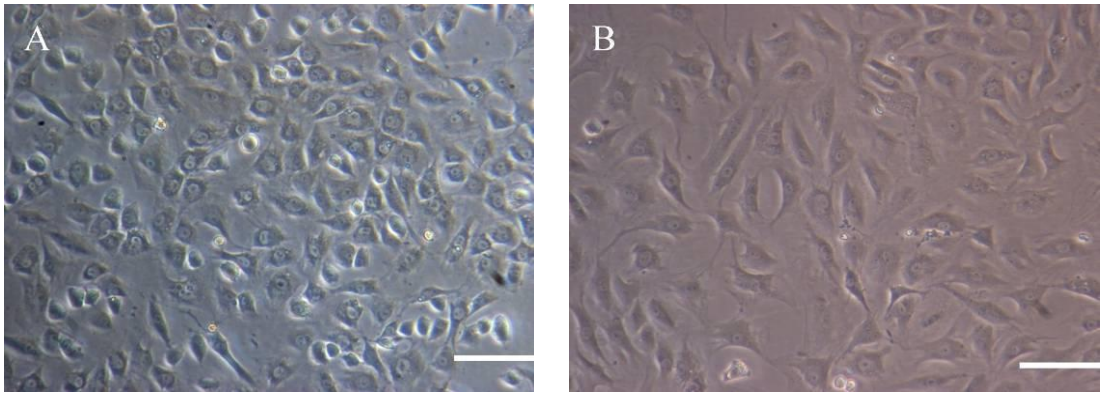
20×10^4 per millilitre rat's primary chondrocytes were cultured in DMEM media without and with 0.1 mg/ml of hyaluronic acid supplementation and incubated at 37˚C. After 24 hours, both control, and HA contained cell cultures were imaged, using a

phase-contrast microscope with 10x magnification. For further analysis both cultures were fed with new media and incubated again until further 48 hours.

After 72 hours chondrocytes from both cultures were detached using 0.25% trypsin-EDTA (Sigma Aldrich, UK). The cells were centrifuged at 17000 rpm, and each pellet was resuspended in 5 ml BSA separately for evaluation of proliferation, and cell viability assay. During the course of each experiment, cell morphology and at the end of each experiment, proliferation rate, and cell viability tests were carried out using a phase-contrast microscope and haemocytometer.

3.4.2 Results and Discussion

Figure 3.10 shows the effect of HA on chondrocyte morphology and some characteristics of monolayer culture, such as cell aggregation, flat, round with less fibroblast-like shape. The control culture shows typical fibroblast-like morphology of chondrocytes in media without HA supplementation (Figure 3.10, B and D).



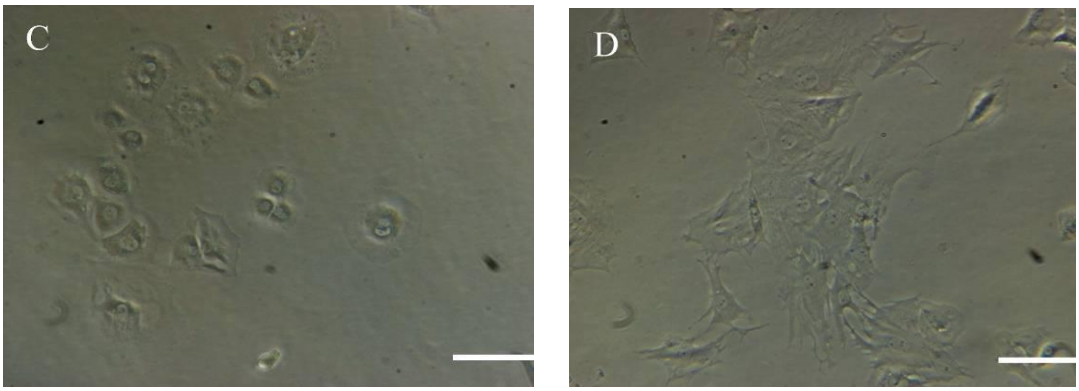


Figure 3.10 A) and C) chondrocytes cultured in medium supplemented with 0.1 mg/ml HA solution; B) and D) chondrocytes in media without HA. (Scale bar = 100 µm).

Hyaluronic acid stimulated the primary chondrocytes to proliferate up to 4.08 fold compared to proliferation rate of control culture being only 3.1 fold (Figure 3.11).

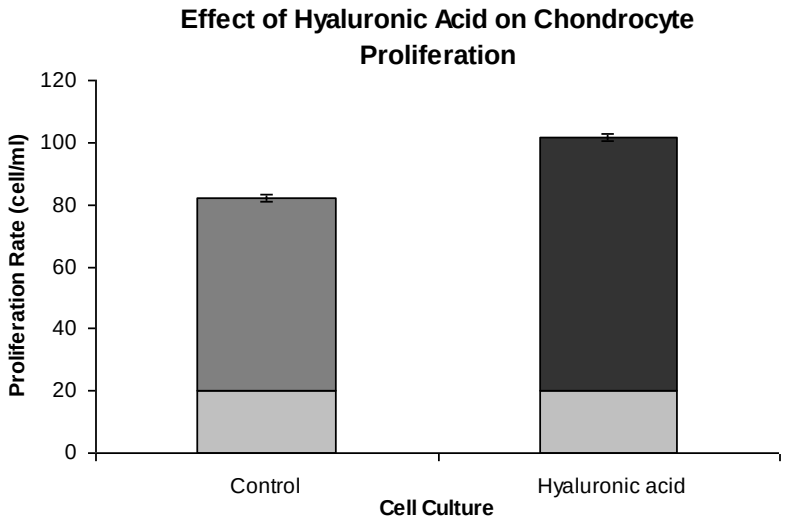


Figure 3.11 Graph showing comparison for chondrocyte cell proliferation in mediums with and without hyaluronic acid.

The proliferation rate of control culture was 372% whereas this rate for HA contained media was 408%. The significant level for rejection of null hypothesis with assumption of equal variance was set as 0.05 in t-test, and the probability of this assumption was less than 0.05 ($p = 2.8 \times 10^{-10} < 0.05$). This meant that the null assumption was rejected.

Monolayer culture of primary chondrocyte cells in a medium with 0.1% hyaluronic acid supplementation increased the proliferation rate, and induced the cells to become more rounded which is close to the cell shape at differentiation stage.

3.5 Culture of Chondrocytes in an Antibiotic-Free (Penicillin and Streptomycin) Environment

Though antibiotics are used to eliminate or control bacterial and fungal contamination in cell culture, it is, however, well known that they negatively affect cell viability (Martinez-Liarte et al., 2007) and cell-membrane synthesis and can modify cell behaviour by interfering with protein synthesis (Cohen et al., 2006). Thus, it was of useful to develop a method of culturing cells in antibiotic-free media. Culturing primary cells in antibiotic-free media is particularly difficult, largely because the extraction of the tissue can often compromise sterility.

The chondrocytes were isolated from the articular cartilage of a Sprague-Dawley rat between one and five days old (the protocol of chondrocyte isolation was described in

Chapter 2). Extra care had to be taken to ensure the sterility of the dissection tools and of the neonate rat.

The aim of this work was to evaluate the effect of Penicillin/Streptomycin on primary chondrocyte cell.

The chondrocytes at 100,000cell/ml was cultured in DMEM media with and without penicillin/streptomycin supplementation and then cell morphology and proliferation rate were investigated over a five-day period.

3.5.1 Results and Discussion

Antibiotics are used to arrest free cell growth while allowing the synthesis and excretion of the products into the medium. Also, Novobiocin (another antibiotic used in cell culture), at concentrations of 100 U/ml, stops free cell growth (Israilides et al., 1989).

The time to reach the confluency level for the culture in antibiotic-free media was shorter than that for cells cultured in media containing antibiotics. Figure 3.12 shows photomicrographs of chondrocytes cultured in both antibiotic-supplemented and antibiotic-free media.

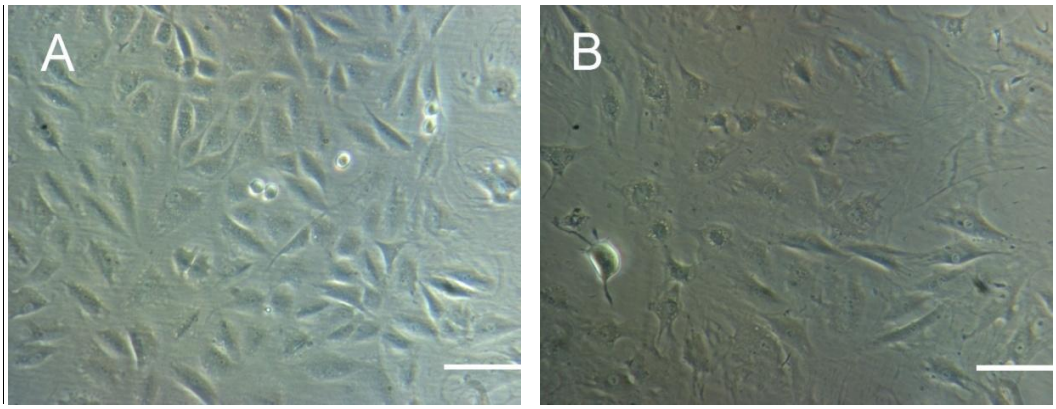


Figure 3.12 a) High cellularity of Chondrocytes cultured in antibiotic-free media; b) in media supplemented with antibiotics (x10 Magnification, Scale bar = 50 µm).

The cell viability, morphology and especially distribution in the antibiotic-free medium were optically compared to the cells cultured in the medium containing antibiotics.

As mentioned earlier, penicillin and streptomycin are widely used antibiotics for cell culture, but, they have a negative effect on cell viability (Martinez-Liarte et al., 2007) via interfering with DNA replication by inhibiting DNA polymerase alpha (Do et al., 1987) as can be seen in Figure 3.12.

3.6 Overall Results and Discussion

Primary chondrocyte cells cultured as monolayer showed dedifferentiated phenotype by expression of stress fibres and fibroblast like morphology. This characteristic was prevented / reduced by culture of chondrocytes in high cell density.

The examination of cell growth in a 3D culture system resulted in some unusual findings. The chondrocyte cells started to grow in size and secrete fibrous extracellular matrix-like material after three days of pellet culture. They produced fibrils and continuously increased in their thickness during the culture procedure, as shown in Figure 3.6. After three weeks of pellet culture, more than 93% cell viability was determined by trypan blue. However, when transferred to a monolayer culture system, the cells delayed to attach, suggesting that the production of the integrins required for cell surface attachment was slowed down. This result indicated that close packing of chondrocytes in a pellet (3D environment) provides insufficient signals to allow the cells to develop their full function and those additional signals, possibly associated with exposure to ECM molecules or cytokines may be required before they acquire their full functionality. Based on the qualitative evidence acquired in this study, it seemed that the chondrocytes in the pellet secreted their own ECM, but failed to interact with it. This

suggests that exposure to ECM alone in a 3D (pellet) environment prevents cells gaining their full functionality and hints at a potential role for other signalling molecules such as cytokine.

The proliferation of cells cultured in high-glucose DMEM media supplemented with hyaluronic acid (HA) was much higher than that in media without HA supplementation. This result confirmed the inhibitory effect of hyaluronic acid on chondrocyte apoptosis (Takahashi et al., 2000).

Also, the morphology of cells grown in HA media showed that they were rounded and flattened and in small clusters of four to six cells with a chondrocytic phenotype, easily visible under a light microscope. This meant that hyaluronic acid had a negative effect on co-cultured fibroblasts and reduced the synthesis of type-1 collagen which is highly expressed by fibroblasts (Akmal et al., 2005).

The results obtained from chondrocyte culture in antibiotic-free media showed that the proliferation rate of chondrocytes was clearly higher than with chondrocyte cultured in media with antibiotics supplementation. This result suggests that the routine use of antibiotics in primary cell culture should be avoided as it reduces the efficiency of the culture system (Figure 3.12).

The set of experiments above and related results revealed that the optimal conditions for culture of primary chondrocyte is pellet culture in high-glucose media with 10% FCS, 0.1% w/v hyaluronic acid and a low level of antibiotic supplementation.

Implementation of a chondrocyte pellet might be the most appropriate scaffold-free tissue for transplantation into a large cartilage defect and/or autologous chondrocyte implantation.

CHAPTER IV

4 Primary Chondrocyte Cell Alignment on ECM Proteins (Collagen, Fibronectin and Laminin)

4.1 Introduction

Proteins are key regulators of cell functions such as cell-cell communications, cell-cell and cell-extracellular matrix adhesion, cell division, and also regulate cellular shape and mobility (Raab et al., 2010).

Understanding the mechanisms controlling cellular function, e.g. cell adhesion, migration and proliferation, is critical for tissue engineering and regeneration as well as directing new tissue formation *ex vivo* (Heungsoo et al., 2003). As cell adhesion and migration regulate division and proliferation, an understanding of the affinity of cell adhesion to proteins is essential for tissue engineering (Rosso et al., 2003).

Cells adhere to the extracellular matrix via integrins, and prominent adhesion molecules expressed by cell at focal contacts (See Figure 4.1). Dependent on the extracellular matrix, the cell expresses various appropriate integrins that bind the cell to its ligand (Woods et al., 2007). For example, chondrocyte differentiation and proliferation require its attachment to type-II collagen-rich matrix (Terpstra et al., 2003). The cooperation of ECM molecules with growth factors and cell adhesion molecules, regulates chondrogenic differentiation and cartilage development (Yang et al., 2006).

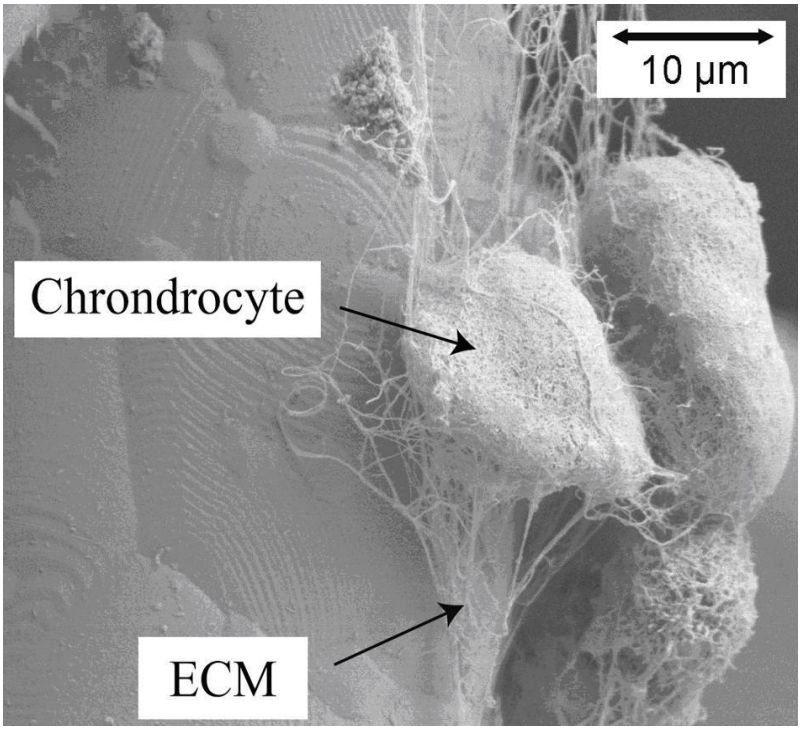


Figure 4.1 Adhesion of chondrocyte via extracellular matrix to the surface (Sobral et al., 2008).

The functionality of cell is dependent on the ECM protein surrounding the cell (Bordeleau et al., 2008). The cell produces specific integrin that can interact with related ligands. However, the affinity, or degree of alignment, is crucial to cell proliferation and migration.

In cartilage, the degree of cell alignment to the ECM plays a central role in formation of tissue, cartilage strength and resistance to mechanical stress (Vinatier et al., 2009).

Laminin, fibronectin and collagen are three prominent ECM proteins whit different integrins that bind to these macromolecules. Their secretion is altered during cell commitment and maturation and the level of expression regulates cell function and progression (Han et al., 2007).

The capability of adherent cell to sense and the response to the mechanical stresses generated at the focal adhesions (FAs) is directly dependent on integrin-mediated interaction between ECM molecules and cytoskeleton (Bordeleau et al., 2008).

Understandably, the lack of cell adhesion molecules causes a reduction in cell attachment and proliferation. For proliferation, a cell needs to come into contact with neighbouring cells and extracellular matrix molecules. By increasing the cell density, this interaction causes a reduction in the spreading of the cell, changing the morphology and decreasing proliferation, as the proliferation rate of cells with round morphology is low (Celeste and Christopher, 2002). Thus, cell seeding on a substrate imprinted with protein microarray (See Figure 4.2) can facilitate study of the degree of alignment of the cell to the patterned protein.

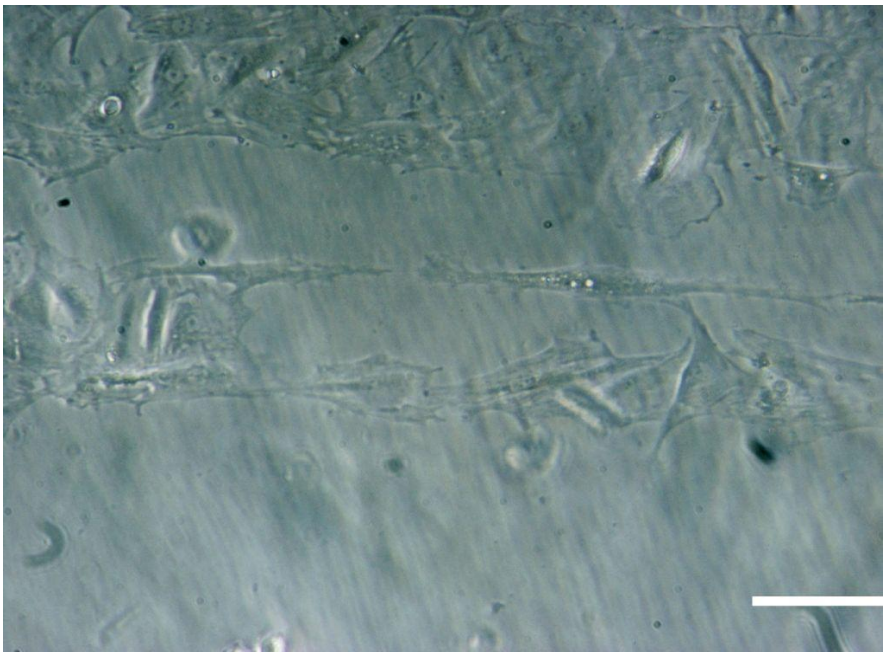


Figure 4.2 Micrograph of primary chondrocyte cells aligned to 100µm collagen pattern imprinted on gold substrate, (Scale bar = 100µm).

Protein microarray substrates contain a highly uniform monolayer of proteins that facilitate cell adhering to the protein lines on the substrate (Li et al., 2006). With this method, the affinity of the cell to adhere to the stamped protein can be studied (Lee et al., 2010).

Microcontact printing (MCP) offers a simple and low-cost surface patterning technique with the capability to form sub-0.1µm patterns of biomaterials (Perl et al., 2009).

There are various types of methodologies for the deposit of materials on surfaces, such as ultra-violet (UV) radiation micropatterning technique, plasma-induced micropatterning, soft llithography and microcontact printing (MCP) techniques (Jamil et al., 2008).

MCP is used in this experiment to deposit protein on the glass coverslips to study the effect of ECM macromolecules on adhesion of primary chondrocyte cells, isolated from the articular joint of neonate rats, to these proteins.

4.2 Materials and Methods

Knowledge of the degree of chondrocyte cell affinity to adhere to laminin, fibronectin, collagen and other cartilage matrix proteins is essential for tissue engineering and regenerative medicine. Thus, for *in vitro* investigation, cells are required to be cultured on the substrates which contain protein patterns. The degree of cell alignment was obtained from the measurement of the angle of cells aligned to the imprinted protein patterns.

For statistical analysis, data acquired from the experiments were tested for normality and then analysed by t-tests or the non-parametric equivalent tests if required. All means are quoted as ± standard error (SE).

4.3 Protocol of fabrication of polydimethylsiloxane (PDMS) stamp

PDMS stamp is a mixture of base silicon elastomer and curing agent. The conventional method for the fabrication of this stamp is based on soft-lithographic technique and rapid prototyping as well as replication modelling (McDonald et al., 1999).

These stamps for microcontact printing are made by micromachined silicon wafers. The templates, with various widths, form part of a mould in which liquid PDMS is polymerised. On demoulding, a flexible transparent stamp is obtained, with structures that can be as small as 100nm (Jamil et al., 2008).

The glass master templates were fabricated at the University of Glasgow through reactive ion etching technique, as described elsewhere (Britland et al., 1996).

Polydimethylsiloxane (PDMS) stamps were fabricated at the University of Bradford from these templates by utilising 184 Sylgard elastomer gel (Dow Corning, 2007).

For fabrication of PDMS, first 9ml of base silicon elastomer was poured in a container and then 1ml of curing agent was added to the base silicon elastomer and mixed together, (See Figure 4.3).

Figure 4.3 Photograph of silicon elastomer and curing agent.

Master stamp was cleaned by leaving in a digicel bath for 48 hours, and 10 minutes in ultrasonic bath containing 70% ethanol. The cleaned (free from any debris) master stamp was placed in a Pyrex Petri dish with a pattern in the upward position. The silicon mixture was poured on the stamp so that the silicon level was 2-3mm above the master stamp. After pouring the silicon mixture, the bobbles were removed, via degassing the mixture several times, and the degassed mixture was left at room temperature for a few days to dry. The silicon was removed carefully from the master stamp and was then cut around the pattern. A schematic PDMS casting diagram is shown in Figure 4.4.

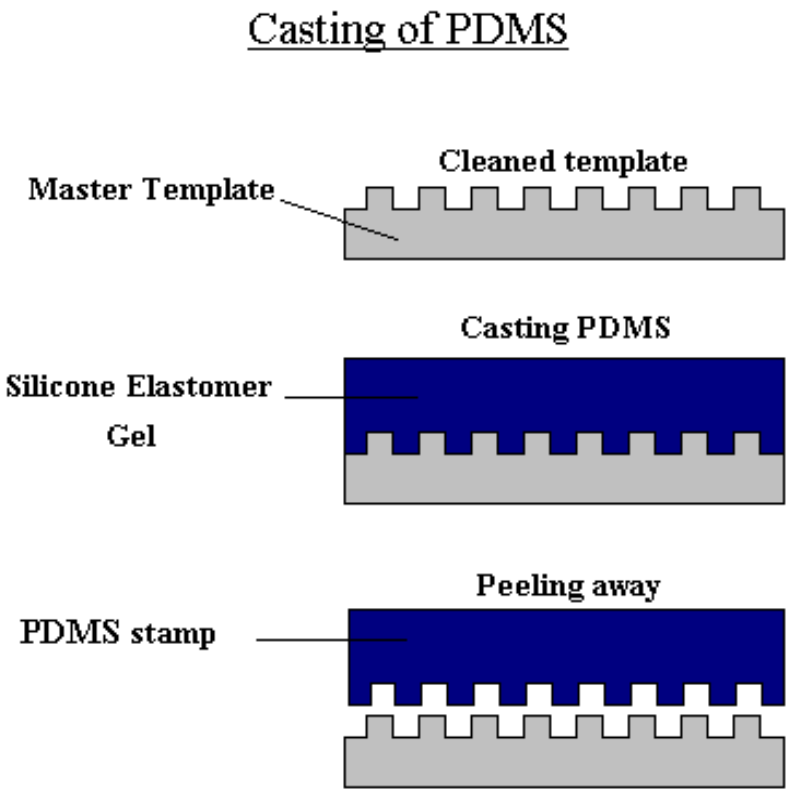


Figure 4.4 Schematic diagram showing casting of PDMS stamp.

Also, Figure 4.5 shows a PDMS stamp with 100µm patterns, fabricated for deposition of ECM proteins on glass coverslip.

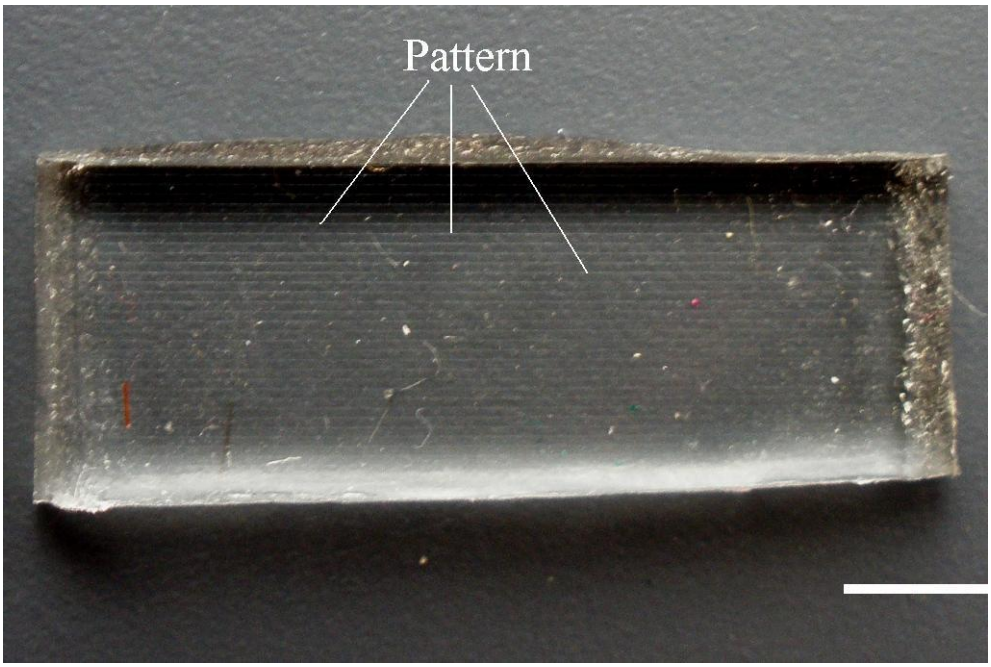


Figure 4.5 Photograph of PDMS stamp with 100 μm patterns, (Scale bar = 3 mm).

For the chondrocyte cell alignment on patterned biomolecules, different PDMS stamps were fabricated. The widths of patterns on the stamps were 5, 12.5, 25, 50 and 100μm.

4.4 Microcontact printing of ECM proteins

Microcontact printing is a very useful method of surface structuring. Patterns can be made of many different materials and on flat or curved surfaces. Repeated printing, using different stamps, can be used to make complex surface patterns of more than one kind of molecule.

The soft PDMS stamp makes conformal contact with the surface and molecules are transferred directly from the stamp to the surface in a few seconds, depending on the type and concentration of molecules (See Figure 4.6).

The prepared polydimethylsiloxane (PDMS) stamps were used for microcontact printing to deposit biomolecules/proteins on the surface of the glass coverslip (Siang et al., 2005).

Collagen type-I (Sigma Aldrich, UK), an extracellular matrix protein, was dissolved in 20mM acetic acid, diluted with Hank's balanced salt solution (HBSS) at the ratio of 1:10, according to the supplier recommendation, and stored at 4ºC for coating and stamping of coverslips.

Fibronectin from bovine plasma (Sigma Aldrich, UK), an adhesive glycoprotein, was dissolved at 1mg/ml in water and diluted with HBSS with ratio of 1:10.

Laminin from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (Sigma Aldrich, UK) was diluted with HBSS with the ratio of 1:10000 and stored at -20°C.

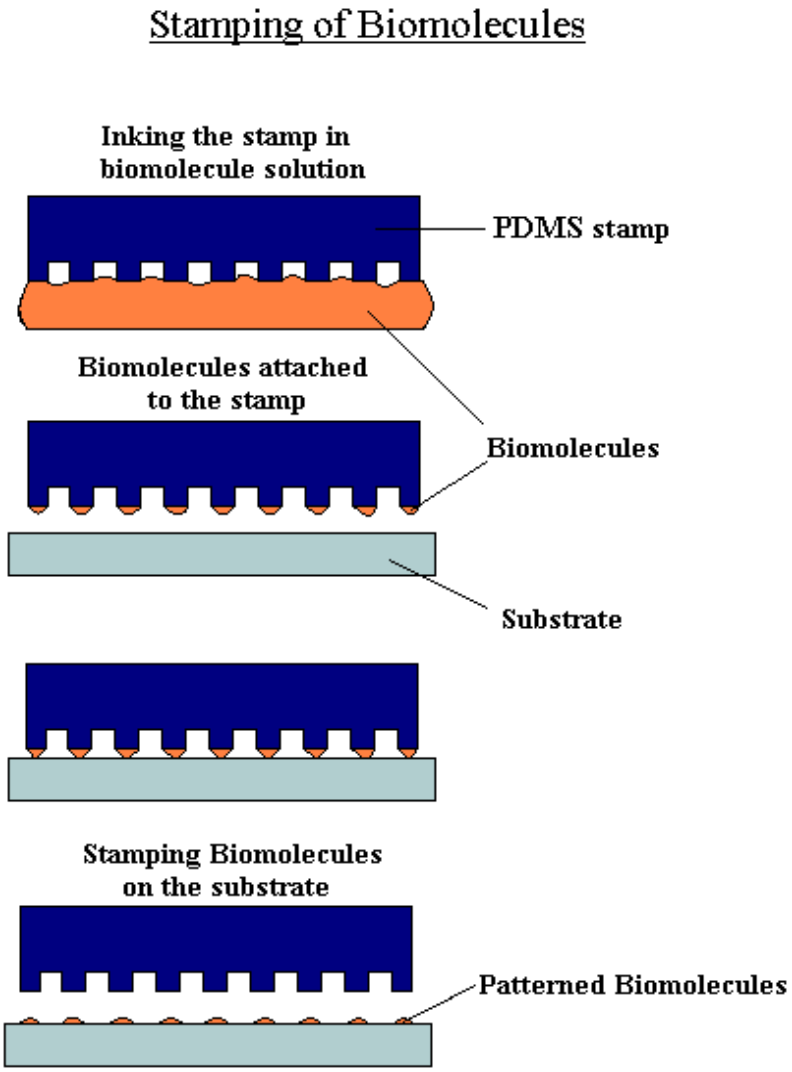


Figure 4.6 Schematic diagram of microcontact printing of biomolecules on substrate.

The stamp was first inked in a solution of extracellular matrix protein, allowing the protein to bind to the stamp and dried after imprinting. The optimal condition for attachment of proteins to the glass coverslip varies depending on the type and dilution ratio of the protein. This can vary from 1-15 minutes for patterning and from 45 minutes to a few hours for substrate coating

The level of pressure and time for the attachment and drying of the biomolecules on the substrate play a central role in microcontact printing. Figure 4.7 shows the photo micrographs of microcontact printed biomolecule with various widths on glass substrate.

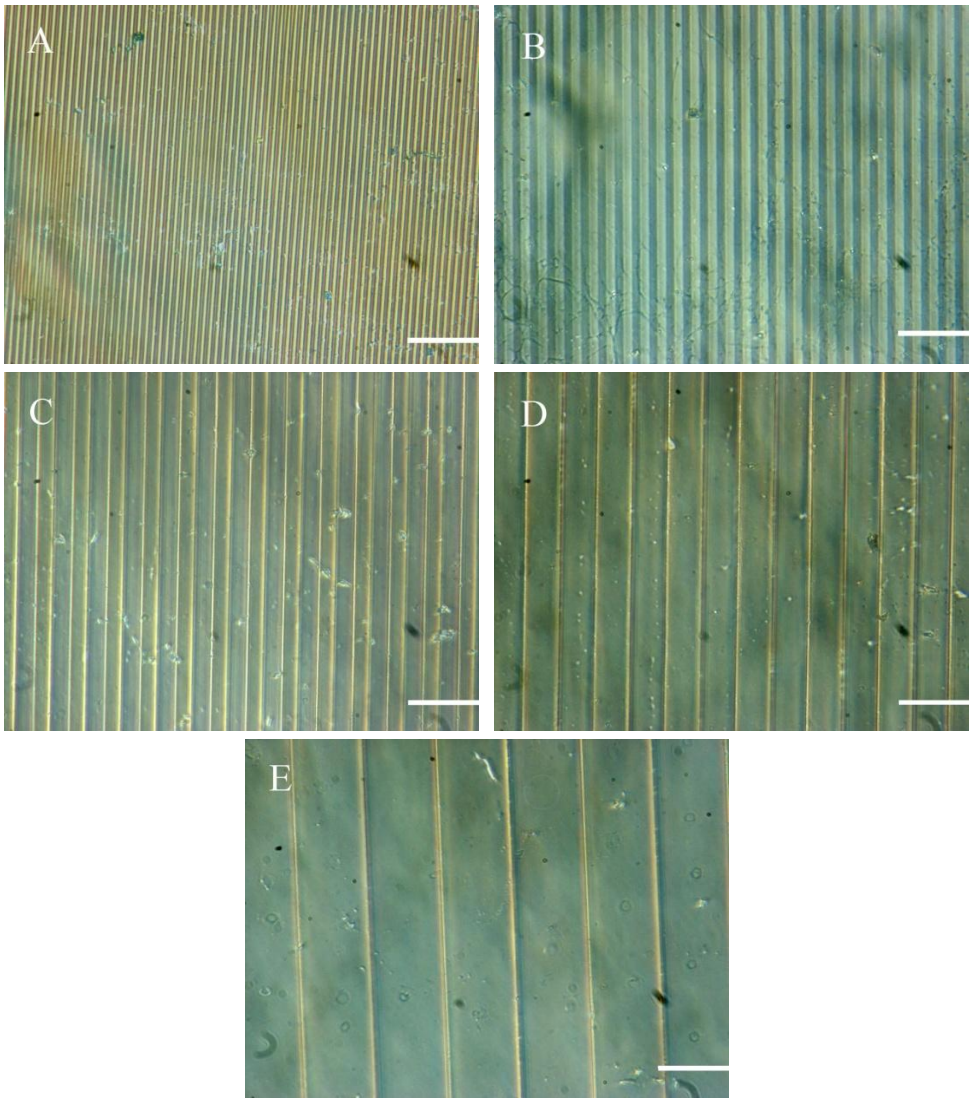


Figure 4.7 Micrographs of patterned biomolecules (collagen) on glass coverslip: A) 5µm, B) 12.5µm, C) 25µm, D) 50µm, E) 100µm PDMS obtained by light microscope with 10 x magnification, (Scale bar = 100 µm).

4.5 Chondrocyte cell alignment

The use of tissue engineering in the treatment of cartilage injuries is in its infancy. To understand how chondrocyte cells behave in the wound environment requires *in vitro* experiments designed to characterise chondrocyte responses to model-guidance cues. This study examined the alignment and spreading of chondrocyte cells derived from 1-5 day-old Sprague-Dawley neonate rats on 5, 12.5, 25, 50 and 100µm wide repeat grating of micro contact-printed fibronectin, laminin and collagen type-1.

4.5.1 Methodology

Primary chondrocyte cells were derived from the digestion of extracted articular cartilage of Sprague-Dawley rats and plated on the patterned substrate with a concentration of 5000 cells/cm². In this experiment five patterned glass coverslips, of 5, 12.5, 25, 50 and 100µm width, were used for each protein. 150 randomly selected cells were subjected to study the effect of these patterned proteins on chondrocyte cell alignment and morphology was examined by using Image-J software and measuring the angle between the line of patterns and cell-elongation direction. The closer this angle is to zero, the better the cells aligned to the pattern. Figures 4.8 - 4.10 show chondrocyte cells alignment for laminin, fibronectin and collagen type-I, respectively.

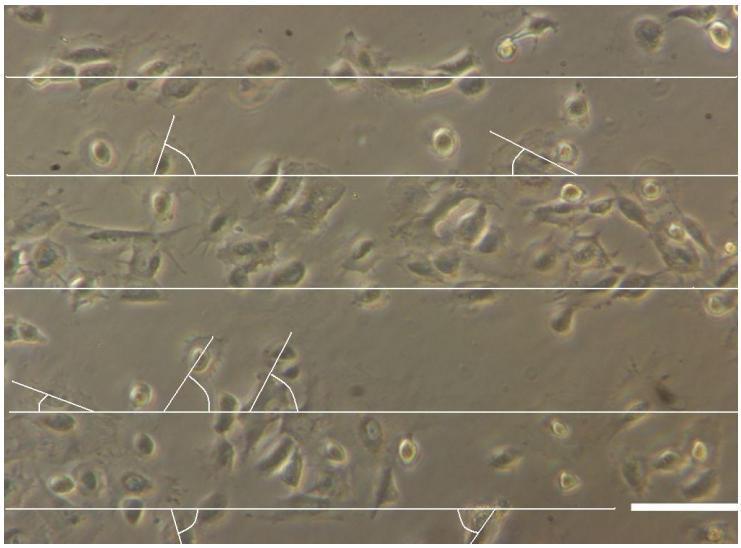


Figure 4.8 Photomicrograph of primary chondrocyte cells on 100µm imprinted laminin pattern, (Scale bar = 100 µm).

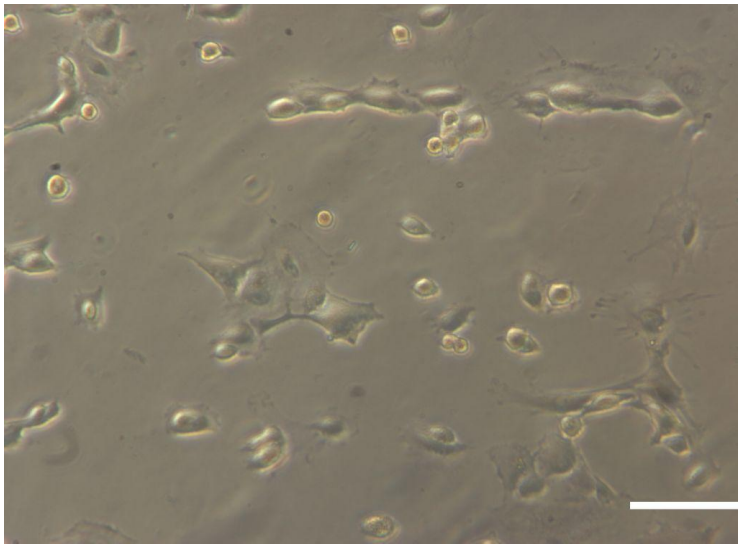


Figure 4.8.1 Photomicrograph of primary chondrocyte cells on 50µm imprinted laminin pattern, (Scale bar = 100 µm).

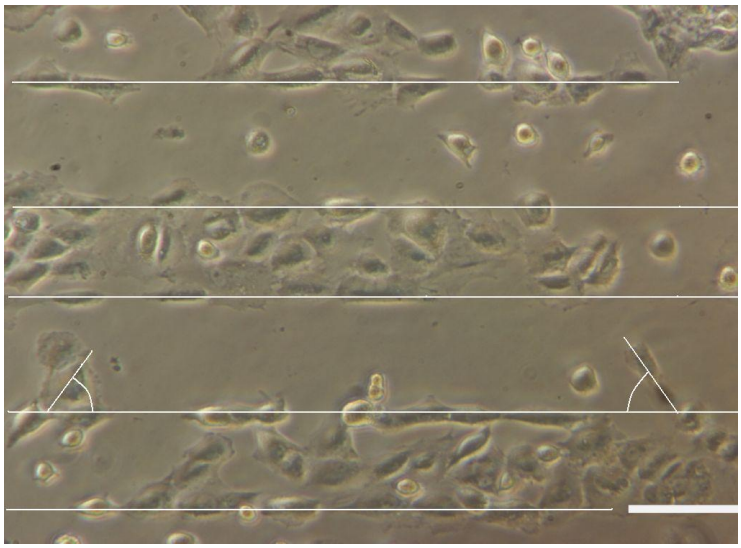


Figure 4.9 Photomicrograph of chondrocyte cells on 100µm fibronectin stamped pattern, (Scale bar = 100 µm).

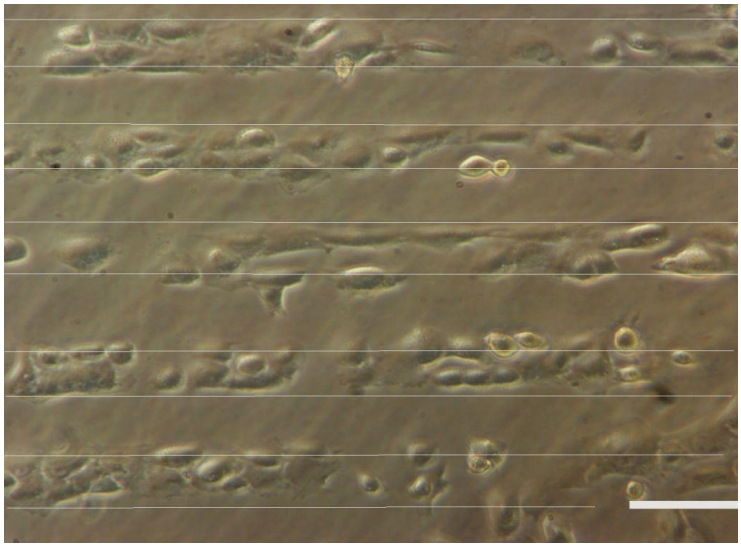


Figure 4.9.1 Photomicrograph of chondrocyte cells aligned to 50µm fibronectin stamped pattern, (Scale bar = 100µm).

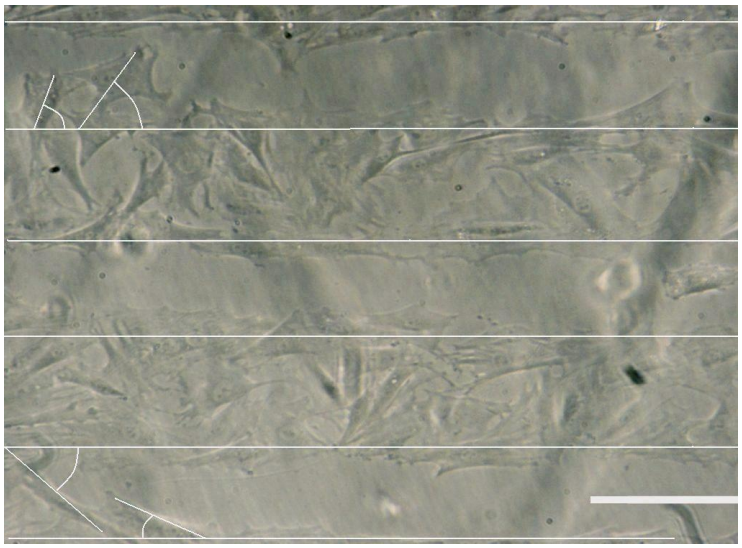


Figure 4.10 Photomicrograph of primary chondrocyte cells on 100µm type-I collagen pattern, (Scale bar = 100µm).

24 hours after cell seeding the non-attached cells were removed and counted by haemocytometer. The shiny, non-attached cells had a rounded shape in the cultures, as can be seen in Figures 4.8 - 4.9.

The experiments were repeated five times and the obtained data were used for further analysis.

4.5.2 Statistical Analysis

For statistical analysis, data acquired from the experiments were test by normality and then analysed by ANOVA-test. All means are quoted ± standard error (SE).

4.6 Results and Discussion

To assess purified chondrocyte behaviour in a planar culture system, cells were cultured on 20x20mm glass coverslips. The substrates were stamped with three different extracellular matrix proteins (collagen, fibronectin and laminin), with various pattern sizes of 5, 12.5, 25, 50, and 100µm, to determine the degree of alignment of the chondrocytes to these proteins. After 24 and 48 hours in culture it was found using Image-J software and linear optimisation, that chondrocytes aligned most readily to the 50µm fibronectin (mean angle of alignment = 1.06º ± 0.7 SE), as shown in Figure 4.11.

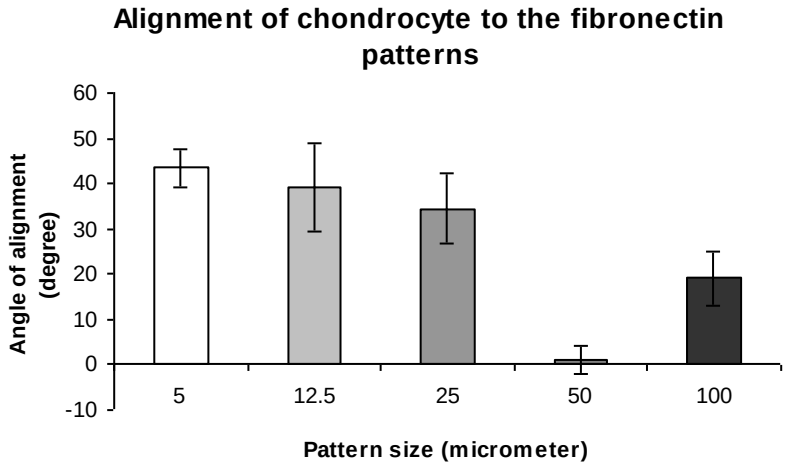


Figure 4.11 Graph of angle of chondrocyte alignment versus pattern size for fibronectin with standard error bar.

However, the graph of chondrocyte alignment to the fibronectin patterns (Figure 4.11) also shows that there was almost no alignment to the 5, 12.5 and 25µm fibronectin patterns. The biggest angle of alignment to the fibronectin was measured on the 5 µm pattern size, with a mean angle of 43.3° ± 4.05 SE.

The highest degree of alignment to collagen patterns was observed with chondrocytes grown on the 100µm-wide type-I collagen pattern (mean angle = 4.84° ± 2.45 SE) shown in Figures 4.10 and 4.12.

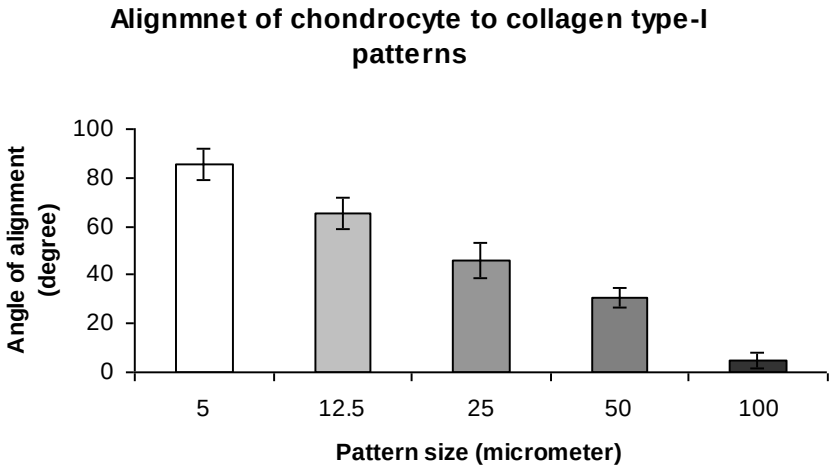


Figure 4.12 Graph of angle of chondrocyte alignment versus pattern size for collagen with standard error bar.

The lowest cell alignment to the collagen patterns was observed on the smallest pattern size (5 µm) with the mean angle = 85.2º ± 6.4 SE. Interestingly, the size of collagen

pattern had a positive relationship with the chondrocyte cell alignment and consequently a negative relationship to the angle of alignment (Figure 4.12).

The 50µm pattern size showed the highest chondrocyte cell alignment to the imprinted laminin patterns on the substrates with the mean angle = 23.4º ± 3.14. The lowest degree of all alignment on the stamped pattern with laminin was on the 5µm and 10 µm width with the mean angle = 40.6º ± 3.14 SE, and 40.6º ± 5.3 SE respectively, as shown in Figure 4.13.

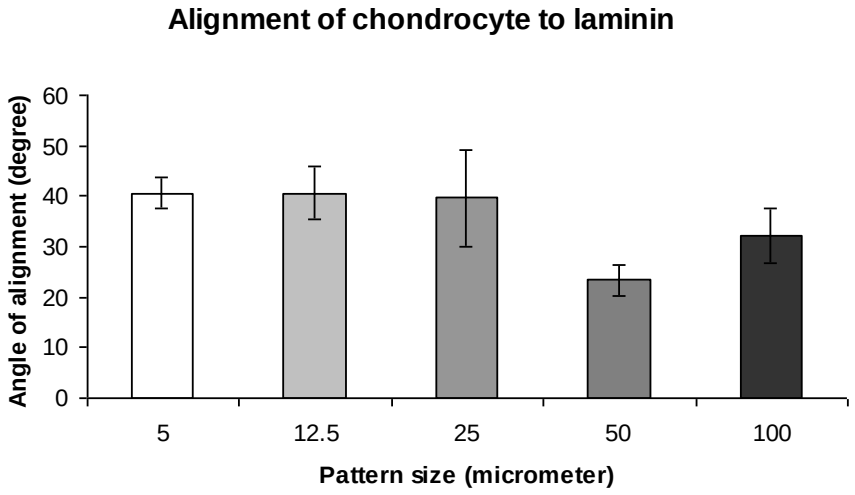


Figure 4.13 Graph of angle of alignment versus pattern size for laminin with standard error bar.

The mean size (Cell length) of the chondrocytes were 27.95µm ± 1.37 µm SE on laminin MCP patterns, 41.01µm ± 3.17 µm SE on collagen patterns, and 28.07µm ± 2.05 µm SE on fibronectin patterns. Also, the null hypothesis using ANOVA test (F

=10.40, df = 2, p= 0.0004, F crit = 3.35) indicated that the chondrocyte cell size was influenced by coating of substrate with collagen, laminin and fibronectin ECM proteins. The chondrocytes on laminin and fibronectin patterns were measurably small in size and rounded. However, on substrate with collagen patterns, they were elongated with fibroblast-like morphology. The best degree of alignment was measured from fibronectin with pattern size of 50μm and collagen 100μm (Figures 4.8- 4.10).

CHAPTER V

5 Effect of Transforming Growth Factor β-1, 2, 3 and their Manipulated Forms on Biological Regulation of Primary Chondrocyte

5.0 Introduction with Aims and Objectives

Cartilage is an avascular tissue (Vincent et al., 2002) with oxygen and nutrients delivered via the synovial fluid (Lauge-Pedersen and Aspenberg, 2003). This is believed to contribute to the poor repair characteristic of (Humphries et al., 2003) cartilage (Erickson et al., 2002). Small lesions in the cartilage can cause severe problems which can affect patient's mobility (Mithoefer et al., 2006). In order to understand how cartilage injuries can be treated *in vivo* (Eltawil et al., 2009), chondrocyte behaviour in relation to model wounds (Bos et al., 2007) in the presence of various cell stimulators (Arend, 2001) such as different growth factors, can be examined *in vitro.*

As described in Chapter 1.6 cytokines are extremely potent biomolecules that regulate cellular functions and play multiple roles in initiation and inhibition of disease. Transforming growth factor-beta (TGF-β) superfamily is a group of pleiotropic cytokines with high molecular weight of 25kD homodimeric peptide (Lawrence, 2001). These highly specialised macromolecules are actively involved in control of cellular proliferation, apoptosis, cell migration and adhesion. TGF-β1, 2, 3, 4 and 5 are involved

in signal transduction between the extracellular environment and the nucleus. Three of these cytokines are potent regulators in cellular development (Raftery and Sutherland, 2002).

