CELL ENGINEERING OF HUMAN BONE MONOLAYERS AND THE EFFECT OF GROWTH FACTORS AND MICROCONTACT PRINTED ECM PROTEINS ON WOUND HEALING

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The Role of ECM Proteins, TGFβ-1, 2 and 3 and HCl/BSA in Cellular Adhesion, Wound Healing and Imaging of the Cell Surface Interface with the Widefield Surface Plasmon Microscope

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STATMENT OF ORIGINALITY

To the best of my knowledge, the material or the contents presented in this dissertation are original except where otherwise noted within the text. None of this research has been submitted in whole or in part for any degree at this or any other University.

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Keywords: Bone Cell Engineering; TGF-β1, 2 and 3; Microcontact Printing; ECM Proteins; Wound Healing

ABSTRACT

Bone repair is modulated by different stimuli. There is evidence that the Transforming Growth Factor-beta (TGF-β) super-family of cytokines have significant effects on bone structure by regulating the replication and differentiation of chondrocytes, osteoblasts and osteoclasts. There is also significant evidence that interactions with extracellular matrix molecules also influence cell behaviour.

This study aimed at determining the role of the TGF-βs, Collagen type I, Fibronectin and Laminin in bone cell behaviour. To do this MG63 bone cells were used to examine cell adhesion and alignment to different micro-contact printed ECM protein patterns of different widths. The study also aimed at examining how TGF-β1, 2 and 3 and their solvent and carrier (HCl and BSA, respectively) effected cell surface interactions, cell morphology, cell proliferation and integrin expression. Finally, this study also aimed at examining how the TGF-βs and their solvent and carrier influenced wound closure in an *in vitro* wound closure model and how TGF-βs influence ECM secretion and integrin expression.

5, 10, 25, 50 and 100μm wide repeat gratings of Collagen type I, Fibronectin and Laminin patterns were stamp patterned onto glass slides and plated with MG63 cells at 50,000 cells per coverslip. Cells on the fibronectin pattern attached and elongated soon after seeding, but did not adhere readily to collagen and laminin and appeared more rounded until 18hrs after seeding. Cells aligned significantly well on the 50μm and 100μm wide fibronectin patterned coverslips with mean angles of alignment ~7.87˚ ± 3.06SD and 6.45˚ ± 5.08SD, respectively, compared to those with smaller width (p<0.001). In comparison, cells aligned less readily to the other two ECM proteins, showing optimal alignments of 9.66˚ ± 4.18SD and 14.36˚ ± 1.57SD to the 50μm wide collagen and laminin patterns, respectively. Differences in cell length mirrored those of alignment, with cells acquiring the greatest length when showing the greatest degree of alignment. The results indicate that MG63 cells responded significantly better to 50 and 100μm wide fibronectin patterns compared to those with smaller width (p<0.001) indicating that the cells may attach mostly via fibronectin specific integrins.

Cell surface attachment was examined via a trypsinisation assay in which the time taken to trypsinise cells from the surface provided a means of assessing the strength of attachment. The results indicated that treatment with the solvent (HCl), TGF-β1, 2 and 3 all decreased cell attachment, but this effect was significantly greater in the case of HCl and TGF-β3 (p<0.001). However, there were significant differences in trypsinisation

rates between HCl and TGF-β3 (p<0.001). The wound healing response to the TGF-βs and their solvent/carrier was also investigated in 300µm ± 10-30µm SD wide model wounds induced in fully confluent monolayers of MG63 bone cells. The results indicated that TGF-β3 and HCl significantly enhance wound closure when compared against negative controls, TGF-β1 and TGF-β2 treatment (p<0.001). It was also found that TGF-β1 and TGF-β2 treatment significantly improved wound closure rate in comparison to the controls (p<0.001).

Experiments were performed to determine if the HCl effects on wound closure were dose dependent. Cells were incubated with 20µM, 40µM, 80µM and 160µM concentrations of HCl prior to wounding and wound closure rates were recorded. Wound closure was dependent on HCl dose with the 80µM and 160µM concentrations inducing increases in wound closure rates that were both significantly greater than those induced by 20µM, 40µM and control treatments (p<0.001). However, there were significant differences in wound closure between the 80µM and 160µM treatment groups after 30hrs of treatment (p<0.001).

The effect of different TGF-β isomers and their combinations on proliferation rate and cell length of human bone cells were also assessed. The results suggest that cell morphology changes were observed significantly more in cells treated with TGF-β(2+3) and TGF-β(1+3) (p<0.001). Any cell treated with TGF-β1, TGF-β(1+2) and TGF-β(1+2+3) showed significantly less elongation compared to the control and other TGF-β isomers. In terms of proliferation rate, TGF-β3 and TGF-β(2+3) increased cell numbers more than TGF-β1, TGF-β2 and other combinations. TGF-β1 and its combinations did not show significant proliferation and attachment compared to the control due to perhaps its inhibitory effect in contact with human bone cells.

Immunostaining indicated that treatment with TGF-β3 significantly promoted the secretion of collagen type I and anti-human fibronectin in addition to integrin (α3 and β1) expression. Statistically TGF-β3 and their combinations showed significant differences in number of cells stained for collagen type I, anti-human fibronectin, α3 and β1integrin. Any cell treated with TGF-β1 or any combination with TGF-β1 showed significantly lower cell number stained with the same proteins and integrins (p<0.001). Imaging with WSPR allowed observation of the focal contacts without the need for immunostaining. WSPR images revealed guided cells with high contrast band like structures at the border of cells distal to the edge of guidance cue to which they aligned and with less concentrically formed band like features across the cell body. It is believed that the high contrast features are associated with the formation of focal contacts on the edge of the cells distal to the edge of fibronectin patterns, which suggests that cell guidance is aided by a decrease in cell attachment along a guidance feature. The WSPR experiments also indicated that TGF-βs influenced the distribution of focal contacts. In the case of TGF-β1 treated cells the bright high contrast regions were intense but only arranged around the periphery of the cell. In TGF-β2 and TGF-β3 cells the bright contrast regions were weaker but again mostly localised around the periphery. These findings supported the earlier trypsinisation results.

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This dissertation is dedicated
to
my parents
Mr. Hossein Sefat,
Mrs. Maryam Molazem
and
my wife Dr. Ehsaneh Daghigh Ahmadi

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1. **SEFAT, F**., YOUSEFFI, M., AND DENYER, M.C.T. (2010) "Imaging via widefield surface plasmon resonance microscope for studying bone cell interactions with micropatterned ECM proteins", Journal of Microscopy, 241, pp 282-290, (doi: 10.1111/j.1365-2818.2010.03430.x).

2. BEGGS, C.B., DENYER, M.C.T., LEMMERZ, A., **SEFAT, F**., WRIGHT, C., YOUSEFFI, M. (2010) "The effect of transforming growth factor beta (TGF-β3) and Sanicle on wound healing", Proceedings of the World Congress on Engineering (WCE): International conference of systems biology and bioengineering (ICSBB), 2010, June 30th –July 2nd, London, UK, Vol II, pp 572-577, (ISBN: 978-988-17012-9-9).

3. **SEFAT, F**., BEGGS, C.B., DENYER, M.C.T., MEAKIN, G.D., YOUSEFFI, M. (2010) "The Effects of different HCl concentrations on wound healing of bone cell monolayer", Proceedings of the World Congress on Engineering (WCE): International conference of systems biology and bioengineering (ICSBB), 2010, June 30th –July 2nd, London, UK, Vol II, pp 542-549, (ISBN: 978-988-17012-9-9).

4. **SEFAT, F**., YOUSEFFI, M., BERENDS, R.F., KHAGHANI, S.A., AND DENYER, M.C.T. (2009) "The Effect of Transforming Growth Factor Beta (TGF-β3), HCl and BSA/HCl on Trypsinisation of Bone Cells Monolayer," Proceedings of the World Congress on Engineering (WCE): International conference of systems biology and bioengineering (ICSBB), 2009, July 1-3, London, UK, Vol II, pp 1859-1862 (ISBN:978-988-18210-1-0).

5. **SEFAT, F**., YOUSEFFI, M., BERENDS, R.F., KHAGHANI, S.A., AND DENYER, M.C.T. (2009) "Wound Closure Properties of TGF-β3, BSA/HCl and HCl in Cultured Human Bone Cell Monolayer", Proceedings of the World Congress on Engineering (WCE): International conference of systems biology and bioengineering (ICSBB), 2009, July 1-3, London, UK, Vol II, pp 1866-1871 (ISBN:978-988-18210-1-0).

6. KHAGHANI, S. A., DENYER, M.C.T., YOUSEFFI, M., BERENDS, R.F., **SEFAT, F**., LOBO, S., AND JAVID, F.A. (2009) "Purification of Primary Chondrocyte Cells isolated from articular joint using neonate Sprague-Dawley Rats," presented at the Winter meeting of Anatomical Society of Great Britain & Ireland (ASGBI): The Art of Anatomy, St Anne's College, Oxford, UK, 6^{th}–8^{th} January 2009; J. Anat.215, pp 711-712. (doi: 10.1111/j.1469-7580.2009.01145.x)

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10. KHAGHANI, S. A., DENYER, M., **SEFAT, F**., YOUSEFFI, M. (2008) "Alignment of rat primary chondrocyte cells to Collagen type-I, Fibronectin and Laminin", Presented at the Winter meeting of Anatomical Society of Great Britain & Ireland (ASGBI): A symposium on Anatomy and Matters Forensic, St Anne's College, Oxford, UK, 3rd–4th January 2008; J. Anat.213, p 351, (doi: 10.1111/j.1469-7580.2008.00949.x).

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1. KHAGHANI, S.A., YOUSEFFI, M., **SEFAT, F**., AND DENYER, M.C.T. (2009) "Does latent transforming growth factor-beta2 play any role in the wound healing of cartilage," presented at the 11th annual meeting of the Surface Science for Biologically Important Interfaces (ssbii), Keele University, Newcastle-under-Lyme, UK, 28 October 2009, p 26.

2. **SEFAT, F**., YOUSEFFI, M., AND DENYER, M.C.T. (2009) "Effect of TGF-β1 on wound closure properties of cultured human bone cell monolayer," presented at the 11th annual meeting of the Surface Science for Biologically Important Interfaces (ssbii), Keele University, Newcastle-under-Lyme, UK, 28 October 2009, p 23.

3. **SEFAT, F**., YOUSEFFI, M., AND DENYER, M.C.T. (2009) "Effect of TGF-β2, BSA/HCl and HCl on wound closure properties of cultured human bone cell monolayer," presented at the 11th annual meeting of the Surface Science for Biologically Important Interfaces (ssbii), Keele University, Newcastle-under-Lyme, UK, 28 October 2009, p 24.

LIST OF ABBREVIATIONS

3D	three dimensional
Au	gold
Ag	aluminium
AFM	atomic force microscopy
ATP	adenosine triphosphate
ATR	attenuated total reflectance
Anti	antibody
BFP	back focal plane
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathies
CCD	charge-coupled device
DIC	differential interference contrast microscope
DNA	deoxyribonucleic acids
Dthiol	1,6-Hexanedithiol
ER	endoplasmic reticulum
EM	electron microscope
ECM	extra-cellular matrix
ESEM	environmental scanning electron microscope
ELISA	enzyme linked immunoabsorbant assay
FCS	foetal calf serum
FGF	fibroblastic growth factor
FIB	fibronectin
GAGs	glycosaminoglycans
HOBs	human osteoblast
HaCaT	Human Keratinocytes
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IgG	immunoglobulin
LAM	laminin
LCD	liquid crystal display
MCP	micro contact printing
MRI	magnetic resonance imaging
MIMIC	micromolding in capillaries
mRNA	messenger ribonucleic acids
NA	numerical aperture
OWLS	optical waveguide lightmode spectroscopy
PCL	polycaprplactone
PEG	poly (ethylene glycol)
PEO	polyethylene oxide
PHA	polyhydroxylkanoate
PHB	polyhydroxybutyrate
PLA	poly(lactic acid)
PLG	poly(glycolic acid)

PMNs	polymorphonuclear leukocytes
PPF	poly(propylene fumarate)
PPO	polypropylene oxide
PDGF	platelet derived growth factor
PDMS	polydimethylsiloxane
PLGA	poly(DL- lactic -co- glycolic acid)
PLLA	Poly (L-lactic acid)
PDLLA	Poly (D,L-lactic acid)
PHSRN	Pro-His-Ser-Arg-Asn
PLLACL	Poly (L-lactic acid-co-ε-caprolactone)
PDLLACL	Poly (D, L-lactic acid-co-ε-caprolactone)
QCM-D	quartz crystal microbalance with dissipation
RER	rough endoplasmic reticulum
RGD	Arg-Gly-Asp
RNA	ribonucleic acids
RPMI	Roswell Park Memorial Institute
RRETAWA	(Arg-Arg-Glu-Thr-Ala-Trp-Ala)
SP	surface plasmon
SPs	surface plasmons
SAM	self assembled monolayer
SER	smooth endoplasmic reticulum
SEM	scanning electron microscope
SFM	scanning force microscope
SPR	surface plasmon resonance
STM	scanning tunnelling microscope
SCOM	scanning confocal optical microscope
TEM	transmission electron microscope
TGF-β	transformation growth factor beta
Thiol	2-Aminoethanethiol
TRITC	tetramethylrhodamine isothiocyanate
UV	ultra violet
WSPR	widefield surface plasmon resonance
IWSPR	interferometer widefield surface plasmon resonance

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CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 OVERVIEW OF BONE

Bone is a living tissue that makes up the body's skeleton. Bones are classified by their anatomical shape as long (e.g. femur, humerus), short (e.g. wrist, ankle), flat (e.g. membrane bone), and irregular (e.g. vertebra). Bone is a specialised form of connective tissue and like other connective tissues including cartilage and blood is derived from mesoderm. Excluding teeth and sesamoid bones, there are a total of 206 bones in the adult human body which are normally separated by various types of cartilage to provide support to the skeletal system and facilitate joint movement (Hollinger, 2004, Bilezikian, 2008, Marolt, 2012). The entire framework of bones and their cartilages together constitute the skeletal system, (see Figure 1.1).

1.1.1 Structure of bone

The outer surface of all bones, except at the joints of long bones, is covered with a membrane consisting of dense irregular connective tissue known as periosteum (Netter, 1987) and a similar tissue, the endosteum, lines the inner surface of all bones. There are three gross types of bone in the human body; compact (cortical), spongy (trabecular or cancellous) and subchondral bone which can be distinguished by their appearance. At the microscopic level, the differences between subchondral, compact and cancellous bone are not easily distinguished (Bilezikian, 2008, Glorieux, 2011).

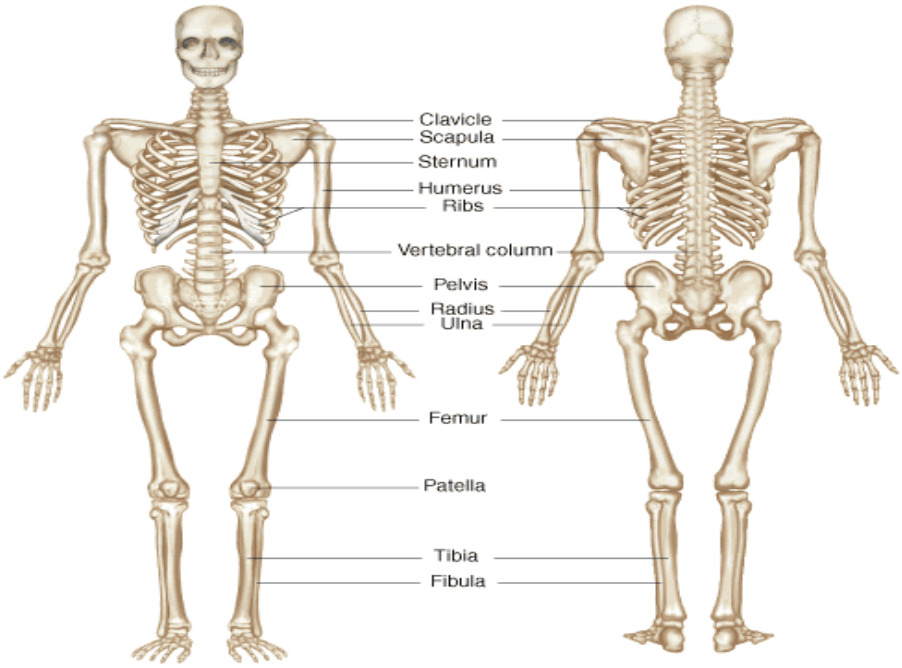


Figure 1.1 *Human body skeleton.*
Adapted from: http://hkeducationcenter.com

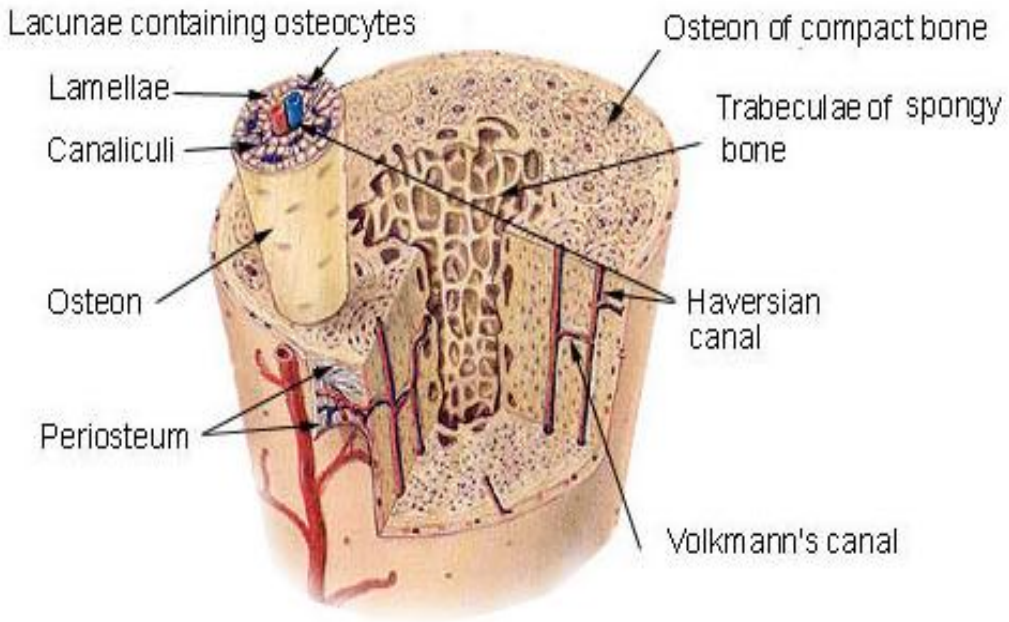


Figure 1.2 *Structure of human bone.*
Adapted from: http://training.seer.cancer.gov

All types of bone have an identical microscopic structure at comparable stages of maturity of bone tissue. The difference between them lies in the relationship of each to the blood supply. Cancellous bone is bony tissue surrounded by blood vessels; compact bone is blood vessels surrounded by bony tissue; and subchondral bone is more vascularised than compact bone and less vascularised than cancellous bone. Compact bone makes up 80% of the skeleton and has a solid, compact structure and 80-90% of its volume is calcified. Spongy bone makes up 20% of the skeleton. This type of bone has more surface area compared to compact bone but it is less dense. It is typically located at the end of long bones and is highly vascularised. It also contains red bone marrow where the production of blood cells (hematopoiesis) occurs (Hollinger, 2004, Glorieux, 2011).

The microscopic structure of bone varies according to its maturity and the speed with which it must be laid down. There are three forms of immature or new bone: woven, laminar, and fine cancellous. Woven bone is deposited in the initial formation of bone in the fetus or where rapid repair of bone is needed. Woven bone is remodelled into mature bone, which has a more ordered structure. In human the remodelling process may be too slow to keep up with growth, so another type of immature bone is formed where greater strength is needed: laminar bone. Laminar bone is composed of sheets, or laminae, of bony tissue seperated from each other by intervening networks of blood vessles. Laminar bone has a smaller volume of vascular canals than other types of bone and is probably stronger in some ways. The third type of rapidly growing bone, fine cancellous bone, is found in places where transient reinforcement of the extant bone is needed. For example, fine cancellous bone may appear on the brow ridge of an animal

when the stresses involved in chewing increase, as when a new tooth is erupting (Bilezikian, 2008).

The differences between immature and mature bone lie mainly in the relationships between the vascular canals and the bony tissue. Mature or adult bone is called lamellar bone. Such bone is arranged in thin sheets or lamellae, which are roughly 3-7 microns thick. In each lamella, the bundles of collagen fibres are arranged parallel to one another, although the predominant orientation may vary from lamella to lamella for strength. This arrangement gives lamellar bone a layered appearance in cross section. A group of lamellae may be organised in one of two ways, depending upon its location within the whole bone. The basic unit of lamellar bone is the osteon (Hollinger, 2004), in which sets of 15-20 lamellae are arranged in concentric tubes around an opening for a blood vessel (See Figure 1.2). The placement of vascular canals within these rings of bone differentiates mature from immature bone. Surrounding each osteon is a cement line, a dense ring that demarcates its boundaries and surrounds the outer lamella throughout the entire length of the osteon. Because osteons are formed by remodelling either immature bone or older mature bone, adjacent osteons may cut across each other, may touch each other at their cement line, or may be separated by regions of less organised bone tissue. These intervening regions of bone, called interstitial bone, are irregular in shape (Marks, 2002, Bilezikian, 2008).

The vascular canal at the centre of each osteon is part of a network of canals, the Haversian system, which brings nourishment to the bone tissue. Within this system are

two types of canals, Haversian and Volkmann's. Haversian canals are oriented more or less longitudinally within the bone. They are connected to each other, to the marrow cavity, and to the surface of the skeletal element by a complex system of oblique and transversely oriented canals, or Volkmann's canals. Volkmann's canals are not surrounded by concentric lamellae and in fact frequently cut through lamellae. Not all mature bone is organised into osteons. Near both the inner and outer surface of long bones, this organisation of bone tissue is replaced by several large-scale lamellae that encircle the entrie perimeter of the shaft; these are called outer and inner circumferential lamellae (Bilezikian, 2008, Glorieux, 2011).

1.1.2 Cellular structure of bone

Bone contains four different types of cells including osteoprogenitor, osteocyte, osteoblast and osteoclast. In general osteoprogenitor cells can be considered as bone stem cells and they are located in the inner portion of periosteum and in endosteum. These cells have the capacity to divide and differentiate into osteoblast bone cells. Osteoprogenitor cells are immature progenitor cells and are induced to differentiate into osteoblast under the influence of growth factors mainly Bone Morphogenetic Proteins (BMP) (Agata, 2007). Bone tissue is formed by osteoblasts, maintained by osteocytes and broken down by osteoclasts (Lee, 1999). The osteoblasts mainly functions in bone formation and these cells secret collagen and ECM substances that constitute unmineralised bone osteoid and subsequently are responsible for calcification and remodelling. Osteoblasts originate in the bone marrow and are derived from mesenchymal stem cells (Mundy, 2008). Osteoblasts are also able to communicate with

one another and with osteocytes by a gap junction. Alkaline phosphatase is an enzyme that is plentiful in osteoblasts and this enzyme is important in the formation of mineral deposits in the matrix (Marks, 2002). Some osteoblasts become embedded in the matrix of bony tissue to become osteocytes. Osteocytes are mature bone cells, and these cells maintain bones' daily metabolism, such as exchanging nutrients and waste with the blood and are completely surrounded by the bone matrix. Lacuna is the place in which octeocytes are located and they are able to communicate with adjacent octeocytes through canaliculi, which are used for the exchange of nutrients and waste (Hollinger, 2004). They are actively involved in the routine turnover of bony matrix, through various mechanosensory mechanisms. The osteoclasts like the osteoblasts originate in the bone marrow. The osteoclasts are derived from the mononuclear/phagocytic cell lineage. Osteoclasts are phagocytic cells responsible for removing bone tissue (Mundy, 2008) via bone resorption. Osteoclasts are huge cells with a diameter of 20-100μm and they migrate across the surfaces of the bone and release enzymes such as collagenase and proteolytic enzymes to remove the organic component of the bone and stabilise minerals by lowering the pH of isolated areas (Lee, 1992). Osteoclasts are found on bone surfaces and are important in the normal growth, maintenance and repair of bone (Marks, 2002).

1.1.3 Molecular structure of bone

Human bone is composed of bone cells and a matrix of collagen fibres impregnated with bone salts (mainly calcium carbonate and calcium phosphate) (Sasano, 2012). The Extracellular Matrix (ECM) makes up 60% of bone and consists of organic and

inorganic parts. The inorganic component (bone minerals) consists of calcium phosphate and hydroxyapetite [Ca_{10} $(PO_4)_6(OH)_2$]. Water makes up 5-8% of bone's composition. The organic component of matrix consists of type I collagen fibers (90%), proteoglycan, osteopontin, osteocalcin, osteonectin, bone sialo protein, alkaline phosphatise, regulatory factors such as cytokines and growth factors (Hollinger, 2004, Ferreira, 2012). Proteoglycan helps the calcification of bone.

The organic matrix consists of a highly vascular connective tissue consists of ECM proteins and enclosed by a fibrocellular layer, the periosteum (Archambault, 2000). One of the main functions of most cells is to produce its own extracellular matrix (ECM). ECM components consist of the mixture of collagens, fibronectin and individual proteoglycans. This is an interactive natural scaffold that provides mechanical stability and cell anchorage, and is also able to control cell functions. In order to manufacture the ECM, cells synthesise mainly high-molecular-weight fibrous proteins. This protein is secreted out of the cell and builds up in the surrounding environment to form an insoluble network. In some tissues like skin and muscle the ECM is soft and elastic, whereas mechanically strong structures are formed in tendon, cartilage and bone. ECM plays an important role in the human body and some of its functions are to provide structural support and tensile strength. It also provides substrates for cell adhesion and cell migration. The ECM also regulates cellular differentiation and metabolic functions.

1.1.3.1 Collagen

Collagen is the most important structural protein of the extracellular matrix (Ferreira, 2012). The collagens are large families of proteins, comprising of at least 19 related glycoproteins, numbered in a series from collagen I to collagen XIX. All are based on a structure called the triple helix, a tight right-handed helix of three individual collagen chains, each individual chain having the structure of a left-handed helix (Davies, 2001). Collagen is synthesized by an extensive number of cell types such as fibroblasts, osteoblasts, chondroblasts, odontoblasts, reticular cells, epithelial cells, endothelial cells, smooth muscle cells, Schwann cells, etc. Cells do not secrete collagens directly, but rather secrete large precursors called procollagens that have 'extra' domains at each end of the peptides of a mature collagen. These domains are cleaved off outside the cell by specific proteinases, and once free of them most collagen chains can associate with each other to form fibrils, which line up with one another to form larger fibres. Collagen types I, II and III account for about 90% of all collagen in the body, and form strong fibrils. In life, most individual fibrils are composed of a mixture of these collagens and may also include small amounts of the other fibrillar collagens (e.g. V and XI), with collagen types IX, XII and XIV coating fibrils to alter their sticky or slippery properties. While these fibrillar collagens are common in connective tissue, the basement membranes of epithelia usually contain a quite different collagen, collagen IV, which forms three-dimensional gels rather than fibrils. In contrast to these common collagens, some types are expressed only in a small number of sites in the body. Collagen type I is the main component of bone (Davies, 2001).

1.1.3.2 Fibronectins

Fibronectins are a family of proteins produced by alternative splicing of a single gene. Some forms of fibronectin remain soluble and may be found in blood plasma, while others associate into disulfide-bonded fibrils in the ECM. Each fibronectin molecule, itself a dimer of two fibronectin peptides, contains binding sites for a variety of other molecules, such as heparan sulphate proteoglycan, integrins and collagens. Fibronectins therefore serve to bind different components of the ECM together, and to serve as sites on the ECM to which cells can bind.

1.1.3.3 Laminin

Laminins are large ECM proteins, typically the shape of a cross, that are composed of three different types of chain, called a, b and g. The three short arms of the cross represent the N-terminal ends of each chain, while the middle and C-terminal portions of the chains run together down the long arm of the cross. There are several types of a, b and g chains, and these can associate promiscuously to generate many types of laminin (e.g. laminin-1has a1, b1 g1chains, laminin-2 has a2, b1, g1chains, etc.). Laminins contain various sites for binding to receptors on the cell surface, and to other components of the cell matrix such as collagens and proteoglycans. Laminin is not normally present in bone (Colucci, 1996), but however, laminin was used in this study as a control and also to verify this ECM protein has any effect on bone cell behaviour.

1.1.3.4 Non-collagenous bone proteins

Osteocalcin, osteonectin, osteopontin and bone sialoprotein are four major non-collagenous bone proteins. Both osteopontin and bone sialoprotein are necessary for the initiation of bone mineralisation (Roach, 1994). Osteocalcin is secreted by osteoblats and plays a role in the body's metabolic regulation and is found in bone and dentin. It is also used as a biochemical marker for the bone formation process. Osteonectin is a glycoprotein in the bone which is secreted by osteoblasts during bone formation, initiating mineralisation and promoting mineral crystal formation. It is an acidic, secreted ECM glycoprotein that plays a major role in bone mineralisation, cell-matrix interactions, and collagen binding (Guweidhi, 2005). Osteopontin is a glycoprotein that was first identified in 1986 in osteoblasts. It is also known as an important factor in bone remodelling.

1.1.4 Osteogenesis

The development of bone tissue (ossification) is known as osteogenesis (Parsh, 2012). There are two types of osteogenesis, intramembranous ossification in which formation of bone occurs directly within the early development of connective tissue and endochondral ossification in which cartilage develops into bone. Both require a solid base and a well-developed vascular supply for the elaboration and mineralization of the exteracellular matrix. Intramembranous ossification occurs during embryonic development by the direct transformation of mesenchymal cells into osteoblasts (Bilezikian, 2008). This type of ossification for entire bones is restricted to those of the cranial vault, some facial bones, and parts of the mandible and clavicle. The flat bones

of the skull grow toward each other from primary ossification centres in each and meet at sutures. This mechanism provides a steady source of osteoblasts and allows bones to expand at their edges. When growth is complete, sutures remain as fibrous connections or disappear, depending on the suture site. Bones that participate in joints and bear weight form by endochondral ossification, a method by which the unique properties of cartilage and bone are exploited to provide a mechanism for formation and growth of the skeleton during growth of the individual (Glorieux, 2011). In such bones the condensed embryonic mesenchyme transforms into cartilage, which reflects in both position and form the eventual bone to be formed at that site. In the central part of such a bone, endochondral ossification provides for a linear, interstitial proliferation of columns of chondrocytes. Their progressive hypertrophy, mineralisation of the intercolumnar cartilage matrix in the long axis of the bone, and the presisence of mineralised cartilage after disapperanace of its cells acts as an elongating scaffold for the deposition of subchondral bone. In the circumference of such a bone, starting initially at the centre and progressing toward the ends, the investing cartilage cells and stroma transform into osteoblast that form a periosteal collar after the underlying chondrocytes have mineralised the matrix. The peripheral osteoblast (periosteum) arrive with a blood supply whose vessles penetrate the central hypertrophied, mineralised cartilage core and carry to the interior the skeletal cell progenitor for the formation and turnover of bone. Thus, peripherally extension of the periosteum and centrally mineralisation of cartilage, hypertrophy, and disappearance of chondrocytes and bone formation on the mineralised cartilaginous scaffold proceed toward the end of each growing long bone (Bilezikian, 2008).

1.1.5 Functions

Bones perform four major functions in the body, having to do with its support, defence, movment, and supplies (Glorieux, 2011). First, the skeleton and its individual elements provide a framework or scaffolding that supports the soft tissues of the body and permits them to function without collapsing. Second, the skeleton functions to protect the body. In one sense it acts as a sort of hard protective device to shield important soft tissue from damage arising from external sources. The ribs, for example, form a partial cage protecting the heart and lungs from injuries, and the skull acts as a bony box surrounding the brain and special sense organs. The joints and spongy bone tissue in the skeleton function as shock absorbers, modulating the impacts produced by locomotion and other movements so as to protect the soft tissue of the body. Third, the bones of the body act as lever, to be pulled upon by muscles, producing movement and locomotion. Finally, bone marrow, located within the medullary cavitiy of long bones and interstices cancellous bone, produces red blood cells (RBC), white blood cells (WBC), and platelets in a process called hematopoiesis (Kale, 2004a). In addition, the bony tissues of the entire body are a huge warehouse of minerals to be trapped by the body in response to different physiological needs. For example pregnant women with an insufficient intake of calcium will resorb part of their own bone tissue to supply the needs of the growing fetus. The principal mineral constituent of bone is calcium, 99% of the body's supply being stored in this way, and phosphorus is also found in large quantities (Kovacevic, 2011). Calcium is concentrated particularly in the dense cortical bone, although the most liable volume is located in cancellous bone (Talmage, 2007). The metabolic functions of bone also consist of storage of growth factors (BMP, TGF-β and Inslulin-like growth factor) and fat. Bone naturalizes the blood against excessive pH

changes by absorbing/releasing alkaline salts. Bone cells also release osteocalcin hormone which regulates blood sugar and fat deposition.

1.2 BONE DAMAGE AND REPAIR

Bone is the main source of calcium (Ca) in the human body. Calcium can be released from bone to the blood or *vice versa*. In this process old bone is removed and resorption occurs in which old bone is removed by bone removing osteoclast cells and new bone is laid down by bone forming osteoblast cells. Bone formation and resorption normally occurs in the body through a lifelong process known as bone remodelling (Lee, 1999, Wolff, 2012). Overall remodelling provides a balance between bone formation and resorption. Any imbalance in the regulation of bone remodelling will result in various metabolic bone diseases such as Osteomalacia which is the softening of the bones caused by defective bone mineralisation (inadequate amounts of available phosphorus and calcium), or because of overactive resorption of calcium from the bone as a result of hyperparathyroidism (Kovacevic, 2011). The control of mineral homeostasis involves both physio-chemical and biological factors. Outstanding among the biological factors affecting calcium level are two hormones, firstly the parathyroid hormone (PTH); and secondly, thyrocalcitonin (TCT) or calcitonin (Wolff, 2012). Vitamin D is also vital for calcium homeostasis and for optimal skeletal health. The main function of vitamin D is to increase the efficiency of calcium absorption from the small intestine. Vitamin D deficiency in children can manifest as rickets, which presents as bowing of the legs (Palmieri, 1989, Schwartz, 1993).

Remodelling takes place very fast in the first year of life and becomes slower in adults. Remodelling is the result of many factors such as mechanical stimuli, metabolic causes, effects of drugs and endocrine changes. Bone healing, or bone repair, is a process in which the body facilitates the repair of bone fractures (Ogasawara, 2008, Kovacevic, 2011). Bone repair is mainly determined by the periosteum. The periosteum is the primary source of precursor cells, which develop into the chondroblast and osteoblast that are essential to the healing of bone. Secondary sources of precursor cells are bone marrow, endosteum, small blood vessels and fibroblasts. Fracture healing can be described in three phases: the reactive phase (fracture inflammatory phase and granulation tissue formation), reparative phase (callus formation and lamellar bone deposition) and remodelling phase (remodelling to original bone contour) (Tosounidisa, 2009, Nakamura, 1998). In the reactive phase bleeding stops due to blood vessel constriction (Brighton, 1997). A few hours later a blood clot is formed by hematoma (extravascular blood cells) and cells within the blood clot die (Brighton, 1991). Inflammatory cells such as macrophages, monocytes, lymphocytes infiltrate the bone. Fibroblasts survive in this area and replicate with granulation tissue formation which is a loose aggregate of cells, distributed with blood vessels (Ham, 1972). This results in the formation of granulation tissue, ingrowth of vascular tissue, and migration of mesenchymal cells. The primary nutrient and oxygen supply of this early process is provided by the exposed cancellous bone and muscle (Kalfas, 2001).

In the reparative phase (days after fracture) periosteum cells replicate and transform. The peristeal cells distal to the fracture gap, develop into osteoblasts and form woven bone (Brighton, 1997). Chondroblast also develop proximal to the fracture gap to form

hyaline cartilage. Fibroblasts begin to lay down a stroma that helps support vascular ingrowths. As vascular ingrowth progresses, a collagen matrix is laid down whilst osteoid (unmineralized organic portion of the bone matrix) is secreted and subsequently mineralized, which leads to the formation of a soft callus around the repair site (Kalfas, 2001, Brighton, 1986). Hyaline cartilage and woven bone provide a bridge within the fracture gap to restore some of its strength. Hyaline cartilage and woven bone are then replaced with lamellar bone referred to as endochondral ossification. Eventually, all Hyaline cartilage and woven bone are replaced by trabecular bone (Brighton, 1986).

Remodelling is the last phase in bone repair and bone fracture healing is completed during the remodelling stage. In this stage the healing bone is restored to its original shape, structure, and mechanical strength (Tosounidisa, 2009). This process occurs slowly over months to years and is facilitated by mechanical stress placed on the bone. Initially, this involves converting the irregular woven bone callus into lamellar bone, although the standard cortical structure is eventually restored. This process is driven by a coupled process of orderly bone resorption followed by the formation of lamellar bone (Teitelbaum, 2000). Osteoclasts are the main cell type involved with the resorption of mineralised bone. Once bone resorption completed, osteoblasts are able to lay down new bone on the eroded surface (Schindeler, 2008).

1.3 CURRENT THERAPIES AND LIMITATIONS FOR BONE HEALING

The processes described above are a part of the natural healing process of human bones. However, many conditions exist where the natural healing process is insufficient and adjunctive measures are needed to assure full regeneration. Examples include infections, fracture mobility, and critical-size defects. This section will discuss treatments that are currently used in these situations to augment natural bone repair (Blitterswijk, 2008, Shapiro, 2008). Tissue substitutes provide a means of treatment in order to repair or replace damaged or diseased tissues/organs. Tissue substitutes include man-made materials and devices, autografting, allografting and xenographting (Banwart, 1995, Finkemeier, 2002a). Other treatments also involve stem cell therapy, gene therapy and drug therapy.

Man-made biomimetic devices and materials are artificial tissue substitutes that replace the function performed by biological systems. They include stainless steel, cobalt chrome, titanium alloys, polyethylene and ceramics as prosthetic hips, knees, etc. (Cássio do Nascimento, 2007). Titanium alloys are also used for cement less joint replacements, screw and paltes for fracture fixation (Grupp, 2010), biodegradable polymers (for ankle and phalangeal fractures) (Eglin, 2008), shape memory allys (for orthopaedic implants specifically for small bone in face) (Kawakita, 2012) and many more. These devices may be limited by factors such as toxicity, initiation of inflammation, wear, fracture, and fatigue failures. Also, they may not remodel with time

(Parikh, 2002), for example, a titanium plate in a child would not deform or remodel as bone grows, which may cause further problems (Matthias Schieker, 2006).

Autografting or autologous bone grafting involves harvesting and transplantation. In this procedure a tissue is harvested from one location in the patient and transplanted into the defect side of the same patient. In autografting rejection immunogenicity is not a major issue as tissue/cells are provided from the patient's own body. Spinal fusions are common autografting procedures (Mummaneni, 2011). Spinal fusion involves grafting a bone from a patient's hip to repair a vertebral defect. Additional surgical problems for harvesting include infection, blood loss and pain at the harvesting site (Parikh, 2002). An autologous cancellous bone graft is the most effective grafting material as it provides the three elements required for bone regeneration: osteoconduction, osteoinduction, and osteogenic cells (Finkemeier, 2002a). Autologous cortical bone grafts provide these three components to a limited extent and also provides the structural integrity important in reconstruction of larger defects (Keating, 2001). There are several shortcomings associated with this form of treatment, including complications in shaping the graft to fill the defect, a need for several procedures, high recovery time, donor site morbidity (Goulet, 1997), risk of disease transmission, loss of biological and mechanical properties and high cost (Parikh, 2002).

Allografting consists of harvesting tissue or an organ from a live or dead donor (same species) and then transplanting it to the patient. Replacing bone damaged by accicdent from a healthy person to a patient with bone fracture is an example of allografts

(Finkemeier, 2002b). The main problems with allografting are shortages of donors and cell/tissue rejection (immunogenicity). Use of immunosuppressant drugs such as sirolimus (SRL) and Cyclosporine A (CsA) (Goodman, 2001) make allografting more common, at the expense of reducing the effectiveness of the immune system (Bruno, 2007).

Xenografting consists of harvesting cell/tissue from individuals of another species. Animal cells are the main source for this and there is still a considerable ethical debate relating to the use of this group with additional problems such as tissue rejection and disease transmission. An example of a xenograft is when bone is taken from a cuttlefish and transplanted into rabbit's femur. At the end of the 30-day postoperative experimental period, bone defects filled with various graft materials were evaluated using macroscopic and radiographic methods (Okumus, 2005). The term zoograft refers to a tissue that is always transferred from an animal to a human while a xenograft can be used either for the transplant of tissue from animal to animal or animal to human (Grove, 2008).

Stem cells are undifferentiated cells, which originate from two different sources, embryonic and adult stem cells. These cells can be found in all multicellular organisms. Stem cells have the ability to renew themselves through mitotic cell division and divide in culture to give rise to different forms of specialized cells (Becker, 1963, Marolt, 2012). In a developing embryo stem cells are able to differentiate into all of the specialized embryonic tissues, whilst in an adult stem cells play a role in the repair and

maintainance of tissues in regenerative organs such as skin, blood or intestinal tissues. Currently stem cells can be grown and differentiated into specific cells through cell culture *in vitro*. Embryonic cell lines and autologous embryonic stem cells are two important candidates for future therapies (Tuch, 2006). Self renewal and potency are two properties of stem cells. Self-renewal is the ability to go through various stages of cell division while maintaining the undifferentiated state (Hans, 2007). Potency is the capability and potential to differentiate into various and certain cell types. In terms of potency stem cells may be totipotent, pluripotent, multipotent, unipotent or oligopotent (Hans, 2007). In the case of totipotent cells the stem cell has the capability to differentiate into any embryonic cell type, thus, can construct a complete organism (Hans, 2007). Pluripotent stem cells are dependent on totipotent cells and can differentiate into a large number of cell types including bone (Mitalipov, 2009). In the case of multipotent, the stem cell can differentiate into a closely related family of cells. Some stem cells can also differentiate into only a few cells, such as lymphoid or myeloid stem cells known as oligopotent stem cells (Hans, 2007), and finally unipotent cells can only produce their own cells like muscle stem cells (Ulloa-Montoya, 2005).

Gene therapies and the harvesting of tissue/organ or cells from cloned transgenic animals are relatively new technologies aimed to help many patients without using conventional therapies (Kofron, 2006, Carbonaro, 2012). These two new methods are still not practical for many different diseases due to limitations such as ethical, economical, technical, scientific and political issues (Vorburger, 2002). Conventional cell culture consists of growing cells in an environment outside the body but cells are not able to differentiate easily due to a lack of natural signals present in the body.

Signals such as mechanical, structural, electrical and chemical are normally present in the body but not outside the body in the cell culture environments (Freshney, 1994). These signals guide the cell and influence cell behaviour via cell signalling pathways (Clarkin, 2012). Thus, lack of these signals *in* vitro may result in cells exhibiting non-normal behaviours (Haj, 1990, Aoudjit, 2012). These structural signals in the body are derived from interactions between cells and cell-ECM. The communication between the cell and ECM affect the cell's shape and function (Einhorn, 1998). Gene therapy or genetic engineering, on the other hand, is a technique to deliver small DNA or RNA sequences to cells or tissues to correct a genetic defect or replace a mutated gene in the treatment of a disease (Marie, 2011). The potential use of gene therapy for bone regeneration is the delivery of physiological levels of therapeutic protein using the natural cellular mechanism (Kofron, 2006). Experimental investigations have verified this approach to yield bone healing equivalent to that achieved via the administration of a recombinant factor or use of bone graft (Laurencin, 1999). Currently the majority of gene therapies focus on adding genetic information to cells rather than alteration of the genome (Lieberman, 1998, Kofron, 2006).

Drug therapy is another treatment option for patients with orthopedic disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammation and severe pain (Kurmis, 2012, Pountos, 2012). Aspirin, Difunisal, Ibuprofen, Fenoprofen, Flurbiprofen, Ketoprofen and many other drugs are common examples of mild NSAIDs (Munson, 1996). NSAID therapy possesses many disadvantages and limitations (Amadio, 1993) and has a temporary effect instead of permanent cure. Chronic dosing with these agents is necessary and they all share a potential for causing serious adverse

effects such as peptic ulceration, bleeding from both the upper (Tamblyn, 1997) and lower digestive tract (Holt, 1991), damage to the liver and promotion of renal impairment, especially in the elderly (Tamblyn, 1997). Bone disorder drugs such as Alendronate can also interact with other drugs stopping them from working correctly (Watts, 2004).

1.4 TISSUE ENGINEERING

Tissue engineering is a field that seeks to replace/repair or enhance the biological function of a tissue or an organ by manipulating cells via their extra-cellular environment (Laurencin, 1999, Ikada, 2006, Rosa, 2012). In reality tissue engineering has a multi-disciplinary approach consisting of cell engineering, molecular biology, biomaterial engineering, design, imaging, etc, to develop materials in order to replace/repair diseased or damaged tissue and restore and improve their function (Parikh, 2002).

1.4.1 Origin of Tissue Engineering and its definition

The concept of directly engineering tissue was articulated in detail in 1985 (Fung, 1985). The term "tissue engineering" was first used during a meeting sponsored by the National Science Foundation (NSF) in 1987. The first true tissue engineering symposium was held in 1988, where a working definition was proposed (Skalak, 1988) in which tissue engineering was defined as "the application of the principles and methods of engineering and life sciences toward the fundamental understanding of

structure-function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain, or improve tissue function" (Skalak, 1988).

1.4.2 The need for bone tissue engineering

Bone and joint diseases cause many people to suffer for years with crippling effects. With the progressive aging of the population, the need for functional tissue substitutes is increasing (Vunjak-Novakovic, 2006). According to new research the cost of fractures in the UK is ~£5.1 billion each year (Strom, 2011). Large bone defects resulting from trauma, tumors, infections or congenital abnormalities often require reconstructive surgery to restore function (Crana, 1995, Sikavitsas, 2002). Organ transplantation and mechanical devices have revolutionised medical practice but have limitations. New bone tissue engineering strategies have been proposed that promise greater bone restoration without many of the limitations of the current therapies (Mooney, 1999). Bone tissue engineering is a different method of treatment as compared to drug therapy, gene therapy or permanent implants since engineered bone becomes incorporated within the patient, giving rise hopefully to a permanent cure for the patients suffering from various bone disorders.

1.4.3 Bone Tissue Engineering

Bone tissue engineering is a promising area in the field 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 bone tissue (Rosa, 2012, Hollinger, 2004). Bone cells (osteoblast, osteocyte, osteoclast), osteoconductive factor (three-dimensional matrices or scaffold) and osteoinductive factor (recombinant signalling molecules or growth factors) are the three main key elements (See Figure 1.3) in the tissue engineering of bone (Ramoshebi, 2002).

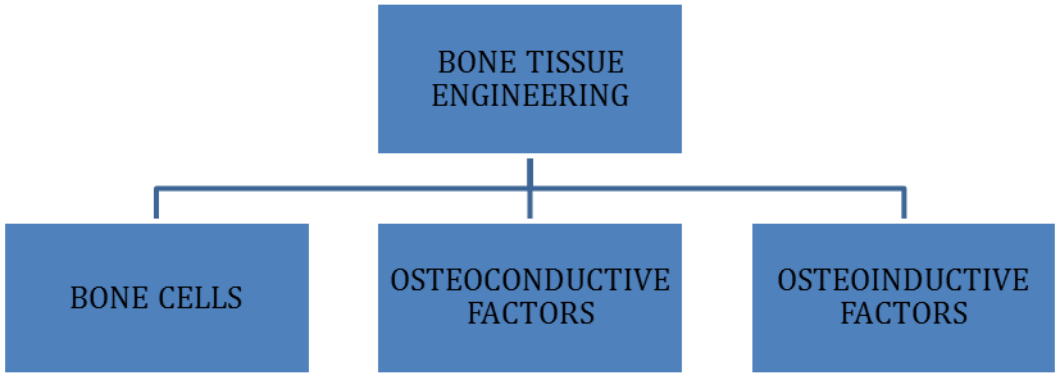


Figure 1.3 *Three main elements of bone tissue engineering (Sefat, 2010a).*

One popular approach in bone tissue engineering involves seeding bone cells in a highly porous biodegradable scaffold in the shape of the required bone. In this system bone cells are grown with growth factors in a bioreactor in which *in vivo* mechanical stresses are mimicked. Once cultured these replacement tissues can then be implanted into the defect site in order to induce and direct the growth of new bone (Jeffrey O. Hollinger, 2004, Vunjak-Novakovic, 2006, Salter, 2012). As a result of this process it is hoped that bone cells from the intact tissue will also grow into the implanted tissue thus allowing full integration of the implant and potentially repair the damaged bone with a high degree of structural integrity. Key in this process is the non-toxic degradation of the implanted scaffold (Hutmacher, 2000, Kawakita, 2012).

Bone repair and regeneration by the method of bone tissue engineering takes place in a series of cellular events that are affected by various biological and mechanical factors. All new formed tissue requires a group of cells known as osteoprogenitor cells capable of forming bone (Fleming, 2000). Cells used for bone tissue engineering are derived from a pluripotent population. These cells are capable of differentiation to different tissue types. Osteoprogenitor cells can be harvested from many sites and can undergo population expansion in *in vitro* cell culture (Freshney, 1994). Bone cells need to be delivered to a bone defect. The bone healing response starts when newly formed bone cells integrate with the surrounding tissue. Bone cells then grow either in a natural matrix or synthetic 3- dimensional scaffolds (Hutmacher, 2000). In this system the natural matrix or synthetic scaffolds are used to support cell attachment, proliferation and differentiation. Growth factors and adhesion molecules are present in or on the surface of the implanted matrix or secreted by cells incorporated in the matrix. Growth factors and adhesion molecules function by controlling cell migration, differentiation and subsequent tissue formation (Ramoshebi, 2002). The stimuli from these growth factors and adhesion molecules are known as osteoinductive signals. These are capable of determining the osseous nature of the tissue produced at the graft site (Fleming, 2000). Mechanical stimulation (environment) is also used to assist bone formation. Distraction osteogenesis (DO) is the process by which application of a tensile force at an optimal rate and frequency controls new bone formation, and this has been used successfully in bone repair (McCarthy, 2001).