The preliminary reports about transforming growth factor beta began with three papers, one published in 1978 and two in 1981 (Derynck and Miyazono, 2008). They described partial purification of polypeptide growth factor secreted by fibroblasts transformed by RNA viruses (Moses and Roberts, 2007), which they called sarcoma growth factor (SGF) (Anzano et al., 1983).

Transforming growth factor-betas are produced by almost all cell types in inactive/latent form (SchwartzBonewald et al., 1993), suggesting that they play a regulatory role in most tissues. Although the function of the TGF-βs in various cell types has been investigated, their function in cartilage repair is as yet not fully understood. Although some researches revealed the effect of transforming growth factor on chondrocyte, the majority of these studies and comments are not related to wound repair of cartilage. The TGF-β superfamily consists of various types of polypeptides effecting up and down-regulation of membrane proteins (Spagnoli et al., 2007) and consequently inducing cell adhesion, proliferation, differentiation, activation, migration and apoptosis (Krauss, 2006). Stimulation by TGF-β1 of the synthesis of cartilaginous matrix suggested (Hickery et al., 2003), (Shah et al., 1999) that this type of growth factor may play an important role in cartilage repair by increasing cell proliferation rates, cell migration and cellular adhesion. This may indicate how cartilage repair is initiated and how this could be accelerated by transforming growth factor-beta1 (Bos et al., 2007). It has also been reported that chondrocytes can be induced to obtain a chondrocytic morphology by

seeding at high cell density (Bashey et al., 2006) and a fibroblastic morphology by culturing in low density after their isolation (Martin et al., 2001).

Members of transforming growth factor-β have been implicated in the morphogenetic transition from scarless fetal repair to adult skin repair with scarring (Rolfe et al., 2007). Fibromodulin, a transforming growth factor-β modulator which is a member of the small leucine-rich proteoglycan family, has been suggested as a biologically significant mediator of fetal scarless repair (Stoff et al., 2007). There is even evidence that fibromodulin may be a biologically relevant modulator of TGF-β activity during scar formation (Soo et al., 2000).

The effect of some cytokines, such as interleukin (IL), transforming growth factor-beta (TGF-β) and bone morphogenic protein (BMP), in the regulation of some other cartilage-related proteins has already been studied (Joosten et al., 2004). However, the effect of these factors in the biological regulation of chondrocyte, proliferation, cell size, cell adhesion and wound repair are still not clearly reported investigated and understood.

Some of these growth factors, such as TGF-β1, are involved in the repair of scarless mature cutaneous tissue (Rolfe et al., 2007). However, there is very little evaluation of the effect of transforming growth factor-betas and their manipulated form on chondrocyte cell proliferation and cartilage wound repair.

Chondrocytes of articular cartilage produce and maintain a matrix consisting of aggregating macromolecules, collagen fibres and 80% water (Balassa, 1987). The effect of some cytokines, such as interleukin (IL), transforming growth factor-beta (TGF-β)

and bone morphogenic protein (BMP), in the regulation of some other cartilage-related proteins has already been studied (Joosten et al., 2004). However, the effect of these factors in the biological regulation of chondrocyte, proliferation, cell size, cell adhesion and wound repair are still not clearly reported investigated and understood.

The limitation of the repair capacity of cartilage is directly related to chondrocyte cell and the adhesion, proliferation and migration of chondrocyte into the wound site. Some of these growth factors, such as TGF-β1, are involved in the repair of scarless mature cutaneous tissue (Rolfe et al., 2007). However, there is very little evaluation of the effect of transforming growth factor-betas and their manipulated form on chondrocyte cell proliferation and cartilage wound repair.

The ability of chondrocyte to produce a correct matrix is inherent (Schuurman et al., 2009), but is also affected by other influences such as mechanical stress (Byers et al., 2008), hormones and specific proteins and growth factors (Maitre et al., 2007).

Although the healing of cartilage can be controlled by gene expression, but also the repair process is controlled by adhesion of chondrocyte to the ECM (Woods et al., 2007). Adhesion of chondrocyte to the ECM (Scaffold), a collagen type-II rich matrix, is dependent on cell adhesion molecules (CAMs) and integrins and their cytoplasmic effectors such as integrin-linked kinase (ILK) (Terpstra et al., 2003). The interaction between chondrocyte and ECM is mediated by integrin linked kinase, scaffolding, and integrins that are recruited to focal adhesions (Grashoff et al., 2003).

The molecule of integrin is composed of two transmembrane glycoprotein subunits called α and β subunit, as can be seen from Figure 5.0.1.

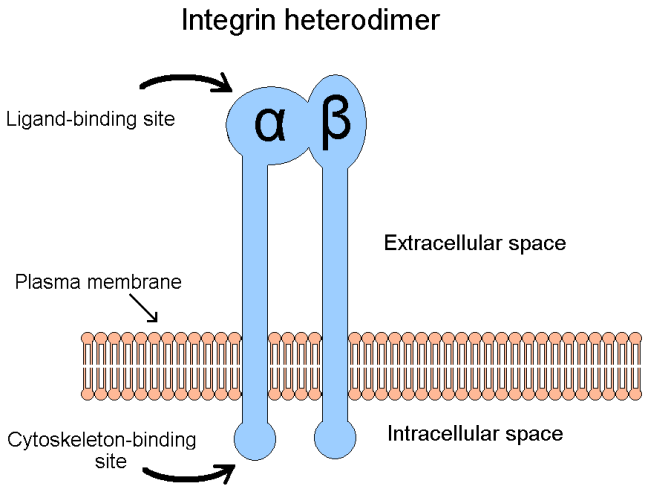


Figure 5.0.1 Schematic diagram of integrin heterodimer (Humphries et al., 2003).

A variety of 24 α-subunits and 9 β-subunits form human heterodimer integrins (Albetrs et al., 1983). Chondrocyte cells express several integrins (Ostergaard et al., 1998) that serve as receptors for fibronectin (α5β1), collagen (α1β1, α2β1, α10β1), and laminin (α6β1) (Loeser, 2000).

Integrin β1, also known as CD29 (Sigma Aldrich, UK), is a 130kD transmembrane glycoprotein that forms receptors with α subunit, and interact with ECM proteins (Shakibaei et al., 2008). Deficiency of integrin β1 (CD29) or blocking of any subunits of integrin inhibits the cell migration and spreading, and consequently causing degeneration of cartilage or decrease in matrix formation (Spiteri et al., 2010). This deficiency or lack of integrin β1 leads to various severities in cartilage, and abnormalities in joints such as chondroplasia in infants and babies (Raducanu et al., 2009).

This work, therefore, investigates the effect of transforming growth factor-beta 1, 2, 3 and their manipulated forms on the biological regulation of chondrocyte and the repair of a created model wound on a planar culture system. Also the effect of this cytokine on expression of chondrocyte specific proteins such as collagen type-I and type-II, chondroitin sulfate proteoglycon (CSPG), S-100 protein, fibronectin and laminin and up- or down-regulation of integrin β1 (CD29) would be investigated.

CHAPTER V

5.1 Transforming Growth Factor β-1

5.1.1 Materials and Methods

5.1.1.1 Cell Culture

Epiphyseal plates were carefully separated from the end of both tibias and femurs derived from 5 days old Sprague-Dawley rats. Tissue was then immersed in 4ml of 0.25% trypsin and 2g per litre EDTA (Sigma Aldrich, UK) and stirred for 15 minutes at 37ºC. The supernatant was aspirated after 15 minutes and transferred into a 15ml centrifuge tube. Trypsin was deactivated by adding 4 ml of 10% FCS contained in DMEM media. The process of digestion by trypsin was repeated three times. The aspirated supernatants and epiphyseal plates were centrifuged at 2000 rpm and the obtained pellet was immersed in 4ml of 0.1% collagenase type-I solution (Sigma Aldrich, UK) for 90 minutes. Following treatment with collagenase type -1 solution the supernatant was mixed with 4ml of FCS containing cell culture media and re-centrifuged at 2000 rpm. The supernatant was discarded and the obtained pellet was resuspended with 5 ml chondrocyte culture media, seeded in a 25 cm^2 tissue culture flask and incubated at 37ºC. After 24 hours the epiphyseal plates and non-attached cells were discarded, fresh media was added to the cell culture and incubated at 37ºC until 70-80% confluency.

To obtain the chondrocytes with chondrocytic morphology the cells were resuspended at cell density of 250,000 cells/ml in 10ml high glucose DMEM (Dulbecco's modified eagles medium) with 10% FCS supplementation and 0.1 mg/ml hyaluronic acid (Sigma Aldrich, UK). Five millilitres of cell suspension were transferred into a 25cm² TC grade falcon cell culture flask.

To acquire chondrocytes with a fibroblast like phenotype 1ml of cell suspension was added to 4 ml chondrocyte culture media to achieve a cell density of 50,000 cells/ml. Both cultures were incubated at 37°C until 70-80% confluency.

5.1.1.2 Cell Proliferation

The effect of TGF-β1 on proliferation rate of chondrocyte cells with both fibroblast like, and chondrocytic morphologies was assessed. The initial chondrocyte cell density for fibroblast like morphology was 50,000 cell/ml and for chondrocytic cells was 250,000 cell/ml. After 72 hours in culture cells were typsinized and counted using a haemocytometer and these cell densities were compared with the initial plating densities. The experiment was repeated three times and the mean proliferation rate (±SE) were calculated.

5.1.1.3 Analysis of Cell Size (Cell Length)

From each cell culture two subcultures were obtained one was supplemented with a bathing concentration of 10 ng/ml (Dan-ning and McCormick, 2005) TGF-β1 (Sigma Aldrich, UK). Non-attached cells were removed after 24 hours and the media was

subsequently renewed every 48 hours. Time related changes in cell length were determined every two days via the acquisition of photomicrographs and measurement using Image J software (NIH) for 150 control and TGF-β1 treated cells. The experiment was repeated 3 times and the mean cell lengths at each time point (±SE) were calculated.

5.1.1.4 Immunocytochemistry

Immunocytochemistry was used to evaluate the effect of TGF-β1 on antibody localization of collagen type-I, collagen type-II and fibronectin. Monoclonal anti-collagen type-I (mouse IgG1 isotype), monoclonal anti-collagen type-IIA1 (mouse IgG1) and monoclonal anti-fibronectin antibodies (Sigma Aldrich, UK) were used as primary antibodies and goat anti-mouse IgG, conjugated to Alexa Fluor 488 SFX Kits (Invitrogen, UK) as secondary antibody.

5.1.1.5 Wound Closure Assay

The capacity of cartilage to repair in the presence of TGF-β1 was assessed by means of creating a wound via scratching a confluent chondrocyte monolayer. Both control and TGF-β1 supplemented monolayers were scratched using the tip of a sterilized 3ml fine tip extended transfer pipette with 1mm tip diameter. The experiments were carried out for both fibroblast like and chondrocytic morphologies. The scratched areas were imaged in 10 different places by phase contrast microscope at 10x magnification every 2hrs to determine the average width of the wound over a period of 72 hours.

5.1.1.6 Cell Adhesion Analysis

Chondrocyte cell surface attachment in both control and TGF-β1 contained environments was assessed at room temperature using a trypsinization assay. Trypsin is a temperature dependant enzyme (Kuzmina and Pervushina, 2004) which stimulates integrin dependent cell adhesion (Miyata et al., 2000) and digests proteins by cleaving the peptide chains at the carboxyl side of amino acids (Olsen et al., 2004). This effect causes breakage of cell-cell and cell-extracellular environment adhesion releasing the cells from the culture flask and/or dissociation from each other. The time taken for detachment of the chondrocytes from the surface allows evaluation of the strength of cell attached to the extracellular environment.

In this case primary chondrocyte cells grown in cell culture medium with and without TGF-β1 supplementation were rinsed 3 times with Hank's Balanced Salt Solution (HBSS) prior to trypsinization and then the cells were removed by adding 2ml of 0.25% Trypsin-EDTA (Sigma Aldrich, UK) to the culture flask. As soon as trypsin was added to the culture flask, the cells started to round up. A sequence of 45 images at 10 second intervals was taken during trypsinization using a digital camera connected to the phase contrast microscope and related software. The cell detachment rate was measured by determining the time taken for all chondrocyte cells to round up.

5.1.1.7 Statistical Analyses

Data acquired from the experiments in this study were tested for normality and then analysed by t-tests or the non-parametric equivalent tests if required. All means in this study are quoted ± standard error (SE).

5.1.2 Results and Discussion

Cell Proliferation: The experiments examining the effect of TGF-β1 on chondrocyte proliferation showed that the proliferation rate of the cells in medium with TGF-β1 supplementation was noticeably lower than that seen in control cultures (See Table 5.1.1). This effect was comparable for both chondrocytic phenotypes and indicates that TGF-β1 controls the production of chondrocytes by decreasing cell proliferation *in vitro*. The proliferation rate of chondrocytes without TGF-β1 supplementation was higher than those cultured with TGF-β1. The average cell number after 72 hours in the control cultures of fibroblastic chondrocytes increased to 152,667±15,147 cells/ml, whereas treatment with TGF-β1 for 72 hours resulted in cell numbers increasing to only 94,000±10,412 cells/ml. It was also found that TGF-β1 treatment for 72 hours induced the fibroblastic chondrocytes to take on a much more flattened and spread morphology. This enabled the cells to cover more of the surface without large increases in cell number (See Figures 5.1.1, and 5.1.2). This change in cell morphology was also seen in the chondrocytic chondrocyte cells, where TGF-β1 also reduced cell proliferation by approximately 1/3 and promoted a de-differentiation into a more fibroblastic morphology.

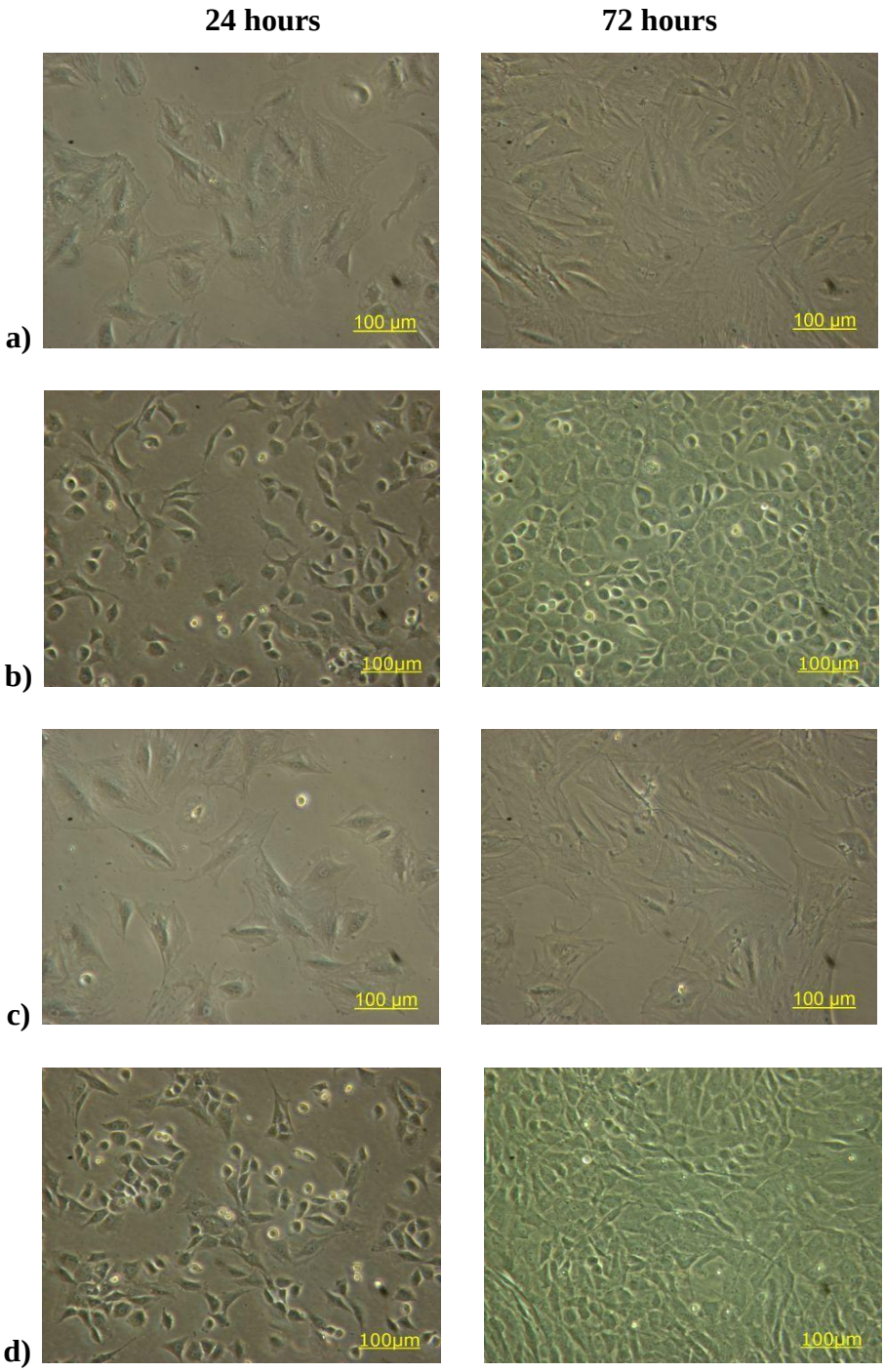
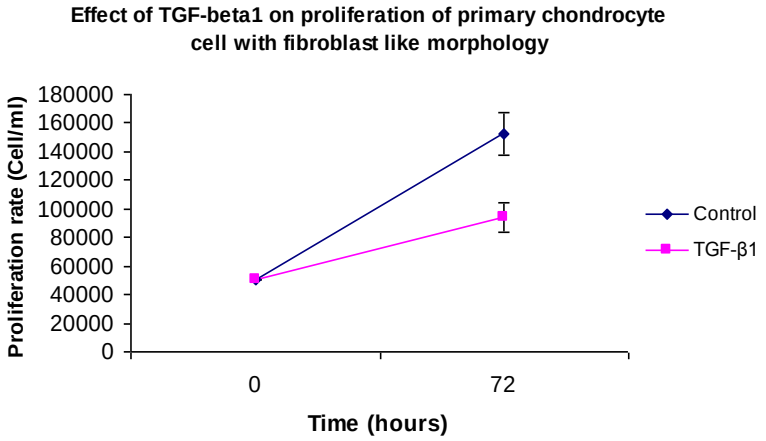


Figure 5.1.1 Chondrocyte cells cultured in monolayer without TGF-β1 supplementation: a) fibroblastic, and b) chondrocytic phenotype; Chondrocyte cells with 10 ng/ml TGF-β1 supplementation: c) fibroblastic, and d) chondrocytic morphology.

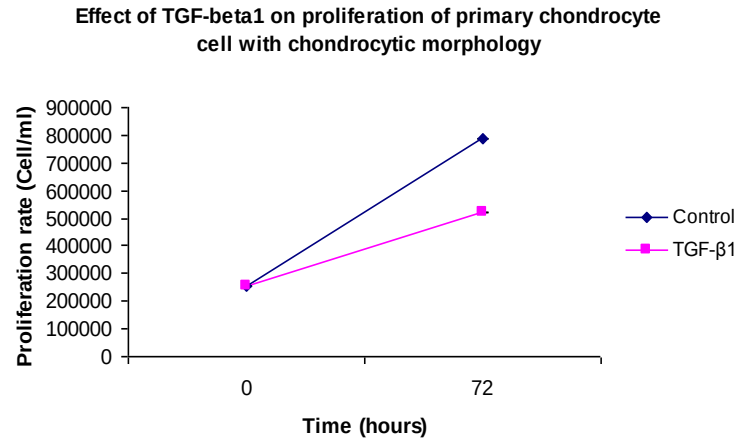
Fibroblastic morphology	Initial cell number (Plotting density)	Mean cell number after 72hrs
Control	50,000	152,667 ± 15,147
TGFβ-1	50,000	94,000 ± 10,412

Chondrocytic morphology	Initial cell number (Plotting density)	Mean cell number after 72hrs
Control	252,000	890,000 ± 4,362
TGFβ-1	252,000	518,667 ± 4,832

Table 5.1.1 Proliferation of chondrocytic and fibroblast like chondrocyte with and without TGF-β1 supplementation (mean cell numbers ± SE).



a)



b)

Figure 5.1.2 Effect of TGF-β1 on the proliferation of primary chondrocyte monolayer: (a) with fibroblast like morphology, and (b) with chondrocytic morphology (mean cell numbers ± SE).

The results of statistical analyses using t-tests supported the view that TGF-β1 significantly ($p<0.05$) decreased fibroblastic and chondrocytic chondrocyte cell proliferation after 72 hours in culture when compared with controls.

Analysis of Cell Size: The sizes of the both fibroblastic and chondrocytic chondrocytes was influenced by treatment with TGF-β1 (See Figure 5.1.3). Cell sizes (length) for fibroblast like chondrocytes cultured in TGF-β1 contained media were 41±0.27μm after 24 hours , 64±0.28 μm after 48 hours and 75±0.46μm after 72 hours, respectively, and for cells without TGF-β1 supplementation were 40±0.7 μm on day one, 48±0.14 μm on day two and 54±0.26 μm on day three, respectively.

In contrast with fibroblastic chondrocytes TGF-β1 had a slightly converse effect on size of cells with chondrocytic phenotype. The average sizes of the chondrocytes in the medium without TGF-β1 supplementation were 22±0.07 μm on day one, 25±0.06 μm on day two and 26±0.18 μm on day three, respectively and 19±0.22 μm on day one, 16±0.32 μm on day two and 21±0.34 μm on day three in the TGF-β1 contained environment.

Statistical analyses of cell size in relation to time in culture indicated that TGF-β1 significantly ($p<0.05$), increased cell size at the 48 hour and 72 hour time point for the

fibroblastic chondrocytes. However, in the case of chondrocytic chondrocytes, TGF-β1 induced a significant decrease in cell length ($p<0.05$) at the 48 hour time point, but no overall increase in cell size after 72 hours in culture. It is highly likely that the decrease in cell size seen at the 48 hour time point in the chondrocytic type cells may be associated with cell division at that time.

These results indicate that TGF-β1 induced a significant increase in fibroblastic cell spreading possibly associated with increased extracellular matrix protein secretion (See Figure 5.1.3). In comparison, TGF-β1 had little effect on chondrocytic cell size after 72 hours in culture, however the cells did change their morphology, becoming more elongated and more fibroblastic.

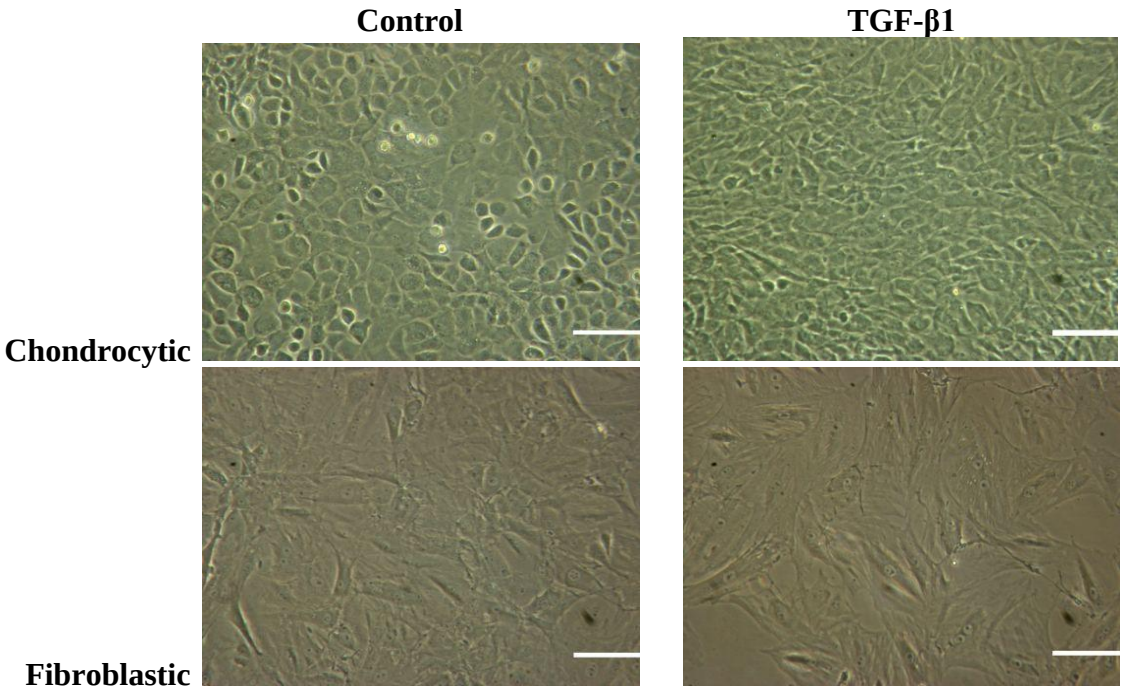


Figure 5.1.3 Cultured chondrocyte cells after 72 hours: (left) without, and (right) with TGF-β1 supplementation (Scale bar=100 μm).

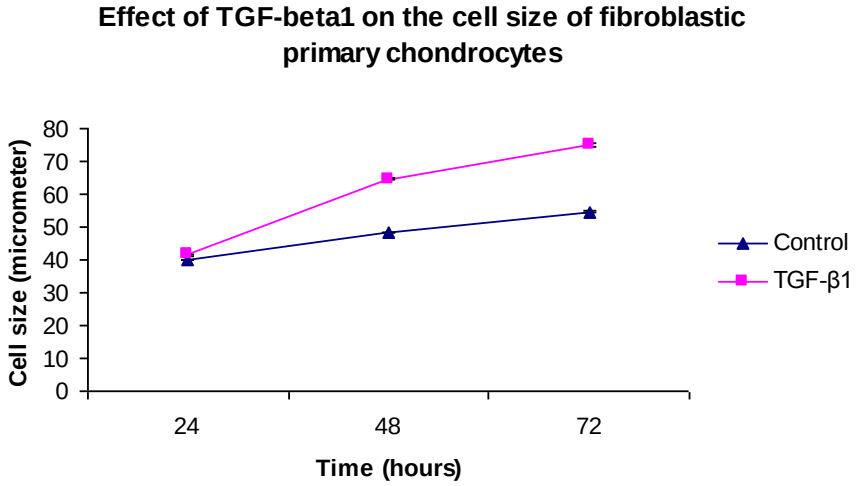
Fibroblastic CHC

Hours	12	36	72
Control	40 µm ± 0.07	48.27 µm ± 0.14	54.69 µm ± 0.26
TGF-β1	41.4 µm ± 0.27	64.64 µm ± 0.28	74.95 µm ± 0.46

Chondrocytic CHC

Hours	12	36	72
Control	22.48 µm ± 0.07	24.15 µm ± 0.06	22.83 µm ± 0.18
TGF-β1	22.40 µm ± 0.22	20.77 µm ± 0.32	23.11 µm ± 0.34

Table 5.1.2 Change in relation to the increase in cell size (cell length) for chondrocytic and fibroblastic chondrocytes (Mean ± SE).



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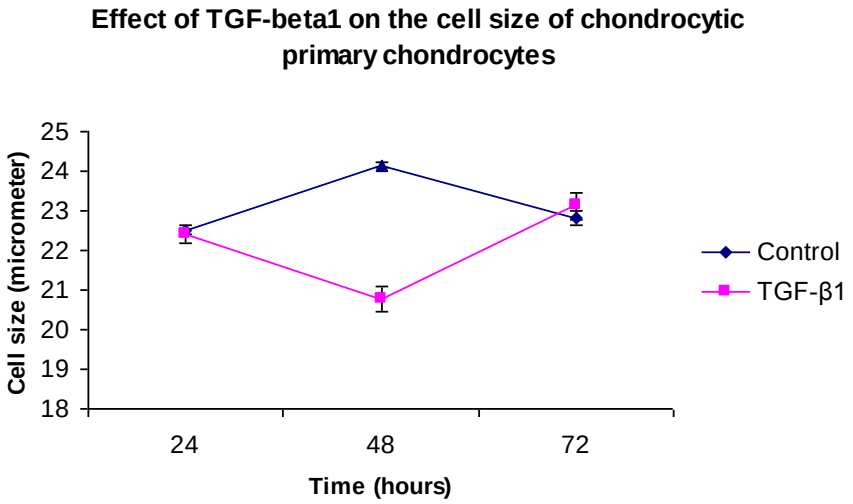


b)

Figure 5.1.4 Cell size of primary chondrocyte monolayers vs time: (a) Fibroblastic, and (b) Chondrocytic. The error bars represent SEM of measured cell sizes.

Immunocytochemistry: Immunostaining of monolayer cultures of chondrocytes revealed that the primary chondrocytes isolated from knee joint of neonate Sprague-Dawley rats form different type of morphologies. Immunofluorescence micrograph of chondrocytes stained for type-I collagen, type-II collagen and fibronectin showed that the chondrocytes with 10 ng/ml TGF-β1 supplementation obtained fibroblast like morphology whereas at the same density of chondrocytes cultured in environment without TGF-β1 exhibited a rounded shape (Figure 5.1.5). The type-II collagen and fibronectin spread around the cell membrane of chondrocytes without TGF-β1 supplementation, whereas this protein was mainly distributed between cell membrane and extracellular matrix in the presence of TGF-β1. There was no significant difference between both cultures stained for collagen type-I (Figure 5.1.5).

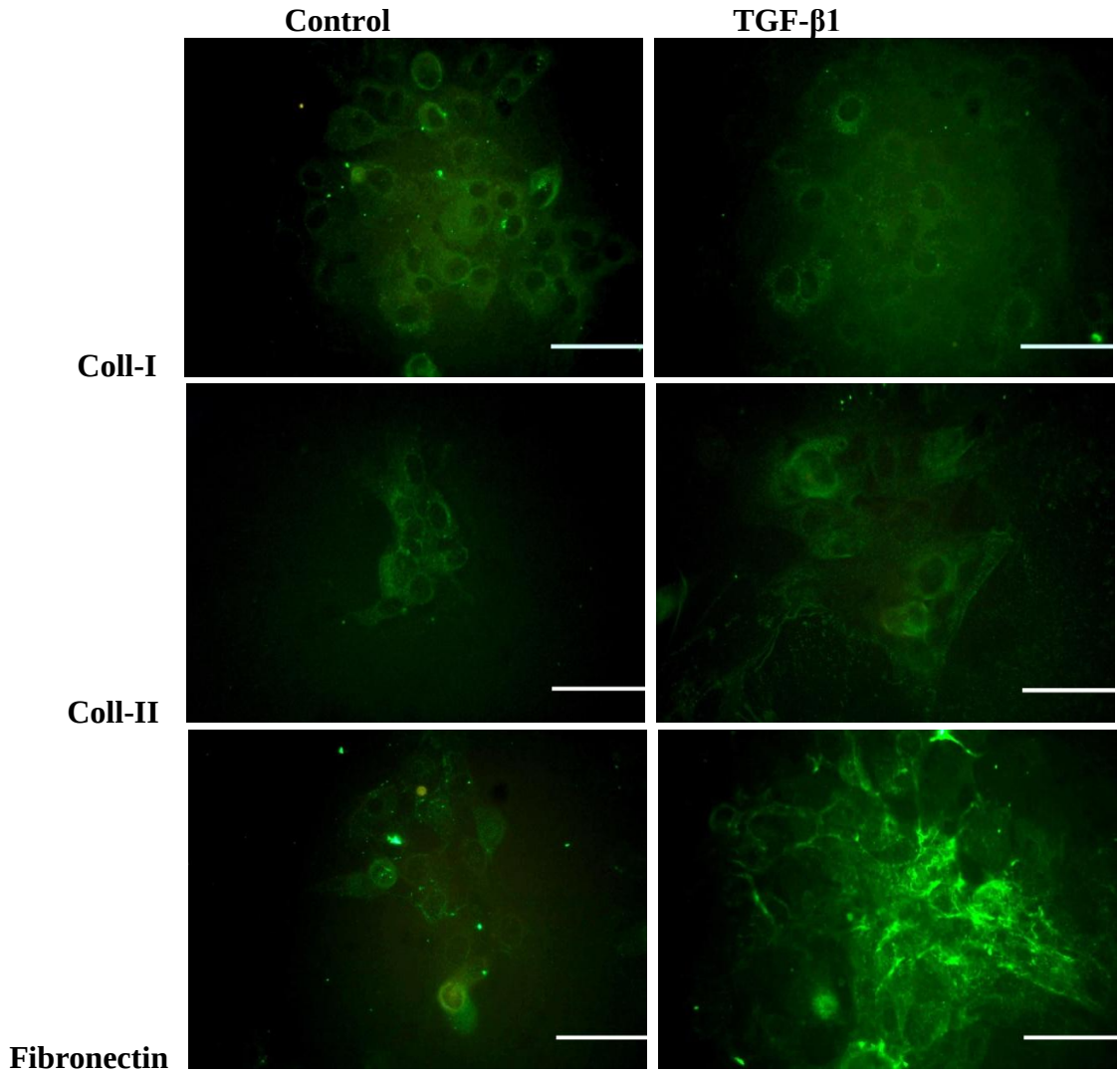


Figure 5.1.5 Immunofluorescence micrographs of primary chondrocytes stained for collagen type-I, collagen type-II and fibronectin without TGF-β1; and for collagen type-I, and collagen type-II (right) with TGF-β1 (Scale bar=100μm).

Wound Closure Assay: The results for the *in vitro* monolayer wound repair assay of primary chondrocyte with fibroblastic and chondrocytic phenotypes showed that the process of healing for a mechanically created wound model in the presence of TGF-β1 was much slower than control cultures (Figures 5.1.6 – 5.1.8). Addition of TGF-β1 to

fibroblastic chondrocytes caused 14.1 % increase in wound size after 24 hours, and 9.8% increase in wound size after 72 hours as compared to control which showed 52.2% wound closure after 24 hours and 96.6% wound closure after 72 hours, respectively.

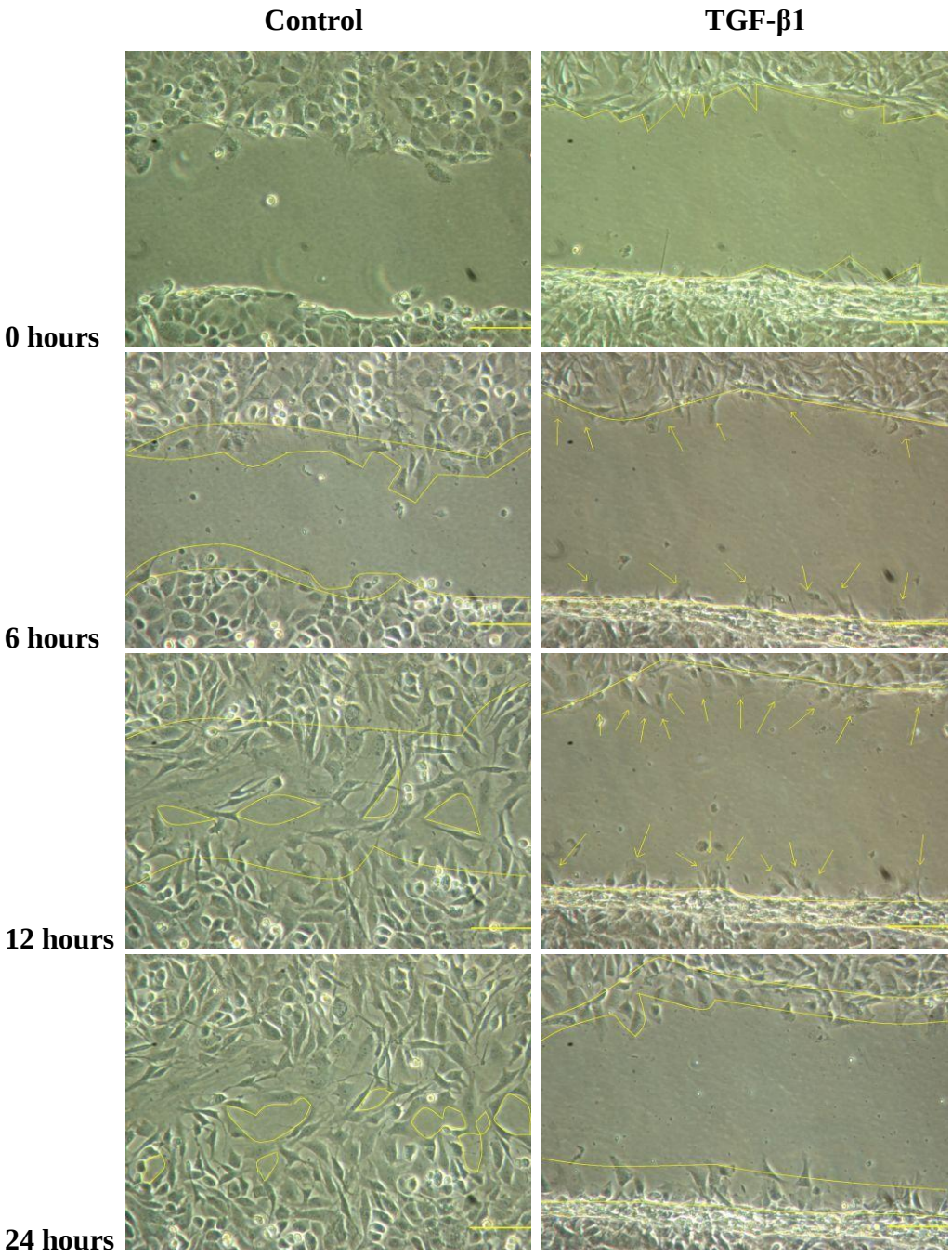
Statistical analyses indicated that wound widths in response to TGF-β1 treatment in fibrobastic chondrocyte cultures remained unchanged after 72 hours in culture ($p=0.94$). However, in control cultures of fibroblastic chondrocytes wound size decreased significantly after 72 hours in culture ($p<0.05$).

In contrast, control culture of chondrocytes with a chondrocytic phenotype showed ~ 28.3% wound closure after 24 hours and 79.1% after 72hours. Addition of TGF-β1 caused 8.4% wound closure after 24 hours and 71.2% after 72 hours (see Figure 5.1.6).

This suggests that TGF-β1 down regulates cell proliferation, adhesion and cell migration which are essential in wound repair. In all experiments the wound width increased during the first 6 hours after creating the wound. Statistical analyses of wound closure in chondrocytic cell cultures indicated that although TGF-β1 seemed to decrease wound closure this decrease in wound closure as determined by comparing wound width after 72 hours was not significant ($p = 0.133$).

In both the fibroblastic and chondrocytic chondrocyte cultures, TGF-β1 seemed to induce a further wound enlargement up to a period of 6 hours. This may indicate that TGF-β1 may play a part in inducing a stress response in chondrocytic cells similar to that described by Lin and Helmke (2009) and production of nitric oxide, causing induction in cell apoptosis (Im and Shin, 2002). This may also play a part in the down regulation of chondrocyte mitogenesis. The nitric oxide secretion by damaged cells will

cause deterioration in matrix proteoglycan in turn affecting the initial wound recovery process.



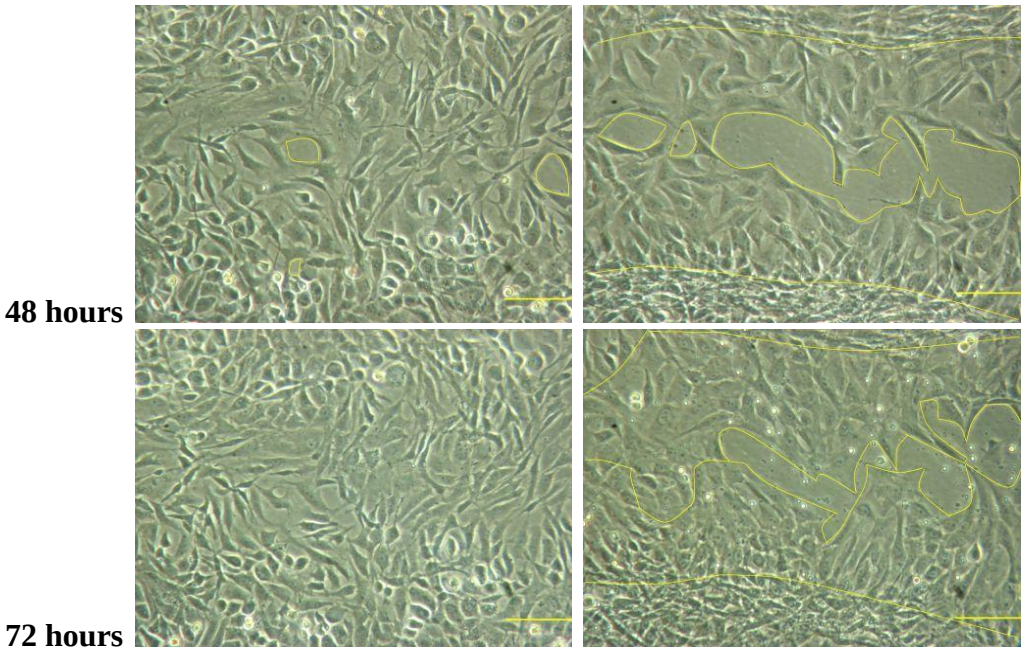


Figure 5.1.6 Images of the wound closure response for primary chondrocytes with chondrocytic phenotype with and without TGF-β1 supplementation (Scale bar=100μm).

Interaction of cells with ligands expressed by neighbouring cells, and cell-cell signalling plays an important role in cell proliferation (Fox et al., 2008). In fact, cell-cell communication can stimulate cell proliferation (Celeste and Christopher, 2002). Interestingly, the chondrocytes monolayer consisting of fibroblast like cells demonstrated an increase in wound size during 72 hours wound closure (See Figures 5.1.7 and 5.18). This may be related to TGF-β1 increasing the secretion of ECM molecules at the wound edge, which functions by capturing cells via the integrin ECM interactions at this edge, thus preventing migration into the wound site. This could go some way towards explaining the poor repair capacity of cartilage *in vivo*. Such a mechanism may be overridden in therapeutic procedures by filling a wound site with large numbers of chondrocytes which would presumably enable the deposition of ECM

across the wound surface enabling cell migration and proliferation into the wound. Such a system would certainly explain the partial success seen in autologous chondrocyte implantation techniques (Knutsen et al., 2008), (Petersen et al., 2008).

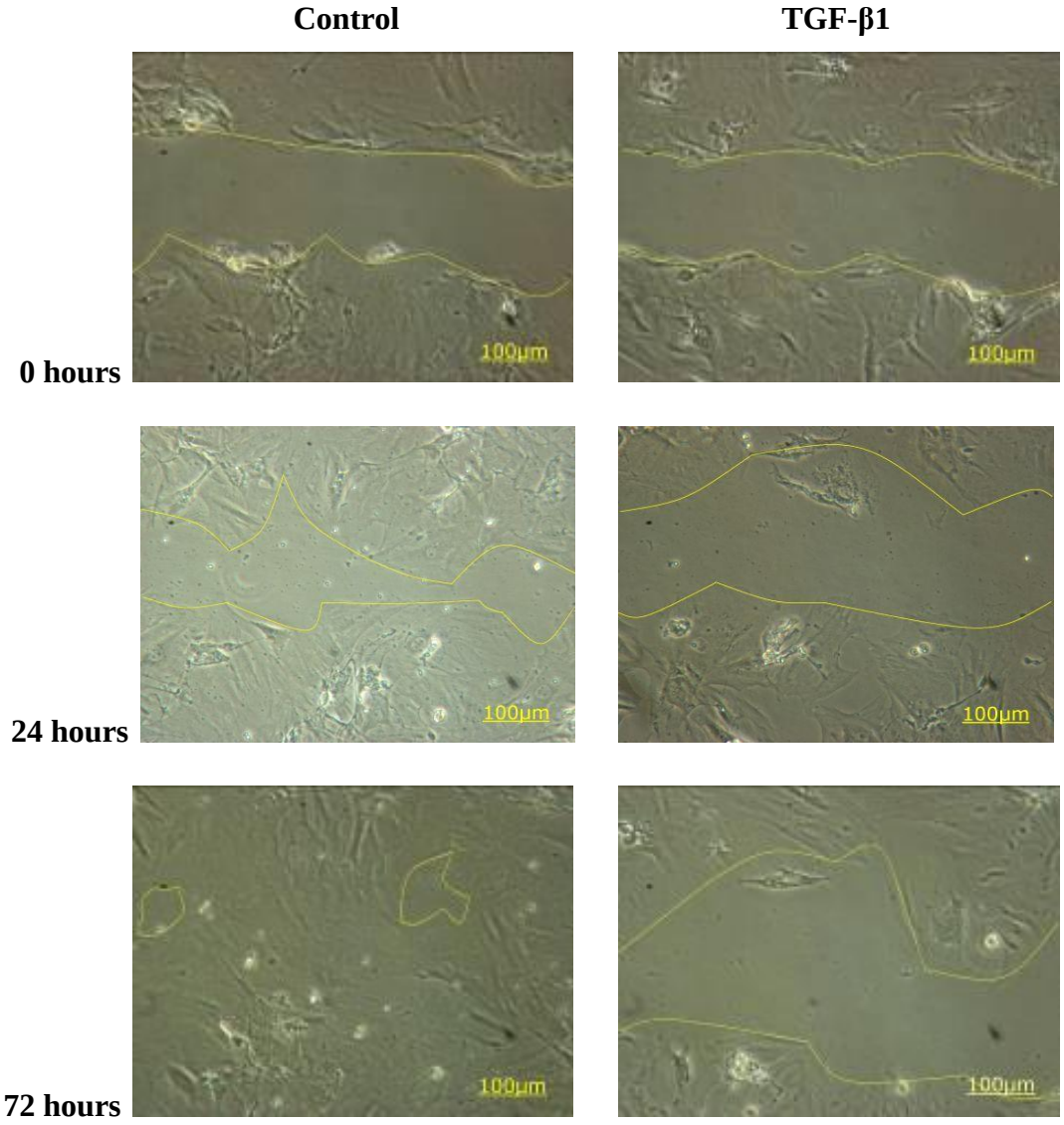
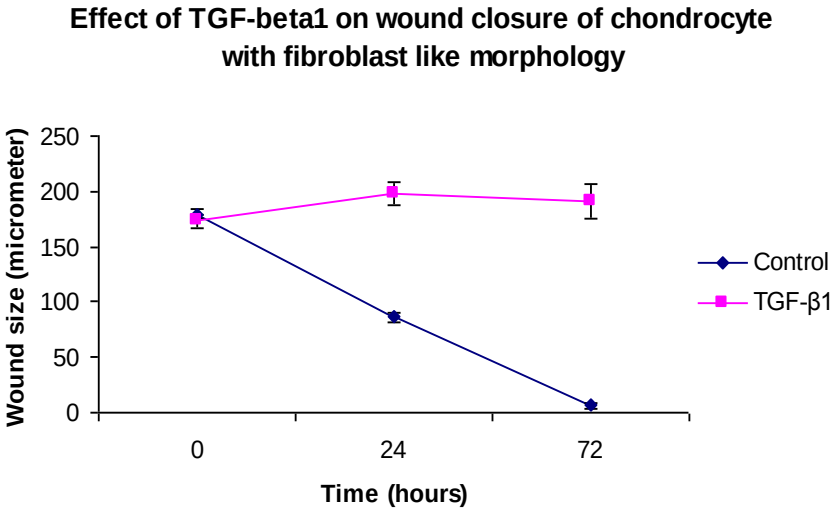


Figure 5.1.7 A comparison in wound closure of chondrocytes with fibroblast like morphology with and without TGF-β1 addition.

Figure 5.1.8 is the graph of wound closure vs. time for the fibroblastic and chondrocytic chondrocytes which shows different cell proliferation rate and cell migration for control culture and treated with TGF-β1. As can be seen clearly, TGF-β1 inhibited wound closure in the fibroblastic monolayer.



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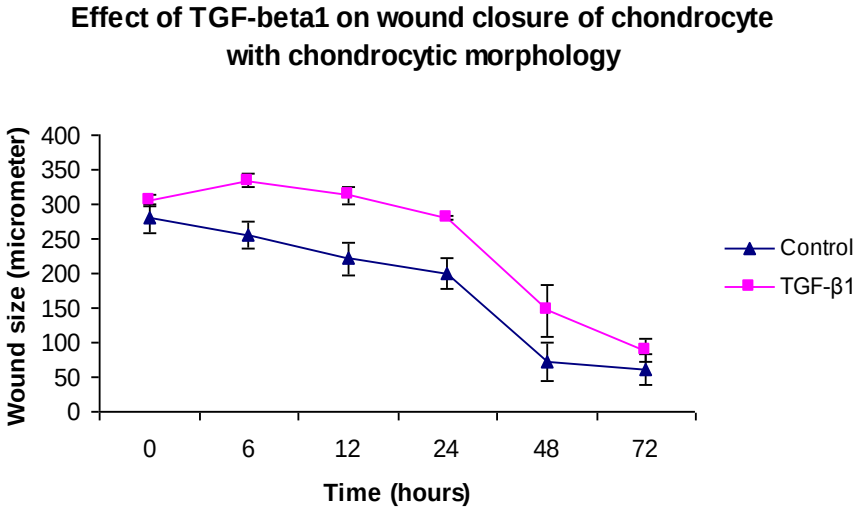

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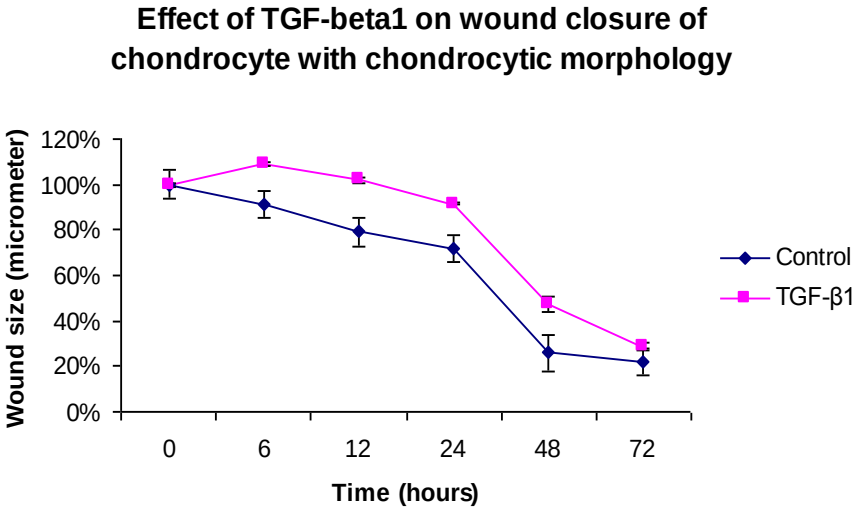


c)

Figure 5.1.8 Wound closure assay for primary chondrocyte with: (a) fibroblast like morphology, (b) chondrocytic morphology, and (c) normalized data of (b). The error bars represent SEM of wound size measurements during the experiment.

One important effect of TGF-β1 on chondrocytes *in vitro* was the rapid differentiation into fibroblast like cells (See Figures 5.1.3, 5.1.6, and 5.1.7). This may be linked to increased synthesis of extracellular matrix and extended fibres causing the cells to occupy larger area which prohibited cell migration and consequently reduction of cell proliferation.

Cell Adhesion Analysis: Trypsinization of chondrocytes monolayers revealed that the detachment time (See Table 5.1.3) from a solid surface for chondrocytes with fibroblast like morphology with TGF-β1 supplementation was longer than those cultured in

medium without TGF-β1. However, this time for cells with chondrocytic morphology and TGF-β1 supplementation was relatively short when compared to the cells without TGF-β1 supplementation (See Table 5.1.3).

From Table 5.1.3 and Figures 5.1.9 and 5.1.10 it can be seen that control culture of fibroblast like cell started to detach after approximately 63 seconds and complete detachment was achieved after 116 seconds, whereas the same cells in the presence of TGF-β1 started to detach after approximately 93 seconds and complete detachment was achieved after about 203 seconds.

In contrast the control culture of chondrocytic cells started detaching after about 85 seconds and showed complete detachment after 263 seconds, whereas the chondrocytes in TGF-β1 contained medium started to detach after about 96 seconds and after approximately 213 seconds full detachment was achieved.

Although the surface of the culture flask was covered completely by cells, the population of fibroblast like chondrocytes was very low as compared to the cells with chondrocytic morphology.

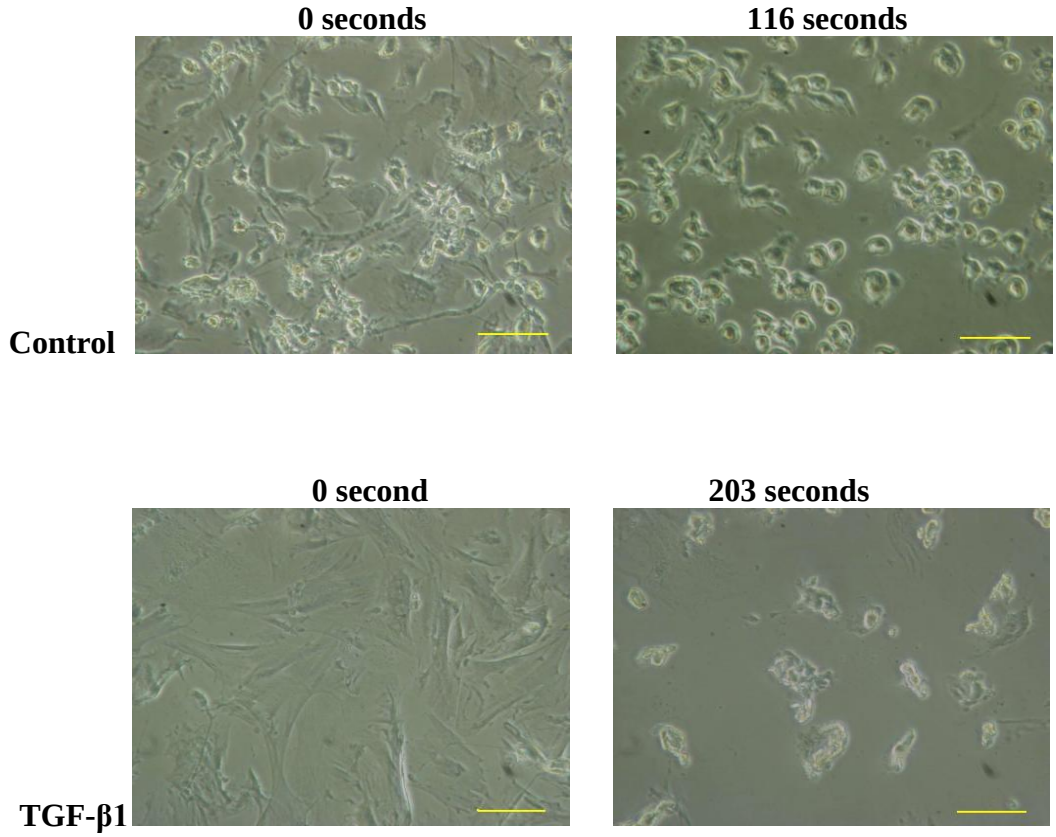


Figure 5.1.9 Detachment of primary chondrocyte cells with fibroblast like morphology from solid surface by trypsinisation (Scale bar=100μm).

The ECM molecules are easily digestible, but the digestion time depends on initial distribution and concentration of proteins. In the case of fibroblast like morphology, excessive production of ECM proteins in the presence of TGF-β1 (Figures 5.1.3, 5.1.6 and 5.1.7) may have caused an increase in detachment time.

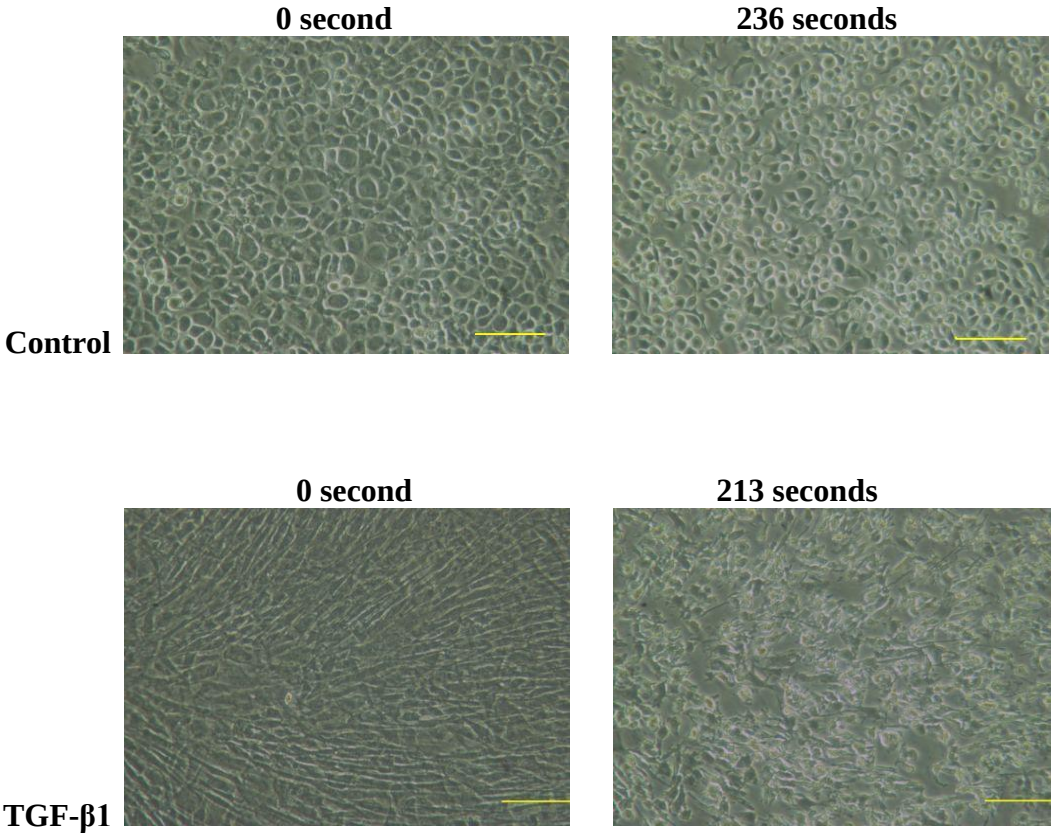


Figure 5.1.10 Detachment of primary chondrocyte cells with chondrocytic morphology from solid surface by trypsinization (Scale bar=100μm).

Detachment times for both fibroblastic and chondrocytic cells (See Table 5.1.3 and Figure 5.1.11) showed that TGF-β1 increases adhesion of chondrocytes with fibroblast like morphology to a solid surfaces, but decreased adhesion of chondrocytic chondrocytes to a solid surface in relation to untreated cells.

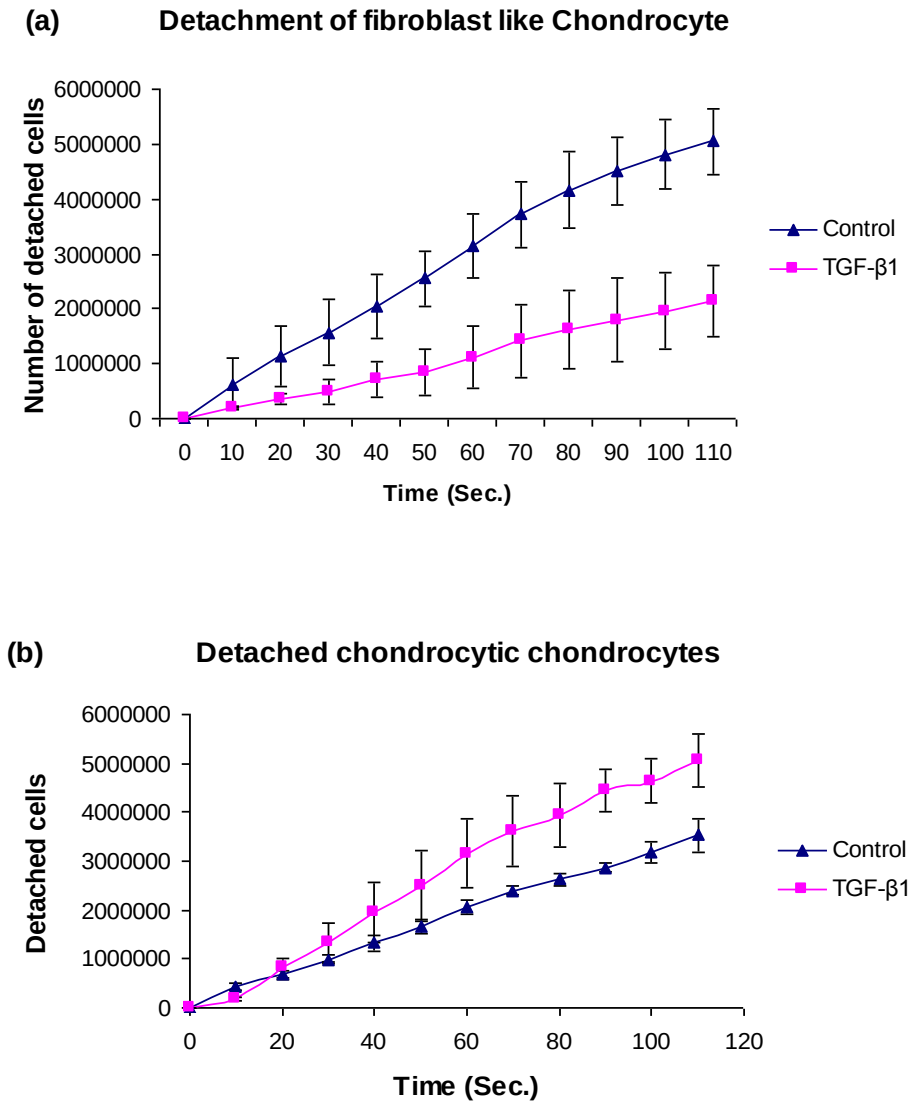


Figure 5.1.11 Detachment of the primary chondrocytes: (a) with fibroblast like morphology, and (b) with chondrocytic morphology, with and without TGF-β1.

Fibroblast like cells

Experiment	Detachment time
Control	116 seconds
TGFβ1	203 seconds
Difference	87 seconds

Chondrocytic cells

Experiment	Detachment time
Control	263 seconds
TGFβ1	213 seconds
Difference	50 seconds

Table 5.1.3 Detachment time in seconds for chondrocytes with fibroblastic morphology (top) and chondrocytic morphology (below).

5.1.3 Overall Discussion and Conclusions

Primary chondrocyte cells acquired a fibroblast like morphology after the first subculture in the 2-dimensional cell culture system. TGF-β1 caused down regulation of chondrocyte proliferation and up-regulation of cell death.

TGF-β1 caused increase in cell size of chondrocyte with fibroblastic phenotype but not with chondrocytic chondrocyte.

TGF-β1 also had a negative effect in wound closure process of chondrocytes *in vitro* suggesting that the inactive TGF-β1 down regulates cell proliferation and cell migration which are essential for tissue repair.

The healing process of scratched chondrocytes monolayer started after ca. 6 hours after creating the wound model. This confirms the stress responsive nature of the cell and delay in the cell activation, particularly the down regulation of chondrocyte mitogenesis (Bordeleau et al., 2008).

TGF-β1 therefore plays a very important role in rapid formation of fibroblast like chondrocyte cells *in vitro*. This could be linked to an up-regulation in the production of extracellular matrix and extended collagen type-I fibres causing the cells to flatten and occupy a larger area which prohibits cells migration and consequently reduces cell proliferation (Chi et al., 2004).

Results of this study also suggest that TGF-β1 stimulates chondrocyte-ECM adhesion but this depends on the cell phenotype. Chondrocytes with a fibroblast like morphology cultured in medium with TGF-β1 supplementation displayed a greater degree of cell/surface attachment than those cultured in medium without TGF-β1. In contrast, the degree of cell surface attachment was decreased in cells with chondrocytic morphology when cultured in TGF-β1 contained media. It is believed that TGF-β1 is involved in chondrocyte cell-ECM adhesion suggesting that TGF-β1 may cause synthesis of different type of CAMs, which may influence cell migration and proliferation in the wound site. However, the negative effect of this cytokine in wound repair of cartilage suggests the hypothesis that the inhibition or activation of TGF-β1 may induce cartilage repair (Davidson et al., 2005).

CHAPTER V

5.2 Transforming Growth Factor-β2

5.2.1 Materials and Methods

5.2.2 Cell Culture

Primary chondrocyte cells from the knee joint of neonate Sprague-Dawley rats were re-suspended in 5ml-high glucose Dulbecco's modified eagle's medium (DMEM) (Sigma Aldrich, UK) with 1% L-glutamine supplementation. In 5ml cell suspension 1,400,000 cells were counted using a haemocytometer, giving a cell density of 280,000 cells/ml. The cell suspension was kept in a 15ml centrifuge tube and incubated at 37ºC until preparation of a transforming growth factor beta-2 (TGF-β2) solution.

Addition of this growth factor to the culture media facilitated the investigation of the effects of TGF-β2 on the chondrocyte cell size, proliferation rate, cell adhesion and on wound repair of the created wound model.

All results from each part of the experiment were standardised using the setting of the initial cell density as 100% and then comparing the remaining cells with initial percentage. Standard error (± SE) is also used to estimate any error in measurements and to determine the accuracy of the results.

5.2.2.1 Reconstitution of Transforming Growth Factor-Beta2 (TGF-β2)

Transforming growth factor-beta2 is a multifunctional peptide soluble in acid solvent. It is also soluble in water; however, the solution would be sticky and difficult to aliquot and utilise. It is also evident that TGF-β2 requires a carrier molecule such as BSA to enable cell uptake. Thus a stock solution of 5 µg/ml TGF-β2 was made by dissolving 2µg of TGF-β2 in 400 µl of sterile 4mM HCl containing 1 µg/ml BSA.

5.2.2.2 Primary chondrocyte culture

The experiment consisted of five tissue culture flasks signed as Control, HCl, HCl/BSA, BSA and TGF-β2. A total of 288,000 cells per culture flask were subjected to this set of experiments. By resuspending this amount of cells into 5 ml cell culture media, each culture flask is supposed to have a cell density of 57,600 cells/ml.

A total of 5ml of cell suspension were poured into the culture flask signed as control. A total of 10µl of 4mM HCl was added into another 5ml cell suspension signed as HCl. 10µl of BSA solution, dissolved in sterilised distilled water with a concentration of 1.5µg/ml, added into 5ml cell suspension and poured into the cell culture flask signed as BSA. Another 5ml cell suspension was supplemented with 10µl of 4mM BSA/HCl prepared earlier and transferred into the culture flask named as BSA/HCl. The final 5ml cell suspension was supplemented with 10µl TGF-β2 solution, poured into the culture flask signed as TGF-β2 and incubated at 37ºC with another four culture flasks. Every 24 hours the cell cultures were checked and imaged using a phase-contrast microscope with

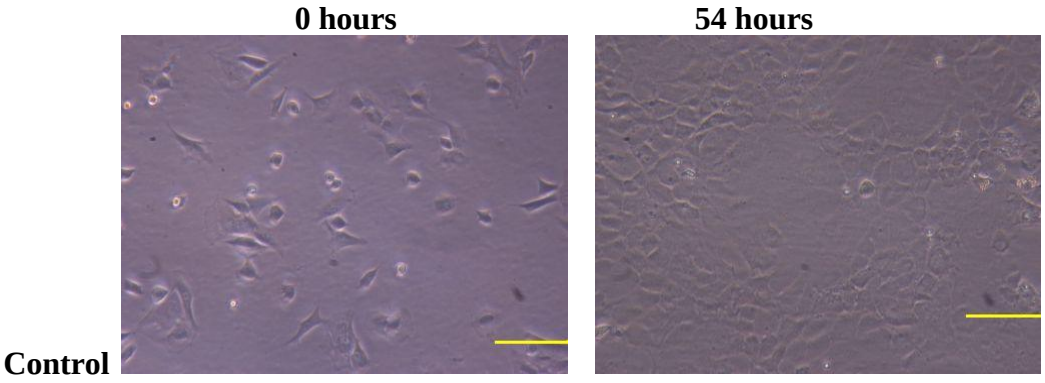
10x magnification. The images were saved for future reference. Also, every 48 hours, the media were aspirated, discarded and replaced with fresh media with the same (10µl/ml v/v) TGF-β2 supplementation.

The experiment was repeated three times to obtain significant data for statistical analysis.

5.2.2.3 Measurement of Cell Size (Cell Length)

Cell-size measurements of all cell cultures supplemented with BSA, BSA/HCl, control and TGF-β2 were performed 12 hours after cell seeding into the tissue culture flasks. This time allowed for primary chondrocytes settling and attaching to the solid surface.

Recording of the cell size continued every 24 hours. A total of 30 randomly selected cells were imaged and saved and their lengths measured using NIH Image J software.



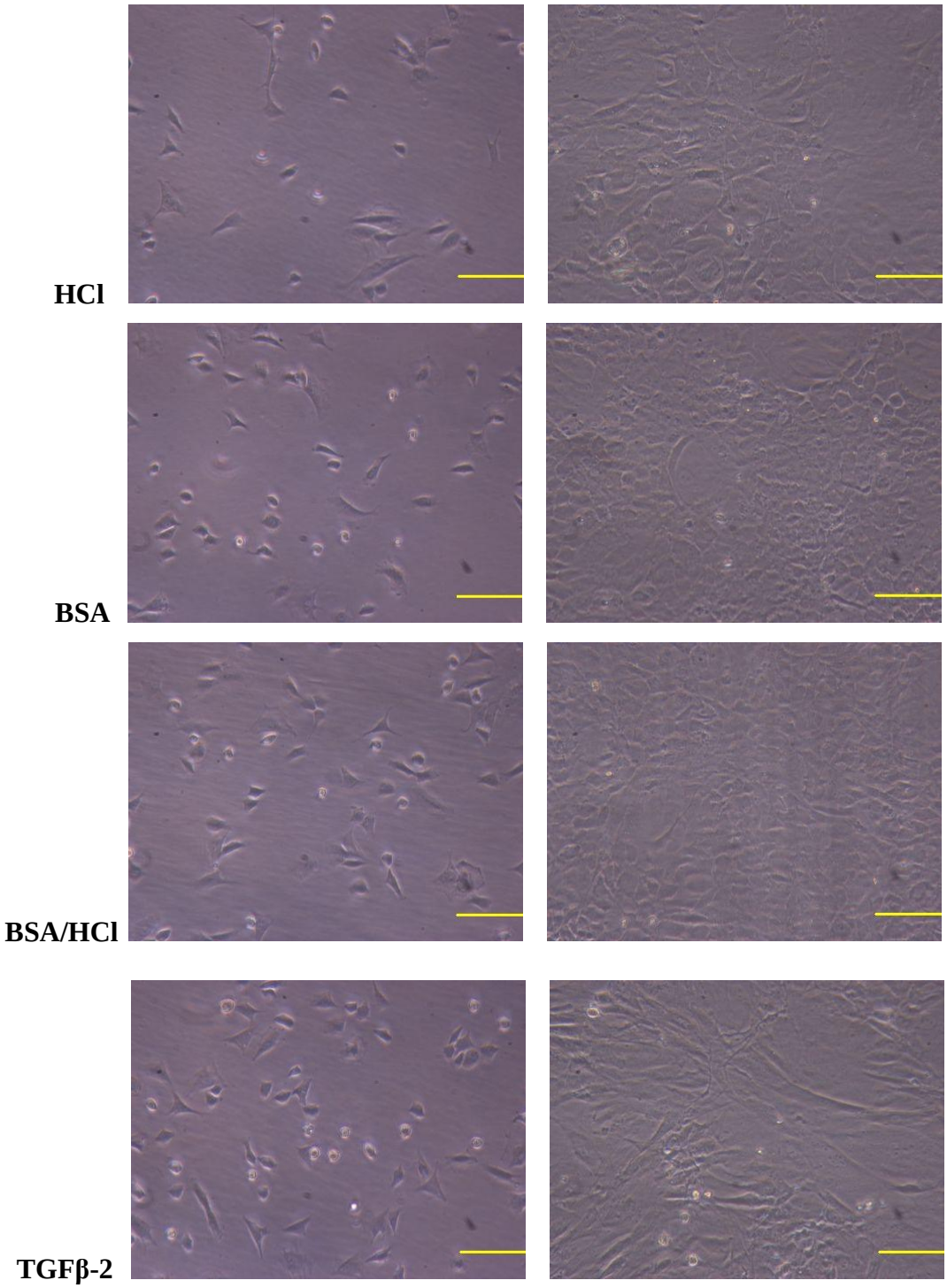


Figure 5.2.1 The chondrocytes cultured in media with different supplementations. The captured images are the first and last pictures, (Scale bar 50μm).

As the primary chondrocytes were isolated from different zones of cartilage they had different shapes, and hence the largest dimension of the cells was considered as the cell size. The average data coming from repeated experiments were calculated and set as average chondrocyte cell size. The mean cell size and standard deviation with error bars were calculated using Microsoft Excel. The standardised cell sizes from each treatment were compared with the standardised dimension of chondrocytes in the control culture to find out whether or not TGF-β2 causes any change in the size of the cells.

Following standardisation of the data, the first measurements were set as 100% and the subsequent data were determined as percentage average.

5.2.2.4 Wound Closure Assay

All five chondrocyte cultures reached 100% confluency after 132 hours.

The wound repair capacity of monolayer primary chondrocyte culture was investigated using wound closure assay. A wound model was created by scratching the cell layer using a fine-tip extended transfer pipette of 1 mm diameter (Sigma Aldrich, UK). Following scratching, wound width was measured via Image J software every two hours at 10 different points along the width until the wound was totally healed except for the model wound treated with TGF-β2, which did not fully heal during the experiment. The re-identification of these points was achieved by drawing 10 lines perpendicular to the scratch in the cell layer on the underside of the culture flask with an alcohol-resistant marker pen. Measurements of wound width were then taken to the right of the point at which each perpendicular line intersected with the model wound. An average wound

width of ~ 131.77 µm was recorded and the normalized % wound closure was used to eliminate different wound width along the wound bed, for future analysis regarding the effect of different growth factor.

5.2.2.5 Primary Chondrocyte Detachment Analysis

The strength of primary-chondrocyte attachment on substrate in the presence of different supplementations was analysed using the trypsinisation assay.

Trypsin is a temperature-dependent digestive enzyme which breaks down the cell-extracellular matrix and cell-cell binding proteins at the carboxyl end (Sigma Aldrich, UK). The cleavage of adhesion proteins results in cell detachment from substratum and/or separation from other cells (See Figure 5.2.2).

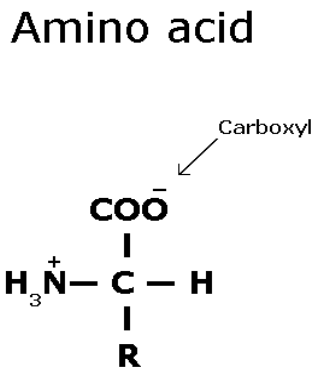


Figure 5.2.2 Structure of basic amino acid and its carboxyl side (Hames and Hooper, 2005).

The assay was carried out at room temperature of 18ºC. The media were aspirated from the tissue culture flask and the flask was rinsed three times with Hank's balanced salt

solution (HBSS) to remove any serum from the confluent layer of cells. After aspiration of the last HBSS, 2ml of trypsin was poured into the culture flask to detach the cells from the culture flask and their ECM. The protein digestion process was imaged by phase-contrast microscope, with a digital camera installed on the microscope, and Image J (NIH) software. A sequence of 60 images with 10-second intervals were recorded and saved for further analysis.

A total of 2ml of serum containing media was added to the cell suspension immediately after detachment of the cell from the culture flask. After trypsinization, the suspension was used for cell count.

The time taken for the chondrocytes to get a round shape and shiny appearance, signs of detachment, was recorded**.**

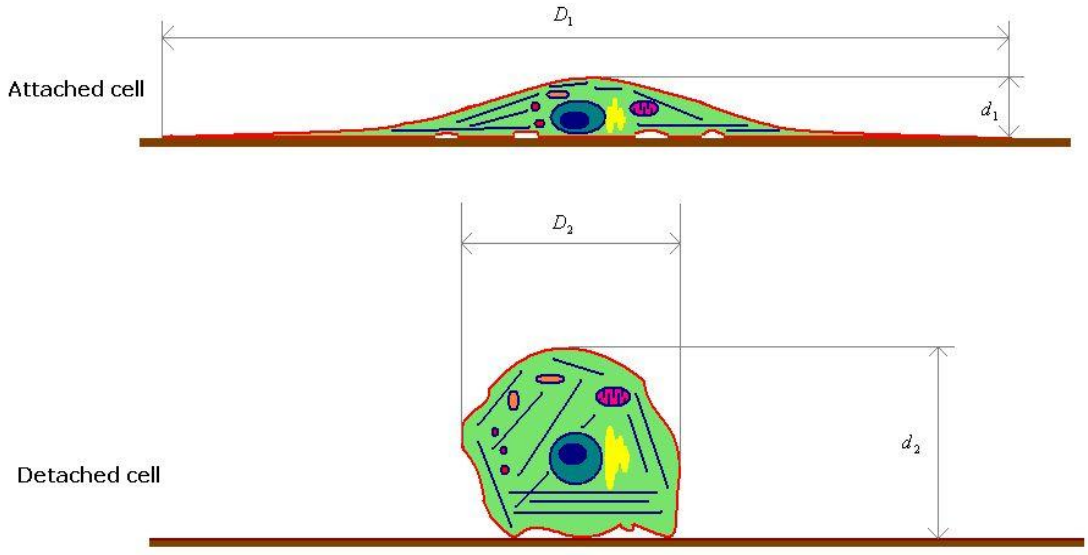


Figure 5.2.3 Morphology of primary chondrocyte during attachment and detachment.

Figure 5.2.3 is the schematic drawing showing how cell spreads when attaching on a surface. The size of D_2 is less than D_1, thus the amount of light scattered from detached cell is much higher than D_1 on an attached cell. This causes the cell in detached position acquire a shiny morphology (See Figure 5.2.4).

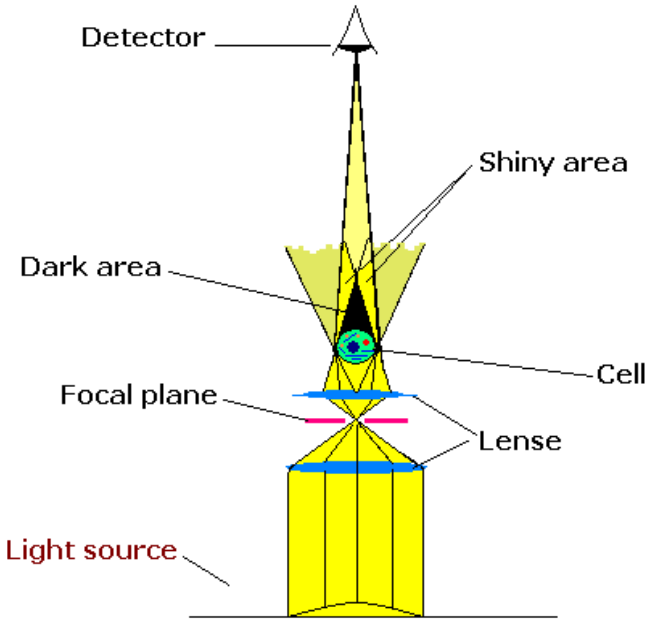


Figure 5.2.4 Simple schematic drawing of scattered light causing the shiny appearance of a detached cell.

The recorded time was multiplied by 10, which was the time interval, and then divided by 60 to obtain the detachment time per second. The time taken for the detaching of a cell in the control culture was converted to percentage average as 100% and the other cell cultures, containing different supplementations, were compared against the control.

Microsoft Excel was used to calculate the mean, standard deviation and a graph was then plotted to demonstrate % detachment time for each supplementation, as shown in Figure 5.2.5. It can be seen that TGF-β2 addition resulted in lower % detachment (~ 70%) whereas the HCl showed a similar result to control followed by BSA & BSA/HCl additions with ~ 85% detachment.

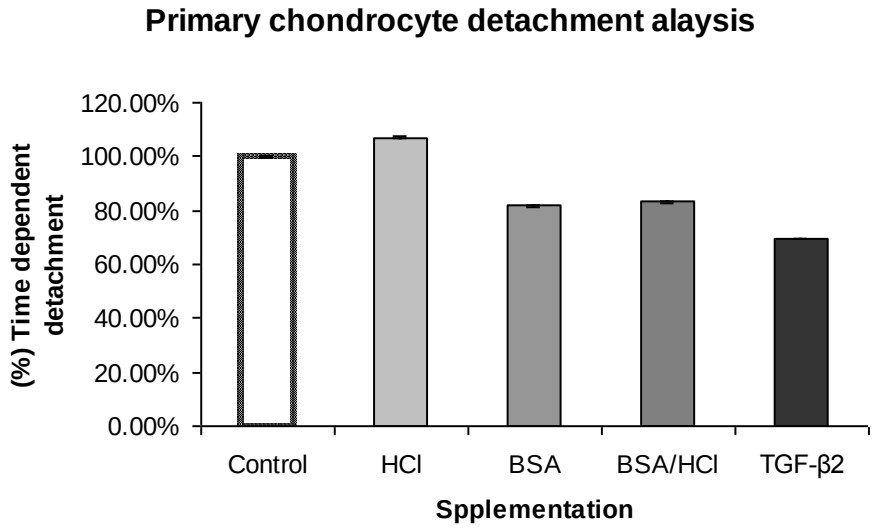


Figure 5.2.5 Graph of primary chondrocyte adhesion strength with a percentage comparison of cell culture mediums with different supplementations against control. The recorded time for chondrocytes to detach is set as 100%.

The final suspensions obtained from trypsinisation were used to evaluate cell proliferation rate. This proliferation rate was achieved by counting the trypsinized cells and comparing with initial seeded cells.

5.2.3 Statistical Analysis

The effect of different supplementations in chondrocyte cell proliferation, cell size, adhesion, and wound repair was statistically analysed by One-way ANOVA test. This test was performed to evaluate the statistically significant difference between the means of the treatments. The difference between the means of the treatments was assumed as Ha, and the similarity was assumed as H0.

H_0: Mean1 = Mean2 = Mean3 = Mean4

H_a: At least one of the means is different

5.2.4 Results and Discussion

Primary chondrocytes, cultured in monolayer in high-glucose DMEM media supplemented with 10% foetal calf serum (FCS), were used as control culture (See Figure 5.2.6). Another four culture environments with addition of HCl, BSA, BSA/HCl and TGF-β2 were compared against the control culture (See Figure 5.2.7).

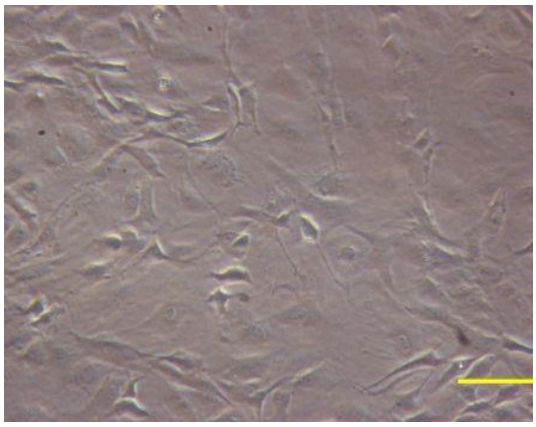


Figure 5.2.6 Control culture of chondrocyte cells (Scale bar = 50 µm).

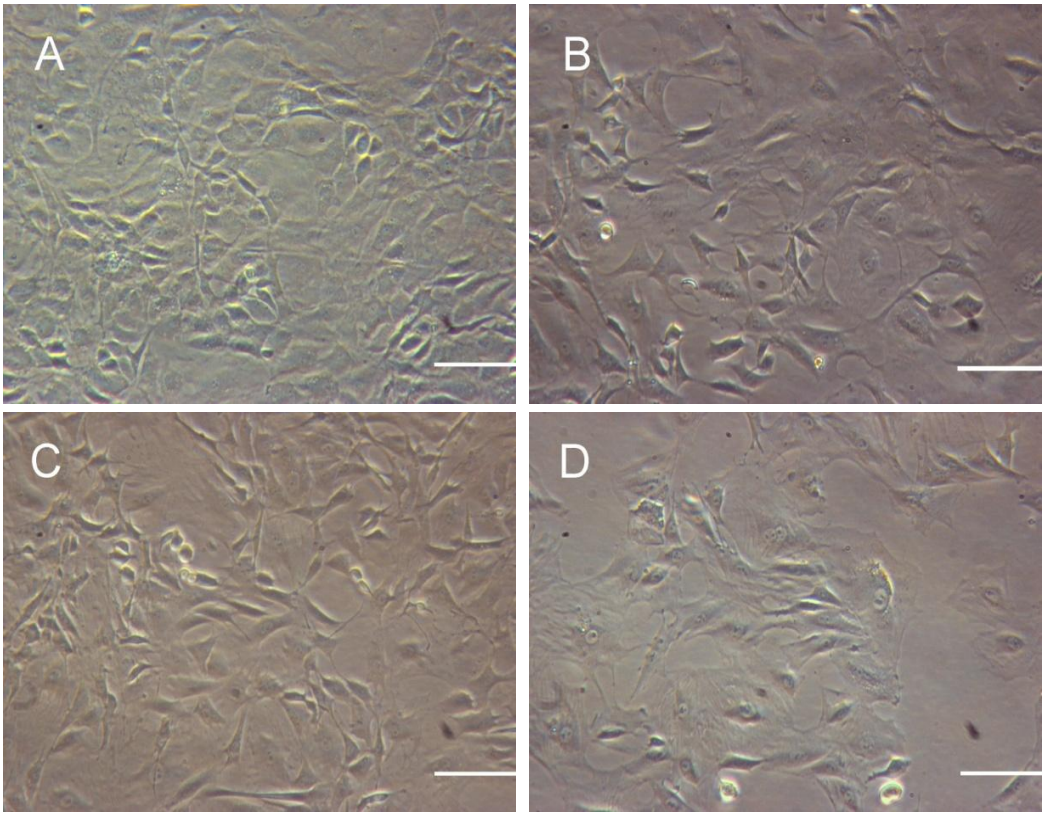


Figure 5.2.7 Culture of chondrocyte cells in media with different supplementations: A) HCl; B) BSA; C) BSA/HCl, and D) BSA/HCl/TGF-β2 (Scale bar = 50 µm).

The cells treated with TGF-β2 developed a well-spread fibroblastic shape acquiring a mean size of 14.20 µm in diameter (Figure 5.2.3). This indicates that TGF-β2 increases

excessive synthesis of extracellular matrix (ECM) proteins which are involved in the formation of fibroblast-type morphology and consequently dedifferentiation of chondrocyte *in vitro*. In comparison, HCl (12.2 µm ± 0.002 SE), BSA (11.18 µm ± 0.002 SE), BSA/HCl (10.45 µm ± 0.002 SE) had no effect on cell length and cells under these treatment regimens resembled cells in the control treatment group (12.51 µm ± 0.002 SE), (See Figure 5.2.7). There was no recognisable difference in cell morphology between HCl, BSA, BSA/HCl and control (See Figure 5.2.7).

The graphs of chondrocyte cell sizes of culture in BSA, BSA/HCl, HCl and TGF-β2 contained mediums are shown below in Figure 5.2.8.

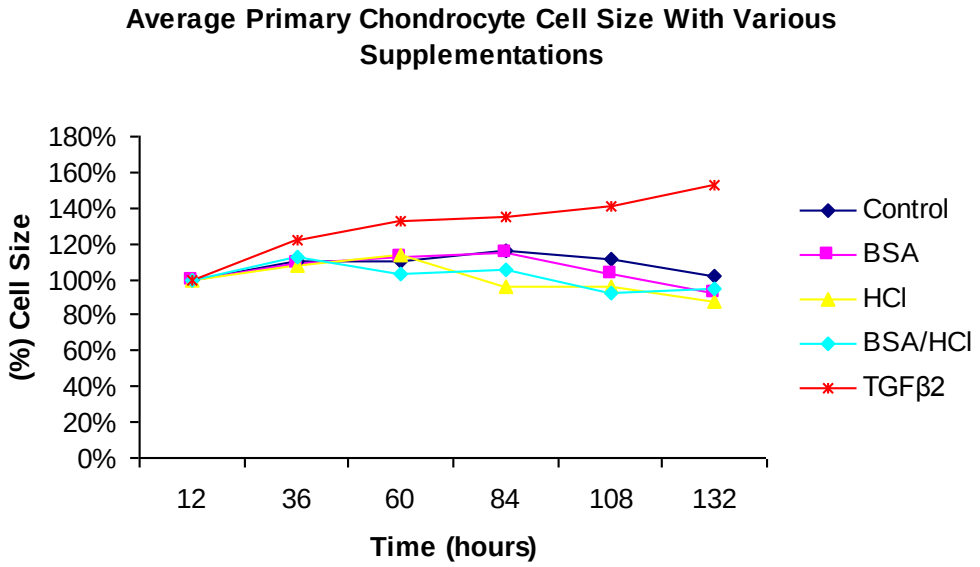


Figure 5.2.8 Graphs of primary chondrocyte cell sizes during 132-hours culture with various supplementations with standard error bar.

Interestingly, each cell culture reached its largest cell size after different times.

TGF-β2 increased the chondrocyte cell size up to 152.9% over a period of 132 hours. This could be related to an increase in the production of components of the ECM, which in turn, via up or down regulation of specific integrins, induced changes in chondrocyte shape. It is well known that the cytoskeleton determines the cell shape by anchoring to the integrin via the actin filament. Enzyme-linked immunosorbent assay (ELISA) could determine the type of integrin binding to the ECM/ligands. In contrast, the smallest cell size was observed in BSA/HCl contained media with 112.85% increase and 10.45μm. The average change of cells in their widest dimension in control was 115.97% with 12.51μm (Figure 4). Only chondrocytes cultured in the TGF-β2 contained medium demonstrated an almost constant increase in cell size with all others showing irregular changes. This is because of low-proliferation capacity of chondrocyte in the presence of TGF-β2 as revealed in determination of the effect of transforming growth factor beta2 on cell proliferation. The initial cell density was set as 100% and all other cultures were compared with this initial setting. The proliferation rate of control culture was 460.35%, corresponding to a 4.6-fold increase in cell density at the end of the experiment.

The evaluation of the effect of different supplementations in chondrocyte proliferation (See Figure 5.2.9) showed that the HCl induced cell proliferation at the highest level with 1026.23% increase. In contrast, TGF-β2 induced apoptosis rather than proliferation. The cell density was lowered at 50.7% when compared with control and the proliferation rate was 2.33-fold. Unlike TGF-β2, HCl induced cell proliferation at the highest level, which was 2.23-fold more than control (See Figure 5.2.9 for more detail).

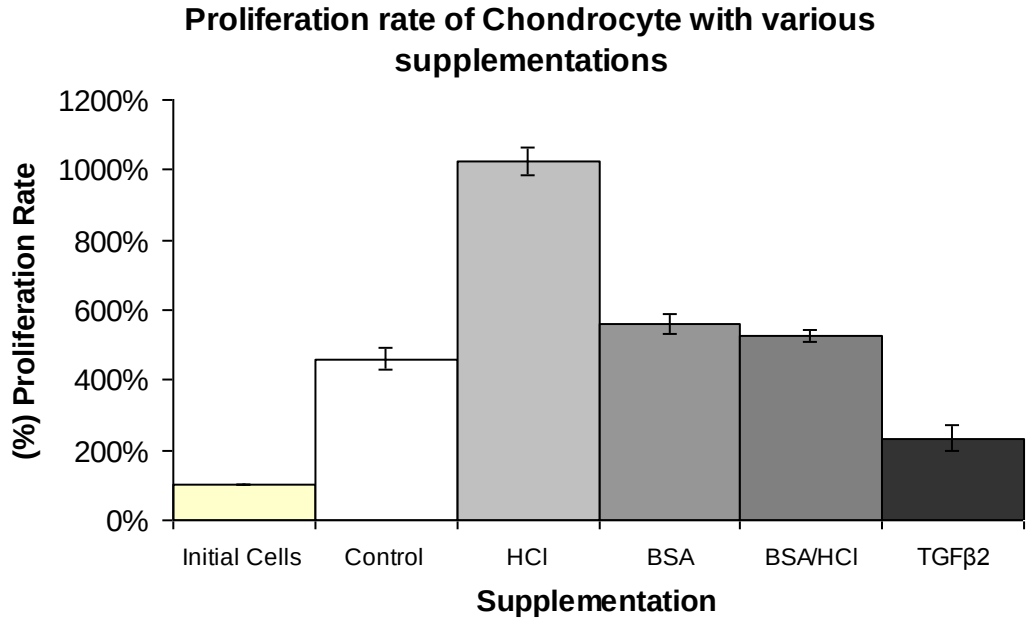


Figure 5.2.9 Graph of primary chondrocyte proliferation cultured in DMEM media in various supplementations. Initial cell concentration, which was 280,000cells/ml, was set as 100%.

During 54 hours wound closure assay (See Figure 5.2.10), only in controls and HCl contained culture was the gap completely closed. The fastest wound healing occurred in an acidic medium with 10µl 4mM HCl. The application of BSA/HCl/TGFβ-2 and the application of BSA containing media appeared to inhibit wound closure. Initially, cell migration was detected circa six hours after scratching. This may suggest that the chondrocyte cell requires a revitalisation time after mechanical stress in the form of scratching.

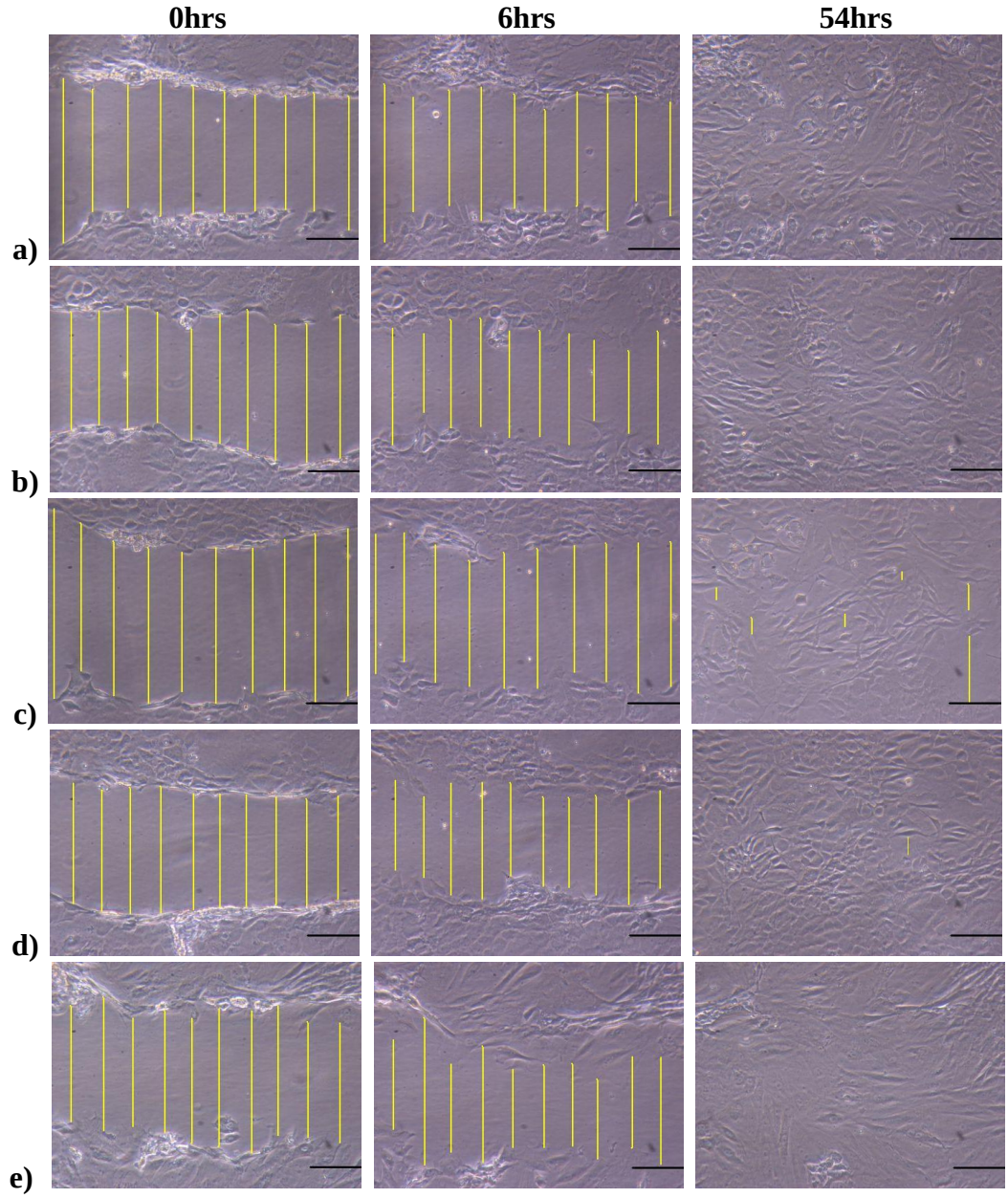


Figure 5.2.10 Microphotographs of wound closure assay for primary chondrocyte: a) Control; b) HCl; c) BSA; d) BSA/HCl, and e) TGFβ-2. Monolayer cultures were scratched by tip of a plastic pipette of 1 mm ϕ and measured using image analysis software. An average wound size of~131.77 µm was recorded after initial scratch at 0 hours (Scale bar = 50µm).

The results of wound closure assays were set as a normalized percentage average. The outcomes were used to plot a graph to demonstrate the comparison of the wound closures of chondrocytes cultured in monolayer with various supplementations, as shown in Figure 5.2.11. From this Figure it can be seen that the wound models of control and HCl contained cultures were closed at a slower rate after 54 hours and those supplemented with BSA (2.88 µm ± 0.4 SE), BSA/HCl (1.38 µm ± 0.4 SE), and TGF-β2 (0.33 µm ± 0.1 SE) closed completely, after 54 hours but at a faster rate, with almost complete closure (~ 98%, Figure 5.2.11) after ~ 30 hours.

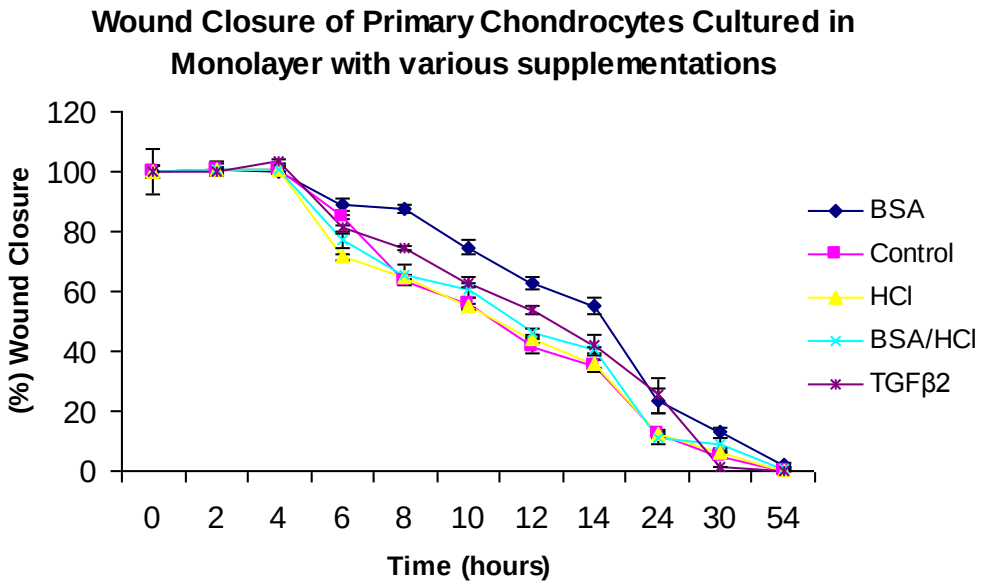
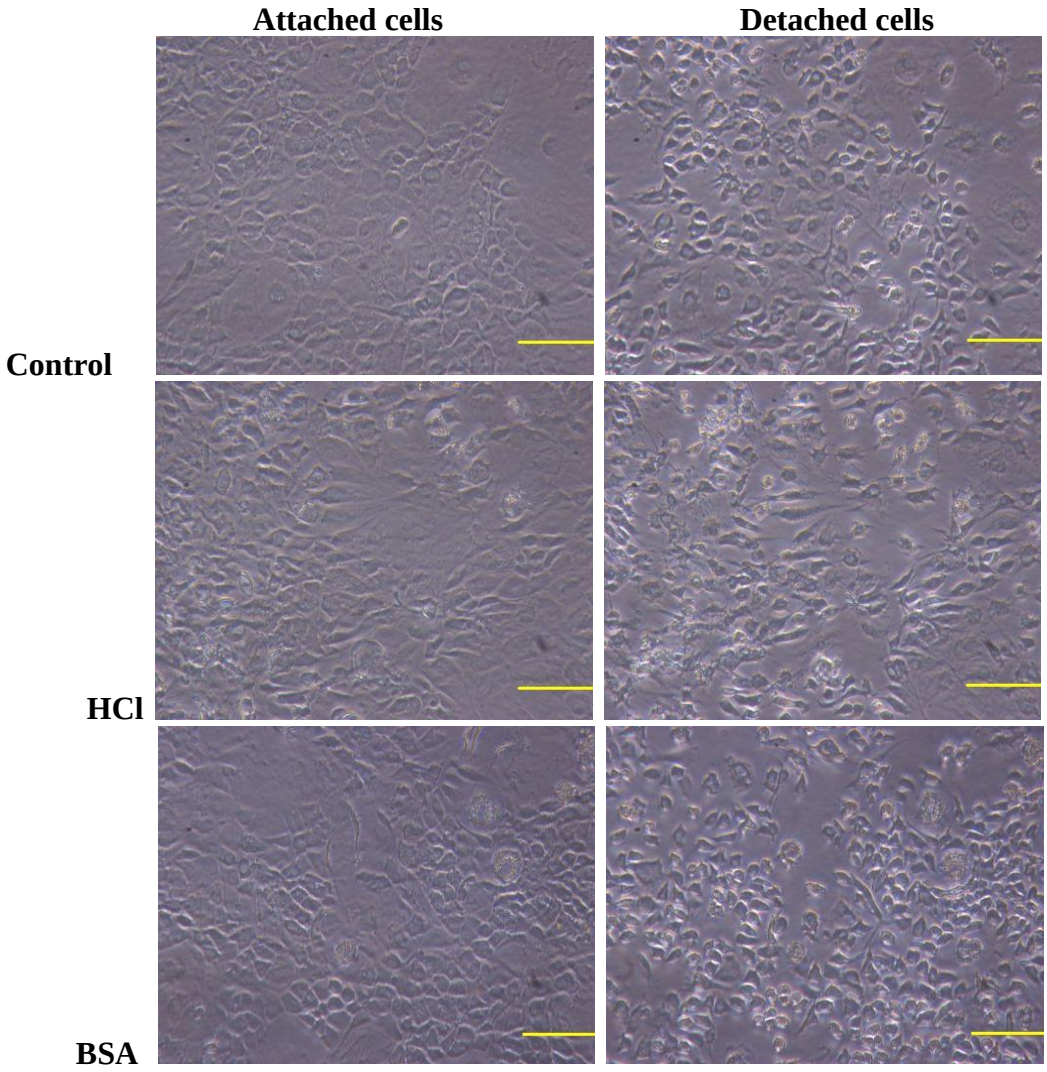


Figure 5.2.11 Graph of wound closure for primary chondrocytes cultured in BSA, BSA/HCl, HCl, and TGF-β2 contained media and control.