The use of a bioreactor is very important. Fully functional tissues cannot be grown in conventional cell culture systems because although they may expand cell numbers they

may also promote inappropriate cell differentiation or de-differentiation (Freshney, 1994, Schnabel, 2002, Salter, 2012). Moreover, cells require external signals or cues before being implanted in the body to grow into functional 3D tissue (Burridge, 1996, Barrientos, 2008). These signals in the body can be mechanical, electrical, chemical or topographic (Logeart-Avramoglou, 2005). Structural cues involve the interaction between cells with their ECM. Firstly, bone cells cause remodelling of the ECM and, in turn, this remodelling of the ECM functions in a feedback mechanism modifying cell function. Thus, the physical communication between cells and ECM impacts cell shape and function (Akiyama, 1990). Cell actions and their responses to different signals (chemical, electrical, mechanical and structural) are mediated by protein based molecules known as growth factors (GF). GFs regulate cell migration, cell morphogenesis (from one cell type to another) and mitogenesis (cellular proliferation) (Ramoshebi, 2002). Growth factors are produced locally and systematically and can have different effects such as autocrine, paracrine, juxtacrine and endocrine effects in terms of the source of secretion, distance they travel in the body and the specific target where they can be effective. Beside their functions such as growth development and bone tissue maintenance, growth factors are also mobilized during bone remodelling and repair (Solheim, 1998).

1.4.4 Integrin receptors and cell adhesion

The interaction of bone cells with their extracellular matrix is of major importance in bone development, repair, and disease (Brighton, 1992, Tosounidisa, 2009). The interaction of bone cells with their surrounding ECM environment influences

physiological functions and pathological processes (Albelda, 1990). These physiological functions are normal skeletal development and bone matrix production. The interaction of bone cells with ECM are mediated by integrins, a family of cell surface receptors (Akiyama, 1990, Aoudjit, 2012). Integrins are a group of membrane spanning receptor proteins which bind to components of the extracellular matrix (Hynes, 1992) and they play key roles in the assembly of the actin cytoskeleton as well as in modulating signal transduction pathways (Srichai, 2010) that control biological and cellular functions including cell adhesion, migration, proliferation, cell differentiation, and apoptosis (Hynes, 1992, Worthington, 2011, Aoudjit, 2012). The physical link between integrins and actin is required for local regulation of actin polymerization as well as for global control of cytoskeletal dynamics.

Integrins exist as two non-covalently bound α and β subunits (Saito, 2004, Srichai, 2010). There are 18α and 8β known subunits which combine to form at least 24 distinct integrin heterodimers (see Figure 1.4) (Hynes, 2002). The α chain conveys ligand specificity and the β chain provides linkage to the cytoskeleton via talin, vinculin, or α actinin. The α and β chains have similar tertiary structures and are linked via their extracellular globular domains (Albelda, 1990, Worthington, 2011). Integrins can be divided into two groups. The first group contains integrins that bind to matrix proteins found in basement membranes. In this group the receptor binds to proteins such as collagen and laminin (Stephansson, 2002). The second group contains integrins that bind to matrix proteins such as fibronectin, vitronectin, osteopontin and fibrinogen, all of which are found during inflammation, wound healing or the development process (Saito, 2004). When integrins are inserted into the leading edge of the cell then cell

surface coupling will predominate in this region, which prevents membrane retraction and provides adhesive traction for cell movement.

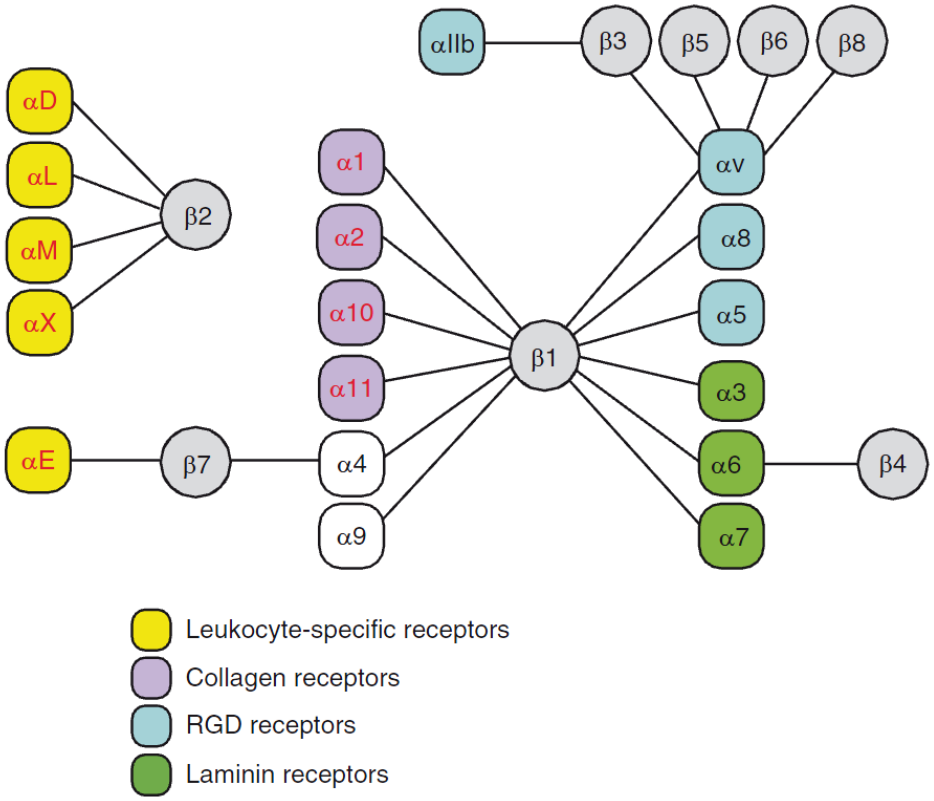


Figure 1.4 *Classification of integrin family of heterodimers. The nine a domains with inserted (I) domains (1, 2, 10, 11, D, L, M, X, E) are indicated in red. Image adapted from (Srichai, 2010).*

Figure 1.5 shows an integrin of the fibronectin receptor with two named subunits. These transmembrane polypeptides associate non-covalently with each other to form a binding site for the ligand on the outer membrane surface and a binding site for a specific cytoskeletal protein on the inner membrane surface. The fibronectin receptor has a binding site for fibronectin on the outer surface and a binding site for talin on the cytoplasmic side of the membrane. In this integrin the alpha subunit is split into two segments held together by a disulfide bond.

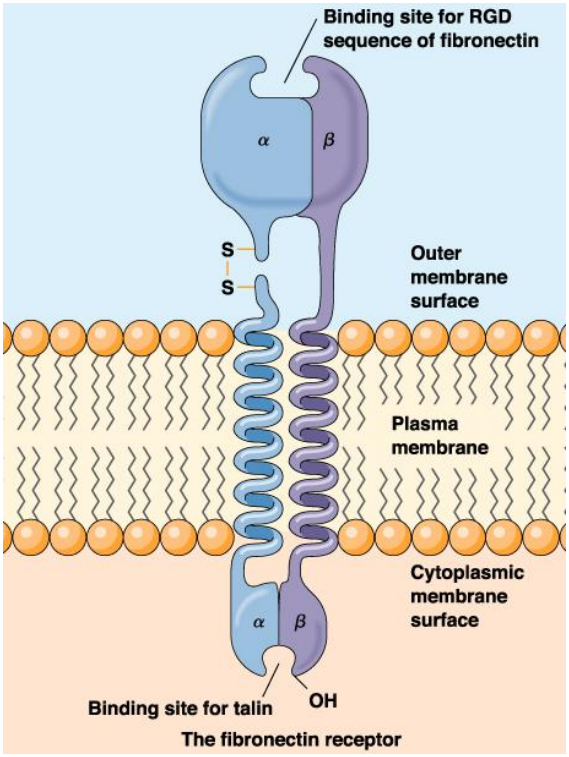


Figure 1.5 *Schematic representation of the integrin subunit structure, showing how both an α and β sub-unit combine to form a matrix binding domain and a cytoskeleton binding domain. Image adapted from (Alberts, 2002)*

Several parallel researches have been carried out for about two decades towards an understanding of the role played by integrins in the growth, maintenance, and repair of bone. For instance, it has been shown that osteoclast isolated from human bone expresses the $\alpha_V\beta_3$ integrin complex, which constitutes the classic vitronectin receptor (Davies, 1989), and α_2 integrin, which combines with β_1 integrin to form the (very late activation) VLA-2 complex, (Davies, 1988) a type I collagen receptor (Staatz, 1989). There is evidence to suggest that the bone matrix protein osteopontin is the ligand for $\alpha_V\beta_3$ expressed on osteoclasts (Reinholt, 1990). The human osteosarcoma-derived cell line MG63 has been shown to express $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins (Dedhar, 1987, Freed, 1989) and a variant of this line showing osteoblastic differentiation expresses increased

levels of cell surface $\alpha_5\beta_1$ but not $\alpha_V\beta_3$ (Dedhar, 1987, Dedhar, 1989a). Furthermore, interleukin-1β induction of osteoblastic differentiation of MG63 osteosarcoma cells results in an induction of expression of $\alpha_5\beta_1$ (Dedhar, 1989b). Inhibition of $\alpha_5\beta_1$ function with subunit-specific monoclonal antibodies inhibits this osteoblastic differentiation (Dedhar 1989c). In order to identify the expression of integrins by bone cells more precisely *in vivo* immunocytochemical studies involving the use of antibodies against $\beta_{1\text{-}4}$, $\alpha_{1\text{-}6}$ and α_V integrins, and the $\alpha_V\beta_3$ dimer (Hughes, 1993) have shown that all cell types present in bone expressed β_1 and α_5 integrins and a subpopulation of osteoblastic cells expressed α_4. The α_V was uniformly expressed by osteoblasts but was heterogeneously expressed by osteocytes. Osteoclasts also expressed α_2, α_V and $\alpha_V\beta_3$. These results (Hughes, 1993) demonstrate differential expression of a restricted range of integrins in bone. This supports the possibility that integrins may mediate the differing interactions of cells of the osteoblast and osteoclast lineages with the matrix of bone (Hughes, 1993). More recent studies have also identified integrins on human bone cells (Saito, 2004). Minced fragments of trabecular bone obtained during total knee arthroplasty were grown in culture until confluent. Bone cells were assayed for alkaline phosphatase activity. Results showed different percentages of attachment for each group of substrates. 60-70% adhered to type I collagen, fibronectin, vitronectin and poly-D-lysine, 40-50% adhered to type-IV collagen, laminin and gelatine and only 10% adhered to fibrinogen (Saito, 2004). It was found that human bone cells revealed high levels of α1β1, α3β1, α5β1 and αvβ5 integrins and lower levels of α2β1, α4β1, αvβ1 and αvβ3 integrins (Saito, 2004). This study also demonstrated that cultured human bone cells adhere to a wide variety of ECM proteins and possess a unique repertoire of integrin receptors that includes relatively large amounts of collagen/laminin receptors (α1β1,

and α3β1 integrins) and fibronectin/ fibrinogen/vitronectin receptors (α5β1, αvβ3, and αvβ5 integrins) (Saito, 2004).

1.4.5 Cell adhesion proteins

The morphology of cells is determined by a sequence of dynamic interactions that are mediated by a heterogeneous population of transmembrane adhesion molecules (Lodish, 2000, Yang, 2010). Besides mediating cell–cell and cell–extracellular-matrix attachment, cell-adhesion molecules facilitate cytoskeletal–membrane interaction and signal-transduction processes, which control some cell's function including, cytoskeletal organisation, cell motility, cell viability and receptor activation. Cell adhesion proteins like selectins and cadherins are often transmembrane receptors. Transmembrane cell adhesion proteins expand across the cell surface membrane and have domains that extend into both the extracellular space and the intracellular space. The extracellular domain of a cell adhesion protein can attach to other molecules that might be either on the surface of neighbouring cells (cell-to-cell adhesion) or part of the ECM (cell-to-ECM adhesion) (Lodish, 2000).

Cell adhesion proteins bind to specific ligands. There are families of cell adhesion proteins that can be categorized in terms of the structure of the adhesion proteins and their ligands. Homophilic binding is an adhesion between similar adhesion protein while heterophilic binding is an adhesion between an adhesion protein and some other molecule. Table 1.1 shows the major cell adhesion proteins (Saito, 2004).

Table 1.1 Major cell adhesion protein families and the corresponding ligands (Saito, 2004).

Major Cell Adhesion Protein Families		
Family	**ligands**	**interactions**
Selectins	Carbohydrates	heterophilic
Integrins	Extracellular matrix	heterophilic
	Ig superfamily proteins	heterophilic
Ig superfamily proteins	Integrins	heterophilic
	Ig superfamily proteins	homophilic
Cadherins	Cadherins	homophilic

1.4.6 Role of Micro-contact Printing (MCP)

Biomaterials can be used to modify the surfaces in cell culturing and so modify cell adhesion, but the complexity and fragility of a biomaterial is an important issue to consider when engineering tissue. Protein patterning can be achieved using a range of techniques (Crozatier, 2005). In the micrometer and sub-micrometer range photo lithographical methods can be used to create patterns, but materials such as organic solvents, UV light and photo resist developer may denature proteins (Tan, 2004). Micro-contact printing (MCP) is a strategy to overcome these problems (Jang, 2012) that was initially used for the creation of alkanethiol patterns on gold. Versatility is the main advantage of this method, since a wide range of biomolecules can be printed onto a variety of different background materials (Offenhäusser, 2007). Collagen type I is the main component of the extracellular matrix (ECM) in bone which acts as a cell adhesion protein and is an ideal candidate for micro-contact printing as it functions by directing cellular adhesion, migration, differentiation and network formation *in vivo*. Fibronectin is present in bone after wounding at the site of injury (Han, 2008). Fibronectin and heparin released from cells during tissue injury participate in blood clotting and play

important roles in initiating tissue repair. In this respect fibronectin provides adhesion sites for tissue repair cells and constitutes a connector for the fibrin glue and heparin (Han, 2008). Despite evidence suggesting that bone cells express laminin receptors, bone ECM contain little laminin and there is little evidence that bone cells adhere to laminin (Colucci, 1996, Gentili, 2009). Thus, laminin was used in this study (as a negative control) in order to compare with main proteins that exist in bone such as collagen type I and fibronectin.

1.4.7 Osteoinductive Factors (Role of Cytokines)

There are various techniques to enhance tissue regeneration via the application of growth factors to the site of regeneration to induce cells to proliferate, differentiate and regenerate. Generally, direct application of growth factors has little effect (Khan, 2000) because the growth factor diffuses out from the site of regeneration very quickly. This is a problem that can be solved by a controlled release of growth factor at the site of action over a long period of time by use of a bioabsorbable scaffold. Growth factors are protein based molecules in the body which are produced by cells and attach to the cell surface. Growth factors bind to membrane receptors, which in turn activate an intracellular signalling pathway. This will activate or inhibit a gene causing either an up regulation or down regulation of a gene product, which then alters the cells behaviour. Growth factors mediate cell actions and their response to different environmental cues. Growth factors may be produced in different ways with different effect such as autocrine effect (growth factors act directly on the cell that produced them), paracrine effect (growth factors act on neighbouring cells surrounding the growth factor), juxtacrine effect

(growth factor communication signal received by one cell to neighbouring cells due to cell-cell interaction), endocrine effect (growth factors act on a cell a distance away from the growth factor by travelling through the blood stream) (Smith, 2003a). Growth factors, therefore, have an important role in growth, development, day-to-day maintenance, mobilised remodelling and injury. Bioabosrbable scaffolds may be seeded with DNA coding for growth factors. Tissue regeneration can be achieved if a growth factor gene is transferred into the cells at the site of regeneration and cause cells to secrete growth factor (Centrella, 1994). Bone cells can be isolated from the recipient (bone marrow/connective tissue), expanded in tissue culture, exposed to bioactive factors, combined with the scaffold and finally implanted into the donor site in order to persuade regeneration. Specific growth factors are synthesized and secreted by developing cell lines and combined with a delivery system for implantation in the skeletal site. Cell differentiation occurs due to the influence of the secreted growth factor (Solheim, 1998).

1.4.8 Previous studies on osteoinductive factor (Cytokines)

Growth factors have direct effects on cell activities such as cell adhesion, proliferation, differentiation, and migration by modulating the synthesis of proteins, other growth factors and receptors (Johnson, 1988). Each growth factor such as bone morphogenetic protein (BMP) (Croteau, 1999), transforming growth factor beta (TGF-β) (Khan, 2000), fibroblast growth factor (FGF) (Hurley, 1993), insulin-like growth factor (IGF) (Thaller, 1993) and platelets-derived growth factor (PDGF) (Solheim, 1998) have different effects on bone cells (Ramoshebi, 2002). For instance, BMP belongs to a group of non-

collagenous glycoproteins that in turn belongs to the TGF-β super family (Guiqian, 2012). BMP is synthesized locally and 15 types of BMP have been discovered (Croteau, 1999). Many studies have been carried out in order to see the effect of BMP on different bone defects (Laflamme, 2008). Research on BMP-2 (Cho, 2002) showed early response genes in the cascade of healing one day after fracture. These discoveries indicate that the BMP family of proteins are actively involved in fracture healing (Matthews, 2005).

Insulin-like growth factor (IGF) makes use of the anabolic effect on bone metabolism. There are two types of IGF known as IGF1 and IGF2 which stimulate osteoblast, osteoclast cell proliferation and matrix synthesis (Khan, 2000). Its ability to promote the healing of critically sized calvarial defects in rats has been studied (Thaller, 1993). Rats receiving IGF-1 subcutaneously showed repair within one week of treatment and complete bone formation by the end of week six. In contrast, controls showed delay osseous repair after eight weeks. The effectiveness of IGF-1 with TGF-β1 compared to autologous bone grafts in cervical fusion (Kandziora, 2002), C3 and C4, under discectomy and stabilisation was achieved by using a titanium cage with IGF-1 and TGF-β1. These results showed that animals treated with IGF-1 and TGF-β1 had significantly higher fusion rates compared with bone grafted animals.

1.4.9 Transforming growth factor-beta (TGF-β)

The TGF-β super family include different proteins such as BMPs, nodals, activins, TGF-β and inhibins (Derynck, 2008). TGF-β is a cytokine produced by different cell

types inside the body and exists in three isomers TGF-β1, 2 and 3 (Worthington, 2011). These isomers can act as either paracrine or endocrine hormones. These isomers are structurally similar and encoded by three distinct genes in mammalian species (Mehta, 2007). The TGF-β superfamily includes a large group of soluble extra-cellular proteins, which regulate development in both vertebrates and invertebrates (Raftery, 2002a). The TGF-β family of cytokines regulate cell functions such as migration, apoptosis, proliferation and differentiation (Krauss, 2006a). It is important to know where the target cells are located as TGF-β can have different effects in different cell environments. For example, in skin cells when fibroblasts are grown in a monolayer in the presence of epidermal growth factor, TGF-β1 causes a decrease in proliferation. In comparison, when these cells are grown in a semi-solid medium, TGF-β1 induces growth (Border, 1994). TGF-β influences a number of cell activities such as differentiating, stimulating mesenchymal stem cell (MSC) growth, acting as a chemotactic factor and also enhances bone cells and ECM production and secretion (Khan, 2000). It has also been used to stimulate bone regeneration (Lind, 1993). Using an animal model and TGF-β2 administered for a period of six weeks in the case of tibial defects it was shown that TGF-β2 had positive effects on fracture repair (Lind, 1993). The effects of TGF-β on bone repair are dependent on the mechanical stability at the site of fracture (Critchlow, 1995). Unilateral tibial fractures were produced in a rabbit animal model and a plate (plastic /steel) was used in order to immobilise the fracture site. TGF-β2 was applied into the calluses 4 days after fracture. Results demonstrated that in an animal with unstable fixation TGF-β2 did not show an effect on callus formation, but the sample with a stable fixation developed enlarged calluses (Critchlow, 1995). The presence of BMP-7 enhanced bone formation when combined with low

dosage of TGF-β1 (Ripamonti, 1997). Mixtures of these two growth factors showed an increase (about 2-3 times) in the cross sectional area of newly generated ossicles compared to BMP-7 alone.

1.4.10 TGF-β signalling

The effects of TGF-β1, 2 and 3 are mediated by a range of signalling pathways (Kale, 2004a, Aoudjit, 2012). Activation of the same signalling cascade follows ligand-receptor interactions at the cell surface. The TGF-β receptor complex is made up of serine-threonine kinase receptor types I and II. These bind to a third receptor, type III; also known as betaglycan. This membrane-anchored proteoglycan binds to the TGF-β ligand and presents it to the type I/II receptor complex allowing signal transduction to occur (Shi, 2003). This betaglycan has been shown (Lo´pez-Casillas, 1994) to act as dual modulator of TGF-β activity. The soluble form of this protein, no longer anchored to the cell membrane, acts as an inhibitor of TGF-β signalling (Lo´pez-Casillas, 1994, Shi, 2003). Furthermore, all TGFβ superfamily members bind to heterodimeric receptor isomers that belong to the same family, i.e. dimeric complexes of various type I receptor serine/threonine kinases (ALK 1-7) and type II receptor proteins (5 isoforms) (Euler-Taimor, 2006). These receptors subsequently activate different intra-cellular Smad proteins to elicit various biological responses (Euler-Taimor, 2006). Besides activation of Smads, TGF-β mediates its effects via activation of a number of signals including mitogen-activated protein kinases (p38 MAPK), extracellular-signal-regulated kinases (Erk), c-Jun N-terminal kinase (JNK), and Transforming growth factor β–activated kinase 1 (TAK1) (Bujak, 2007).

Apart from TGF-β itself, the biological functions of the other TGF-β superfamily ligands are poorly understood such as BMP which share the same signalling cascade. To initiate intracellular signaling, BMP binds as a dimer to a complex of two transmembrane receptors (type I and II) and starts a phosphorylation cascade upregulating bone-specific differentiation genes (e.g., runx2, osteocalcin, bone sialoprotein, type I collagen, and alkaline phosphatase). Interestingly, BMP-2 receptors type I and II are cascaded by two independent signalling pathways (Hollinger, 2005, Massague, 1996, Palcy, 1999, Kale, 2004a).

The first pathway involves BMP-2 dimerization and binding to a preformed receptor complex, initiating a phosphorylation cascade. The type I receptor phosphorylates Smad1, Smad5, and Smad8. A complex of these three is formed with Smad4 in the cytoplasm. The complex translocates to the nucleus and functions as a transcriptional regulator of bone-specific genes. In contrast Smad6 and Smad7 have an inhibitory effect for such formation by bending to type I receptor and blocking the phosphorylation of Smad1, Smad5, and Smad8 (See Figure 1.6). The second signalling pathway is activated when the BMP-2 dimer binds to receptor type II only. This binding event recruits receptor type I and initiates a phosphorylation cascade via the MAPK pathway involving p38 and Erk1/2. This pathway culminates with activation of AP-1 in the nucleus.

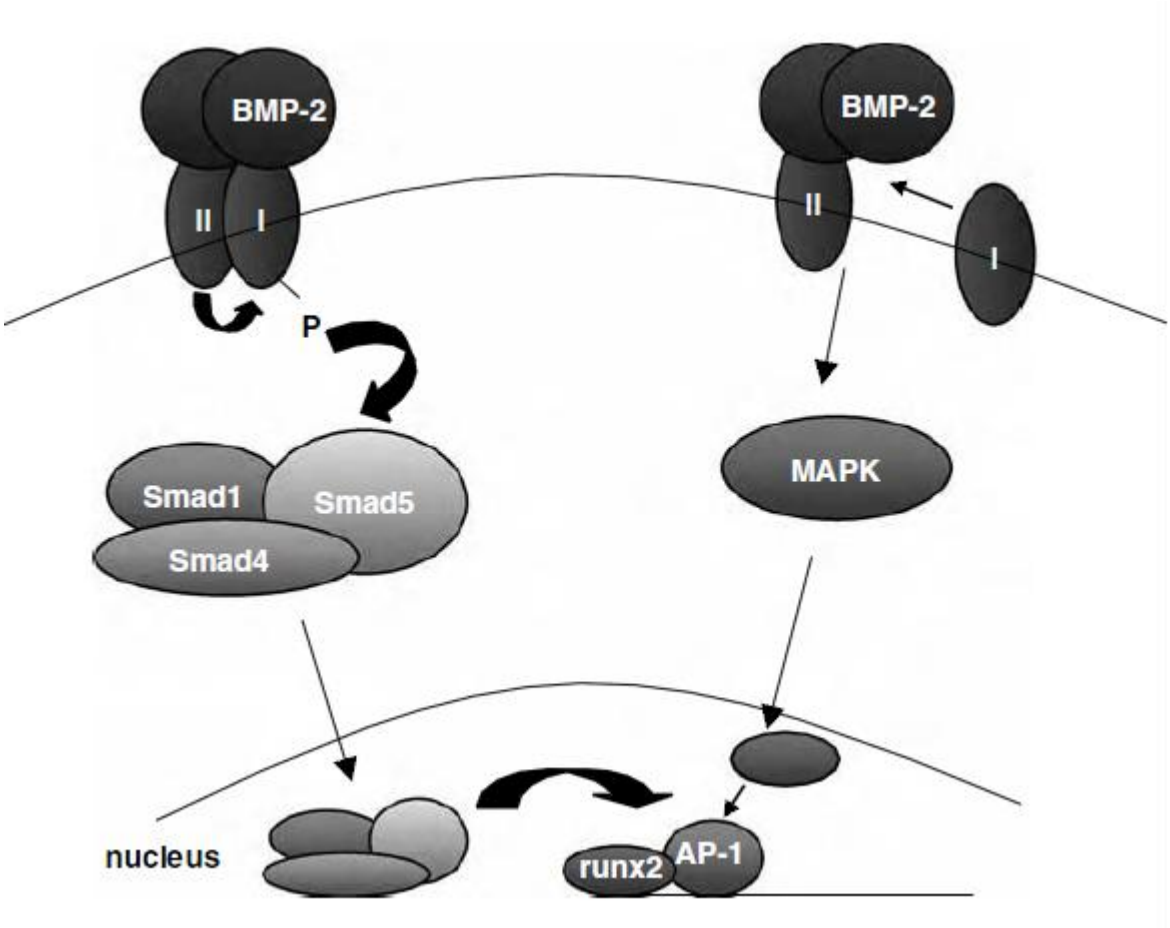


Figure 1.6 *Two different pathways characterized for Runx2 activation via BMP's (Hollinger, 2004).*

TGF-βs are among the most multi-functional cytokines known; they affect a variety of cell types and modulate multiple biological processes (Mehta, 2007). The overall effect of TGF-β depends on the cytokine milieu and the state of cell differentiation (Souchelnytskyi, 1996). Previous studies regarding the effects of different TGF-β families and their combinations in the wound healing of bone are poorly understood or have only been partly addressed (Khan, 2000). For instance, many studies have been carried out in the presence of bone with various growth factors such as bone morphogenetic protein (BMP) (Croteau, 1999), platelets-drived growth factor (PDGF),

insulin-like growth factor (IGF) (Thaller, 1993) and fibroblast growth factors (FGF) (Hurley, 1993). The current work, related to the use of TGF-βs, investigates the effect of different TGF-β isomers and their combinations on bone cell detachment via the trypsinization process in addition to their effect on the wound closure response on dish-cultured bone monolayers, using a simple scratch assay.

In order for TGF-β to be administered exogenously it needs to be dissolved with HCl and with BSA as carrier of TGF-β. It is suggested that HCl also contributes to the rate of wound repair (Centrella, 1988). This is particularly important since there is evidence that the pH of injured bone can decrease and that this may play a role in activating TGF-β (Oursler, 1994, Dallas, 2002). Thus, it is important to investigate the role played by HCl in wound closure in a simple bone cell culture model. It is also very important for the growth factor to be delivered properly to ensure a stable form and without losing its bioactivity. To overcome these problems HCl is added to the TGF-β to stabilize the solution and reconstitute it from the powder form. Upon cell culturing further HCl is added to TGF-β, which prevents losses allowing a final concentration ranging from 0.1ng/ml to 10ng/ml in most culture systems (Lind, 1993). Other groups have also investigated that latent TGF-β1 can be activated by heat, acidification, alkalinisation or the action of chaotropic agents in vitro (Annes, 2003). The Zamora research group compared the effectiveness of acidification versus heat treatment in activating latent TGF-β1 in a cell-free system. ELISA assay was carried out using recombinant human latent TGF-β1 (Zamora, 2007). It was found that while both heat and HCl led to the activation of latent TGF-β1, heat treatment was significantly more effective than acidification (Zamora, 2007). On the other hand ascorbic acid has been reported to

increase collagen deposition and tensile strength in experimental models of wound repair (Wendt, 1997). The role of ascorbic acid in the healing of injured tissues has been investigated in a variety of studies (Cevikel, 2008). Other groups have shown that the administration of ascorbic acid expedites wound healing in rats (Kamer, 2010). Ascorbic acid could bestow benefits to tissue healing by significantly enhancing tissue hydroxyproline levels, neovascularization, fibroblast maturation, and collagen deposition. It is therefore important to examine the role played by HCl in wound closure of bone. Previous studies have shown (Khan, 2000) an overall poor understanding regarding the use of TGF-β isomers in bone cells in terms of fracture repair and healing compared to other cell type, for example, keratinocyte, and, also, compared to other growth factors such as bone morphogenetic protein (BMP) (Croteau, 1999), platelets-derived growth factor (PDGF), insulin-like growth factor (IGF) (Thaller, 1993) and fibroblast growth factors (FGF) (Hurley, 1993). It is therefore important to examine the role of each TGF-β isomer in cultured bone cell monolayers.

1.5 AIMS AND OBJECTIVES

The overall aim of this study was to investigate the role of ECM proteins (collagen type I, fibronectin and laminin), transforming growth factor beta isomers (TGF-β1, 2 and 3), and HCl/BSA in cellular adhesion, cell behaviour and wound healing in cultured bone cell monolayers.

The main objectives of this work were to:

1) Compare Collagen type I, Fibronectin and Laminin in the adhesion and alignment of the human bone (MG63) cell to these proteins printed via micro-contact printing with various widths (sizes).

2) Investigate the effects of different transforming growth factor beta isomers (TGF-β1, 2 and 3) on bone cell detachment via a trypsinization assay. This will provide information about the degree of cell surface attachment.

3) Investigate and compare the wound closure properties of bone cell monolayers using different TGF-β isomers, HCl and BSA/HCl in cultured dish environments by creating a simple 2D wound model. This will provide information about how different TGF-β isomers influenced wound repair. Also, to identify various integrins that may inhibit wound repair, it was necessary to examine the combination of TGF-βs and use specific antibodies. This has the potential for therapeutic combinations of cytokines and cell attachment inhibitors in the *in vivo* wound.

4) Examine the effect of HCl on cell behaviour used as TGF-β solvent and to distinguish its effect compared to the actual growth factor effects on wound healing.

5) Investigate other cellular responses such as proliferation and detachment along with different stages of cell behaviour and morphology (cell length and cell number) during wound healing.

6) Use immunostaining to identify which integrins are up or down regulated and how TGF-β modified ECM production. It is hoped the above investigation will allow the identification of the major integrins that influencing wound repair. To have a better understanding about cell-surface interfacial interactions, a newly developed WSPR microscope will used for imaging and analysing the cell surface interactions.

CHAPTER TWO

2. STUDYING BONE CELL INTERACTIONS WITH MICROPATTERNED ECM PROTEINS

2.1 INTRODUCTION

Micro-contact printing (MCP) is a strategy that enables a surface to be readily functionalised with a material such as an extra cellular matrix (ECM) protein, in a defined pattern (Sefat, 2010a, Ricoult, 2012, Sefat, 2008, Berends, 2009, Khaghani, 2008). MCP also allows control of cell adhesion geometry on a surface. Micro-contact printed substrates may be used to examine cell behaviour in relation to a wide range of extracellular matrix (ECM) molecules including collagen type I, fibronectin and laminin (Kumar, 1994). These cell adhesion proteins are the main components of ECM and ideal candidates for MCP as they can direct cellular adhesion, migration, differentiation and network formation *in vitro* (Carter, 1965). These cellular behaviours may then be used to aid in deciphering the cell signalling pathways associated with the control of cell surface interactions (Kleinman, 1990). Collagen is a group of naturally occurring proteins and one of the most important structural proteins of the extracellular matrix. It is the major element of connective tissue and is the most abundant protein in mammals (Viguet-Carrin, 2006). 25 to 35% of the entire body consists of collagen protein. Collagen in the form of elongated fibril is typically found in skin, tendon and ligament, which are known as fibrous tissue. Collagen is also plentiful in bone, cartilage, cornea, blood vessels, intervertebral disc and the gut. Fibronectin is a transmembranous glycoprotein that is found naturally in its soluble form in plasma and its insoluble form

in connective tissue and the basal lamina (Pankov, 2002). It is a high molecular weight protein that binds to collagen, fibrin, fibroblasts in the ECM and the plasma membrane of the cells. This protein plays a major role in cell adhesion and re-organiation of the ECM (Salber, 2007). Fibronectin and collagen can bind together at the region called FN1/2, while the FN3 region takes up contact with the respective cells (Pankov, 2002). The contact point consists of the amino acid sequence RGD (Arg-Gly-Asp) which binds to integrins. The α5β1-integrin has a particularly high affinity for fibronectin. Completely different kinds of fibronectin are formed by alternative splicing. The liver, for example, builds fibronectin, which then circulates as a dissolved component of the serum. However, stationary fibronectin is also formed in different tissues (Pankov, 2002).

Laminin is another important protein present in the body and a major protein in basal lamina. Laminins are large molecules, which occur mainly in the basement membrane of the epithelium. Laminin is very important in influencing cell differentiation, migration, adhesion as well as phenotype and survival (Timpl, 1979). It consists of α, β and γ chain. Laminin can bind to two groups as it has a binding site for the cell receptor and a binding site to other components of ECM. The laminin molecules are named by looking at their chain composition, e.g. laminin-511 contains α5, β1 and γ1 chains (Aumailley, 2005). This protein has a cross shape with three shorter arms and a long arm at the bottom. The shorter arms are suitable for binding to other laminin molecules in order to form a sheet, while the long arm can bind to cells (Haralson, 1995). Laminin is not normally found in the ECM of bone (Colucci, 1996, Gentili, 2009).

The major ECM components of bone consist of collagen type I, fibronectin and individual proteoglycans (Gentili, 2009). This is an interactive natural scaffold that provides mechanical stability and cell anchorage, and is also able to control cell functions. There is not enough laminin in bone and therefore poorely expressed. We were motivated to use laminin in this study (as a control) to enable a comparison of this protein with other ECM proteins such as collagen type I and fibroenctin, (Sefat, 2010a). The aim of this study was to compare osteoblast behaviour grown on micro-contact printed laminin against collagen type I and fibronectin. Therefore, osteoblast responses to these three ECM proteins were investigated.

In natural skeletal tissues, such as bone, ligaments and tendons, the cells and the structures are found to align at spatial and oriented geometries and patterns (Zhu, 2005). When designing an implant material, it is important to mimic natural bone tissue, where cells are orientated at specific directions, by providing directional cues in regulating the cell alignment in a preferential direction and in specific locations to create organized structures (Zhang, 2011). In recent years, considerable efforts have been made to understand cell surface interaction and cell guidance for applications in biomedical systems (Feinberg, 2008, Hamilton, 2006). The potential applications of such systems include the micro-textures that have been introduced to enhance tissue integration and wound healing (Dalby, 2003, Khang, 2006, Berends, 2009, Khaghani, 2008). On the other hand, many polymer and metal materials have been commercially developed as implants for bone and cartilage repair. These materials often exhibit poor and uncontrolled interactions with proteins and cells, making them difficult to integrate into the body tissues. Therefore, efforts have been made to deposit bioactive coatings onto

implant surfaces to improve the bioactivity and biocompatibility. For instance, the uses of chemical guidance cues have been shown to promote “contact guidance” (Folch, 1998, Clark, 1990), which is a phenomenon that involves the alignment of cells, as they spread across micro-patterns. Another investigator (Su, 2007) studied the effects of micro-patterned geometries on cell alignment. Polydimethylsiloxane (PDMS) has been extensively used in microfluidic devices, BioMEMS and lab-on-a-chip applications (Whitesides, 2006, Cecchini, 2008, Yea, 2006). The increasing attention on PDMS has been due mainly to its excellent combination of biocompatibility and mechanical properties (Peterson, 2005, Griscom, 2002). Some investigators also studied the effects of micro-pattern on cell spreading and alignment on PDMS (Chaw, 2007, Salber, 2007).

Recently collagen type I and gelatin were stamped on modified glass and titanium surfaces to create patterns with varying shapes and sizes (Zhang, 2011). Osteoblast-like cells were seeded onto these patterned surfaces and the cell attachment, proliferation, and spatial alignment were investigated. Their results showed that the alignment of cells was dependent on the width of the protein stripes and the spacing distance between the stripes. Their results also showed that the osteoblast-like cells exhibited a precise alignment along the stripe patterns at low stripe widths of 10, 25, and 50μm. As the width of the stripes increased to 100, 150 and 200μm, the cells on the protein stripes tended to become more randomly orientated (Zhang, 2011).

This method can be used to modify implantable medical devices to provide an ECM-mimicking environment for better cell attachment, proliferation and tissue growth.

Creation of precise surface patterns on the glass, polymer or metal materials could provide entirely new insights into the factors that control cell adhesion onto material surfaces, cell proliferation, differentiation and molecular signalling pathways (Ogaki, 2010, Diener, 2005). It is expected that these surface-engineered materials will have the ability to control the shape of the attached cells and the cell-cell contacts through the formation and dimensions of the cell-adhesive patterns. The methods and results in the present study could find applications in surface engineering of implant materials for bone replacement and repair (Zhang, 2011, Sefat, 2010a).

The aims of this chapter is to investigate the effect of micro-contact printed ECM proteins, namely fibronectin, collagen type I and laminin, with different pattern width (5, 10, 25, 50 and 100μm), on cell alignment, attachment, density and morphology of MG63 bone cells cultured *in vitro*. ECM proteins were stamped on plain (un-modified) glass cover slips to create patterns with varying sizes. Our approach to cell patterning has the advantages of simple preparation, high stability, tunable pattern sizes and facilitation of controlling the cell alignment, and this could find wide applications in tissue engineering, such as in bone implants, where precise control of cell structures would be preferable.

2.2 MATERIALS AND METHODS

2.2.1 Cell Culture

Bone cells were cultured in a low glucose (1g/L-D-Glucose) HEPES (25mM) buffered DMEM (Dulbecco's Modified Eagle Media, Sigma Aldrich) culture media supplemented with L-glutamine (2.5 mM U/mL), Penicillin (100 U/mL), Streptomycin (0.1 mg/mL), Amphotericin B or a fungizone (250µg/ml), (Sigma Aldrich-UK) and 10% FCS v/v (fetal calf serum- PromoCell - UK). The bone cells were plated in 25cm^2 tissue culture grade cell culture flasks at a cell density of 250,000 cells/culture flask and bathed in the culture media. All supplements were purchased from Sigma Aldrich-UK except those mentioned. Media was changed every 2 days for normal cell culture procedure. Cells were incubated at 37°C and split upon reaching confluency, usually every 3-4 days (Vunjak-Novakovic, 2006). Sterilisation was a key aspect in performing the experiment, and therefore a sterilised environment was essential when working with cell culture to avoid contamination by using 70% ethanol. Cells were seeded on glass surface patterned 5, 10, 25, 50 and 100µm wide repeat gratings of collagen type I, fibronectin or laminin in order to identify the role of these proteins in the adhesion and alignment of the human bone cell (MG63) to different micro-contact printed ECM patterns.

2.2.2 Type of cell used

Human bone cell line MG63 (Osteoblast-like cells originally isolated from a human osteosarcoma) was used in this study (Sefat, 2008, Sefat, 2010a). MG63 human

osteoblasts, is a widely used cell line (Morris, 2006) because of its ability to retain a differentiated phenotype in culture conditions (Declercq, 2004). MG63 cells have also been used previously in the study of the integrin-mediated cell-matrix interactions and regulated by cytokines and hormones (Broberg, 1996, Riikonen, 1995). Moreover, the MG63 cell line has been used as a convenient source for purification and identification of known and novel osteoblast transcription factor complexes (Newberry, 1997).

2.2.3 Stamp Fabrication

Master stamps (glass template) repeat gratings consisting of 5, 10, 25, 50 and 100μm wide ridges (width) with 5μm deep channels were micro-fabricated by the Department of Electronics and Electrical Engineering, at University of Glasgow. Polydimethylsiloxane (PDMS) stamps were then produced at the University of Bradford from these templates by firstly coating the templates with 2% dimethylchlorosilane mixed with 98% trichloroethylene. This mixture made the surface hydrophobic. Next, 9ml of sylgard (silicon-based elastomer gel) and 1ml of curing agent were poured onto the templates and allowed to cure overnight. Vacuum processing was carried out to remove any trapped gases. After curing, the sylgard gel was removed from the template enabling sylgard stamps exhibiting a negative relief of the template to be produced. These stamps were then used to functionalize plain glass slides and gold coated test substrates with different proteins.

2.2.4 Micro contact printing (MCP) Technique

Prior to MCP all working environments (surfaces), stamps and coverslips were sterilized with 70% ethanol and left to dry completely in a laminar airflow cabinet. Plain glass coverslips and gold coated glass coverslips were then micro-contact printed with collagen type I, fibronectin and laminin. The dilution used for collagen, fibronectin and laminin was 3mg/ml (SIGMA). The following stamping method was then employed: stamps were inked by dipping in each protein solution for duration of 60 seconds (Tan, 2004, Khaghani, 2008). The stamp was then removed from the protein solution and air-dried for 30 seconds and placed immediately in contact with the substrate and pressed slightly using another glass cover slip for 60 seconds. This allowed the substrate to be patterned with 5, 10, 25, 50 and 100μm wide protein coated tracks separated by 5, 10, 25, 50 and 100μm wide uncoated tracks. The same procedure was used for stamping each ECM protein. This process has been depicted in Figure 2.1 and Table 2.1. MCP patterns were reasonably transferred to the substrates, although imperfections in the stamp and the application of manual pressure resulted in some variation in the track widths of the stamped extracellular matrix proteins.

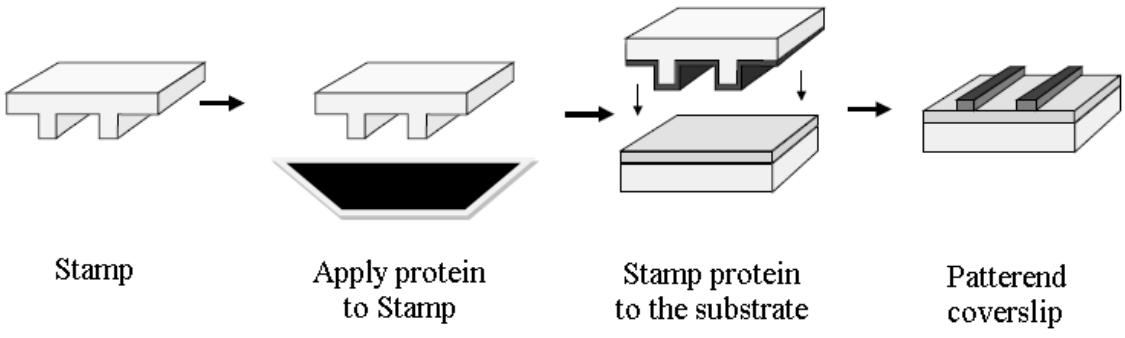


Figure 2.1 *Illustration of the stamping process. Adapted: (Thibault, 2005).*

The following table shows a summary of the micro-contact printing process.

Table 2.1 Illustration of the steps involved in micro-contact printing.

Steps	Process
1	The elastomeric stamp is wet with the inking solution (biomolecules).
2	Dried in flow hood, leaving a thin layer of material on the stamp surface.
3	The stamp is then pressed to the substrate surface, leaving the biomolecules only on the regions defined by the raised structures of the stamp.
4	The stamp is removed from the surface

Figure 2.2 show images of the master stamps with repeat gratings of five different sizes (5, 10, 25, 50 and 100μm width).

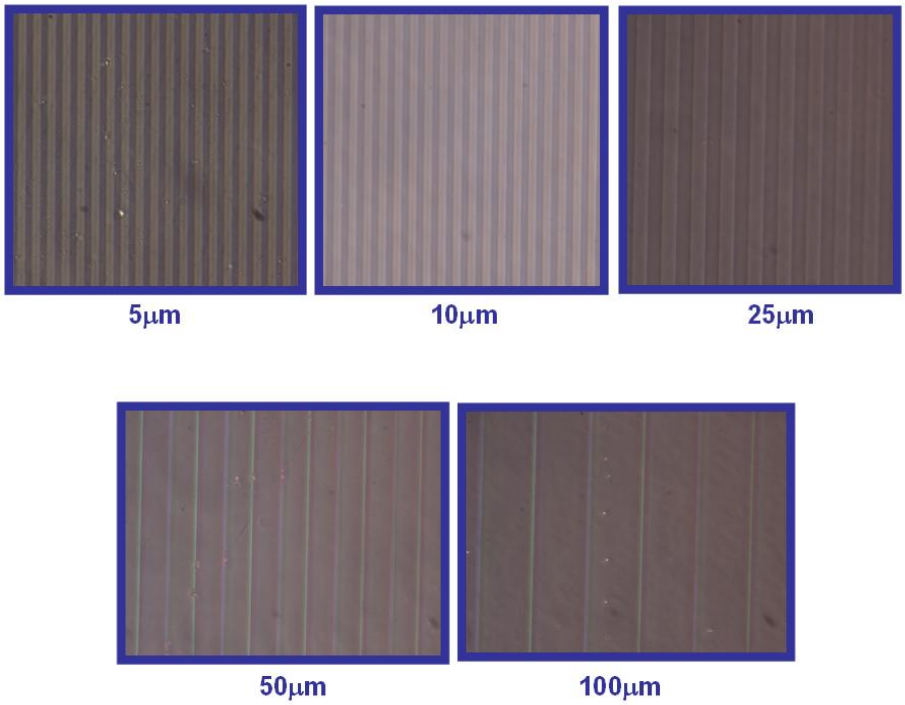
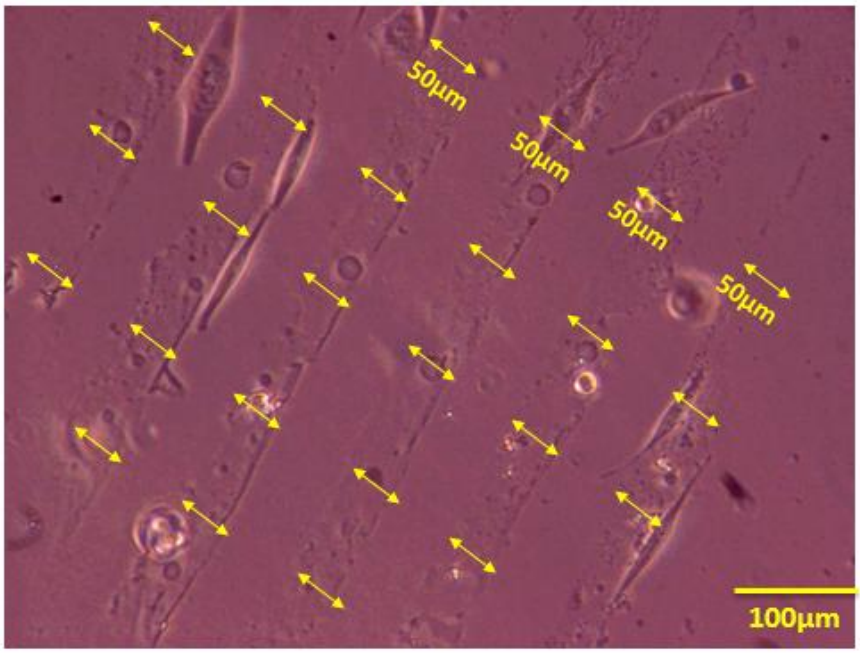
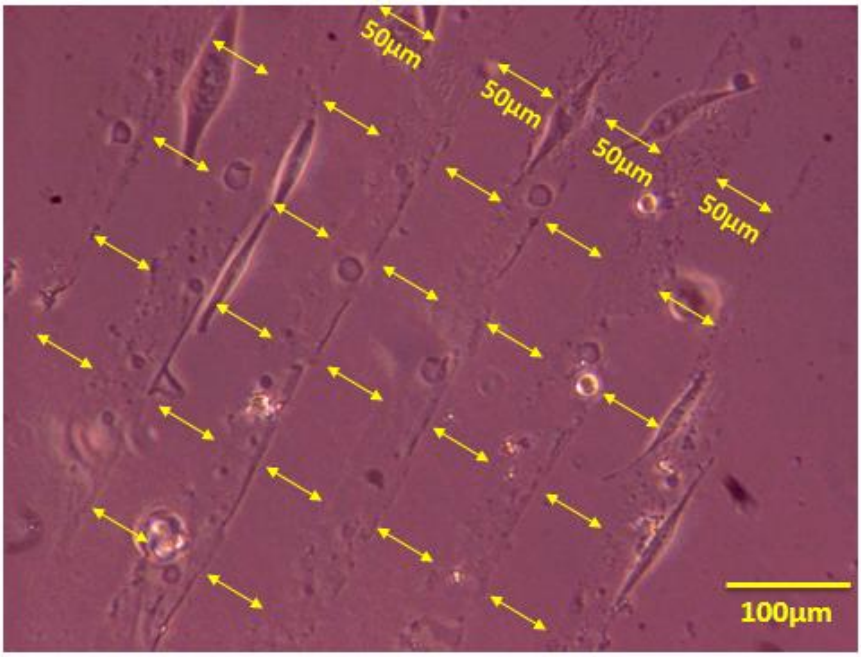


Figure 2.2 *Actual stamps with five different sizes (5, 10, 25, 50 and 100 μm width) (Sefat, 2008).*

Figures 2.3a and 2.3b show the images of collagen protein patterns printed on coverslips.