Results from trypsinisation assay (See Figure 5.2.12) demonstrated the time required for detachment of chondrocyte cells from substrate and separation from each other. This

time shows the strength of cell-substrate and/or cell-cell adhesion. Chondrocytes cultured in the presence of 1% v/v of 4mM HCl were detached in 7.778 minutes, which corresponds to being 6.87% longer than control culture. This time for TGFβ-2 was the lowest with 5.056 minutes, which caused the cells to detach 2.22 minutes faster than the cells in the control culture. The cells in BSA and BSA/HCl contained mediums showed recognisable reductions in detachment time of 5.94 and 6.05 minutes, respectively.



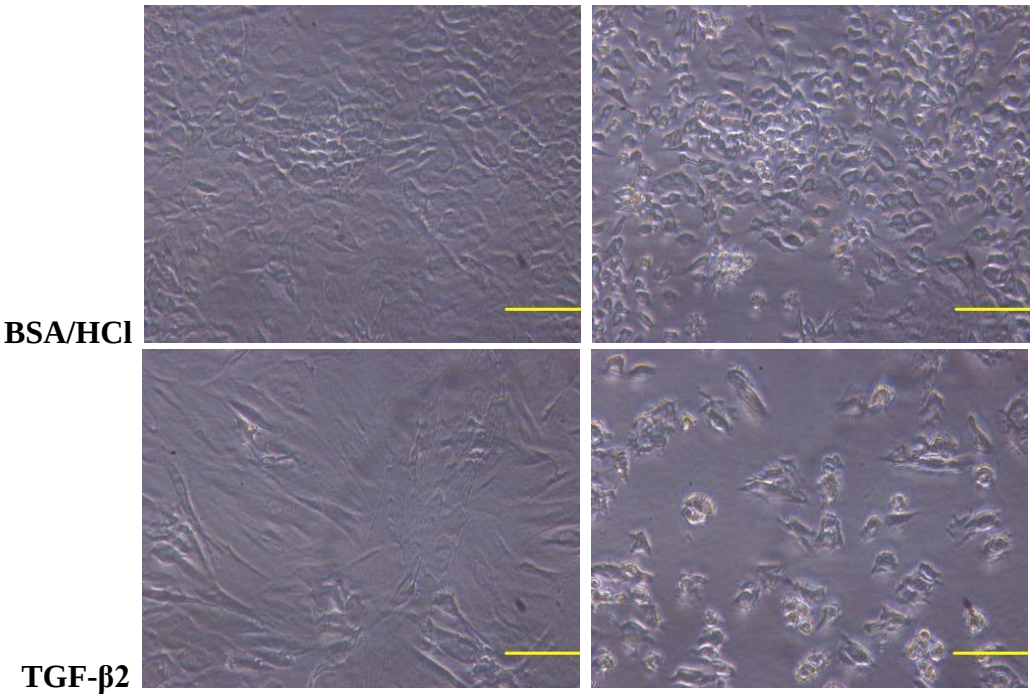


Figure 5.2.12 Trypsinization assay before (left) and after (right) detachment, (Scale bar = 50μm).

The above set of experiments revealed that TGFβ-2 has an inhibitory effect on chondrocyte cell proliferation and reduces cell density in the presence of 10% FCS in planar culture system. This type of growth factor induces an increase in cell size by synthesis of extracellular matrix proteins and by stimulating the cell to acquire fibroblastic morphology. Excessive ECM molecules capture a large area in the culture flask and consequently cause the cells to spread well in monolayer with weak cell adhesion ability. In contrast, 1% v/v 4mM HCl contained media promoted cell proliferation up to 10.12-fold, which is very close to the required cell density for autologous chondrocyte implantation (ACI).

The results of statistical analysis revealed that the null hypothesis was rejected in all tests, as in all tests the F > Fcrit and the P < 0.05, except for wounds repair, which showed that the model wounds on the chondrocyte monolayers with different supplementations were almost similar in terms of wound closure response after 54 hours (Figure 5.2.11 and Table 5.2.1).

ANOVA (Proliferation)					
Source of Variation	df	MS	**F**	**P-value**	**F crit**
Between Groups	5	8.56E+11	**96.86329**	**2.27E-33**	**2.323126**
Within Groups	84	8.84E+09			

ANOVA (Cell size)					
Source of Variation	df	MS	**F**	**P-value**	**F crit**
Between Groups	4	12.21093	**8.685231**	**0.000154**	**2.75871**
Within Groups	25	1.405942			

ANOVA (Cell adhesion)					
Source of Variation	df	MS	**F**	**P-value**	**F crit**
Between Groups	4	3.6	**9.436893**	**0.001996**	**3.47805**
Within Groups	10	0.381481			

ANOVA (Wound repair)					
Source of Variation	df	MS	**F**	**P-value**	**F crit**
Between Groups	4	750.0941	**0.303277**	**0.874411**	**2.557179**
Within Groups	50	2473.298			

Table 5.2.1 Evaluation of variation in the cell proliferation, size, adhesion, and wound repair of chondrocyte cells cultured in DMEM media with different supplementations using One-way ANOVA.

CHAPTER V

5.3 Transforming Growth Factor-β3

5.3.1 Materials and Methods

The following method was designed to investigate the effect of transforming growth factor-beta3 on the biological regulation of chondrocyte.

Primary chondrocyte cells from the fourth passage were cultured in monolayer, expanded and prepared for this study. The cells were cultured for a period of 132 hours to measure cell sizes and evaluate the proliferation rate. Wound-closure assessment was performed by cultivation of chondrocyte on a planar culture system and performing a scratch assay.

Microstructural analysis was performed regularly on daily basis using light microscopy, image J software and statistical analysis using Excel and SPSS.

The experiments were performed three times identically to ensure repeatability and for reliable results.

5.3.1.1 Culture of Chondrocyte Cells

Primary chondrocyte cells were isolated from the articular joint of six five-day old neonate Sprague-Dawley rats and purified according to our protocol described in Chapters two and three.

Isolated chondrocytes from the fourth passage were cultured and high glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, UK), supplemented with 10% fetal calf serum (Promocell, UK) and 0.1% w/v hyaluronic acid (Sigma Aldrich, UK) in planar culture system after expansion, were utilised for these experiments.

5.3.1.2 Solvent

Transforming growth factor beta3 (TGF-β3) is a type of protein produced by almost all types of cell (Schwartz et al., 1993). It is soluble in an acidic environment. It is also soluble in water; however, the result would be a sticky jelly (Wallace and Rosenblatt, 2003). Hence, it is not appropriate to dissolve it in water and aliquot in micro-litre scope, as the final solution is less than 1ml.

Hydrochloric acid is an appropriate solvent for all types of transforming growth factor that promises to provide a TGF-β solution and proper aliquot. The solvent also contained 1mg/ml bovine serum albumin (BSA) (Sigma Aldrich, UK) as a protein carrier.

5.3.1.3 Preparation of Solutions

Human recombined transforming growth factor (Sigma Aldrich, UK) was utilised for this experiment. It was diluted, according to supplier recommendation, with hydrochloride acid (HCl), and bovine serum albumin (BSA).

The dilution equation was used to prepare 4mM hydrochloric acid (HCl) from an available 2.5M HCl.

According to the supplier instructions, 10mg bovine serum albumin was dissolved in 10ml of 4mM hydrochloric acid to obtain 1mg/ml HCl/BSA. Following this, the solution was sterilised, using a 0.22µm filter.

2µg of TGF-β3 was dissolved in 0.4ml of 1mg/ml HCl/BSA, aliquoted into forty 10µl vials and stored at -20ºC.

5.3.1.4 Cell Size Analysis

350.000 chondrocyte cells were resuspended in 5ml high glucose (4500 mg/L) Dulbecco's modified eagle medium (DMEM) (Sigma Aldrich, UK), supplemented with FCS 10% v/v (Promocell, UK), 2.5 mM U/ml L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1µg amphoterysin B (Fungizone) (Sigma Aldrich, UK), and signed as control.

The cell suspension was seeded in a 25cm² cell culture flask and incubated at 37ºC until 70-80% confluency.

50µl of prepared TGF-β3 was added to another chondrocyte cell suspension with the same volume and cell density as control to make a concentration of 10ng/ml transforming growth factor-beta3. After 12 hours the non-attached cells were removed, fresh media were added to both control and TGF-β3 contained culture flasks. Every 24 hours the process of confluency was microstructurally analysed and imaged by light microscope. The sizes of 270 randomly selected cells were measured for analysis, and culture flasks were incubated again at 37°C until full confluency.

5.3.1.5 Cell Proliferation Analysis

To evaluate the effect of TGF-β3 on chondrocyte cell proliferation, primary chondrocytes were cultured in monolayer without and with TGF-β3 supplementation.

10ml cell suspension with a cell density of 70000 cell/ml was subjected to this experiment. 50µl of human, recombined TGF-β3 (Sigma Aldrich, UK), with a dilution ratio of 10µg/ml, was added to 4.950µl cell suspension to obtain a final concentration of 10ng/ml and seeded in a 25cm² tissue culture flask. The remaining 5ml cell suspension was seeded in a similar culture flask without addition of TGF-β3. Both cell cultures were incubated at 37ºC for 132 hours. The media were replaced with fresh media every 48 hours.

After 132 hours both cell cultures were 100% confluent. The media were removed and the culture flasks washed three times with Hank's balanced salt solution (HBSS). After aspiration of third HBSS, 2ml of 0.25% Trypsin-EDTA was added to the culture flask to dissociate chondrocyte cells from the surface of the culture flask. Detached cells were counted using a haemocytometer and compared with the initial cells. The amount of initial cells at the beginning and at the end of the experiment were standardised and a graph was produced to show how TGF-β3 affected proliferation of chondrocyte in a planar cell culture system.

5.3.1.6 Wound Healing Assay

The primary chondrocyte cells were cultured in 25 cm² tissue culture flasks without and with 10 ng/ml TGF-β3 supplementation. The cultures were incubated at 37ºC until full confluency. The media were changed every 48 hours.

To assess the effect of TGF-β3 on the cartilage repair capacity, a wound model was created via scratching the confluent cell layer of chondrocyte monolayer cultured in both culture flasks. The scratch was performed by the tip of a polyethylene plastic pipette with a tip size of 1mm in diameter. The average od initial wound size was 261.3 µm.

The model wounds were imaged using ImageJ (NIH) software every two hours until the first gap was closed, which was the sign of a complete wound closure. The earliest wound repair was observed after 48 hours for control. All images were recorded and saved for future analysis.

For precise statistical analysis, the experiment was repeated three times and the mean wound size (± SE) was calculated.

5.3.1.7 Cell Adhesion Analysis

The chondrocyte cells were cultured in 25 cm^2 TC grade culture flasks without and with 10ng/ml transforming growth factor-beta3 and incubated at 37°C until full confluency. To analyse the strength of chondrocyte attachment on the solid surface, the confluent monolayer chondrocyte culture was trypsinized by 0.25% Trypsin-EDTA solution (Sigma Aldrich, UK). Before trypsinization, the media were removed and the cell culture was washed three times with PBS or alternatively with Hank's balanced salt solution (HBSS) (Sigma Aldrich, UK). HBSS was aspirated and 4ml of trypsin was added to the cell culture. Following the trypsinization process, the chondrocyte culture was imaged by light microscope for further microstructural and statistical analysis.

The chondrocyte detachment time in both control and TGF-β3 treated cultures was measured and compared. The trypsinisation assay was performed at room temperature, the data was used to evaluate cell adhesion and a graph was drawn to demonstrate the different cell detachment time.

5.3.2 Statistical Analysis

Statistical analysis was performed to determine the normality and accuracy of the data, graphs were drawn and results compared. The means of all data were quoted ± standard error (SE).

5.3.3 Results and Discussion

5.3.3.1 Cell Size Analysis

Addition of transforming growth factor-beta3 to the cell culture media did not influence the size of the primary chondrocytes. The initial size of the chondrocyte cells in both TGF- β3 contained media and the control culture were 15.68 μm ± SE. However, the size of the cells in the control culture after 12, 36, 60, 84, 108, and 132 hours were 29.29 ± 0.46, 39.14 ± 0.54, 45.34 ± 1.43, 44.99 ± 2.91, 40.45 ± 2.45, 44.05 ± 0.33 μm respectively. These sizes for chondrocytes in the TGF-β3-contained environment were 33.11± 0.54, 39.83 ± 0.46, 40.36 ± 0.28, 44.04 ± 0.64, 48.55 ± 1.92, 49.76 ± 1.58 μm , respectively (Figures 5.3.1-5.3.3 and Table 5.3.1). One-way ANOVA test was used to compare the size of the cells in both cultures. Statistical analyses of variation of cell size versus time indicated that the assumption of null hypothesis was accepted. Null hypothesis was an assumption of equal mean sizes of chondrocytes cultured in control,

and TGF-β3 supplemented media. The results of ANOVA test (F = 0.086, Fcrit = 4.747, P = 0.774) showed that F < F crit, and P > 0.05, which is an acceptance of the null hypothesis.

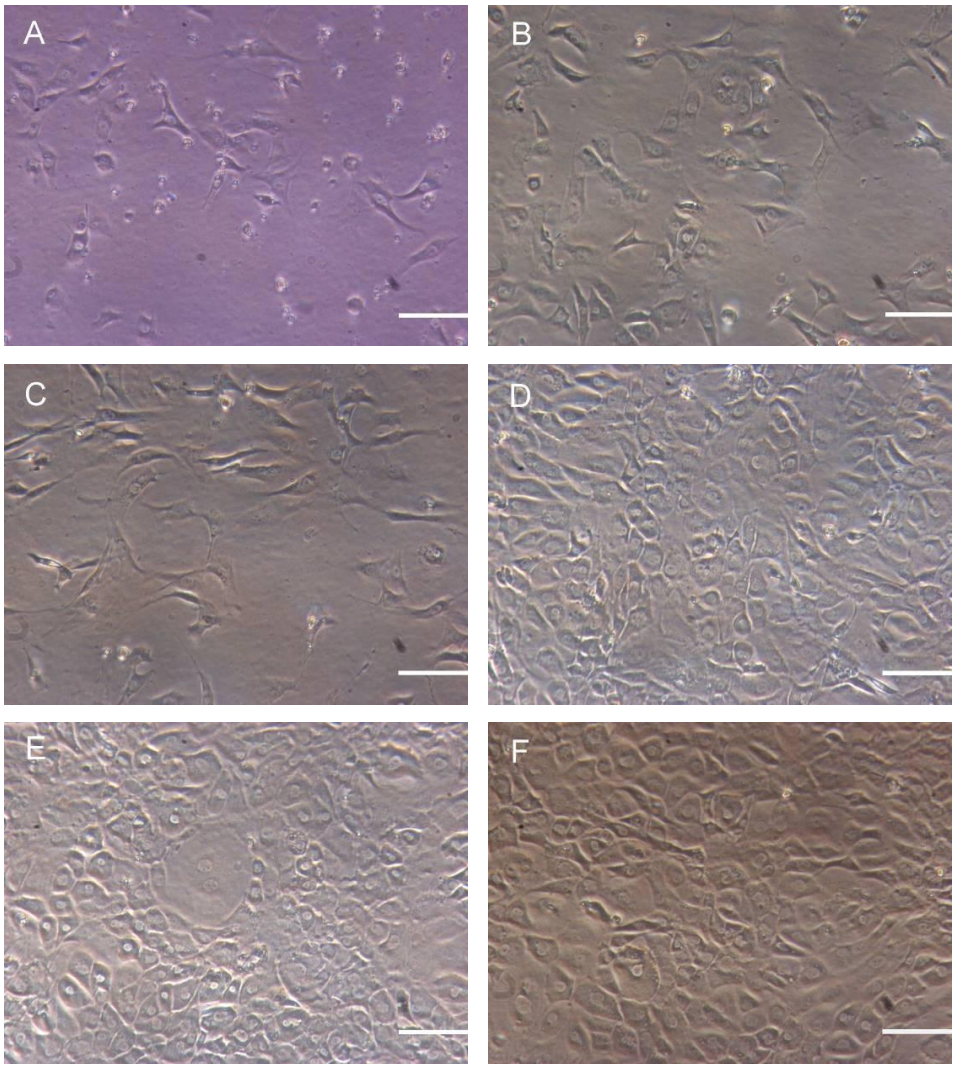


Figure 5.3.1 Culture of primary chondrocyte without TGF-β3 addition after: a) 12, b) 36, c) 60, d) 84, e) 108 and f) 132 hours (Scale bar = 100μm).

Primary chondrocyte culture without addition of transforming growth factor-beta3 exhibited a rounded morphology, which was a sign of chondrocyte at differentiation stage (Kim et al., 2009).

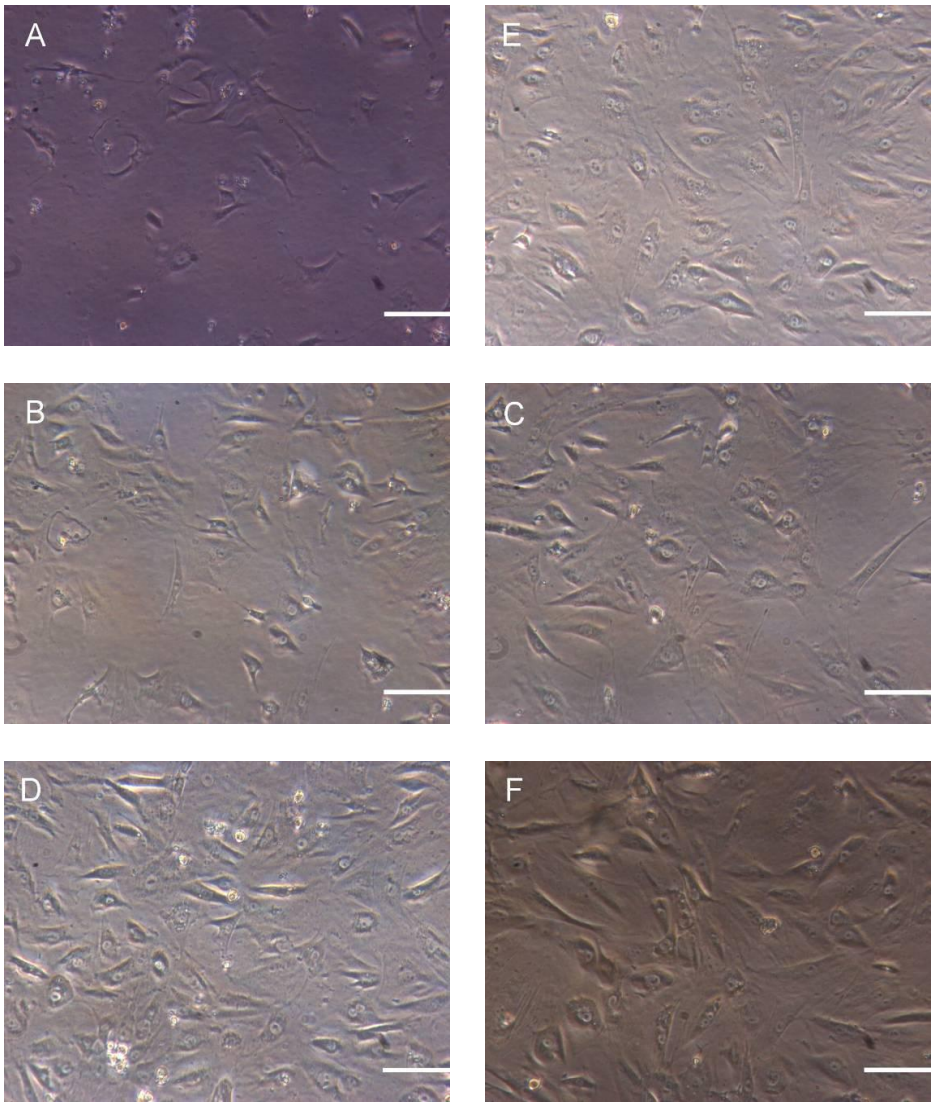


Figure 5.3.2 Chondrocyte cell size, supplemented with TGF-β3 after: a) 12, b) 36, c) 60, d) 84, e) 108 and f) 132 hours (Scale bar = 100μm).

Control culture of chondrocytes over 132 hours also showed an increase in cell size, (See Table 5.3.1). However, after 60 hours the cell size decreased from 45.34μm to 40.45μm. This reduction may have happened at the stage of chondrocyte mitosis. In contrast, the cells cultured in media with TGF-β3 supplementation revealed an increasing cell size up to 132 hours (Table 5.3.1).

Hours	0	12	36	60	84	108	132
Control	15.68µm ± 0.63	29.29µm ± 0.46	39.14µm ± 0.54	45.34µm ± 1.43	44.99µm ± 2.91	40.45µm ± 2.45	44.05µm ± 0.33
TGF-β3	15.68µm ± 0.63	33.11µm ± 0.54	39.83µm ± 0.46	40.36µm ± 0.28	44.04µm ± 0.64	48.55µm ± 1.92	49.76µm ± 1.58

Table 5.3.1 Alteration of cell size of chondrocytes cultured without and with TGF-β3 supplementation during 132 hours (Mean ± SE).

The statistical data was used to plot a graph to compare the change in cell size with the addition of TGF-β3 to the culture (Figure 5.3.3).

Results from the one-way ANOVA test ($F = 268.16$, $P = 0.0001$, $F\ crit = 5.31$) confirmed a significant difference between the proliferation rate of the control culture and the TGF-β3 contained culture, as $F > F\ crit$ and $P < 0.05$.

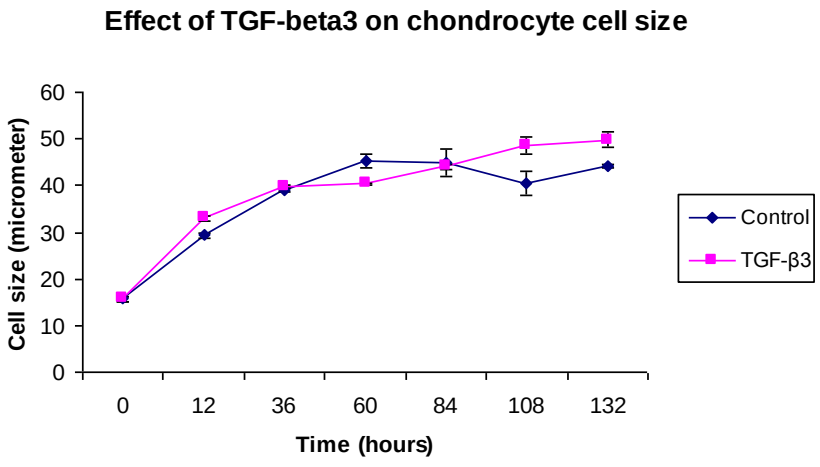


Figure 5.3.3 Graph showing change in chondrocyte cell size cultured for 132 hours without and with TGF-β3.

5.3.3.2 Cell Proliferation Analysis

The result of the cultivation of primary chondrocyte culture without and with TGF-β3 supplementation showed that transforming growth factor-beta3 reduces chondrocyte cell proliferation by increasing cell apoptosis (Figure 5.3.4).

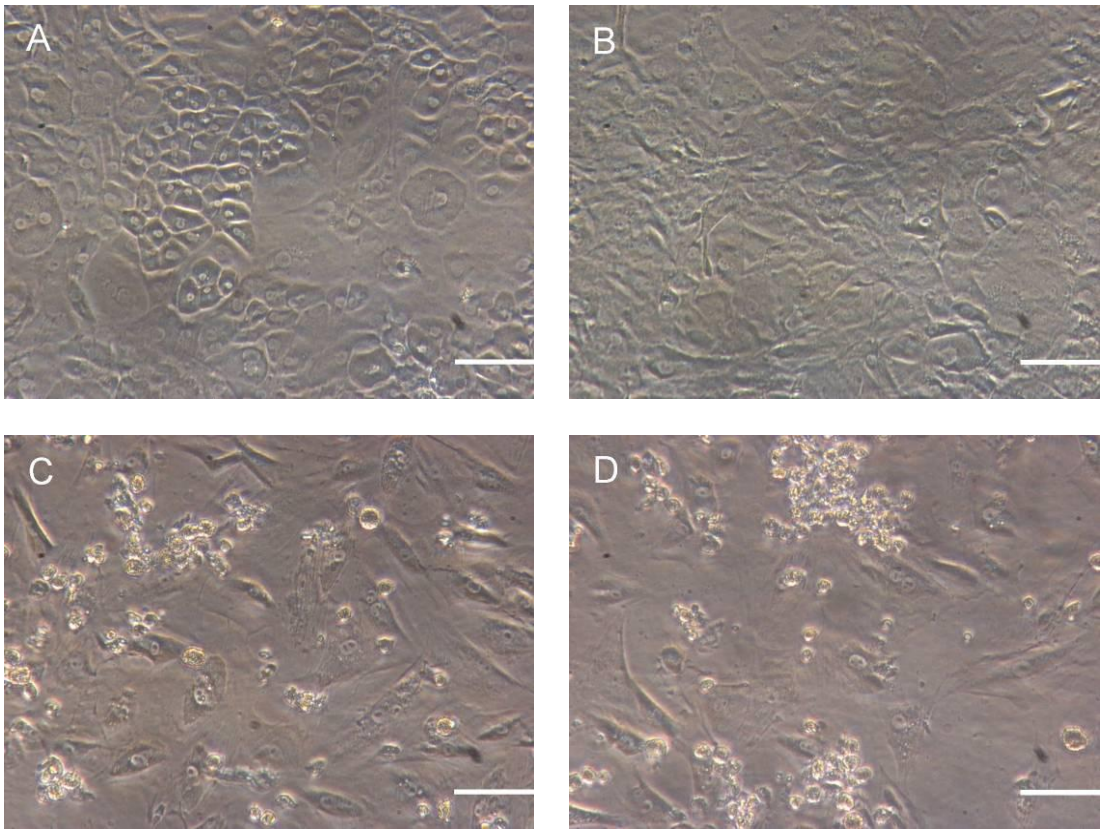


Figure 5.3.4 Proliferation and apoptosis of chondrocyte cells a) and b) without, c) and d) with TGF-β3 supplementation (Scale bar = 100μm).

Microstructural analysis of chondrocyte cells cultured in transforming growth factor-beta3 contained medium showed partially rounded and shiny cells, which are the sign of detached, dead cells (Figure 5.3.4).

After 132 hours of chondrocyte cell culture, the number of initial seeded cells, 70000 cell/ml ± 4000 SE, was increased to 270000 cell/ml ± 13.416 SE. In contrast, the number of initial cultured chondrocytes, 70000 cell/ml ± 4000 SE, was decreased to 46000 cell/ml ± 2666 SE for TGF-β3 contained culture flask (See Table 5.3.2).

Hours	**0**	**132**
Control	70000 ± 4000	270000 ± 13416
TGF-β3	70000 ± 4000	46000 ± 2666

Table 5.3.2 Comparison of chondrocyte cell proliferation between control and TGF-β3 contained media.

The statistical data was used to plot a graph showing the number of cells for initial, control and TGF-β3 contained culture after 132 hours, as shown in Figure 5.3.5.

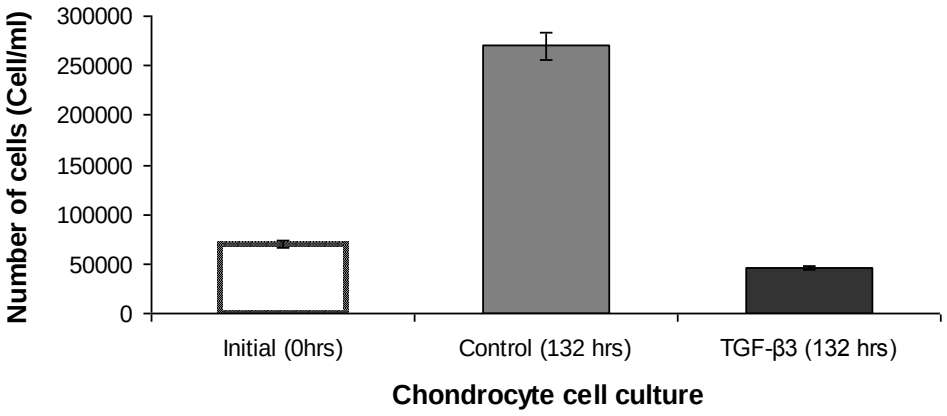


Figure 5.3.5 Graph showing the initial number of chondrocyte cell and after 132 hours in medium without and with TGF-β3 supplementation.

5.3.3.3 Wound-Healing Assay

Wound-closure assay of primary chondrocyte cells cultured on planar culture system showed that the modified wound model on control culture was repaired after 48 hours. The created wound width was initially 238.07 μm ± 5.37 μm and after 48 hours this wound was closed completely (Figure 5.3.6).

There was ca. 4-6 hours' delay in the wound-closure process and in the migration of cells into the wound area after creation of the model wound. The cells started to migrate into the gap after partial removal of the dead cells from the wound edge. This delay was a demonstration of stress-responsive cells.

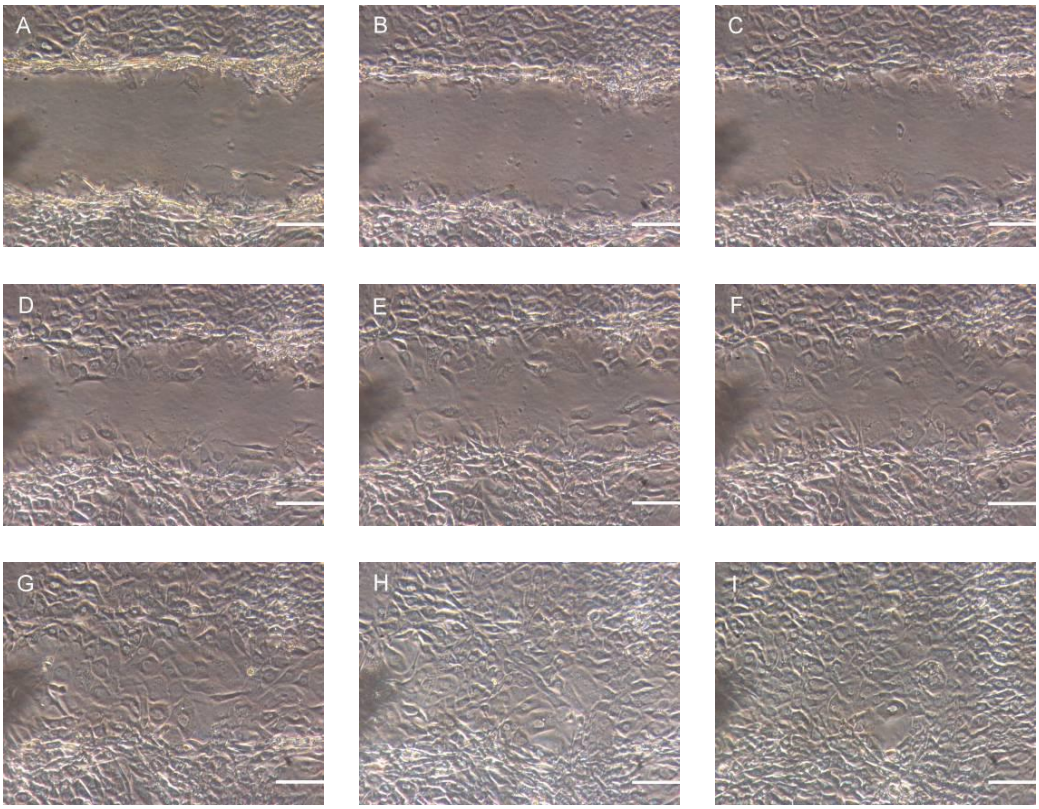


Figure 5.3.6 Images of wound closure of monolayer for the control culture of chondrocyte monolayer: a) 0, b) 2, c) 4, d) 6, e) 8, f) 10, g) 18, h) 24 and i) 48 hours respectively (Scale bar = 100μm).

In contrast, the generated model wound on chondrocytes monolayer with TGF-β3 supplementation showed no complete repair after 48 hours. The initial created wound width was 283.95 μm ± 12.19 μm and after 48 hours reduced to only 130.11 μm ± 22.05 μm (Figure 5.3.7).

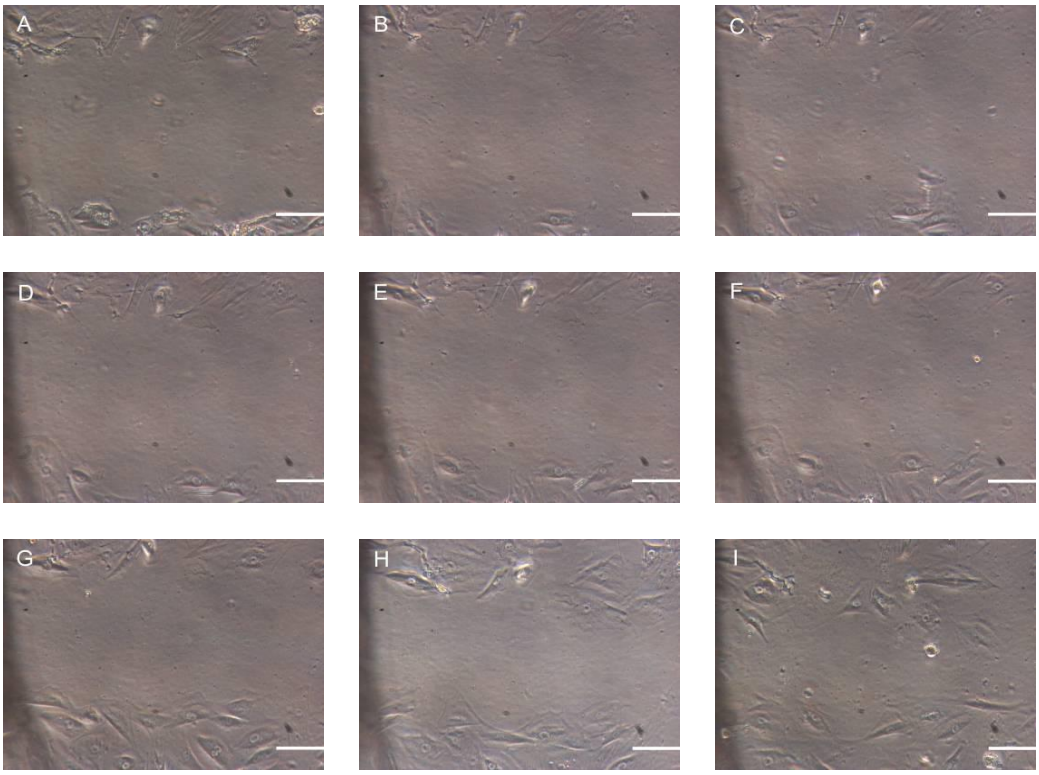


Figure 5.3.7 Images of wound closure assay of chondrocyte monolayer culture with 10 ng/ml TGF-β3 supplementation: a) 0, b) 2, c) 4, d) 6, e) 8, f) 10, g) 18, h) 24 and i) 48 hours (Scale bar = 100 μm).

Figure 5.3.8 also shows and compares the progression of wound healing of chondrocyte in a planar culture system without and with TGF-β3 supplementation. As can be seen from the graph in Figure 5.3.8, the wound width created on control culture is zero after 48 hours, as compared to the wound of the TGF-β3 culture showing only 60% closure and no sign of complete wound closure, even after 48 hours. Down regulation of chondrocytes proliferation by TGF-β3 (See Table 5.3.2 for detail) might be a reason for not completely wound closure. High level of nitric oxide in wound edge produced by

damaged cells could be another cause increase of apoptosis and consequently slowing down of the wound repair process.

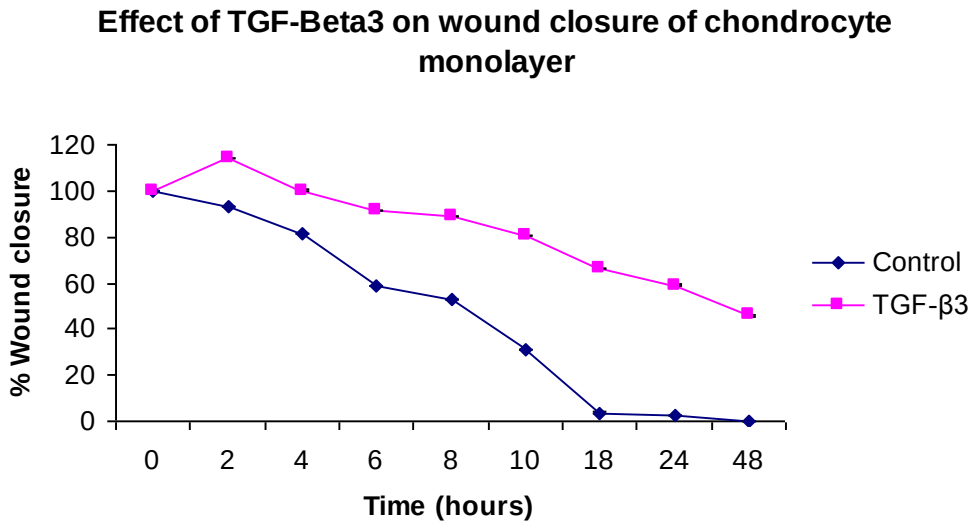


Figure 5.3.8 Graph of %wound closure vs time for the chondrocyte monolayer without and with TGF-β3 supplementation.

A one-way ANOVA test was used to examine the wound-width difference of both wound-closure assays. The results of statistical data analysis showed that the F (5.63) is greater than F crit (4.49) and P (0.03) < 0.05. According to these results, the null hypothesis, the assumption of equal wound closure process, was rejected.

5.3.3.4 Cell-adhesion assay

Results from trypsinization assay showed that the chondrocyte cells of the control culture detached completely after 240 seconds. In contrast, the entire cells from the surface of culture flask with TGF-β3 supplementation detached in 90 seconds (Figure 5.3.9-5.3.10).

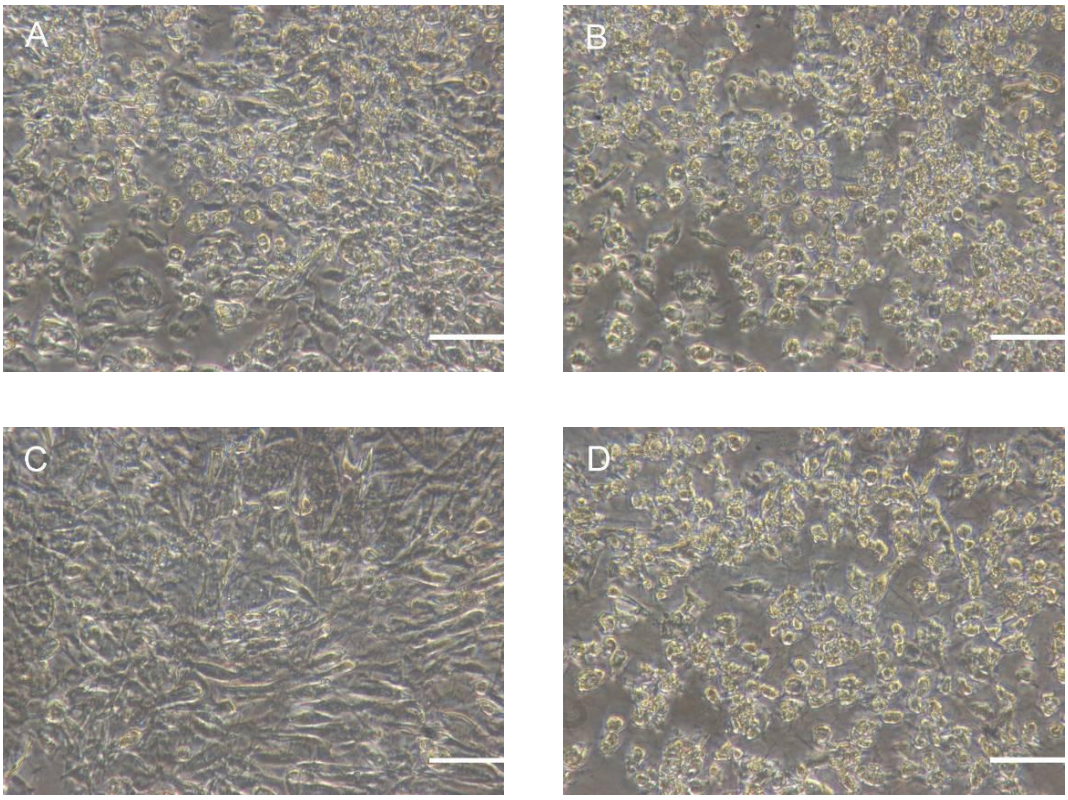


Figure 5.3.9 Images of chondrocyte cells' detachment: a) control at 0 seconds, b) control after 240 seconds, c) TGF-β3 contained culture at 0 seconds, and d) TGF-β3 after 90 seconds (Scale bar = 100 µm).

After about 90 seconds, chondrocyte cells were detached from the surface of the tissue culture flask, which was supplemented with 10ng/ml TGF-β3. Unlike the chondrocyte culture with TGF-β3 addition, the cells of the control culture required 240 seconds to detach from the surface of the culture flask (Figure 5.3.10).

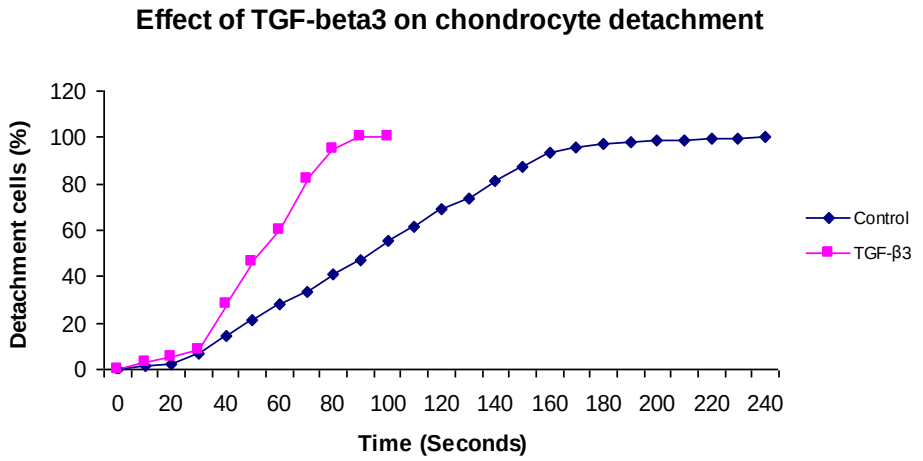


Figure 5.3.10 Graph of trypsinization assay for chondrocyte cell cultured without and with TGF-β3 supplementation.

The difference between chondrocytes detached from the control culture and the TGF-β3 contained media was ~150 seconds. This time difference revealed that the transforming growth factor-beta3 facilitated the detachment of chondrocyte cells from a solid surface, by almost 1.5 times.

5.3.4 Overall Discussion

Results from cell-size measurement revealed that presence of transforming growth factor-beta3 had no significant effect on chondrocyte cell size. However, this cytokine caused regulation of chondrocyte apoptosis and therefore facilitated the process of cells occupying larger area, spreading around and getting a flatter shape. This occurrence caused the cells to develop a fibroblast-like morphology. Although the chondrocytes with fibroblastic shape occupied a larger area, the mean size of the chondrocytes in the TGF-β3 contained culture was only 5μm larger than the mean cell size in the control culture.

The culture of primary chondrocytes in planar culture system, and with the addition of TGF-β3, showed that this growth factor induced cell apoptosis and consequently caused down regulation of cell proliferation to 65.7% and a reduction of ~34.3% in initial cell density.

Wound-healing assay revealed that the process of wound repair for the chondrocytes monolayer culture with transforming growth factor-beta3 supplementation was much slower than wound repair progression for the control culture. The effect of TGF-β3 on down regulation of chondrocyte proliferation may be the reason for the non-completion of wound healing. Although the model wounds in both control and TGF-β3 contained media were created by the same pipette tip, the wound width on the TGF-β3 supplemented chondrocytes monolayer was 283.95 μm ± 12.19 μm, whereas the wound width for control culture was 238.07 μm ± 5.37 μm, and thus a normalized wound width was necessary.

The ability of adherent cells to sense and adapt to the mechanical stress created at the focal adhesions (FAs) is directly dependent on integrin-extracellular matrix and ECM-cytoskeleton interaction (Bordeleau et al., 2008). This might be the explanation for the delay in the wound-repair process (increase in wound width) after the creation of the model wound.

The measurement of cell-adhesion strength on the solid surface showed that TGF-β3 had a negative effect on cell-adhesion strength. Chondrocytes in TGF-β3 cell culture flask started to detach after ca. 30 seconds and after ~90 seconds complete detachment took place. In contrast, the cells from the control culture required ca. 240 seconds to detach from the surface of the culture flask. This suggests that transforming growth factor-beta3 induces down regulation of expression of some types of integrins.

CHAPTER V

5.4 Manipulated Transforming Growth Factor β-1, 2, and 3

5.4.1 Materials and Methods

5.4.1.1 Cell Culture

Primary chondrocyte cells were isolated from the articular cartilage of Sprague-Dawley neonate rats (See Chapter 2, Subsection 2.2.3, Protocol of isolation of chondrocyte cells).

Twenty-five millilitres of chondrocyte cell suspension with 80,000 cell/ml were prepared with DMEM cell culture media, supplemented with 10% FCS (Sigma Aldrich, UK).

Transforming growth factor-beta 1, 2 and 3 were reconstituted according to the supplier's recommendation (See Sub-chapters 5.1 - 5.3).

Five millilitres of the prepared chondrocyte cell suspension was transferred into a 25cm² tissue culture flask and labelled as control.

A total of 50ng of TGF-β1 and 50ng of TGF-β2 were added to 5ml cell suspension and incubated at 37°C. The same amount of TGF-β1 and TGF-β3 were added to another five-millilitre cell suspension. Similar manipulation process was performed to produce

5ml of cell suspensions containing TGF-β2&3 and TGF-β1&2&3. All culture flasks were labelled and incubated at 37°C.

5.4.1.2 Cell Size (Cell Length) Analysis

Photomicrographs of all five chondrocyte cultures, control, TGF-β1&2, TGF-β1&3, TGF-β2&3 and TGF-β1&2&3 were acquired every 24 hours to analyse the variation of cell size. The size of 30 randomly selected cells was measured by Image J (NIH) software immediately after the cells were attached on the surface of culture flasks. After 72 hours, the size of 30 randomly selected cells was measured again and compared with the initial calculated sizes. The difference between initial and final cell size was regarded as the change of chondrocyte cell size.

The experiment with all five chondrocyte cultures, without and with manipulated TGF-βs, was repeated three times and the mean cell size of each culture was (±SE) calculated.

5.4.1.3 Proliferation Rate Analysis

The effect of manipulated transforming growth factor-beta 1, 2 and 3 on the proliferation of primary chondrocytes was analysed. All five chondrocyte cultures were trypsinised after 72 hours and the detached cells were counted using a haemocytometer. The initial cell density, 80,000 cells/ml, was compared to the final counted cells.

The experiment was repeated three times to acquire accurate repeatable data and the mean (±SE) was calculated.

5.4.1.4 Wound Repair Analysis

To evaluate the effect of manipulated transforming growth factor-beta 1, 2 and 3 on the wound-repair capacity of cartilage, primary chondrocyte cells were cultivated in a planar culture system, and scratch assay was performed. To create the model wound, the confluent monolayer of cultured chondrocyte cells was scratched by the tip of a sterilised 3ml fine-tip extended transfer pipette with 1mm tip diameter.

To evaluate the mean size of the wound over a period of time, the wound width was imaged every two hours at the same point by a light microscope with 10x magnification until the modified model wound in the control culture flask was closed. The size of each wound was measured in different places with the same intervals and the mean (±SE) was calculated.

5.4.1.5 Cell Adhesion Analysis

To evaluate the strength of chondrocyte cell adhesion without and with manipulated transforming growth factor-βs, trypsinisation assay was performed at room temperature. Trypsin is a time-dependent proteinase (Tsybina et al., 2005), (Kuzmina and Pervushina, 2004) which digests the proteins by cleavage of peptide chains at the carboxyl side of amino acids (Olsen et al., 2004). These set of experiments were performed at 18°C, room temperature.

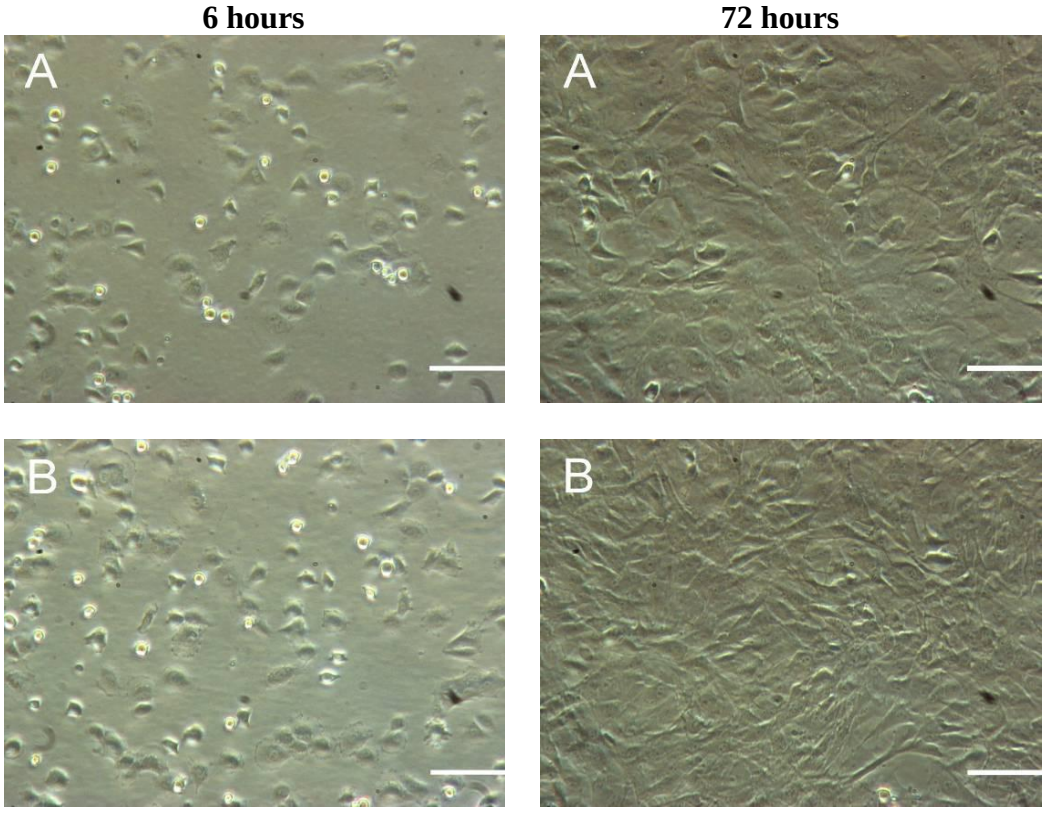
The media was removed from the culture flask and washed three times with Hank's balanced salt solution (HBSS) (Sigma Aldrich, UK). After aspiration of HBSS, 2ml of 0.25% trypsin-EDTA was added to the flask and the process of cell detachment was

imaged by light microscope. Detached cells appeared round and shiny under light microscopy. A set of images were taken at 10-second intervals until all cells developed a round and shiny morphology and detached from the culture flask.

5.4.2 Results and discussion

5.4.2.1 Cell size analysis

The results obtained from cell-size measurement of 30 randomly selected chondrocytes in different manipulated TGF-βs-contained medias revealed that the cell sizes varied between cell cultures (Figure 5.4.1).



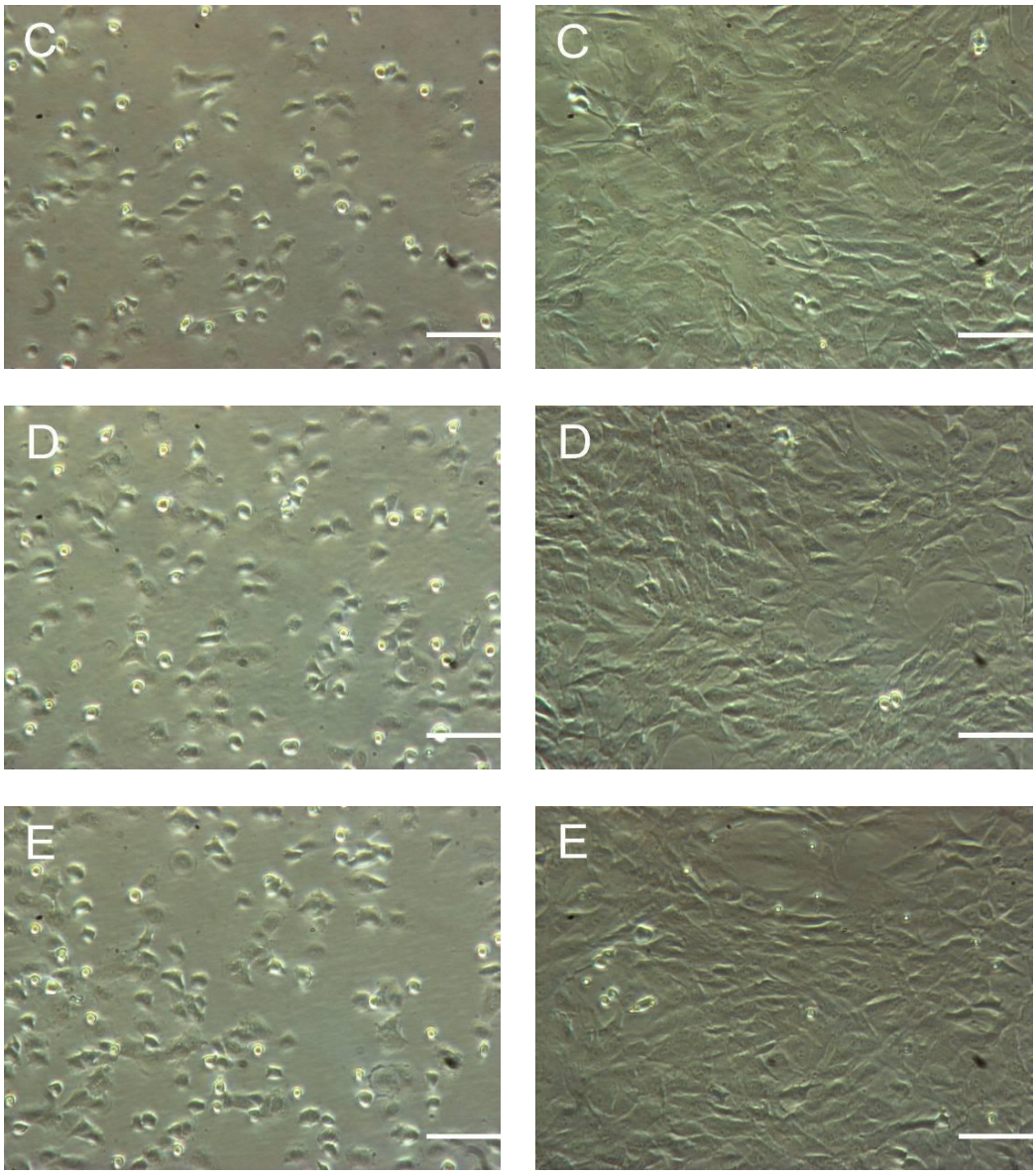


Figure 5.4.1 Photomicrographs of primary chondrocyte cells after six hours (left) and 72 hours (right): a) control culture; b) with TGF- β1&2; c) with TGF- β1&3; d) with TGF- β2&3; and e) with TGF- β1&2&3 additions, (Scale bar = 100 μm).

The mean size of the chondrocytes labelled as control culture was 25.4μm ± 0.85μm. This size for manipulated TGF- β1&2 was 22.33μm ± 0.69μm, for TGF- β1&3 23.24μm ± 0.69, for TGF- β2&3 22.88μm ± 0.59 and for TGF- β1&2&3 22.5μm ±

0.51µm. The cells in the control and all manipulated culture flasks revealed different sizes. The greatest cell-size difference after six hours was ~3µm between control with 25.4µm and TGF- β1&2 with 22.33µm cell size. In contrast, after 72 hours, the cell-size difference between control with 24.98µm and TGF- β1&2&3 with 20.36µm was 4.62µm (see Figure 5.4.2 and Table 5.4.1).

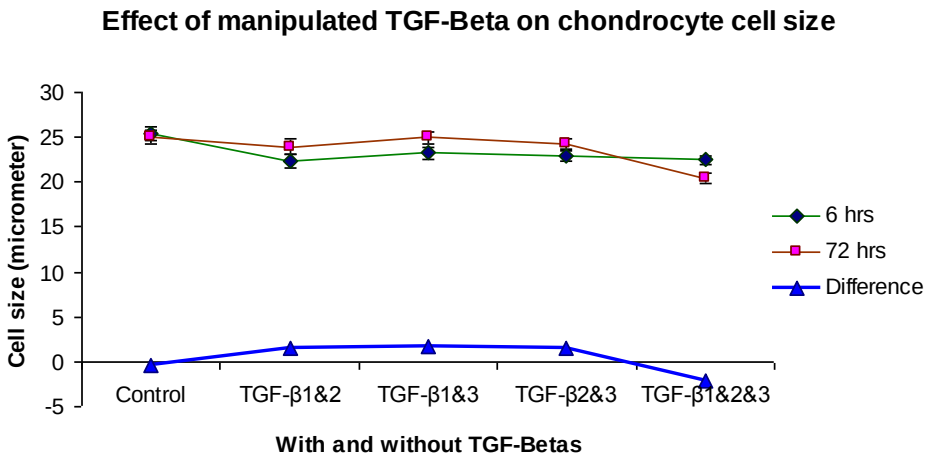


Figure 5.4.2 Graph of a comparison of chondrocyte cell size in DMEM media without and with different TGF-β manipulations.

The results from the analysis of variances (ANOVA) showed F = 3.3 > F crit = 2.43, and P = 0.01 < α = 0.05. As F > Fcrit, and P < α, these results indicated that the null hypothesis of variance could not be accepted. This meant that the mean size of the chondrocytes in each culture was unequal.

	Control	TGF-β1&2	TGF-β1&3	TGF-β2&3	TGF-β1&2&3
6 hrs	25.40	22.33	23.24	22.88	22.50
72 hrs	24.98	23.90	24.97	24.34	20.36
Difference	-0.42	1.57	1.73	1.45	-2.13

Table 5.4.1 The mean (μm) size of chondrocyte cells cultured without and with TGF-β manipulations.

As can be seen from Table 5.4.1, the mean size of chondrocytes in the control culture was reduced after 72 hours with a size difference of 0.42μm. Also, TGF-β1&2&3 showed reductions in cell size after 72 hours' culture. This reduction might be a consequence of cell division, apoptosis or down regulation of mammalian target of rapamycin (mTOR) which regulate cell size and proliferation (Fumarola et al., 2005). In contrast, the chondrocytes from manipulated TGF-β1&2, TGF-β1&3 and TGF-β2&3 showed an increase in cell size after 72 hours' cell culture (see Figure 5.4.2 and Table 5.4.1).

5.4.2.2 Proliferation Rate Analysis

The results of primary chondrocyte culture for 72 hours without and with manipulated TGF-β1, 2 and 3 showed significant difference in cell proliferation (See Figure 5.4.3). The control culture showed an increase in proliferation of chondrocytes up to ~ 5.9-fold. The maximum increase in cell numbers was found in the medium with manipulated TGF-β1&2 with a mean rate of 510,083 cell/ml ± 1303 SE, whereas TGF-β2&3 had a minimum effect on cell proliferation with a mean rate of 296,833 cell/ml ± 16427 (SE).

The results were standardised as percentages and used to plot a graph shown in Figure 5.4.3.

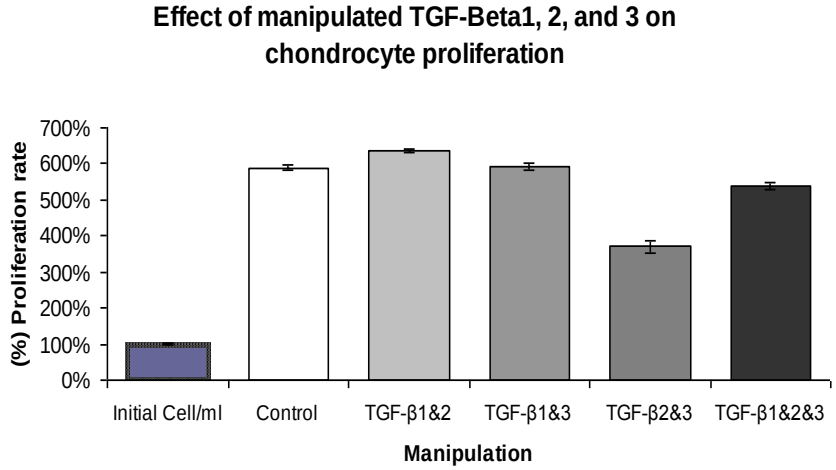


Figure 5.4.3 A comparison of chondrocyte proliferation rates between manipulated TGF-β and control culture with ± SE.

Statistical analysis of variation with the assumption of equal cell proliferation rate between control culture and all manipulated cell cultures was tested using ANOVA-test shown in Table 5.4.2.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	8.84333E+11	5	1.77E+11	**109.0778**	**2.55E-26**	**2.39295264**
Within Groups	84316550000	52	1.62E+09			
Total	9.6865E+11	57				

Table 5.4.2 Results of analysis of variation in the cell proliferation rate between control culture and manipulated TGF-β1, 2 and 3.

As can be seen from Table 5.4.2, the F = 109 > F crit = 2.39, and P = 0.001 < α = 0.05. These results proved significant variation to reject null hypothesis, which meant that the proliferation rates of the experiments were not similar. In terms of detail of ANOVA, ("SS") = Sum of Squares, ("df") = degree of freedom, which is one less than number of groups, ("MS") = Mean Square, ("F") = F-value, ("P") = P-value, and ("F crit") = F-critical.

The above experiments with manipulated TGF-βs revealed that the cell culture media containing 10ng/ml TGF-β1 and 10ng/ml TGF-β2 stimulated the primary chondrocytes to proliferate up to six-fold up to 72 hours.

5.4.2.3 Wound Repair Analysis

The results obtained from scratch assay of primary chondrocyte monolayer without and with manipulated TGF-β1, 2 and 3 showed that the best wound closure was achieved with manipulated TGF-β1&2 within 24 hours (mean wound width = 1.05μm ± 0.1 (Figure 5.4.5). Whereas the wound created in the culture flask with TGF-β2&3 supplementations demonstrated almost no wound repair after 30 hours (mean wound width = 3.2μm ± 1.0), as can be seen from Figure 5.4.8.

The cells started to migrate into the modified wounds after ~2 hours. This delay could be related to the cellular response to the mechanical stress generated and also to the time taken to remove the dead and damaged cells from the wound edges, as can be seen from the A and B parts of Figures 5.4.4-5.4.8.

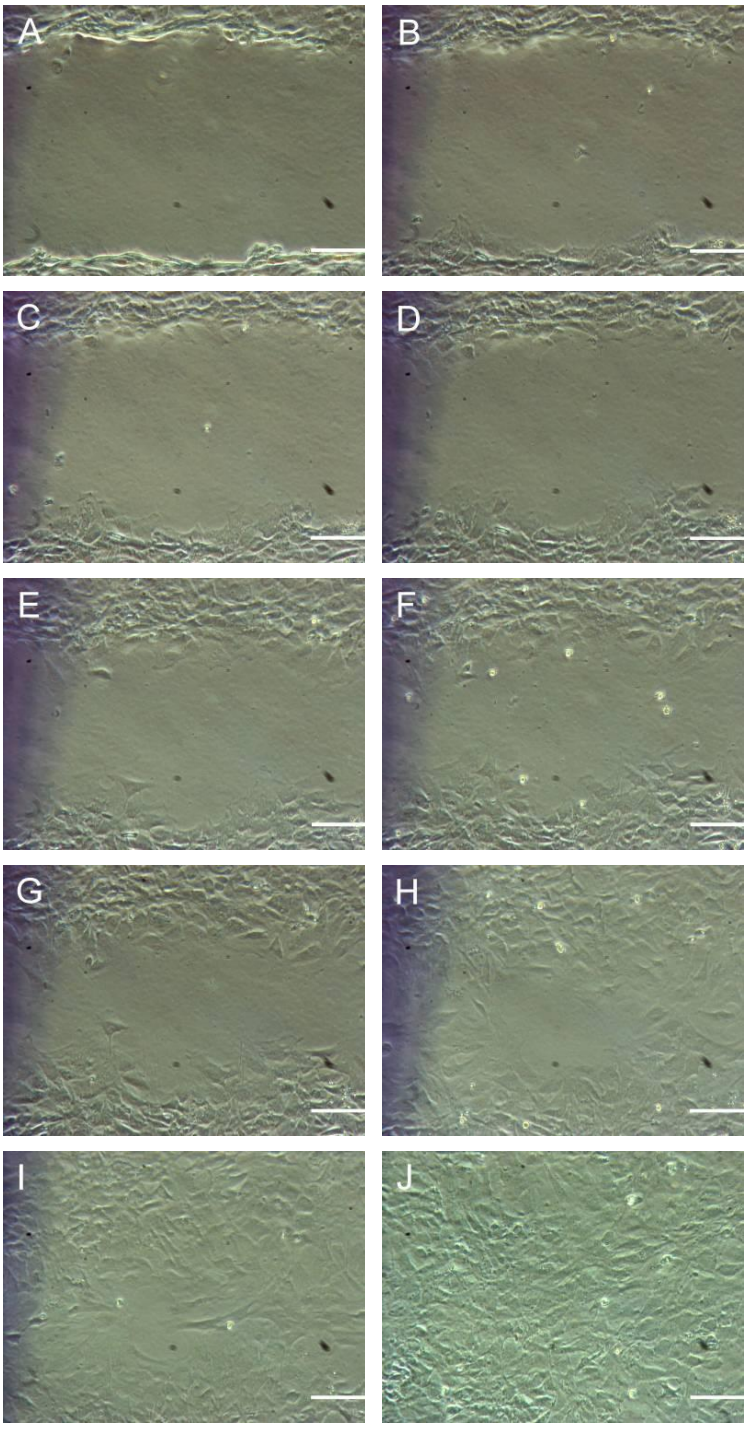


Figure 5.4.4 Micrographs of the wound-repair process for chondrocytes monolayers without addition of TGF-β after: a) 0; b) 2; c) 4; d) 6; e) 8; f) 10; g) 12; h) 18; i) 24; j) 30 hours, (Scale bar = 100 μm).

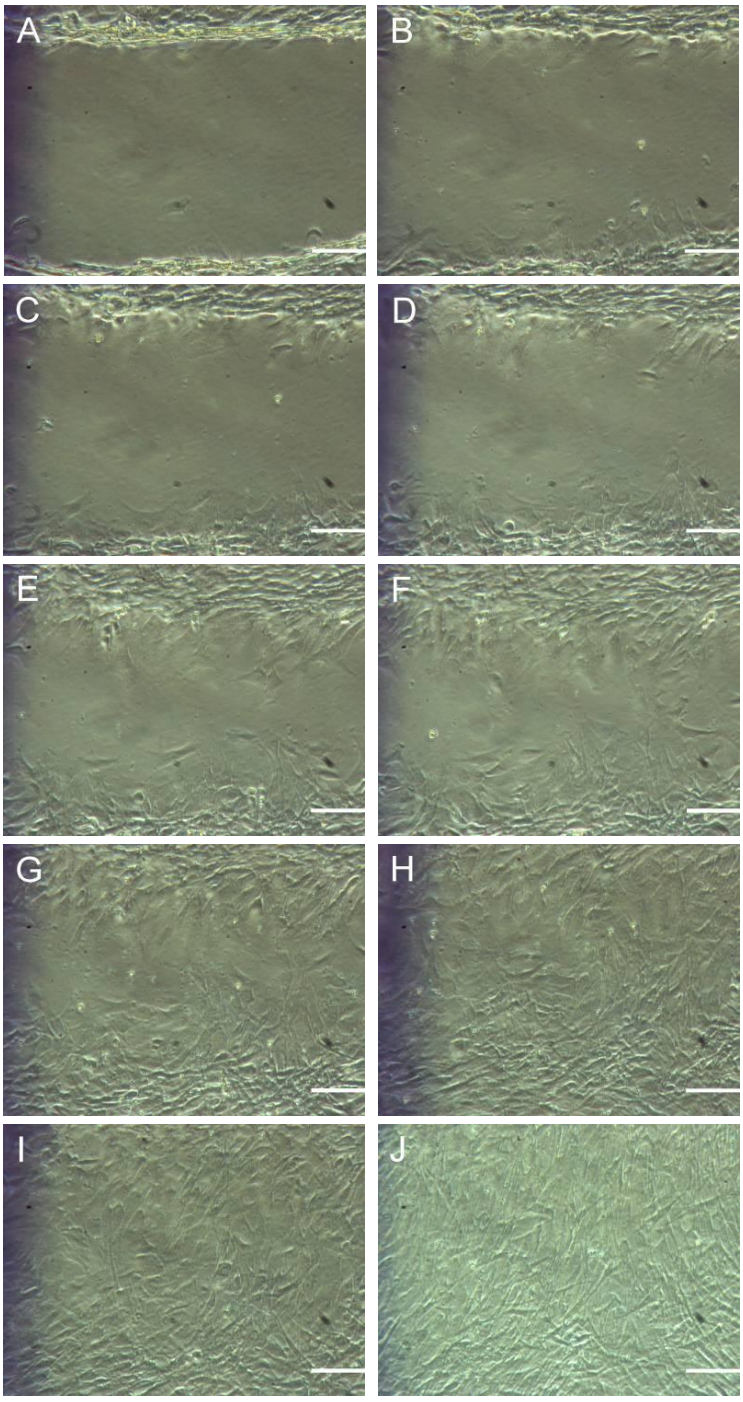


Figure 5.4.5 Micrographs of the wound-repair process for chondrocyte monolayers with manipulated TGF-β1&2 after: a) 0; b) 2; c) 4; d) 6; e) 8; f) 10; g) 12; h) 18; i) 24; j) 30 hours, (Scale bar = 100 μm).

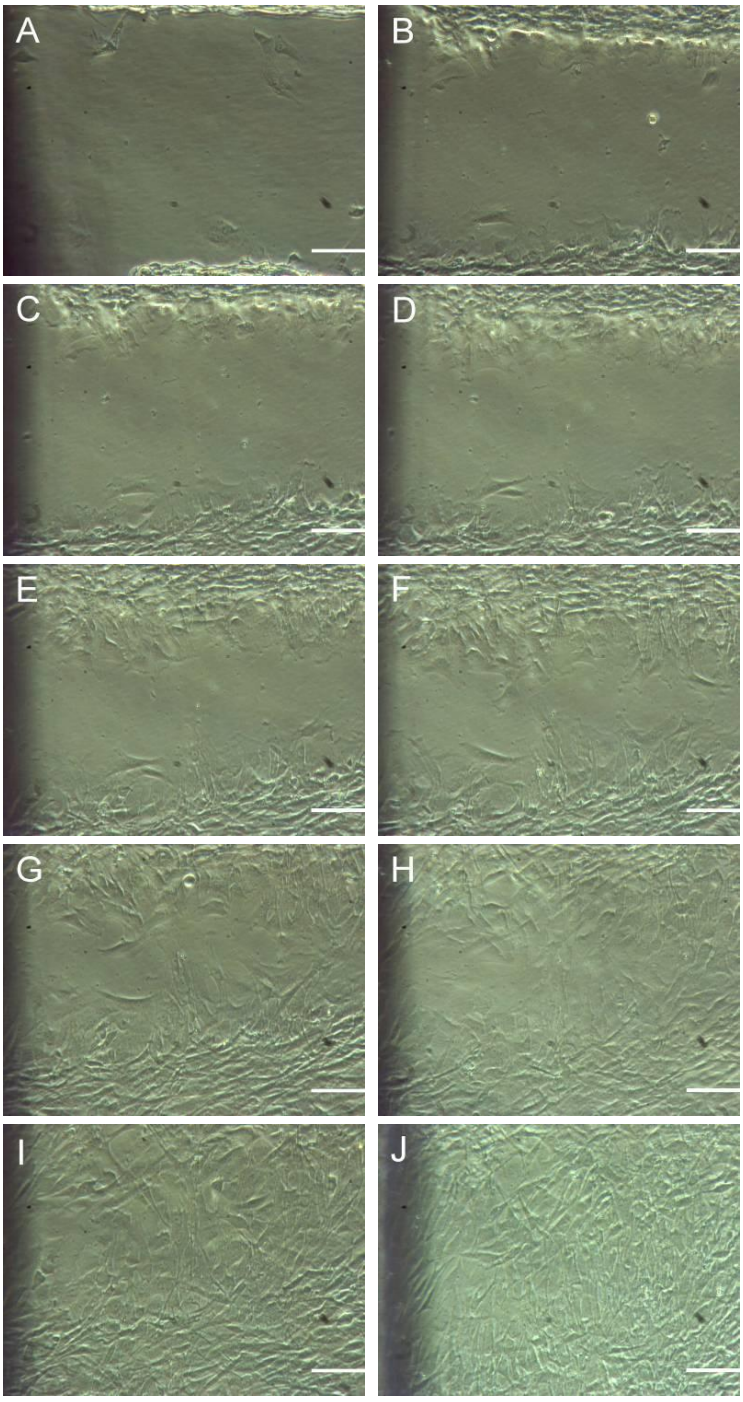


Figure 5.4.6 Micrographs of the wound-repair process for chondrocytes monolayers containing manipulated TGF-β1&3 after: a) 0; b) 2; c) 4; d) 6; e) 8; f) 10; g) 12; h) 18; i) 24; j) 30 hours, (Scale bar = 100 μm).

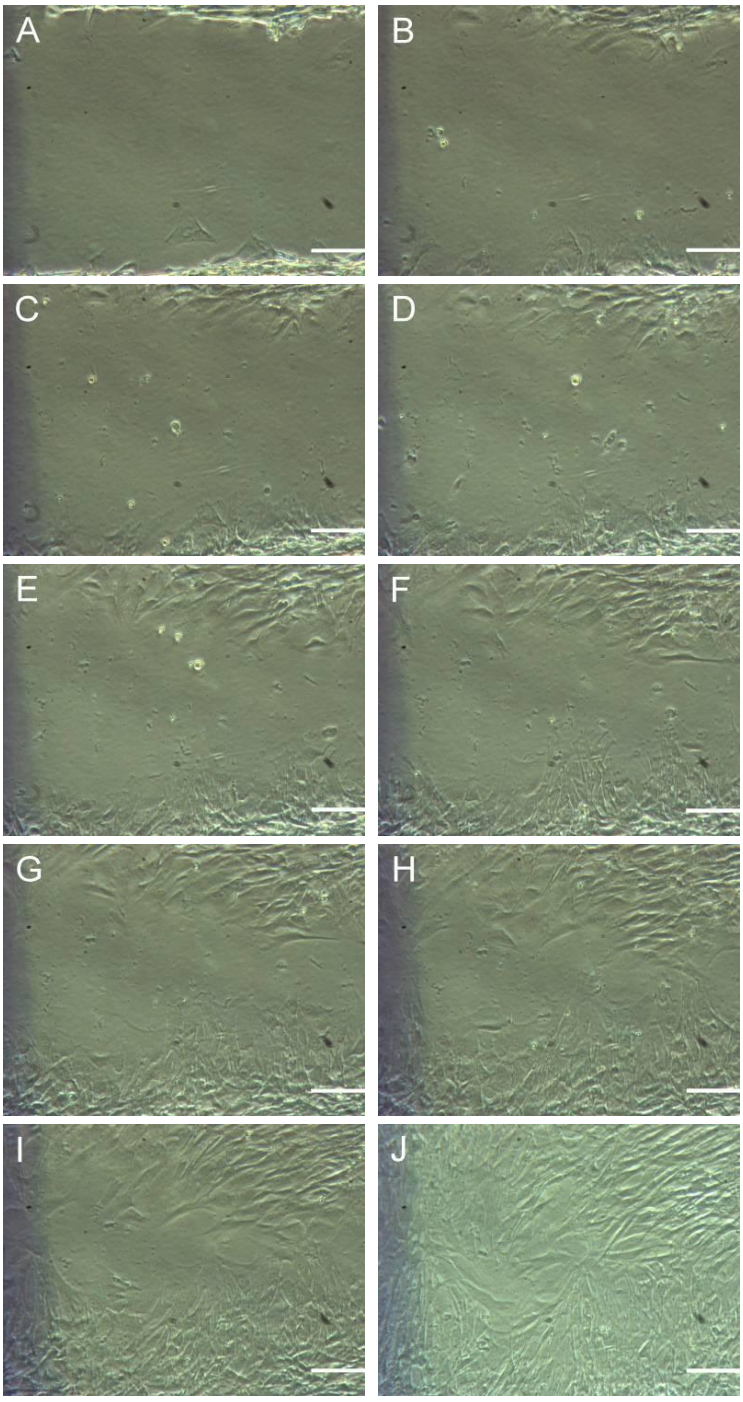


Figure 5.4.7 Micrographs of the wound-repair process for chondrocyte monolayers with addition of TGF-β2&3 after: a) 0; b) 2; c) 4; d) 6; e) 8; f) 10; g) 12; h) 18; i) 24; j) 30 hours, (Scale bar = 100 μm).

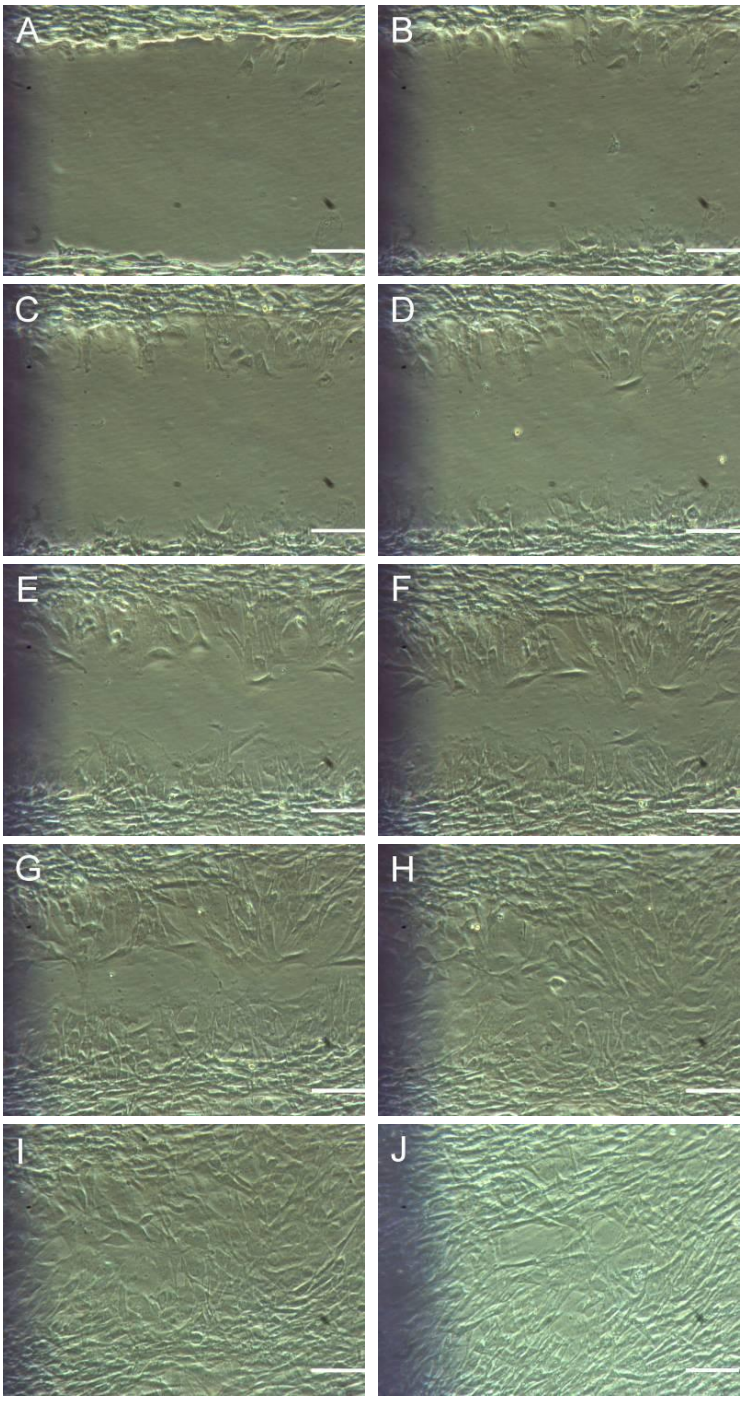


Figure 5.4.8 Micrographs of the wound-repair process for chondrocyte monolayers with TGF-β1&2&3 supplementation after: a) 0; b) 2; c) 4; d) 6; e) 8; f) 10; g) 12; h) 18; i) 24; j) 30 hours, (Scale bar = 100 μm).

The widths of all model wounds, measured with Image-J (HIN) software, showed that addition of manipulated TGF-β 1 and 2 reduced the rate of wound-repair, when compared to the control (See Figure 5.4.9), but generally, these growth factors did not affect the wound repair negatively and a complete closure were achieved after ~ 30 hours.

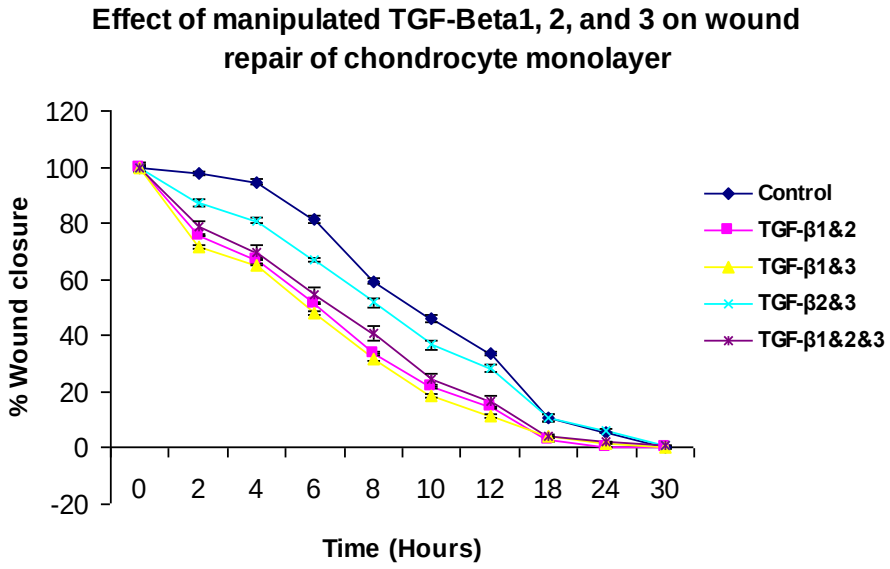


Figure 5.4.9 Graph of percentage wound closure for the chondrocyte monolayers without and with manipulated TGF-βs.

The results from statistical analysis using t-test for two samples with the assumption of unequal variances showed: P (TGF-β1&2) = 0.36, P (TGF-β1&3) = 0.41, P (TGF-β2&3) = 0.99, and P (TGF-β1&2&3) = 0.51. These P-values are all greater than α = 0.05, which meant that the null hypothesis for all groups was accepted. Although the wound-repair process of TGF-β1 & 2-contained sample was faster than control, the non-repaired section of the wound in the control culture was only 21.8 μm ± 2.18 μm (SE).

This difference for the non-repaired wound for the TGF-β1&3-contained culture flask was 6.8μm ± 0.35μm (SE), for TGF-β2&3 = 27.7μm ± 1μm (SE) and for TGF-β1&2&3 = 10.37μm ± 0.48 μm (SE). According to the mean cell size of chondrocyte wound width in mediums with different manipulated TGF-βs, these sizes of non-repaired wound could be negligible (See Figure 5.4.9).

5.4.2.4 Cell Adhesion Analysis

The evaluation of the strength of primary chondrocyte cell adhesion to the surface of the tissue culture flask revealed that the chondrocytes without addition of any types of manipulated transforming growth factor-beta 1, 2 and 3 attached to the solid surface more strongly than any type of manipulated TGF-βs. This time for chondrocytes in medium with addition of TGF-β2&3 was the shortest (See Table 5.4.3).

	Control	**TGF-β1&2**	**TGF-β1&3**	**TGF-β2&3**	**TGF-β1&2&3**
Detachment time (seconds)	207 ± 6.6	167 ± 8.8	170 ± 11.5	133 ± 3.3	137 ± 8.8

Table 5.4.3 Measured time for primary chondrocyte cell to detach from solid surface without and with manipulated TGF-β1, 2 and 3.

As can be seen from Figures 5.4.10-5.4.14, only chondrocytes cultured without addition of any type of TGF-β showed rounded morphology when starting to detach from the

surface. Chondrocytes cultured in the presence of manipulated TGF-β exhibited an elongated morphology which is a sign of fibroblast-like phenotype. It has already been shown in Chapter 5.1 that chondrocytes with fibroblastic morphology detach faster than those with chondrocytic morphology.

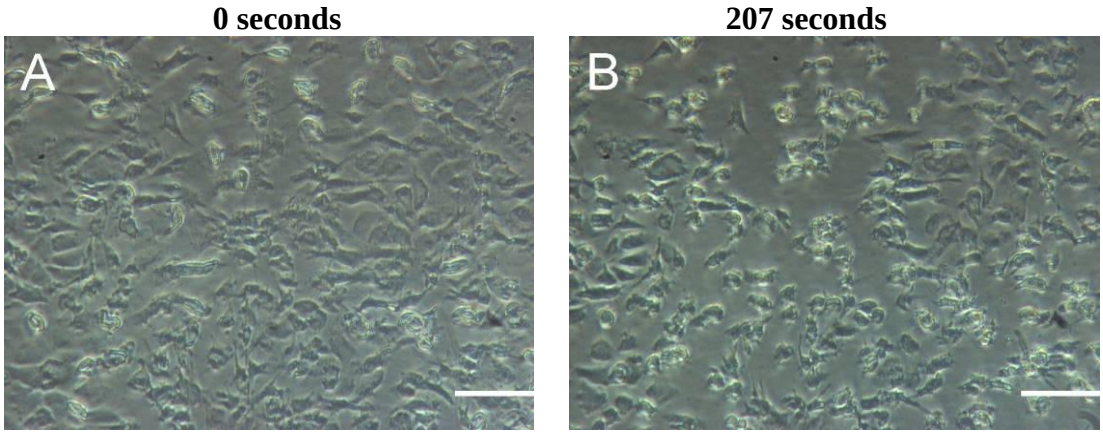


Figure 5.4.10 Chondrocytes detachment from solid surface without addition of any type of TGF-β, (Scale bar = 100 μm).

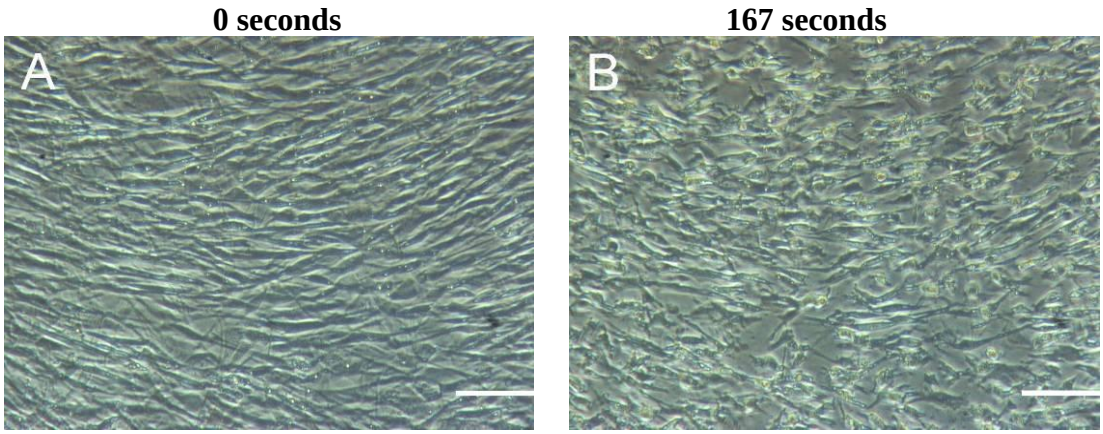


Figure 5.4.11 Detachment of chondrocytes from solid surface. The cells were cultured in the presence of manipulated TGF-β1&2, (Scale bar = 100 μm).

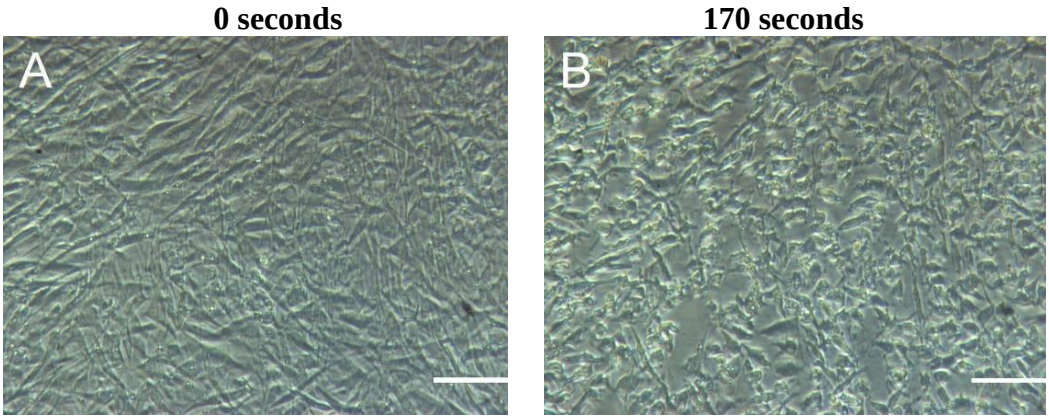


Figure 5.4.12 Detachment assay of chondrocytes from solid surface, cultured in manipulated TGF-β1&3-contained media, (Scale bar = 100 μm).

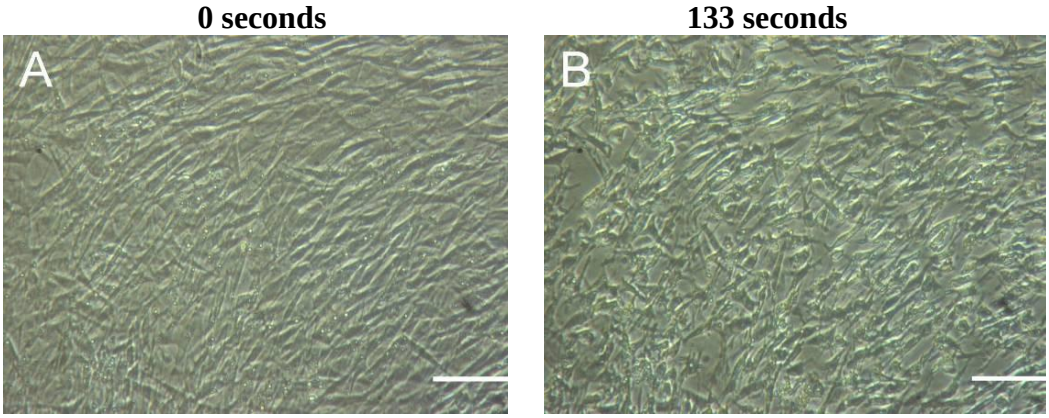


Figure 5.4.13 Detachment of chondrocytes from solid surface without addition of any type of TGF-β2&3 using trypsinisation assay, (Scale bar = 100 μm).

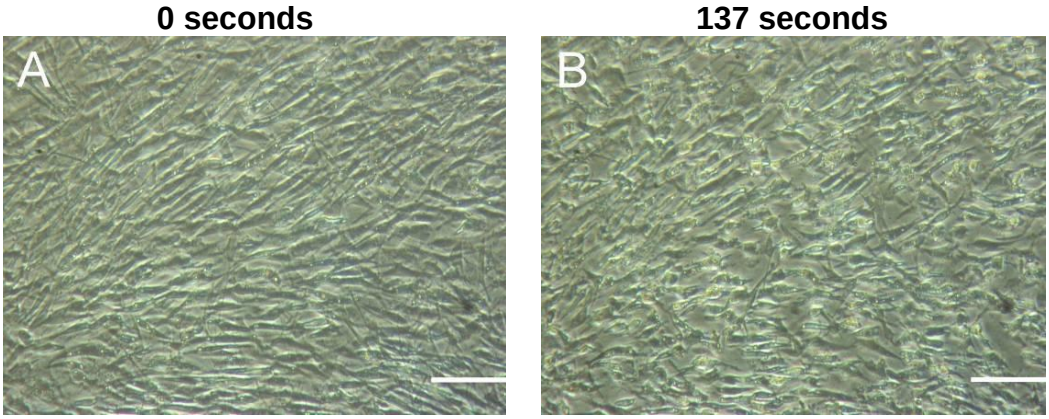


Figure 5.4.14 Detachment of chondrocytes from surface of tissue culture flask cultured in the presence of manipulated TGF-β1, 2 and 3, (Scale bar = 100 μm).

Different morphologies of chondrocytes could be distinguished from Figures 5.4.10-5.4.14. The cells with fibroblast-like phenotype detached faster than those with rounded morphology.

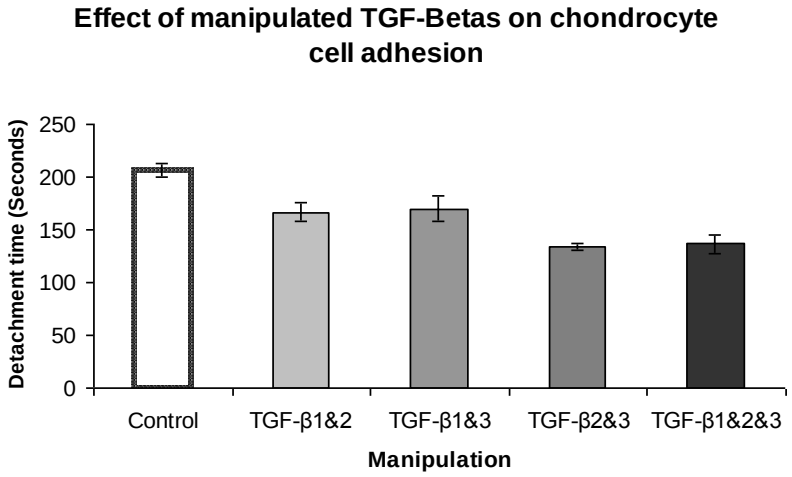


Figure 5.4.15 Graph of detachment time for chondrocyte from solid surface without and with manipulated TGF-β1, 2 and 3.

Statistical analysis of variance (ANOVA) was used to test the significance of null hypothesis. The obtained results showed that the $F = 12.85 > F\ crit = 3.47$, and $P = 0.0005 < \alpha = 0.05$. According to these results, the null hypothesis was rejected. Null hypothesis was the assumption of the same detachment time for chondrocyte in media without and with addition of manipulated transforming growth factor-beta 1, 2 and 3.

5.4.3 Overall Discussion

Manipulated transforming growth factor-beta in the forms of TGF-β1&2, TGF-β1&3, TGF-β2&3 and TGF-β1&2&3 reduced the size of the primary chondrocyte cell during 72 hours' monolayer culture.

Chondrocyte cultured in medium with a mixture of TGF-β1&2 with a content of 10ng/ml stimulated the cells to proliferate up to 638%, whereas the control culture could promote the cells to proliferate up to 590%. The combination of TGF-β2 and TGF-β3 had a negative effect on cell proliferation with a reduction of up to 37% as compared to the proliferation rate of the control culture. Thus, this combination induced cell apoptosis of primary chondrocyte.

Manipulated transforming growth factor did not effect the progression of wound repair of chondrocyte monolayer. Although the created model wound with manipulated TGF-β1&2 supplementation exhibited faster wound repair, the wound size difference was less than a chondrocyte cell size (23.25 µm ± 0.66 µm SE) and negligible.

Trypsinisation assay for evaluation of the effect of manipulated transforming growth factor-beta 1, 2 and 3 on chondrocyte cell adhesion to a solid surface revealed that the manipulated TGF-β reduced cell adhesion. These results suggested that different combinations of TGF-β1, 2 and 3 reduced the synthesis of cell-adhesion molecules.

CHAPTER V

5.5 Role of Transforming Growth Factor-β in Up/Down Regulation of Chondrocyte Markers

5.5.1 Materials and methods

Chondrocyte cells, isolated from the knee joint of a neonate Sprague-Dawley rat (See Chapter 2, Subsection 2.2.3, Protocol of isolation of chondrocyte cells) were cultivated in a planar cultured system. The cells were expanded 200-fold through four passages and utilised for this experiment.

One sterilised 22 mm² glass coverslip was placed into a sterile Petri dish. A total of 300µl of chondrocyte cell suspension with a cell density of 200,000 cell/ml and 10ng/ml TGF-β1 supplementation was seeded on a glass coveslip, sealed with parafilm and incubated at 37°C for 24 hours (see Chapter-5.1-5.3 for reconstitution and preparation of TGF-β). The same amount of cell suspension with the addition of TGF-β2, TGF-β3 and manipulated forms of TGF-β (TGF-β1&2, TGF-β1&3, TGF-β2&3 and TGF-β1&2&3) were prepared and seeded on glass coverslips and incubated at 37°C (see Chapter-5.4 for more detail about manipulated TGF-β). As positive control, 300µl cell suspension without addition of any type of TGF-β was seeded on a glass coverslip and incubated at 37°C. For each treatment a separate positive control culture was prepared to monitor and compare with other results.

After 24 hours each chondrocyte culture was fixed with 1% formaldehyde (See Chapter 2, Subsection 2.2.5.2, Cell fixation) and immunocytochemically stained for collagen type-I, collagen type-II, chondroitin sulfate proteoglycon (CSPG), S-100 protein and fibronectin (See Chapter 2, Subsection 2.2.5.3.2, Immunostaining procedure).

Laminin is a membrane protein regulating cell adhesion and migration (Hashimoto et al., 2005). Peroxidase staining kit for laminin (Sigma Aldrich, UK) was used to stain the localisation of laminin.

5.5.1.1 Peroxidase staining protocol

Prior to staining the laminin, the primary chondrocytes cultured on 22cm² glass coverslips were fixed by 1% formaldehyde solution. The substrates were treated with two drops of rabbit anti-laminin antibody (primary antibody) for one hour. The substrates were washed three times with PBS. The PBS was completely aspirated and two drops of biotinylated secondary anti-laminin antibody (Sigma Aldrich, UK) was added. The coverslips were washed three times for 15 minutes with PBS, treated with peroxidase and incubated at room temperature for 20 minutes.

According to the supplier's instructions, a mixture of solution containing 4ml deionised water, two drops of acetate buffer, one drop of AEC chromogen and one drop of 3% hydrogen peroxide (Sigma Aldrich, UK) was prepared.

After 20 minutes' incubation the substrates were washed only once with PBS and two drops of prepared solution was added to the substrates and incubated for 10 minutes. During 10 minutes' incubation the staining was monitored under a light microscope. The samples were washed with PBS, imaged and saved for future analysis.

The experiment was performed in a sterile condition (vertical laminar hood) and repeated three times to prove the precision of the results.

The level of stained cells was compared to the control for evaluation of the effect of various forms of TGF-β in up-or-down regulation of stained proteins.

5.5.2 Results

Collagen type-I: Chondrocytes cultured in monolayer at low density produce collagen type-I instead of collagen type-II (see Chapter-2). On the other hand, at high density and 3D culture, these cells showed the synthesis ability of type-II collagen, a chondrogenic marker (Bernstein et al., 2009), (Gigout et al., 2008).