(a)



(b)

Figure 2.3 *Actual images of 50µm width protein patterns printed on glass coverslip, (a) Show protein ridges with widths of 50µm (coated) (b) 50µm wide channels (un-coated) (Scale bar=100 µm).*

The stamp consisted of ridges with widths of 50μm separated by 50μm wide channels (uncoated). This allowed the substrate to be patterned with 50μm wide collagen coated tracks separated by 50μm wide uncoated tracks. The distances between coated and uncoated channels have been marked by the yellow arrows in Figures 2.3a and 2.3b. However, in applying the stamps to the surface the pattern was distorted by the stamping process probably because of nanometric scale stamp imperfections causing the point of contact between the stamp and the surface to decrease in some places.

2.2.5 Plating of cells on Patterned Coverslips

The glass slides micro-contact printed with 5 different gratings were then seeded with bone cells (50,000 cells/coverslip) for 18hrs. Figure 2.4 shows the method of cell seeding on stamped coverslips. After 18hrs, cells were then fixed with 0.1% formaldehyde diluted in Hank's Balanced Salt Solution (HBSS, SIGMA) for 5 minutes and imaged with standard phase contrast light microscope. The effects of initial cell attachment on different substrates were investigated by recording cell images up to 18hrs after cell culture using Image J software. Cell alignment to the patterns was measured by measuring the angle of the long axes of the cells in relation to the stamp pattern, such that an angle of 0° represents 100% alignment to the pattern (Figures 2.5 and 2.6). Figure 2.6 shows cells attached to a 100μm stamped protein pattern. The stamp pattern is clearly shown by arrows for areas without bone cells. Stamp pattern may not be visible in the some areas as it is coverd by bone cells. The actual pattern is visible as a very fine line which is shown by an arrow in Figure 2.6. Image J software were also used to measure cell length, width and number of cells.

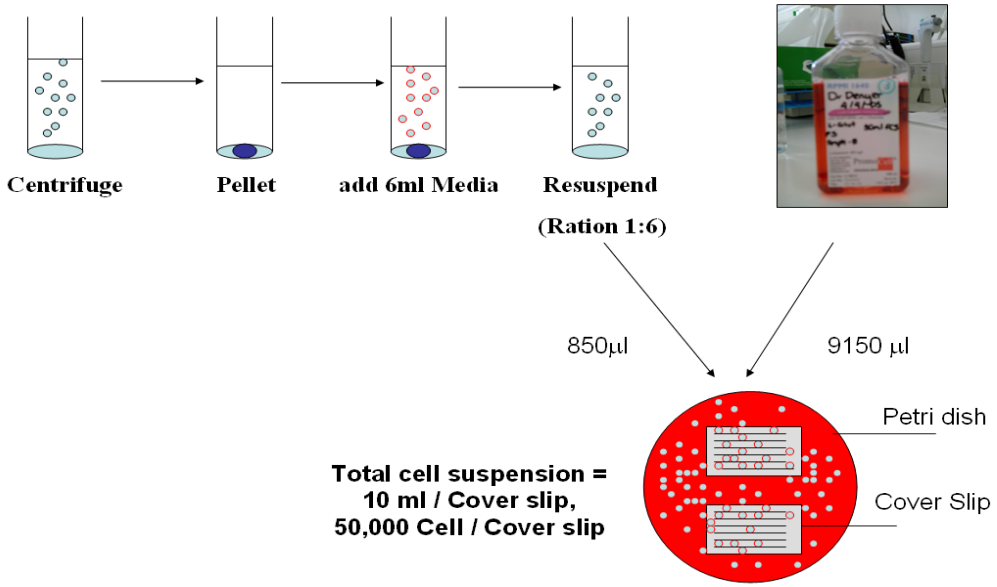


Figure 2.4 *schematic drawing showing the method of cell seeding on stamp patterned coverslips.*

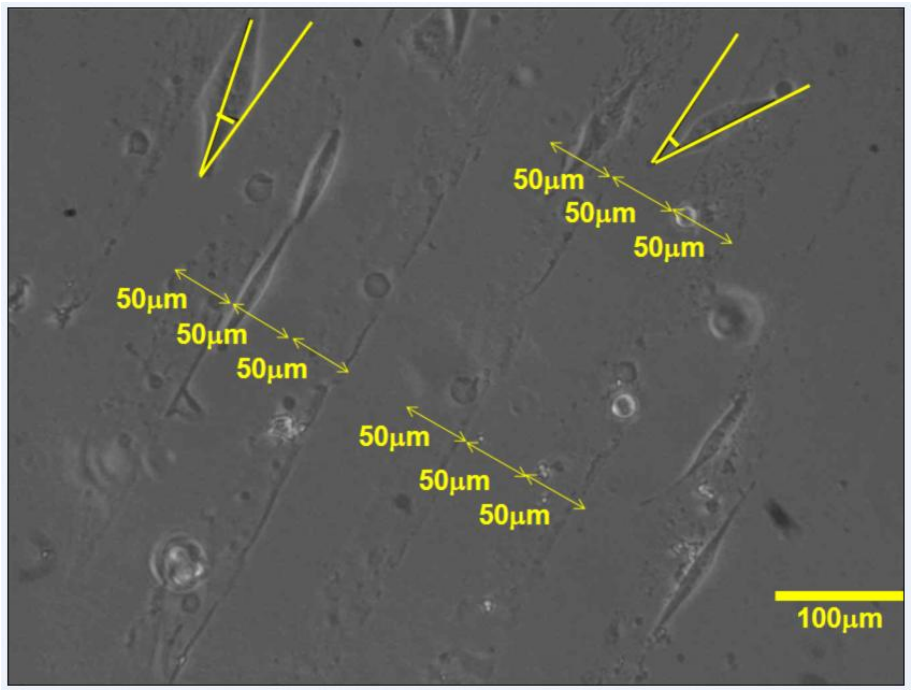


Figure 2.5 *Measuring the angle between cell axis and 50µm collagen stamp patterned coverslip (glass); image taken with standard phase contrast microscope, (Scale bar=100 µm) (Sefat, 2010a).*

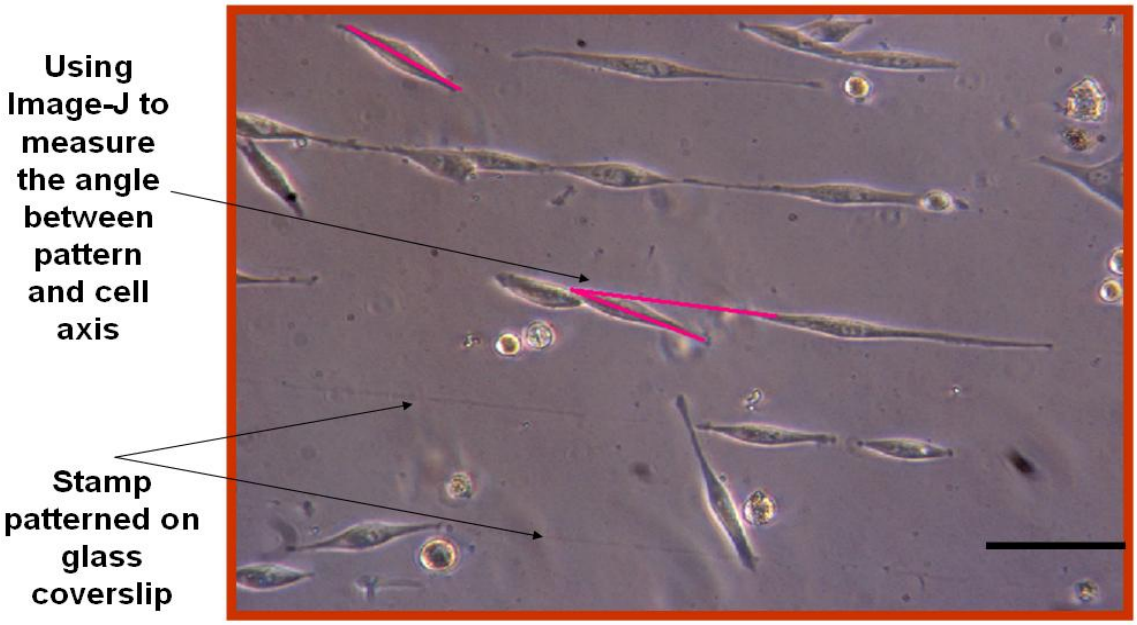


Figure 2.6 *angles measured between cell axis and stamp patterned glass cover slip. The edges of the stamp pattern are indicated by the arrows (Scale bar=100 µm) (Sefat, 2008).*

2.2.6 Control experiment

Cells were also seeded on three ECM coated substrates (collagen type I, fibronectin, laminin) and plain un-coated substrates as control. These four substrates either ECM coated or un-coated provided were used to compare to different micro-contact printed ECM patterns. All experiment either controls or micro-contacted printed were repeated three times. In each repeat 10 fields of view were captured. Cell number, cell alignment and cell length were measured from 10 fields of view. We were motivated to use laminin in this study as a control due to the lack of laminin present in bone. Therefore, it enabled a comparison of this protein with other ECM proteins such as collagen type I and fibroenctin (Sefat, 2010a, Colucci, 1996). Finally, the degree of cell alignment on un-coated/un-patterend substrates was measured (using image J software) by measuring the angle of the long axes of the cells in relation to the parallel lines drawn on each field

of view such that an angle smaller than 45° represents good alignment to the parallel lines (See Figure 2.7).

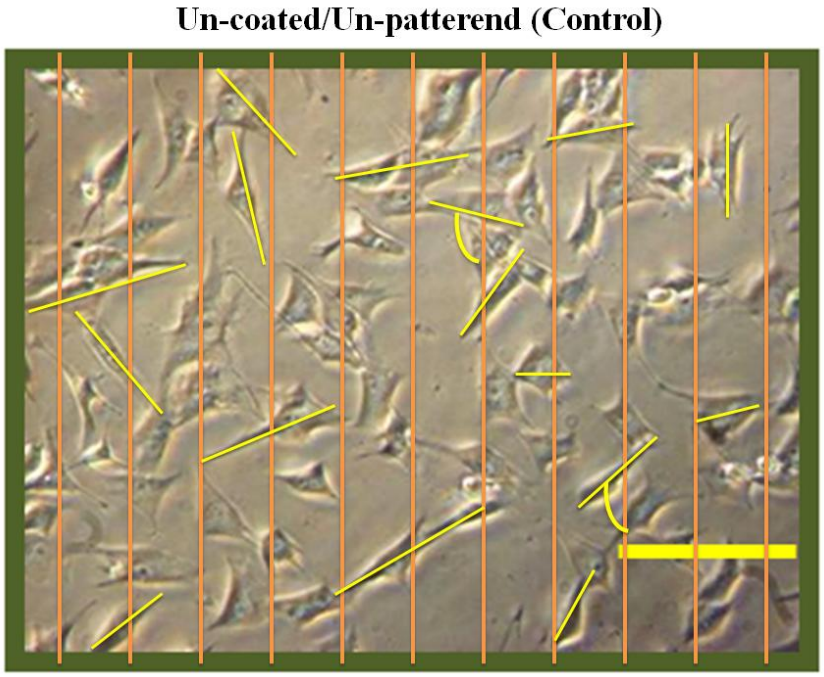


Figure 2.7*Angles measured between cell axis and parralle lines drawn by image J software on un-patterend/un-coated glass cover slip (Scale bar=100 µm).*

2.3 STATISTICAL ANALYSIS

Mean angle of cell alignment and cell length were tested for normality using a Kolmogorov Smirnov test. Results that showed normal distribution ($p > 0.05$) were analysed using SPSS via a Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed results ($p < 0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

2.4 RESULTS

2.4.1 Effect of ECM protein coating on cell morphology

Figure 2.8 shows the morphology of bone cells on four different substrates including ECM coated substrates (collagen type I, fibronectin, laminin) and plain un-coated substrates as control. These four substrates either ECM coated or un-coated provided a negative control to compare different micro-contact printed ECM patterns in the next set of results. Although, these four coverslips are similar in terms of initial cell number and passage number, they show different cell adhesion capacity. There seems to be a very poor cell adhesion and cell number on laminin coated coverslips (mean cell number ~ 62.00/field of view ± 3.96SD) while fibronectin coverslips show better adhesion (with mean cell number ~ 150.73 ± 3.65SD). According to the collected data (See Tables 2.4 and 2.5) bone cells on laminin coated coverslips show poor attachment with not much elongation (with mean cell length ~ 67.10μm ± 2.16SD). There were also lots of rounded cells which imply bone cells with less attachment to this type of ECM protein while substrates with fibronectin showed better attachment with larger number of attached and elongated cells (with mean cell length ~ 92.09μm ± 6.02SD). Collagen coated and un-coated (control) substrates show similar result to fibronectin in terms of cell number, but the cell morphology was different. Cells grown on collagen coated coverslip had a more elongated morphology (with mean cell length ~ 84.10μm ± 2.17 SD), while bone cells on un-coated control substrates had shorter lengths of ~ 77.33μm ± 2.05SD). Tables 2.2-2.5 show the descriptive statistics and statistical test for bone cell number and cell length of three proteins coated substrates (collagen type I, fibronectin and laminin) and un-coated substrate.

Table 2.2 Descriptive statistics of bone cell number of various substrates with addition of protein coated (collagen, fibronectin, laminin) and un-coated (n= 3times, 10 samples for each repeat).

Treatment (Coating)	Mean Cell Number ± Std. Deviation (SD)	Std. Error (SE)
Collagen	73.13 ± 2.29	0.59
Fibronectin	150.73 ± 3.65	0.94
Laminin	62.00 ± 3.96	1.02
Plain (Un-coated)	78.53 ± 2.38	0.61

Table 2.3 P value derived from pair wise comparisons of bone cell number for various substrates with addition of protein coated (collagen, fibronectin, laminin) and un-coated (n=3, 10 samples/repeat). Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment (Coating)	*p*-value	*p*-value (significant difference)
Collagen – Fibronectin	0.001	***
Collagen – Laminin	0.001	***
Collagen – Plain (Un-coated)	0.001	***
Fibronectin – Laminin	0.001	***
Fibronectin – Plain (Un-coated)	0.001	***
Laminin – Plain (Un-coated)	0.001	***

In all cases the data acquired regarding cell length was found to be normaly distributed and were thus analysed using a Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test (See Tables 2.4 and 2.5). Results showed that there are significant differences in cell length between fibronectin and laminin coated substrates (p<0.01). These results indicate that MG63 cells respond better to fibronectin coated substrates with the highest cell length (with mean cell length ~92.09 ± 6.02SD), which indicate the cells attach mostly via fibronectin specific integrins.

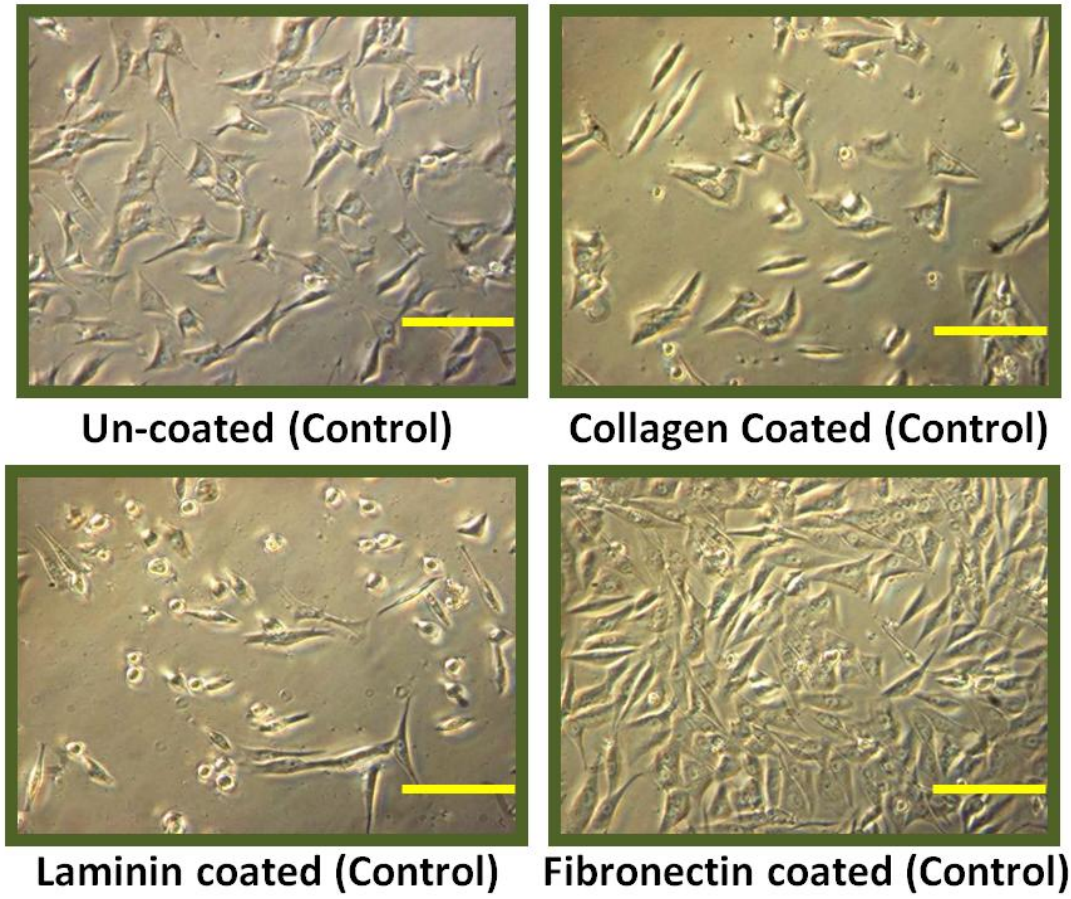


Figure 2.8 *Initial attachment and cell morphology evaluated with collagen, fibronectin, laminin coated glass coverslips and un-coated (control) at 18hrs of seeding; scale bar=100μm.*

Table 2.4 Descriptive statistics of bone cell length of various substrates with addition of protein coated (collagen, fibronectin, laminin) and un-coated (n= 3times, 10 samples for each repeat).

Treatment (Coating)	**Mean Cell Length (μm) ± Std. Deviation (SD)**	**Std. Error (SE)**
Collagen	84.10 ± 2.17	7.01
Fibronectin	92.09 ± 6.02	1.55
Laminin	67.10 ± 2.16	0.55
Plain (Un-coated)	77.33 ± 2.05	7.50

Table 2.5 P values derived from pair wise comparisons for bone cell length of various substrates with addition of protein coated (collagen, fibronectin, laminin) and un-coated (n= 3times, 10 samples for each repeat). Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment (Coating)	***p*-value**	***p*-value (significant difference)**
Collagen - Fibronectin	0.699	
Collagen - Laminin	0.108	
Collagen – Plain (Un-coated)	0.794	
Fibronectin - Laminin	0.007	**
Fibronectin – Plain (Un-coated)	0.198	
Laminin – Plain (Un-coated)	0.511	

2.4.2 Studying Bone Cell Interactions with Micro-patterned ECM Proteins

The aim of this study was to identify the role of collagen type I, fibronectin and laminin, in the adhesion and alignment of the human bone cell (MG63) to different micro-contact printed ECM patterns (Sefat, 2010a). An additional plain (un-coated/un-patterend) coverslip was seeded with cells as a control. As described earlier an angle of 45° suggests no alignment. i.e., random orientation to the pattern, whilst mean angles of alignment less than 45° suggest alignment to the pattern such that a mean angle of 0° indicates complete alignment to the pattern. Figure 2.9 shows photomicrographs of MG63 bone cells after 18hrs of seeding on 5, 10, 25, 50 and 100μm micro-contact printed substrates with three ECM proteins. Patterned cells exhibited an elongated morphology in the direction of the patterned guidance cue. It was found that after 18hrs in culture cells grown on the patterned coverslips show more elongation (and better adhesion) on fibronectin-patterned substrates than substrates patterned with collagen and Laminin. The cells aligned well on fibronectin patterned coverslips especially to 50

and 100μm with a mean angle of 7.87˚± 3.06SD and 6.45˚ ± 5.08SD, respectively. Poorly aligned cells were evident on laminin patterns where some cells still remained rounded even after 18hrs (50 and 100μm with a mean angle of 14.36˚ ± 1.57SD and 15.58˚ ± 1.94SD, respectively). By comparison, cells on almost all fibronectin patterns attached and elongated at early stages (after about 2hrs of seeding), whereas with collagen and laminin patterns, cells did not adhere readily and appeared more rounded until about 18hrs after seeding. The best cell alignment to collagen and laminin patterns occurred after 18hrs in culture on the 50μm wide patterns (mean angle of 9.66˚ ± 4.18SD and mean angle 14.36˚ ± 1.57SD, respectively). Photomicrographs of MG63 bone cells after 18hrs of culture on 10 and 25μm showed very poor cell alignment in the presence of all three types of proteins, e.g. the angle of alignment for the 10μm patterned collagen, fibronectin and laminin was 31.24˚ ± 5.6SD, 20.82˚ ± 5.7SD and 33.83˚ ± 4.36SD, respectively, and in the case of 25μm patterned collagen, fibronectin and laminin was 26.03˚ ± 16.29SD, 23.33˚ ± 9.03SD and 32.19˚ ± 11.54SD, respectively. MG63 bone cells showed no alignment on control (Un-patterend) coverslip with mean angle of 46.11˚ ± 3.96SD. Overall, of the three ECM proteins and five stamped patterns bone cells, the best response was obtained for the 50μm and 100μm wide stamp patterns. Statistical results showed (See Tables 2.6-2.8) that there are highly significant differences in cell alignment between larger (50 and 100μm) and smaller (5, 10 and 25μm) stamp sizes ($p<0.001$). Mean angle of alignment versus stamp size is shown in Figure 2.10 which confirms that fibronectin patterns with 50 and 100 μm width gave the best cell alignment.

Table 2.6 Descriptive statistics of mean angle of alignment for three ECM proteins (Collagen, Fibronectin and Laminin) and un-coated (control) versus stamp sizes (5, 10, 25, 50 and 100μm) of MG63 bone cells after 18hrs of seeding (n= 3times, 10 samples for each repeat).

Treatment	Stamp Size (μm)	Mean Angle of Alignment ± Std. Deviation (SD)	Std. Error (SE)
Collagen	5μm	22.33° ± 4.34	1.37
	10μm	31.24° ± 5.60	1.77
	25μm	26.03° ± 6.29	5.15
	50μm	9.66° ± 4.18	1.32
	100μm	19.12° ± 5.77	1.82
Fibronectin	5μm	17.11° ± 9.00	2.84
	10μm	20.82° ± 5.70	1.80
	25μm	23.33° ± 9.03	2.85
	50μm	7.87° ± 3.06	0.97
	100μm	6.45° ± 5.08	1.60
Laminin (Control)	5μm	24.27° ± 6.35	2.00
	10μm	33.83° ± 4.36	1.37
	25μm	32.19° ± 11.54	3.64
	50μm	14.36° ± 1.57	0.49
	100μm	15.58° ± 1.94	0.61
Un-patterend (Control)	----------	46.11° ± 3.96	1.25

Table 2.7 P values derived from pair wise comparisons of mean angle of alignment for three ECM proteins (Collagen, Fibronectin and Laminin) and un-coated/un-patterend (control) versus stamp sizes (5, 10, 25, 50 and 100μm). Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	Collagen		Fibronectin		Laminin	
5μm - 10μm	0.155		0.059		0.012	*
5μm - 25μm	0.869		0.028	*	0.054	
5μm - 50μm	0.015	*	0.001	***	0.009	**
5μm - 100μm	0.918		0.001	***	0.028	*
10μm - 25μm	0.656		0.762		0.977	
10μm - 50μm	0.001	***	0.001	***	0.001	***
10μm - 100μm	0.022	*	0.001	***	0.001	***
25μm - 50μm	0.001	***	0.001	***	0.001	***
25μm - 100μm	0.385		0.001	***	0.001	***
50μm - 100μm	0.116		0.597		0.992	

Table 2.8 P values derived from pair wise comparisons of mean angle of alignment for three ECM proteins (Collagen, Fibronectin and Laminin) and un-coated/un-patterend (control) versus stamp sizes (5, 10, 25, 50 and 100μm). Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	5μm	10μm	25μm	50μm	100μm
Collagen – Fibronectin	0.002**	0.002**	0.762	0.290	0.001***
Collagen – Laminin	0.002**	0.001***	0.123	0.001***	0.001***
Collagen – Control	0.001***	0.001***	0.001***	0.001***	0.001***
Fibronectin – Laminin	0.002**	0.001***	0.019*	0.001***	0.001***
Fibronectin – Control	0.001***	0.001***	0.001***	0.001***	0.001***
Laminin – Control	0.001***	0.001***	0.001***	0.001***	0.001***

Differences in cell length mirrored those of alignment, with cells acquiring the greatest length when showing the greatest degree of alignment (see Figure 2.11) (Sefat, 2010a). Tables 2.6-2.11 show descriptive statistics and statistical test results comparing the mean angle of alignment and cell length for three ECM proteins (Collagen type I, Fibronectin and Laminin) versus stamp sizes (5, 10, 25, 50 and 100μm) of MG63 bone cells after 18hrs of seeding. In the case of 5μm micro contact printed substrates fibronectin compared to collagen induced better cell elongation (mean cell length ~ 121.6μm ± 5.61SD and 115.23μm ± 5.11SD, respectively). Cells grown on the patterned coverslips after 18hrs showed more elongation for fibronectin followed by collagen and laminin (see Figure 2.9 for details). Statistical results showed that there are significant differences between 5μm and all others stamp sizes (p<0.001). Photomicrographs of MG63 bone cells after 18hrs of culture on 10 and 25μm showed very poor cell elongation in the presence of all three types of proteins, e.g. the cell length for the 10μm patterned collagen, fibronectin and laminin was 85.86μm ± 7.73SD, 98.31μm ± 6.60SD and 80.78μm ± 6.55SD, respectively, and in the case of 25μm patterned collagen, fibronectin and laminin was 81.22μm ± 5.99SD, 88.65μm ±

8.46SD and 77.42μm ± 5.05SD, respectively. Mean cell length was tested statistically using SPSS. Results demonstrated that there are significant differences in cell length between 10μm-50μm and 10μm-100μm stamp sizes ($p<0.001$). Photomicrographs of MG63 bone cells after 18hrs of culture on 50 and 100μm showed better cell elongation in the presence of all three types of proteins, e.g. the cell length for the 50μm patterned collagen, fibronectin and laminin was 138.23μm ± 5.53SD, 160.85μm ± 8.18SD and 122.58μm ± 9.88SD, respectively, and in the case of 100μm patterned collagen, fibronectin and laminin was 153.26μm ± 7.91SD, 162.37μm ± 6.62SD and 124.19μm ± 5.68SD, respectively (Sefat, 2010a).

Table 2.9 Descriptive statistics of mean cell length for three ECM proteins (Collagen, Fibronectin and Laminin) and un-coated (control) versus stamp sizes (5, 10, 25, 50 and 100μm) of MG63 bone cells after 18hrs of seeding (n= 3, 10 samples/repeat).

Treatment	Stamp Size (μm)	Mean cell length(μm) ± Std. Deviation (SD)	Std. Error (SE)
Collagen	5μm	115.23 ± 5.11	0.89
	10μm	85.86 ± 7.73	1.34
	25μm	81.22 ± 5.99	1.04
	50μm	138.23 ± 5.53	0.96
	100μm	153.26 ± 7.91	1.37
Fibronectin	5μm	121.6 ± 5.61	0.97
	10μm	98.31 ± 6.60	1.15
	25μm	88.65 ± 8.46	1.47
	50μm	160.85 ± 8.18	1.42
	100μm	162.37 ± 6.62	1.15
Laminin (Control)	5μm	101.0 ± 7.06	1.23
	10μm	80.78 ± 6.55	1.14
	25μm	77.42 ± 5.05	0.88
	50μm	122.58 ± 9.88	1.72
	100μm	124.19 ± 5.68	0.98
Un-patterned (Control)	----------	77.33 ± 2.65	0.46

Table 2.10 P value derived from pair wise comparisons of mean cell length for three ECM proteins (Collagen, Fibronectin and Laminin) and un-coated/un-patterend (control) versus stamp sizes (5, 10, 25, 50 and 100μm). Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	Collagen		Fibronectin		Laminin	
5μm - 10μm	0.001	***	0.001	***	0.001	***
5μm - 25μm	0.001	***	0.001	***	0.001	***
5μm - 50μm	0.001	***	0.001	***	0.001	***
5μm - 100μm	0.001	***	0.001	***	0.001	***
10μm - 25μm	0.013	*	0.001	***	0.305	
10μm - 50μm	0.001	***	0.001	***	0.001	***
10μm - 100μm	0.001	***	0.001	***	0.001	***
25μm - 50μm	0.001	***	0.001	***	0.001	***
25μm - 100μm	0.001	***	0.001	***	0.001	***
50μm - 100μm	0.001	***	0.248		0.887	

Table 2.11 P value derived from pair wise comparisons of mean cell length for three ECM proteins (Collagen, Fibronectin and Laminin) and un-coated/un-patterend (control) versus stamp sizes (5, 10, 25, 50 and 100μm). Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	5μm	10μm	25μm	50μm	100μm
Collagen – Fibronectin	0.001***	0.001***	0.001***	0.001***	0.001***
Collagen – Laminin	0.001***	0.001***	0.001***	0.001***	0.001***
Collagen – Control	0.001***	0.001***	0.001***	0.001***	0.001***
Fibronectin – Laminin	0.001***	0.001***	0.001***	0.001***	0.001***
Fibronectin – Control	0.001***	0.001***	0.001***	0.001***	0.001***
Laminin – Control	0.001***	0.005**	0.573	0.001***	0.001***

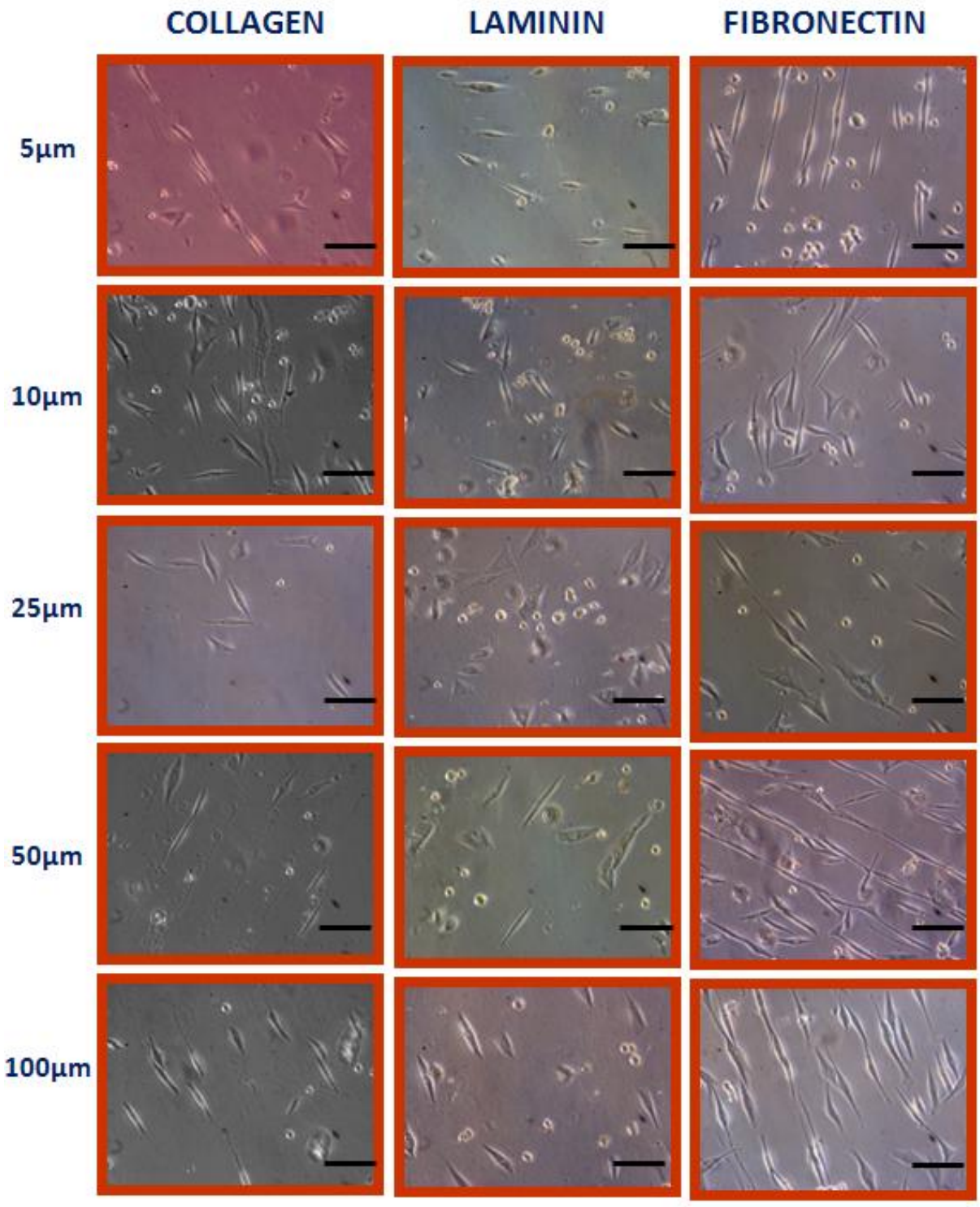


Figure 2.9 *Photomicrographs of MG63 bone cells after 18hrs of seeding on 5, 10, 25, 50 and 100µm micro-contact printed glass substrates: (a) Collagen, (b) Laminin, and (c) Fibronectin; Images taken with standard phase contrast microscope; (Scale bar=100 µm) (Sefat, 2010a).*

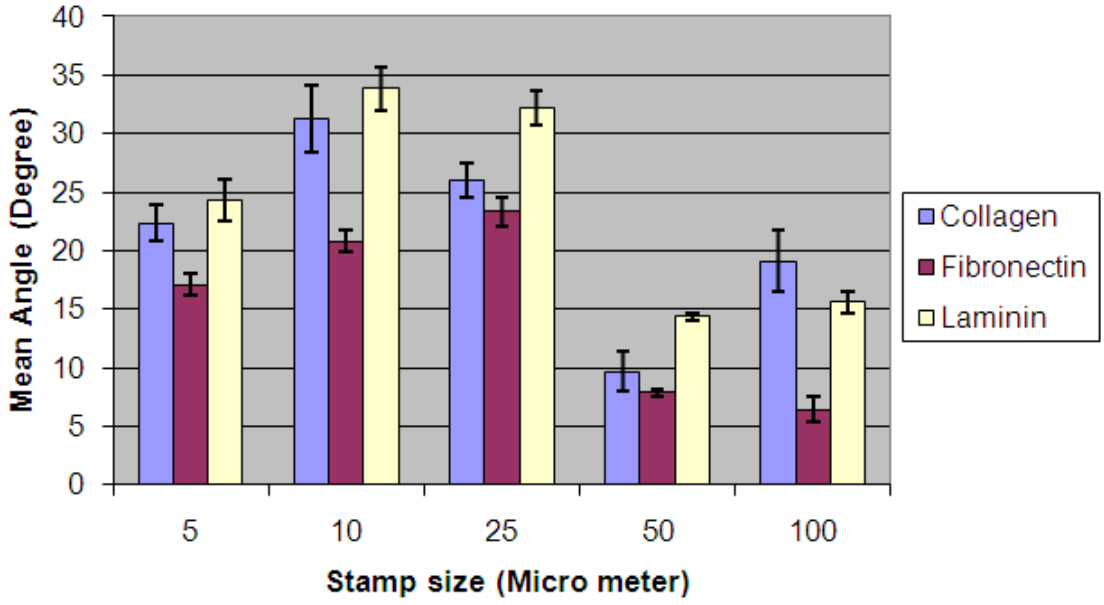


Figure 2.10 *Comparison between three patterned ECM proteins (Collagen, fibronectin and laminin) in terms of cell alignment on five different stamp sizes (5, 10, 25, 50 and 100μm). (Error bar= Standard error) (Sefat, 2008, Sefat, 2010a).*

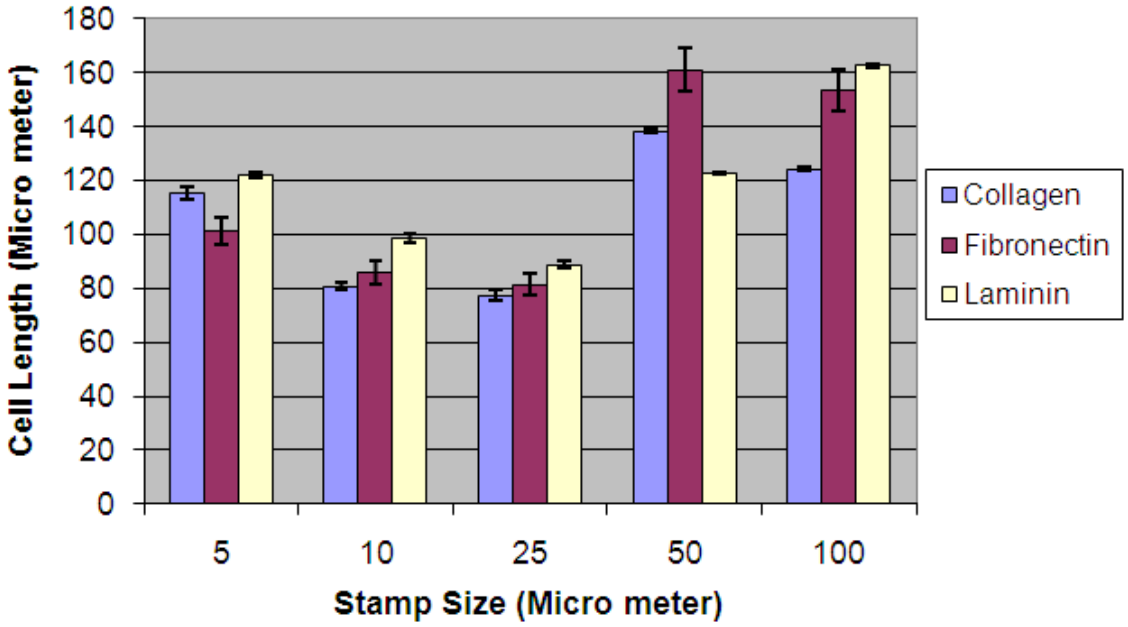


Figure 2.11 *Comparison between three patterned ECM proteins (Collagen, fibronectin and laminin) in terms of cell elongation with five different stamp sizes (5, 10, 25, 50 and 100μm). (Error bars = Standard error) (Sefat, 2008, Sefat, 2010a).*

2.5 DISCUSSION

2.5.1 Effect of MCP on bone cell behaviour

The results indicate that cells align, elongate and attach better along specific patterns depending on their width and make-up in terms of ECM protein. In the last decade or so the effects of surface micro- and nano-textures on cell adhesion, alignment, migration and morphology have attracted much attention and various models/mechanisms exist to explain alignment and attachment (Clark, 1990, Curtis, 1997, Flemming, 1999, Teixeira, 2006, Lim, 2007, Feinberg, 2008, Zhang, 2011). Substrates printed with fine chemical cues cause various cell responses such as cell alignment and migration along the cues (Dalton, 2001, Diehl, 2005, Su, 2007, Sefat, 2010a, Sefat, 2008, Khaghani, 2008), they promote adhesion of osteoblast (Hasenbein, 2002, Charest, 2006) and, more recently patterned substrates have been found to enable differentiation of stem cells (Charest, 2007). Many researchers have assessed cell alignment after cells have been fixed in formaldehyde, methanol or glutaraldehyde (Fischer, 2008). Static/fixed observations are useful for visualizing intracellular protein fiber alignment in detail, but they provide little information about dynamic/live cell behaviour on surfaces with MCP or nanopatterns. Present studies were carried out on both fixed and time-lapse dynamic cell behaviours and hence more detailed information on the mechanism of cell alignment on MCP patterns was obtained. Figure 2.9 shows that the differentiation direction of cells can be controlled by the surface chemistry of cell culture substrates with a major effect on the morphology of cells. In this study the time dependence of cell alignment was analysed to study the mechanism of the chemical guidance effect of the substrate on cell responses. It was found that fibronectin, rather than collagen and

laminin, promoted MG63 cells adhesion (Sefat, 2010a). Cell orientation angles were also quantified by the analysis of low-magnification time-lapse phase contrast images using image J software. The orientation angle of an individual cell was determined as the angle between the pattern direction and the direction of the longer axis of each cell. It was found that cells on the MCP plate started to align along the patterns within a few hours (or even less) of cell seeding, depending on the type of MCP protein, as seen in Figure 2.9. Cells extended/elongated in parallel with the MCP pattern and aligned with each other during the subsequent 18hrs of culture. Cell alignment was maintained for at least 3-4 days following this period. To express cell alignment quantitatively, the orientation angles of individual cells against the pattern direction were measured together with standard deviation (SD) and the distribution of the orientation angles at each time point. After 18hrs in culture (Figure 2.9) cells were poorly aligned to the 5µm, 10µm and 25µm wide repeat patterns of collagen, fibronectin and laminin, but at widths of 50µm and 100µm alignment improved significantly, particularly for cells cultured on the fibronectin patterns (See Tables 2.6-2.11 for full statistical analysis). However, cells seemed to align to the edge of these larger patterns indicating that they were in fact responding to the small feature of the interface between the fibronectin patterned region and the fibronectin free regions.

A large number of studies have examined the effects of surface chemistry on cell behaviour or contact guidance. One group (Teixeira, 2003) employed human corneal epithelial cells and they found that filopodia aligned along the patterns and that cells attached to the ridge and aligned along the nano-patterns. From these findings, they speculated that filopodia sense differences in surface chemistry and induce cell

alignment. In their other report, however, filopodia aligned perpendicular to the guidance cues (Teixeira, 2006). They concluded that these two contradictory results reflected a difference in culture media. Also it was reported (Wo´jciak-Stothard, 1996) that filopodia extending perpendicular to the groove direction were more frequently observed than those along the pattern, even though they were in cells that had aligned along the pattern. The role of filopodial probing remains controversial. MCP have also been used (Hasenbein, 2002) to pattern circles with N [3-(trimethoxysilyl) propyl] diethylenetriamine (DETA) in presence of osteoblasts. Diameters of 10, 50, 100, and 200μm were used with inter-pattern spacings of 10μm in the study. The DETA patterned regions were further modified by cell-adhesive peptides Arginine–Glycine–Aspartic Acid–Serine (RGDS) and Lysine–Arginine–Serine–Arginine (KRSR) or non-adhesive peptides Arginine–Aspartic Acid–Glycine–Serine (RDGS) and Lysine–Serine–Serine–Arginine (KSSR). After four hours under standard cell culture conditions, adhesion of either osteoblasts or fibroblasts on surfaces patterned with the non-adhesive peptides was random and low (Hasenbein, 2002). In contrast, both osteoblasts and fibroblasts adhered and formed clusters onto circles modified with the adhesive peptide RGDS, whereas only osteoblasts adhered and formed clusters onto the circles modified with KRSR, a peptide that selectively promotes adhesion of osteoblasts.These results provide evidence that patterning of selected peptides can direct adhesion of specific cell lines exclusively to predetermined regions on material surfaces (Hasenbein, 2002). Another group also presented a hot embossing imprint lithography technique to form mechanical topography and microcontact printing to surface chemical patterns on polymer cell substrates in order to investigate the response of osteoblast cells to these surface patterns (Charest, 2006). The resulting substrate had surface

features consisting of micro-contact printed adhesive lanes 10μm wide with spacing that ranged from 10 to 100μm. Results showed that the cells significantly aligned to the pattern presented. As the chemical pattern spacing was increased, osteoblasts remained aligned to the mechanical topography (Charest, 2006). However, at this moment in time, it is difficult to identify what is the major mechanism in cell alignment on the MCP patterns. However, our results suggest that guidance to ECM proteins such as fibronectin and collagen with good cell alignment represent great attachment to the edge of micor-contact printed area which means there are high levels of integrins present and expressed in this region. It has been reported that human bone cells revealed high levels of α1β1, α3β1, α5β1 and αvβ5 integrins and lower levels of α2β1, α4β1, αvβ1 and αvβ3 integrins (Saito, 2004). This study also demonstrated that cultured human bone cells adhere to a wide variety of ECM proteins and possess a unique repertoire of integrin receptors that includes collagen/laminin receptors (α1β1, and α3β1 integrins) and fibronectin/fibrinogen/vitronectin receptors (α5β1, αvβ3, and αvβ5 integrins) (Saito, 2004). Therefore, in chapter 7, we have tried to examine some important intgrins such as α3 and β1 in order to see which integrins are most important for the attachment and migration of bone cells.

On the other hand, laminin is not normally expressed in bone (Colucci, 1996) and our results show that bone cells poorly aligned to the laminin, but however, to verify that laminin has little effect on bone cell behaviour additional experiment was performed without using any ECM protein (control). Our results show a significant difference between laminin micro-contact printed and un-patterend (control). Cells showed a better

alignment to laminin than the unpatterned surface which indicates either a response to laminin or a response to the nanoscale topography.

The natural process of healing a fracture starts when the injured bone and surrounding tissues bleed, forming a fracture hematoma. The blood coagulates to form a blood clot situated between the broken fragments. It is well established that collagen type I, major ECM protein, mainly exist in intact bone (Tosounidisa, 2009). After few days of injury blood vessels develop into the matrix of the blood clot. The blood vessels bring fibroblasts in the walls of the vessels and these multiply and produce collagen fibres. Therefore, the blood clot is replaced by a matrix of collagen. However, collagen type I is not the only ECM protein present in bone while fibronectin accumulates in the site of injury (Canavan, 2005b). This further supports this finding in which MG63 cells responded mostly to the fibronectin patterns suggesting that these cells attach mostly via fibronectin specific integrins. This in term suggests that cells take on a more migratory phenotype when cultured on fibronectin. This, therefore, suggests that specific integrins associated with attachment to fibronectin could be important in bone wound repair.

2.5.2 Effect of various substrates and topography on cell behaviour

There has been a challenge in recent years to understand the principle of surface chemistry and topography. Techniques of protein patterning can be used to produce patterns that guide cell movement (Clark, 1990, Britland, 1992a, Britland, 1996). Since these protein patterns have edges, it is possible to reverse the criticism and then ask whether cells can react to edges which may be a single molecule in height or are simply

discontinuities in molecular ordering. This problem has been answered by Pritchard et al who used patterning systems to fill adjacent spaces with different proteins to level up the surface so that any step at the junction is less than a molecule in height (Pritchard, 1995). Result shows that extending cells appear to be localized more frequently at the region of junction or discontinuity than would be expected if cell attachment to the patches of protein was random. Laminin protein was used by Britland et al as a chemical cue which oriented at right angles to a topographic one (Britland, 1996). When the grooves were 500nm deep or less, the cells reacted mostly to the chemical cue. On deeper grooves the topographic cue over-rode the chemical one and at 5µm depth the topographic effect oriented about 80% of the cells and the chemical one 7%. Curtis et al created a method for applying very small areas of chemically specified substratum to a cell using a bead technique. Although these cues often modified or activated cells leading to increased neurite extension, they did not lead to a polarized extension or any other obvious reaction resembling a topographic one (Curtis, 1997). Den Braber et al modified silicone grooved and plane surfaces by exposure to UV light, which affected surface energies but did not alter topographic reaction (Den Braber, 1995). This result suggests that surface chemistry plays a small role in topographic phenomena. It is also consistent with the findings that cells react to topography in a broader similar way on very many different materials bearing topographic features.

It is always useful to bear in mind all other factors that may have effect on cell attachment. The ideal substrate provides a framework and initial support for the cells to attach, proliferate and differentiate, and form an ECM (Agrawal, 2001). It should be noted that substrate surface topography and chemistry (wettability, softness, stiffness

and roughness); microstructure (porosity, pore size, pore shape, interconnectivity, specific surface area) (O'Brien, 2005) and mechanical properties (Peyton, 2005, Engler, 2006) have been shown to influence cell behaviours. When cells adhere to surface of a substrate, a sequence of physico-chemical reactions will happen between cells and the substrate. Immediately after a substrate comes into contact with cell culture environments, protein adsorption to its surface occurs and which mediates the cell adhesion, and also provides signals to the cell through the cell adhesion receptors, mainly integrins. The interaction between cells and substrates is called focal adhesion. In this study ECM proteins such as fibronectin, collagen and laminin were used in order to investigate adhesion, alignment and elongation of MG63 bone cells. In here we are going to briefly discuss the other factors which have direct effect on cell adhesion.

2.5.2.1 Protein adsorption

Since cell adhesion to a substrate (*in-vivo* or *in-vitro*) requires a series of cytoplasmic, transmembranal and ECM proteins that assemble into stable contact sites (Geiger, 1987), cell adhesion and behaviours is likely involved the adsorption onto the material surface of serum and ECM proteins (Brynda, 1990). Many proteins, including vitronectin, fibronectin and fibrinogen adsorb onto substrate (biomaterial) surfaces immediately upon contact with physiological fluids and modulate subsequent inflammatory responses. Therefore, according to the literature various cell behaviours, related to different hydrophobicities, may be mediated by protein absorption. For this reason the effect of fibronectin, collagen and laminin were investigated in this study. Surface wettability also has effect on adsorption of cell adhesion molecules. Hydrophobic surfaces tend to adsorb more proteins, while hydrophilic surfaces tend to

resist protein adsorption (Xu, 2007). In this study we have used different substrates including plain glass and glass coated with gold in order to investigate cell alignment and elongation *in vitro*. Bone cells represented similar alignment and elongation in both substrates; however, as a future work it is beneficial to investigate the characteristics of these two surfaces in detail. Tissue culture (TC) plastic was also used in the next chapters. The inside surface of TC plastic provides an optimal growth surface on the flask base for the most varied matrix-dependent tissue cultures. TC treatment cross links carboxyl and amine groups and gives the plastic its net negative charge. TC surface modification is usually done by ionizing radiation or physio-chemical methods.