The effect of TGF-β1, 2 and 3, and also manipulated forms of this growth-factor superfamily (TGF-β1&2, TGF-β1&3, TGF-β2&3 and TGF-β1&2&3), on synthesis of collagen type-I showed that the manipulated TGF-β1&2&3 stained for this protein at a higher level than other cultures in this experiment. The minimum staining was observed in the chondrocyte culture with the addition of manipulated TGF-β1&2 (see Figure 5.5.1).

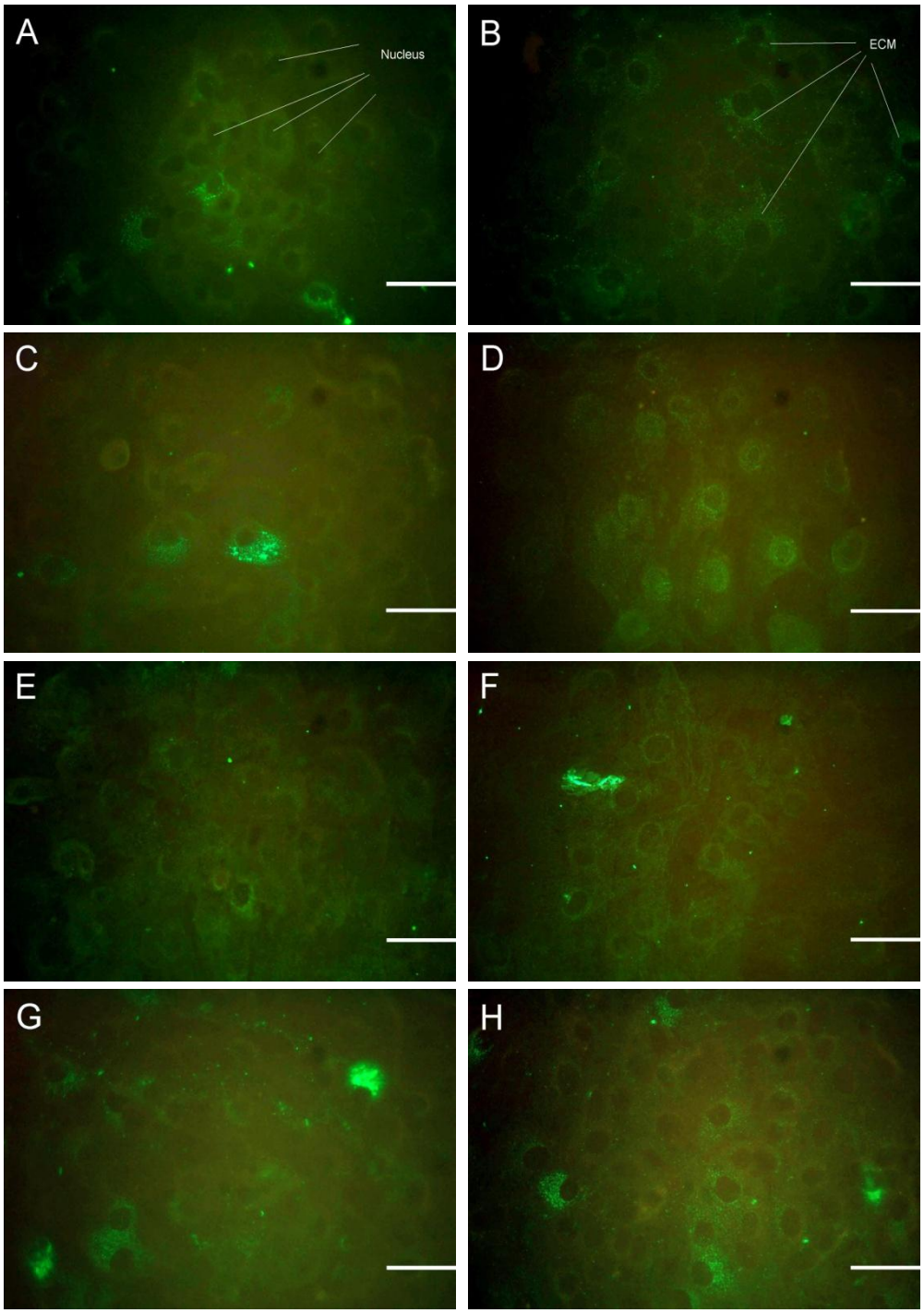


Figure 5.5.1 Immunofluorescence micrographs of chondrocytes stained for collagen type-I: a) control; b) TGF-β1; c) TGF-β2; d) TGF-β3; e) TGF-β1&2; f) TGF-β1&3; g) TGF-β2&3; h) TGF-β1&2&3, (x 20 magnification; Scale bar = 50μm).

In all cases the staining was around the cell membrane and spread on the extracellular matrix.

Collagen type-II: Up/down regulation of collagen type-II production, a predominant protein, in formation of cartilage (Cameron et al., 2009) was analysed in the presence of transforming growth factor-beta. TGF-β cooperates with extracelluar matrix (ECM) molecules to regulate chondrogenic differentiation and development of cartilage (Yang et al., 2006). The effect of transforming growth factor-beta and its manipulated forms in synthesis of collagen type-II, a chondrogenic marker (Pei et al., 2008)), showed that the primary chondrocyte was strongly stained for collagen type-II cultured in media supplemented with mixture of TGF-β1&2&3, as can be seen from Figure 5.5.2-A and H. There was no significant difference between control and TGF-β1, TGF-β2 or TGF-β3. The manipulation of TGF-β2&3 showed a reduction in collagen type-II staining (see Figure 5.5.2-G).

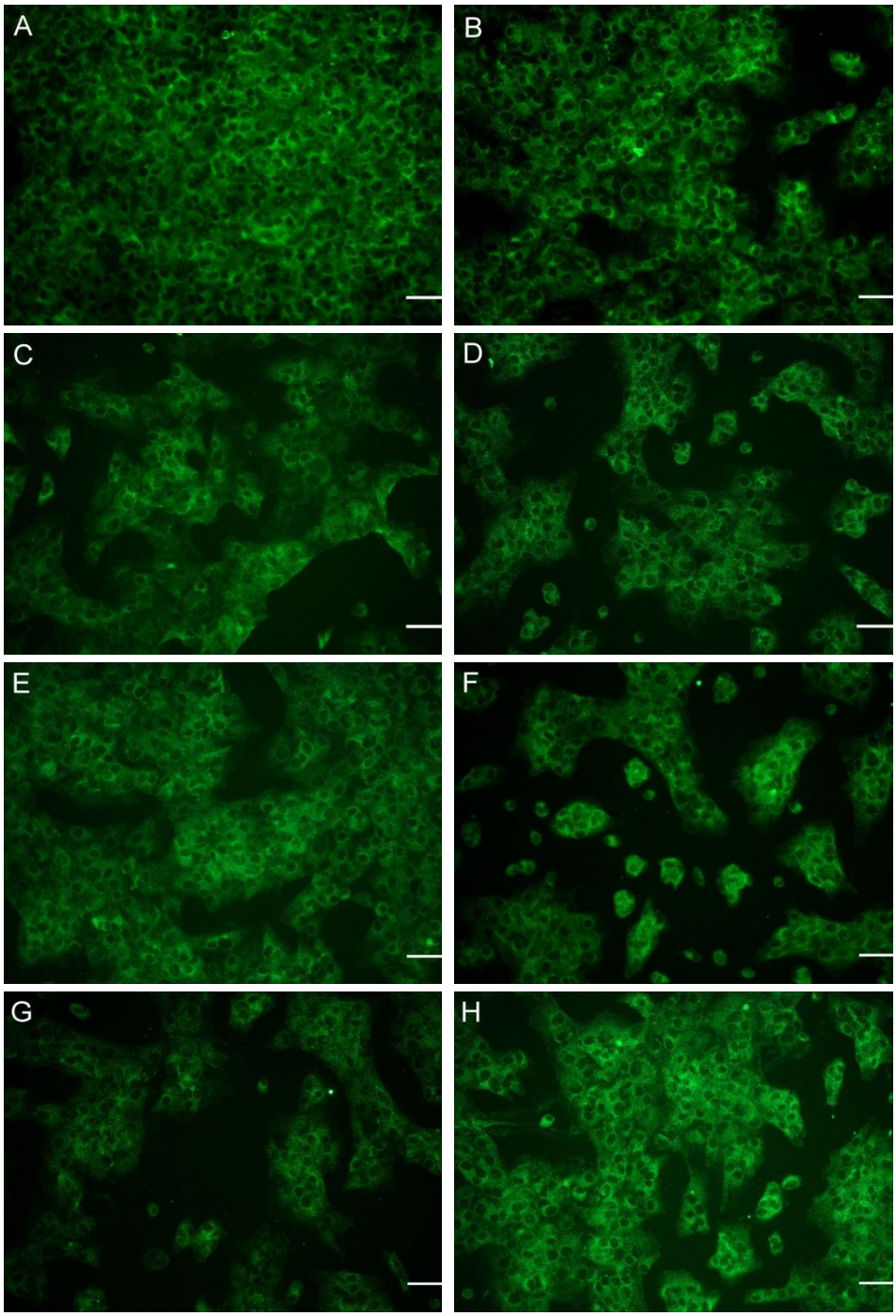
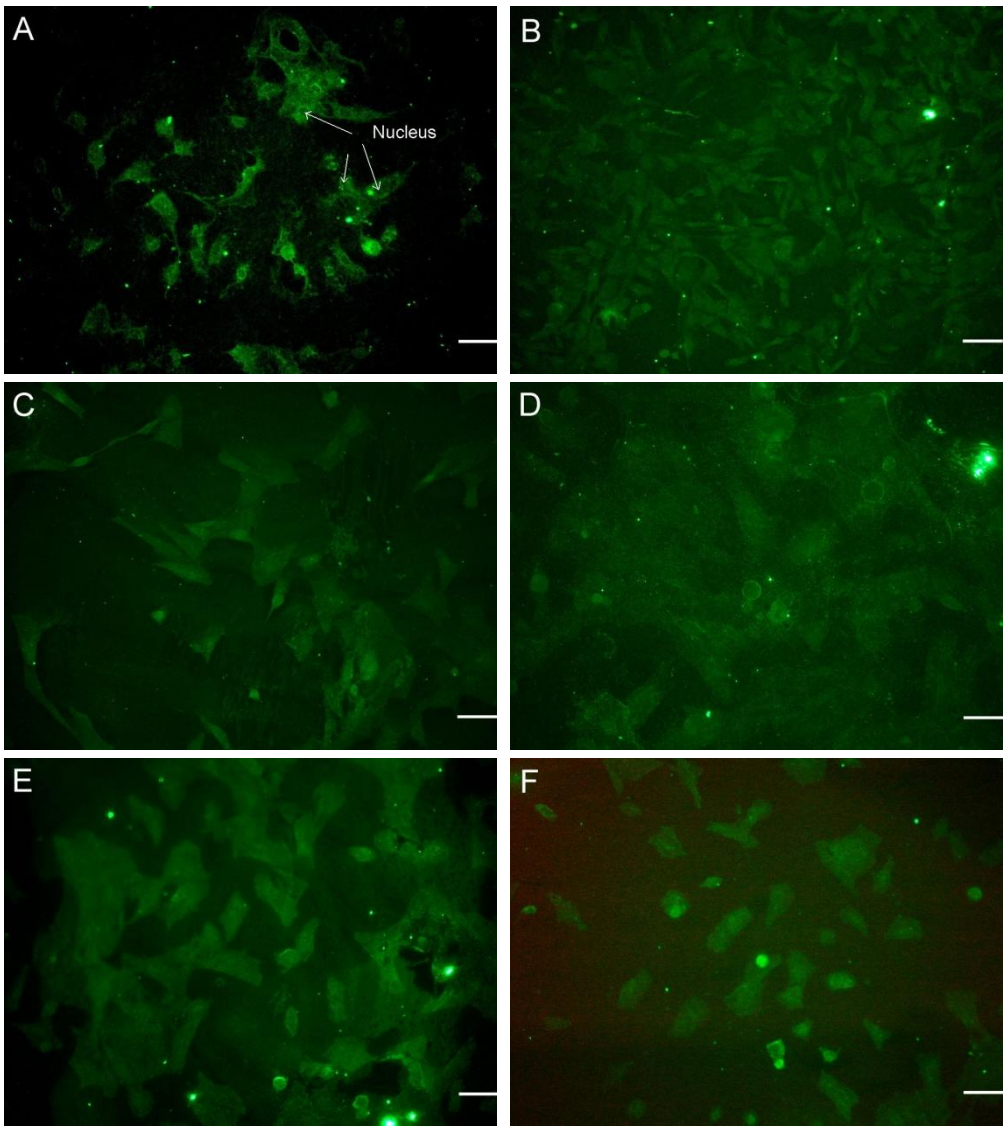


Figure 5.5.2 Immunofluorescence staining of high-density chondrocyte for collagen type-II: a) control; b) with TGF-β1; c) with TGF-β2; d) with TGF-β3; e) with TGF-β1&2; f) with TGF-β1&3; g) with TGF-β2&3; and h) with TGF-β1&2&3, (x10 magnification; Scale bar = 50μm).

Chondroitin sulfate proteoglycon (CSPG): The results obtained from the immunofluorescence staining of (CSPG) showed that the staining of CSPG was spread on ECM of the chondrocyte, once cultured in media with manipulated TGF-β2, TGF-β3, TGF-β1&2 and TGF-β2&3 supplementation (see Figure 5.5.3-C, -D, -E, and -G). Strong CSPG staining was observed in media without any addition of TGF-β, as can be seen from Figure 5.5.3-A. In contrast, the TGF-β1 and TGF-β2-contained mediums showed less staining of CSPG (see Figure 5.5.3-B, and –C).



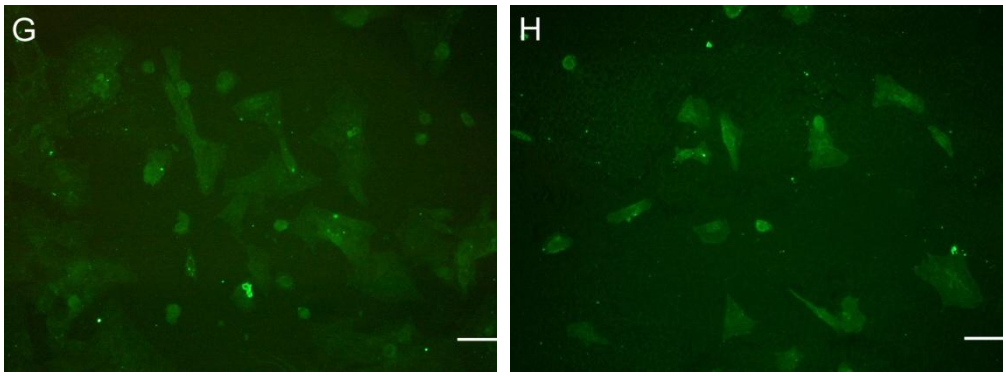


Figure 5.5.3 Immunofluorescence staining of high-density chondrocyte for CSPG: a) control; b) with TGF-β1; c) with TGF-β2; d) with TGF-β3; e) with TGF-β1&2; f) with TGF-β1&3; g) with TGF-β2&3; and h) with TGF-β1&2&3, (x10 magnification; Scale bar = 50μm).

Immunofluorescence staining of CSPG showed the localisation of this protein in the chondrocyte nucleus in all cases, as can be seen from Figure 5.5.3.

S-100: Evaluation of the effect of different types of TGF-βs and their manipulated forms on synthesis of S-100, a chondrocyte marker, showed that this protein was distributed along with ECM. There was no significant effect on up/down regulation of CSPG, except that slightly stronger staining was observed around the nucleus and cell membrane, as can be seen in Figure 5.5.4.

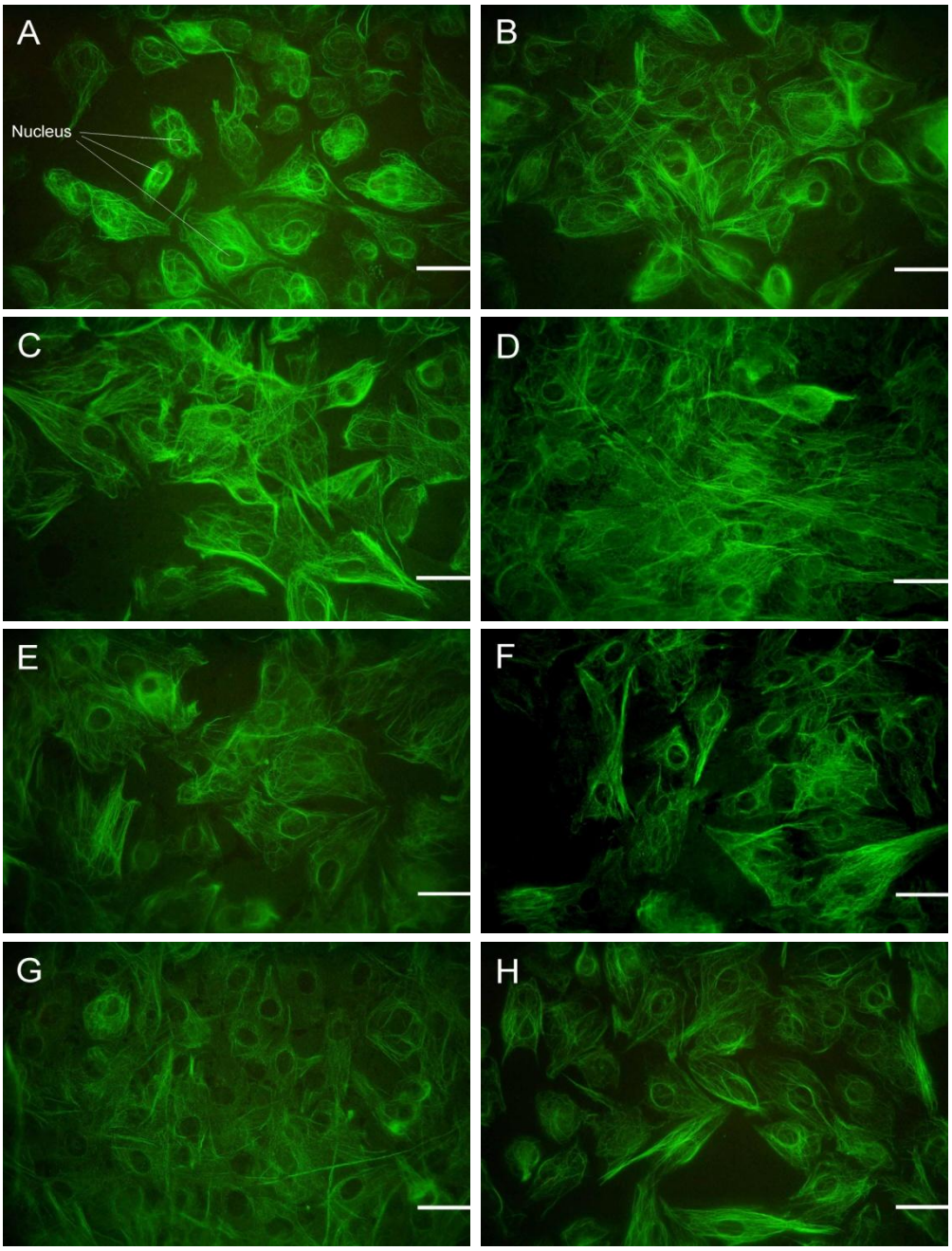
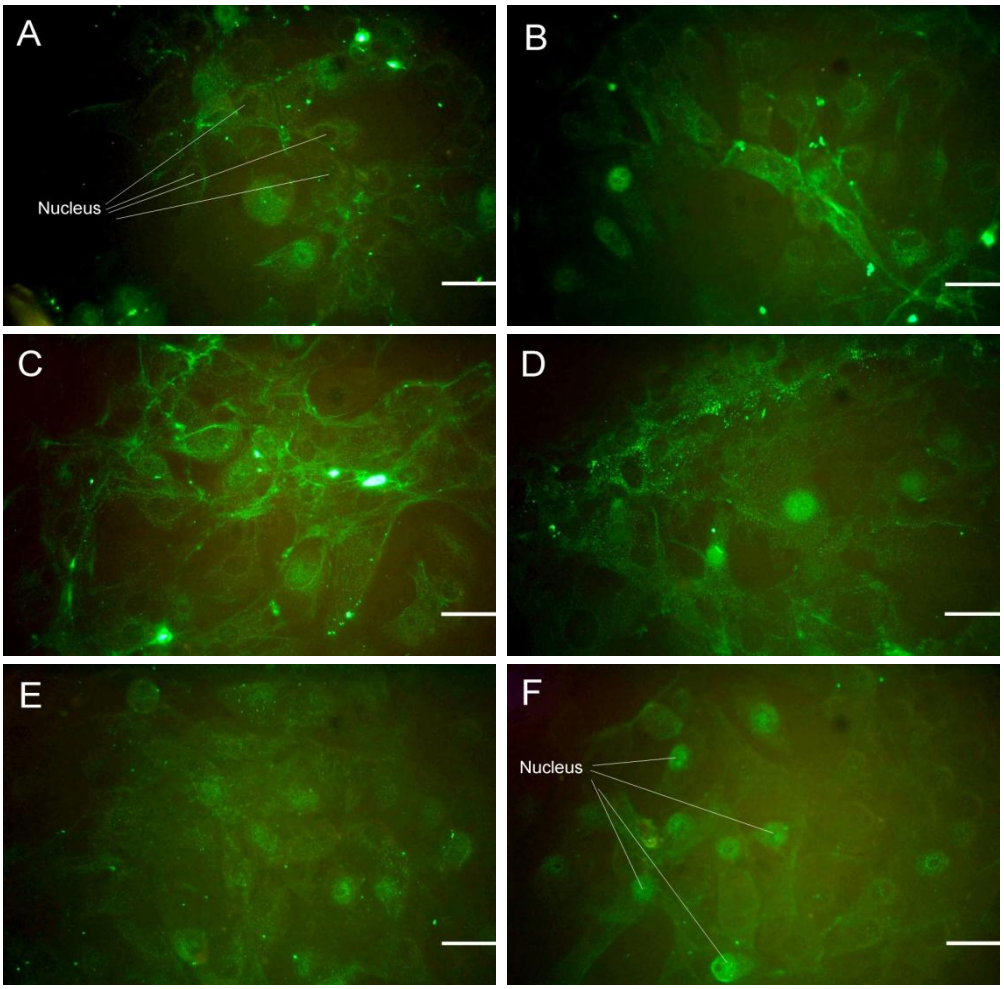


Figure 5.5.4 Immunocytochemical staining of high-density primary chondrocyte for S-100 protein: a) control; b) with TGF-β1; c) with TGF-β2; d) with TGF-β3; e) with TGF-β1&2; f) with TGF-β1&3; g) with TGF-β2&3; and h) with TGF-β1&2&3 (x20 magnification; Scale bar = 50μm).

Fibronectin: The effect of TGF-β on expression of fibronectin by primary chondrocyte was assessed by immunocytochemistry. The results showed no significant variation for fibronectin expression in chondrocyte cultures with and without TGF-β. However, some chondrocytes cultured and supplemented with TGF-β3, TGF-β1&2 and TGF-β1&3 displayed clear nuclear staining (See Figure 5.5.5 -D, -E, -F).



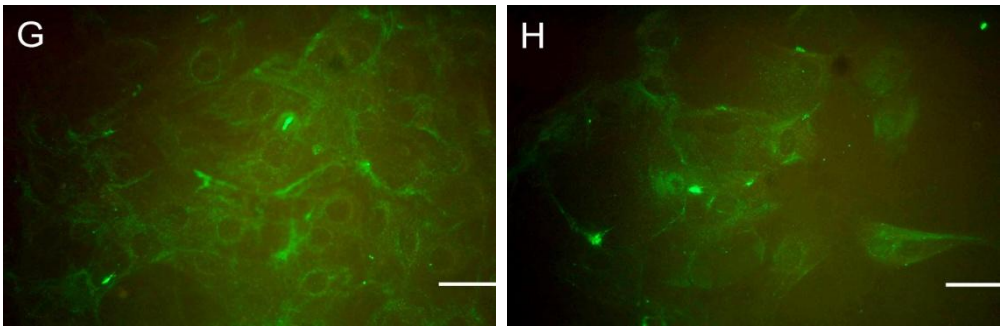


Figure 5.5.5 Immunocytochemical localisation of fibronectin in high-density primary chondrocytes cultured: a) control; b) with TGF-β1; c) with TGF-β2; d) with TGF-β3; e) with TGF-β1&2; f) with TGF-β1&3; g) with TGF-β2&3; and h) with TGF-β1&2&3 (x20 magnification; Scale bar = 50µm).

Laminin: The results acquired from immunocytochemical staining for localisation and expression of laminin showed that the TGF-β and any manipulated form of this growth factor, slightly reduced laminin expression in the chondrocytes monolayer. The highest level of staining was observed in the control culture and the lowest was expressed by chondrocytes cultured in TGF-β1&2, TGF-β1&3, and TGF-β1&2&3-contained media, as can be seen in Figure 5.5.6 -F, -G, -H. The chondrocytes cultured in media with addition of TGF-β2&3 showed fibroblast-like morphology and stained slightly more for laminin. Laminin was mainly localised and spread in the extracellular matrix of the cell (See Figure 5.5.6).

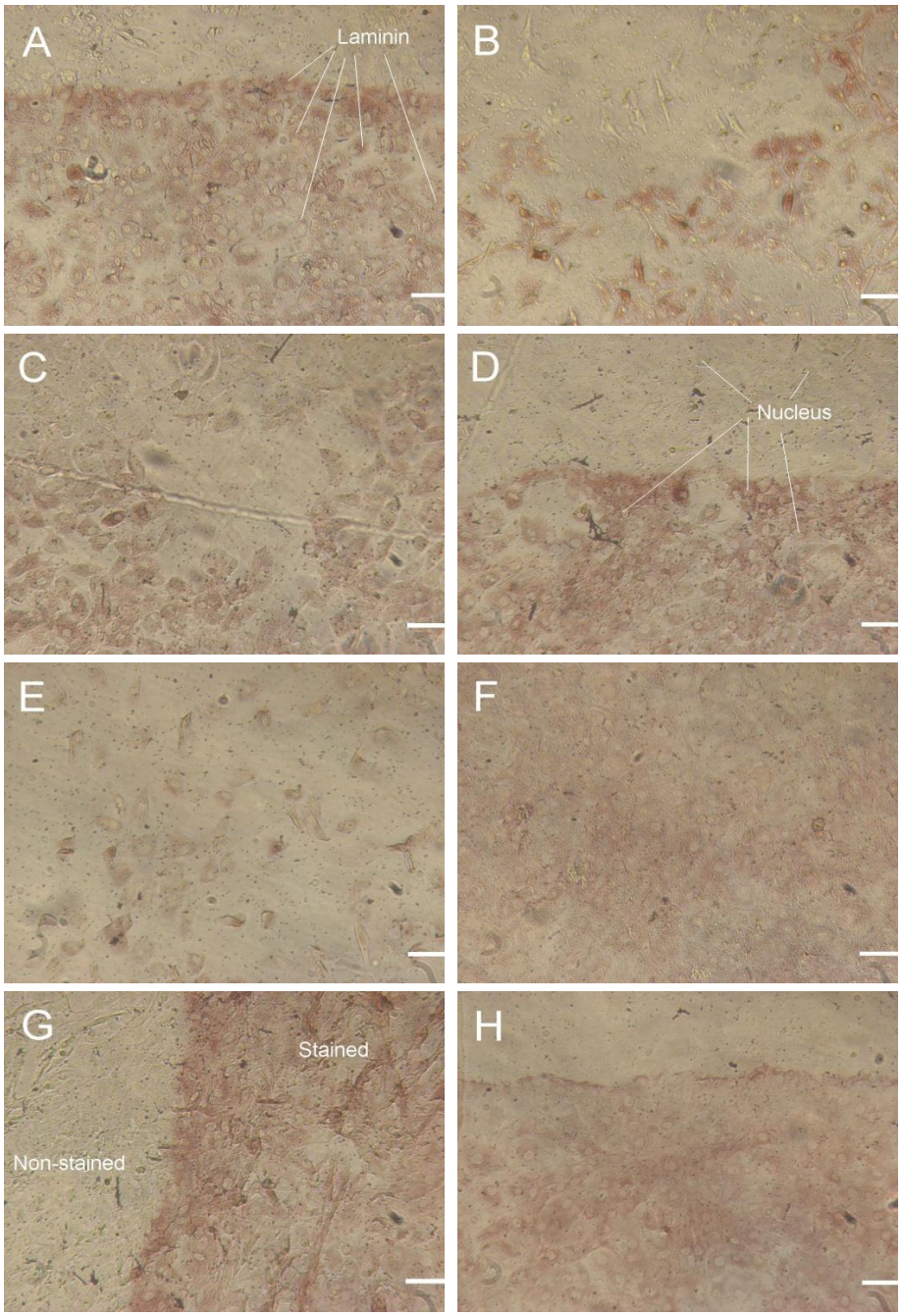


Figure 5.5.6 Standard staining for localisation of laminin in high-density monolayer primary chondrocytes cultured: a) control; b) with TGF-β1; c) with TGF-β2; d) with TGF-β3; e) with TGF-β1&2; f) with TGF-β1&3; g) with TGF-β2&3; and h) with TGF-β1&2&3 (x10 magnification; Scale bar = 50μm).

5.5.3 Overall Discussion

Expression of collagen type-I, type-II, S-100 protein, chondroitin sulfate proteoglycon (CSPG), fibronectin and laminin was detected by immunocytochemistry of high density primary chondrocytes, cultured in medium with and without various types of manipulated and TGF-βs.

TGF-β1 reduced expression of collagen type-I by high density chondrocytes when compared to control culture, as can be seen from Figure 5.5.1. Seeding chondrocytes at high density provided chondrogenic condition, and subsequently decreased collagen type-I synthesis.

Collagen type-II expression was increased by manipulated TGF-β1&2&3 and slightly decreased by TGF-β2&3, which is a sign of the role of TGF-β in synthesis of ECM and subsequently formation of cartilage.

Primary chondrocyte showed nuclear staining, as immunostained for CSPG in media with and without TGF-β. Generally, and mixture of transforming growth factor-beta reduced the expression of chondroitin sulfate glycoprotein.

TGF-β did not induce the chondrocytes regarding expression of S-100 protein.

Expression of fibronectin by high density chondrocytes was not significantly affected by any type, or by manipulated TGF-β.

Manipulated TGF-β2&3 showed slightly increased laminin expression and spread along ECM, but generally TGF-β decreased the synthesis of laminin by primary chondrocyte cultured in planar system.

CHAPTER V

5.6 Effect of Transforming Growth Factor-β on Up/Down Regulation of Integrin-β1 in Primary Chondrocyte

5.6.1 Materials and Methods

Monolayer-expanded primary chondrocyte cells derived from forth passage were used in this work. The cells were isolated from knee joint of neonate Sprague-Dawley rat, and the protocol is described in Chapter-2, (See Chapter 2, Subsection 2.2.3, Protocol of isolation of chondrocyte cells).

Eight 22 mm^2 sterilised glass coverslips were sterilised, and eight Petri dishes were labelled as Control, TGF-β1, TGF-β2, TGF-β3, TGF-β1&2, TGF-β1&3, TGF-β2&3, and TGF-β1&2&3.

Chondrocyte cells were cultured, expanded in monolayer culture system, and 8000000 cells were resuspended in 40 ml DMEM media supplemented with 10% FCS. Five ml of cell suspension subjected to seeding on each coveslip. 5 ml of cell suspension was seeded on coverslip labelled as control. Remaining cell suspensions were aliquoted in 5 millilitres, and supplemented with TGF-β1, TGF-β2, TGF-β3, TGF-β1&2, TGF-β1&3, TGF-β2&3, and TGF-β1&2&3 (See Chapter 5-3 and 5-4 for reconstitution protocol). All cell cultures were incubated at 37°C for 24 hours.

After 24 hours, cells were fixed by 1% formaldehyde and immunocytochemically stained for integrin β1 (CD29).

5.6.1.1 Reconstitution of Anti Integrin β1 (Anti-CD29)

According to the supplier's recommendation, the antibody was diluted with 1%BSA and a dilution ratio of 1:200 for cell staining.

Immnuocytochemical staining was performed with the same method as described in Chapter 2, (See Chapter 2, Subsection 2.2.5.3.2, Immunostaining procedure). After two weeks the cells were imaged with immunofluorescence microscope.

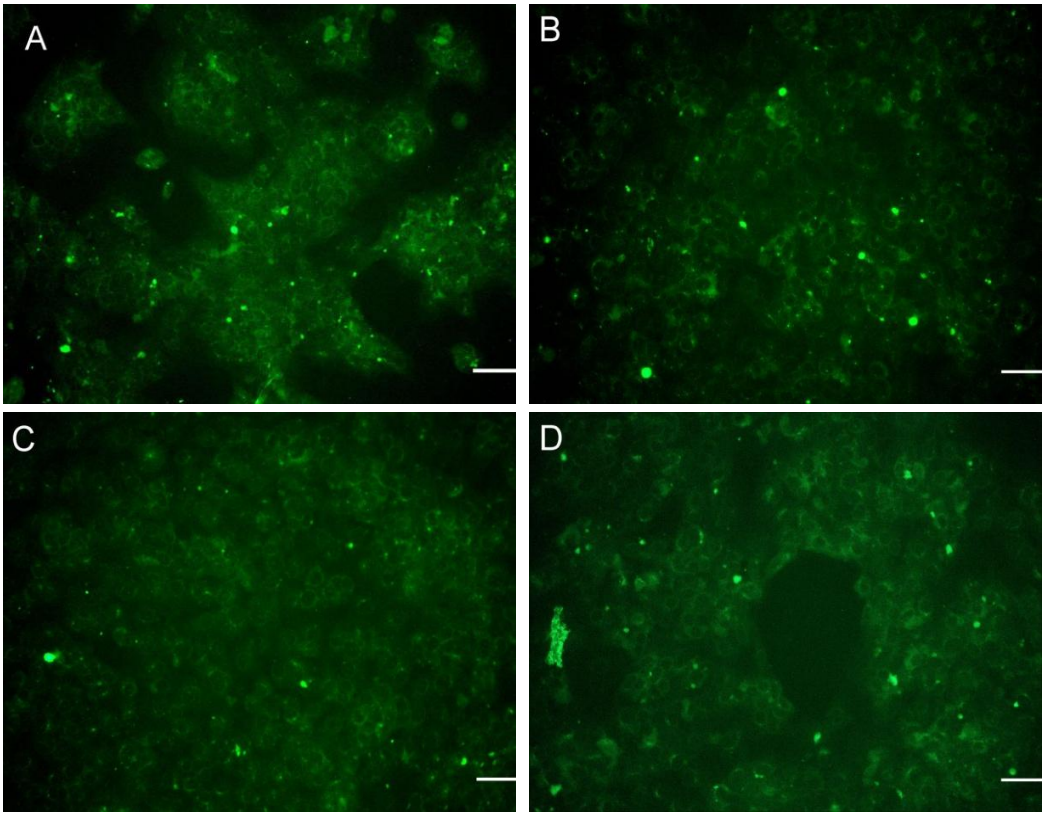
5.6.2 Results and Discussion

Immunocytochemistry was performed to stain integrin β1 in primary chondrocytes cultured in medium supplemented with and without TGF-β1, 2, and 3, and their manipulated forms. The acquired results showed that all cultured chondrocyte stained positively, but with different intensity for integrin β1.

Primary chondrocytes cultured in DMEM media without addition of any type of TGF-β showed strong staining for integrin β1 (See Figure 5.6.1-A). Also TGF-β2, TGF-β3, and manipulated TGF-β2&3 exhibited almost similar staining (Figure 5.6.1-C, -D and -G). Chondrocyte attaches to ECM glycoproteins, collagen, laminin, and fibronectin (Gigout

et al., 2008), via integrin β1, and therefore strong staining confirmed the synthesis of these predominant cartilage matrix proteins.

In contrast, the cells cultured in media with addition of TGF-β1, TGF-β1&2, TGF-β1&3, and TGF-β1&2&3 showed poor staining, as can be seen from Figure 5.6.1-D, E, -F, and –H. The weak presence of integrin β1 revealed that these forms of transforming growth factor-β and their manipulated forms down-regulated integrin β1 leading possibly down regulation of cartilage formation (Thorp et al., 1992), (Motoyama et al., 2010).



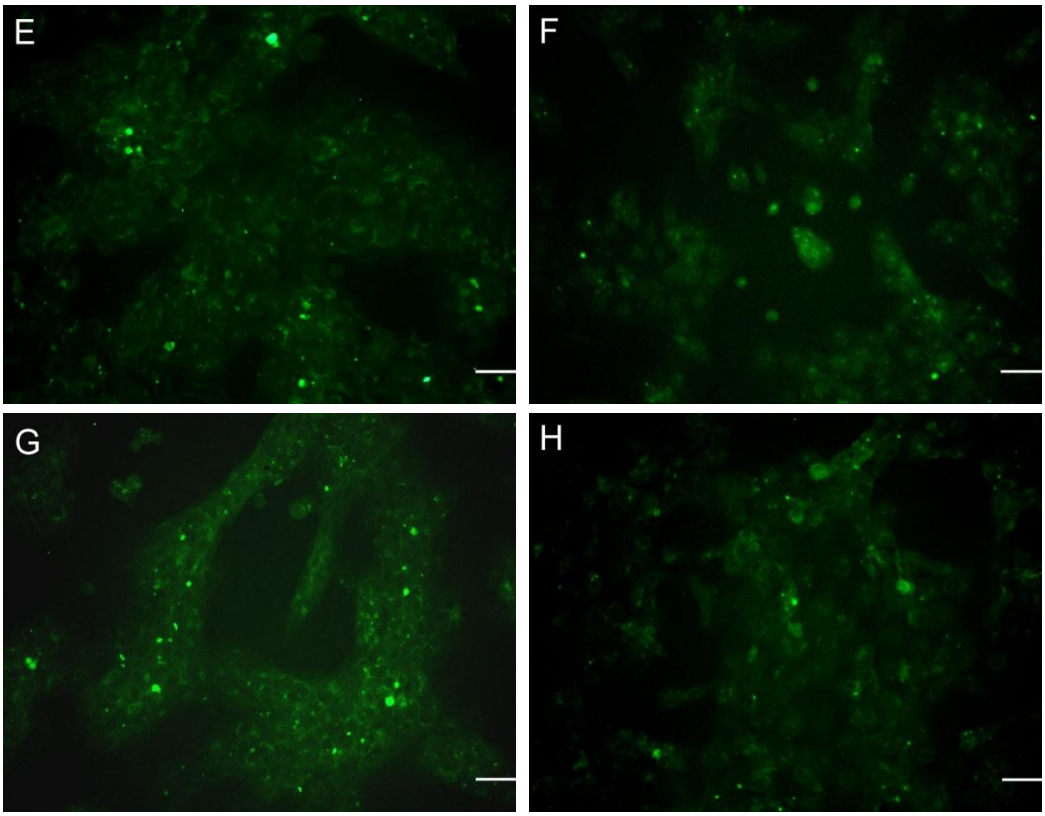


Figure 5.6.2 Immunofluorescence micrographs of chondrocytes stained for integrin-β1 subunit: a) control; b) TGF-β1; c) TGF-β2; d) TGF-β3; e) TGF-β1&2; f) TGF-β1&3; g) TGF-β2&3; h) TGF-β1&2&3, (x 10 magnification; Scale bar = 50μm).

This experiment revealed that none of the three types of transforming growth-β, and none of their manipulated forms could up-regulate the expression of integrin β1. As chondrocytes are embedded within a collagen, proteoglycons, and non-collagenous contained extracellular matrix, appropriate expression of integrin β1 is required to bind the chondrocyte to ECM to migrate, proliferate, and repair defected cartilage. The obtained results proved that the utilization of transforming growth factor-β down-regulates the expression of integrin β1 and therefore have negative effect on cell adhesion properties.

CHAPTER VI

6 Overall Discussion and Conclusions

Cartilage, a highly specialized tissue, sustains compressive load by cushioning the bones at joints, and by providing almost frictionless condition for joints to reduce wear. Cartilage as an avascular tissue has poor repair capacity and thus any damage or degradation on this tissue can cause pain, complexity in mobility and adversely affect daily life.

The isolation and purification of cartilage cell, chondrocyte, and cultivation of this cell in planar culture system showed that cells with low density produced fibroblast like morphology, and synthesised collagen type-I instead of collage type-II (Chapter 2, Subsection, 2.2.5 Immunocytochemistry). Monolayer culture of the chondrocyte resulted in dedifferentiation of cells and production of stress fibres. This characteristic was prevented by high density and 3D chondrocyte culture (Chapter 3, Subsection, 3.2 Monolayer Culture). Primary chondrocytes cultured in pellet showed 93% cell viability after three weeks; however, cells delayed to attach on substrate. This result proved that pellet culture of chondrocyte could be appropriate to store primary chondrocyte cells (Chapter 3, Subsection, 3.3 Three Dimensional (3D)-Pellet Culture).

This work clearly demonstrated a successful and typical cell engineering of chondrocyte monolayers with our own model using isolated primary rat articular chondrocytes (RAC) by cell seeding, 2D cell expansion and differentiation *in vitro* and without the

use of bioreactor. Therefore, the same model can be used for isolation of human articular chondrocyte (HAC) from clinically approved human biopsies for autologous chondrocyte implantation, and/or monolayer production for, e.g. cytotoxicity tests. Our model can also be used for direct expansion of the limited number of chondrocytes obtained from RAC or HAC, reaching higher densities similar to those used in conventional cartilage tissue engineering based on cells expanded by 2D cultures.

To our knowledge, so far, there are only few surgical options for treatment of chondral or osteochondral lesions of the hip. One such treatment is that of autologous chondrocyte implantation (ACI) which has been used frequently for treatment of knee cartilage defects with good outcomes, but there is probably only one or no use of ACI for the hip. It is our recommendation that collagen (type-I) gel (or patches) be used for prior chondrocyte implantation.

Hyaluronan, an anionic glycosaminoglycan, is an important factor in formation and maintenance of cartilage. Addition of 0.1mg/ml hyaluronic acid in chondrocyte culture media resulted in higher proliferation of chondrocyte and better maintenance of chondrocytic phenotype (Chapter 3, Subsection, 3.4 Effect of Hyaluronic Acid (HA) on Regulation of Primary Chondrocyte).

Although antibiotics prevents the cell culture from any possible contamination, but use of the antibiotics caused a reduction in proliferation of primary chondrocyte in monolayer culture. Thus, routine use of penicillin/streptomycin should be avoided in cell culture (Chapter 3, Subsection, 3.5 Culture of Chondrocytes in an Antibiotic-Free (Penicillin and Streptomycin) Environment).

Another application of this research output/findings is that of 3D scaffold design which is a critical and essential part of tissue engineering. Our results suggested clearly that collagen (type-I) as matrix will increase mechanical strength and biological interactions. Collagen (type-I) with different patterns can be used for the design of 3D scaffolds with presence of HA and hence for the engineering of various tissues such as cartilage, vascular and nerve.

Investigation of major/key matrix proteins expressed by chondrocytes has helped us to understand their roles better in modulating the surrounding environment of the ECM.

Chondrocytes isolated from knee joint of rats and cultured at $2x10^5$cells/ml as monolayers on glass coverslips allowed comparison between three different ECM proteins. It was found that the primary chondrocyte cells showed best alignment to the fibronectin stamped pattern with 50µm pattern width. Also, the 100µm wide collagen type-I patterns induced chondrocytes to align to this protein very well. However, chondrocyte cells did not show good alignment to the laminin stamped pattern (Chapter 4, Subsections, 4.5 Chondrocyte cell alignment). These results revealed that fibronectin and collagen were useful for chondrocyte adhesion and can be used in fabrication of biodegradable scaffolds for chondrocyte culture and also autologous chondrocyte implantation (ACI).

Transforming growth factor-β1 caused weak adhesion of primary chondrocyte to solid surface. The healing process with created model wounds on chondrocyte monolayers were negatively affected by TGF-β1 (Chapter 5.1), i.e. the wounded monolayer with chondrocytic morphology showed slower wound closure as compared to control, and

TGF-β1 did not close the wound gap of the fibroblast like morphology, compared to control with complete healing after 72 hours.

Transforming growth factor-β2 increased cell size of chondrocyte, but decreased its proliferation rate and the wound healing process. TGF-β2 also decreased cell adhesion ability to the surface of the culture flask (Chapter 5.2).

Transforming growth factor-β3 caused the chondrocytes to obtain fibroblast like and flattened morphology which occupied larger area without increase in cell size. TGF-β3 induced chondrocyte cell apoptosis up to 34.3%. Model wound created on primary chondrocyte monolayer was not completely repaired after 48 hours of treatment with TGF-β3 and only ~ 54% wound closure occurred compared to control with 100% wound closure after 24 hours. Also, this growth factor reduced adhesion strength of chondrocyte on solid surface (Chapter5.3).

Manipulated transforming growth factor-β, TGF-β1&2, TGF-β1&3, TGF-β2&3 and TGF-β1&2&3, decreased primary chondrocyte cell size. TGF-β1&2 increased cell proliferation up to 638% during 72 hours monolayer culture, and accelerated the repair of created model wound of primary chondrocyte culture (Chapter 5.4). None of the manipulated TGF-βs increased cell adhesion of chondrocyte. These results suggested that TGF-β1&2 could be utilized in fabrication of biodegradable scaffolds for 3D chondrocyte culture, due to its ability to induce cell proliferation.

Transforming growth factor-β1, 2, 3, and their manipulated forms showed reduction in synthesis of collagen type-I. However, the combination of TGF-β1&2&3 increased

production of collagen type-II. Also manipulated TGF-β1&2&3 negatively regulated cell proliferation (Chapter 5.4, Subsection, 5.4.3.2, and Chapter 5.5, Figure 5.4.3).

The amount of chondroitin sulfate proteoglycon (CSPG), and laminin were reduced by all three types of transforming growth factor-βs and their manipulated forms, but the expression of fibronectin and S-100 proteins were not significantly affected by TGF-β isomers (Chapter 5.5).

TGF-β2, TGF-β3, and manipulated TGF-β2&3 exhibited similar synthesis of integrin-β1 (CD29) to control, but TGF-β1, TGF-β1&2, TGF-β1&3, and TGF-β1&2&3, decreased the expression of integrin-β1 (Chapter 5.6). As integrin activates the TGF-β activator (Neurohr et al., 2006), up/down regulation of this cell adhesion molecule plays a crucial role in adhesion of cells to ECM and migration which result in progression of wound closure.

It is our understanding that, in particular, TGF-β1 addition causes down regulation of cell proliferation, adhesion and migration which are essential for wound repair. It was also noticeable that the wound width increased initially up to a period of ~ 6 hours which is believed to be due to the secretion of nitric oxide as a result of damage to the cell periphery and cell membrane. The nitric oxide secretion will certainly damage the matrix proteoglycon (Schwentker et al., 2002) affecting survival of healthy and damaged cells which are gradually removed as dead cells, and hence this is typically a recovery process of the wound width which may take up to ~ 6 hours as seen in this work and depending on the type of growth factor which affects this incubation (recovery) time before the start of wound closure.

It is our believe that a regenerative approach based on cartilage cell therapy in combination with growth factors (TGF-β1&2) as stimulant could promote cartilage repair.

It is also our believe that wound healing of articular cartilage due to injury or disease, could be achieved by producing/providing good quality matrix that helps the repair process, and hence tissue engineered cartilage is one such approach, using isolated chondrocytes and the use of biomimetic or bioresponsive hydrogels. It is clear that scaffolds are used to deliver cells and propagate the formation of cartilage matrix, using biodegradable scaffolds.

6.1 Summary

As discussed in the introduction in Chapter 1, this work consisted of two main sections. First part, dealt with isolation, purification, and culture of primary chondrocytes (Chapters 2, Subsection 2.2.3 Protocol of Isolation of Chondrocyte Cells, Subsection, 2.2.4 Purification of Chondrocyte Cells Isolated from Articular Cartilage, and Chapter 3) and the second part dealt with the effect of TGF-β isomers in chondrocyte behaviour in monolayer culture system (See Chapter 5). The aim has been to develop appropriate condition and supplementations for chondrocyte to maintain its differentiation state and promote proliferation, and to identify the effect of TGF-β isomers in biological regulation of primary chondrocyte. Quite a large number of experiments were developed, and several conclusive results have been found during this research.

Chapters 2 and 3 focused on cell engineering and cultivation of primary chondrocyte *in vitro*. In chapter 2, a novel method for isolation of cartilage from knee joint of neonate Sprague-Dawley rat was developed. This chapter had also implications for the primary chondrocyte culture methods discussed in chapter 3. Development of an appropriate method for primary chondrocyte culture was discussed in this chapter which intends to help the reader to understand how challengeable this research was! The results of these chapters showed that utilization of hyaluronic acid (HA) in chondrocyte culture and fabrication of a biodegradable scaffolds should not be avoided.

Chapter 4 focused on tissue engineering of cartilage by aligning chondrocytes to collagen, fibronectin and laminin, three prominent extracellular matrix proteins which play important role in adhesion of cell to its extracellular matrix. The results from alignment of chondrocyte cells to these cartilage glycoproteins showed how and which protein could be utilized more effectively for fabrication of biodegradable scaffolds for cartilage tissue engineering.

As growth factors play important role in cell behaviour such as proliferation, apoptosis, motility, the investigation on the effect of cytokines was necessary to understand how chondrocyte cell was affected by these growth factors. Chapter 5 consisted of a set of experiments investigating the role of the transforming growth factor-β isomers in regulation of chondrocyte in planar culture system. It was found that TGF-β1, 2 and 3 down regulate cell proliferation and caused the chondrocytes get fibroblast like morphology. They also showed negative effect on the process of wound closure of scratched chondrocyte monolayers. Fibroblastic morphology of the chondrocytes caused

the cells to occupy larger area and resulting in increased contact inhibition which led to decrease in cell proliferation.

6.2 Future Further Work

The results from six individual experiments in chapter 5 seem to have raised more questions than it has answered. There are several lines of future research arising from this thesis which should be pursued.

Primary RAC, using our model of isolation, can therefore be seeded and expanded in 3D scaffolds allowing for dense cartilage tissue production.

Further immunostaining for detection of Ki67 antigen, proliferation marker, should be carried out to confirm the presence of proliferating cells after ~ 2 weeks following the chondrogenic conditions on engineered constructs using a bioreactor-based approach.

6.2.1 Manipulation of TGF-β

The first line of further research, following chapter 5, is to investigate different manipulation techniques for TGF-β. One of the most important functions of TGF-β is control of cellular differentiation, and one of the most challenges in chondorcyte culture is rapid dedifferentiation of this cell, and thus understanding of how these growth factors control differentiation of chondrocyte would be the key point in cartilage tissue engineering. Some techniques regarding activation of transforming growth factor-β

have already been discovered. However, the cartilage repair still is one of the greatest challenges in orthopaedics and tissue engineering. Investigation on interaction and activation of TGF-β with other proteins, cytokines, protease, certainly would lead to determine the most effective combination of these multi functional proteins.

6.2.2 Biodegradable Scaffolding

Depending on the type of cartilage lesion, engineering of cartilage tissue and culture of chondrocytes *in vitro* could require a biodegradable scaffold. The scaffold should attract the chondrocytes to attach on to it and facilitate cell migration and growth. The results from chapter 4 showed that the chondrocytes were best aligned to the fibronectin and collagen stamped patterns. Regarding these outcomes collagen and fibronectin could be recruited in fabrication of a biodegradable scaffold. Also, the role of hyaluronic acid on proliferation and maintenance of chondrocytic phenotype should be taken into consideration.

6.2.3 Bioreactor Application

Dedifferentiation of chondrocytes cultured *in vitro* is well documented as seen also in this work. To maintain this cell in differentiation state similar to an *in vivo* condition, a bioreactor can be made for allowing better cell growth, differentiation and adhesion. Therefore, a bioreactor is necessary to provide *in situ* conditions for cells to grow, secret ECM proteins, and form a cartilage tissue on scaffold that mimics native tissue. This bioreactor should facilitate gas exchange of the culture environment, control of flow

rate of the media, temperature and pH level, and also provide mechanical and biomechanical conditions required for regeneration of cartilage tissue.

6.2.4 Anti-inflammatory medicine

Widely avaiable anti-inflammatroy drugs can relieve pain in some arthritises but most of them cannot repair diseased or injured cartilage (Jaffré et al., 2003). *In vitro* study on the effect of anti-inflammatory herbal medicine (e.g. cannabinoid extracts to reduce the proteoglycan breakdown via nitric oxide secretion) on chondrocyte proliferation and wound repiar can shed some light on the cartilage therapy.

References:

AKMAL, M., SINGH, A., ANAND, A., KESANI, A., ASLAM, N., GOODSHIP, A. & BENTLEY, G. 2005. The effects of hyaluronic acid on articular chondrocytes. *Journal of Bone and Joint Surgery - British Volume,* 87**,** 1143-9.

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. & WATSON, J. D. 1983. *Molecular biology of the cell,* Abingdon/UK, Garland Publishing.

ALBETRS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. & WATSON, J. D. 1983. *Molecular biology of the cell,* Abingdon/UK, Garland Publishing.

APPLETON, C. T. G., MCERLAIN, D. D., PITELKA, V., SCHWARTZ, N., BERNIER, S. M., HENRY, J. L., HOLDSWORTH, D. W. & BEIER, F. 2007. Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. *Arthritis Research & Therapy,* 9**,** R13.

AREND, W. P. 2001. Cytokines and cellular interactions in inflammatory synovitis. *Journal of Clinical Investigation,* 107**,** 1081-2.

ATESHIAN, G. A., MOW, V. C., HUNG, T. C. & WALHMU, W. B. 2001. *Bioreactor for generating functional catilaginous tissue*. US patent application PCT/US2001/007815.

BAGROV, A. Y., SHAPIRO, J. I. & FEDOROVA, O. V. 2009. Endogenous Cardiotonic Steroids: Physiology, Pharmacology, and Novel Therapeutic Targets. *Pharmacological Reviews,* 61**,** 9-38.

BALASSA, L. L. 1987. *Method of processing animal cartilage*. USA patent application PCT/US1987/000118.

BALKWILL, F. R. (ed.) 2000. *Cytokine molecular biology: a practical approach,* New York: Oxford University press, 2000.

BANNING, M. 2005. The principles of inflammation in the development of rheumatoid arthritis. *British Journal of Nursing,* 14**,** 277-283.

BASHEY, R. I., IANNOTTI, J. P., RAO, V. H., REGINATO, A. M. & JIMENEZ, S. A. 2006. Comparison of morphological and biochemical characteristics of cultured chondrocytes isolated from proliferative and hypertrophic zones of bovine growth plate cartilage. *International Society for Differentiation,* 46**,** 199-207.

BELL, F., BRYDSON, G., CONWAY, B. A., SHAW-DUNN, J., MCDONALD, S., NICOL, A. C., PAYNE, A. P. & TAVERNER, G. 2009. Histology of the growth plate. *In:* BIOMECHANICS, F. A. A. (ed.) *Functional Anatomy and Biomechanics/Tutorial/Bone.* Glascow: .

BENCHABANE, H. & WRANA, J. L. 2003. GATA- and Smad1-dependent enhancers in the Smad7 gene differentially interpret bone morphogenetic protein concentrations. *Moelcular and Cell Biology,* 23**,** 6646-61.

BENTLEY, G., BIANT, L., CARRINGTON, R., AKMAL, M., GOLDBERG, A., WILLIAMS, A., SKINNER, J. & PRINGLE, J. 2008. A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee. *Journal of Bone and Joint Surgery - British Volume,* 85B**,** 223-230.

BERG, W. B. V. D. 2001. Anti-cytokine therapy in chronic destructive arthritis. *Arthritis Research and Therapy,* 3**,** 18-26.

BERNSTEIN, P., DONG, M., CORBEIL, D., GELINSKY, M., GÜNTHER, K.-P. & FICKERT, S. 2009. Pellet culture elicits superior chondrogenic redifferentiation than alginate-based systems *American Institute of Chemical Engineers AIChE* 25**,** 1146 - 1152.

BHATNAGAR, R. S. & NICOLL, S. B. 2001. *Chondrocyte-like cells useful for tissue engineering and methods*. USA patent application No. 204824 filed on 12/03/1998 07.12.1998.

BHOSALE, A. & RICHARDSON, J. 2008. Articular cartilage: structure, injuries and review of management. *British Medical Bulletin,* 87**,** 77-95.

BILODEAU, K. & MANTOVANI, D. 2004. Bioreactors for tissue engineering: Focus on mechanical consrtuction. A comparative review. *Tissue Engineering,* 12**,** 2367-83.

BILODEAU, K. & MANTOVANI, D. Nov. 2004. Bioreactors for tissue engineering: Focus on mechanical consrtuction. A comparative review. *Tissue Engineering*, 12.

BORDELEAU, N., BESSARD, J., SHENG, Y. & MARCEAU, N. 2008. Keratin contribution to cellular mechanical stress response at focal adhesions as assayed by laser tweezers. *Journal of Biochemistry and Cell Biology,* 86**,** 352-9.

BOS, P. K., VERHAAR, J. A. N. & OSCH, G. J. V. M. V. 2007. Age-Related Differences in Articular Cartilage Wound Healing: A Potential Role for Transforming Growth Factor β1 in Adult Cartilage Repair *In:* FISHER, J. P. (ed.). Springer US.

BOUBRIAK, O. A., BROOKS, J. T. S. & URBAN, J. P. G. 2009. Cytochrome c oxidase levels in chondrocytes during monolayer expansion and after return to three dimensional culture. *Osteoarthritis and Cartilage,* 17**,** 1084-1092.

BRITLAND, S., MORGAN, H., WOJIAK-STODART, B., RIEHLE, M., CURTIS, A. & WILKINSONA, C. 1996. Synergistic and Hierarchical Adhesive and Topographic Guidance of BHK Cells. *Experimental Cell Research,* 228**,** 313-325.

BRODKINB, K. R., GARCÍAA, A. J. & LEVENSTON, M. E. 2004. Chondrocyte phenotypes on different extracellular matrix monolayers. *Biomaterials,* 25**,** 5929-5938

BRODY, J. E. 1986. Knee microsurgery: Boon to some, but overuse is a growing concern. NYTimes.

BYERS, B., MAUCK, R., CHIANG, I. & TUAN, R. 2008. Transient Exposure to Transforming Growth Factor Beta 3 under Serum-Free Conditions Enhances the Biomechanical and Biochemical Maturation of Tissue-Engineered Cartilage. *Tissue Engineering, Part A,* 14**,** 1821-34.

CAILOTTO, F., BIANCHI, A., SEBILLAUD, S., VENKATESAN, N., MOULIN, D., JOUZEAU, J.-Y. & NETTER, P. 2007. Inorganic pyrophosphate generation by transforming growth factor-beta-1 is mainly dependent on ANK induction by Ras/Raf-1/extracellular signal-regulated kinase pathways in chondrocytes. *Arthritis Research & Therapy,* 9**,** R122.

CAMERON, T., BELLUOCCIO, D., FARLIE, P., BRACHVOGEL, B. & BATEMAN, J. 2009. Global comparative transcriptome analysis of cartilage formation in vivo. *BMC Development Biology,* 9**,** 20.

CANDRIAN, C., VONWIL, D., BARBERO, A., BONACINA, E., MIOT, S., FARHADI, J., WIRZ, D., DICKINSON, S., HOLLANDER, A., JAKOB, M.,

LI, A., ALINI, M., HEBERER, M. & MARTIN, I. 2008. Engineered cartilage generated by nasal chondrocytes is responsive to physical forces resembling joint loading. *Arthritis and rheumatism* 58**,** 197-208.

CARLSON, M., ALT-HOLLAND, A., EGLES, C. & GARLICK, J. 2008. Three-dimensional tissue models of normal and diseased skin. *Current Protocol in Cell Biology,* Chapter 19**,** Unit 19.9.

CARRIER, L. R., PAPADAKI, M., RUPNICK, M., SCHOEN, F. J., BURSAC, N., LANGER, R., FREED, L. E. & VUNJAK-NOVAKOVIC, G. 2000. Cardiac tissue engineering: Cell seeding, cultivation parameters, and tissue construct characterization *Biotechnology and Bioengineering,* 64**,** 580-589.

CELESTE, M. N. & CHRISTOPHER, S. C. 2002. Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal. *Federation of European Biochemical Societies,* 514**,** 238-242.

CHEN, F. H., ROUSCHE, K. T. & TUAN, R. S. 2006. Technology Insight: adult stem cells in cartilage regeneration and tissue engineering. *Nature Clinical Practice Rheumatology* 2**,** 373-82.

CHI, S. S., RATTNER, J. B. & MATYAS, J. R. 2004. Communication between paired chondrocytes in the superficial zone of articular cartilage. *Journal of Anatomy,* 205**,** 363-370.

CHOI, J. B., YOUN, I., CAO, L., LEDDY, H. A., GILCHRIST, C. L., SETTON, L. A. & GUILAK1, F. 2008. Zonal changes in the three-dimensional morphology of the chondron under compression: The relationship among cellular, pericellular, and extracellular deformation in articular cartilage. *Journal of Biomechanics,* 40**,** 2596-603.

CHUANG, C.-K., LIN, K.-J., LIN, C.-Y., CHANG, Y.-H., YEN, T.-C., HWANG, S.-M., SUNG, L.-Y., CHEN, H.-C. & HU, Y.-C. 2009. Xenotransplantation of Human Mesenchymal Stem Cells into Immunocompetent Rats for Calvarial Bone Repair. *Tissue Engineering, Part A,* 15**,** 479-88.

CHUBINSKAYA, S., HAKIMIYAN, A., RAPPOPORT, L., YANKE, A., DC, D. R. & COLE, B. 2008. Response of human chondrocytes prepared for autologous implantation to growth factors. *The Journal of Knee Surgery,* 21**,** 192-9.

CLEELAND, C. S., GONIN, R., HATFIELD, A. K., EDMONSON, J. H., BLUM, R. H., STEWART, J. A. & PANDYA, K. J. 1994. Pain and Its Treatment in Outpatients with Metastatic Cancer. *New England Journal of Medicine,* 330**,** 592-596.

COHEN, K., DIEGELMANN, R. F. & LANDBLAD, W. J. 1992. *Wound healing, biochemical and clinical aspects*, W.B. Saunders Company

COHEN, S., SAMADIKUCHAKSARAEI, A., POLAK, J. M. & BISHOP, A. E. 2006. Antibiotics Reduce the Growth Rate and Differentiation of Embryonic Stem Cell Cultures. *Tissue Engineering,* 12**,** 2025-30.

DAN-NING, H. & MCCORMICK, S. 2005. *Chondrocyte culture formulationns*. USA patent application PCT/US2005/013515 10/11/2005.

DARLING, E. & ATHANASIOU, K. 2003. Articular cartilage bioreactors and bioprocesses. *Tissue Engineering,* 9**,** 9-26.

DAVIDSON, E. B., SCHARSTUHL, A., VITTERS, E., KRAAN, P. V. D. & BERG, W. V. D. 2005. Reduced transforming growth factor-beta signaling in cartilage of old mice: role in impaired repair capacity. *Arthritis Research & Therapy,* 7**,** R1338–R1347.

DAVIDSON, E. N. B., VITTERS, E. L., BERG, W. B. V. D. & KRAAN, P. M. V. D. 2006. TGF β-induced cartilage repair is maintained but fibrosis is blocked in the presence of Smad7. *Arthritis Research & Therapy,* 8**,** R65.

DAVIES, L. C., BLAIN, E. J., GILBERT, S. J., CATERSON, B. & DUANCE, V. C. 2008. The Potential of IGF-1 and TGFβ1 for Promoting "Adult" Articular Cartilage Repair: An In Vitro Study. *Journal of Tissue Engineering,* 14**,** 1251-1261.

DEARBORN, J. D. & JERGESEN, H. E. 2005. The evaluation and initial management of arthritis. *Primary Care,* 23**,** 215-40.

DESJARDINS, M. R. & HURTIG, M. B. 1990. Cartilage healing: A review with emphasis on the equine model. *Canadian Veterinary Journal,* 31**,** 565-572

DINGLE, J. T. 2007. The effect of NSAIDs on human articular cartilage glycosaminoglycan synthesis *European journal of rheumatology and inflammation* 16**,** 47-52.

DO, U. H., NEFTEL, K. A., SPADARI, S. & HÜBSCHER, U. 1987. Betalactam antibiotics interfere with eukaryotic DNA-replication by inhibiting DNA polymerase alpha. *Nucleic Acids Res,* 15**,** 10495–10506.

DUTTON, M. 2004. Orthopaedic Examination, Evaluation, and Intervention. McGraw-Hill Professional.

EDWARDS, R. B. 2002. Debridement of Chondral Defects of the Knee. *American journal of sports medicine,* 30**,** 90-97.

ELTAWIL, N. M., BARI, C. D., ACHAN, P., PITZALIS, C. & DELL'ACCIO, F. 2009. A novel in vivo murine model of cartilage regeneration. Age and strain-dependent outcome after joint surface injury. *Osteoarthritis and Cartilage,* 17**,** 695-704.

ERICKSON, G. R., GIMBLE, J. M., FRANKLIN, D. M., RICE, H. E., AWAN, H. & GUILAK, F. 2002. Chondrogenic Potential of Adipose Tissue-Derived Stromal Cells in Vitro and in Vivo. *Biochemical and Biophysical Research Communications,* 290**,** 763-769.

FERGUSON, M. W. J. & O'KANE, S. 2004. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 359**,** 839–850.

FILOVÁ, E., F, J., HANDL, M., LYTVYNETS, A., RAMPICHOVÁ, M., VARGA, F., CINÁTL, J., SOUKUP, T., TRČ, T. & AMLER, E. 2008. Novel composite hyaluronan/type I collagen/fibrin scaffold enhances repair of osteochondral defect in rabbit knee. *Journal of Biomedical Materials Research, Part B, Applied Biomaterials,* 87**,** 415-24.

FOTY, R. & STEINBERG, M. 2005. The differential adhesion hypothesis: a direct evaluation. *Development Biology,* 278**,** 255-263.

FOX, V., GOKHALE, P., WALSH, J., MATIN, M., JONES, M. & ANDREWS, P. 2008. Cell-cell signaling through NOTCH regulates human embryonic stem cell proliferation. *Journal of Stem Cell,* 26**,** 715-23.

FREYRIA, A.-M., RONZIÈRE, M.-C., CORTIAL, D., GALOIS, L., HARTMANN, D., HERBAGE, D. & MALLEIN-GERIN, F. 2009. Comparative Phenotypic Analysis of Articular Chondrocytes Cultured within Type I or Type II Collagen Scaffolds. *Tissue Engineering, Part A,* 15**,** 1233-1245.

FUMAROLA, C., MONICA, S. L., ALFIERI, R., BORRA, E. & GUIDOTTI, G. 2005. Cell size reduction induced by inhibition of the mTOR/S6K-signaling pathway protects Jurkat cells from apoptosis. *Cell death and differentiation,* 12**,** 1344-57.

GALVANI, D. W. & COWLEY, J. C. (eds.) 1992. *Cytokine therapy,* Cambridge: University of Cambridge.

GARDNER, D., MAZURYK, R., O'CONNOR, P. & ORFORD, C. 1987. Anatomical changes and pathogenesis of OA in man, with particular reference to the hip and knee joints. Edited by: Lott DJ, Jasani MK, Birdwood GFB. Chichester: . *Research and Veterinary Science***,** John Wiley & Sons; 1987:21-48.

GIANNETTI, B., BEHRENS, P. & ASCULAI, S. 2005. *Methods, instruments and materials for chondrocyte cell transplantation*. Germany patent application.

GIGOUT, A., JOLICOEUR, M., NELEA, M., RAYNAL, N., FARNDALE, R. & BUSCHMANN, M. 2008. Chondrocyte aggregation in suspension culture is GFOGER-GPP and beta 1 integrin dependent. *The Journal of Biologycal Chemistry,* 283**,** 31522-30.

GILLOGLY, S. D., VOIGHT, M. & BLACKBURN, T. 1998. Treatment of articular cartilage defects of knee with autologous chondrcoyte implantation, . *Journal of Orthopaedic and Sports Physical Therapy,* 28**,** 241-251.

GOLDBERG, A. J., LEE, D. A., BADER, D. L. & BENTLEY, G. 2005. Autologous chondrocyte implantation: Culture in a TGF-ß- containing medium enhances the expression of a chondrocyte phenotype in passaged human chondrocytes in pellet culture. *Journal of Bone and Joint Surgery - British Volume* 87-B**,** 128-134.

GOLDRING, M. B. 2007. Osteoarthritis and cartilage: The role of cytokines *Current Rheumatology Report,* 2**,** 459-465.

GOOCH, K., KWON, J., BLUNK, T., LANGER, R., FREED, L. & VUNJAK-NOVAKOVIC, G. 2001. Effects of mixing intensity on tissue-engineered cartilage. *Biotechnology and Bioengineering,* 72**,** 402-7.

GORVY, D. A., HERRICK, S. E., SHAH, M. & FERGUSON, M. W. J. 2005. Experimental Manipulation of Transforming Growth Factor-β Isoforms Significantly Affects Adhesion Formation in a Murine Surgical Model. *American Journal of Pathology,* 167**,** 1005-19.

GRASHOFF, C., ASZÓDI, A., SAKAI, T., HUNZIKER, E. B. & FÄSSLER, R. 2003. Integrin-linked kinase regulates chondrocyte shape and proliferation. *EMBO Reports,* 4**,** 432-438.

GUILAK, F. 2003. *Functional Tissue Engineering,* New York, Springer.

HAN, F., GILBERT, J. R., HARRISON, G., ADAMS, C. S., FREEMAN, T., TAO, Z., ZAKA, R., LIANG, H., WILLIAMS, C., TUAN, R. S., NORTON, P. A. & HICKOK, N. J. 2007. Transforming Growth Factor β1 regulates fibronectin isoform expression and splicing factor SRp40 expression during ATDC5 chondrogenic maturation. *Experimental Cell Research,* 313**,** 1518–1532. .

HARMS, S., LARSON, R., SAHMOUN, A. E. & BEAL, J. R. 2007. Obesity incearses the likelihood of total joint replacemant surgery among younger adults. *International Orthopaedics,* 31**,** 23–26.

HARRISON, R. G., PICKFORD, G. E. & HUTCHINSON, G. E. 2004. Are Research Schools Necessary? Contrasting Models of 20th Century Research at Yale. *Journal of the History of Biology*.

HASHIMOTO, J., OGAWA, T., TSUBOTA, Y. & MIYAZAKI, K. 2005. Laminin-5 suppresses chondrogenic differentiation of murine teratocarcinoma cell line ATDC5. *Experimental Cell Research,* 310**,** 256-69.

HENRIKSON, R. C., KAYE, G. I. & MAZURKIEWICZ, J. E. 1997. Cartilage Growth. *In:* NIEGINSKI, E. A. (ed.) *Histology.* 1 ed. Pennsylvania: Lippincott Williams & Wilkins.

HENSON, F. M. & VINCENT, T. 2007. Chondrocyte outgrowth into a gelatin scaffold in a single impact load model of damage/repair – effect of BMP-2. *Arthritis Research & Therapy,* 8**,** 120.

HEUNGSOO, S., SEONGBONG, J. & MIKOS, A. G. 2003. Biomimetic materials for tissue engineering. *Biomaterilas,* 24**,** 4353-436.

HICKERY, M., BAYLISS, M., DUDHIA, J., LEWTHWAITE, J., EDWARDS, J. & PITSILLIDES, A. 2003. Age-related changes in the response of human articular cartilage to IL-1alpha and transforming growth factor-beta (TGF-beta): chondrocytes exhibit a diminished sensitivity to TGF-beta. *The Journal of Biologycal Chemistry,* 278**,** 53063-71.

HIRAI, F., NAKAYAMADA, S., OKADA, Y., SAITO, K., KUROSE, H., MOGAMI, A. & TANAKA, Y. 2007. Small GTPase rho signaling is involved in β1 integrin-mediated up-regulation of intercellular adhesion molecule 1 and receptor activator of nuclear factor kb ligand on osteoblasts and osteoclast maturation. *Biochemical and biophysical research communications,* 356**,** 279-285.

HORAS, U., PELINKOVIC, D., HERR, G. & SCHNETTLER, R. 2003. Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial. *The journal of Bone and Joint Surgery, American Volume,* 85**,** 185-92.

HOU, J., HOLMES, M., LAI, W. & MOW, V. 1989. Boundary conditions at the cartilage-synovial fluid interface for joint lubrication and theoretical verifications. *Journal of Biomechanical Engineering,* 111**,** 78-87.

HU, J. & ATHANASIOU, K. 2006. Chondrocytes from different zones exhibit characteristic differences in high density culture. *Connective Tissue Research,* 47**,** 133-40.

HUMPHRIES, M. J., MCEWAN, P. A., BARTON, S. J., BUCKLEY, P. A., BELLA, J. & MOULD, A. P. 2003. Integrin structure: heady advances in ligand binding, but activation still makes the knees wobble. *Trends in Biochemical Sciences,* 28**,** 313-320.

IBOLD, Y., LÜBKE, C., PELZ, S., AUGST, H., KAPS, C., RINGE, J. & SITTINGER, M. 2009. Effect of different ascorbate supplementations on in vitro cartilage formation in porcine high-density pellet cultures. *Tissue and Cell,* 41**,** 249-256.

IKADA, Y. 2006a. Challenges in tissue engineering. *Journal of The Royal Society,* 3**,** 589–601.

IKADA, Y. 2006b. Tissue enginneeirng: Fundamental and applications, First Edition. *In:* IKADA, Y. (ed.). Academic Press; 1 Edition (September 13, 2006).

IM, G.-I. & SHIN, S.-R. 2002. Changes in the production and the effect of nitric oxide with aging in articular cartilage: An experimental study in rabbits *Acta Orthopaedica,* 73**,** 6-10.

INOUE, L. 1997. *Sydney Ringer Field Note for the 100st memorial year in 2010* [Online]. Tokyo: Lisa Inoue. [Accessed 12 Aug. 2007 2007].

IRVINE-JR., T. F. & PARK, N.-A. 1987. *Apparatus and method for viscosity measurements for Newtonian and non-Newtonian fluids.* USA patent application 06/694747.

ISHIDA, O., TANAKA, Y., MORIMOTO, I., TAKIGAWA, M. & ETO, S. 1997. Chondrocytes are regulated by cellular adhesion through CD44 and hyaluronic acid pathway. *Journal of bone and mineral research,* 12**,** 1657-1663.

ISRAILIDES, C. J., WEIR, A. N. C. & BULL, A. T. 1989. Effect of antibiotics on lysine production in free and immobilized cells of Bacillus subtilis *Applied Microbiology and Biotechnology,* 32**,** 134-136.

JAFFRÉ, B., WATRIN, A., LOEUILLE, D., GILLET, P., NETTER, P., LAUGIER, P. & SAÏED, A. 2003. Effects of antiinflammatory drugs on arthritic cartilage: a high-frequency quantitative ultrasound study in rats. *Arthritis and Rheumatism,* 48**,** 1594-601.

JAMES, C.-B. & UHL, T. L. 2001. A Review of Articular Cartilage Pathology and the Use of Glucosamine Sulfate. *Journal of Athletic Training,* 36**,** 413-419.

JAMIL, M. M. A., YOUSEFFI, M., TWIGG, P. C., BRITLAND, S. T., LIUD, S., SEED, C. W., ZHANG, J., SOMEKH, M. G. & DENYER, M. C. T. 2008. High resolution imaging of bio-molecular binding studies using a widefield surface plasmon microscope *Sensors and Actuators B: Chemical* 129**,** 566-574.

JAMTVEDT, G., DAHM, K. T., HOLM, I. & FLOTTORP, S. 2008. Measuring physiotherapy performance in patients with osteoarthritis of the knee: A prospective study. *BMC Health Services Research,* 8**,** 145.

JOOSTEN, L. A. B., SMEETS, R. L., KOENDERS, M. I., BERSSELAAR, L. A. M. V. D., HELSEN, M. M. A., OPPERS-WALGREEN, B., LUBBERTS, E., IWAKURA, Y., LOO, F. A. J. V. D. & BERG, W. B. V. D. 2004. Interleukin-18 Promotes Joint Inflammation and Induces Interleukin-1-Driven Cartilage Destruction. *the American Journal of Pathology,* 165**,** 959-67.

KARVONEN, R., NEGENDANK, W., TEITGE, R. & REED, A. 1994. Factors affecting articular cartilage thickness in osteoarthritis and aging. *The Journal of Rheumatology,* 21**,** 1310-8.

KATO, Y., IWAMOTO, M., KOIKE, T., SUZUKI, F. & TAKANO, Y. 1988. Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: regulation by transforming growth factor beta and serum factors *National Academy of Sciences,* 85**,** 9552-9556.

KAVUNCU, V. & EVCIK, D. 2004. Physiotherapy in Rheumatoid Arthritis. *MedGenMed,* 6**,** 3.

KERMANI, M. G. & PHAM, S. H. 2001. Role of Cytokines and Cytokine Therapy in Wound Healing and Fibrotic Diseases. *Current Pharmaceutical Design,* 7**,** 1083-103.

KHOURY, M., ESCRIOU, V., COURTIES, G., GALY, A., YAO, R., LARGEAU, C., SCHERMAN, D., JORGENSEN, C. & APPARAILLY, F. 2008. Efficient suppression of murine arthritis by combined anticytokine small interfering RNA lipoplexes. *Arthritis and Rheumatism,* 58**,** 2356-67.

KIM, M.-H., KINO-OKA, M., MORINAGA, Y., SAWADA, Y., KAWASE, M., YAGI, K. & TAYA, M. 2009. Morphological regulation and aggregate formation of rabbit chondrocytes on dendrimer-immobilized surfaces with d-glucose display. *Journal of Bioscience and Bioengineering,* 107**,** 196-205.

KINGSLEY, R. C. & MEHTA, S. 2007. Othopedic key review concepts. Lippincott Williams & Wilkins, 165-167.

KNUTSEN, G., DROGSET, J., ENGEBRETSEN, L., GRØNTVEDT, T., ISAKSEN, V., LUDVIGSEN, T., ROBERTS, S., SOLHEIM, E., STRAND, T. & JOHANSEN, O. 2008. A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. *The journal of Bone and Joint Surgery, American Volume,* 89**,** 2105-2112.

KOLI, K., SAHARINEN, J., HYYTIÄINEN, M., PENTTINEN, C. & KESKI-OJA, J. 2001. Latency, activation, and binding proteins of TGF-beta. *Journal of electron microscopy technique.,* 52**,** 354-62.

KOPACEK, K. B. 2007. Administration and Kinetics of Drugs. Merck & Co., Inc.

KOPLOV, H. M. 1994. Cell culture and purification process. *Annals of Hematology,* 68**,** 15-20.

KRAUSS, G. 2006. Biochemistry of Signal Transduction and Regulation (Third Edition). Published by Wiley-VCH.

KUZMINA, V. V. & PERVUSHINA, K. A. 2004. Effects of Temperature and pH on Activity of Proteinases of Intestinal Mucosa and Enteral Microbiota of Fish. *Journal of Evolutionary Biochemistry and Physiology,* 40**,** 264-270.

KWOK-SUI LEUNG, L. Q., WING-HOI CHEUNG 2008. A Practical Manual for Musculoskeletal Research. *In:* KWOK-SUI LEUNG, L. Q., WING-HOI CHEUNG (ed.). World Scientific, 2008.

LAGANÀ, K., MORETTI, M., DUBINI, G. & RAIMONDI, M. 2008. A new bioreactor for the controlled application of complex mechanical stimuli for cartilage tissue engineering. *Journal of engineering in medicine,* 222**,** 705-15.

LALAN, S., POMERANTSEVA, I. & VACANTI, J. P. 2001. Tissue Engineering and its Potential Impact on Surgery. *World Journal of Surgery,* 25**,** 1458-66.

LAUGE-PEDERSEN, H. & ASPENBERG, P. 2003. Synovial fluid depletion: successful arthrodesis without operative cartilage removal *Journal of Orthopaedic Science,* 8**,** 591-595.

LAWRENCE, D. 2001. Latent-TGF-beta: an overview. *Moelcular and Cellular Biochemistry,* 219**,** 163-70.

LEE, H.-U., PARK, S.-Y., KANG, Y.-H., JEONG, S.-Y., CHOI, S.-H., JAHNG, K.-Y. & CHO, C.-R. 2010. Surface modification of and selective protein attachment to a flexible microarray pattern using atmospheric plasma with a reactive gas. *Acta Biomaterialla,* 6**,** 519-525.

LEONIDAS G. ALEXOPOULOSA, SETTONA, L. A. & GUILAK, F. 2005. The biomechanical role of the chondrocyte pericellular matrix in articular cartilage. *Acta Biomaterialla,* 1**,** 317-325.

LEVIN, E. R. 2008. Rapid signaling by steroid receptors. *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology,* 295**,** 1425-30.

LI, H., LIU, D., ZHAO, C.-Q., JIANG, L.-S. & DAI, L.-Y. 2008. High glucose promotes collagen synthesis by cultured cells from rat cervical posterior longitudinal ligament via transforming growth factor-β1. *European Spine Journal,* 17**,** 873–881.

LI, Y., LEE, H. J. & CORN, R. M. 2006. Fabrication and characterization of RNA aptamer microarrays for the study of protein–aptamer interactions with SPR imaging. *Nucleic Acids Research,* 34**,** 6416–6424.

LIBERA, J., GRALLA, A., HÖLL, T., JOSIMOVIC-ALASEVIC, O. & MEISEL, H. J. 2004. Regenerative capability of human disc derived chondrocytes *The Spine Journal* 4**,** 2609-2620.

LO, C. Y. & CHANG, Y. P. 2003. Osteochondral grafting of the Metacarpophalangeal joint in rheumatoid arthritis. *Journal of hand surgery,* 28**,** 94-7.

LOESER, R. 2000. Chondrocyte integrin expression and function. *Biorheology,* 37**,** 109-16.

LOWE, C. J. M., BARKER, K. L., DEWEY, M. & SACKLEY, C. M. 2007. Effectiveness of physiotherapy exercise after knee arthroplasty for osteoarthritis: systematic review and meta-analysis of randomised controlled trials. *BMJ (Clinical research ed.),* 335**,** 812.

MACNEIL, S. 2008. Biomaterials for tissue engineering of skin. *Materials Today,* 11**,** 26-35.

MADRY, H. & PAPE, D. 2008. Autologous chondrocyte transplantation. *Der Orthopäde,* 37**,** 756-63.

MAITRE, C. L. L., HOYLAND, J. A. & FREEMONT, A. J. 2007. Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1β and TNFα expression profile. *Arthritis Research & Therapy,* 9**,** R77.

MAJUMDAR, M. K., BANKS, V., P.PELUSO, D. & MORRIS, E. A. 2000. Isolation, characterization and chondrogenic potential of human bone marrow-derived multipotential stromal cells. *Journal of cellular physiology,* 185**,** 98-106.

MARTIN, I., SUETTERLIN, R., BASCHONG, W., HEBERER, M., VUNJAK-NOVAKOVIC, G. & FREED, L. E. 2001. Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation. *Journal of Cellular Biochemistry,* 83**,** 121-128.

MARTIN, I., WENDT, D. & HEBERER, M. 2004. The role of bioreactors in tissue engineering. *Trends in Biotechnology,* 22**,** 80-6.

MARTINEZ-LIARTE, J. H., SOLANO, F. & LOZANO, J. A. 2007. Effect of Penicillin-Streptomycin and Other Antibiotics on Melanogenic Parameters in Cultured B16/F10 Melanoma Cells. *Pigment Cell & Melanoma Research,* 8**,** 83 - 88.

MCDONALD, J. C., DUFFY, D. C., ANDERSON, J. R., CHIU, D. T., WU, H., SCHUELLER, O. J. A. & WHITESIDES, G. M. 1999. Fabrication of microfluidic systems in poly(dimethylsiloxane) *Electrophoresis,* 21**,** 27-40.

MCILWRAITH, C. W. 2007. *Anatomy and Physiology of Equine Joints* [Online]. Colorado: Colorado state University. [Accessed 01 July 2007].

MENDELSON, K. & SCHOEN, F. J. 2006. Heart Valve Tissue Engineering: Concepts, Approaches, Progress, and Challenges. *Annals of Biomedical Engineering,* 34**,** 1799–1819.

METCALFE, A. D. & FERGUSON, M. W. J. 2006. Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. *Journal of the Royal Society Interface,* 4**,** 413–437.

MEYER, U. & WIESMANN, H. P. 2006. *Bone and Cartilage Engineering,* Pages 25-33. Springer Verlag.

MI, R., LUO, Y., CAI, J., LIMKE, T. L., RAO, M. S. & HÖKE, A. 2005. Immortalized neural stem cells differ from nonimmortalized cortical neurospheres and cerebellar granule cell progenitors *Journal of Experimental Neurology,* 194**,** 301-319.

MICHOT, M., CONEN, D., HOLTZ, D., ERNI, D., ZUMSTEIN, M. D., RUFLIN, G. B. & RENNER, N. 2002. Prevention of deep-vein thrombosis in ambulatory arthroscopic knee surgery. *The Journal of Arthroscopic & Related Surgery,* 18**,** 257-263.

MILLER, B. S., STEADMAN, J. R., BRIGGS, K. K., RODRIGO, J. J. & RODKEY, W. G. 2004. Patient Satisfaction and Outcome After Microfracture of the Degenerative Knee. *Journal of knee surgery,* 17**,** 13-7.

MINAS, T. & PETERSON, L. 1999. Advanced techniques in autologous in chondrocyte transplantation. *Clinics in Sport Medicine,* 18**,** 13-44.

MINAS, T. & PETERSON, L. 2005. Advanced techniques in auotologous in chonrocyte transplantation. *Elsevier*.

MITHOEFER, K., WILLIAMS, R., WARREN, R., WICKIEWICZ, T. & MARX, R. 2006. High-impact athletics after knee articular cartilage repair: a prospective evaluation of the microfracture technique. *The American Journal of Sports Medicine,* 34**,** 1413-18.

MIYATA, S., KOSHIKAWA, N., YASUMITSU, H. & MIYAZAKI, K. 2000. Trypsin Stimulates Integrin α5β1-dependent Adhesion to Fibronectin and Proliferation of Human Gastric Carcinoma Cells through Activation of Proteinase-activated Receptor-2. *Journal of Biological Chemistry,* 275**,** 4592-4598.

MONROY, A., KOJIMAC, K., GHANEMA, M. A., PAZC, A. C., KAMILD, S., VACANTIC, C. A. & EAVEYA, R. D. 2007. Tissue engineered cartilage "bioshell" protective layer for subcutaneous implants *International Journal of Pediatric Otorhinolaryngology,* 71**,** 547-552

MOODY, H. R., BROWN, C. P., BOWDEN, J. C., CRAWFORD, R. W., MCELWAIN, D. L. S. & OLOYEDE, A. O. 2006. In vitro degradation of articular cartilage: does trypsin treatment produce consistent results? *Journal of Anatomy,* 209**,** 259 - 267.

MORONI, L., HAMANN, D., PAOLUZZI, L., PIEPER, J., WIJN, J. R. D. & BLITTERSWIJK, C. A. V. 2008. Regenerating Articular Tissue by Converging Technologies. *PLoSONE,* 3**,** e3032.

MOTOYAMA, M., DEIE, M., KANAYA, A., NISHIMORI, M., MIYAMOTO, A., YANADA, S., ADACHI, N. & OCHI, M. 2010. In vitro cartilage formation using TGF-beta-immobilized magnetic beads and mesenchymal stem cell-magnetic bead complexes under magnetic field conditions. *Journal of Biomaterial Research Part A,* 92**,** 196-204.

MOW, V. C. & HUISKES, R. 2004. Basic Orthopaedic Biomechanics & Mechano-biology. *In:* HURLEY, R. (ed.). Lippincott Williams & Wilkins

MÜLLER, B. 2002. Cytokine Imbalance in Non-Immunological Chronic Disease *Cytokine,* 18**,** 334-339

MURRAY, K., GROM, A., THOMPSON, S., LIEWEN, D., PASSO, M. & GLASS, D. 1998. Contrasting cytokines profile in the synovium of different forms of juvenile rheumatoid arthritis and spondyloarthropathy: prominence of interleukin 4 in restricted disease. *The Journal of Rheumatology,* 25**,** 1388-98.

NESIC, D., WHITESIDE, R., BRITTBERG, M., WENDT, D., MARTIN, I. & MAINIL-VALERT, P. 2006. Cartilage tissue engineering for degenerative joint disease. *Advanced Drug Delivery Reviews,* 58**,** 300-22.

NEUGEBAUER, V., HAN, J. S., ADWANIKAR, H., FU, Y. & JI, G. 2007. Techniques for assessing knee joint pain in arthritis. *Molecular Pain,* 3**,** 8.

NEUROHR, C., NISHIMURA, S. L. & SHEPPARD, D. 2006. Activation of Transforming Growth Factor-by the Integrin v8 Delays Epithelial Wound Closure. *American Journal of Respiratory and Molecular Biology,* 35**,** 252-256.

NICHOLAS, J. S. 1960. Ross Granville Harrison 1870-1959. *Yale Journal of Biology and Medicine,* 32**,** 407.b1–412.

OKA, K., OKA, S., SASAKI, T., ITO, Y., BRINGAS, P., JR., NONAKA, K. & CHAI, Y. 2007. The role of TGF-β signaling in regulating chondrogenesis and osteogenesis during mandibular development. *Developmental Biology,* 303**,** 391-404.

OKAZAKI, R., SAKAI, A., NAKAMURA, T., KUNUGITA, N., NORIMURA, T. & SUZUKI, K. 1996. Effects of transforming growth factor beta s and basic fibroblast growth factor on articular chondrocytes obtained from immobilised rabbit knees. *Annals of Rheumatic Disease,* 55**,** 181-186.

OLSEN, J., ONG, S. & MANN, M. 2004. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular and Cell Proteomics,* 3**,** 608-614.

ONG, C. K. S., LIRK, P., TAN, C. H. & SEYMUR, R. A. 2007. An evidence-based update on nonstroidal anti-inflammatory drugs. *Clinical medicine and Research,* 5**,** 19-34.

OSSENDORF, C., KAPS, C., KREUZ, P. C., BURMESTER, G. R., SITTINGER, M. & ERGGELET, C. 2007. Treatment of posttraumatic and focal osteoarthritic cartilage defects of the knee with auotologous polymer-based three dimensional chondrocyte grafts: 2-year clinical results. *Arthritis research & therapy,* 9**,** R41.

OSTERGAARD, K., SALTER, D., PETERSEN, J., BENDTZEN, K., HVOLRIS, J. & ANDERSEN, C. 1998. Expression of α and β subunits of the integrin superfamily in articular cartilage from macroscopically normal and osteoarthritic human femoral heads. *Annals of the Rheumatic Diseases,* 57**,** 303–308.

PAVESIO, A., ABATANGELO, G., BORRIONE, A., BROCCHETTA, D., HOLLANDER, A., KON, E., TORASSO, F., ZANASI, S. & MARCACCI, M. 2003. Hyaluronan-based scaffolds (Hyalograft C) in the treatment of knee cartilage defects: preliminary clinical findings. *Novartis Foundation Symposium,* 249**,** 229-33.

PEI, M., LUO, J. & CHEN, Q. 2008. Enhancing and maintaining chondrogenesis of synovial fibroblasts by cartilage extracellular matrix protein matrilins. *Osteoarthritis and Cartilage,* 16**,** 1110-1117.

PERL, A., REINHOUDT, D. N. & HUSKENS, J. 2009. Microcontact Printing: Limitations and Achievements. *Advanced Materials,* 21**,** 2257 - 2268.

PETERSEN, W., ZELLE, S. & ZANTOP, T. 2008. Arthroscopic implantation of a three dimensional scaffold for autologous chondrocyte transplantation. *Archives of Orthopaedic and Trauma Surgery,* 128**,** 505-508.

PETERSON, L. & RENSTROEM, P. 2004. Sport Injuries: Their Preventation and Treatment. Informa health care.

PETTY, K. 2009. Biology 160: Human Anatomy and Physiology, San Diego Miramar College. http://faculty.sdmiramar.edu/KPETTI/Bio160/TissueHistology/Chondrocyte.jpg.

PETTY, R., SOUTHWOOD, T., BAUM, J. & BHETTAY, E. 1998. Division of propsed classification criteria for juvenile idiopathic arthritis. *Journal of Rheumatology,* 25**,** 1991-4.

POHLERS, D., BEYER, A., KOCZAN, D., WILHELM, T., THIESEN, H.-J. & KINNE, R. W. 2007. Constitutive upregulation of the transforming growth factor-β pathway in rheumatoid arthritis synovial fibroblasts. *Arthritis Research & Therapy,* 9**,** R59.

POUR, A. E., PARVIZI, J., SHARKEY, P. F., HOZACK, W. J. & ROTHMAN, R. H. 2003. Minimally Invasive Hip Arthroplasty: What Role Does Patient Preconditioning Play? *The Journal of Arthroplasty,* 89**,** 1920-1927.

PRITZKER, K. P. H., GAY, S., JIMENEZ, S. A., OSTERGAARD, K., PELLETIER, J.-P., REVELL, P. A., SALTER, D. & BERG, W. B. V. D. 2006. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis and Cartilage,* 14**,** 13-29.

RAAB, M., DAXECKER, H., EDWARDS, R., TREUMANN, A., MURPHY, D. & MORAN, N. 2010. Protein interactions with the platelet integrin alpha(IIb) regulatory motif. *Proteomics,* 10**,** 2790 - 2800.

RADUCANU, L., HUNZIKER, E., DROSSE, I. & ASZODI, A. 2009. beta 1 Integrin Deficiency Results in Multiple Abnormalities of the Knee Joint. *JOURNAL OF BIOLOGICAL CHEMISTRY* 284**,** 23780-23792.

RAFIQUE, M. 2010. Histology of CArtilage. 2/5/2010 ed.: http://www.docstoc.com/docs/24583628/Histology-of-Cartilage.

RAFTERY, L. A. & SUTHERLAND, D. J. 2002. TGF-β Family Signal Transduction in Drosophila Development: From Mad to Smads *Development Biology,* 210**,** 251-268.

ROLFE, K., RICHARDSON, J., VIGOR, C., IRVINE, L., GROBBELAAR, A. & LINGE, C. 2007. A role for TGF-beta1-induced cellular responses during wound healing of the non-scarring early human fetus? *The Journal of Investigative Dermatology,* 127**,** 2656-67.

ROSSO, F., GIORDANO, A., BARBARISI, M. & BARBARISI, A. 2003. From Cell-ECM interactions to tissue engineering. *Journal of Cellular Physiology,* 199**,** 174 - 180.

SCHLEICHER, M., WENDEL, H. P., FRITZE, O. & ULRICH A STOCK 2009. In vivo tissue engineering of heart valves: evolution of a novel concept *Regenerative Medicine,* 4**,** 613-619.

SCHNABEL, O., MARLOVITS, S., ECKHOFF, G., FICHTEL, I., GOTZEN, L., VÉCSEI, V. & SCHLEGEL, J. 2002. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture *Osteoarthritis and Cartilage,* 10**,** 62-70.

SCHUURMAN, W., GAWLITTA, D., KLEIN, T. J., HOOPE, W. T., RIJEN, M. H. P. V., DHERT, W. J. A., WEEREN, P. R. V. & MALDA, J. 2009. Zonal Chondrocyte Subpopulations Reacquire Zone-Specific Characteristics During In Vitro Redifferentiation. *The American Journal of Sports Medicine,* 33**,** 97-104.

SCHWARTZ, Z., BONEWALD, L., CAULFIELD, K., BROOKS, B. & BOYAN, B. 1993. Direct effects of transforming growth factor-beta on chondrocytes are modulated by vitamin D metabolites in a cell maturation-specific manner. *The Endocrine Society,* 132**,** 1544-1552.

SCHWENTKER, A., VODOVOTZ, Y., WELLER, R. & BILLIAR, T. R. 2002. Nitric oxide and wound repair: role of cytokines? *Journal of Nitric Oxide Society,* 7**,** 1-10.

SELMI, T., VERDONK, P., CHAMBAT, P., DUBRANA, F., POTEL, J., BARNOUIN, L. & NEYRET, P. 2008. Autologous chondrocyte implantation in a novel alginate-agarose hydrogel: outcome at two years. *The journal of Bone and Joint Surgery, British Volume,* 90**,** 597-604.

SHAH, M., REVIS, D., JR., HERRICK, S., BAILLIE, R., THORGEIRSON, S., FERGUSON, M. & ROBERTS, A. 1999. Role of Elevated Plasma Transforming Growth Factor-β1 Levels in Wound Healing. *The American Journal of Pathology,* 154**,** 1115–1124.

SHAKIBAEI, M., CSAKI, C. & MOBASHERI, A. 2008. Diverse roles of integrin receptors in articular cartilage. *Advances in anatomy, embryology, and cell biology,* 197**,** 1-60.

SIANG, J., YANG, J. & COLAS, A. 2005. *Silicon coatings for urinary catheters* [Online]. Dow Coring. [Accessed Aug. 2008].

SIGMA 2007. Sigma Aldrich, UK.

SILBERBERG, R., AUFDERMAUR, M. & HASLER, M. 2005. Ultrastructural changes in articular chondrocytes of mice fed a lard-enriched diet. *The Anatomical Record,* 166**,** 447-67.

SMITH, C. & WOOD, E. J. 1991. *Cell Biology-Molecular and cell Biochemistry,* Springer.

SNIEKERS, Y., OSCH, G. V., JAHR, H., WEINANS, H. & LEEUWEN, J. V. 2009. Estrogen modulates iodoacetate-induced gene expression in bovine cartilage explants. *Journal of Orthopaedic Research,* 28**,** 607-15.

SOBRAL, J., O'NEILL, B. & CLYNE, T. W. 2008. Cell Growth on Metallic Substrates with Tailored Topographies Produced by Laser Processing and Heat Treatments. University of Cambridge.

SOO, C., HU, F.-Y., ZHANG, X., WANG, Y., BEANES, S. R., LORENZ, H. P., HEDRICK, M. H., MACKOOL, R. J., PLAAS, A., KIM, S.-J., LONGAKER, M. T., FREYMILLER, E. & TING, K. 2000. Differential Expression of Fibromodulin, a Transforming Growth Factor-β Modulator, in Fetal Skin Development and Scarless Repair. *The American Journal of Pathology,* 157**,** 423-433.

SPAGNOLI, A., O'REAR, L., CHANDLER, R. L., GRANERO-MOLTO, F., MORTLOCK, D. P., GORSKA, A. E., WEIS, J. A., LONGOBARDI, L., CHYTIL, A., SHIMER, K. & MOSES, H. L. 2007. TGF-β signaling is essential for joint morphogenesis. *The Journal of Cell Biology,* 177**,** 1105-17.

SPITERI, C., RAIZMAN, I., PILLIAR, R. & KANDE, R. 2010. Matrix accumulation by articular chondrocytes during mechanical stimulation is influenced by integrin-mediated cell spreading. *Journal of Biomaterial Research Part A,* 94A**,** 122-129.

STEINERT, A. F., PROFFEN, B., KUNZ, M., HENDRICH, C., GHIVIZZANI, S. C., NÖTH, U., RETHWILM, A., EULERT, J. & EVANS, C. H. 2009. Hypertrophy is induced during the in vitro chondrogenic differentiation of human mesenchymal stem cells by bone morphogenetic protein-2 and bone morphogenetic protein-4 gene transfer. *Arthritis Research & Therapy,* 11**,** R148.

STEPHENS, T. D. & SEEGMILLER, R. E. 2005. Normal production of cartilage glycosaminoglycan in mice homozygous for the chondrodysplasia gene. *Birth Defects Research Part A: Clinical and Molecular Teratology,* 13**,** 317-25.

STEVENS, M. M., MARINI, R. P., SCHAEFER, D., ARONSON, J., LANGER, R. & SHASTRI, V. P. 2005. In vivo engineering of organs: The bone bioreactor. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 11450–11455.

STOFF, A., RIVERA, A. A., MATHIS, J. M., MOORE, S. T., BANERJEE, N. S., EVERTS, M., ESPINOSA-DE-LOS-MONTEROS, A., NOVAK, Z., VASCONEZ, L. O., BROKER, T. R., RICHTER, D. F., FELDMAN, D., SIEGAL, G. P., STOFF-KHALILI, M. A. & CURIEL, D. T. 2007. Effect of adenoviral mediated overexpression of fibromodulin on human dermal fibroblasts and scar formation in full-thickness incisional wounds. *Journal of molecular medicine* 85**,** 481-496.

STOOP, R., ALBRECHT, D., GAISSMAIER, C., FRITZ, J., FELKA, T., RUDERT, M. & AICHER, W. K. 2007. Comparison of marker gene expression in chondrocytes from patients receiving autologous chondrocyte transplantation versus osteoarthritis patients. *Arthritis Research & Therapy,* 9**,** R60.

SURGERYINFORMATION. 2008. *Autologous chondrocytes implantation knee surgery operation* [Online]. surgery-information.org. [Accessed].

TAKAHASHI, K., HASHIMOTO, S., KUBO, T., HIRASAWA, Y., LOTZ, M. & AMIEL, D. 2000. Effect of hyaluronan on chondrocyte apoptosis and nitric oxide production in experimentally induced osteoarthritis. *Journal of Rheumatology,* 27**,** 1713-1720

TCHETINA, E. V., ANTONIOU, J., TANZER, M., ZUKOR, D. J. & POOLE, A. R. 2006. Transforming Growth Factor-β2 Suppresses Collagen Cleavage in Cultured Human Osteoarthritic Cartilage, Reduces Expression of Genes Associated with Chondrocyte Hypertrophy and Degradation, and Increases Prostaglandin E2 Production. *The American Journal of Pathology,* 168**,** 131–140.