2.5.2.2 Surface roughness

Surface roughness (or topography) is another important factor influencing cell adhesion and behaviour. Roughness modulates the biological response of cells in contact with the substrates. Material surface roughness has a direct influence *in vitro* as well as *in vivo* on cellular morphology, proliferation, and phenotype expression. Literature papers have been reported that cells grown on micro-rough surfaces were stimulated towards differentiation in comparison with cells growing on smooth surfaces. For instance, primary rat osteoblasts and human foetal osteoblastic cells had higher proliferation, cell spreading and osteocalcin expression on the rough surface in comparison with smooth one (Hatano, 1999, Lim, 2007). Depending on the scale of irregularities of the material surface, surface roughness can be divided to macroroughness (100μm – milimeters), microroughness (100nm – 100μm), and nanoroughness (less than 100 nm), each with its specific influence (AGASKÁ, 2010). The response of cells to roughness is different depending on the cell type. For larger cells, such as osteoblasts (MG63), macroscopic

descriptions of the surface roughness could be reasonable (Donoso, 2007, Lee, 2004). It seems that the cell adhesion and proliferation were progressively inhibited as the substrate had micropores. Therefore, the selectivity of cells on surface roughness could be highly advantage on the development of implanted devices. In this study surface chemistry was mainly considered and it is also beneficial to consider surface topography in order to investigate more precise cell behaviour.

2.5.2.3 Effects of depth

It has been documented that on groove/ridge topography the extent of reaction is related to groove width as well to depth and probably also to the number of adjacent grooves (Clark, 1990). There is general evidence that the extent of orientation increases with groove depth up to about 25μm from topographies of about 1μm relief. Below this degree of relief results are less available, partly owing to the difficulty of quantifying the etch depth in earlier years and perhaps partly because of a naive belief that there would not be any effect on the cells. Other group have discovered that macrophage-like cells react down to dimensions at least as small as 44nm (Wojciak, 1995). Such small topographies approach the dimensions of large molecules and in the very near future the excitement generated by the suspicion that the sensing mechanisms of cells for topography are perhaps as sensitive as those of the atomic force microscope may lead to further discoveries.

2.5.2.4 Groove width

According to the literature when the grooves or ridges are noticeably wider than the cells effects on orientation are not very marked, although cells may align to one edge (Clark, 1991). On the other hand, as the groove/ridge width is reduced to the width of the cells and less, effects on orientation become more marked. When more than two discontinuities underlay a cell, lines of actin condensation mark each discontinuity. It also showed that cells will react to groove/ridge topography with a pitch (repeat) of 260 nm. These grooves were relatively deep at 500nm. No work has yet discovered the minimum width of topography to which a cell can react.

2.6 CONCLUSIONS

In summary, it can be concluded that human bone cells (MG63) were cultured successfully in the presence of different protein patterned substrates using micro contact printing. Cells on the fibronectin patterns attached, aligned 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 18hrs after seeding. The results indicated that MG63 cells responded mostly to the edge of 50 and 100μm wide fibronectin patterns suggesting that these cells attach mostly via fibronectin specific integrins.

CHAPTER THREE

3. TRYPSINISATION ASSAY:

THE EFFECT OF TRANSFORMING GROWTH FACTOR BETA (TGF-β), BSA, HCl AND BSA/HCl ON CELL DETACHMENT OF HUMAN BONE CELLS

3.1 INTRODUCTION

Cells are not found in isolation and they usually adhere to other cells or the surrounding extracellular matrix (ECM) environment *in vivo* and substrate or a surface *in vitro* (Critchlow, 1995, Freshney, 1994, Sefat, 2009a, Sefat, 2010c, Eapen, 2012, Beggs, 2008). Osteoblasts and other bone cells produce many growth factors including TGF-β and this growth factor has clear functions in regulation of bone formation and their actions on bone formation have been examined extensively in vivo (Linkhart, 1996, Mohan, 1995, Chin, 2004, Kovacevic, 2011, Guiqian, 2012). Application of TGF-β promoted bone healing in skull defects and tibia1 fractures (Nielsen, 1994, Critchlow, 1995) and continuous infusion of TGF-β increased bone formation and fracture healing (Tosounidisa, 2009), suggesting TGF-β as a potential therapeutic agent for fracture repair. The effect of TGF-β is mediated by a range of signalling pathways (Hollinger, 2004) and the interaction of bone cells with their surrounding ECM environment influence physiological functions and pathological processes (Erlebacher, 1996). These physiological functions are normal skeletal development and bone matrix production (Tosounidisa, 2009(Guiqian, 2012). These interactions are mediated by integrins, which

are capable of transducing the signals from ECM to the cells which results in migration, differentiation and specific protein synthesis. Bone repair and wound healing comprises an ordered sequence of events including cell migration and proliferation, synthesis of extracellular matrix, angiogenesis and remodelling. TGF-β regulates many of these processes such as cell migration during bone repair. Degree of cell attachment in presence of different isomers of TGF-β is also important issue that needs to be considered because enhanced cell migration is associated with decresed attachment and there is evidence that TGF-β may promote decreased attachment in some cell types (Loeser, 1994). For instance, Boland et al. investigated the effects of TGF-β1 on rabbit tracheal epithelial cells in primary culture, with respect to cell proliferation and differentiation (Boland, 1996). TGF-β1 treatment produced a negative effect on cell proliferation, but in contrast, promoted a marked enhancement of cell migration and increase in cell spreading (Boland, 1996). Therefore, degrees of cell detachment using different TGF-β isomers is an important issue that needs to be consided in order to understand how fast cells are able to migrate to the site of injury during wound healing. This can be easily measured by a process called "Trypsinisation". This is a technique in cell culture to detach the cells from the culture flask. Trypsin is an enzyme which can be used to digest the proteins that assist adhesion between culture flask and cells (Freshney, 1994). Therefore, the aim of this study was to investigate the effect of different TGF-β isomers (TGF-β1, TGF-β2, and TGF-β3), TGF-β combinations, BSA, HCl and BSA/HCl on bone cell detachment via a trypsinisation assays (Sefat, 2009a, Sefat, 2010c). HCl and BSA were also tested, as they are the solvent and carrier for TGF-β and thus it was important to investigate if these carriers have positive effect on cell detachment.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

Bone cells were cultured in a low glucose (1g/L-D-Glucose) HEPES (25mM) buffered DMEM (Dulbecco's Modified Eagle Media, Sigma Aldrich) culture media supplemented with L-glutamine (2.5 mM U/mL), Penicillin (100 U/mL), Streptomycin (0.1 mg/mL), Amphotericin B or a fungizone (250µg/ml), (Sigma Aldrich-UK) and 10% FCS v/v (fetal calf serum- PromoCell - UK). The bone cells were plated in a 12 multiwell plates at a cell density of 50,000 cells/well and bathed in the culture media. All supplements were purchased from Sigma Aldrich-UK, except those otherwise mentioned.

3.2.2 Reconstitutions

TGF-β1 was reconstituted in a 10mM HCl solution containg 2mg/ml BSA to give a stock solution containing 50ng/ml TGF-β1. Further dilutions were made into PBS containing 2 mg/ml albumin to give a working concentration of 5ng/ml. This solution was then stored at working aliquate at -20°C. TGF-β2 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 2 mg/ml BSA to give a working concentration of 10ng/ml. This solution was then stored at working aliquate at -20°C. TGF-β3 was diluted, according to supplier recommendation, with HCl and BSA. 10mg BSA was dissolved in 10ml of 4mM HCl 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 to give a working concentration of 50ng/ml and aliquoted into forty 10μl vials and stored at -20ºC. Combinations of TGF-β1, 2 and 3 along with solutions of HCl (4mM), BSA (2mg/ml) and distilled water were prepared. A total of 5ng/ml of TGF-β1 and 10ng/ml of TGF-β2 were added to 5ml cell suspension and incubated at 37°C. A total of 5ng/ml of TGF-β1 and 50ng/ml TGF-β3 were added to another 5 ml cell suspension. Similar manipulation process was performed to produce 5ml of cell suspensions containing TGF-β (2+3) and TGF-β (1+2+3). All culture flasks were labelled and incubated at 37°C. Final concentrations of 50ng/ml (80μM) were used for HCl and BSA (as a control).

3.2.3 Trypsinisation Method

Bone cells were cultured in a 12 well petridish (50,000 cells/well) and left for 3 days to become confluent. Cells were checked after 3 days to observe their confluency. The 12 well cultured dishes were imaged using an inverted phase contrast microscope (Optika, XDS-2). Old media was aspirated and cells were washed with Hank's balanced salt solution (HBSS) and trypsin (0.5ml) was added to each well. Cells were imaged every 20 second for a duration of 14 minutes (42 frames in total). This method was repeated for bone cell monolayer treated with BSA, HCl, HCl/BSA, TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and bone with media as negative control (Sefat, 2009a, Sefat, 2010c). Detatchment was measured over 14 minutes as 100% cell detachment was recorded in control culture flask (with no treatment) and this time frame is good enough to compare with other treatments. The

aim of this study was to investigate the effect of the named solutions on cell detachment. Cells detaching from a surface take on a spherical morphology (Canavan, 2005a). Thus, the degree of cell detachment was determined by counting the number of rounded cells at each timepoint and expressing this as a percentage of cells in the field of view. HCl and BSA were used, as they are solvent and carrier for TGF-β. This method was repeated three times and the mean % of rounded cells was determined at each timepoint. Figures 3.1, 3.2a and 3.2b show the schematic of the 12 well petridish for different TGF-β isomers, TGF-β combinations, BSA, HCl, HCl/BSA and bone cell only as control.

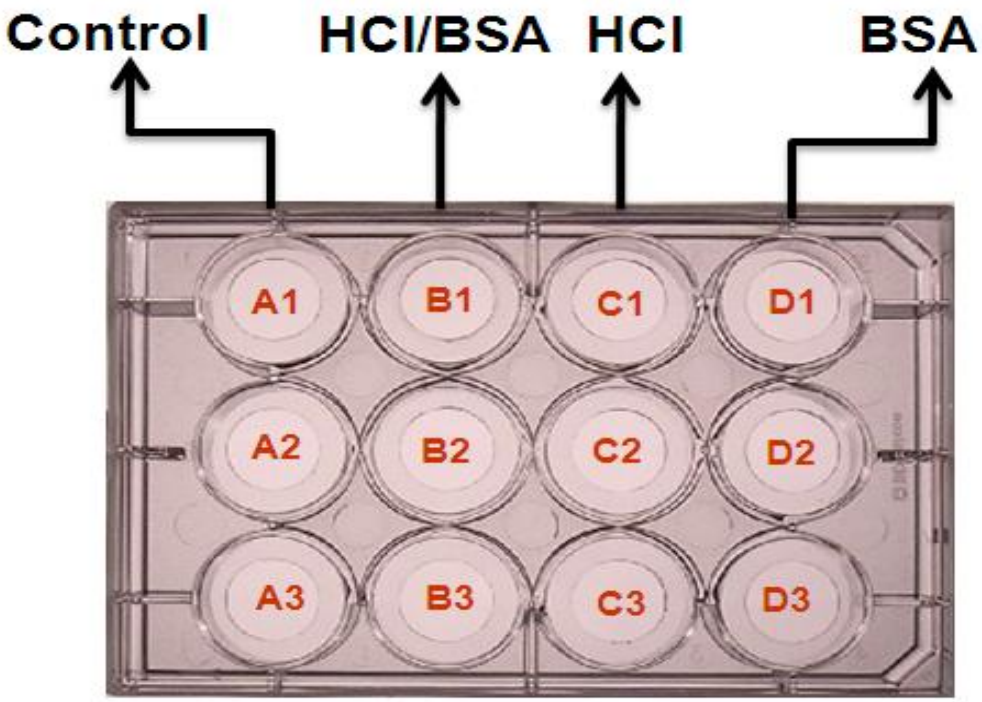
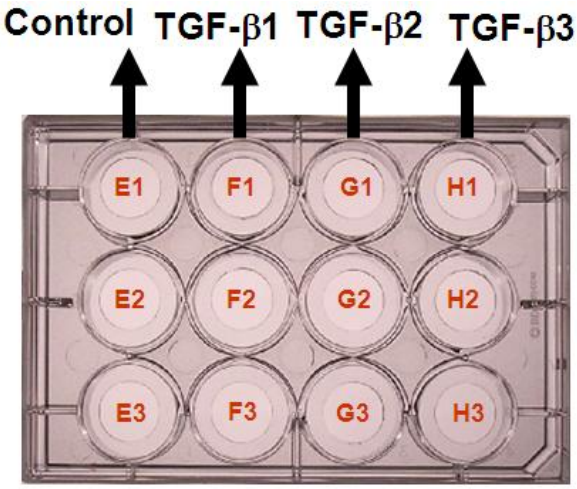
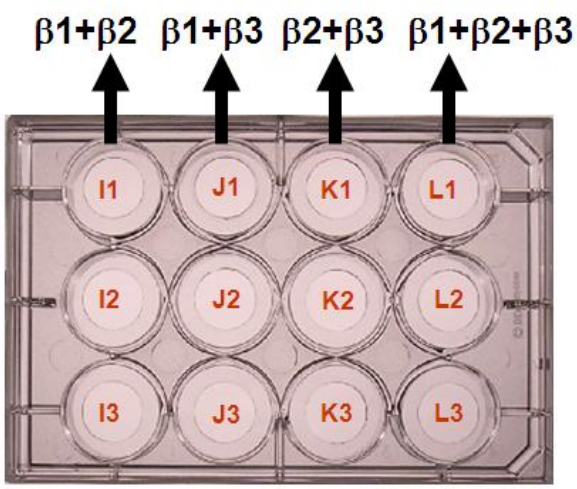


Figure 3.1 *– 12 well culture dish for trypsinisation of (A1-A3) Control; (B1-B3) HCl/BSA; (C1-C3) HCl and (D1-D3) BSA (Sefat, 2009a, Sefat, 2010c).*



(a)



(b)

Figure 3.2a: *12 well culture dish for trypsinisation of different TGF-β isomers: (E1-E3) Control; (F1-F3) TGF-β1; (G1-G3) TGF-β2; (H1-H3) TGF-β3;* ***Figure 3.2b:*** *12well culture dish for trypsinisation of different TGF-β combinations: (I1-I3) TGF-β(1+2); (J1-J3) TGF-β(1+3); (K1-K3) TGF-β(2+3) and (L1-L3) TGF-β(1+2+3).*

3.3 STATISTICAL ANALYSIS

The mean % of rounded cells was tested for normality using a Kolmogorov Smirnov test. Results that showed normal distribution ($p > 0.05$) were analysed using SPSS via a Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed results ($p < 0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

3.4 RESULTS

3.4.1 Trypsinisation Assessment: Control, HCl, BSA, BSA/HCl

Figures 3.3-3.6 show the trypsinisation process for control, BSA/HCl, HCl and BSA over a period of 14 minutes. In the case of control, cells start detaching from the surface about 6 minutes after applying trypsin. Cell detachment was 43% ± 2.23SD after 6 minutes, which suggests that control cells detached relatively slowly during the trypsinisation process. Cells in the presence of BSA/HCl started detaching from the surface faster than control cells, about 4-5 minutes after applying trypsin. Cell detachment was about 63.20% ± 5.89SD after 6 minutes which is faster as compared to the control. Figure 3.5 shows the trypsinisation process for bone cells cultured with HCl alone which shows that cells started to detach from the surface about 2 minutes after application of trypsin. Cell detachment was about 46% ± 6.14SD after 4 minutes, which is faster when compared to the control and BSA/HCl (with mean % rounded cells ~ 19% ± 2.54SD and 41.6% ± 3.50SD, respectively). Cell detachment was about 59.20% ± 12.13SD after 6 minutes which was faster as compared to the control. Figure 3.6 shows the trypsinisation process for bone cells cultured with BSA indicated that cells started to detach from the surface about 4 minutes after application of trypsin but that detachment occurred at a very slow rate. Cell detachment was 11% ± 3.56SD after 4 minutes and 13.00% ± 3.16SD after 6**,** which is slower when compared to all other treatments. Complete detachment was achieved for cells cultured with HCl and un-treated (control) after 14 minutes while cells remained attached to culture flask treated with BSA and BSA/HCl with mean percentage rounded cells about 22.20% ± 3.27SD and 88.40% ± 2.40SD, respectively.

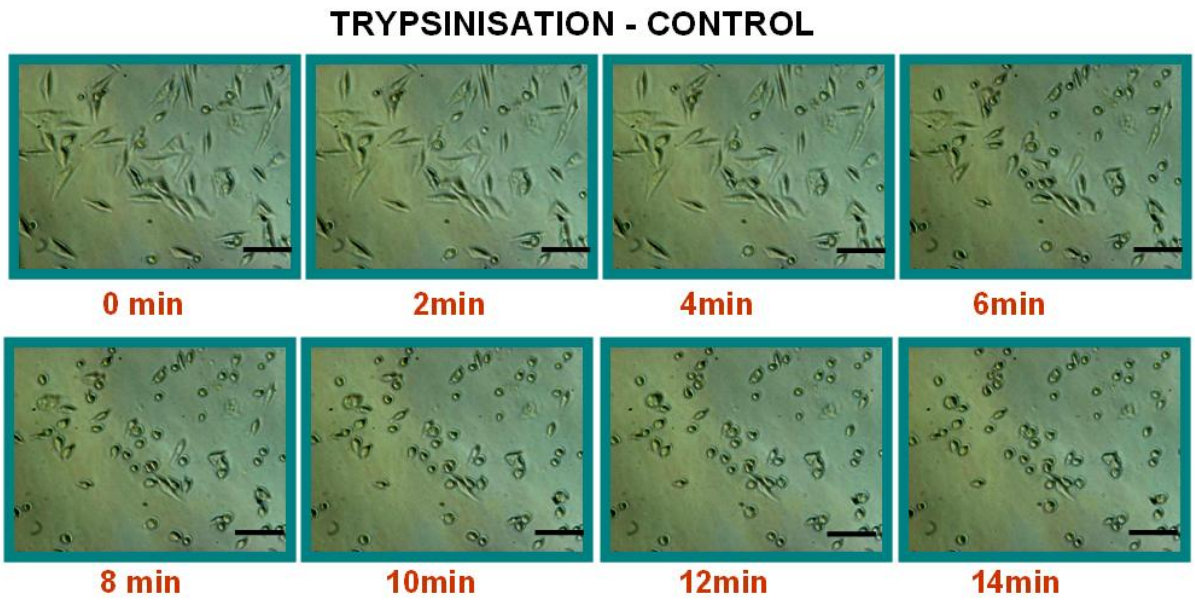


Figure 3.3 *Trypsinisation process for the control over a period of 14 minutes; (Scale bar=100 μm) (Sefat, 2009a, Sefat, 2010c).*

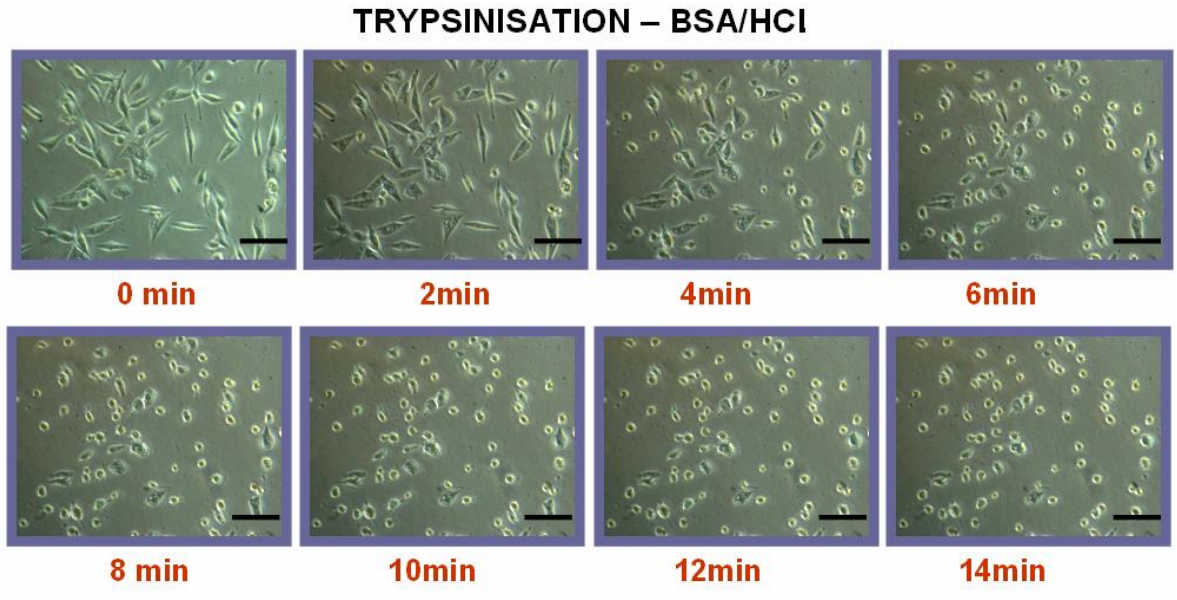


Figure 3.4 *Trypsinisation process in the presence of BSA/HCl for the period of 14 minutes; (Scale bar=100 μm) (Sefat, 2009a, Sefat, 2010c).*

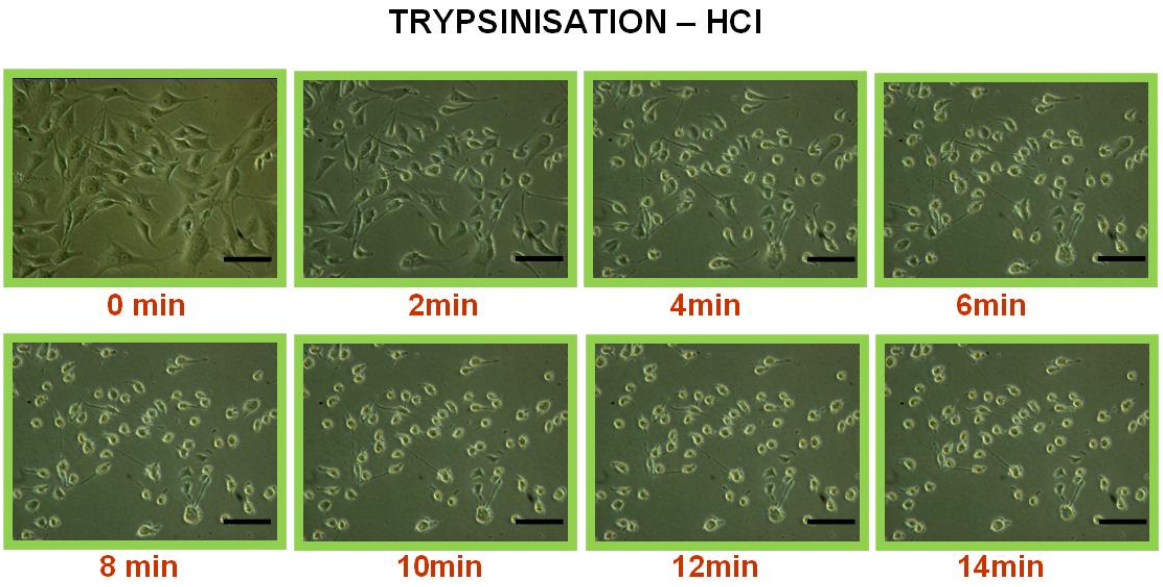


Figure 3.5 *Trypsinisation process in the presence of HCl for the period of 14 minutes; (Scale bar=100 μm) (Sefat, 2009a, Sefat, 2010c).*

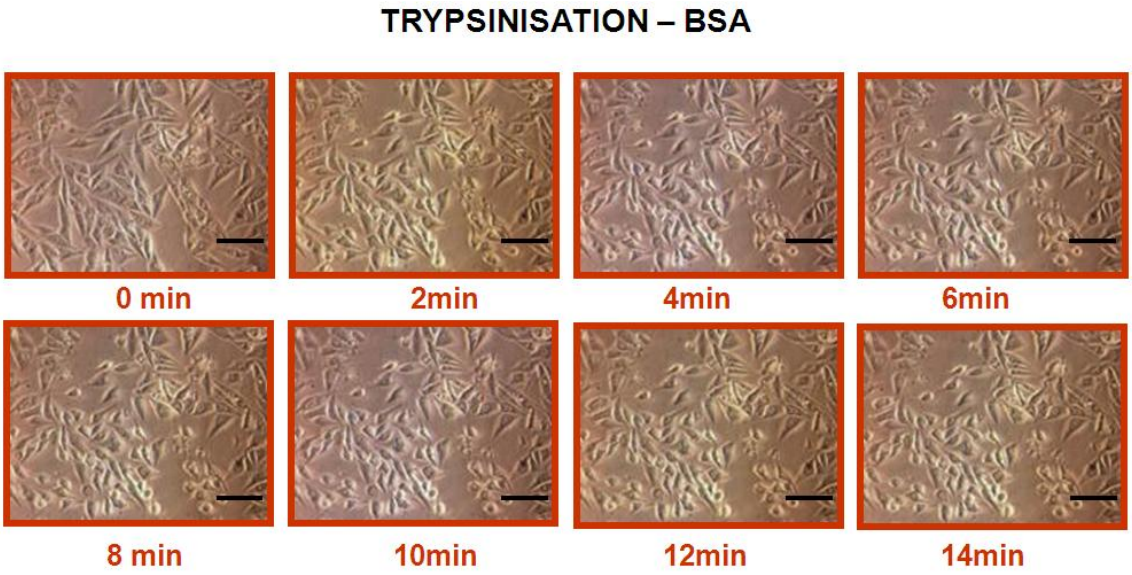


Figure 3.6 *Trypsinisation process in the presence of BSA for the period of 14 minutes; (Scale bar=100 μm) (Sefat, 2009a, Sefat, 2010c).*

Table 3.1 Descriptive Statistics for trypsinisation (Percentage of rounded cells) of bone cell treated with control, BSA, HCl and BSA/HCl over period of 14 minutes.

Treatment	Trypsinisation Time (min)	Mean % Rounded Cells ± Std. Deviation (SD)	Std. Error (SE)
Control	**0min**	0.00	0.00
	2min	14.00 ± 1.58	0.71
	4min	19.00 ± 2.54	1.14
	6min	43.00 ± 2.23	1.00
	8min	57.00 ± 3.80	1.70
	10min	79.00 ± 4.52	2.02
	12min	87.40 ± 2.88	1.28
	14min	100.00	0.00
HCl	**0min**	0.00	0.00
	2min	24.20 ± 7.04	3.15
	4min	46.20 ± 6.14	2.74
	6min	59.20 ± 12.13	5.42
	8min	74.60 ± 11.19	5.00
	10min	87.60 ± 4.15	1.86
	12min	96.20 ± 2.16	0.96
	14min	100.00	0.00
BSA	**0min**	0.00	0.00
	2min	5.20 ± 1.30	0.58
	4min	11.00 ± 3.56	1.59
	6min	13.00 ± 3.16	1.41
	8min	17.20 ± 2.04	0.91
	10min	20.00 ± 2.44	1.09
	12min	21.60 ± 2.50	1.12
	14min	22.20 ± 3.27	1.46
BSA/HCl	**0min**	0.00	0.00
	2min	14.00 ± 2.23	1.00
	4min	41.60 ± 3.50	1.56
	6min	63.20 ± 5.89	2.63
	8min	81.60 ± 3.97	1.77
	10min	81.00 ± 4.63	2.07
	12min	85.40 ± 1.67	0.74
	14min	88.40 ± 2.40	1.07

Table 3.1 shows descriptive statistics of percentage of rounded cells during trypsinisation process treated with control, BSA, HCl and BSA/HCl.

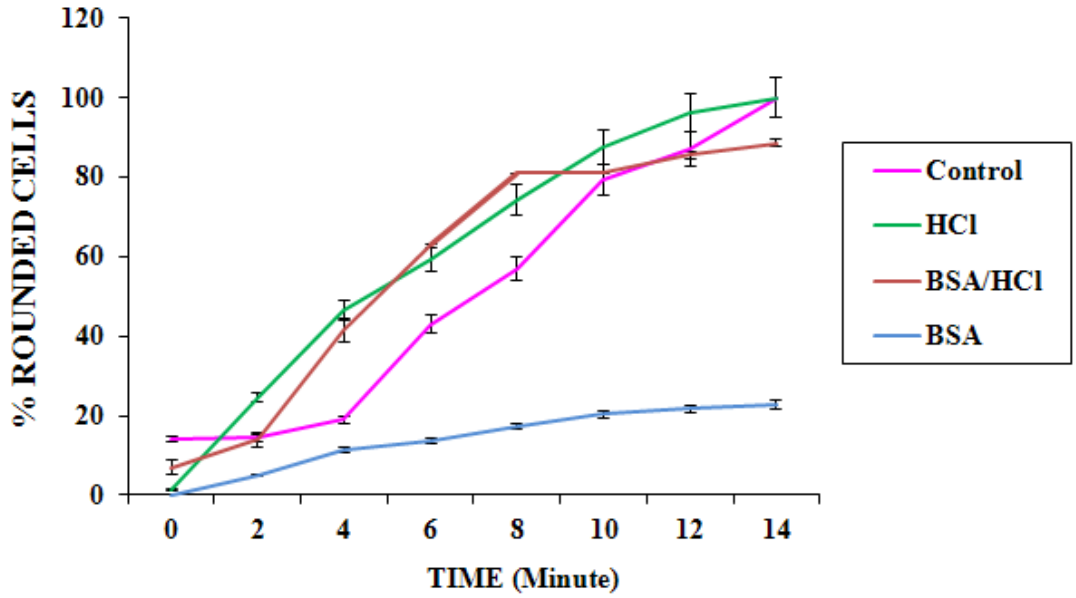


Figure 3.7 *Comparison between percentages rounded cells during trypsinisation process treated with control, HCl, BSA and BSA/HCl (Error bars represent standard deviation) (Sefat, 2009a, Sefat, 2010c).*

3.4.2 Trypsinisation - Different TGF-β Isomers

In the previous section, it was observed that HCl and BSA as a solvent and carrier for TGF-β influenced cell detachments compared to the control ($p < 0.05$). In this section the effect of TGF-β isomers (TGF-β1, TGF-β2 and TGF-β3) on cell detachment were investigated. Figure 3.8 shows the trypsinisation process for bone cells cultured with TGF-β1 which shows that cells started to detach from the surface about 1 minute after application of trypsin and majority of cells were detached by the forth minute (with a mean % rounded cells ~ 77.60 ± 5.54SD). In comparison, as seen in previous Figures (e.g. Figure 3.3-3.6), a completely different response was recorded with the bone cells

plated in negative controls (without any TGF-β), i.e. the rate of detachment was much slower in control even after 6-8 minutes. In the case of TGF-β1, cell detachment was about 84% after 6 minutes, which was significantly faster when compared to the controls ($p < 0.001$), BSA ($p < 0.001$), HCl ($p < 0.001$) and HCl/BSA ($p < 0.001$). Figure 3.9 shows the trypsinisation process for bone cells cultured with TGF-β2 which shows that cells started to detach from the surface about 1 minute after application of trypsin and even more cells were detached by the forth minute compared to the TGF-β1. In the case of TGF-β2 cell detachment was approximately 88% after 4 minutes, which was faster as compared to the control ($p<0.001$), BSA ($p<0.001$), HCl ($p<0.001$) and HCl/BSA ($p<0.001$). TGF-β2 also induced faster cell detachment after 4 minutes compared to TGF-β1 ($p=0.463$) but there was not significance differences in cell detachment. Figure 3.10 shows the trypsinisation process for bone cells cultured with TGF-β3 which shows that cells started to detach from the surface about 1 minute after application of trypsin and were mostly detached by the third minute. In comparison, as seen in the previous Figures (e.g. Figure 3.3-3.6), a completely different response was recorded with the bone cells plated without TGF-β3, i.e. the rate of detachment was much slower in control even after 6-8 minutes. Cell detachment with TGF-β3 was about 85% after 4 minutes, which is faster as compared to the control, BSA, HCl and HCl/BSA. For comparison, as seen in Table 3.2, it was found that treatment with TGF-β1, TGF-β2 and TGF-β3, enhanced the bone cell detachment rate by statistically similar amounts when applied in islolation.

Table 3.2 Descriptive statistics for trypsinisation (Mean percentage of rounded cells) of bone cells monolayer treated with TGF-β1, TGF-β2 and TGF-β3 over period of 14 minutes.

Treatment	Trypsinisation Time (min)	Mean % Rounded Cells ± Std. Deviation (SD)	Std. Error (SE)
TGF-β1	**0min**	0.00	0.00
	2min	22.20 ± 1.92	0.86
	4min	77.60 ± 5.54	2.48
	6min	84.20 ± 1.30	0.58
	8min	92.00 ± 4.35	1.94
	10min	95.40 ± 1.14	0.50
	12min	100.00	0.00
	14min	100.00	0.00
TGF-β2	**0min**	0.00	0.00
	2min	39.40 ± 3.71	1.66
	4min	88.40 ± 3.04	1.36
	6min	91.00 ± 1.22	0.54
	8min	95.00 ± 5.24	2.34
	10min	96.00 ± 3.08	1.37
	12min	100.00	0.00
	14min	100.00	0.00
TGF-β3	**0min**	0.00	0.00
	2min	21.40 ± 4.61	2.06
	4min	85.60 ± 3.43	1.53
	6min	96.00 ± 3.08	1.37
	8min	96.00 ± 3.08	1.37
	10min	96.60 ± 2.50	1.12
	12min	98.20 ± 0.83	0.37
	14min	100.00	0.00

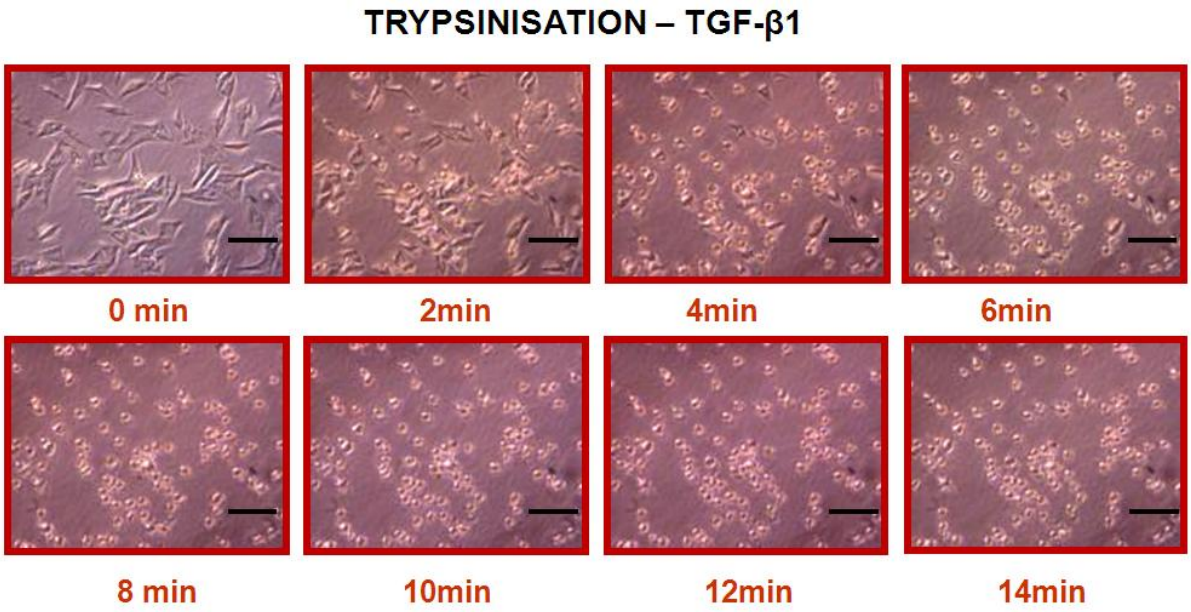


Figure 3.8 *Trypsinisation process in the presence of TGF-β1 for the period of 14 minutes; (Scale bar=100 μm).*

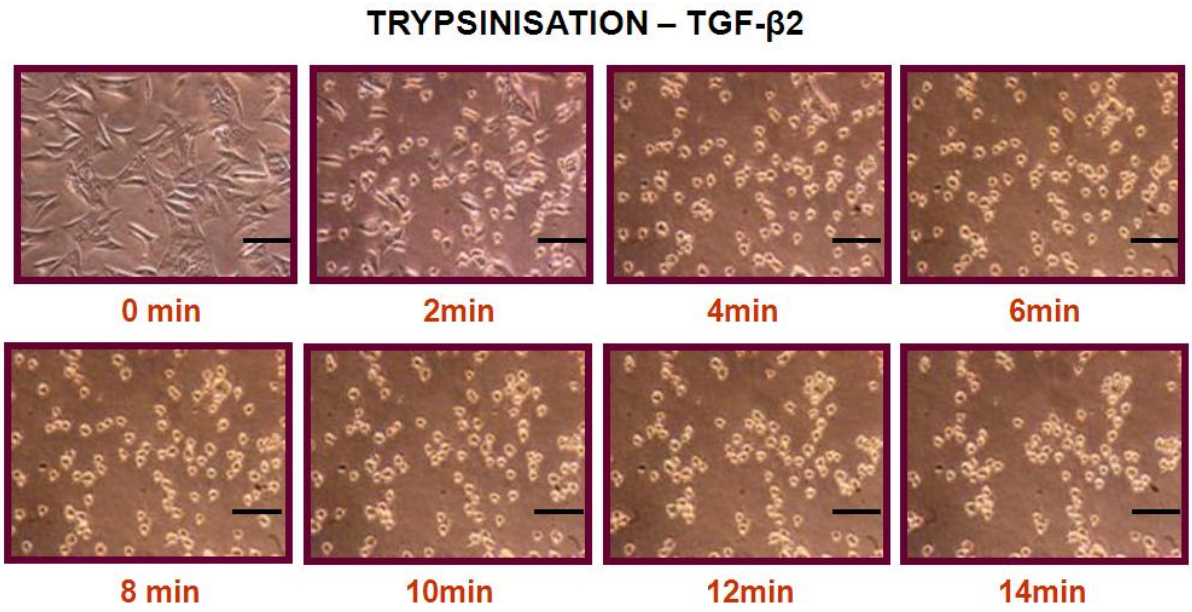


Figure 3.9 *Trypsinisation process in the presence of TGF-β2 for the period of 14 minutes; (Scale bar=100 μm).*

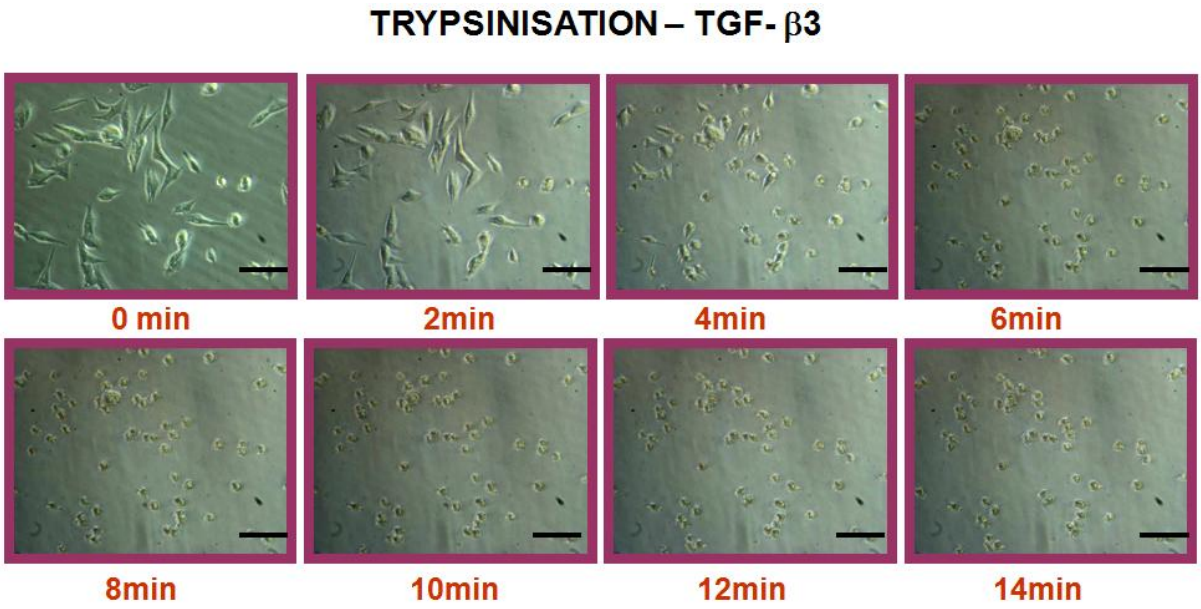


Figure 3.10 *Trypsinisation process in the presence of TGF-β3 for the period of 14 minutes; (Scale bar=100 μm) (Sefat, 2009a, Sefat, 2010c).*

3.4.3 Trypsinisation - Different TGF-β Combinations:

In vivo it is unlikely that cells will be exposed to just one cytokine, thus the aim of this part of the thesis was to determine the effects of different TGF-β combinations such as TGF-β(1+2), TGF-β(1+3), TGF-β(2+3) and TGF-β(1+2+3) on cell detachment. Figure 3.11 shows the trypsinisation process for bone cells treated with TGF-β(1+2). In response to treatment with TGF-β(1+2) cells started to detach from the surface about 1 minute after application of trypsin but not much detachment was observed upto 6 minutes 30.20 ± 2.48SD, which was extremely slow compared to all three isomers of TGF-β ($p<0.01$). After treatment with TGF-β(1+2) cell detachment was not completed even after 14 minute (with a mean % rounded cells ~ 85.80% ± 2.86SD).

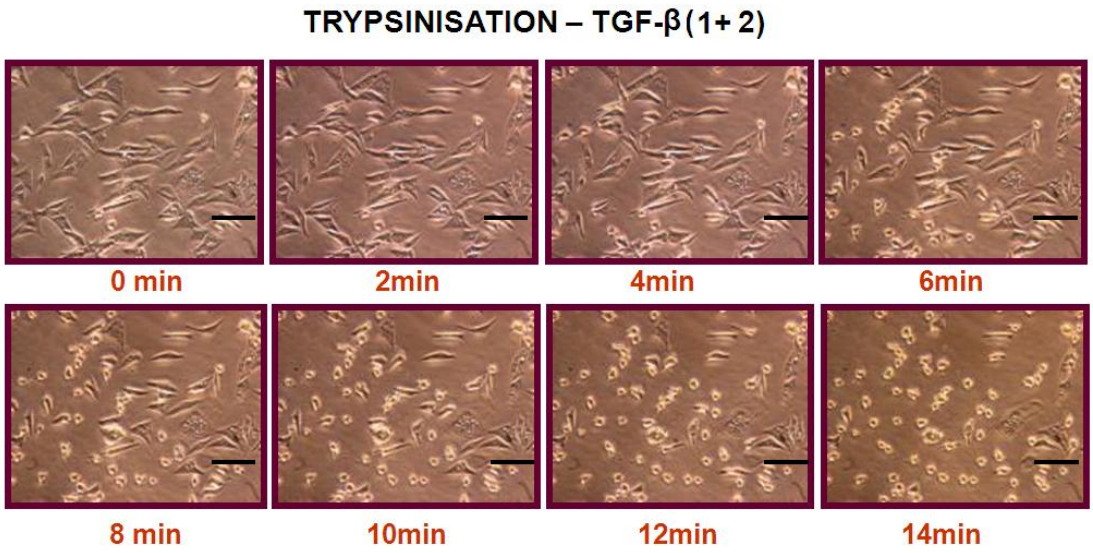


Figure 3.11 *Trypsinisation process in the presence of TGF-β(1+2) for the period of 14 minutes; (Scale bar=100 μm).*

Figure 3.12 shows the trypsinisation process for bone cells cultured treated with TGF-β(1+3), which shows that cells started to detach from the surface about 2 minute after application of trypsin and good detachment were observed from up to 4 minutes (cell detachment was 16% ± 2.54SD after 2 minutes and suddenly increased to 67.6% ± 2.70SD at 4 minute). Cell detachment was unchanged after 8 minutes and no more cell detachments were observed even after 14 minutes (cell detachment was 88%). TGF-β(1+3) showed better cell detachment compared to TGF-β(1+2) ($p < 0.01$) but complete detachment was not observed even after 14 minutes.

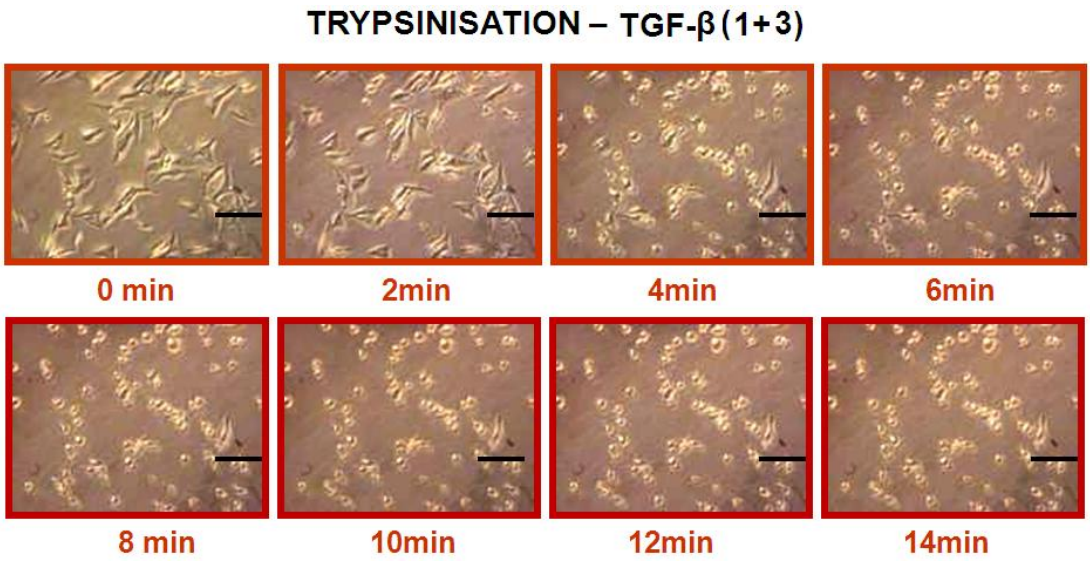


Figure 3.12 *Trypsinisation process in the presence of TGF-β(1+3) for the period of 14 minutes; (Scale bar=100 μm).*

Figure 3.13 shows the trypsinisation process for bone cells cultured treated with TGF-β(2+3), which shows that cells started to detach from the surface about 1 minute after application of trypsin and great detachment were observed up to 4 minutes (cell detachment was 30% after 2 minutes and increased to 74% at 4 minutes). Cell detachment continued after 4 minutes and complete detachment was observed at 12 minutes (100% cell detachment). Petridish treated with TGF-β(2+3) showed the best result in terms of cell detachment compared to all other TGF-β combinations.

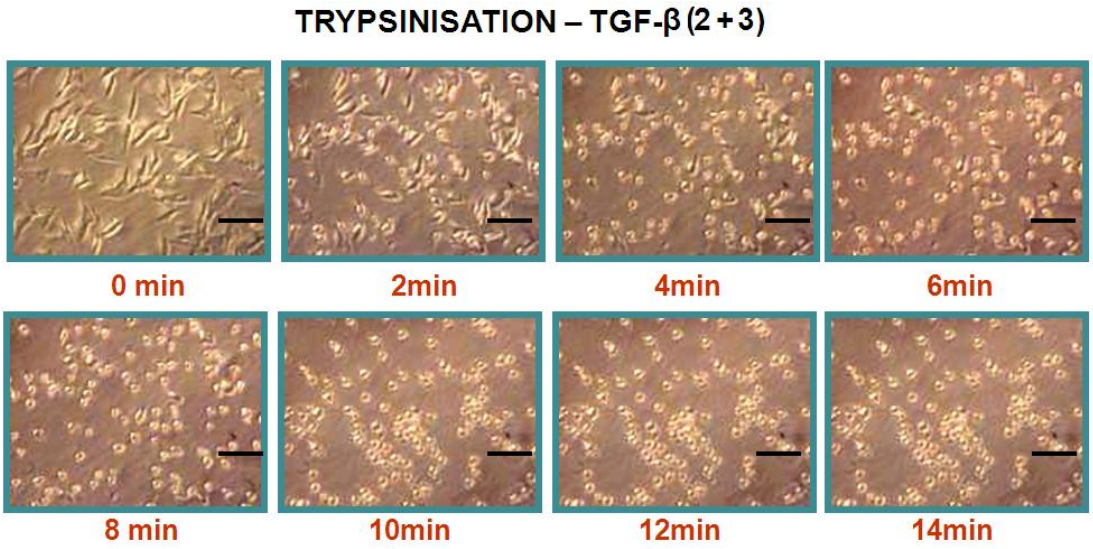


Figure 3.13 *Trypsinisation process in the presence of TGF-β(2+3) for the period of 14 minutes; (Scale bar=100 μm).*

Figure 3.14 shows the trypsinisation process for bone cells cultured treated with TGF-β(1+2+3), which showed that cells started to detach from the surface about 2 minutes after application of trypsin and good detachment was observed up to 4 minutes (cell detachment was 18% after 2 minutes and increased to 64% after 4 minutes). Cell detachment was increased gradually after 4 minutes but complete detachment was not achieved even after 14 minutes (cell detachment was 85%). TGF-β(1+2+3) showed similar results to TGF-β(1+3).