TERPSTRA, L., PRUD'HOMME, J., ARABIAN, A., TAKEDA, S., KARSENTY, G., DEDHAR, S. & ST-ARNAUD, R. 2003. Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes *Journal of Cell Biology,* 162**,** 139-148.

TETLOW, L. C. & WOOLLEY, D. E. 1995. Mast cells, cytokines, and metalloproteinases at the rheumatoid lesion: dual immunolocalisation studies. *Annals of the Rheumatic Diseases,* 54**,** 896–903.

THORP, B., ANDERSON, I. & JAKOWLEW, S. 1992. Transforming growth factor-beta 1, -beta 2 and -beta 3 in cartilage and bone cells during endochondral ossification in the chick. *Development Company of Biologists* 114**,** 907-911.

TOGNANA, E., BORRIONE, A., LUCA, C. D. & PAVESIO, A. 2007. Hyalograft C: hyaluronan-based scaffolds in tissue-engineered cartilage. *Cell, Tissue, Organs,* 186**,** 97-103.

TRATTNIG, S., MILLINGTON, S. A., SZOMOLANYI, P. & MARLOVITS, S. 2007. MR imaging of osteochondral grafts and autologous chondrocyte implantation. *European Radiology,* 17**,** 103-118.

TSAI, C.-L. & LIUA, T.-K. 2002. Inhibition of estradiol-induced early osteoarthritic changes by tamoxifen. *Life Sciences,* 50**,** 1943-1951.

TSYBINA, T. A., DUNAEVSKY, Y. E., BELOZERSKY, M. A., ZHUZHIKOV, D. P., OPPERT, B. & ELPIDINA, E. N. 2005. Digestive proteinases of yellow mealworm (Tenebrio molitor) larvae: Purification and characterization of a trypsin-like proteinase. *Biochemistry (Moscow),* 70**,** 300-305.

TUAN, R. S. 2007. A second-generation autologous chondrocyte implantation approach to the treatment of focal articular cartilage defects. *Arthritis Research & Therapy,* 9**,** 109.

TULI, R., LI, W.-J. & TUAN, R. S. 2003. Current state of cartilage tissue engineering. *Arthritis research & therapy,* 5**,** 235–238.

UNDERHILL, C., ANDREWS, P. & ZHANG, L. 1998. Hypertrophic chondrocytes are surrounded by a condensed layer of hyluronan. *Cell and Materials,* 8**,** 63-71.

VASITA, R. & KATTI, D. S. 2006. Nanofibers and their applications in tissue engineering. *International of Nanomedicine,* 1**,** 15-30.

VINATIER, C., MRUGALA, D., JORGENSEN, C., GUICHEUX, J. & NOËL, D. 2009. Cartilage engineering: a crucial combination of cells, biomaterials and biofactors. *Trends in Biotechnology,* 27**,** 307-314.

VINCENT, T., HERMANSSON, M., BOLTON, M., WAIT, R. & SAKLATVALA, J. 2002. Basic FGF mediates an immediate response of articular cartilage to mechanical injury. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 8259–8264.

VINDIGNI, V., CORTIVO, R., IACOBELLIS, L., ABATANGELO, G. & ZAVAN, B. 2009. Hyaluronan Benzyl Ester as a Scaffold for Tissue Engineering. *International Journal of Molecular Science,* 10**,** 2972–2985.

VUNJAK-NOVAKOVIC, G. & FRESHNEY, R. I. (eds.) 2006. *Culture of Cells for Tissue Engineering,* New Jersey: Wiley-Liss.

WAKEFIELD, T. W., CAPRINI, J. & COMEROTA, A. J. 2008. Thromboembolic Diseases. *Current problems in surgry,* 45**,** 844-899.

WALKER, E. A., VERNER, A., FLANNERY, C. R. & ARCHER, C. W. 2005. Cellular responses of embryonic hyaline cartilage to experimental wounding in vitro *Journal of Orthopaedic Research,* 18**,** 25-34.

WALLACE, D. G. & ROSENBLATT, J. 2003. Collagen gel systems for sustained delivery and tissue engineering *Advanced Drug Delivery Reviews,* 55**,** 1631-1649.

WALLACE, S. 2001. Newer antiepileptic drugs: advantages and disadvantages. *Brain and Development,* 23**,** 277-83.

WANG, X. & MAO, J. J. 2002. Chondrocyte Proliferation of the Cranial Base Cartilage upon in vivo Mechanical Stresses *Journal of dental research,* 81**,** 701-705.

WEIDENBECHER, M., HENDERSON, J. H., TUCKER, H. M., BASKIN, J. Z., AWADALLAH, A. & DENNIS, J. E. 2007. Hyaluronan-Based Scaffolds to Tissue-Engineer Cartilage Implants for Laryngotracheal Reconstruction. *The Laryngoscope,* 117**,** 1745-9.

WEISS, L. 1998. *Cell and Tissue Biology a Text Book of Histology*

WIDJAJA, A., TRAN, A., BAILEY, M. & PROPER, S. 2006. Correlation between Bankart and Hill-Sachs lesions in anterior shoulder dislocation. *ANZ journal of Surgery,* 76**,** 436-8.

WOODS, A., WANG, G. & BEIER, F. 2007. Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. *Journal of Cell Physiology,* 213**,** 1-8.

YAMASHITA, A., YONEMITSU, Y., OKANO, S., NAKAGAWA, K. & IRISA, T. 2002. Fibroblast growth factor-2 determines severity of joint disease in Adjuvant-Induced arhtiritis in rats. *Journal of Immunology,* 168**,** 450-7.

YANG, T. N. W., GOMES, R. R., BROWN, A. J., BURDETT, A. R., ALICKNAVITCH, M., FARACH-CARSON, M. C. & CARSON, D. D. 2006. Chondrogenic Differentiation on Perlecan Domain I, Collagen II and Bone Morphogenetic Protein 2-Based Matrices. *Tissue Engineering,* 12**,** 2009-2024.

YATES, K. E., ALLEMANN, F. & GLOWACKI, J. 2005. Phenotypic analysis of bovine chondrocytes cultured in 3D collagen sponges: effect of serum substitutes. *Cell and Tissue Banking,* 6**,** 45-54.

YE, X. J., TANG, B., MA, Z., KANG, A. H., MYERS, L. K. & CREMER, M. A. 2004. The roles of interleukin-18 in collagen-induced arthritis in the BB rat. *The Journal of Translational Immunology,* 136**,** 440-7.

YONGZHONG, W., BLASIOLI, D. J., KIM, H. J., HYUN, S. K. & KAPLAN, D. L. 2006. Cartilage tissue engineering with silk scaffolds and human articular chondrocytes. *Biomaterilas,* 27**,** :4434-42.

YOSHIZUMI, Y., SUGITA, T. & KAWAMATA, T. 2002. Clyndrical osteochondral graft for osteochondritis dissecans of knee. *The American Journal of Sports Medicine,* 30**,** 441-5.

ZHANG, M. 2004. Biomaterials and tissue engineering. *In:* SHI, D. (ed.) *Biocompatibilty of materials.* Springer.

ZHANG, W., JOENS, A. & DOHERTY, M. 2004. Does paracetamol (acetaminophen) reduce the pain of osteoarthritis. *Annals of Rheumatic Disease,* 63**,** 901-7.

Appendix: Associated publications

Cell imaging with the Widefield Surface Plasmon Microscope

M. Mahadi Abdul Jamil[1], S. B. Lobo[3,4], F. Sefat[2,4], S.A. Khaghani[2, 4], F. A. Javid [3], M. Youseffi [2], S.T. Britland [3], S. Liu [5], C.W. See [5], M.G. Somekh [5] and M.C.T. Denyer [3, 4]

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Abstract- **Imaging interfacial environment has proved challenging using standard imaging systems. This is a problem that may be circumvented using the widefield surface plasmon microscope (WSPR). Surface plasmon microscopy relies on the excitation of electron oscillations at a conductor/dielectric interface by P polarised light striking that interface at a specific surface plasmon resonance (SPR) angle. The SPR angle can be changed by application of a molecular species to the conductor, which modifies the mean refractive index at that interface and thus alters the coupling efficiency between the conductor and the P-polarised light. Commercial SPR microscopes unfortunately have poor lateral resolutions, but the WSPR uses a high numerical aperture lens to excite surface plasmons, and thus not only enables nanometric Z axes imaging of interfacial molecular interactions but also enables SPR imaging at micron to submicron lateral resolutions. Initial work has shown that this system can be used to image cell/surface interactions. This paper focuses on looking at the use of the WSPR microscope in the imaging of a human keratinocyte cell line (HaCat cells), bone cells, neonatal rat intestinal smooth muscle cells and neonatal rat knee joint derived chondrocytes. Of these cell types the HaCat cells couple tightly to the cell culture surface, and this is reflected by clear band like arrangements of focal contacts, in comparison chondrocytes, smooth muscle cells and bone cells couple less strongly to the surface and this is reflected by less clearly defined arrangements of focal contacts. In all cases WSPR microscopy also enabled identification of internal cellular features, specifically the nucleus and in the case of smooth muscle cells contractile filament like structures.**

Keywords– Surface plasmon, cell imaging, interfaces.

I. INTRODUCTION

Cells respond to a wide range of guidance cues, include, including surface chemistry, topography, chemical gradients, electric fields and magnetic fields [1-5]. However, all of these guidance cues illicit a cellular response via altered gene upregulation and by promoting modifications in the cytoskeleton. Many of these gene upregulations also result in modifications in cell surface receptors, which in turn enable cells to respond differently to the cellular environment. In wound repair for example the TGF beta family of cytokines not only promote migration of macrophages and neutrophils into a wound site, but also modify the migratory and proliferative ability of keratinoctes and fibroblasts [6-7]. To understand these mechanisms it is of some importance to be able to image what exactly happens between a cell and the surface upon which it grows. This is difficult using standard microscopy, and generally involves fixation the use of immunostaining techniques aimed at identifying the location and distribution of focal contacts. What is required is a means of imaging cell surface interface in live cells without the need for labeling techniques.

Surface plasmon microscopy has been enabled by a modification of the standard Kretchmann configuration [8], where p-polarised light is applied to a conductor/dialectric interface at a fixed angle.

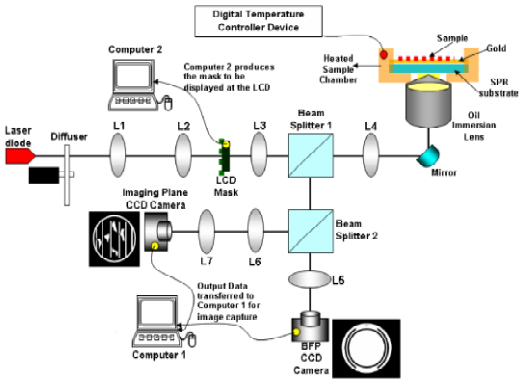

Fig. 1 Schematic of the WSPR microscope.

The deposition of molecular species is then detected by these molecules changing the surface plasmon resonance angle and changing the efficiency of surface plasmon generation resulting in the loss of energy and light via refraction. This alters the intensity of reflected light and thus generates a contrast map of the observed surface [9, 10].

Unfortunately traditional surface plasmon systems have lateral resolutions limited to a few µm [9] because of the distance propagated by surface plasmon at the interface between the dialectric and the conductor. However, recent work has resulted in the development of a new kind of surface plasmon microscope, the widefield surface plasmon microscope (WSPR) [11]. The WSPR microscope (Fig. 1) is Köhler illuminated and uses a high numerical aperture oil immersion lense, 1.45 NA, to couple *p*-polarised light at an angle capable of exciting surface plasmons into a gold-coated slide microscope. This allows near diffraction limited lateral resolution imaging of interfacial interactions. This potentially provides a tremendous opportunity, that of directly imaging the cell surface interface and determining the location of focal contacts and the degree of cell surface attachment without the need for immunostaining.

Preliminary experiments have shown that the WSPR microscope can be used to image antibody antigen interactions at lateral resolutions of about 1µm without the need for labeling techniques [12] and changes in ketatinocyte/substrate attachment in relation to application of the cytokine TGFβ3 [13] In this present study we aim to show that the WSPR microscope can be used to examine the way that different cell types interface with a surface.

The Anti-Scarring (Wound Closure) Properties of TGF-β3, BSA/HCl and HCl in Cultured Human Bone Cell Monolayer

F. Sefat, M. Youseffi, R.F. Berends, S.A. Khaghani, and M.C.T Denyer

Abstract- **Bone repair can be modulated by different stimulus including growth factors. TGF-β3 is a cytokine known to be associated with the scarless healing of skin and it is highly probable that it may play a role in the repair of other tissues. Thus the aims of this study were to investigate the effect of TGF-β3 on closure of a model wound in cultured monolayers of the MG63 human bone cells. This *in vitro* work examined and compared the anti-scarring (wound closure) properties of TGF-β3, and its dosage carriers, HCl and BSA/HCl. The wound healing response was investigated in TC grade culture flasks by creating a wound (with average scratch width of 300μm±10-30μm SD, 1.7-5μm SEM) on confluent monolayer of MG63 human bone cell. After wounding cultures were then treated with 50ng/ml TGF-β3 at concentration of 4mM HCl and 1mg/ml BSA and Distilled water. Also the same method was applied for cell cultured monolayer with no growth factor as control and with HCl/BSA and HCl only solutions. After wounding, wound width was measured every 5 hours over a 30-hour period. The results showed that TGF-β3 (with addition of HCl and BSA/HCl) enhances the rate of wound repair in a monolayer of MG63 bone cells. After careful observation it was observed that after 20 hours all the culture flasks treated with TGF-β3 (with 15.5% of wound remained open), HCl (with 16% of wound remained open) and finally BSA/HCl (with 17.7% of wound remained open) had resulted in faster wound healing compared to control (with 85% of wound remained open). These results indicated that wound closure in model MG63 wound with TGF-β3 (with addition of HCl and BSA/HCl) is higher than the control. TGF-β3, HCl alone and HCl/BSA all enhanced the rate of wound repair in relation to the negative controls.**

Index Terms: **Bone cell engineering, BSA/HCl, HCl, TGF-β3, Wound healing.**

Manuscript received March 5, 2009. This work was supported by school of Engineering and School of Pharmacy, University of Bradford.

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I. INTRODUCTION

Bone Tissue Engineering is a promising field in the area of medicine and involves principles of biology and biomedical engineering with the aim of developing a viable tissue substitute that can restore the function of human tissue [1]. Despite healing of soft tissues, bone healing has features of degeneration, and usually no scar can be find after healing. As soon as the fracture has been bridged by new bone, it remodels. Bone repair (wound healing of bone) can be manipulated by different stimulus such as growth factors, distraction osteogenesis and electrical stimulation. TGF-β3 is a cytokine produced by different cell types inside the body and influences a number of cell activity such as differentiating, stimulating mesenchymal stem cell (MSC) growth, acting as a chemotactic factor and also enhances bone cells and extracellular matrix (ECM) product secretion [2].

II. Aims AND objectives

Main objective was to investigate the effect of TGF-β3 on wound closure in cultured monolayers of MG63 bone cells. The lab-based experimental work investigated and compared the anti-scarring (wound closure) properties of TGF-β3, HCl and BSA/HCl in cultured dish environment using cultured monolayers of human bone cells. Other cellular responses such as proliferation, differentiation and detachment have also been investigated along with different stages of cell behaviour and morphology during wound healing.

III. Materials AND Methods

Bone cells were cultured in a low glucose culture medium known as Dulbecco's Modified Eagle Medium (DMEM, from SIGMA) containing various supplements such as L-glutamine (4mM), Penicillin-Streptomycin (5ml), Amphotericin or a fungizone (1ml), HEPES buffered culture medium and 'fetal calf serum' (50ml). The bone cells were cultured inside culture flasks and bathed in the culture media. The cells attached to form a layer at the bottom of the culture flasks. A 'wound' was made using a disposable long nosed plastic pipette. The tip was bent downwards so that it could be inserted into the flasks. The tip was then drawn across the cells on the cultured surface creating the wound.

The Effect of Transforming Growth Factor Beta (TGF-β3), HCl and BSA/HCl on Trypsinisation of Bone Cells Monolayer

F. Sefat, M. Youseffi, R.F. Berends, S.A. Khaghani, and M.C.T Denyer

***Abstract*- In order to investigate wound healing in bone it is essential to know the process of bone cell attachment and detachment to the surface. Cells are not found in isolation and they usually adhere to other cells or surrounding extracellular (ECM) environment in vivo and substrate or a surface in vitro.**

Trypsinisation was carried out in order to investigate its effect on cell detachment, in the presence of TGF-β3, HCl or BSA/HCl solutions. Trypsin was therefore added to four groups of bone cells with addition of TGF-β3, HCl, HCl/BSA solutions and additional flask as control. These results further confirmed that application of TGF-β3, HCl and HCl/BSA at 50ng/ml decreased the degree of cell attachment on surface of culture flasks.

Cell detachment in control is about 43% after 6 minutes, which is slow. Bone cells in presence of BSA/HCl have start detaching from the surface faster than control (about 4-5 minutes after applying trypsin). Cell detachment is about 63% after 6 minutes which is faster as compared to the control. Bone cells in presence of HCl alone have start detaching from the surface faster than control and BSA/HCl (about 2 minutes after applying trypsin). Cell detachment is about 69% after 6 minutes which is faster compared to the BSA/HCl and control.

Trypsinisation experiments for bone cells cultured with TGF-β3 shows that cells started to detach from the surface about 1 minute after application of trypsin and were completely detached by the third minute. Cell detachment is about 85% after 4 minutes, which is faster as compared to the control, HCl and BSA/HCl. Trypsinisation results indicated that application of TGF-β3 at 50ng/ml decreased the degree of cell attachment.

***Index Terms*: Bone cells monolayer, BSA/HCl, HCl, TFG-β3, Trypsinisation**

Manuscript received March 5, 2009. This work was supported by school of Engineering and School of Pharmacy, University of Bradford.

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INTRODUCTION

Cells are not found in isolation and they usually adhere to other cells or surrounding extracellular matrix (ECM) environment in vivo and substrate or a surface in vitro [1,2].

The effect of TGF-β3 is mediated by a range of signalling pathways. The interaction of bone cells with their surrounding ECM environment influence some physiological function and pathological processes [3]. These interactions are mediated by integrins. Integrins are capable of transducing the signals from ECM to the cells in which results in migration, differentiation and specific protein synthesis. To determine which integrins are involved flow cytometric analysis and immunoprecipitation need to be carried out.

AIM AND OBJECTIVES

The aim of this study was to investigate the effect of TGF-β3, HCl and BSA/HCL on bone cell detachment via Trypsinization process.

MATERIAL AND METHODES

Trypsinisation was carried out to investigate the effect of TGF-β3 on cell detachment. To establish the appropriate dilution at which to plate cells, a 1 in 3, 1 in 6 and 1 in 12 dilution was plated into the three rows of a 12 well plate. For TGF-β3 to have sufficient time to influence cells in culture, cells were grown for at least 2 days prior to the attachment assay. The 1 in 3 dilution was confluent by day 3 and was therefore chosen for this assay.

In order to reconstitute the vile containing TGFβ3, a solution of HCl (4mM), BSA (1mg/ml) and Distilled water was prepared.

Trypsin was added to four groups of cultured bone cells with four different solutions including TGF-β3, HCl, HCl/BSA solution and bone cell only as control to study the effect of these solutions on cell detachment. HCl and HCL/BSA solutions were used, as they are carrier for TGF-β3.

Alignment of Rat primary chondrocyte cells to Collagen type-I, Fibronectin and Laminin

Seyedali Khaghani [1,2,3], **Morgan Denyer** [2,3], **Farshid Sefat** [1,2,3] **and Mansour Youseffi** [1,3]

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Abstract:

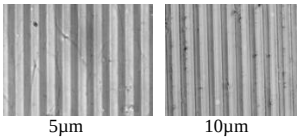
The use of tissue engineering in the treatment of cartilage injuries is in its infancy. To understand how chondrocyte cells behave in the wound environment requires in vitro experiments designed to characterise chondrocyte responses to model guidance cues. This study examined the alignment and spreading of chondrocyte cells derived from 1-5 day old Sprague Dawley rats on 5, 10, 25, 50 and 100µm wide repeal grating of microcontact printed Fibronectin, Laminin Collagen type-1.

Methodology:

The primary chondrocyte cells were derived from digestion of extracted articular cartilage of Sprague Dawley rats and plated on the patterned substrate of concentration of 5000 cells/cm². In this experiment five patterned glass cover slips of 5, 10, 25, 50 and 100µm wide were used for each protein. The effect on cell alignment and morphology of these patterned proteins was examined, after 48 and 72 hours of seeding.

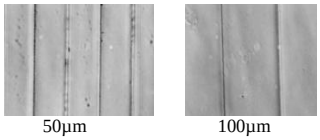
Microcontact Printing (MCP) and cell seeding:

Polydimethylsiloxane (PDMS) stamp of sizes 5, 10, 25, 50 and 100µm were made during this work and used to deposit cells on cover slips as shown in Figure 1. The stamp was first dyed (inked) with a solution of protein and after one minute as the stamp was dried, it was then pressed onto the surface for patterning. The soft PDMS stamp makes conformal contact with the surface and molecules are transferred directly from the stamp to the surface in the space of about one minute [1].



5µm 10µm

25µm

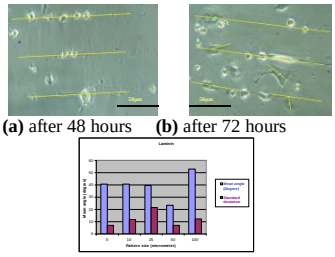


50µm 100µm

Figure 1. 5-100µm PDMS Stamps made for MCP of various proteins in this work

Results and discussion:

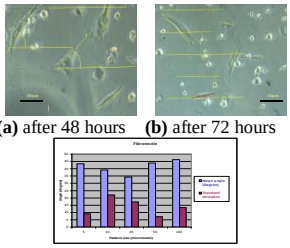
It was found, using Image-J software and linear optimization, that chondrocytes aligned to the 50µm laminin (mean angle of 23.4 ±7.02 SD) shown in Figure 2. There were no alignments to the fibronectin patterns as shown in Figure 3. The highest degree of alignment was observed with chondrocytes grown on the 100µm wide collagen type-I pattern (mean angle of alignment 18.9 ±6.797 SD) as shown in Figure-4.



(a) after 48 hours **(b)** after 72 hours

(c)

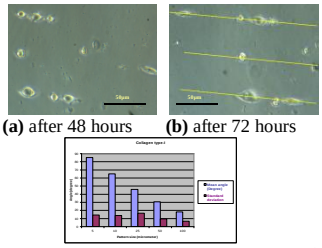
Figure 2. Photomicrograph of chondrocyte cells on 50µm Laminin patterns: (a) after 48 hours of seeding and (b) after 72 hours of seeding.



(a) after 48 hours **(b)** after 72 hours

(c)

Figure 3. Photomicrograph of chondrocyte cells on 100µm Fibronectin pattern. (c) Graph of mean angle versus stamps size for Laminin after 48 and 72 hours of seeding.



(a) after 48 hours **(b)** after 72 hours

(c) Figure 4. (a) and (b) photomicrograph of chondrocyte cells on 50µm Type-I Collagen patterns after 48 hours after 72 hours of seeding.

Chondrocyte cell imaging:

Using our Widefield surface Plasmon resonance (WSPR) microscope, individual cells were imaged after fixation (using formaldehyde and ethanol). Figure 5 shows chondrocyte cells having circular/rounded shapes attached strongly to the culture substrate. Each cell shows typical morphology with circular concentric rings around the nucleus. The bright rings show the focal contacts whereas the dark space between these bright rings are non-focal contact areas of the cell membrane. Also evident is the presence of extended cytoplasm (footlets) at the periphery of each cell.

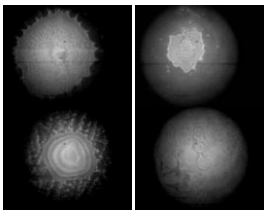


Figure 5. Typical image of (fixed) individual chondrocyte cells cultured on gold coated SPR substrate showing morphological details obtained via Widefield surface plasmon microscope (WSPR). The circular/rounded flat cells suggest the presence of ball/sphere like chondrocyte cell in culture media.

Conclusions:

In all cases the chondrocyte cells were small (mean size of 36.2µm ±14.4 on Laminin MCP patterns, mean size 34.03µm ±7.2 on Collagen patterns, and mean size of 33.87µm ±11.9 on Fibronectin Patterns) and rounded. This was obtained from the first passage.

Of the cells plated on all the substrates only 50% - 60% were firmly attached. There was no significant change of proliferation rate in relation to different proteins. See figures and rounded images obtained by using our Surface plasmon resonance microscope showing a chondrocyte cell.

Future work:

The results of this research would help to design suitable scaffolding for 3D chondrocyte cell culture.

References:

[1]http://www.chm.colostate.edu/vanorden/Research/Micro%20and%20Nanoscale%20Patterning.htm

☒ Oral ☒ Poster

Effect of Transforming Growth Factor-Beta (TGFβ-1) in Biological Regulation of Primary Chondrocyte

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Abstract. In planar cell culture systems primary chondrocyte cells develop a fibroblastic morphology. This study examined the effect of transforming growth factor beta-1 (TGF-β1) on fibroblastic and chondrocytic type primary chondrocyte, size, proliferation rate, capacity of model wound repair and cell adhesion. The results showed that (TGF-β1) had no effect on cell size, but decreased cell migration and proliferation and thus wound repair, particularly in the fibroblastic type cells. However, trypsinization assays indicated that chondrocyts with a fibroblast morphology adhered to culture substrates more strongly than did cells with a typically chondrocytic morphology after treatment with (TGF-β1).

Keywords: TGF-β**,** Chondrocyte, Wound repair, Cell adhesion.

MATERIALS AND METHODS

Chondrocytes with chondrocytic and fibroblast like morphologies were resuspended separately in DMEM with and without supplementation of 10ng/ml TGF-β1, cultured in monolayer with 100.000cells/ml at *37ºC and imaged up to 72 hrs. The sizes of 150 randomly selected cells were measured using Image J (NIH). The capacity of cartilage repair in presence of TGF-β1 was assessed by means of monitoring closure of a model wound in confluent cultures of chondrocytes.*

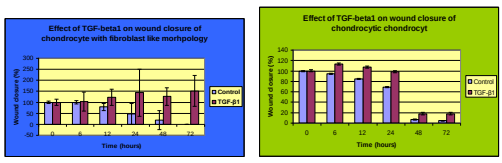

FIGURE 1. Wound closure assay of chondrocyte with left; fibroblast like cells and right; chondrocytie morphology.

RESULTS AND DISCUSSION

Transforming growth factor-beta1 had no effect on cell size, however after 72 hours in culture, the proliferation rate of the cells in TGF-β1 containing medium was noticeably less than in control cultures without TGF-β1.

The initial number of cells was considered as being 100% and the following increases were calculated as % increase beyond that (see Table 1 and Figure 1 for details).

TABLE 1. Proliferation Of Primary Chondrocyte Cell With And Without TGF-β1

Experiment	Fibroblast Like Morphology	Chondrocytic Morphology
Control	305%	313%
TGF-β1	188%	207%

The results of the wound repair assay revealed that the process of wound healing in presence of TGF-β1 was much slower than in controls. The trypsinization experiments also indicated that TGF-β1 increase fibroblastic type chondrocyte attachment and thus inhibit cell proliferation and migration. This probably explains the inhibitory effect of TGF-β1 on wound closure.

Acknowledgments

I would like to thank my supervisors Dr. M.Youseffi and Dr. M.C.T. Denyer for their continuous support and assistance in this PhD project and the University of Bradford for sponsoring my postgraduate research programme.

References

1. GOLDBERG, A. J., LEE, D. A., et al. (2005b) Culture in a TGF-ß-containing medium enhances the re-expression of a chondrocytic phenotype in passaged human chondrocytes in pellet culture. *Journal of Bone and Joint Surgery - British,* **87** (1)**,** 128-134

Purification of Primary Chondrocyte Cells isolated from Articular Joint using from neonate Sprague-Dawley Rats

S.A. Khaghani[1,2,3], **M.C.T. Denyer**[2,3], **M. Youseffi**[1,3], **R.F. Berends**[1,2,3], **F. Sefat**[1,2,3], **S. Lobo**[2,3] **and F.A. Javid**[4]

1- School of Engineering,Design and Technolog-Medical Engineering, 2- School of Life Science;, 3- Institute for pharmaceutical innovation (IPI); University of Bradford, Bradford, BD7 1DP, U;, 4- School of Applied Sciences, University of Huddersfield, HD1 3DH Huddersfield, UK

Abstract:

Cartilage is mostly made up of chonrocytes, but in harvesting cartilage other tissues arise from the synovial membranes, tendons and even nerves. This makes acquisition of a pure chondrocyte culture challenging. Harvesting chondrocytes from neonatal rat joints is even more difficult because the larger joints such as the knee joint is roughly about 2 mm in diameter. Cells derived from this tissue will therefore need to be purified.

In this study, different adhesion affinity of dissimilar cells on solid surface was used to separate chondrocytes from other cell types. This was achieved by plating primary cell suspensions in tissue culture grade (TC) cell culture flasks and removing those unattached cells after 20 minutes. The unattached cells were then re-plated in a new TC grade cell culture flask. This process was repeated a further 6 times, after which the final cell suspension, was allowed to attach to the surface for 100 minutes. Immunocytochemical staining with monoclonal Anti S-100 (β-subunit) antibody and monoclonal anti collagen type-I antibody (Sigma,UK) showed that control cultures consisted of a mixture of chondrocytes and other cell types. However, of those cultures derived from the serial attachment process, chondrocytes only became evident after 100 minutes of cell attachment.

Methodology:

Knee joint cartilage was extracted from neonate Sprague-Dawley rats, enzymatically digested resulting in chondrocyte cell suspension.

During extraction of cartilage, bone cells were also acquired (Figure 1). Initial cell suspension had a cell density of approximately 109 x 10^4 cells/ml. These cell suspensions required purification to remove endothelial type cells and bone cells. The sequence of purification and relevant results are shown in Figures 1-8. Cells were initially plated in TC grade cell culture flasks and incubated for twenty minutes. After twenty minutes unattached cells were removed, replated and allowed to attach for a further 20 minutes. This process was repeated 7 times and at the final plating the cells attached slowly. Consequently the cell suspension was incubated for 100 minutes to allow adhesion to the flasks.

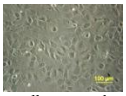

Figure 1. Various cell types such as chondrocytes, tenocytes, osteocytes and fibroblasts before cell separation

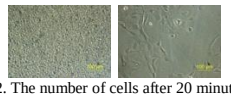

Figure 2. The number of cells after 20 minutes was 646,000 cells/ml

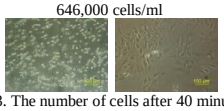

Figure 3. The number of cells after 40 minute was 210,000 cells/ml

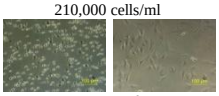

Figure 4. The cell number after 60 minutes was 124,000 cells/ml

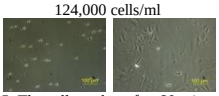

Figure 5. The cell number after 80 minutes was 100,000 cells/ml

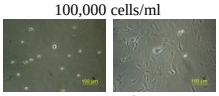

Figure 6. The cell number after 100 minutes was 68,000 cells/ml

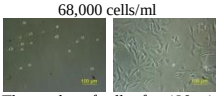

Figure 7. The number of cells after 120 minutes was 64,000 cells/ml

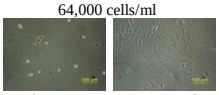

Figure 8. Very few cells were attached after 140 minutes. The number of cells was 61,000 cells/ml

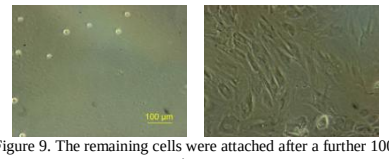

Figure 9. The remaining cells were attached after a further 100 minutes

The statistical data obtained from cell separation were used for calculation of mean cell densities (cell/ml) +/- the standard deviation, (Figures 10).

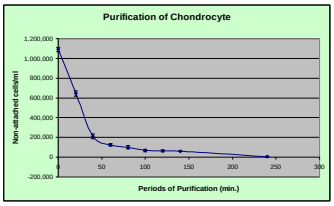

Figure 10. Graph of Purification of Chondrocyte Cells isolated from knee joints of neonate rats

Immunocytochemical staining:

72 hours after purification process the cells were detached by trypsin, seeded on sterile glass coverslips and incubated at 37ºC for 48 hours. Cells were then fixed and stained with Monoclonal Anti S-100 (β -subunit) (mouse IgG1 isotype) and Monoclonal Anti-Collagen type-I (mouse IgG1 isotype).

Type-I Collagen staining was localizing around the cell membrane as part of extracellular matrix (ECM) shown in Figure

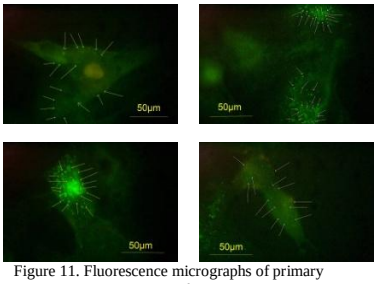

Figure 11. Fluorescence micrographs of primary chondrocytes immunostained for Type-I Collagen. The arrows show the regions positively labelled by the Anti-collagen type-I antibodies.

The stained S-100 was localized around the nuclei and was also found in ECM, which confirmed the existence of chondrocytes as shown in Figure 12.

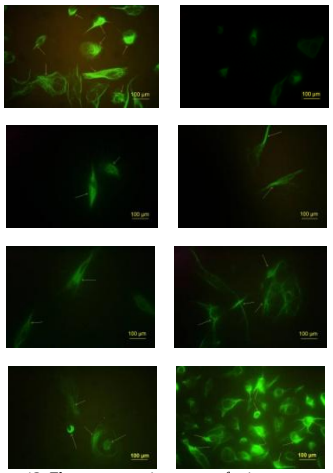

Figure 12. Fluorescence microscopy of primary culture of cartilage showing green stained S-100 antigen around the nuclei. Before purification (top left), during purification and the final stage with purified chondrocytes (down right)

Those cells that attached within the first 20 minutes displayed little or no immunostaining. A small proportion of cells at the second plating did, however, display staining, but the proportion of stained cells decreased until the final plating. Those cells plated and subsequently incubated for 100 minutes were almost all stained via the collagen type 1 and S-100 immunostaining process (Figure 13).

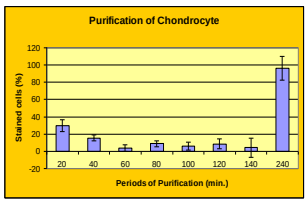

Figure 13. Graph of stained cells to S-100 antibody during the purification process.

Conclusions:

Cell populations without purification contained low numbers of positively immunostained chondrocytes. However, the proportion of chondrocyets increased to almost 100% after final plating following purification. Chondrocytes started attaching on solid surface after about 80 minutes and their full attachment took at least 240 minutes. These results suggest that chondrocytes can be readily isolated and purified by a prolonged differential adhesion technique with a primary incubation extending for 140 minutes

Investigating Keratinocyte cell responses to ECM proteins using Microcontact printing

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Introduction

Following injury to the skin, keratinocytes within the epidermis migrate and proliferate to fill the wound bed, a process known as re-epithelialisation. These cellular activities rely on specific interactions with the changing extracellular matrix (ECM). Major proteins within the extracellular matrix include collagen type I, fibronectin and laminin. Type I collagen is a major component of the dermis and its exposure following injury is central to the wound repair process. In response to exposure of collagen type I haemostasis begins causing the formation of the provisional matrix or fibrin clot. This fibrin clot is also rich in fibronectin and provides a temporary matrix onto which keratinocytes can migrate. As cells from the edges of the wound (leading keratinocytes) migrate into the wound bed they begin to restore the basement membrane. The leading keratinocytes deposit proteins including laminin. Keratinocytes behind the leading edge respond to these proteins and proliferate to fill the wound bed.

Microcontact printing has been commonly used for the assembly of protein monolayers onto surfaces (Yeung 2001, Liu 2006). The specific patterning of protein on substrate allows cellular adhesion, migration and proliferation to be studied.

In this study, a human keratinocyte cell line (HaCat cells) was used to study the response to different ECM proteins central to wound repair. Microcontact printing was used to pattern proteins onto substrates in order to analyse cell alignment.

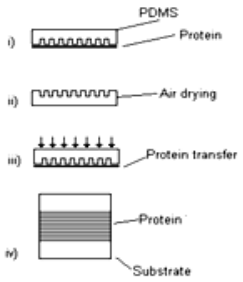
Keywords: **Micro-Contact Printing, Fibronectin, Laminin, Collagen Type I, Cell Adhesion and Guidance**

Aims and Objectives

To use microcontact printing to study the response of a human keratinocyte cell line (HaCaT) when cultured with different ECM proteins patterned on glass substrate. More specifically, to determine whether HaCaT cells align to different proteins and at which pattern size this is optimised.

Materials and methods

Microcontact printing was used to pattern fibronectin, laminin and collagen type I onto glass substrates. A variety of PDMS stamps with gratings ranging from 1.8 to 100µm in width were fabricated from pre-cleaned glass temples. Cells were seeded at 5.4 x10^4/ Petri dish and cultured with protein patterned substrates at 37°C for 24 hours. Cell responses were analysed using light microscopy and Image J software.



Results and Discussion

HaCaT cells were successfully cultured on each microcontact printed protein. Cell responses were imaged and data was collected on cell length, width and angle of alignment. For each of the three proteins, fibronectin, laminin and collagen type I the smallest average angle of alignment was seen with the 50µm pattern (7.52°±1.73, 6.52°±1.33 and 17.97°±3.42 respectively). Fibronectin and laminin achieved the best cell alignment indicating that HaCat cells adhere via integrins that favour these proteins. In general, cell length increased when cells responded well to the pattern. Figure 1 shows examples of cell alignment for each protein. Figures 2 and 3 are bar charts showing angle of alignment and cell length for each protein respectively. Standard error bars are also shown.

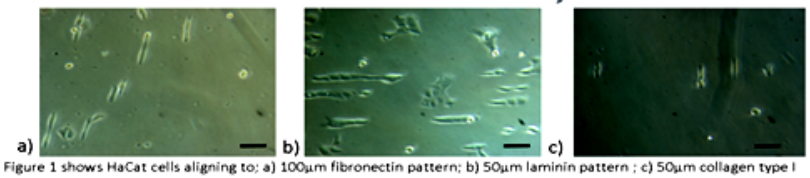


Figure 1 shows HaCat cells aligning to; a) 100µm fibronectin pattern; b) 50µm laminin pattern ; c) 50µm collagen type I pattern. The scale bar represents a distance of approximately 80µm.

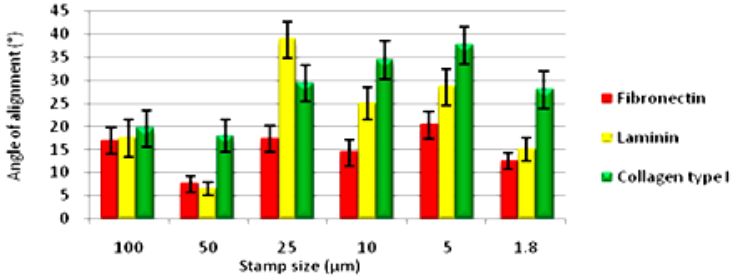


Figure 2: Chart showing angle of alignment for each protein and stamp size. Standard error bars shown.

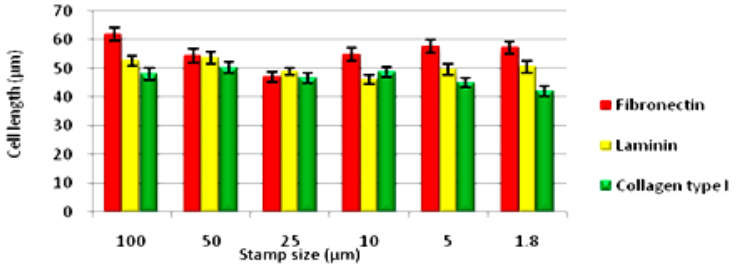


Figure 3: Chart showing cell length (µm) for each protein and stamp size. Standard error bars shown.

Conclusions

These experiments have shown that HaCat cell adhesion and alignment can be influenced by the patterning of proteins on glass substrate. The microcontact printing of extracellular matrix proteins will provide a useful tool for examining the effects of chemical mediators on cell adhesion to proteins important in wound repair.

Does transforming growth factor-beta2 play any role in the wound healing of cartilage!?

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Abstract:

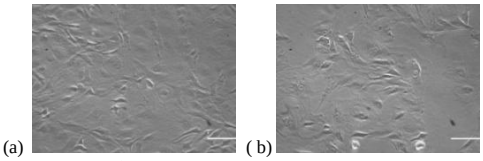
Articular cartilage repair is still one of the most challengeable fields in regenerative medicine and tissue engineering and is not yet successfully applied clinically. Transforming growth factor-betas are produced by almost all cell types in inactive/latent form suggesting that they play a regulatory role in most tissues. Though the potential effect of TGF-β2 in function of the cell and it's response of the extracellular environment is widely studied, but the role of these cytokines is not fully understood (Cohen et al., 2006). To determine the effect of TGF-β2 on wound healing of cartilage a monolayer culture of chondrocyte was scratched by tip of a pipette. Cell migration into the wound area was observed and imaged microscopically. This study revealed that transforming growth factor beta-2 has an inhibitory effect on chondrocyte wound healing process.

Methodology

To investigate the effect of TGF-β2 on cartilage repair, primary chondrocyte cells were isolated from articular joint of six neonate Sprague-Dawely rats and cultured in high glucose DMEM (Sigma, 2007) with 10% FCS, with and without 10ng/ml transforming growth factor-beta2 (Promokine, 2008) at a cell density of 57,600 cell/ml incubated at 37ºC. The cell monolayer was scratched by the tip of a pipette to create a model wound. Following scratching, wound width was measured via Image J software every 2 hours at 10 different points until the wound was totally healed. The results were plotted as % wound closure against time to demonstrate the comparison for both control and with TGF-β2.

Results and discussion

The cells treated with TGF-β2 developed a well-spread fibroblastic shape acquiring a mean size of 14.20µm in diameter (Figure 1). This indicated that TGF-β2 may have caused excessive synthesis of extracellular matrix (ECM) proteins which are involved in the formation of fibroblast type morphology leading to dedifferentiation of chondrocyte *in vitro*.



(a) (b)

Figure 1: Culture of chondrocyte cell in media: (a) without and (b) with TGF-β2; Scale bar=50µm.

TGF-β2 increased the chondrocyte cell size up to 152.9% over a period of 132 hours (Figure 2). This could be related to an increase in the production of the ECM components which in turn via up or down regulation of specific integrins induce changes in chondrocyte shape.

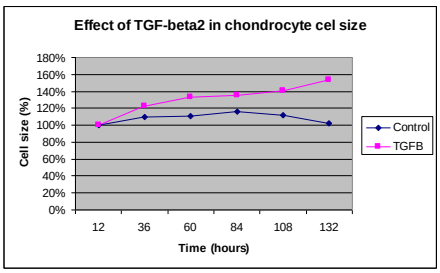


Figure 2. Graphs of primary chondrocyte cell size during 132 hours culture with and without TGF-β2.

Initial cell migration (see Figure 3) was detected ~ 6 hours after scratching. This may suggest that the chondrocyte cells require a revitalization time after introduction of mechanical stress in the form of scratching.

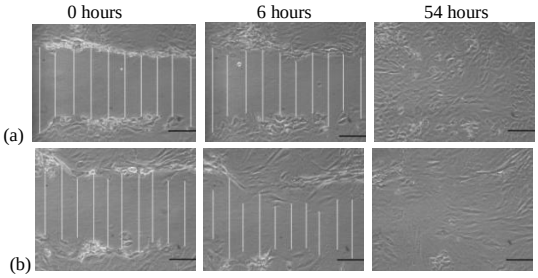


Figure 3. Microphotographs of the wound model for primary chondrocyte: (a) Control, (b) TGF-β2; Scale bar=50µm.

The results of the wound closure assay are shown in Figure 4 as (averaged) percentage wound closure versus time for both control and with TGF-β2 for comparison.

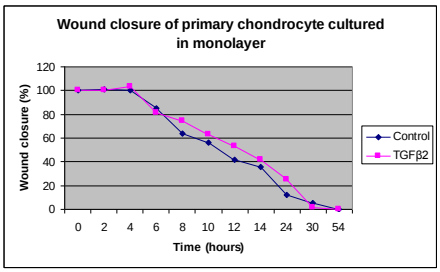


Figure 4. Graph of % wound closure for primary chondrocytes cultured in TGFβ-2 contained media and control.

This type of growth factor increased cell size by stimulating fibroblastic type morphology. Excessive ECM molecules occupied large areas in the culture flask and caused the cells to spread well within the monolayer and filled the wound bed relatively fast, but with ECM molecules and not with chondrocyte cells.

References

COHEN, S., SAMADIKUCHAKSARAEI, A., et al. (2006) Antibiotics Reduce the Growth Rate and Differentiation of Embryonic Stem Cell Cultures. *Mary Ann Liebert*, **12** (7).

The role of ECM protein (collagen, fibronectin and laminin) in cellular adhesion (cell alignment) and cell engineering of bone

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Abstract: Tissue Engineering may help in the restoration of tissue function. Cells in the body attach to the normal scaffold known as extracellular matrix. The *in-vitro* environmental allows the examination of the way in which cell behave in relation to the ECM. Fibronectin, Laminin and collagen are the main components of extracellular matrix. These are cell adhesion proteins and ideal candidates for printing biomolecules as they can direct cellular adhesion, migration, differentiation and network formation in vitro [1]. The aim of this study was to identify the role of Collagen, Fibronectin and Laminin, in the adhesion and alignment of the human bone cell line (MG63) to different micro-contact printed ECM patterns. ECM proteins were stamp patterned onto glass slides using templates consisting of 5, 10, 25, 50 and 100µm wide repeal grating. Cells were seeded at 15,000 cells per patterned coverslip. The effects of initial cell attachment on different substrates were investigated by recording cell images at different time points. Image J software was used in order to find the mean length and angle between the long axis of cells and the patterns. The results indicate that MG63 cells respond most to 50 and 100µm wide fibronectin patterns which indicates the cells attach mostly via fibronectin specific integrins.

Introduction

Micro-contact printing (MCP) allows deposition of molecule at precise and predetermined geometry on a surface and is thus ideal for examining directed cellular adhesion, migration, differentiation and network formation in vitro [1].

Aims and Objectives

The aim of this study was to identify the role of Collagen, Fibronectin and Laminin, in the adhesion and alignment of the human bone cell line (MG63) to different micro-contact printed ECM patterns.

Materials and Methods

Collagen, Fibronectin and Laminin were stamp patterned onto glass slides using templates consisting of 5, 10, 25, 50 and 100µm wide repeal grating as shown in Figure 1.

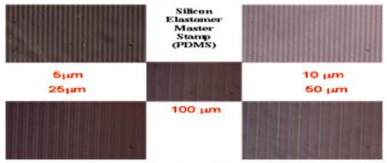

Figure 1. Images of the stamps with various sizes used for micro contact printing of the ECM proteins.

Cells were seeded at 15,000 cells per patterned coverslip. The effects of initial cell attachment on different substrates were investigated by recording cell images at different time points after cell seeding (2, 5 and 18hours). Image J software was used in order to measure the mean length of cells and the angle between the long axis of cells as shown in Figure 2 for the fibronectin patterned substrate after 18 hrs of seeding.

Results and Discussion

Cells on the MCP fibronectin patterns attached and elongated at early stages (about 2hrs) after seeding. In the case of collagen and laminin, cells did not adhere readily and appeared more rounded until about 18 hrs after seeding.

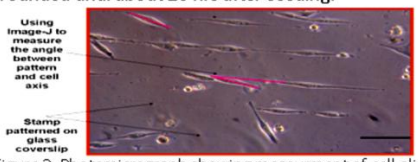

Figure 2. Photomicrograph showing measurment of cell alignment and cell length using image J software on stamp patterned fibronectin substrate(100 µm) after 18 hrs of seeding; (Scale bar=100 µm)

The cells aligned very well on fibronectin patterned coverslips especially to the 50µm (mean angle of 7.87±3.07SD) and 100µm patterns (mean angle of 6.46±5.01SD). In contrast, the alignment of the cells to the collagen and laminin patterns was poor, with the best mean angle of alignment (9.67±4.19SD) occurring in the 50µm collagen wide MCP pattern and the best alignment on the laminin patterns (mean angle of 14.36±1.57SD) occurring also in the 50µm wide MCP as can be seen from the photomicrographs in Figure 3 and graph of mean angle vs stamp size in Figure 4.

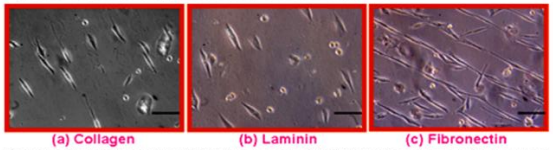

Figure 3. Photomicrograph of MG63 bone cells after 18hrs of seeding on 100µm micro contact printed substrate: (a) Collagen, (b) Laminin and (c) Fibronectin; (Scale bar=50 µm)

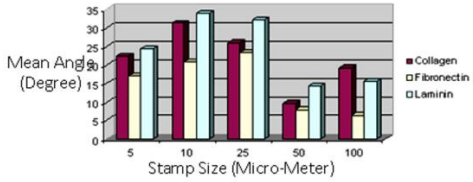

Figure 4. Comparison between three patterned ECM proteins in terms of cell alignment with five different stamp sizes.

Differences in cell length mirrored those of alignment, with cells acquiring the greatest length when showing the greatest degree of alignment as can be seen in Figure 5.

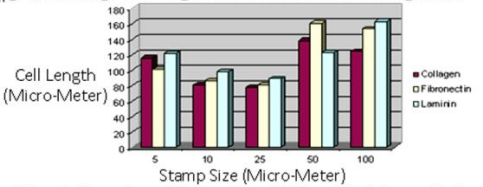

Figure 5. Comparison of three patterned ECM proteins in terms of cell elongation with five different stamp sizes.

Conclusions

The results indicate that MG63 cells responded mostly to 50 and 100µm wide fibronectin patterns which indicates that these cells attach mostly via fibronectin specific integrins.

Reference

[1] Bone tissue engineering, Jeffrey O. Hollinger, 2004, first edition.

[2] Culture of cells for tissue engineering, R. Ian Freshney, 2006, first edition.

Paper in preparation

1. SEYED ALI KHAGHANI, MORGAN C.T. DENYER, MANSOUR YOUSEFFI. 2010. Effect of transforming growth factor beta-1 in biological regulation of primary chondrocyte. (**In preparation for submission**).