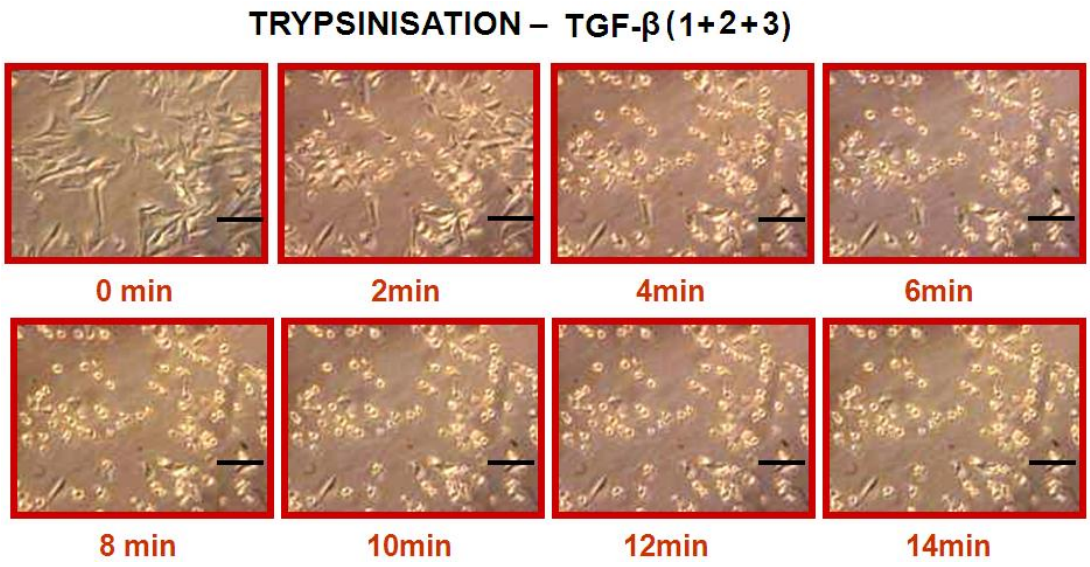


Figure 3.14 *Trypsinisation process in the presence of TGF-β (1+2+3) for the period of 14 minutes; (Scale bar=100 μm).*

Table 3.3 shows mean percentage of rounded cells during trypsinisation process for all TGF-β combinations (these experiments were repeated three times). Table 3.4 also shows *P* values derived from pair wise comparisons of different treated TGF-βs, HCl, BSA, BSA/HCl and control for trypsinisation of bone cell monolayer over period of 14 minutes. For comparison, as seen in Figures 3.8-3.14, it was found that treatment with TGF-β1, TGF-β2 and TGF-β3, enhanced the bone cell detachment rate by statistically similar amounts when applied in islolation but when applied in combination induced statistically different rates of detachment (See Table 3.4).

Table 3.3 Descriptive statistics for trypsinisation (Mean percentage of rounded cells) of bone cell monolayer treated with TGF-β combinations over period of 14 minutes.

Treatment	Trypsinisation Time (min)	Mean % Rounded Cells ± Std. Deviation (SD)	Std. Error (SE)
TGF-β1+2	**0min**	0.00	0.00
	2min	4.60 ± 1.14	0.50
	4min	10.00 ± 1.58	0.70
	6min	30.20 ± 2.48	1.11
	8min	46.60 ± 3.04	1.36
	10min	63.60 ± 2.30	1.02
	12min	72.40 ± 3.20	1.43
	14min	85.80 ± 2.86	1.28
TGF-β1+3	**0min**	0.00	0.00
	2min	16.00 ± 2.54	1.14
	4min	67.60 ± 2.70	1.20
	6min	79.20 ± 3.34	1.49
	8min	88.60 ± 1.51	0.67
	10min	88.00 ± 1.58	0.70
	12min	88.20 ± 3.42	1.52
	14min	88.20 ± 1.30	0.58
TGF-β2+3	**0min**	0.00	0.00
	2min	30.00 ± 2.23	1.00
	4min	74.20 ± 1.64	0.73
	6min	83.00 ± 3.80	1.70
	8min	87.20 ± 1.78	0.80
	10min	97.40 ± 1.51	0.67
	12min	100.00	0.00
	14min	100.00	0.00
TGF-β1+2+3	**0min**	0.00	0.00
	2min	18.00 ± 2.23	1.00
	4min	64.20 ± 3.11	1.39
	6min	72.80 ± 1.64	0.73
	8min	76.40 ± 2.07	0.92
	10min	81.00 ± 2.54	1.14
	12min	83.60 ± 4.82	2.15
	14min	85.20 ± 2.16	0.96

Table 3.4 P values derived from pair wise comparisons of different treated TGF-βs, HCl, BSA, BSA/HCl and control for trypsinisation of bone cell monolayer over period of 14 minutes. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	0min	2min	4min	6min	8min	10min	12min	14min
Control - HCl	1.000	0.001***	0.001***	0.001***	0.036*	0.002**	0.008**	1.000
Control - BSA	1.000	0.004**	0.047*	0.001***	0.009**	0.001***	0.008**	0.001***
Control - BSA/HCl	1.000	1.000	0.001***	0.001***	0.008**	0.992	0.239	0.001***
Control - TGF-β1	1.000	0.010*	0.001***	0.001***	0.009**	0.001***	0.005**	1.000
Control - TGF-β2	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.005**	1.000
Control - TGF-β3	1.000	0.028*	0.001***	0.001***	0.009**	0.001***	0.008**	1.000
Control - TGF-β1+2	1.000	0.002**	0.011*	0.004**	0.009**	0.001***	0.008**	0.001***
Control - TGF-β1+3	1.000	0.996	0.001***	0.001***	0.009**	0.001***	0.589	0.001***
Control - TGF-β2+3	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.005**	1.000
Control - TGF-β1+2+3	1.000	0.681	0.001***	0.001***	0.009**	0.992	0.112	0.001***
HCl - BSA	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.009**	0.001***
HCl - BSA/HCl	1.000	0.001***	0.640	0.956	0.169	0.040*	0.009**	0.001***
HCl - TGF-β1	1.000	0.996	0.001***	0.001***	0.009**	0.007**	0.005**	1.000
HCl - TGF-β2	1.000	0.001***	0.001***	0.001***	0.012*	0.003**	0.005**	1.000
HCl - TGF-β3	1.000	0.950	0.001***	0.001***	0.009**	0.001***	0.088	1.000
HCl - TGF-β1+2	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.009**	0.001***
HCl - TGF-β1+3	1.000	0.010*	0.001***	0.001***	0.009**	1.000	0.009**	0.001***
HCl - TGF-β2+3	1.000	0.181	0.001***	0.001***	0.021*	0.001***	0.005**	1.000
HCl - TGF-β1+2+3	1.000	0.119	0.001***	0.002**	0.675	0.040*	0.009**	0.001***
BSA - BSA/HCl	1.000	0.004**	0.001***	0.001***	0.008**	0.001***	0.009**	0.001***
BSA - TGF-β1	1.000	0.001***	0.001***	0.001***	0.008**	0.001***	0.005**	0.001***
BSA - TGF-β2	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.005**	0.001***
BSA - TGF-β3	1.000	0.001***	0.001***	0.001***	0.008**	0.001***	0.008**	0.001***
BSA - TGF-β1+2	1.000	1.000	1.000	0.001***	0.009**	0.001***	0.009**	0.001***
BSA - TGF-β1+3	1.000	0.001***	0.001***	0.001***	0.008**	0.001***	0.009**	0.001***
BSA - TGF-β2+3	1.000	0.001***	0.001***	0.001***	0.008**	0.001***	0.005**	0.001***
BSA - TGF-β1+2+3	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.009**	0.001***
BSA/HCl - TGF-β1	1.000	0.010*	0.001***	0.001***	0.015*	0.001***	0.005**	0.001***
BSA/HCl - TGF-β2	1.000	0.001***	0.001***	0.001***	0.015*	0.001***	0.005**	0.001***
BSA/HCl - TGF-β3	1.000	0.028*	0.001***	0.001***	0.008**	0.001***	0.008**	0.001***
BSA/HCl - TGF-β1+2	1.000	0.002**	0.001***	0.001***	0.008**	0.001***	0.009**	0.363
BSA/HCl - TGF-β1+3	1.000	0.996	0.001***	0.001***	0.025*	0.023*	0.173	1.000
BSA/HCl - TGF-β2+3	1.000	0.001***	0.001***	0.001***	0.044*	0.001***	0.005**	0.001***
BSA/HCl - TGF-β1+2+3	1.000	0.681	0.001***	0.076	0.026*	1.000	0.292	0.124
TGF-β1 - TGF-β2	1.000	0.001***	0.001***	0.463	0.401	1.000	1.000	1.000
TGF-β1 - TGF-β3	1.000	1.000	0.038*	0.011*	0.202	1.000	0.005**	1.000
TGF-β1 - TGF-β1+2	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.005**	0.001***
TGF-β1 - TGF-β1+3	1.000	0.119	0.003**	0.838	0.07	0.013*	0.005**	0.001***
TGF-β1 - TGF-β2+3	1.000	0.017*	0.916	1.000	0.035*	0.992	1.000	1.000
TGF-β1 - TGF-β1+2+3	1.000	0.617	0.001***	0.016*	0.009**	0.001***	0.005**	0.001***
TGF-β2 - TGF-β3	1.000	0.001***	0.976	0.838	0.916	1.000	0.005**	1.000
TGF-β2 - TGF-β1+2	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.005**	0.001***
TGF-β2 - TGF-β1+3	1.000	0.001***	0.001***	0.011*	0.115	0.005**	0.005**	0.001***
TGF-β2 - TGF-β2+3	1.000	0.002**	0.001***	0.240	0.059	1.000	1.000	1.000
TGF-β2 - TGF-β1+2+3	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.005**	0.001***
TGF-β3 - TGF-β1+2	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.009**	0.001***
TGF-β3 - TGF-β1+3	1.000	0.263	0.001***	0.001***	0.008**	0.002**	0.009**	0.001***
TGF-β3 - TGF-β2+3	1.000	0.005**	0.001***	0.003**	0.009**	1.000	0.005**	1.000
TGF-β3 - TGF-β1+2+3	1.000	0.847	0.001***	0.001***	0.009**	0.001***	0.008**	0.001***
TGF-β1+2 - TGF-β1+3	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.009**	0.480
TGF-β1+2 - TGF-β2+3	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.005**	0.001***
TGF-β1+2 - TGF-β1+2+3	1.000	0.001***	0.001***	0.001***	0.009**	0.001***	0.009**	1.000
TGF-β1+3 - TGF-β2+3	1.000	0.001***	0.159	0.968	0.163	0.001***	0.005**	0.001***
TGF-β1+3 - TGF-β1+2+3	1.000	0.996	0.916	0.552	0.009**	0.023*	0.116	0.184
TGF-β2+3 - TGF-β1+2+3	1.000	0.001***	0.003**	0.046*	0.009**	0.001***	0.005**	0.001***

HCl and HCl/BSA induced similar rates of detachment for up to 8 minutes with no significant differences ($P < 0.05$) compared to the control. TGF-β1, TGF-β2 and TGF-β3 treatments were significant differences ($P < 0.05$) in term of cell detachment compared to the control, BSA and BSA/HCl. Multiple comparisons with respect to different treatments at each time point were performed (See Table 3.4). TGF-β3 treated cells appeared to detach faster than did HCl/BSA and BSA treated cells, although TGF-β3 and HCl induced detachment times were not significantly different ($P = 0.088$) after 12 minutes of trypsinisation. Tables 3.1-3.4 show that there are no significant differences between all TGF-β isomers with TGF-(β2+3) (p=1.000) which means combination of TGF-β2 and TGF-β3 induce responses similar to those associated with the treatment of cells with the different TGF-β isomers in isolation. However, there are significantly difference in percentage rounded cells between cells treated with TGF-β isomers in isolation and other TGF-β combinations ($p<0.05$). It can be concluded that combination of TGF-β3 with any other TGF-β isomer other than TGF-β2 causes better cell detachment whilst a lack of TGF-β3 decreased cell detachment. Exceptionally, TGF-(β1+2+3) caused less detachment compared to those with TGF-β3 ($p<0.01$). Also in all cases with addition of TGF-β isomers better cell detachments were observed compared to the control, BSA, HCl and BSA/HCl. These results suggest that the application of TGF-β3 at 50ng/ml decreased the degree of cell attachment on surface as also shown graphically in Figure 3.7. BSA represented slowest degree of cell detachment compared to all other solutions ($p<0.05$).

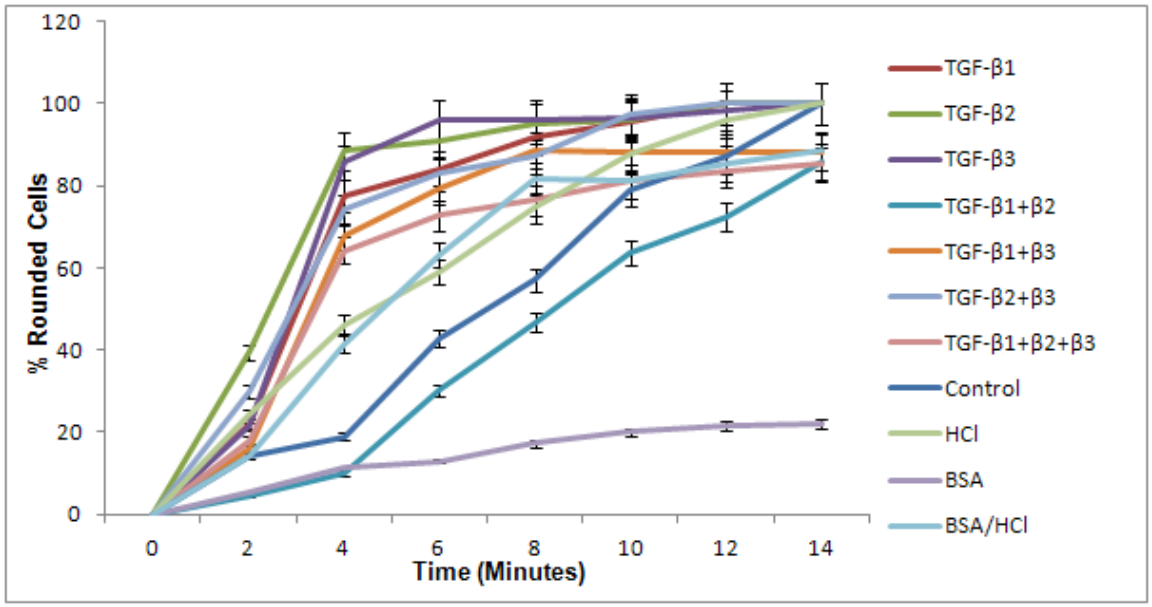


Figure 3.15 *Comparison between mean percentages rounded cells during trypsinisation process treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3)and un-treated as control (Error bars represent standard deviation).*

3.5 DISCUSSION

The natural process of healing a fracture starts when the injured bone and surrounding tissues bleeds, forming a fracture hematoma. The blood coagulates to form a blood clot situated between the broken fragments. It is well established that collagen type I, the major ECM protein of bone, mainly exist in intact bone (Tosounidisa, 2009). Within a few days of injury blood vessels develop into the matrix of the blood clot. The blood vessels bring fibroblasts in the walls of the vessels and these multiply and produce collagen fibres. Therefore, the blood clot is replaced by a matrix of collagen. However, collagen type I is not the only ECM protein present in bone and fibronectin also accumulates in the site of injury (Canavan, 2005b). This further supports the finding in the previous chapter in which MG63 cells responded mostly to the fibronectin patterns

suggesting that these cells attach mostly via fibronectin specific integrins. This is in term represents that cells take on a more migratory phenotype when cultured on fibronectin. This indicates that specific integrins associated with attachment to fibronectin could be important in bone wound repair. For this reason trypsinisation assay were carried out in this chapter in order to assess the effect of various TGF-βs treatment on cell detachment. Cell detachment using the trypsinisation assay has been investigated for different purposes in many cell types; however, in the case of osteoblasts the amount of information for cell detachment behaviour is limited. For instance, MG63 cell adhesion was measured using a trypsinisation assay (Abed, 2009). MG63 cells were seeded in 12-well plates for 4 days and treated with platelet-derived growth factor (PDGF) for 16hrs. Then cells were incubated with 1 ml of 0.05% trypsin-EDTA for 4 minute to promote cell detachment. Detached cells were collected by adding media. The number of detached cells under limited trypsinisation was then manually counted using a haemocytometer. Results showed that cell adhesion induced by PDGF was prevented when cells were transfected with melastatin-like transient receptor potential 7 (TRPM7) compared to the control (Abed, 2009). Another group also showed osteoblast-like cell adhesion on bioactive glasses (Foppiano, 2007a, Foppiano, 2007b). Surface reactions and resistance to trypsinisation have been studied. Osteoblast attachment and detachment phenomenon were also investigated for the successful integration of an orthopaedic implant into bone (Moutzouri, 2010). Human osteoblast was used to study the development of attachment for coated and uncoated surfaces. Current literature, or similar research, showed adhesion assay of osteoblast cells without the presence of any treatment and it was necessary for our research studies to know the detachment rate of MG63 over a period of time with the presence of various

TGF-βs treatments. In addition, many cell types such as osteoblast take on a migratory phenotype during wound repair and this can be associated with a change in the degree of cell surface attachment (Boland, 1996, Moutzouri, 2010). However, a decrease in cell surface attachment may be beneficial to changes in cell behaviour during wound healing and repair with the presence of different TGF-β isomers. Trypsinisation was also carried out in order to investigate its effect on cell detachment in the presence of HCl, BSA, BSA/HCl (as a TGF-β solvent and carrier) (Sefat, 2009a, Sefat, 2010c). HCl decreased cell attachment and this could be associated with cells taking on a migratory phenotype. This is supported in the literature by Dong et al, 2005. The aim of their study was to investigate the effect of taspine hydrochloride (TA/HCl) on skin wound healing in rats (Dong, 2005). The closure time of the skin wounds was significantly shorter in the TA/HCl treated groups than that in the control. TA/HCl has the ability of promoting skin wound healing in rats, and it can also accelerate the angiogenesis and raise the production of collagen in wound tissue. It has also been proposed (Oreffo, 1989) that TGF-β may not be the only reason for increasing the rate of wound repair in bone. In order for TGF-β to be administered exogenously it needs to be dissolved with HCl and BSA used as the carriers of TGF-β. It is suggested that HCl also contributes to the rate of wound repair (Centrella, 1988, Sefat, 2010b). This is particularly important seeing as there is evidence that the pH of injured bone can decrease and that this may play a role in activating TGF-β (Oursler, 1994, Dallas, 2002). These results also suggest that the application of TGF-β3 and HCl decreases the degree of cell attachment. It can be concluded that combination of TGF-β3 with any other TGF-β isomer decreases cell attachment whilst a lack of TGF-β3 increases cell attachment. This could be associated with changes in integrin expression. For this reason we will investigate the effects of

various TGF-βs and HCl in wound closure in a simple bone cell culture model in the next two chapters.

3.6 CONCLUSIONS

In conclusion, TGF-β3, HCl and HCl/BSA enhanced the rate of cell detachment in relation to the negative controls indicating perhaps that TGF-β3 does not act alone in cell detachment, but instead functions synergistically with signalling pathways that are dependent on the availability of hydrogen ions. Such a mechanism would depend on signalling molecules undergoing a conformational change on binding hydrogen ions, which is not a new concept. For comparison, a completely different response was recorded with the bone cells plated with TGF-β1, TGF-β2 and TGF-β3 as all three TGF-β isomers enhanced the cell detachment rate on their own, but induced different rates of detachment when combined together. It can also be concluded that the combination of TGF-β3 with any other TGF-β isomer causes better cell detachment, while TGF-β3 absence showed less cell detachment.

CHAPTER FOUR

4. EFFECTS OF DIFFERENT TRANSFORMING GROWTH FACTOR BETA (TGF-β) ISOMERS ON WOUND CLOSURE OF BONE CELL MONOLAYERS

4.1 INTRODUCTION

Bone repair can be modulated by different stimuli including growth factors (Hollinger, 2004, Guiqian, 2012). TGF-β is a cytokine known to be associated with the scarless healing of skin (Khan, 2000) and it is highly probable that it may play a role in the repair of other tissues including bone (Sefat, 2009b, Sefat, 2010d, Beggs, 2010). TGF-β can be found in three isomers: TGF-β1, TGF-β2 and TGF-β3. TGF-β has also been used to stimulate bone regeneration (Lind, 1993). TGF-β2 has been administrated for a period of 6 weeks in animal models with tibial defects (Lind, 1993). It was shown that TGF-β2 induced a higher healing rate compared to the control (Lind, 1993). The anabolic effects of TGF-β on bone repair are also dependent on the mechanical stability at the site of fracture (Critchlow, 1995). Unilateral tibial fractures were produced in a rabbit animal model and a plate (plastic /steel) was used in order to immobilise the fracture site. TGF-β2 was applied to the calluses 4 days after fracture. Results demonstrated that in animals with unstable fixation, TGF-β2 did not show an anabolic effect on callus formation, but animals with stable fixation enlarged calluses were developed (Critchlow, 1995). It is not clear whether or not TGF-β2 will always induce a positive response since different dosages of TGF-β2 have different effects (Duque,

2007). However, some believe that the anabolic effect of TGF-β may be due to its potentiation of bone morphogenic protein (BMPs) (Centrella, 1994). BMP-7 has been reported (Ripamonti, 1997) as having positive effects on bone formation when co-administered with low dosages of TGF-β1. Mixing these two growth factors induced a 2-3 fold increase in the cross sectional area of newly generated ossicles compared to treatment with BMP-7 alone. Previous studies have shown (Khan, 2000, Tosounidisa, 2009) that there is limited research regarding the use of TGF-β isomers in bone cells in terms of fracture repair and healing compared to other cell types such as keratinocytes (Sun, 2009, Shirakata, 2010, Mauvie, 2009), and also compared to other growth factors such as BMP (Croteau, 1999), platelets-derived growth factor (PDGF), insulin-like growth factor (IGF) (Thaller, 1993) and fibroblast growth factors (FGF) (Hurley, 1993). Therefore, there is a need to carry out further research on the effect of different TGF-β isomers and their combinations on bone repair and its related mechanism.

4.2 AIMS AND OBJECTIVES

The main objective of this chapter was to investigate the effect of different TGF-β isomers and their combinations on wound closure in cultured monolayers of MG63 bone cells. The lab-based experimental work investigated and compared the wound closure properties of three TGF-β isomers, HCl and BSA/HCl in cultured monolayers of human bone cells. Other cellular responses such as proliferation and detachment were also investigated along with morphology changes during wound closure in an *in vitro* model wound.

4.3 MATERIALS AND METHODS

4.3.1 Cell Culture

Bone cells were cultured in a low glucose (1g/L-D-Glucose) HEPES (25mM) buffered DMEM (Dulbecco's Modified Eagle Media, Sigma Aldrich) culture media supplemented with L-glutamine (2.5 mM U/mL), Penicillin (100 U/mL), Streptomycin (0.1 mg/mL), Amphotericin B or a fungizone (250µg/ml), (Sigma Aldrich-UK) and 10% FCS v/v (fetal calf serum- PromoCell - UK). The bone cells were plated in 25cm^2 tissue culture grade cell culture flasks at a cell density of 250,000 cells/culture flask and bathed in the culture media. All supplements were purchased from Sigma Aldrich-UK, except those otherwise mentioned.

4.3.2 Creating wound on cell monolayers

MG63 human bone cells were cultured and when confluent the cell monolayer was wounded (scratched) using a disposable plastic pipette of 1mm diameter. 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 underside of the flask was then marked with 3 parallel lines using a water resistant marker pen such that the lines intersected with the wound drawn in the monolayer of cells at 90^o. These markings facilitated orientation and allowed the same points to be repeatedly monitored and photographed every 5hrs (see Figure 4.1). A ‘test experiment’ was performed in order to determine the time frame of wound closure in the wounded cultures. The duration of this test experiment was 30hrs. This was similar to another experiment on wound closure using NIH/3T3 fibroblast monolayers (Hollinger, 2004, Sefat, 2009b) for which

wound closure was achieved after ~300 minutes. In their study, cell monolayers were wounded with “the corner of a piece of Mylar film which is commonly used in copy machines”.

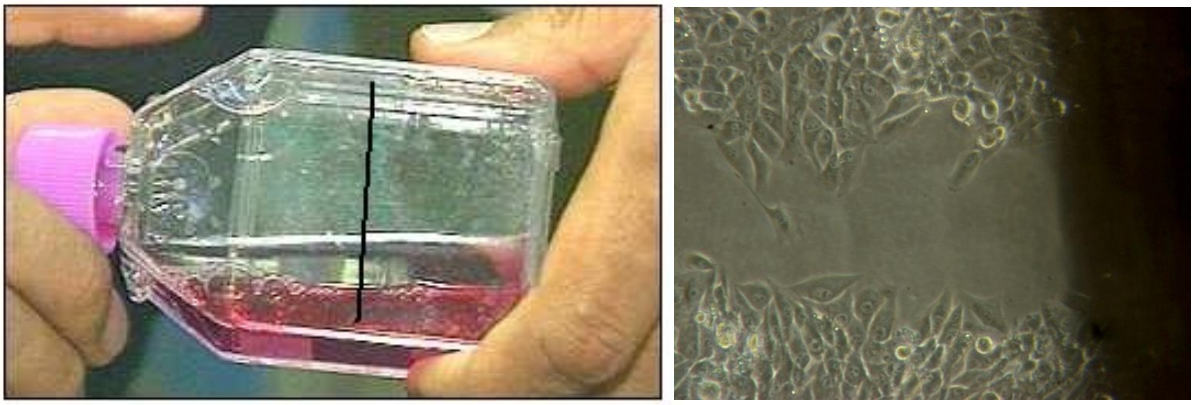


Figure 4.1 *Marked line determining point of reference on cultured monolayer for scratch assay.*

Scratching the cell monolayer resulted in wounds of approximately 300μm; however, there was some variability in wound widths. To normalise for this the mean starting wound width was calculated per treatment and each starting wound width was expressed as a percentage of that mean starting wound width. Wound width at each time point was in turn expressed as a percentage of the mean starting wound width per treatment. During our ‘test experiment’ (20hrs), it became clear that complete wound closure was not achieved during this time frame and thus the time frame for our experiment was set to 30hrs with data collection every 5hrs. Figure 4.2 (a-c) diagrammatically shows the steps involved in these experiments. All experiments in this chapter were repeated three times.

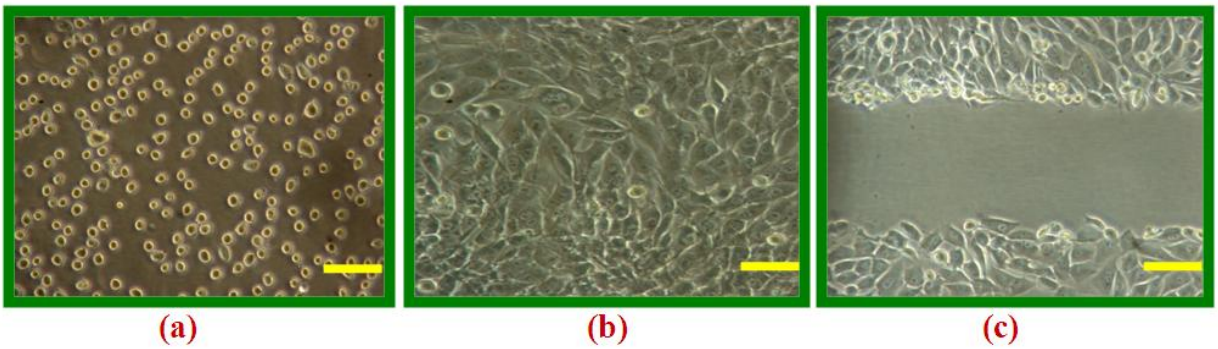


Figure 4.2 *Growth of human bone cells (MG63) in culture flask: (a) after cell seeding (b) confluence cell monolayer (after 3 days in culture) and (c) wounded cells ready for treatment; X100 magnification (Scale bar = 100 µm).*

Different TGF-β isomers (TGF-β1, TGF-β2 and TGF-β3) and their combinations (TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3)) were applied with the same concentrations described in Chapter 3 to investigate their effect on wound healing (Sefat, 2009b, Sefat, 2010d). The same method was applied for culturing the cells with HCl and BSA/HCl as TGF-β solvent (HCl) and carrier (BSA) with working concentrations of 50ng/ml, and without growth factor as a control.

4.3.3 Data acquisition

After wounding, culture flasks were stored inside the incubator and wound width was imaged and measured every 5hrs over a 30hrs period. The cells were photographed using a phase contrast light microscope and a digital camera. Pictures were captured and stored as tif images. Image J software was used in order to measure the distance between the wound edges. Ten vertical lines at approximately equal distances apart and the distances between the intersections of the lines with the wound edges were measured as shown in Figure 4.3. The measurements began at a scratch width of 300µm ± 10-30µm at 0mins. Graphs of percentage wound closure versus time were plotted for

the different TGF-β treatments and control (with no treatment). Comparison was made in terms of percentage wound closure. The whole procedure was repeated three times and the data acquired was combined to acquire mean % wound width at each timepoint.

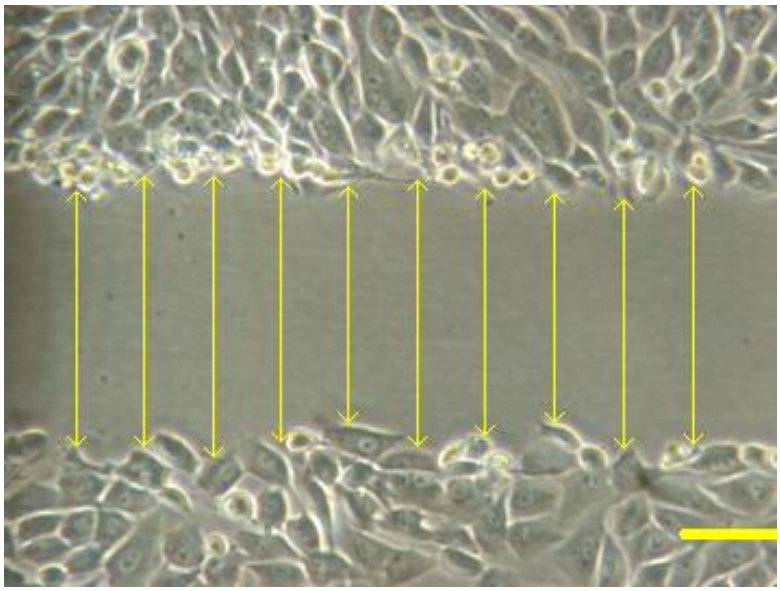


Figure 4.3 *Measurement of wound closure width on cultured MG63 bone cell monolayer using 'Image J' software; (Scale bar = 100µm)(Sefat, 2009b, Sefat, 2010d).*

4.4 STATISTICAL ANALYSIS

Mean % of wound remained open was tested for normality using a Kolmogorov Smirnov test. Results that showed normal distribution ($p > 0.05$) were analysed using SPSS via a Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed results ($p < 0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference. There was some variability in initial wound widths therefore, % wound closure was normalised as described previously.

4.5 RESULTS

4.5.1 The wound closure properties of HCl, BSA/HCl and control in cultured human bone cell monolayer

Figure 4.4 shows the entire wounded bone cell monolayers together during 30hrs of healing treated with HCl, BSA/HCl and control. Results for the control showed that there were only small changes in % wound remained open even after 10hrs (Sefat, 2009b, Sefat, 2010d). Cell morphology changes could be observed only in the cells at the wound edges after 15hrs (with mean % of wound remained open ~ 85.67% ± 1.31SD). These cells had elongated but without migration to the wound site. After 20hrs there were still gaps between the cells in the wound site. The cells in the control flask did not seem to form bridges with cells from the opposite wound margin to stabilize the wound site until about 25hrs (with mean % of wound remained open ~ 63.47% ± 0.56SD). Cells met each other after 25hrs and started making bridges but without complete closure. It was clear that wounds remained open even after 25hrs and that after 30hrs ~57.86% ± 0.62SD of the wound still remained open (See Table 4.1). For the HCl treatment, it was found that after 10hrs wound size had decreased by 50%. After 20hrs the cells treated with HCl showed a high percentage of wound closure (only 15.17% ± 0.40SD remained open). By comparison between control and HCl treated cultures, it became clear that after 30hrs the wound in the control remained open by ~ 57.86% ± 0.62 whereas HCl treated cultures seemed to almost reach full closure (p<0.001). This indicates that HCl even in buffered media had a significant effect on the wound healing process compared to the control. Treatment with BSA/HCl induced a similar wound closure response to treatment with HCl. From figures 4.4 and 4.5, it can be seen that

after 15hrs, the cells had migrated into the wound site and had started to meet cells from the opposite side of the wound similar in a way similar to those cells treated with HCl previously. These cells then formed bridges to connect the wound edges and closed the wound. As soon as the bridges were formed in the wound site the wound closed quite rapidly. During bridge formation the cells a distance away from the wound edge aligned behind those bridges to organize the cell structure and this seemed to aid wound closure after 25hrs (Sefat, 2009b, Sefat, 2010d).

Table 4.1 Descriptive statistics for mean % of wound remaining open against time for wounded monolayers treated with HCl, BSA/HCl and control.

Treatment	Wound Closure Time (hrs)	Mean % of Wound Remaining Open ± Std. Deviation (SD)	Std. Error (SE)
Control	0hrs	100.00 ± 1.10	0.34
	5hrs	94.13 ± 0.64	0.20
	10hrs	92.25 ± 0.9	0.28
	15hrs	85.67 ± 1.31	0.41
	20hrs	82.15 ± 0.73	0.23
	25hrs	63.47 ± 0.56	0.17
	30hrs	57.86 ± 0.62	0.19
HCl	0hrs	100.01 ± 1.48	0.46
	5hrs	93.44 ± 0.80	0.25
	10hrs	48.69 ± 0.6	0.18
	15hrs	31.87 ± 0.61	0.19
	20hrs	15.17 ± 0.40	0.12
	25hrs	6.19 ±1.00	0.31
	30hrs	0.00	0.00
BSA/HCl	0hrs	100.00 ± 1.32	0.42
	5hrs	71.49 ± 0.61	0.19
	10hrs	49.83 ± 0.89	0.28
	15hrs	41.92 ± 0.41	0.13
	20hrs	17.68 ± 0.52	0.16
	25hrs	9.96 ± 0.85	0.27
	30hrs	0.00	0.00

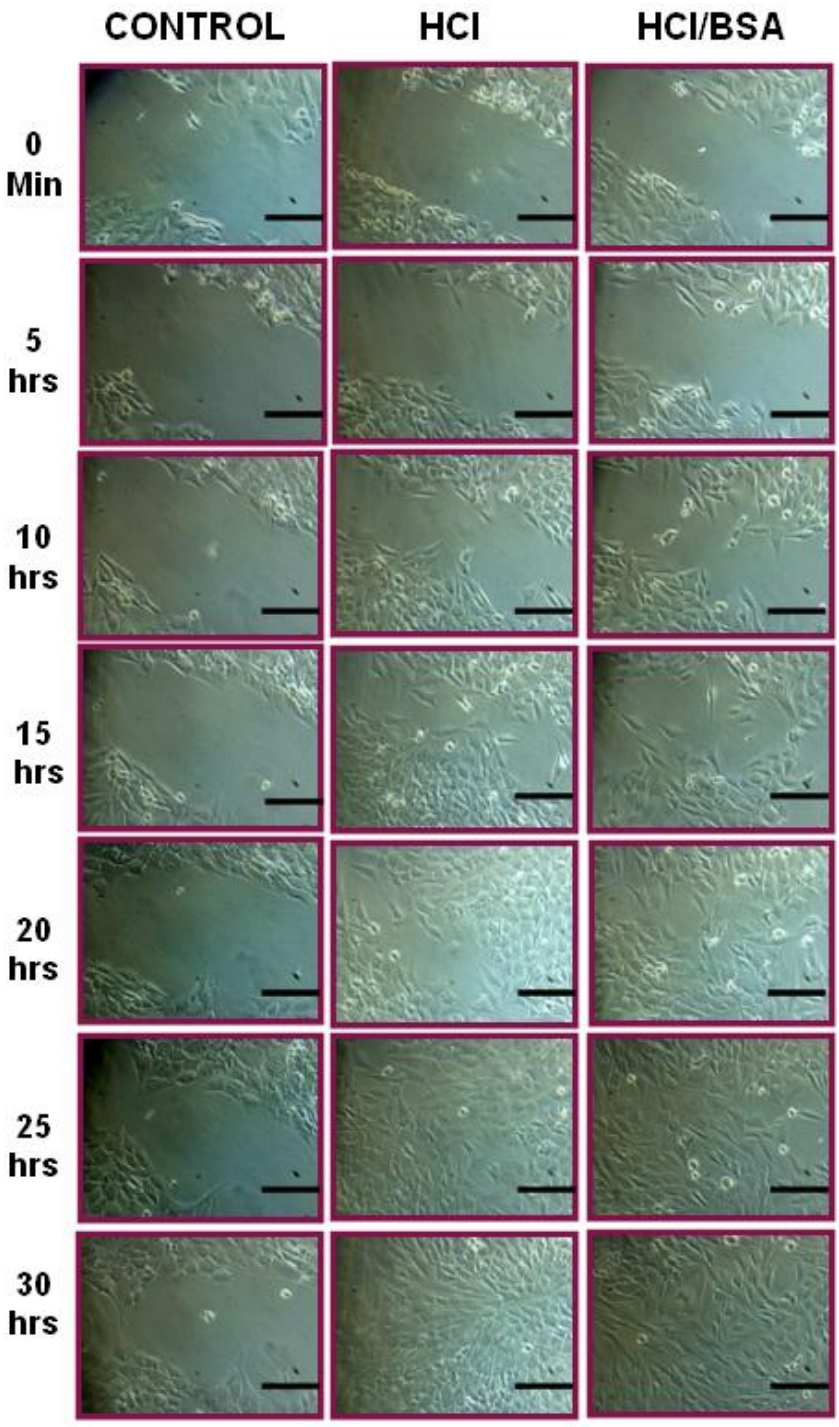


Figure 4.4 *Images of the wound healing process for the bone cell monolayers treated with 50ng/ml of HCl, BSA/HCl and un-treated as control for the period of 30hrs (Scale bar=100μm)(Sefat, 2009b, Sefat, 2010d).*

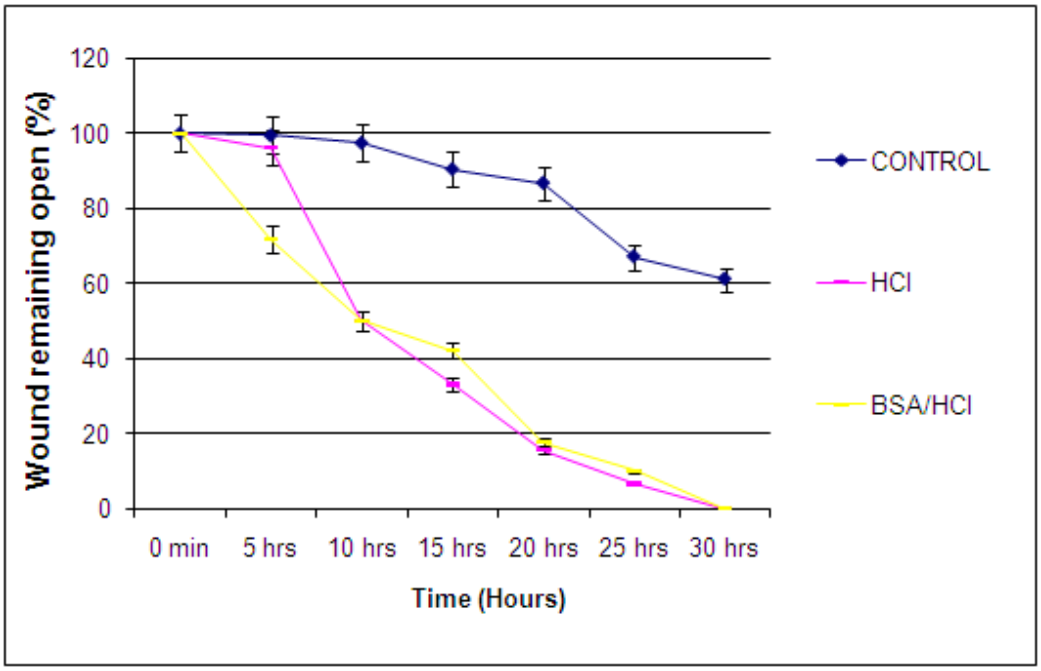


Figure 4.5 *Graph of normalised % wound remaining open against time for wounded bone cell monolayers treated with HCl, BSA/HCl, and un-treated as control (Error bars represent standard deviation).*

However, the control culture flasks did not perform well with 82.15% ± 0.73SD of the wound remaining open after 20hrs. In contrast, the HCl treated culture flask had only 15.17% ± 0.40SD of the wound still open. This indicates that HCl had significant effect on wound closure ($p<0.001$, See Table 4.1). Also, in cultures treated with BSA/HCl the % of wound remained open was 17.68% ± 0.52D after 20hrs. Statistical analysis shows that there were a significant differences between HCl and BSA/HCl treated flasks at 15, 20 and 25hrs ($p<0.001$).

4.5.2 The Wound Closure Properties of TGF-β1, TGF-β2 and TGF-β3 in Cultured Human Bone Cell Monolayer

Figure 4.6 shows all of the wounded bone cell monolayers together during 30hrs of healing treated with TGF-β1, TGF-β2, TGF-β3 and un-treated as control. In the case of TGF-β1 it was observed that after 5hrs wound closure was faster compared to the control (with mean % wound remained open ~ 77.48% ± 1.11SD for TGF-β1 and 94.13% ± 0.64SD for control) (Sefat, 2009c). Cells from both wound edges had elongated in order to contact the opposite cells and form bridges after ~15hrs of healing process (with mean % wound remained open ~ 48.92% ± 1.38SD). Cells had migrated along these bridges and a distance away from the wound edge they were aligned to reorganize the cell structure. These cells had elongated in order to fill the gaps created by the migrated and elongated cells at the wound edges. After 20hrs, cells covered the wound site, but there were still some gaps between cells. It was observed that after 20hrs all the cells treated with TGF-β1 showed a high percentage of wound closure (with mean % of wound remained open ~ 29.77% ± 1.20SD). Elongation and bridging of the cells inside and outside the wound site was also observed and wound healing still continued even after 30hrs. The remaining wound following TGF-β1 healed very fast after 25hrs and the percentage of wound remaining open was ~13.93% ± 2.46SD after 30hrs which meant that wound healing was not complete even after 30hrs. The results indicate that TGF-β1 does indeed speed up the wound closure process in a pure cell culture environment. Statistical analysis show that TGF-β1 treated flasks at 25hrs has significantly improved the wound closure response as compared to the control (p<0.001) and reflected by % wound remained open (19.63% for TGF-β1 and 63.47% for control).

In the case of TGF-β2 it can be seen that after 5hrs the morphology of the cells at the wound edges have changed and that the cells seems to be elongated. After 15hrs the cells had migrated into the wound site and had begun to meet cells from the opposite side of the wound. As soon as the bridges formed in the wound site the wound was closed quite rapidly. It is useful to note that this process of wound closure is very similar to that following treatment with TGF-β1 (Sefat, 2009c, Sefat, 2009d). After 20hrs, cells covered the wound site (with percentage of wound remained open ~ 31.89% ± 1.20SD) but there were still some gaps between cells. It was seen that after 25hrs all the culture flasks treated with TGF-β2 showed high percentage of wound closure (only ~ 15.43% ± 2.76SD of the wound remained open). Wound healing was not completed even after 30hrs and there were still some gaps between cells (only 8.67% ± 1.34SD of wound remained open). Statistical results (Table 4.2) show that TGFβ2 treatment caused model wounds to heal faster than control (p<0.001), but induced a significantly slower rate of wound closure when compared to treated flasks with HCl (p<0.001) and BSA/HCl (p<0.001) (Sefat, 2009d).

Despite cultures treated with TGF-β1 and TGF-β2 it was observed that cells treated with TGF-β3 had already elongated and started to migrate to the wound axis after 5hrs (~62.25% ± 1.65SD wound remained open). After 10hrs the cells had already covered a great part of the wound site (~41.95% ± 0.76SD wound remained open). It was seen that after 20hrs all the cells treated with TGF-β3 showed a high percentage of wound closure (only ~16.19% ± 1.54SD wound remained open) and the remaining wound healed very fast after 25hrs and the percentage of wound remaining was almost zero which meant

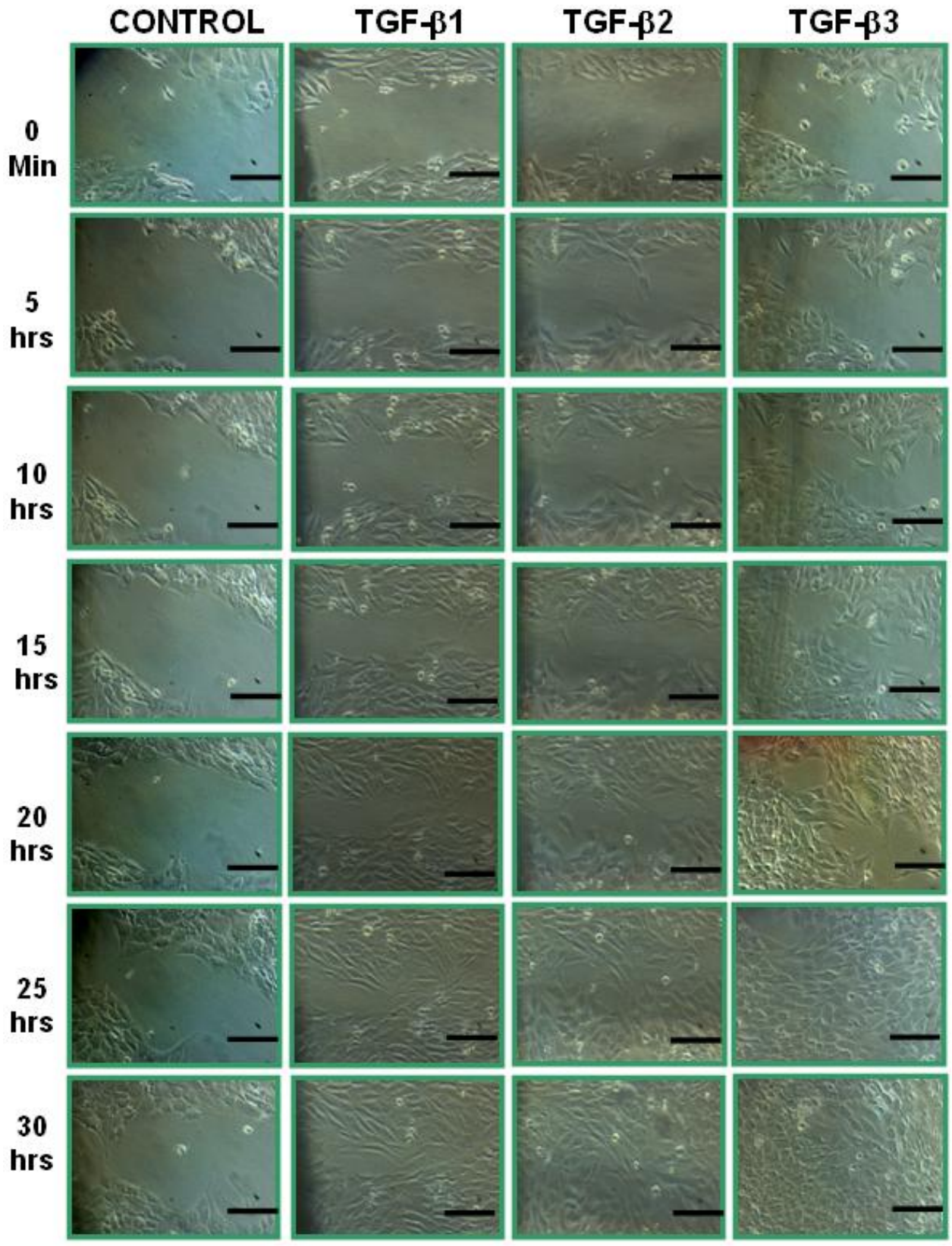


Figure 4.6 *Photomicrographs of the wound healing process for the bone cell monolayers treated with TGF-β1, TGF-β2, TGF-β3 and control for the period of 30hrs (Scale bar=100μm)(Sefat, 2009b, Sefat, 2009c, Sefat, 2009d).*

Table 4.2 Descriptive statistics for % of wound remaining open against time for wounded monolayers treated with TGF-β1, TGF-β2, TGF-β3 and un-treated as control.

Treatment	Wound Closure Time (hrs)	Mean % of Wound Remaining Open ± Std. Deviation (SD)	Std. Error (SE)
Control	0hrs	100.00 ± 1.10	0.34
	5hrs	94.13 ± 0.64	0.20
	10hrs	92.25 ± 0.9	0.28
	15hrs	85.67 ± 1.31	0.41
	20hrs	82.15 ± 0.73	0.23
	25hrs	63.47 ± 0.56	0.17
	30hrs	57.86 ± 0.62	0.19
TGFβ1	0hrs	100.00 ± 1.52	0.48
	5hrs	77.48 ± 1.11	0.35
	10hrs	63.35 ± 0.86	0.27
	15hrs	48.92 ± 1.38	0.43
	20hrs	29.77 ± 1.20	0.38
	25hrs	19.63 ± 2.82	0.89
	30hrs	13.93 ± 2.46	0.77
TGFβ2	0hrs	100.00 ± 1.80	0.57
	5hrs	69.86 ± 2.64	0.83
	10hrs	61.55 ± 2.27	0.72
	15hrs	48.94 ± 1.73	0.54
	20hrs	31.89 ± 1.20	0.38
	25hrs	15.43 ± 2.76	0.87
	30hrs	8.67 ± 1.34	0.42
TGFβ3	0hrs	99.99 ± 1.55	0.49
	5hrs	62.25 ± 1.65	0.52
	10hrs	41.95 ± 0.76	0.24
	15hrs	21.07 ± 0.75	0.23
	20hrs	16.19 ± 1.54	0.48
	25hrs	0.00	0.00
	30hrs	0.00	0.00

that the wound healed completely after 25hrs. These results indicate that TGF-β3, HCl, and BSA/HCl treatments show significant faster wound healing compared to the cultures treated with TGF-β1 (p<0.001), TGF-β2 (p<0.001) and un-treated as control (p<0.001). Table 4.2 and Figures 4.6 and 4.7 show results for the % wound remained

open against time for wounded bone cell monolayers treated with TGF-β1, TGF-β2, TGF-β3 and un-treated as control.

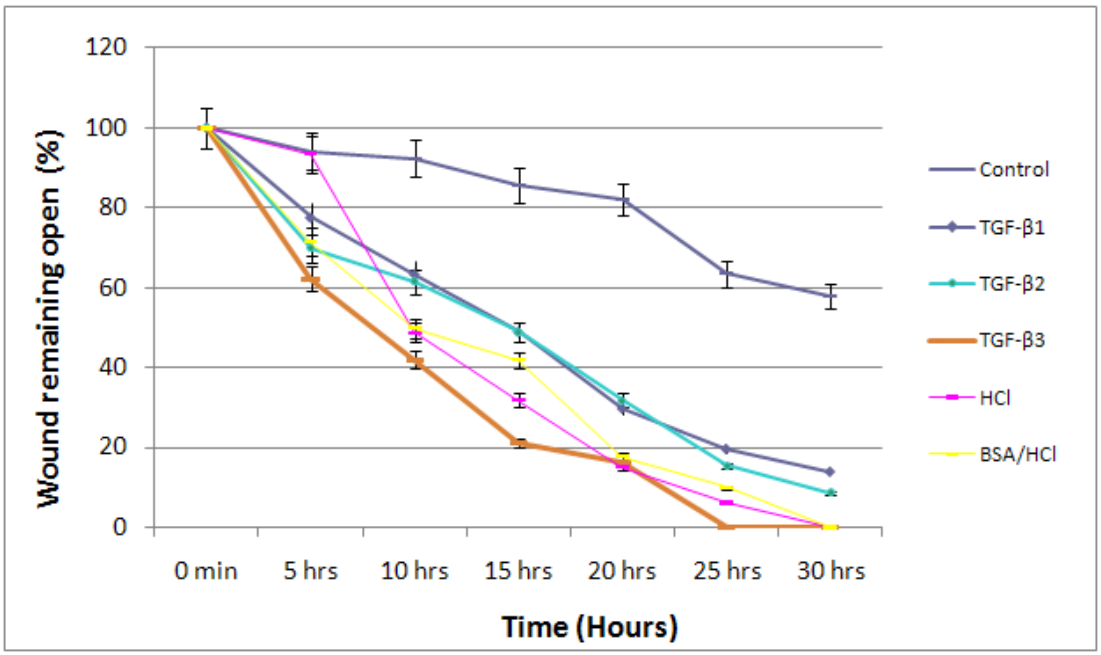


Figure 4.7 *Graph of normalised % wound remaining open against time for wounded bone cell monolayers treated with TGF-β1, TGF-β2, TGF-β3, HCl, BSA/HCl, and control (Error bars represent standard deviation).*

4.5.3 The Effect of Combinations of Transforming Growth Factor Beta Isomers TGF-β(1+2), TGF-β(2+3), TGF-β(1+3) and TGF-β(1+2+3) On Wound Closure of Bone Cell Monolayers

Figure 4.8 shows wounded bone cell monolayers during 30hrs of healing treated with various combinations of three TGF-β isomers including TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and untreated flask as control. In the case of TGF-β(1+2) wound healing was not observed after 5hrs, while after 15hrs the cells replication had increased at the wound edge. After 20hrs the cells did not cover a great part of the

wound site (~ 63.19% ± 1.55SD of wound remained open) and showed slower healing compared to the other TGF-β combinations but closed faster than control wounds. Cells finally formed bridges at 30hrs and about 37.77% ± 1.90SD of wound remained open, for the TGF-β(1+2) combination. Cells in culture flasks treated with TGF-β(1+2+3) showed similar behaviours up to 20hrs and formed bridges 25hrs after wounding (with mean % of wound remained open ~ 40.83% ± 0.52SD) which was significantly faster compared to TGF-β(1+2) (with mean % of wound remained open ~ 47.88% ± 1.94SD) (p<0.001). More cells could be seen in the middle of wound after 25hrs of healing but similar to TGF-β(1+2) there were still some gaps between cells (~ 33.60% ± 1.12SD of the wound still remained open at 30hrs). In the case of TGF-β(1+3) it was observed that after 5hrs of incubation the cells had already elongated and started to migrate perpendicularly to the wound. After 25hrs cells started forming bridges and full wound closure did not occur at 30hrs but the rate of wound repair was significantly faster (25.33% ± 0.73SD of wound remained open at 30hrs) compared to TGF-β(1+2) (p<0.001) and TGF-β(1+2+3) (p<0.001). Figure 4.8 also shows the images for the wound closure process with TGF-β(2+3) treated culture flask. It was observed that after 5hrs the cells had already enlarged and elongated. After 25hrs the cells had already covered a great part of the wound site and wound healing was not completed even after 30hrs (23.87% ± 0.94SD of wound remained open). However, compared to all other TGF-β combinations, TGF-β(2+3) showed the fastest rate of wound closure. Table 4.3 show descriptive statistics for the % wound remained open against time for wounded bone cell monolayers treated with TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and untreated as control. Results indicated that there was no significant difference in % wound closure between TGF-β(1+3) and TGF-β(2+3), while there were

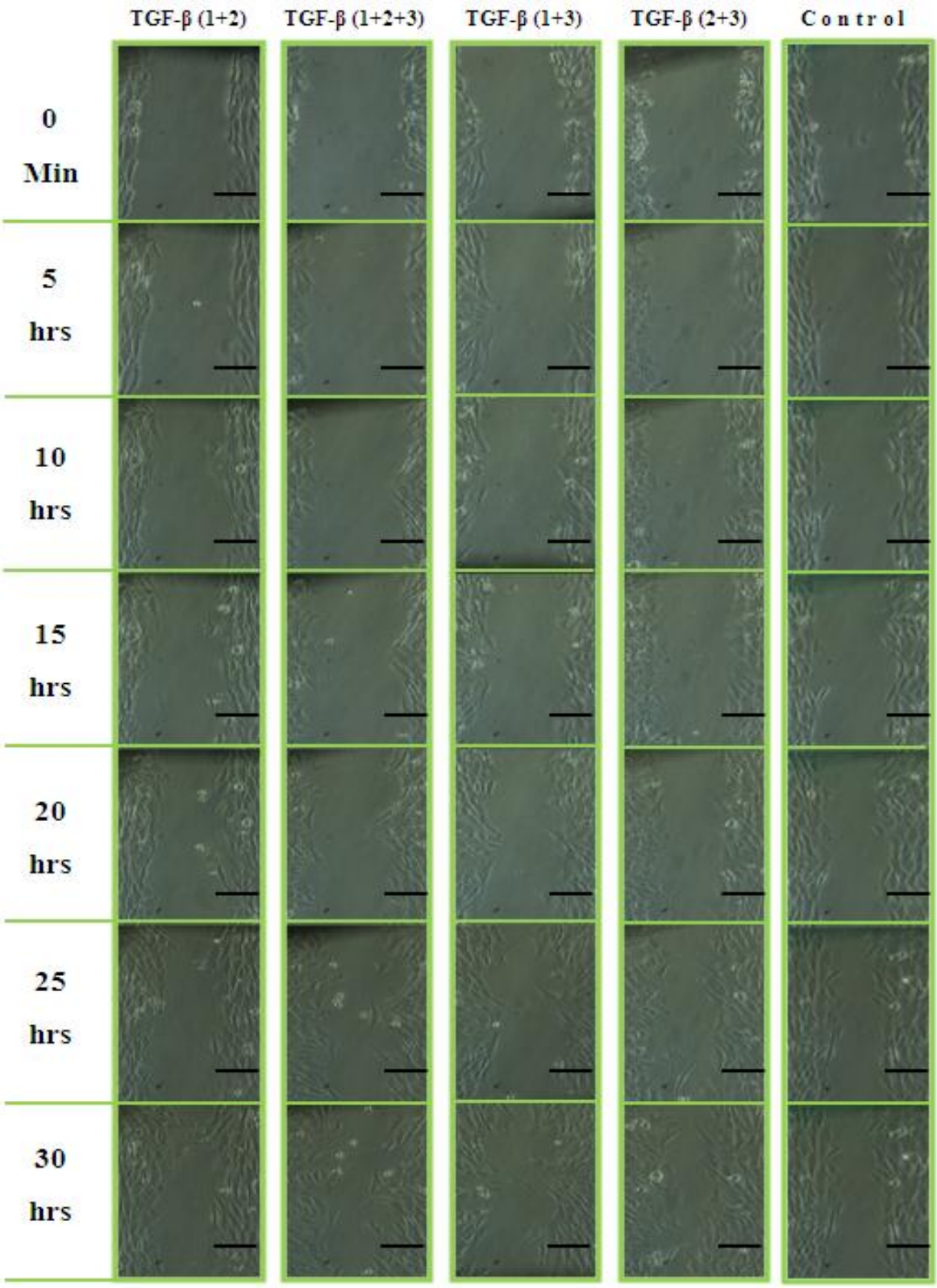


Figure 4.8 *Photomicrographs of the wound healing process for the bone cell monolayers treated with TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control for the period of 30hrs (Scale bar=100μm).*

significance differences in % wound closure between other possible TGF-β combinations (p<0.001) (See Table 4.4). The results also showed that TGF-β(2+3) increased the wound closure rate in a cell culture environment faster than the other TGF-β combinations. TGF-β(1+2) showed the slowest healing rate, but it enhanced wound closure as compared to the control (p<0.001). Overall comparison between all treatments represent that cells treated with HCl and BSA/HCl induced a decrease in the % wound remained open compared to all TGF-β combinations (p<0.001) whereas complete wound closure was achieved after 30hrs of healing in both HCl and BSA/HCl. This result also suggested that cells treated with TGF-β3, TGF-β2 and TGF-β1 showed significant faster wound healing compared to all TGF-β combinations (p<0.001) (See Figures 4.8, 4.9 and Table 4.3).

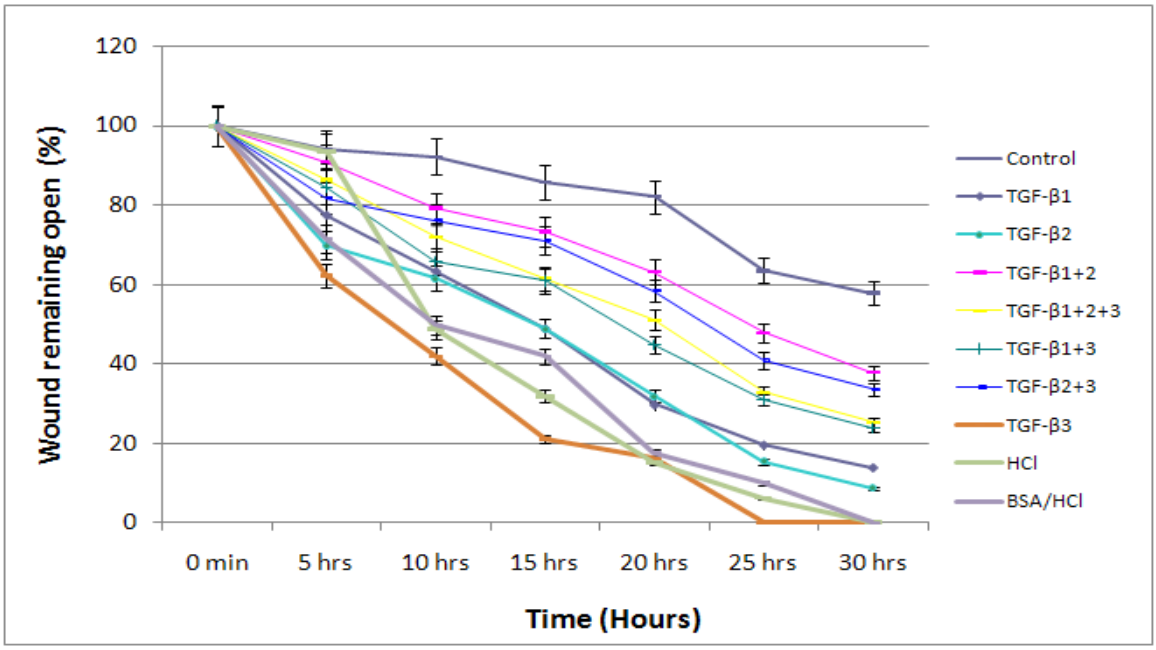


Figure 4.9 *Graph of normalised % wound closure against time for wounded bone cell monolayers treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control (Error bars represent standard deviation).*

Table 4.4 is the tabulated results to show the P value for the % of wound remained open against time for wounded bone cell monolayers treated with all TGF-β isomers and their combinations including TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3), HCl, BSA/HCl and un-treated as control.

Table 4.3 Descriptive statistics for mean % of wound remaining open against time for wounded monolayers treated with TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3)and TGF-β(2+3).

Treatment	Wound Closure Time (hrs)	Mean % of Wound Remaining Open ± Std. Deviation (SD)	Std. Error (SE)
TGFβ1+2	0hrs	100.01 ± 1.25	0.39
	5hrs	90.92 ± 1.33	0.42
	10hrs	79.22 ± 2.07	0.65
	15hrs	73.26 ± 2.12	0.67
	20hrs	63.19 ± 1.55	0.49
	25hrs	47.88 ± 1.94	0.61
	30hrs	37.77 ± 1.90	0.60
TGFβ1+3	0hrs	99.98 ± 1.77	0.56
	5hrs	86.35 ± 0.84	0.26
	10hrs	71.98 ± 1.07	0.34
	15hrs	61.42 ± 1.18	0.37
	20hrs	50.99 ± 1.38	0.43
	25hrs	32.76 ± 1.36	0.43
	30hrs	25.33 ± 0.73	0.23
TGFβ2+3	0hrs	100.00 ± 2.17	0.68
	5hrs	84.60 ± 1.16	0.36
	10hrs	65.79 ± 1.2	0.38
	15hrs	60.92 ± 0.96	0.30
	20hrs	44.86 ± 1.40	0.44
	25hrs	30.95 ± 0.93	0.29
	30hrs	23.87 ± 0.94	0.29
TGFβ1+2+3	0hrs	99.98 ± 0.578	0.18
	5hrs	81.83 ± 1.47	0.46
	10hrs	76.24 ± 0.61	0.19
	15hrs	71.07 ± 0.83	0.26
	20hrs	58.43 ± 0.97	0.30
	25hrs	40.83 ± 0.52	0.16
	30hrs	33.60 ± 1.12	0.35

Table 4.4 P values derived from pair wise comparisons of treatment related % wound remained open. Pairs that are significantly different from one another showing marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	Wound Closure 0hrs	Wound Closure 5hrs	Wound Closure 10hrs	Wound Closure 15hrs	Wound Closure 20hrs	Wound Closure 25hrs	Wound Closure 30hrs
Control – HCl	1.000	0.041*	0.001***	0.001***	0.001***	0.001***	0.001***
Control – BSA/HCl	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ1	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
HCl - BSA/HCl	1.000	0.001***	0.587	0.001***	0.001***	0.001***	1.000
HCl - TGFβ1	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
HCl - TGFβ2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
HCl - TGFβ3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	1.000
HCl - TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
HCl - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
HCl - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
HCl - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
BSA/HCl - TGFβ1	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
BSA/HCl - TGFβ2	1.000	0.023*	0.001***	0.001***	0.001***	0.001***	0.001***
BSA/HCl - TGFβ3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	1.000
BSA/HCl - TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
BSA/HCl - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
BSA/HCl - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
BSA/HCl - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ2	1.000	0.001***	0.056	0.705	0.705	0.013*	0.001***
TGFβ1 - TGFβ3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ1+2+3	1.000	0.001***	0.001***	0.008**	0.008**	0.001***	0.001***
TGFβ1+3 - TGFβ2+3	1.000	0.003**	0.001***	0.226	0.226	0.005**	0.190
TGFβ1+3 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2+3 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***

The overall results show that complete healing did not occur in cell cultures treated with four different TGF-β combinations, whereas TGF-β3 on its own induced complete healing in a similar time frame (Sefat, 2009b, Sefat, 2010d). TGF-β1 and TGF-β2 showed faster rate of wound closure compared to any TGF-β combinations (p<0.001). In all culture flasks with combined TGF-βs, healing rate was significantly better than that seen in the control treatment groups (p<0.001). Figure 4.9 also showed that the presence of TGF-β3 in any combination caused faster closure rates when compared to others without presence of TGF-β3. For instance, TGF-β(1+2) had much slower rate of closure compared to the other combined culture flasks. The presence of TGF-β1 in any TGF-β combination reduced the healing rate, while presence of TGF-β2 or TGF-β3 showed increase in bridge formation and wound healing. It was also seen that even after 30hrs none of the cells treated with TGF-β1, either alone or combined showed complete closure. These results suggested that presence of TGF-β1 had some inhibitory effect on bone cell elongation, proliferation and healing, while TGF-β3 did not show these effects in a pure cell culture environment. The data in Figure 4.9 showed that as time of the healing increased up to 25hrs, the rate of wound closure increased in the case of all TGF-β isomers either alone or in combined form. There are also significant differences between TGF-β3 and the other two isomers (p<0.001) while there were no significant difference in % wound closure between TGF-β1 and TGF-β2 (See Tables 4.2 and 4.4). However, there were significant differences in % wound closure between TGF-β1, 2 and 3 with all TGF-β combinations (P < 0.001), (See Table 4.4). These results suggest that wound healing took place in all culture flasks but at different rates while TGF-β3 caused the fastest wound closure followed by HCl (p<0.001) and BSA/HCl (p<0.001) whereas control induced the slowest rate of healing. All TGFβ combinations resulted in

faster healing particularly those treated with TGFβ (2+3) (p<0.001). TGFβ1 and TGFβ2 treated culture flasks induced faster healing compared to all TGFβ combinations but complete closure were not achieved. In summary, TGF-β3 had the most significant effect followed by HCl, BSA/HCl, TGF-β2, TGF-β1, TGF-β(2+3), TGF-β(1+3), TGF-β(1+2+3), TGF-β(1+2) and control.

4.6 DISCUSSION

Following scratching of the bone cell monolayers, and after 5 hrs of incubation, the % wound remained open showed different rates of healing depending on different treatments. This rate was significantly higher for all TGF-β isomer treated cells compared to un-treated controls. There is significant enhancement in the % of wound remaining open for TGF-β3 (P < 0.001) and TGF-β2 (P < 0.001). As the time of healing increased up to 25 hrs, the rate of wound closure also increased in the case of TGF-β1 and TGF-β2 compared to TGF-β3. There were no significant differences in the % wound remaining open between TGF-β1 and TGF-β2 for the first 20 hrs. These rates increased and then became gradually similar to each other in the case of TGF-β3, HCl and HCl/BSA. In the case of combined TGF-β isomer treated cells this rate was significantly higher (P < 0.001) (See Table 4.4) compared to the un-treated control. However, the wound width showed a significant increase in healing rate for TGF-β(2+3) (P < 0.001) and TGF-β(1+3) (P < 0.001) compared to the control and other TGF-β combinations. As the time of healing increased up to 25 hrs the rate of wound closure increased in all cases of TGF-β isomers, however, there were highly significant differences in % wound closure between TGF-β1, 2 and 3 with all TGF-β combinations

($P < 0.001$) (See Table 4.4). It is important to know why cell monolayers respond differently to each TGF-β. Cytokines are known as potent biomolecules that regulate cellular functions and play multiple roles in the initiation and inhibition of disease (Hynes, 1992, Solheim, 1998). The transforming growth factor-beta (TGF-β) superfamily is a group of cytokines (Lawrence, 2001) which are actively involved in the control of some cellular activities such as cellular proliferation, cell migration, adhesion and apoptosis. TGF-β isomers are involved in signal transduction between the extracellular environment and the nucleus. Some of these isomers are potent regulators in cellular development (Raftery, 2002b). TGF-β was initially identified in 1978 (Derynck, 2008) and is produced by almost all cell types in an inactive/latent form (Schwartz, 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 bone repair is, as yet, not fully understood. There have been studies on the effect of TGF-β on bone (Solheim, 1998, Zande, 2007, Dallas, 2002), the majority of these studies are not related to the bone's wound repair. The TGF-β seems to influence changes in cell behavior by up and down regulatory membrane proteins (Spangoli 2007) and, consequently, inducing cell adhesion, proliferation, differentiation, activation, migration and apoptosis (Krauss, 2006b). TGF-β has been used to stimulate bone regeneration (Lind, 1993). Using an animal model with TGF-β2 administered for a period of 6 weeks, in the case of tibial defects, it was shown that TGF-β2 had a positive effect on fracture repair (Lind, 1993). In terms of wound healing, cells can behave differently under various conditions. It is important to note that the interaction of cells (via membrane receptors) with ligands expressed by neighboring cells, and cell-cell signaling play an important role in cell proliferation (Fox, 2008). In fact, cell-cell

communication can stimulate cell proliferation (Celeste, 2002). Interestingly our studies indicated that the bone monolayers treated with TGF-β (mainly TGF-β3, TGF-β2+3, TGF-β1+2+3) demonstrated a rapid decrease in wound width during 25hrs of wound healing. This may be related to TGF-β3 increasing the secretion of ECM molecules at the wound edge, which functions by capturing cells via the integrin ECM interactions at the wound edge, thus helping migration into the wound site (Celeste, 2002). Such a mechanism may be useful in therapeutic procedures by filling a wound site with a large number of bone cells, which would presumably enable the deposition of ECM across the wound surface enabling cell migration and proliferation into the wound. The results of this study (Sefat, 2009b, Sefat, 2009c, Sefat, 2009d, Sefat, 2010d) together with the results in chapter 3 (Sefat, 2009a, Sefat, 2010c) also suggest that TGF-β2 and TGF-β3 do not stimulate bone-ECM adhesion. Bone cells cultured in a medium with TGF-β2 and TGF-β3 supplementation separately displayed a greater degree of cell/surface detachment. In contrast, the degree of cell surface attachment was increased in bone cells cultured with TGF-β1. However, the negative effect of this cytokine in the wound repair of bone suggests the hypothesis that the inhibition or activation of TGF-β1 may induce bone repair (Davidson, 2005, Sefat, 2009a). TGF-β1 induced a slower healing effect *in vitro* compared to TGF-β3 and TGF-β2 suggesting that the TGF-β1 down-regulates cell proliferation and cell migration. The healing process of a scratched bone monolayer started after 2 hrs, which was mainly due to what appears to be a stress shock effect or stress responsive delay in cell activation. Similar results have been reported with chondrocytes and keratinocytes cells (Bordeleau, 2008, Khaghani, 2009a, Khaghani, 2009b). TGF-β1 in chondrocytes plays a very important role in rapid formation of fibroblast like chondrocyte cells *in vitro* (Chi, 2004). 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 (Annes, 2003, Chin, 2004).

Growth factors can also be combined in order to target specific cell types, which results in certain functions such as proliferation, differentiation and migration. Previous studies (Zande, 2007) have used a similar approach with different growth factors in order to stimulate osteogenic expression *in vitro* by using a combination of different cytokines such as TGF-β1 and BMP-2. It is well established that the TGF-β super-family, mainly TGF-β1, 2 and 3, fulfil specific roles in the formation of new bones (Zande, 2007). It is therefore important to understand the effect of a combination of TGF-β isomers in wound closure response. This work concurs with what was found in the literature (Ackermann, 2011). This research group evaluated that combinations of growth factors such as VEGF, bFGF and PDGF have been shown to improve wound healing in streptozotocin-induced Diabetes in mice. Results showed that complete closure was reached in combined growth factors whereas the PDGF alone needed more time for complete closure. However, no clinical research has yet been undertaken on TGF-β3, thus this work was a first step for evaluating the effects of TGF-β3, HCl and HCl/BSA *in vitro* in relation to wound closure and the healing process for bone cell monolayers. Interestingly, there was little difference on the rate of wound repair between cells treated with TGF-β3 and HCl. It is possible that HCl directly influences bone cell behaviour or that HCl/free hydrogen ions could activate latent signalling proteins in the serum within the culture media. The availability of hydrogen ions could be essential for these signalling pathways to accelerate wound repair through administration of HCl. For

this reason the effect of different HCl concentrations on wound healing of bone cell monolayer were investigated in the next chapter.

4.7 CONCLUSIONS

In terms of wound healing, all TGF-β treatments caused different rates of wound closure *in vitro*. TGF-β3 gave the best wound closure (with 0% of the wound remaining open after 30hrs), followed by TGF-β2 (with 8.67% ± 1.34SD of the wound remaining open after 30hrs) and TGF-β1 (with 13.93% ± 2.46SD of the wound remaining open after 30hrs). Also, complete wound closure was achieved after 30 hrs by the presence of HCl as the solvent and BSA/HCl as the carrier and solvent, whereas the wound in control remained open within the same time period. This indicated that TGF-β3 does have a significant effect on the wound healing process and its healing rate was found to be higher than the control (p<0.001), TGF-β1 (p<0.001), TGF-β2 (p<0.001), BSA/HCl (p<0.001) and HCl (p<0.001) in ascending order. Combinations of TGF-β isomers behaved similarly *in vitro* but with different rates of wound closure. All TGF-β combinations showed a faster healing rate than the control (p<0.001). It was expected that the healing rate following treatment with TGF-β combinations would be greater than those healing rates following treatments with TGF-β isomers alone, but this was not the case. No complete wound closure occurred after treatment with any TGF-β combinations while TGF-β3 on its own induced complete healing in a similar time frame. TGF-β1, TGF-β2 and TGF-β3 showed a significantly faster rate of wound closure compared to a culture flask treated with TGF-β combinations (P < 0.001). Combining TGF-β3 with any other TGF-β isomer also resulted in a faster closure rate

(p<0.001) while the presence of TGF-β1 in any TGF-β combination reduced the healing rate (P<0.001). It can therefore be concluded that the presence of TGF-β1 has some inhibitory effect on bone wound healing while TGF-β3 had the opposite effect and increased the rate of wound closure in a pure cell culture environment. There was a significant difference in the rate of wound repair between cells treated with TGF-β3 and HCl (P < 0.001). For this reason the effect of different HCl concentrations on wound healing of bone cell monolayer were investigated in the next chapter. Changes in wound closure could be associated with variations in cell proliferation, elongation and migratory phenotype of cells in the presence of different TGF-β isomers which carried out in chapter 6.

CHAPTER FIVE

5. THE EFFECTS OF DIFFERENT HCl CONCENTRATION ON WOUND CLOSURE OF CULTURED HUMAN BONE CELL MONOLAYER

5.1 INTRODUCTION

Bone is unique in nature since it has the ability to heal without the development of a fibrous scar (Centrella, 1988, Glowacki, 1998, Guiqian, 2012). The regeneration of bone follows a complex set of four overlapping phases, which are characterised by specific molecular and cellular events (Gerstenfeld, 2003, Einhorn, 1998, Bolander, 1992). The four phases include: haematoma formation/inflammatory phase, soft callus formation, hard callus formation and the remodeling process. Bone has the ability to repair itself using different methods depending on the biophysical environment. Ultimately bone synthesis is achieved through osetoblasts via woven and/or lamellar matrix. Investigations have shown that with an increased ageing population healthcare in the United Kingdom is set to cost millions of pound each year and a large percentage of that will go towards the treatment of orthopedic problems (Strom, 2011, Sefat, 2010b). According to new research presented at the European Congress on Osteoporosis and Osteoarthritis the cost of fractures and different orthopeadic problems in six major European countries shows that the UK loses £5.1 billion as a result of the problem (Strom, 2011).

There are many factors that affect bone repair including age, nutrients, hormones, and growth factors, etc. The transforming growth factor-Beta (TGF-β) superfamily has been reported as a bone formation stimulus (Janssen, 2005). They also showed that TGF-β has detrimental effects on bone construction and resorption. Other groups have claimed that bone formation is induced *in vivo* by osteoblasts in response to TGF-β injected into the fracture site (Noda, 1989, Marcelli, 1990). TGF-β has been identified in the fracture haematoma and surrounding periosteal mesenchymal cells with staining techniques (Joyce, 1990), and may orchestrate the cascade of cellular events resulting in fracture repair. All three isoforms (TGF-β1, -β2, -β3) have been found (Sodeka, 1994) to have overlapping functions *in vivo* with different biological activities in vitro. TGF-β1 is more potent throughout adult development, whereas TGF-β3 is more potent in tissues with mesenchymal origin. The three isoforms signal through the same kinase receptor with different binding abilities (Massague, 1996, Barrientos, 2008, Sefat, 2010b). In order to understand how TGF-β regulates bone repair and formation, *in vitro* experiments using osteoblast cell lines, which are mainly derived from osteosarcomas, and share phenotype similarities to osteoblast cells, can be performed. *In vitro* experiments have indicated that TGF-β stimulates osteoblast chemotaxis, DNA synthesis, cell division, and promotion of bone matrix proteins (Rodan, 1987, Janssen, 2005).

TGF-β is difficult to manufacture due to its poor physical and chemical stability in aqueous solution and in powder form. When TGF-β is ready to be administered it is reconstituted into a liquid form before use. A number of problems can exist with TGF-β in liquid formulations, e.g. aggregation can take place under certain conditions in which

TGF-β can lose its biological activity due to oxidation. Also, TGF-β has a high binding affinity for the surfaces of containers, which will reduce the percentage of TGF-β that will be administered reducing the actual dose delivered (Sefat, 2010b). It is, therefore very important for the growth factor to be delivered properly to ensure a stable form and prevent loss of its bioactivity. To overcome these problems HCl is added to the TGF-β to stabilise the solution and reconstitute it from the powder form. Upon cell culturing further HCl is added to TGF-β, which prevents losses allowing a final concentration ranging from 0.1ng/ml to 10ng/ml in most culture systems (Lind, 1993). A physiological carrier is also added, e.g. Bovine Serum Albumin (BSA), which acts as a carrier for the TGF-β hindering adherence to the surfaces of equipment. There are many different delivery systems by which TGF-β can be administered to a wound site, e.g. by gradual release from a scaffold (usually biodegradable which releases TGF-β intermittently) or it can be administered directly by injection into the wound site. Administration of TGF-β adjacent to the periosteum has been found to increase bone thickness and chondrogenesis (Marcelli, 1990, Joyce, 1990). TGF-β can be secreted from bone in its latent form where TGF-β can be found in abundance. The latent form of TGF-β has a non-covalent association with LAP (Latency Associated peptide). Activation of the TGF-β can be achieved through acidification with dissociation of the LAP protein that unmasks TGF-β binding receptors and hence activates the TGF-β mechanism (Lawrence, 1985). Other groups showed that latent TGF-β in a medium conditioned by cultured cells can be activated by transient treatment with either acids, bases, heat, or chaotrophic agents (Lawrence, 1985, Flaumenhaft, 1993).

This post-translational control of TGF-β1 through activation is arguably the most potent regulatory mechanism for this cytokine (Annes, 2003). Once activated, TGF-β1 binds to its signalling receptor complex (type I, type II, and type III in concert) (Shi, 2003). An enzyme-linked immunosorbent assay (ELISA) is a routine method for the detection of TGF-β1, which detects active TGF-β1 after activation by transient acidification or treatment with urea (Danielpour, 1989).

Due to its simplicity, specificity and sensitivity this method is the best option when working on a cell-free system (Zamora, 2007). Other groups also mentioned that latent TGF-β1 can be activated by heat, acidification, alkalinisation or the action of chaotropic agents *in vitro* (Annes, 2003), but some discrepancies can be found in the literature regarding this process. The Zamora research group compared the effectiveness of acidification vs. heat treatment in activating latent TGF-β1 in a cell-free system. The ELISA assay was carried out using recombinant human latent TGF-β1 (Zamora, 2007). It was found that, while both heat and HCl lead to activation of latent TGF-β1, heat treatment was significantly more effective than acidification. Interestingly, neither treatment induced full activation and the mechanism of activation is not fully understood (Zamora, 2007). However, it has been proposed (Oreffo, 1989) that TGF-β may not be the only reason for increasing the rate of wound repair in bone. In order for TGF-β to be administered exogenously it needs to be dissolved with HCl and BSA used as the carriers and solvent of TGF-β. It is suggested that HCl also contributes to the rate of wound repair (Centrella, 1988, Sefat, 2010b). This is particularly important seeing as there is evidence that the pH of injured bone can decrease and that this may play a role

in activating TGF-β (Oursler, 1994, Dallas, 2002). Thus it is important to investigate the role played by HCl in wound closure in a simple bone cell culture model.

5.2 SCOPE

Wound closure experiments were carried out using MG63 bone cells in the presence of different HCl concentrations, i.e. 20µM, 40µM, 80µM, 160µM and in control cultures without HCl. The experiments were all repeated three times for each concentration. Each culture flask was incrementally photographed every 5hrs.

5.3 WORKING HYPOTHESIS

HCl as the solvent is believed to bind to serum proteins (Kohn, 2002, Sefat, 2010b) that will change their molecular structure possibly turning the serum protein into a signalling molecule assisting in the bone repair process. The Null Hypothesis to be tested in this work is that administered of HCl to a wounded MG63 bone cell monolayer has no impact on rate of wound repair compared to wound repair in control cultures.

5.4 AIMS AND OBJECTIVES

The purpose of this chapter was, therefore, to investigate the effect of different HCl concentrations on wound closure in cultured monolayers of MG63 bone cells. This work investigated and compared the wound closure properties of various HCl concentrations in the cultured dish environment using in the cultured monolayers of

human bone cells. Other cellular responses such as proliferation, differentiation and detachment were also investigated.

5.5 MATERIALS AND METHODS

5.5.1 Cell Culture

Bone cells were cultured in a low glucose (1g/L-D-Glucose) HEPES (25mM) buffered DMEM (Dulbecco's Modified Eagle Media, Sigma Aldrich) culture media supplemented with L-glutamine (2.5 mM U/mL), Penicillin (100 U/mL), Streptomycin (0.1 mg/mL), Amphotericin B or a fungizone (250µg/ml) (Sigma Aldrich-UK) and 10% FCS v/v (fetal calf serum- PromoCell - UK). The bone cells were plated in 25cm^2 tissue culture grade cell culture flasks at a cell density of 250,000 cells/culture flask and bathed in the culture media. The cells attached to form a layer at the bottom of the culture flasks. Once confluent, a 'wound' was made using a disposable long nosed plastic pipette of 1mm diameter as described in the previous chapter.

5.5.2 Treatment with HCl Dilutions

This *in vitro* work examined and compared wound closure properties of the TGF-β solvent "HCl" in various concentrations. The wound healing response was investigated in TC grade culture flasks by creating a wound (with average scratch width of 300µm±10-30µm SD, 2.1-8µm SEM) on confluent monolayer of MG63 human bone cells. After wounding, cultures were then treated with 20µM, 40µM, 80µM and 160µM final bathing concentrations of HCl. As a control wound healing was monitored in

cultures without HCl treatment. After wounding, culture flasks were stored inside the incubator and wound width was imaged and measured every 5hrs over a 30hrs period as described previously. The whole procedure was repeated three times. Wound widths were normalised as percentages and then mean percentage wound widths were plotted against time for each HCl treatment. Addition to the percentages of wound closure, the rate of wound closure was also calculated.

5.6 STATISTICAL ANALYSIS

Mean % and mean rate of wound closure were tested for normality using a Kolmogorov Smirnov test. Normaly distributed data ($p > 0.05$) were analysed using SPSS via a Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed data ($p < 0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

5.7 RESULTS

5.7.1 Wound healing

Images of the wound closure process for the 80µM and 160µM HCl (see Figure 5.1) showed that after 5hrs the morphology of the cells at the wound edges had changed and that the cells had elongated, spread and migrated into the wound site perpendicularly to the wound axis (% wound remained open 93.00% ± 7.39SD and 91.38% ± 2.31SD, respectively). The vertically elongated cells looked different (more elongated) to the

cells a distance away from the wound edges (more rounded). The cells seemed to be less dense and more spread out at the wound edges. After 15hrs the cells had migrated into the wound site and elongated in order to meet cells from the opposite side of the wound. Observations induced that bone cells replicated and divided forming daughter cells that form a complete and new wound margin (Sefat, 2010b). It also appeared that bone cells migrate to the centre of the wound as shown in Figure 5.1 below.

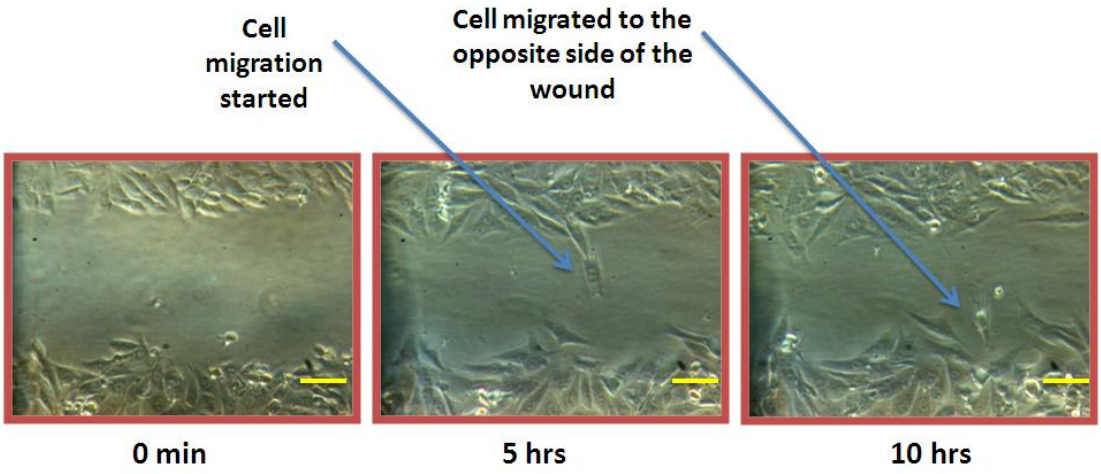


Figure 5.1 *Bone cells migration to the centre of the wound; magnification X50, (Scale bar=100μm).*

The daughter cells then form a component of the wound margin and seem to be subject to a partial contact inhibition and then divide again to form a new wound margin. It also appeared that bone cells migrate to the centre of the wound. These cells then formed bridges to connect the wound edges and close the wound gap. As soon as the bridges formed in the wound site the wound was closed quite rapidly. The 20µM and 40µM concentrations of HCl and control induced slower wound closure rates compared to the higher HCl concentrations with full confluency not achieved even after 30hrs. Images and collected data for the wound closure process in the 20µM and 40µM HCl culture flasks (Figure 5.2 and Table 5.1) showed that there were small changes in wound width

even after 10hrs (% wound remained open 85.29% ± 9.83SD and 82.73% ± 10.64SD, respectively). Wound closure changes could be observed after 15hrs. The percentage of wound remained open after 15hrs of culture was 69.05% ± 11.82SD in the case of cells treated with 20µM HCl and 58.25% ± 13.22SD in those trated with 40µM HCl. After 20hrs there were still huge gaps between the cells in the wound site, for the 20µM and 40µM of HCl (p=0.375). The cells in the 20µM and 40µM HCl culture flasks did not seem to form bridges with cells from the opposite wound margin to stabilise the wound site until about 25hrs (% wound remained open 38.03% ± 15.66SD and 34.62% ± 10.18SD, respectively). Cells met each other after 25hrs and started making bridges but without complete closure. It was clear that the wound remained open even after 25hrs. Wound healing occurred very slowly in control culture flasks (percentage of wound remained open after 15hrs of culture was 56.29% ± 1.20SD) (Sefat, 2010b). Figure 5.2 shows all four wounded bone cell monolayers during 30hrs of healing treated with different HCl concentrations and control. These experiments showed that cells treated with 80µM and 160µM concentrations of HCl did indeed speed up the wound closure process in a pure cell culture environment. HCl enhanced wound closure as compared to the control (See Figures 5.2). At higher concentrations of HCl it was noticeable that bone cells looked longer and elongated and had more projections than that of the control.

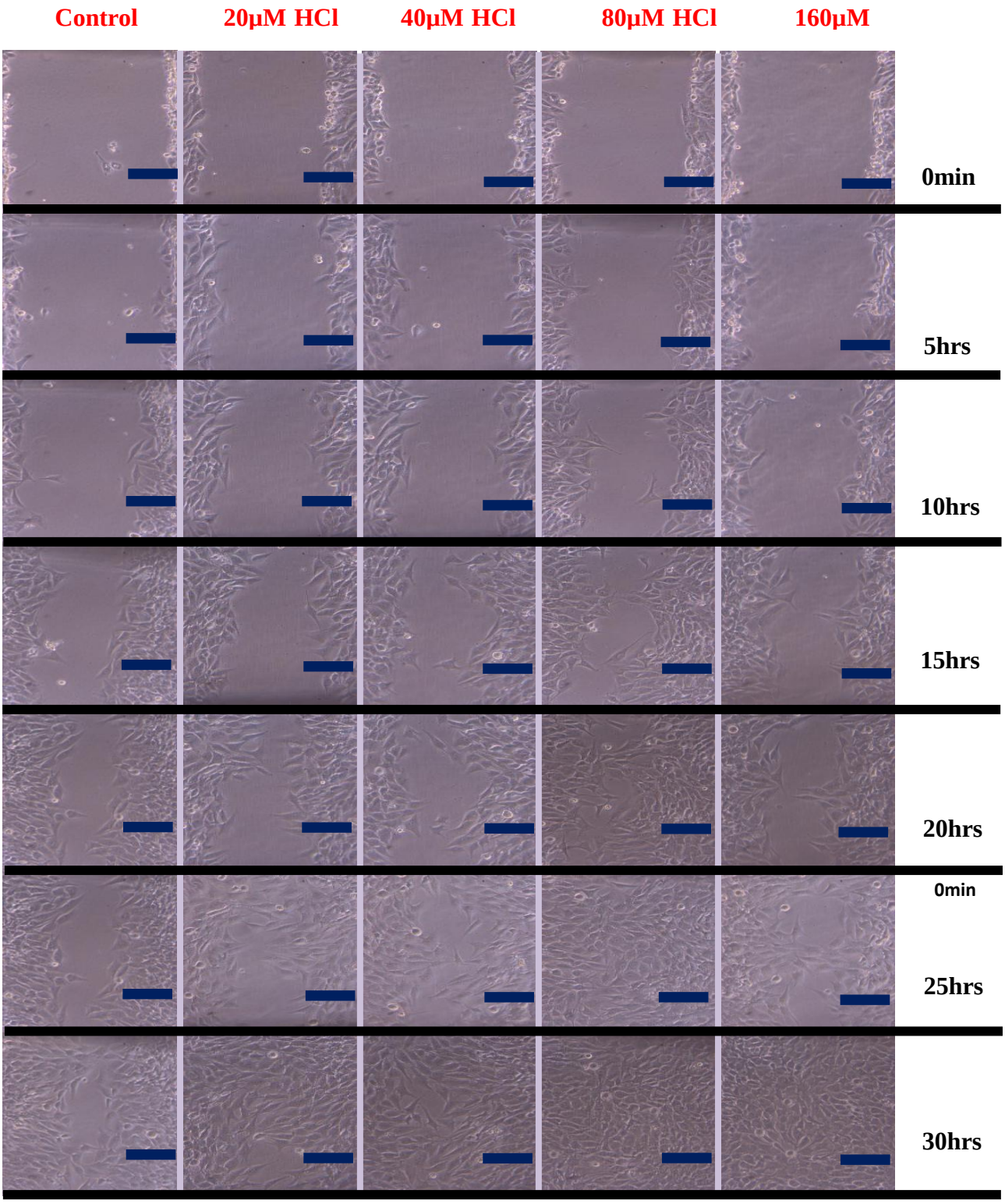


Figure 5.2 *Images of the wound healing process for the bone cell monolayers with four different HCl concentrations and control for the period of 30hrs, (Scale bar=100µm)(Sefat, 2010b).*

Table 5.1 Descriptive statistics for mean % of wound remaining open against time for wounded monolayer treated with different concentration of HCl and control over period of 30 hrs.

Treatment	Wound Closure Time (hrs)	Mean % of wound remaining open ± Std. Deviation (SD)	Std. Error (SE)
Control	0hrs	100.00 ± 1.68	0.30
	5hrs	88.06 ± 0.91	0.16
	10hrs	82.03 ± 1.15	0.21
	15hrs	56.29 ± 1.20	0.22
	20hrs	37.97 ± 1.23	0.22
	25hrs	38.00 ± 1.22	0.22
	30hrs	20.99 ± 0.91	0.16
20µM	0hrs	100.00 ± 0.49	0.09
	5hrs	96.81 ± 6.54	1.19
	10hrs	85.29 ± 9.83	1.79
	15hrs	69.05 ± 11.82	2.15
	20hrs	42.67 ± 18.63	3.40
	25hrs	38.03 ± 15.66	2.85
	30hrs	24.56 ± 10.39	1.89
40µM	0hrs	99.99 ± 1.07	0.19
	5hrs	93.28 ± 3.90	0.71
	10hrs	82.73 ± 10.64	1.94
	15hrs	58.25 ± 13.22	2.41
	20hrs	46.05 ± 12.33	2.25
	25hrs	34.62 ± 10.18	1.85
	30hrs	16.03 ± 4.35	0.79
80µM	0hrs	100.00 ± 1.21	0.22
	5hrs	93.00 ± 7.39	1.35
	10hrs	72.03 ± 10.75	1.96
	15hrs	44.31 ± 13.76	2.51
	20hrs	27.31 ± 8.79	1.60
	25hrs	0.00	0.00
	30hrs	0.00	0.00
160µM	0hrs	100.00 ± 1.38	0.25
	5hrs	91.38 ± 2.31	0.42
	10hrs	48.97 ± 0.83	0.15
	15hrs	31.32 ± 0.99	0.18
	20hrs	14.59 ± 0.73	0.13
	25hrs	6.18 ± 0.54	0.09
	30hrs	0.00	0.00

Table 5.2 P value derived from pair wise comparisons of treatment related % wound remained open of bone cell monolayer over period of 30hrs. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	Wound Closure 0hrs	Wound Closure 5hrs	Wound Closure 10hrs	Wound Closure 15hrs	Wound Closure 20hrs	Wound Closure 25hrs	Wound Closure 30hrs
Control - 20µM	0.408	0.001***	0.526	0.001***	0.965	0.175	0.054
Control - 40µM	0.605	0.001***	0.997	0.859	0.001***	0.139	0.002**
Control - 80µM	0.647	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
Control - 160µM	0.756	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
20µM - 40µM	0.506	0.003**	0.736	0.003**	0.375	0.894	0.001***
20µM - 80µM	0.399	0.069	0.001***	0.001***	0.001***	0.001***	0.001***
20µM - 160µM	0.515	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***
40µM - 80µM	0.848	0.352	0.001***	0.001***	0.001***	0.001***	0.001***
40µM - 160µM	0.657	0.032*	0.001***	0.001***	0.001***	0.001***	0.001***
80µM - 160µM	0.636	0.028*	0.001***	0.001***	0.001***	0.001***	1.000

In Table 5.2 those pairs that are significantly different from one another are marked by an asterisk (*) in which *** represents highest significant difference between each pair (p<0.001). The experiments showed that there was no difference in the % wound width at the start of the experiments. However after 5hrs in culture the percentage wound width between the control cultures and the treatment cultures was quite different (See Figure 5.3). Mean percentage wound width in control cultures decreased to 88.06% ± 0.91SD, but in HCl treated cultures this decrease was not significantly reduced in comparison to the control (P<0.001), with the percentage wound widths only decreasing to 96.81% ± 6.54SD, 93.28% ± 3.90SD, 93.00% ± 7.39SD and 91.38% ± 2.31SD in the 20µM, 40µM, 80µM and 160µM HCl treatments groups, respectively. 10hrs after treatment the percentage wound widths had decreased further, however, in the 20µM HCl treated cultures the percentage wound width had decreased to 85.29% ± 9.83SD. This represented a significantly smaller decrease in wound closure compared to the other treatment groups. Control treated cultures and 40µM HCl treated cultures exhibited wound percentage wound closures of 82.03% ± 1.15SD and 82.73% ±

10.64SD, these wound closures were not significantly different (p=0.997). In comparison the percentage wound widths in the 80µM and 160µM HCl treated cultures were significant decreased in comparison to both the control treated cultures and the 20µM HCl treated cultures (p<0.001). Percentage wound width in the 80µM and 160µM HCl treated cultures decreased to 72.03% ± 10.75SD and 48.97% ± 0.83SD, respectively, with the percentage wound closure induced by treatment with 160µM HCl being significantly greater (p<0.001) than that seen in any other treatment group. At the 15hrs time point the trend seen after 5hrs was continued, with the 20µM HCl treatment group displaying the lowest degree of wound closure (69.05% ± 11.82SD) and the control and 40µM HCl treatment groups showing insignificantly different (p=0.859) mean percentage wound widths of 56.29% ± 1.20SD and 58.25% ± 13.22SD. In comparison the 80µM and 160µM HCl treated cultures exhibited significantly lower percentage wound widths of only 44.31% ± 13.76SD and 31.32% ± 0.99SD. Again the 160µM HCl treated cultures had % wound widths that were significantly lower than any of the other treatment groups ($p < 0.001$). This treatment related pattern in percentage wound width was followed at the 20hrs time point although after 20hrs the percentage wound width had decreased to 37.97% ±1.23SD, 42.67% ± 18.63SD, 46.05% ± 12.33SD, 27. 31% ± 8.79SD and 14.59% ± 0.73SD in the control, 20µm, 40µm, 80µM and 160µM HCl treatment groups, respectively. At this time point all the percentage wound widths were significantly different from one another, other than the wound widths between the controls and the 20µM HCl treated cultures (p=0.965) and the 20µM and 40µM HCl treated cultures (p=0.894).

Once again treatment with 160µM HCl resulted in a significantly greater decrease in the % wound width when compared with the other treatments. By the 25hrs time point the % wound width in the 80µM HCl treated cultures had dropped to zero% whilst the % wound width in the 160µM had decreased further to 6.18% ± 0.54SD, and the control, 20µM and 40µM HCl treated cultures had insignificantly different percentage wound widths of 38.00% ± 1.22SD, 38.03% ± 15.66SD and 34.62% ± 10.18SD, respectively. The cells in the control flask showed significantly slower wound closure when compared with the higher concentrations of HCl ($p<0.001$).

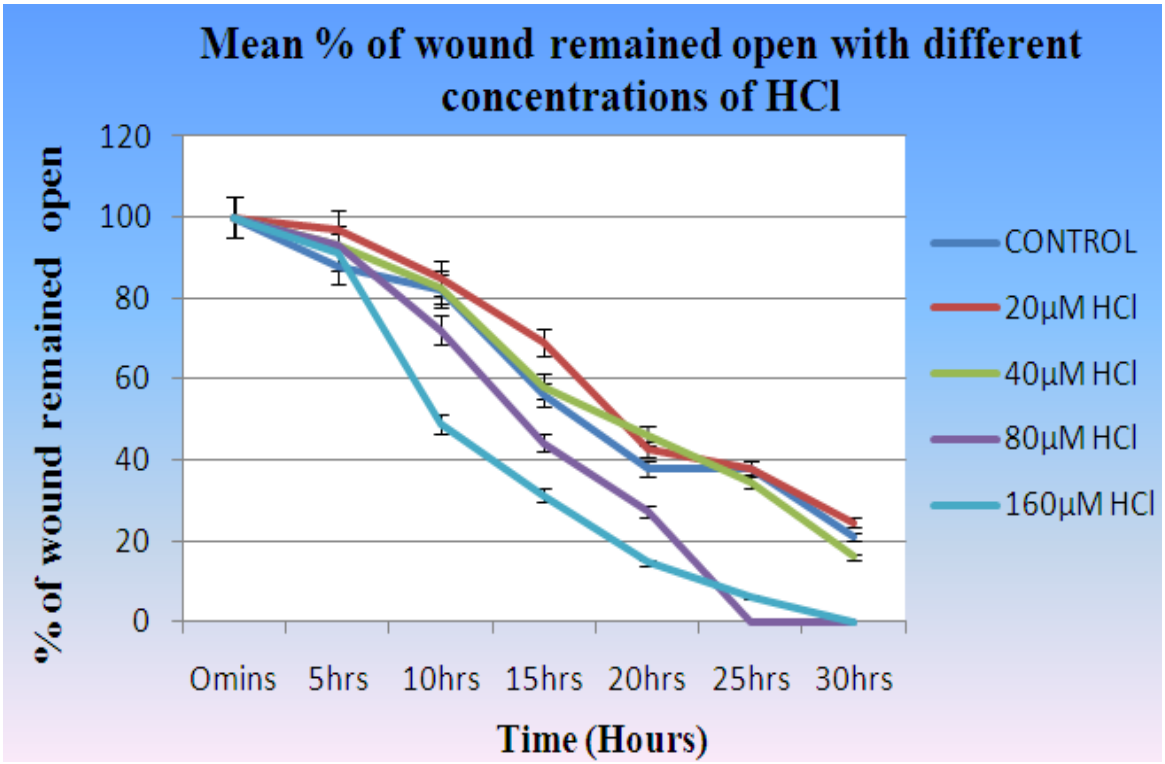


Figure 5.3 *Graph of % wound closure width against time for wounded bone cell monolayers treated with different HCl concentrations and control (Error bars represent standard deviation)(Sefat, 2010b).*

5.7.2 Rate of wound closure

In addition to the % of wound closure, the rate of wound closure was calculated. These results showed that wound healing took place in all five culture flasks but at different rates. Statistical analyses demonstrated that 80µM and 160µM HCl concentrations caused model wounds to heal fully after 25hrs whilst wounds with 20µM, 40µM HCl concentrations and control remained open even after 30hrs (with mean rate of wound closure ~ 7.66µm/hrs ± 0.18SD, 7.91µm/hrs ± 0.22SD and 11.26µm/hrs ± 0.22SD, respectively). The rate of wound closure for each HCl concentration was calculated and a graph of wound closure rate vs. time was plotted (Sefat, 2010b), as presented in Figure 5.4 and Table 5.3. It became apparent that the higher the HCl concentration the faster the rate of wound closure up to a peak value after which the rate decreased.

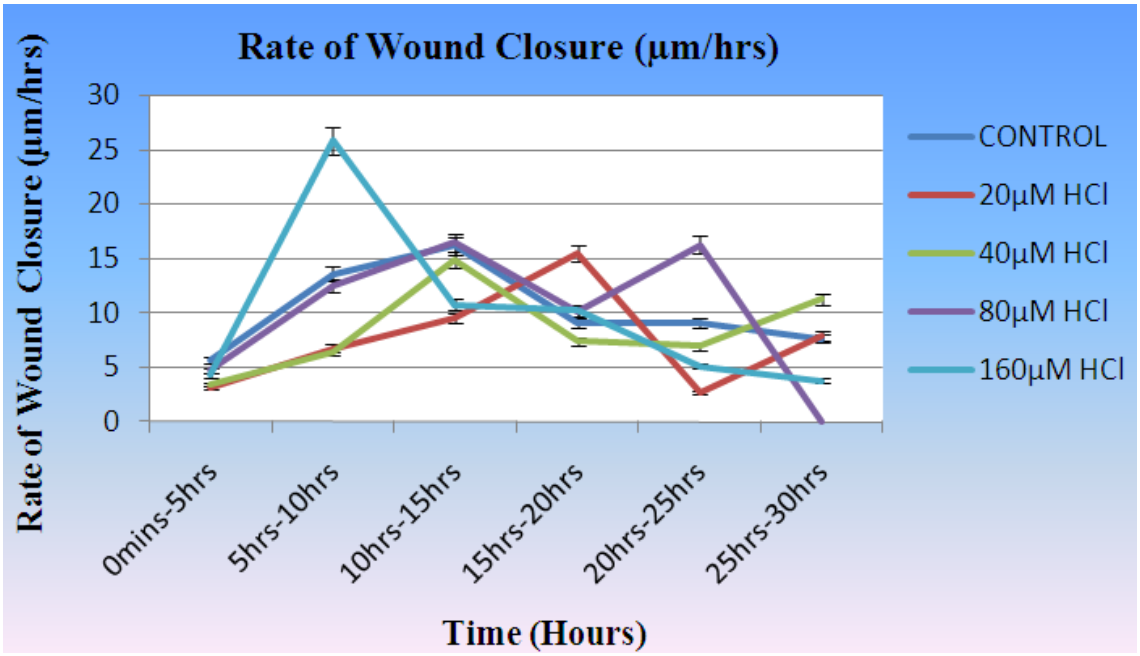


Figure 5.4 *Graph of rate of wound closure width against time (µm/hrs) for wounded bone cell monolayers treated with different HCl concentrations and control (Error bars represent standard deviation)(Sefat, 2010b).*

Table 5.3 Descriptive statistics for wound closure rate of bone cell monolayer with different treatments of HCl and control over period of 30hrs.

Treatment	Rate of Wound Closure (hrs)	Mean Rate of Wound Closure (µm/hrs) ± Std. Deviation (SD)	Std. Error (SE)
Control	0min-5hrs	5.67 ± 0.26	0.11
	5hrs-10hrs	13.58 ± 0.32	0.14
	10hrs-15hrs	16.20 ± 0.27	0.12
	15hrs-20hrs	9.06 ± 0.04	0.01
	20hrs-25hrs	9.06 ± 0.04	0.01
	25hrs-30hrs	7.66 ± 0.18	0.08
20µM	0min-5hrs	3.13 ± 0.15	0.06
	5hrs-10hrs	6.75 ± 0.16	0.07
	10hrs-15hrs	9.53 ± 0.36	0.16
	15hrs-20hrs	15.49 ± 0.09	0.04
	20hrs-25hrs	2.72 ± 0.02	0.00
	25hrs-30hrs	7.91 ± 0.22	0.10
40µM	0min-5hrs	3.45 ± 0.20	0.08
	5hrs-10hrs	6.39 ± 0.1	0.04
	10hrs-15hrs	14.84 ± 0.20	0.09
	15hrs-20hrs	7.39 ± 0.18	0.08
	20hrs-25hrs	6.93 ± 0.23	0.10
	25hrs-30hrs	11.26 ± 0.22	0.09
80µM	0min-5hrs	4.70 ± 0.19	0.08
	5hrs-10hrs	12.46 ± 0.07	0.03
	10hrs-15hrs	16.48 ± 0.10	0.04
	15hrs-20hrs	10.10 ± 0.31	0.14
	20hrs-25hrs	16.24 ± 0.11	0.05
	25hrs-30hrs	---------	----------
160µM	0min-5hrs	4.25 ± 0.22	0.09
	5hrs-10hrs	25.87 ± 0.20	0.09
	10hrs-15hrs	10.76 ± 0.14	0.06
	15hrs-20hrs	10.20 ± 0.29	0.13
	20hrs-25hrs	5.12 ± 0.15	0.07
	25hrs-30hrs	3.77 ± 0.06	0.02

Table 5.4 P values derived from pair wise comparisons of treatment related wound closure rate. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	Wound Closure Rate 0min-5hrs	Wound Closure Rate 5hrs-10hrs	Wound Closure Rate 10hrs-15hrs	Wound Closure Rate 15hrs-20hrs	Wound Closure Rate 20hrs-25hrs	Wound Closure Rate 25hrs-30hrs
Control - 20µM	0.001***	0.001***	0.001***	0.009**	0.009**	0.147
Control - 40µM	0.001***	0.001***	0.001***	0.009**	0.009**	0.001***
Control - 80µM	0.001***	0.001***	0.372	0.009**	0.009**	0.001***
Control - 160µM	0.001***	0.001***	0.001***	0.009**	0.009**	0.001***
20µM - 40µM	0.166	0.057	0.001***	0.009**	0.009**	0.001***
20µM - 80µM	0.001***	0.001***	0.001***	0.009**	0.009**	0.001***
20µM - 160µM	0.001***	0.001***	0.001***	0.009**	0.009**	0.001***
40µM - 80µM	0.001***	0.001***	0.001***	0.009**	0.009**	0.001***
40µM - 160µM	0.001***	0.001***	0.001***	0.009**	0.009**	0.001***
80µM - 160µM	0.020*	0.001***	0.001***	0.465	0.009**	0.001***

Results also showed that there was significant difference in wound closure rate from time zero to 5hrs of the experiments. However after 5hrs in culture the rate of wound closure between the control cultures and the treatment cultures was quite different. Mean rate of wound closure in control cultures increased to 13.58µm/hrs ± 0.32SD, but this was significantly increased in HCl treated cultures with higher concentration (P<0.001), with the rate of wound closure increased to 6.75µm/hrs ± 0.16SD, 6.39µm/hrs ± 0.1SD, 12.46µm/hrs ± 0.07SD and 25.87µm/hrs ± 0.2SD in the 20µM, 40µM, 80µM and 160µM HCl treatments groups, respectively (P<0.001). This indicates that the highest rate of wound closure was induced by 160µM HCl. From time 10hrs to 15hrs of culture the rate of wound closure increased slightly further for all cases except 160µM HCl (Rate of wound closure 10.76µm/hrs ± 0.14SD). However, in the 20µM HCl treated cultures the rate of wound closure had increased only to 9.53µm/hrs ± 0.36SD. This was a represented a significantly smaller increase in wound closure rate

compared with the other treatment groups (P<0.001). The rate of wound closure between time points 15hrs-20hrs was decreased for all cases except 20µM HCl (15.49µm/hrs ± 0.09SD). At time points 20hrs-25hrs decrease rate of wound closure were observed for control treated cultures and all other HCl treated flasks except 80µM HCl with rate of 16.11µm/hrs ± 0.11SD which was significantly greater that others (p=0.009). This rate was unchanged for control flasks (9.06µm/hrs ± 0.04SD). This rate was 2.72µm/hrs ± 0.02SD, 6.93µm/hrs ± 0.23SD and 5.12µm/hrs ± 0.15SD for 20µM, 40µM and 160µM HCl concentrations, respectively, which is quite slow compared with the 80µM HCl (16.24µm/hrs ± 0.11SD) (P<0.009). At time point between 25hrs-30hrs no rate of wound closure recorded as the wounds had healed completely for 80µM HCl, however, rate of wound closure for 40µM HCl was ~ 11.26µm/hrs ± 0.22SD representing higher rate of wound closure and significantly different compare to others (p<0.001).

5.8 DISCUSSION

From observation and upon an initial scratch it was clearly seen that there were a number of rounded cells at the edges of the wound. It took a while for these rounded cells to become detached. After 15hrs and 20hrs of applying HCl it could be seen that, at certain points along the wound width, peaks started to form so that bridges were made across the width of the wound as cells extended to meet cells on the opposite side of the wound. This is a crucial part of the healing process when contact bridging has been accomplished (Hom, 2007). This closed the area of the wound further with the cells being able to make contact more easily in a smaller space. Cells such as 3T3

fibroblasts (Hollinger, 2004) migrate into a model wound site and are known to replicate exponentially (1 to 2, 2 to 4, 4 to 8, etc.) until contact inhibition takes place, which then slows the rate of replication (Heckman, 1994, Sefat, 2010b). Our results suggest that wound healing took place in all concentrations of HCl but at different rates. The 80μM concentration of HCl fully healed after 25hrs ($p<0.001$) whilst others remained open even after 30hrs. The 20μM and 40μM concentrations of HCl failed to close the wound after 30hrs with 24.56% ± 10.39SD and 16.03% ± 4.35SD of the wound remaining open respectively ($p<0.001$). The 80μM concentration of HCl was closely followed by the 160μM and with respect to the rate of wound closure up to the point of 20hrs this occurred with accelerated healing. This was slower for the 20μM and 40μM of HCl (with a mean rate of wound closure ~ 6.75μm/hrs ± 0.16SD and 6.39μm/hrs ± 0.1SD respectively). The 160μM concentration of HCl induced the quickest rate of repair but took 30hrs for the wound to become fully confluent with a large rate of closure between 5-10hrs (25.87μm/hrs ± 0.20SD) indicating that this concentration increased wound closure rate as compared to the control. It is interesting to note that the greatest percentage of wound closure was between 5 and 15hrs for the 80μM and 160μM concentrations of HCl. This indicates that the bone cells made a connection across the wound width by successful bridging with additions of HCl accelerating the process (Sefat, 2010b). It can be speculated that some of the hydrogen ions from the HCl bind to the serum proteins and cause a change in amino acid molecular structure and hence its function as a signalling molecule. Those hydrogen ions are known to bind to the negative sites on amino acids (Yeung, 1992) supports this view, and suggest that interaction could alter molecular structure and encourage changes in the folding of proteins. Protein structure influences function (Yeung, 1992) thus

hydrogen ion/plasma protein interactions or hydrogen ion/cell membrane protein interactions could activate signalling pathways altering gene expression and thus influence cell motility. There is evidence supporting this in the literature (Cheng, 2006). For example platelet activating factor (PAF) and interleukin 6 (IL-6) are both upregulated in oesophageal smooth muscle cells in response to treatment with HCl. There is also evidence in the literature the growth and development of different cells, including osteoblasts, is linked to the regulation of pH and the acidity of the extracellular microenvironment (Kohn, 2002). Skeletal tissue repair and mineralisation are influenced by the ionic and molecular composition of the extracellular fluids (Chakkalakal, 1994) and it is well established that Ca-P salts are precipitated in a pH dependent manner (Johnson, 1992, Chakkalakal, 1994, Posner, 1985). As a result, it has been suggested that bone repair and mineralisation are tissue pH dependent (Chakkalakal, 1994). On a cellular level even modest reductions in extracellular pH have an effect on osteoblast function *in vitro* (Kaysinger, 1998). It can be concluded that the cellular mechanisms involved in bone formation and resorption may therefore be responsive to the acid-base balance. It is necessary to know how pH affects bone metabolism, therefore some studies showed that during metabolic acidosis the activity of osteoblasts declines, whereas during metabolic alkalosis osteoblast activity increases (Kaysinger, 1998). Similarly, the pH in the immediate microenvironment of osteoblasts, in addition to the intracellular spaces of differentiating bone, varies with progressive stages of osteogenesis and may be linked to the regulation of gene expression (Ramp, 1994). An experimental wound in vitro was examined to investigate the effects of pH for human fibroblast. The wounded monolayers were cultured at pH ranging from 7.2 to 8.4. A significant decrease was recorded both in cell migration, and DNA synthesis in,

the experimental wounds as a result of pH increases (Lenghedeit, 1995). Finally Kohn et al. showed that small shifts in extracellular pH result in significant changes in bone marrow stromal cells (MSCs), which express markers of the osteoblast phenotype (Kohn, 2002). These pH effects potentially relate to the microenvironment that bone marrow stromal cells would likely be exposed to. This further confirms findings regarding the effect of HCl on MG63 bone cells. It was observed in our investigation that different concentrations of HCl did not affect the pH level of the media indicating that HCl was being sequestered away. However, this is inconclusive as the pH value was not properly evaluated through testing. Observation of the colour of the media indicated to the naked eye that the pH value was not affected.

5.9 CONCLUSIONS

Bone repair is a complex process with interconnecting phases through cell management with many factors affecting the promotion of a successful bone repair. Many areas are investigated to promote bone repair and increase the rate of closure. *In vitro* studies allow us to understand how cells behave and react to different parameters. This study examined the role of HCl in wound closure rates. It was found that higher concentrations of HCl, in particular 80µM and 160µM, promoted a faster rate of wound closure compared with the control. The 160µM concentration of HCl showed the fastest rate of closure between 5-10 hrs (with the mean rate of wound closure ~ 25.87µm/hrs ± 0.20SD). Once bridging had occurred the rate of wound repair decreased at ~15hrs up to confluency. A culture flask treated with 80µM HCl showed bridge formation at the wound site at 15hrs of healing and complete healing achieved at 25hrs. This meant early

bridging is an important factor and enhances the rate of wound closure. We assume that the serum proteins present in the media act as signalling molecules due to the surface charge changes from the release of hydrogen ions via the HCl. The availability of hydrogen ions is essential for these signalling pathways to accelerate wound repair through administration of HCl.

CHAPTER SIX

6. THE EFFECT OF TGF-β ISOMERS ON THE CELL MORPHOLOGY (SIZE AND NUMBER) OF HUMAN BONE CELLS

6.1 INTRODUCTION

Cell adhesion and protein adsorption are thought to be very important factors in cell culture and tissue engineering of any functional tissue (Fleming, 2000, Yang, 2010, Worz, 2012). It is well established that cells attach to the extracellular matrix (ECM) *in vivo* and that the presence of ECM is vital for any cell type within the body (Hollinger, 2004, Yang, 2010). Much of the R&Ds in the 80's and 90's focused on the interaction of biomedical materials with proteins and cultured cells (Cooper, 1982, Ludwicka, 1984, Flemming, 1999). In recent years, there has been an increased interest in how growth factors (Khan, 2000, Logeart-Avramoglou, 2005), mechanical stimulation (Hardiman, 2005, Kesavan, 2005), surface stiffness (Giesen, 2004, Maina, 2010, Foppiano, 2007b) and drugs (Barcew, 2008, Joshi, 2008) influence cell morphological changes. For instance, human bone cells were compared to marrow-derived human mesenchymal stem cells (HMSCs) for a duration of 12 weeks in order to investigate their capacity to proliferate and differentiate into osteoblasts under various culture conditions (Krattinger, 2011). Cell treated with FGF-2 and BMP2 were also compared. Cultures treated with FGF-2 increased cell proliferation of both cell types. BMP-2 increased cell numbers and reduced osteoblastic differentiation in human bone cells

with the opposite effect seen in HMSCs (Krattinger, 2011). In 2009, Yang et al. analysed the influence of substrate topography on cell morphology of MG63 (Yang, 2009). They found that cells spread faster on the nano-patterned surfaces than on the flat surface, suggesting that surface anisotropic features facilitate initial cell extension along its direction.

Other groups investigated the role of extracellular Ca^{2+} and Mg^{2+} in osteoblast morphology and adhesion in the absence or presence of PDGF (Abed, 2009). They showed that the stimulation of osteoblast (MG63) proliferation and migration by PDGF was significantly reduced under culture conditions of low extracellular Mg^{2+} concentrations (Abed, 2009). Therefore, the morphology of cells can be important in many contexts. In culture the morphology indicates the status of the cells, both in terms of the health of the cells and cell adhesion capacity (Yang, 2010, Worz, 2012). Variations in cell morphology can results from changes in underlying cytoskeletal organization and dynamics. For example, different protein coatings on a surface or chemical topography of the surface (e.g. micro-contact printing) can cause changes in morphology (Lo, 2004). There is evidence that TGF-β isomers influence wound repair by modifying cell function and morphology in a number of tissues (Wu, 1997, Critchlow, 1995, Derynck, 2008, Janssen, 2005). Therefore, the aim of this study was to examine the effects of different TGF-β isomers on MG63 cell size, morphology and proliferation *in vitro.*

6.2 MATERIALS AND METHODS

6.2.1 Cell culture

MG63 cells were cultured as described previously in a low glucose (1g/L-D-Glucose) Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich) supplemented with L-glutamine (2.5 mM U/mL), Penicillin (100 U/mL), Streptomycin (0.1 mg/mL), Amphotericin B and fungizone (250µg/ml), HEPES (25mM) buffer (Sigma Aldrich-UK) and 10% FCS v/v (fetal calf serum- PromoCell - UK). The bone cells were cultured inside culture flasks at a cell density of 50,000 cell/ml and bathed in the culture media. Culture flasks were incubated at 37°C for 48hrs. All supplements were purchased from Sigma Aldrich-UK except those mentioned.

6.2.2 Cell size (length)

TGF-β1, 2, and 3 were reconstituted as described in chapter 3. In order to investigate the effect of each TGF-β growth factor on cell length seven culture flasks were treated with the different TGF-β isomers including TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and one culture flask remained un-treated as a control. Human bone cells were seeded at cell density of 50,000 cells/ml and photographs were taken every 6hrs for the first 24hrs and final imaging took place at 36hrs. The cells were photographed using a light microscope and a digital camera. Pictures were captured and stored as TIF images using Scion Image Visi Capture from Scion Corporation. Image J software was used to measure the cell length. In order to measure cell length, 10 images were captured from randomly selected regions of each

culture flasks and the lengths of 100 cells were measured. Mean average cell length was calculated. There was some variability in initial cell length for each treatment. To normalise, the mean starting cell length was calculated per treatment and each starting cell length was expressed as a percentage of that mean. Cell length at each time point was therefore expressed as a percentage of the normalised mean cell length per treatment.

6.2.3 Proliferation (cell number)

The effect of different TGF-β isomers and their combinations including TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) on proliferation rate of human bone cells was assessed. Seven culture flasks were seeded at cell densities of 50,000 cells/ml treated with different TGF-β isomers and one culture flask remained un-treated as a control. Photomicrographs were taken as described previously and cell numbers were determined from randomly selected regions of each culture flasks**.** The whole experiment was repeated three times and mean average cell number was calculated. There was some variability in initial cell number for each treatment. To normalise, the mean starting cell number was calculated per treatment and each starting cell number was expressed as a percentage of that mean. Cell number at each time point was therefore expressed as a percentage of the normalised mean cell number per treatment.

6.3 STATISTICAL ANALYSIS

The mean % cell numbers and cell length were tested for normality using a Kolmogorov Smirnov test. Normally distributed ($p > 0.05$) were analysed using SPSS via a Oneway Analysis of Variance (ANOVA) followed by post Hoc Bonferroni tests. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed results ($p < 0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

6.4 RESULTS

Figures 6.1 – 6.8 show the images for the bone cells after seeding in Tissue Culture (TC) grade falcon cell culture flask after treatment with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated (control).

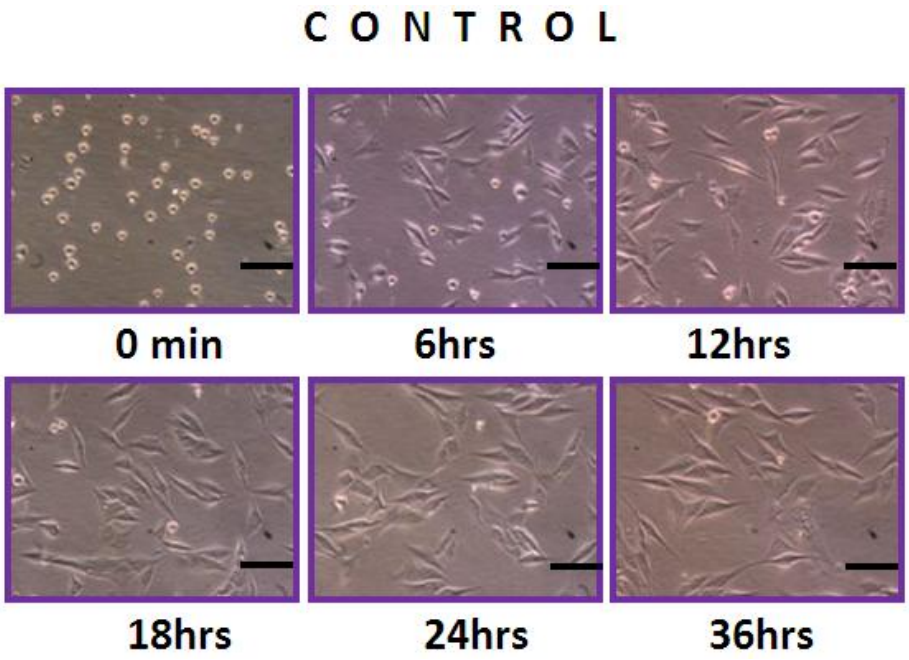


Figure 6.1 *Images of human bone cells after seeding in TC grade falcon cell culture flask with no growth factor (Control) over a period of 36hrs (Scale bar=100μm).*

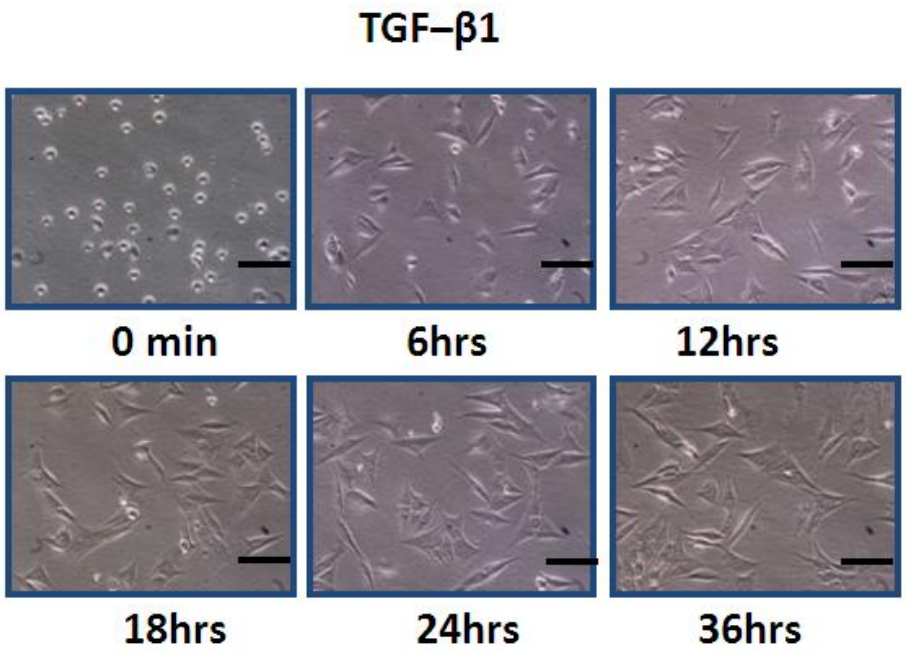


Figure 6.2 *Images of human bone cells after seeding in TC grade falcon cell culture flask treated with TGF-β1 over a period of 36hrs (Scale bar=100μm).*

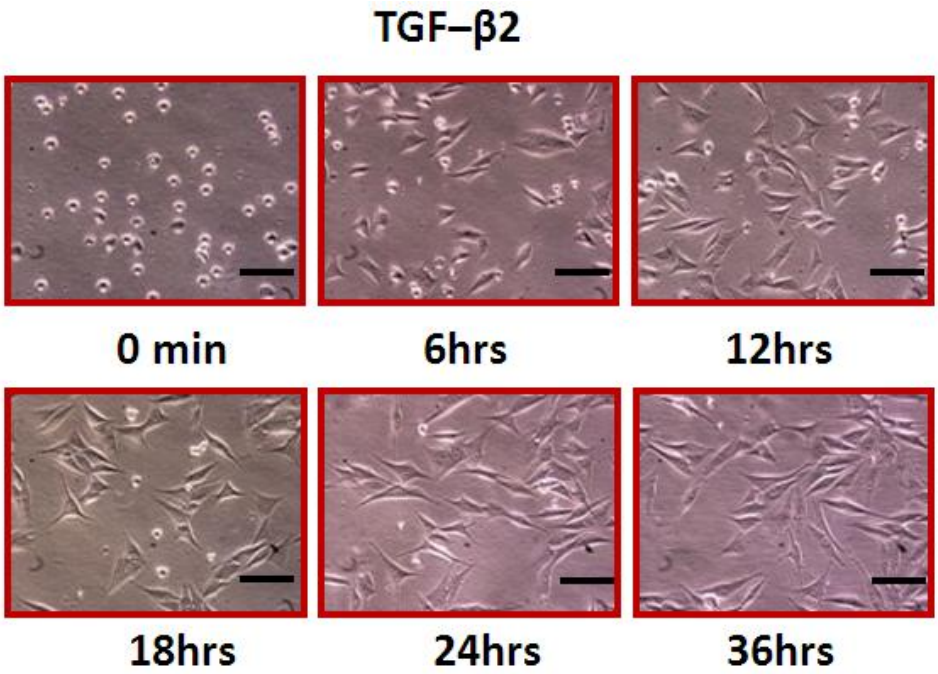


Figure 6.3 *Images of human bone cells after seeding in TC grade falcon cell culture flask treated with TGF-β2 over a period of 36hrs (Scale bar=100μm).*

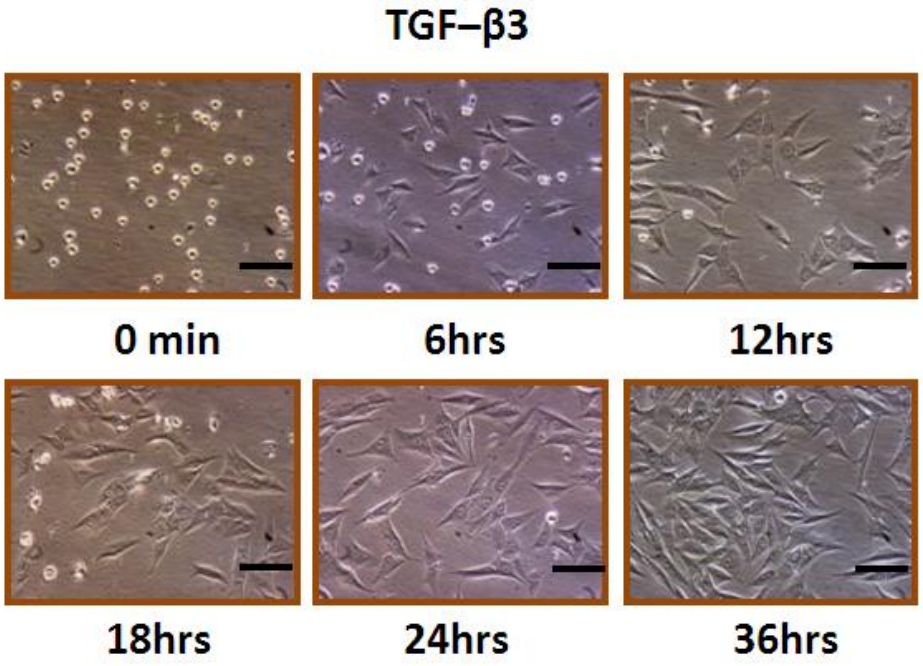


Figure 6.4 *Images of human bone cells after seeding in TC grade falcon cell culture flask treated with TGF-β3 over a period of 36hrs (Scale bar=100μm).*

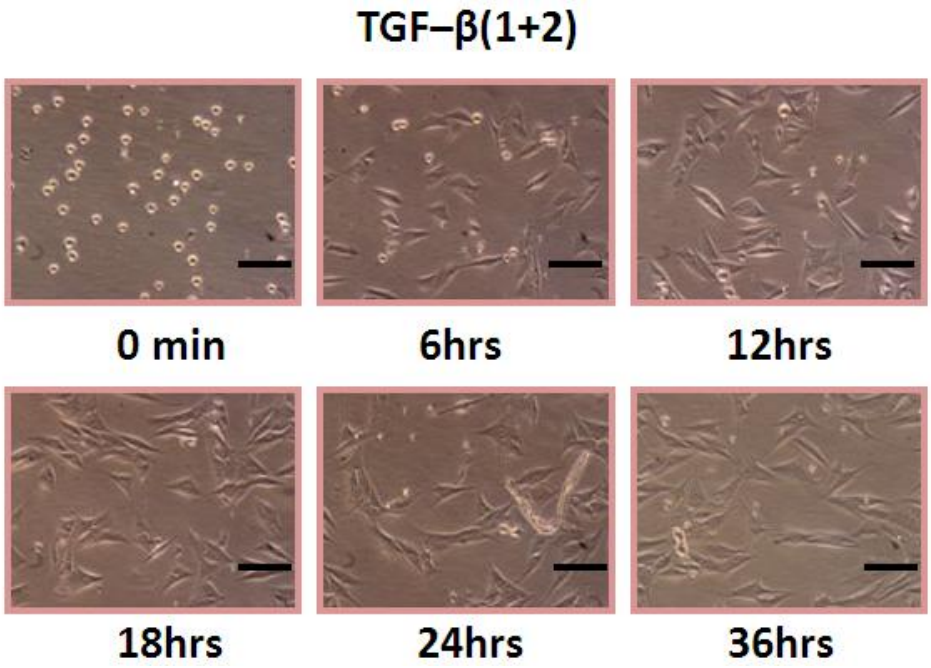


Figure 6.5 *Images of human bone cells after seeding in TC grade falcon cell culture flask treated with TGF-β(1+2) over a period of 36hrs (Scale bar=100μm).*

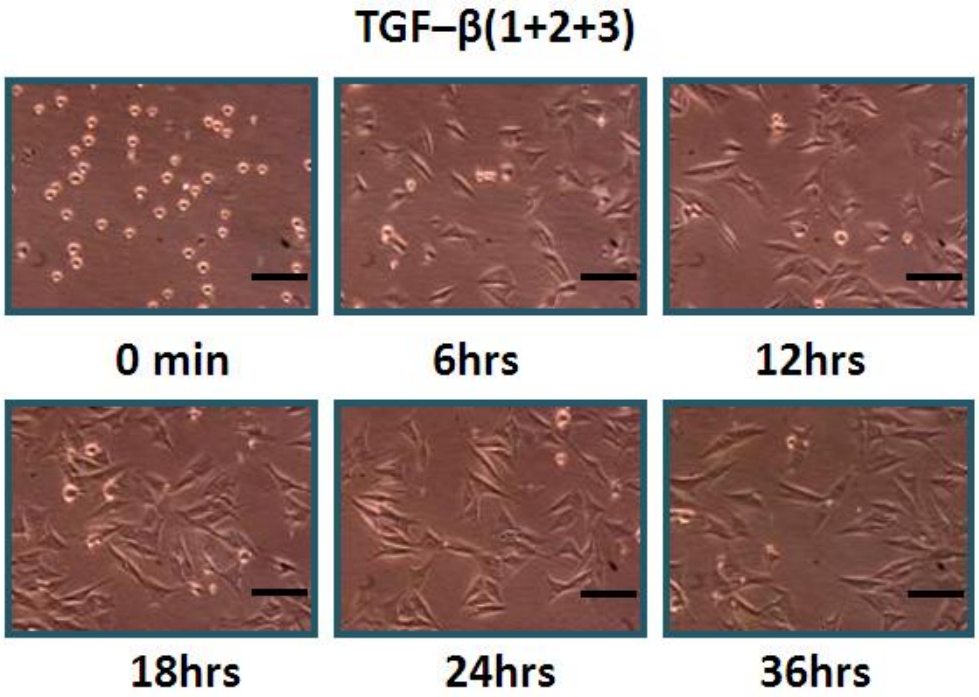


Figure 6.6 *Images of human bone cells after seeding in TC grade falcon cell culture flask treated with TGF-β(1+2+3) over a period of 36hrs (Scale bar=100μm).*

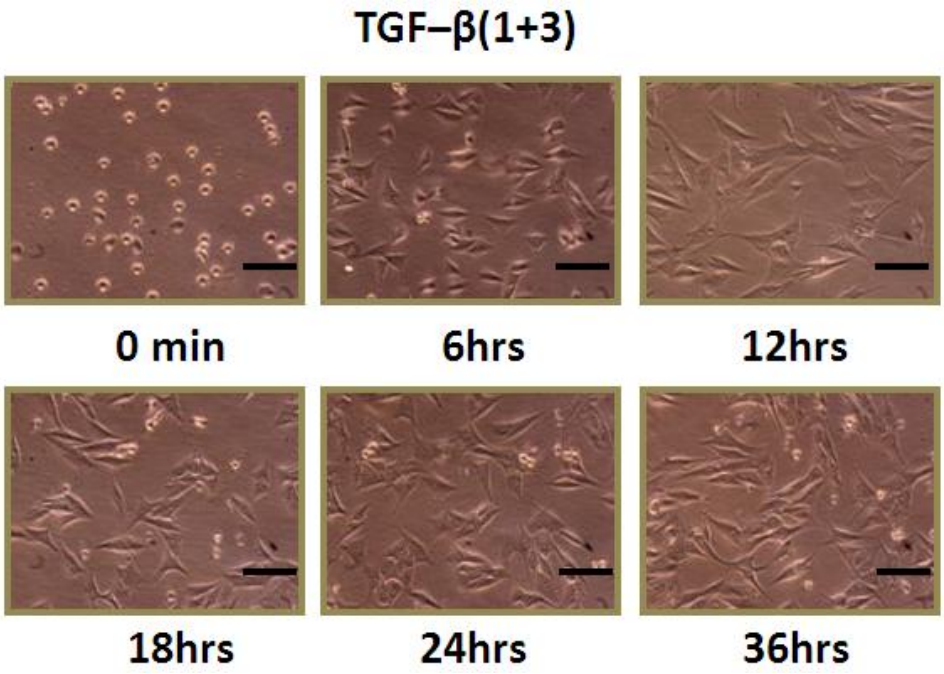


Figure 6.7 *Images of human bone cells after seeding in TC grade falcon cell culture flask treated with TGF-β(1+3) over a period of 36hrs (Scale bar=100μm).*

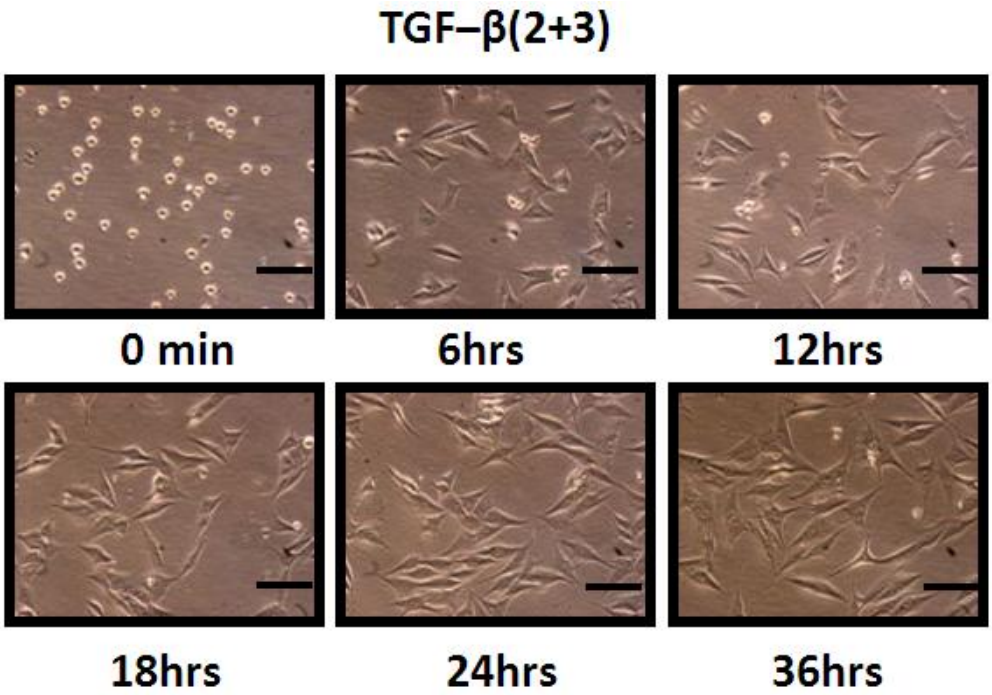


Figure 6.8 *Images of human bone cells after seeding in TC grade falcon cell culture flask treated with TGF-β(2+3) over a period of 36hrs (Scale bar=100μm).*

6.4.1 Cell length analysis

The experiments examined the effect of different TGF-β isomers and their combinations on bone cell morphology including cell length and shape. Table 6.1 below shows the tabulated results for the human bone cell length against time for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control. Mean cell size at time zero was 18.32μm ± 2.54SD. From the results in Figure 6.9, it was found that cells treated with TGF-β(2+3) and TGF-β(1+3) after 36hrs (mean % cell lengths ~551.57% ± 9.14SD and 490.86% ± 9.62SD, respectively, $p < 0.001$) elongated more than all other treatments. Cells treated with TGF-β3, TGF-β2 and control showed less elongation (mean % cell length ~ 469.91% ± 9.44SD, 470.87% ± 12.90SD and 469.34 ± 8.41SD, respectively) compared to TGF-β(2+3) and TGF-β(1+3) after 36 hours in culture. There were no significant differences in mean % cell length between TGF-β2, TGF-β3 and control after 36hrs of culture but there were significant differences at earlier time points (6hrs). It was found that TGF-β2 and TGF-β3 cause a faster elongation 6hrs after seeding. This concurs with wound closure results in chapter 4. After 36hrs, TGF-β2 and TGF-β3 did not have any effect on cell elongation when compared with the control. Cells in TGF-β1 treated culture flasks elongated less (with mean % cell length ~447.24% ± 24.30SD) compared with the cultures treated with TGF-β2, TGF-β3 and un-treated control cells but elongated more than cells treated with TGF-β(1+2) and TGF-β(1+2+3). The initial cell length for all culture flasks were similar and all treated culture flasks elongated after 36hrs except TGF-β(1+2) and TGF-β(1+2+3) with mean % cell length ~ 401.48% ± 13.01SD and 311.34% ± 30.24SD, respectively, while cells treated with TGF-β(1+3), TGF-β(2+3) showed greatest elongation. These results, therefore, suggest that TGF-β3 results in

more elongated cells when combined with either of the other two isomers to form TGF-β(1+3) and TGF-β(2+3) (See Tables 6.1-6.3). In comparison, cells treated with TGF-β(1+2), TGF-β(1+2+3) showed less elongation. TGF-β1 in contrast to TGF-β2 and TGF-β3 seemed to significantly ($p < 0.05$) (See Table 6.3) decrease cell elongation.

Table 6.1 Descriptive statistics of mean % cell length against time for cells treated with TGF-β1, TGF-β2, TGF-β3 and un-treated as control.

Treatment	Time (hrs)	Mean % Cell length ± Std. Deviation (SD)	Std. Error (SE)
Control	**0hrs**	99.98 ± 7.43	2.35
	6hrs	207.30 ± 11.17	3.53
	12hrs	331.57 ± 11.32	3.58
	18hrs	415.08 ± 10.18	3.22
	24hrs	438.84 ± 6.35	2.01
	36hrs	469.34 ± 8.41	2.66
TGFβ1	**0hrs**	99.98 ± 7.37	2.33
	6hrs	268.94 ± 8.71	2.75
	12hrs	330.06 ± 8.66	2.74
	18hrs	385.52 ± 14.38	4.55
	24hrs	429.00 ± 10.46	3.31
	36hrs	447.24 ± 24.30	7.68
TGFβ2	**0hrs**	99.98 ± 5.08	1.61
	6hrs	256.85 ± 9.53	3.01
	12hrs	335.86 ± 9.15	2.89
	18hrs	381.76 ± 15.85	5.01
	24hrs	414.64 ± 7.90	2.50
	36hrs	470.87 ± 12.90	4.08
TGFβ3	**0hrs**	99.99 ± 12.58	3.98
	6hrs	214.49 ± 5.49	1.74
	12hrs	328.99 ± 10.74	3.40
	18hrs	369.98 ± 11.89	3.76
	24hrs	403.25 ± 17.45	5.52
	36hrs	469.91 ± 9.44	2.99

Table 6.2 Descriptive statistics of mean % cell length against time for cells treated with TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3) and TGF-β(2+3).

Treatment	Time (hrs)	Mean % Cell length ± Std. Deviation (SD)	Std. Error (SE)
TGFβ1+2	**0hrs**	99.99 ± 15.76	4.98
	6hrs	211.11 ± 6.49	2.05
	12hrs	238.50 ± 12.15	3.84
	18hrs	252.16 ± 9.29	2.94
	24hrs	292.02 ± 8.60	2.72
	36hrs	401.48 ± 13.01	4.11
TGFβ1+3	**0hrs**	99.98 ± 8.86	2.80
	6hrs	232.42 ± 11.65	3.69
	12hrs	300.40 ± 8.18	2.59
	18hrs	337.11 ± 8.75	2.77
	24hrs	456.74 ± 10.55	3.34
	36hrs	490.86 ± 9.62	3.04
TGFβ2+3	**0hrs**	100.01 ± 7.15	2.26
	6hrs	273.70 ± 8.42	2.66
	12hrs	336.78 ± 9.34	2.95
	18hrs	385.51 ± 12.07	3.82
	24hrs	526.33 ± 12.94	4.09
	36hrs	551.57 ± 9.14	2.89
TGFβ1+2+3	**0hrs**	100.01 ± 11.35	3.59
	6hrs	139.56 ± 7.22	2.28
	12hrs	182.22 ± 13.03	4.12
	18hrs	221.65 ± 9.62	3.04
	24hrs	249.14 ± 15.69	4.96
	36hrs	311.34 ± 30.24	9.56

Table 6.3 P value derived from pair wise comparisons of mean % bone cell length vs. time treated with different TGF-β isomers and their combinations. Pairs that are significantly different from one another showing marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	Cell length 0hrs	Cell length 6hrs	Cell length 12hrs	Cell length 18hrs	Cell length 24hrs	Cell length 36hrs
Control – TGFβ1	1.000	0.001***	1.000	0.001***	0.580	0.023*
Control – TGFβ2	1.000	0.001***	0.983	0.001***	0.001***	0.880
Control – TGFβ3	1.000	0.608	0.999	0.001***	0.001***	0.939
Control – TGFβ1+2	1.000	0.978	0.001***	0.001***	0.001***	0.001***
Control – TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.024*	0.001***
Control – TGFβ2+3	1.000	0.001***	0.952	0.001***	0.001***	0.001***
Control – TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ2	1.000	0.058	0.917	0.996	0.134	0.013*
TGFβ1 - TGFβ3	1.000	0.001***	1.000	0.076	0.001***	0.028*
TGFβ1 - TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ2+3	1.000	0.927	0.837	1.000	0.001***	0.001***
TGFβ1 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ3	1.000	0.001***	0.821	0.340	0.390	0.879
TGFβ2 - TGFβ1+2	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.004**
TGFβ2 - TGFβ2+3	1.000	0.001***	1.000	0.996	0.001***	0.001***
TGFβ2 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ1+2	1.000	0.989	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ2+3	1.000	0.001***	0.709	0.076	0.001***	0.001***
TGFβ3 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ1+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+3 - TGFβ2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+3 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2+3 - TGFβ1+2+3	1.000	0.001***	0.001***	0.001***	0.001***	0.001***

Statistical analysis (See Table 6.3) showed that after 36hrs of culture there were significant differences in mean % cell length between cells treated with TGF-β(1+2), TGF-β(1+2+3) and TGF-β1 which represented less elongation (p<0.001). Cell treated with TGF-β(1+2+3) showed least elongation compared to all other treatments. On the other hand, there were significant differences in mean % cell length between TGF-β(2+3) and TGF-β(1+3) after 36hrs of culture (p<0.001). Figure 6.9 show graph of

mean % cell length against time for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control. It is clear that there are significant differences (p<0.001) in mean % cell length between TGF-β(1+3) and TGF-β(2+3) with all other treatments after 36hrs of culture. Cells treated with TGF-β(2+3) showed most elongation compared to all other treatments.

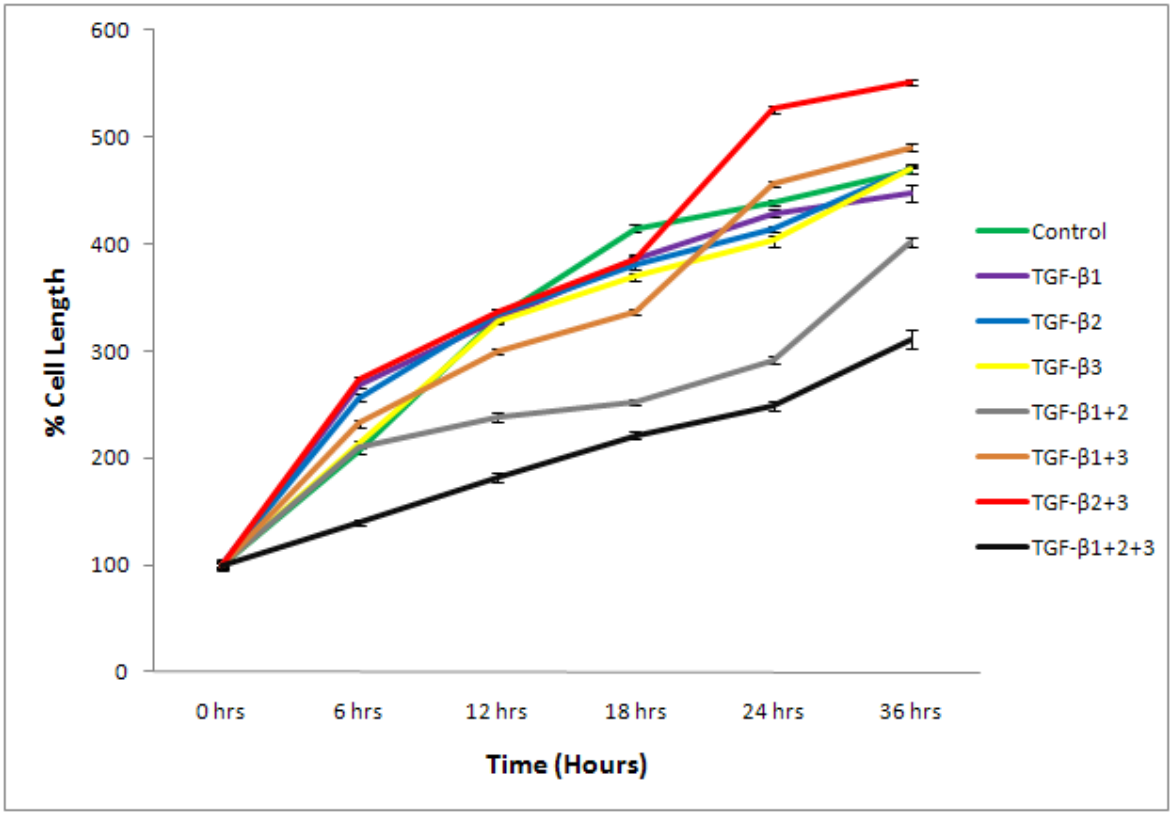


Figure 6.9 *Graph of mean % cell length against time for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control (Error bars represent standard deviation).*

6.4.2 Analyses of Cell Proliferation (Cell number)

Proliferation rate was also studied in order to investigate the effect of different TGF-β isomers on cell number of MG63 cells (See Figures 6.1 – 6.8). Tables 6.4 and 6.5 also give the statistical results for the human bone cell number against time for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control.

Tables 6.4-6.6 and Figure 6.10 show the highest proliferation occurred in cultures treated with TGF-β3 after 36hrs of culture (mean % cell number ~ 213.33% ± 7.41SD) followed by TGF-β(2+3) (mean % cell number ~ 181.40% ± 6.58SD). This suggests that there were more cell division (and cell attachment) in cell cultures treated with TGF-β3 and TGF-β(2+3). In comparison, cells treated with TGF-β1 and TGF-β(1+2) show less proliferation after 36hrs of culture (mean % cell number ~ 138.30% ± 3.88SD and 134.78% ± 7.32SD, respectively). Results also showed that after 36hrs of culture there were significant differences in mean % cell number between cells treated with TGF-β3 and TGF-β(2+3) (p=0.001) indicating that TGF-β3 treated cells proliferated more than the other cells. On the other hand, there were no significant differences in mean % cell number between cells treated with TGF-β1 and TGF-β(1+2) (p=0.171) (See Table 6.6). Cells treated with TGF-β(1+3), TGF-β(1+2+3) and control showed similar results and there were no significant difference between them (mean % cell number ~ 162.79% ± 8.35SD, 163.04 ± 6.15SD and 160.00% ± 8.31SD, respectively). These results, therefore, suggest that any culture flask treated with TGF-β3 or any combination with TGF-β3 induced an increase in cell proliferation compared to all other

treated culture flasks. Control cultures and all cultures treated with TGF-β3 including their combinations also showed higher proliferation rates compared to the cultures treated with TGF-β1 and TGF-β2 (with mean % cell number ~ 138.30% ± 3.88SD for TGF-β1 and 147.14% ± 5.03SD for TGF-β2). This indicates that both TGF-β1 and TGF-β2 decreased cell proliferation compared to the control**.** There were significant differences in mean % cell number between cells treated with TGF-β1 and TGF-β2 (p<0.001). Results also showed that at 36hrs of culture there were significant differences in mean % cell number between cells treated with TGF-β3 and all other treatments (p<0.001) indicating that cells treated with TGF-β3 proliferated more than any of the other cells.

Table 6.4 Descriptive statistics of mean % bone cell number against time for cells treated with TGF-β1, TGF-β2, TGF-β3 and un-treated as control (10000 Cells/Flask).

Treatment	Time (hrs)	Mean % Cell Number ± Std. Deviation (SD)	Std. Error (SE)
Control	**0hrs**	100.00 ± 8.11	2.27
	6hrs	113.33 ± 6.11	1.93
	12hrs	120.00 ± 4.57	1.44
	18hrs	135.56 ± 5.13	1.62
	24hrs	153.33 ± 8.57	2.71
	36hrs	160.00 ± 8.31	2.63
TGFβ1	**0hrs**	100.00 ± 5.76	1.82
	6hrs	108.51 ± 8.57	2.71
	12hrs	117.02 ± 12.88	4.07
	18hrs	121.27 ± 3.17	1.00
	24hrs	131.92 ± 7.30	2.31
	36hrs	138.30 ± 3.88	1.23
TGFβ2	**0hrs**	100.00 ± 3.77	1.19
	6hrs	107.55 ± 3.33	1.05
	12hrs	122.64 ± 3.88	1.23
	18hrs	139.63 ± 3.77	1.19
	24hrs	141.51 ± 5.18	1.64
	36hrs	147.17 ± 5.03	1.59
TGFβ3	**0hrs**	100.00 ± 3.63	1.15
	6hrs	137.78 ± 7.98	2.52
	12hrs	142.22 ± 4.91	1.55
	18hrs	151.11 ± 4.57	1.44
	24hrs	173.33 ± 2.34	0.74
	36hrs	213.33 ± 7.41	2.34

Table 6.5 Descriptive statistics of mean % bone cell number against time for cells treated with TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3) and TGF-β(2+3) (10000 Cells/Flask).

Treatment	Time (hrs)	Mean % Cell Number ± Std. Deviation (SD)	Std. Error (SE)
TGFβ1+2	**0hrs**	100.00 ± 5.12	1.62
	6hrs	104.35 ± 5.61	1.77
	12hrs	108.70 ± 6.06	1.92
	18hrs	117.39 ± 6.23	1.97
	24hrs	123.91 ± 10.20	3.22
	36hrs	134.78 ± 7.32	2.31
TGFβ1+3	**0hrs**	100.00 ± 4.10	1.30
	6hrs	116.28 ± 6.20	1.96
	12hrs	132.56 ± 4.65	1.47
	18hrs	141.86 ± 7.44	2.35
	24hrs	151.16 ± 5.59	1.77
	36hrs	162.79 ± 8.35	2.64
TGFβ2+3	**0hrs**	100.00 ± 4.52	1.43
	6hrs	111.63 ± 6.85	2.17
	12hrs	127.91 ± 6.58	2.08
	18hrs	139.54 ± 8.13	2.57
	24hrs	162.79 ± 7.35	2.33
	36hrs	181.40 ± 6.58	2.08
TGFβ1+2+3	**0hrs**	100.00 ± 7.32	2.31
	6hrs	102.17 ± 5.32	1.68
	12hrs	115.22 ± 10.09	3.19
	18hrs	141.30 ± 6.40	2.02
	24hrs	154.35 ± 9.45	2.99
	36hrs	163.04 ± 6.15	1.94

Table 6.6 P value derived from pair wise comparisons of mean % bone cell number vs. time treated with TGF-β isomers and their combinations. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	Cell No. 0hrs	Cell No. 6hrs	Cell No. 12hrs	Cell No. 18hrs	Cell No. 24hrs	Cell No. 36hrs
Control – TGFβ1	0.545	0.702	0.984	0.001***	0.001***	0.001***
Control – TGFβ2	0.494	0.481	0.992	0.069	0.014*	0.002**
Control – TGFβ3	0.540	0.001***	0.001***	0.001***	0.001***	0.001***
Control – TGFβ1+2	0.493	0.05	0.020*	0.001***	0.001***	0.001***
Control – TGFβ1+3	0.543	0.969	0.006**	0.041*	0.998	0.325
Control – TGFβ2+3	0.320	0.999	0.251	0.495	0.097	0.001***
Control – TGFβ1+2+3	0.544	0.005**	0.826	0.068	1.000	0.325
TGFβ1 - TGFβ2	0.940	1.000	0.678	0.001***	0.088	0.001***
TGFβ1 - TGFβ3	0.820	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ1+2	0.940	0.832	0.196	0.129	0.248	0.171
TGFβ1 - TGFβ1+3	0.940	0.139	0.001***	0.001***	0.001***	0.001***
TGFβ1 - TGFβ2+3	0.761	0.958	0.029*	0.001***	0.001***	0.001***
TGFβ1 - TGFβ1+2+3	1.000	0.362	0.999	0.001***	0.001***	0.001***
TGFβ2 - TGFβ3	0.939	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ2 - TGFβ1+2	0.819	0.952	0.002**	0.001***	0.001***	0.002**
TGFβ2 - TGFβ1+3	0.939	0.062	0.064	0.363	0.084	0.001***
TGFβ2 - TGFβ2+3	0.702	0.845	0.745	0.705	0.001***	0.001***
TGFβ2 - TGFβ1+2+3	0.879	0.576	0.327	0.196	0.005**	0.001***
TGFβ3 - TGFβ1+2	0.730	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ3 - TGFβ1+3	1.000	0.001***	0.078	0.003**	0.001***	0.001***
TGFβ3 - TGFβ2+3	0.465	0.001***	0.001***	0.005**	0.042*	0.001***
TGFβ3 - TGFβ1+2+3	0.761	0.001***	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ1+3	0.819	0.002**	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ2+3	0.672	0.199	0.001***	0.001***	0.001***	0.001***
TGFβ1+2 - TGFβ1+2+3	0.939	0.995	0.496	0.001***	0.001***	0.001***
TGFβ1+3 - TGFβ2+3	0.847	0.738	0.845	0.493	0.017*	0.001***
TGFβ1+3 - TGFβ1+2+3	1.000	0.001***	0.001***	1.000	0.978	0.880
TGFβ2+3 - TGFβ1+2+3	0.703	0.032	0.005**	0.447	0.191	0.001***

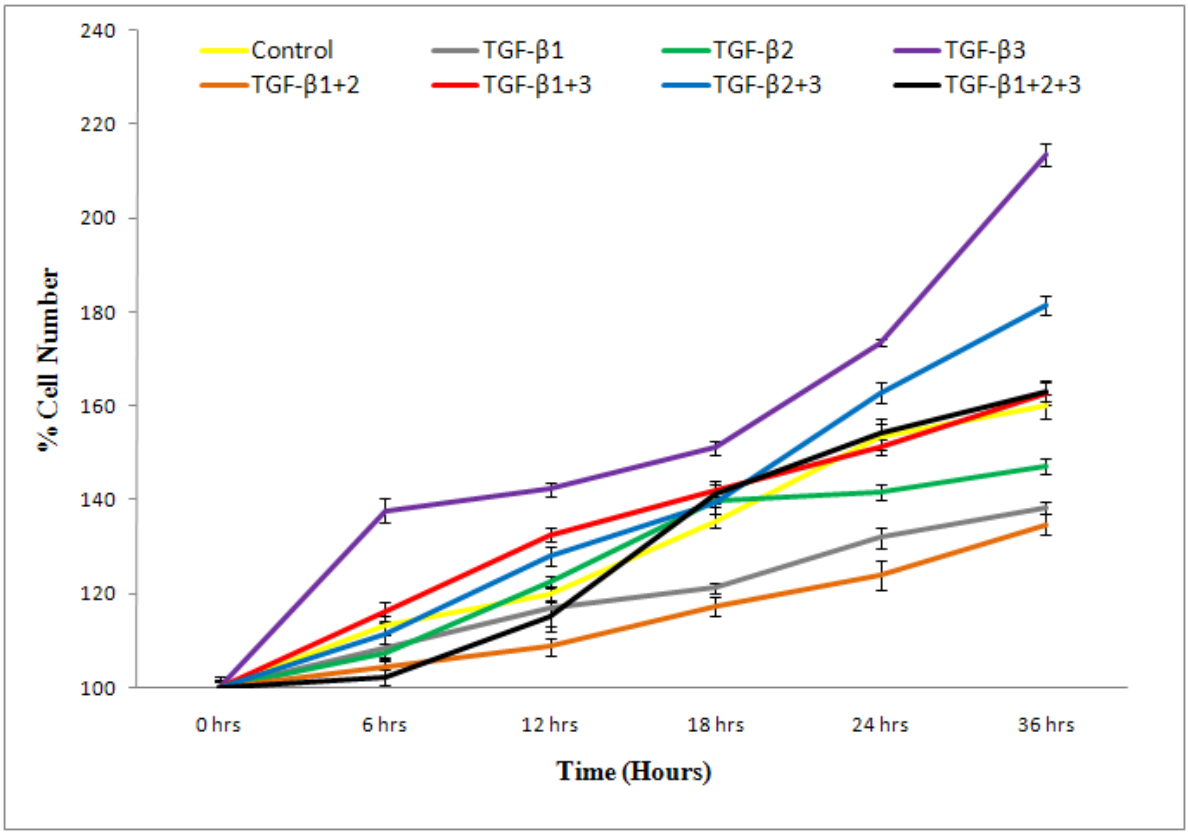


Figure 6.10 *Graph of mean % cell number against time for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control (Error bars represent standard deviation).*

6.5 DISCUSSION

The Transforming growth factor-β (TGF-β) is a powerful modulator of bone metabolism and both its anabolic and catabolic effects on bone have been investigated (Karsdal, 2001, Ehnert, 2010). TGF-β is a cytokine that profoundly alters osteoblast shape (Hurley, 1994). It is one of the most abundant growth factors in skeletal tissues (Bonewald, 1990, Sun, 2009) and, in mammals, comprises three isoforms; TGF-β1, TGF-β2, and TGF-β3, all of which are expressed by bone cells (Horner, 1998, Chin, 2004) and interact with the known TGF-β receptors types I, II, and III (betaglycan) (Hartsough, 1997). All TGF-β isoforms display different biological activities

(Bonewald, 1996) although one isoform may be more potent than another in a given assay (Joyce, 1990, Chin, 2004). Since the role of TGF-β induced changes in osteoblast morphology is largely unknown various investigations suggest that TGF-β has multiple functions in bone metabolism (Karsdal, 2001). *In vivo* studies show that TGF-β can increase bone formation and promote fracture healing (Bonewald, 1996, Rosier, 1998). TGF-β1 knock-out mice display approximately a 30% decrease in tibial length and a reduction in bone mineral content (Geiser, 1998), consistent with the idea that TGF-β functions as a bone-forming agent. On the other hand, over expression of TGF-β2 controlled by the osteoblast-specific osteocalcin promoter leads to bone loss (Erlebacher, 1996), and mice expressing a dominant-negative TGF-β type II receptor have no obvious skeletal defects apart from joint abnormalities that are probably due do chondrocytes malfunction (Serra, 1997, Khaghani, 2009a). These apparently conflicting results are also reflected in numerous studies in cell and tissue cultures where TGF-β seems to modulate various bone cell activities in opposite ways. For instance, both increases and decreases in osteoclast formation, bone resorption, osteoblast proliferation, and osteoblast differentiation have been reported (Bonewald, 1996, Centrella, 1994, Ehnert, 2010). TGF-β has various actions on bone cells while the intracellular signals triggered by the binding of TGF-β to its receptor are vast and complex. They include; phosphorylation and activation of Smad transcription factors; regulation of Ras, Rho, and other small G-proteins; activation of protein kinases such as TGF-β-activated kinase-1 (TAK1); mitogen-activated protein (MAP) kinases, and Src, recruitment of adaptor proteins and regulation of ion channels (Visser, 1998). In this chapter the hypothesis that different TGF-β isomers induced changes in osteoblast morphology and cell proliferation (cell number) was tested. Our results suggested that

cell morphology changes were observed more in cells treated with TGF-β(2+3) and TGF-β(1+3) followed by TGF-β3 and TGF-β2 and control treatments. A culture flask treated with TGF-β1 showed less elongation compared to cells treated with TGF-β3 and TGF-β2. In terms of proliferation cells treated with TGF-β3 and TGF-β(2+3) showed a higher % cell number followed by TGF-β(1+2+3), TGF-β(1+3) and the control. TGF-β1 and TGF-β2 did not induce proliferation. Regulation of cell shape is an important process that controls tissue morphogenesis, cell migration, differentiation, proliferation, and survival, presumably by regulating the expression of genes central to these processes (Boudreau, 1999, Lukashev, 1998, Nakamura, 2007). However, it is possible that TGF-β induced osteoblast elongation contributes to several aspects of TGF-β function in bone. Previous literature suggests that the increased bone resorption in response to TGF-β is due to the shape alterations of osteoblasts and is not mediated through the direct action of TGF-β on osteoclasts or by the production of osteoclast-activating factors produced by MC cells in response to TGF-β (Hartsough, 1997, Nakamura, 2007). It has been demonstrated in epithelial cells that secretion of TGF-β can stimulate MAP kinase pathways and that they mediate some of the effects of TGF-β on gene expression (Hartsough, 1997). TGF-β induced osteoblast elongation was dependent on p38 MAP kinase activity, as assessed by using the p38 MAP kinase inhibitor. This is the first report functionally linking the p38 MAP kinase pathway to TGF-β in osteoblasts (Kozawa, 1999, Suzuki, 1999). Osteoblast shape changes induced by bFGF were only slightly inhibited, indicating that TGF-β and bFGF alter osteoblast morphology by using different intra-cellular signalling cascades. This finding is also consistent with the fact that the morphology of osteoblasts in response to TGF-β is obviously different from the morphology of osteoblasts treated with bFGF (Suzuki,

1999). At present it is not clear in which situations TGF-β could facilitate bone resorption/absorbtion *in vivo* by altering osteoblast shape (Karsdal, 2001, Serganova, 2009). One possibility is that active TGF-β is produced during bone remodeling within the basic multi-cellular unit (BMU) of the osteon. Both osteoblasts and osteoclasts are able to synthesise TGF-β (Oursler, 1994, Oreffo, 1989, Bonewald, 1991, Selvamurugan, 2004). Although osteoblasts are responsible for production of most of the TGF-β entrapped in bone matrix (Bonewald, 1994, Selvamurugan, 2004), the majority of active TGF-β appears to be generated by bone resorbing osteoclasts either through the release of TGF-β stored in bone matrix or by biosynthesis of new TGF-β (Pfeilschifter, 1987). Active TGF-β may then be made accessible to osteoblasts through a process involving transcytosis (Mostov, 1997, Ruan, 2010). The selective activation of TGF-β by osteoclasts may fulfill a function by inducing the elongation of bone lining cells (Lucas, 1989, Pfeilschifter, 1990, Ruan, 2010). It also well known that calcium ion present in bone matrix at the tendon-bone repair site is associated with new bone formation and improved collagen organisation at the healing tendon-bone interface (Kovacevic, 2011). The addition of TGF-β3 significantly improved the strength of the bone repair at 4 weeks. There is an assumption that TGF-β3 is able to shift calcium ions into the wound area due to the need for this ion during osteoblast proliferation. The required calcium ion will be obtained from a source close to the wound. Bone itself is a rich and close source for calcium. On the other hand, decalcification causes bone to become less dense. These findings concur with our wound healing result in chapter 4 and current proliferation results in the current chapter. Our results showed an increase in wound healing, cell proliferation and cell length in present of TGF-β3. Thus, the precise role and mechanism of TGF-β with regard to osteoblast elongation remains to be

established. However, our results suggest that various TGF-β causes an increase or decrease in % cell number and cell length. The Focal adhesions activate signalling pathways, such as MAPK and JNK that regulate transcription factor activity and direct cell growth and differentiation (Giancotti, 1999). Many of these integrin-activated signalling cascades are required for osteoblast differentiation (Lai, 2001, Xiao, 2002). Alternatively as a future work it is useful to use other cell viability assays such as alamar blue, formazan based assay (MTT) and live/dead assay to quantitatively measure cell viability and proliferation using fluorescent or colorimetric detection strategies. In the next chapter we will look at the integrin expression and protein secretion of bone cells in the presence of TGF-β.

6.6 CONCLUSIONS

The results suggested that cell morphology changes were observed more in cells treated with TGF-β(2+3) and TGF-β(1+3) followed by TGF-β2 and TGF-3 and un-treated as the control. A culture flask treated with TGF-β1 showed less elongation compared to the control and other TGF-β isomers. In terms of proliferation cells treated with TGF-β3 and TGF-β(2+3) induced the greatest cell division followed by TGF-β(1+2+3), TGF-β(1+3) and the control. TGF-β3 and its combinations showed a higher proliferation compared to the control. TGF-β1, TGF-β2 and TGF-β(1+2) showed less proliferation compared to the control.

CHAPTER SEVEN

IMMUNOCYTOCHEMISTRY AND CELL IMAGING WITH THE WIDEFIELD SURFACE PLASMON RESONANCE (WSPR) MICROSCOPE

7.1 INTRODUCTION

In the previous chapters the effect of TGF-βs (either TGF-β isomers in isolation or in combined form), HCl and BSA (as a TGF-β solvent and carrier) were investigated in relation to wound healing, degree of cell detachment, cell size and cell number. TGF-β3 and HCl enhanced the rate of cell detachment, proliferation and wound healing. The combination of TGF-β3 with any other TGF-β isomer seemed to cause better cell detachment and wound healing, while the absence of TGF-β3 was associated with less cell detachment and wound healing. All previuos findings are directly related to the way cells interact with the surface. These findings suggest that TGF-β isoforms modify cell surface interaction which could influence integrin expression. Cell-surface and cell-cell interactions are mediated by integrins and cell adhesion molecules (Akiyama, 1990, Yang, 2010, Eapen, 2012). Integrins are a group of membrane bound receptor protein which bind to components of the ECM (Hynes, 1992, Aoudjit, 2012) and they are capable of transducing signals from the ECM to the cells which modulate cell behaviour such as migration, differentiation and the synthesis of specific proteins (Hynes, 1992, Keselowsky, 2004). When integrins are inserted into the front of the cell focal adhesions form, these focal adhesions prevent membrane retraction and provide

adhesive traction for cell movement (Bordeleau, 2008). Integrins are heterodimeric glycoproteins consisting of an α and β subunit in which the α chain conveys ligand specificity and the β chain provides linkage to the cytoskeleton via talin, vinculin, or α-actinin (Saito, 2004, Clëzardin, 2009). The α and β chains have similar tertiary structures and are non-covalently linked via their extracellular globular domains. Integrins can be divided into two groups. The first group contains integrins that bind to matrix proteins found in basement membranes. In this group receptors bind to proteins such as collagen and laminin (Stephansson, 2002). The second group contains integrins that bind to extracellular matrix proteins such as fibronectin, vitronectin, osteopontin and fibrinogen, all of which are found during inflammation, wound healing or the development process (Saito, 2004). Integrins also play a part in the formation of hemidesmosomes. Hemidesmosomes are very small structures on the inner basal surface of keratinocytes and they link one cell to the extracellular matrix. Human bone cells are known to express high levels of α1β1, α3β1, α5β1 and αvβ5 integrins and lower levels of α2β1, α4β1, αvβ1 and αvβ3 integrins (Saito, 2004). All cell types in bone express β1 and α5 as integrins (Hughes, 1993, Saito, 2004, Clëzardin, 2009). The human osteosarcoma-derived cell line MG-63 has been shown (Hughes, 1993) to express α5β1 and α5β3 integrins and a variant of this line showing osteoblastic differentiation expresses increased levels of cell surface α5β1 integrin but not αVβ3. Furthermore, interleukin-Iβ induction of osteoblastic differentiation of MG-63 osteosarcoma cells results in an induction of the expression of α5β1. Inhibition of the α5β1function with subunit-specific monoclonal antibodies inhibits this osteoblastic differentiation (Clëzardin, 2009). However, acquired expression of these receptors is a

common phenomenon in cultured cells and *in vitro* phenotypes may not reflect the pattern of expression *in vivo*.

Stimulation of cell migration during wound repair depends on the presence of growth factors such as TGF-β (Sun, 2009, Chin, 2004). TGF-β is a cytokine that may play a role in the repair of tendon and bone (Kovacevic, 2011, Kandziora, 2002, Chin, 2004, Sefat, 2010d). TGF-β1 and, to a lesser extent, bone morphogenetic protein 2 (BMP-2) have been reported to regulate the gene expression of different osteoblast markers *in vitro* (Palcy, 1999, Bodoa, 2010, Ehnert, 2010). TGF-β functions to stimulate alterations in integrin expression as well as altering binding affinity (Chin, 2004). To investigate the expression of ECM proteins and integrins by bone cells treated with various TGF-β isomers *in vitro*, this work aims at undertaking an immunohistochemical study using monoclonal anti-collagen type I, monoclonal anti-Human fibronectin, monoclonal CD49c (Integrin α3) and monoclonal CD29 (Integrin β1) antibodies.

In addition, one of the major problems associated with analysing cell guidance, alignment and cell behaviour is that of imaging the cell surface interface at Z sub-micron resolutions sufficient to interrogate the nanometric scale spatiotemporal interfacial interactions in real time (Abdul Jamil, 2008a, Abdul Jamil, 2008b, Sefat, 2010a). To date, these processes have been mostly examined using standard phase contrast light microscopy, confocal microscopy and immunofluorescence staining techniques. Unfortunately the Z resolution of phase contrast microscope systems is poor and, although the confocal system, by optically sectioning a sample, can allow imaging

at high Z resolutions, this requires the sample to be fixed and taken through immunostaining processes. This means that the imaging of interfacial interactions at high Z resolutions of live cells cannot be achieved. In order to overcome some of these deficiencies in interfacial imaging it is necessary to have a non-invasive mode of imaging at the interface. This may be achieved using surface plasmon (SP) microscopy. The pioneering work of Kretschmann and his colleagues in the 1960s indicated that attenuated total reflectance (ATR) prism based on SPs excitation could be induced in a configuration known as the Kretschmann configuration (Kretschmann, 1968). In this configuration (See Figure 7.1) p-polarised light striking the metallic coated surface from a region of high refractive index at a specific angle will resonantly excite the electrons in the metallic film (Kano, 1998). This resonance will propagate along the conductor, giving rise to a surface Plasmon wave, which decays resulting in the re-immission of light with no loss of energy. The SPR angle is changed when bio-molecular species bind to the coated metal layer and this changes the angle required to excite surface plasmons. This means that inefficient coupling between the incident light and the interface occurs and energy (light) is lost by diffraction. Thus, the binding of a molecular species to an interface in a surface plasmon microscope results in a change in the intensity of the reflected light. This feature gives rise to what has become the surface Plasmon microscope (Kano, 1998). The drawback with these systems are that surface plasmons can travel several microns before decaying and this limits the lateral resolution of surface plasmons to 10-20μm. The Widefield Surface Plasmon Resonance (WSPR) system was developed to circumvent these difficulties. In the WSPR, the surface Plasmon substrate consisting of a gold coated cover slip is illuminated with p-polarised light via a high NA objective lens at an angle sufficient to excite surface plasmons.

However, before reaching the objective lens the light passes through a rotatable diffuser. When stationary the transmission of the light through the diffuser illuminates the surface Plasmon substrate with a sub micron speckled pattern, but when rotated the speckled pattern of illumination is averaged giving rise to a wide field surface plasmon image with a lateral resolution that is no longer dependent on the propagation of surface plasmons, but that is dependent on the NA and wavelength of the illuminating P-polarised light (Somekh, 2003, Abdul Jamil, 2008b). The aim of using this system in this thesis is to directly image cell surface interactions such as the formation of focal contacts or antibody/antigen interactions without the need for immunoflourescent techniques (Abdul Jamil, 2008a, Abdul Jamil, 2008b, Abdul Jamil, 2007a, Abdul Jamil, 2007b, Sefat, 2010a).

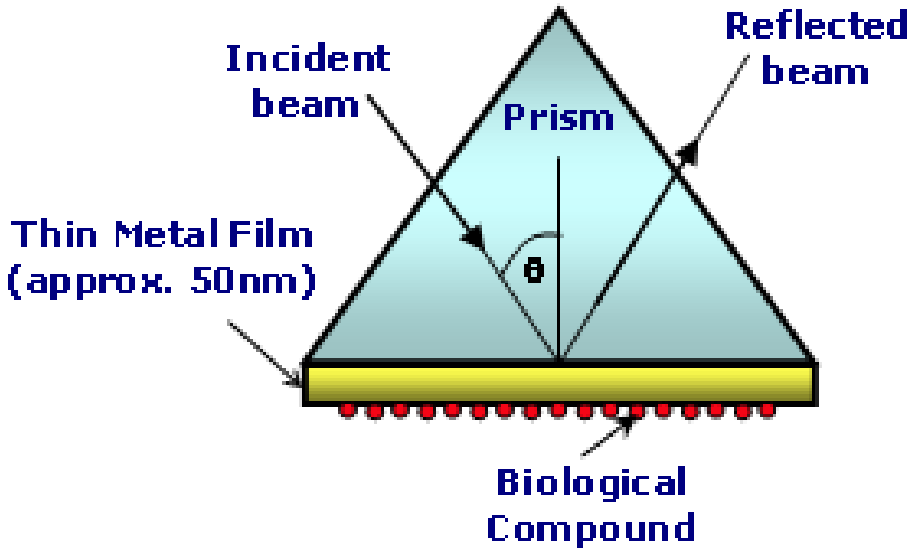


Figure 7.1 *Schematic drawing of the typical Kretschmann configuration used to excite surface plasmons (Kano, 1998, Abdul Jamil, 2007b).*

7.2 AIMS AND OBJECTIVES

In previous chapters the effects of different TGF-β isomers and their combinations on cell behaviour in terms of cell morphology (size and shape), attachment and wound repair were investigated. These experiments indicated that TGF-β3 enhanced bone cell guidance to fibronectin, and therefore, immunostaining studies in this chapter will concentrate on determining which integrins are up regulated by TGF-β3. Immunostaining will also be used to determine if TGF-β3 modifies ECM secretion by the bone cells. In addition, imaging with WSPR will allow observation of the focal contacts without the need for immunostaining which that mediate the regulatory effects such as cell anchorage and direct numerous signaling proteins at sites of integrin binding and clustering. This could provide information on how TGF-βs effect the formation of focal contact which could allow the acquisition of data that supports the findings of the trypsinisation study. The study using the WSPR is also hoped to shed light on how cells on guidance by micro patterened ECM proteins.

7.3 MATERIALS AND METHODS

7.3.1 Collagen Type I Immunostaining

Coverslips were sterilised with 70% ethanol for at least 30 minutes and left in a laminar flow hood to dry. Human bone cells (MG63) and transforming growth factor beta (TGF-β) isomers were prepared. Three dry coverslips were placed into petridishes and 5ml of cell suspension (10,000 cells/ml) was used along with 50µl of TGF-β1, 2 and 3 to give final TGF-β1, 2 and 3 bathing concentrations of 5ng/ml, 10ng/ml and 50ng/ml,

respecticvley. The petridishes were sealed and incubated for 24-48hrs. After 48hrs the old media was aspirated and cells were washed three times with Hanks Balanced Salt Solution (HBSS). Fixation was carried out by bathing the slides in a 4% formaldehyde solution containing 1% BSA for 5-7 minutes at room temperature. Fixation was followed by three 5 minute washes in HBSS, the second of which contained 0.1M Glycine in order to prevent auto-fluorescence. Triton X-100 (0.1%) was used to permeabilize cells for 3-5 minutes. Coverslips were washed with HBSS three times. Cells were then treated with blocking solution (Goat serum or Image-iT™ FX signal enhancer) for 30 minutes and were again washed three times in HBSS. Cells were then incubated in 1:500 dilution of Monoclonal Anti-Collagen Type I primary antibody for 1 hour in a humidity chamber at room temperature. Following incubation with the primary antibody, the coverslips were washed with HBSS three times at 15 minute intervals. Following washing, a secondary antibody (Alexa Fluor 488 Goat Anti-mouse IgG SFX kit, 5µg/ml, SIGMA) was applied for 1 hour in the dark at room temperature. The secondary antibody was washed off with HBSS three times at 15 minute intervals. Prior to imaging, cells were washed in Dapi (SIGMA) solution (10%) for 15 minutes and then imaged using a fluorescence microscope. Finally the total number of cells per micrograph was determined and number of cells stained with anti-collagen type I was counted per micrograph. Cell number was then expressed as the percentage number of stained cell per micrograph. Additional two samples were prepared without any TGF-β treatment as controls. One of them was stained for anti-collagen type I and the other was treated with the secondry antibody only without pre-incubation with the only collagen type I antibody.

7.3.2 Fibronectin Immunostaining

Cells were cultured and treated with TGF-β 1, 2 and 3 as described in section 7.3.1. Cells were washed, fixed and permeabalised as previously described. The cells were then incubated in Monoclonal Anti-Human Fibronectin (working dilution of 1:5000) primary antibody for 1 hour in a humidity chamber at room temperature. Following this incubation the cells were washed with HBSS three times at 15 minute intervals. The cells were then incubated with a secondary antibody (Alexa Fluor 488 Goat Anti-mouse IgG SFX kit, 5µg/ml) for 1 hour in a dark area at room temperature. The cells were then washed with HBSS three times in 15 minute intervals and stored at 4°C for 3-4 days. Cells were finally washed in Dapi solution (10%) for 15 minutes and then imaged using a fluorescence microscope. Numbers of cells stained with anti-human fibronectin were measured. The % number of stained cells was then calculated as described in section 7.3.1. Additional two samples were also prepared without any TGF-β treatment as controls. One of them was stained for anti-human fibronectin and the other one was treated with the secondry antibody only without pre-incubation with the only fibronectin antibody.

7.3.3 Integrin Immunostaining

Cells were cultured and treated with TGF-β 1, 2 and 3 and then were washed, fixed and permeabilized as described in section 7.3.1. Cells were incubated in CD49c (Integrin α3) and CD29 (Integrin β1) primary antibodies for 1 hour in humidity chamber at room temperature (with the dilution ratio of 1:500 and 1:1000, respectively). After this incubation, the coverslips were washed with HBSS three times in 15 minute intervals.

Secondary antibody (Alexa Fluor 488 Goat Anti-mouse IgG SFX kit, 5μg/ml) was applied for 1 hour in a dark area at room temperature. All incubations were performed in a humidity chamber to avoid the evaporation of reagents. The cells were then washed with HBSS three times in 15 minute intervals. Cells were then washed in Dapi solution (10%) for 15 minutes and finally imaged using a fluorescence microscope. The number of cells stained with α3 and β1 integrin was measured. Cell number was then expressed as percentage number of stained cells per micrograph.

7.3.4 Control

Cell cultures treated with no growth factor and no primary antibody (Ab) were used as negative controls while positive control represents absence of TGF-β only with addition of primary antibody. Additional samples were also prepared for each TGF-β treatment with and without applying the primary antibody.

7.3.5 SPR substrate

A prefabricated coverslip (0.18mm thick and 22mm diameter), coated with 50nm gold on top of 1nm chromium (for better adherence) (See Figure 7.2) were stamp patterned with fibronectin protein by using a 50μm stamp. The substrate was then plated with MG63 bone cells (10,000 cells/coverslip). After fixing the cells, the substrates were dehydrated in serial alcohol and then imaged using a phase contrast light microscope. The substrates were then mounted in the sample holder of the WSPR microscope and imaged further. Additional gold substrates seeded with MG-63 bone cells were treated

with all TGF-β isomers and their combinations in order to be imaged using WSPR microscope.

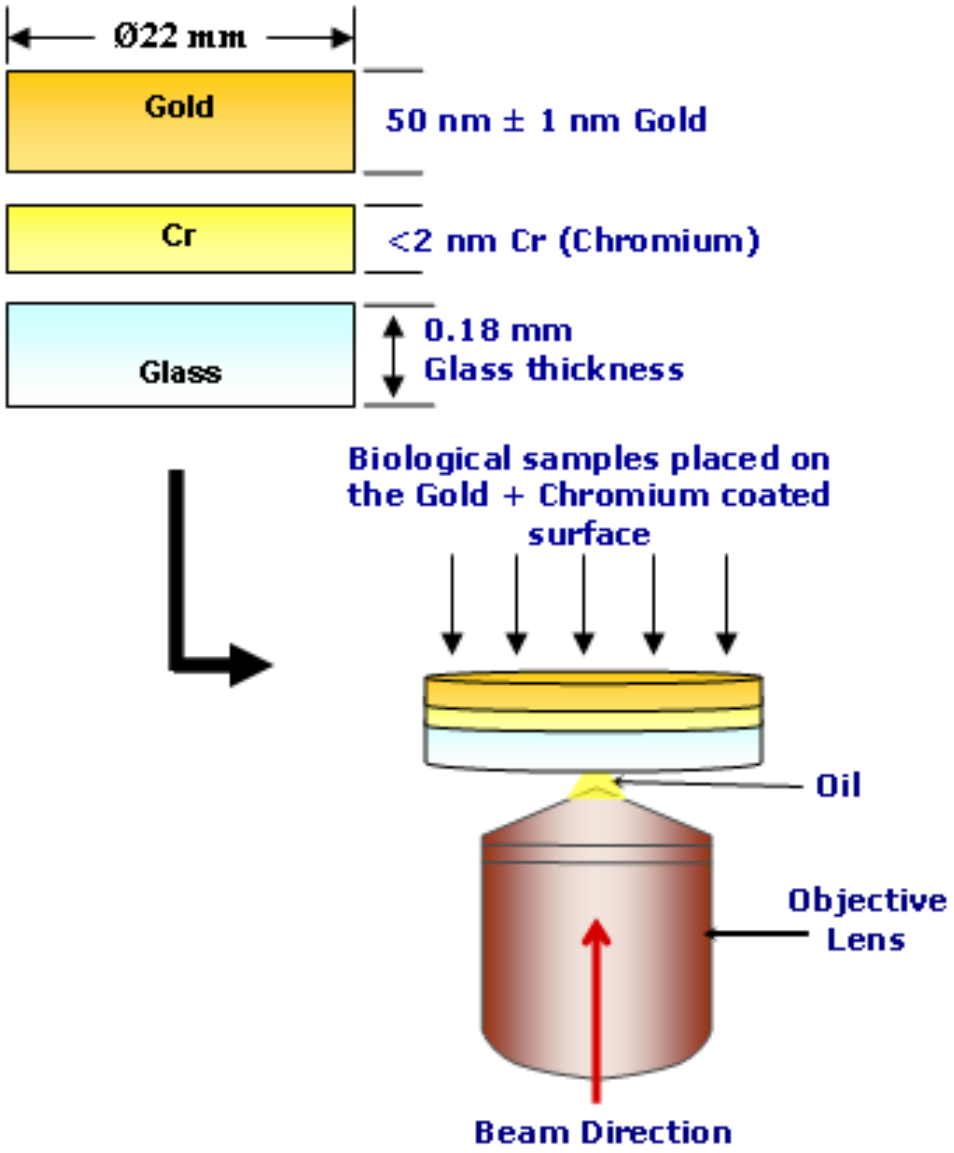


Figure 7.2 *Illustration of the SPR substrate structures and sample orientation in the system during imaging with high NA objective lens. Diagram also indicates on which side the samples were placed (Sefat, 2010a, Sefat, 2010d).*

7.4 STATISTICAL ANALYSIS

The mean % number of stained cells per treatment group was tested for normality using a Kolmogorov Smirnov test. Normally distributed data ($p > 0.05$) was analysed using SPSS via a Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed results ($p < 0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

7.5 RESULTS

7.5.1 Immunostaining

7.5.1.1 Collagen type I immunostaining

The mean % number of stained cells per treatment group for immunocytochemistry with anti-collagen type I antibodies were normally distributed and thus analysed using SPSS via Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test (See Tables 7.1 and 7.2). Immunofluorscence micrographs of human bone cells stained for anti collagen type I showed that the bone cells treated with TGF-β2 and TGF-β3 produced more collagen protein compared to the control (see Figure 7.3) (with mean % number of cells stained per micrograph ~ 100% ± 11.26SD after TGF-β2 treatment, 93.10% ± 6.70SD for after TGF-β3 treatment and 37.93% ± 6.50SD for control cultures with no TGF-β treatment. Statistically, there are no significant differences in mean % number of cells stained per micrograph with collagen type I between TGF-β2 and TGF-β3 isomers (p=0.504). In these micrographs, cell surface

appears quite green with TGF-β3 treatment indicating release of high amount of collagen type I protein compared to the control with no TGF-β treatment (see Figure 7.3). No specific staining was observed in the micrographs with no primary antibody confirming the specificity of the primary antibody and blue dapi staining represents live cells. Bone cells treated with TGF-β(1+2+3) also exhibited strong staining of cells with mean % number of cells stained per micrograph with anti-collagen type I ~ 79.31% ± 2.27SD and this was similar to the staining observed after TGF-β2 and TGF-β3 treatment. This suggests that both TGF-β2 and TGF-β3 may increase the secretion of collagen type I in MG63 bone cells. Cells treated with TGF-β1 (see Figure 7.3) shows only partial (weak) staining for collagen type I with mean % number of cells stained per micrograph with anti-collagen type I ~ 62.06% ± 6.50SD. This represents a significantly lower collagen type I expression in comparison to treatment with either TGF-β2 or TGF-β3 (p<0.001) (See Tables 7.1, 7.2 and Figure 7.4). Cells in control cultures with no TGF-β also show weak staining with mean % number of anti-collagen type I stained cells per micrograph ~ 37.93% ± 6.50SD when compared with cultures treated with TGF-β1 (mean % number of anti-collagen type I stained cells per micrograph ~ 62.06% ± 6.50SD). The immunofluorscence micrographs (Figure 7.3) of human bone cells stained for anti collagen type I treated with TGF-β(2+3) and TGF-β(1+3) also show strong staining with mean % number of cells stained per micrograph with anti-collagen type I ~ 68.96% ± 8.60SD and 55.17% ± 5.62SD, respectively. However, there is a significant difference in the mean % number of cells stained per micrograph for collagen type I between these two cultures (p<0.01) and no significant difference was seen between TGF-β(2+3) and TGF-β(1+2+3) (p=0.074) treated cells (See Tables 7.1, 7.2 and Figure 7.4). In the case of TGF-β(1+2) the mean % number of stained cells

(34.48% ± 8.45SD) was not significantly different from the control treated cells (p=0.975). Strong staining was seen in cultures treated with TGF-β2 and TGF-β3 isomer combinations while culture flasks treated with TGF-β1 combinations demonstrated weak staining (See Figure 7.3). This suggests that the presence of TGF-β1 in combination with other TGF-β isomers had an inhibitory effect on bone cell protein secretion (Sefat, 2009c). These results showed that there are significant differences in the mean % number of cells stained per micrograph for collagen type I between control and all TGF-β isomers (p<0.001) except for TGF-β(1+2) (p=0.975) (See Tables 7.1 and 7.2). Overall, the staining results showed that TGF-β2 and TGF-β3 increased the mean % number of cells stained per micrograph with anti-collagen type I antibodies indicating that treatment with these cytokines enhanced collagen type I expression.

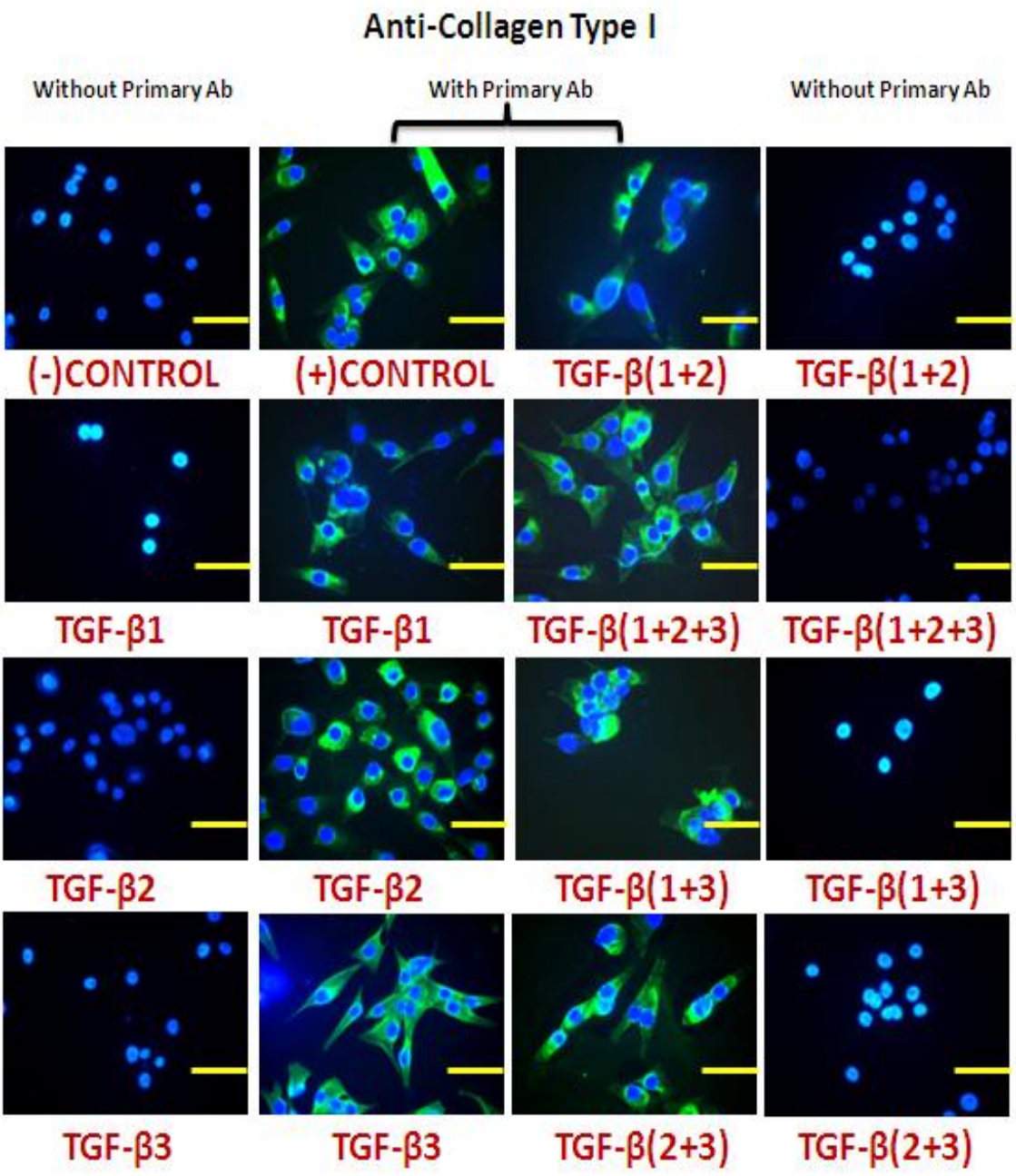


Figure 7.3 *Immunofluorescence micrographs of human bone cell (MG63) treated with different TGF-β isomers and their combinations stained with dapi solution (blue) and anti-collagen type I (green). Negative control represents absence of both TGF-β and primary antibody (Ab) while positive control represents absence of TGF-β only with addition of primary antibody. Images in the right and left columns show dapi stained negative control bone cells following immunostaining in which the primary antibody was omitted (Scale bar = 100μm).*

Table 7.1 Descriptive statistics for mean % numbers of bone cells stained with anti-collagen type I in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control.

Treatment	Mean % numbers of bone cells stained with anti-collagen Type I ± Std. Deviation (SD)	Std. Error (SE)
Control	37.93 ± 6.50	2.06
TGFβ1	62.06 ± 6.50	2.06
TGFβ2	100.00 ± 11.26	3.56
TGFβ3	93.10 ± 6.70	2.11
TGFβ1+2	34.48 ± 8.45	2.67
TGFβ1+3	55.17 ± 5.62	1.78
TGFβ2+3	68.96 ± 8.60	2.72
TGFβ1+2+3	79.31 ± 2.27	2.29

Table 7.2 P value derived from pair wise comparisons of mean % numbers of bone cells stained with anti-collagen type I in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	*P*-value	Sig diff	Treatment	*P*-value	Sig diff
Control – TGFβ1	0.001	***	**TGFβ2 - TGFβ1+2**	0.001	***
Control – TGFβ2	0.001	***	**TGFβ2 - TGFβ1+3**	0.001	***
Control – TGFβ3	0.001	***	**TGFβ2 - TGFβ2+3**	0.001	***
Control – TGFβ1+2	0.975		**TGFβ2 - TGFβ1+2+3**	0.001	***
Control – TGFβ1+3	0.001	***	**TGFβ3 - TGFβ1+2**	0.001	***
Control – TGFβ2+3	0.001	***	**TGFβ3 - TGFβ1+3**	0.001	***
Control – TGFβ1+2+3	0.001	***	**TGFβ3 - TGFβ2+3**	0.001	***
TGFβ1 - TGFβ2	0.001	***	**TGFβ3 - TGFβ1+2+3**	0.004	**
TGFβ1 - TGFβ3	0.001	***	**TGFβ1+2 - TGFβ1+3**	0.001	***
TGFβ1 - TGFβ1+2	0.001	***	**TGFβ1+2 - TGFβ2+3**	0.001	***
TGFβ1 - TGFβ1+3	0.504		**TGFβ1+2 - TGFβ1+2+3**	0.001	***
TGFβ1 - TGFβ2+3	0.504		**TGFβ1+3 - TGFβ2+3**	0.004	**
TGFβ1 - TGFβ1+2+3	0.001	***	**TGFβ1+3 - TGFβ1+2+3**	0.001	***
TGFβ2 - TGFβ3	0.504		**TGFβ2+3 - TGFβ1+2+3**	0.074	

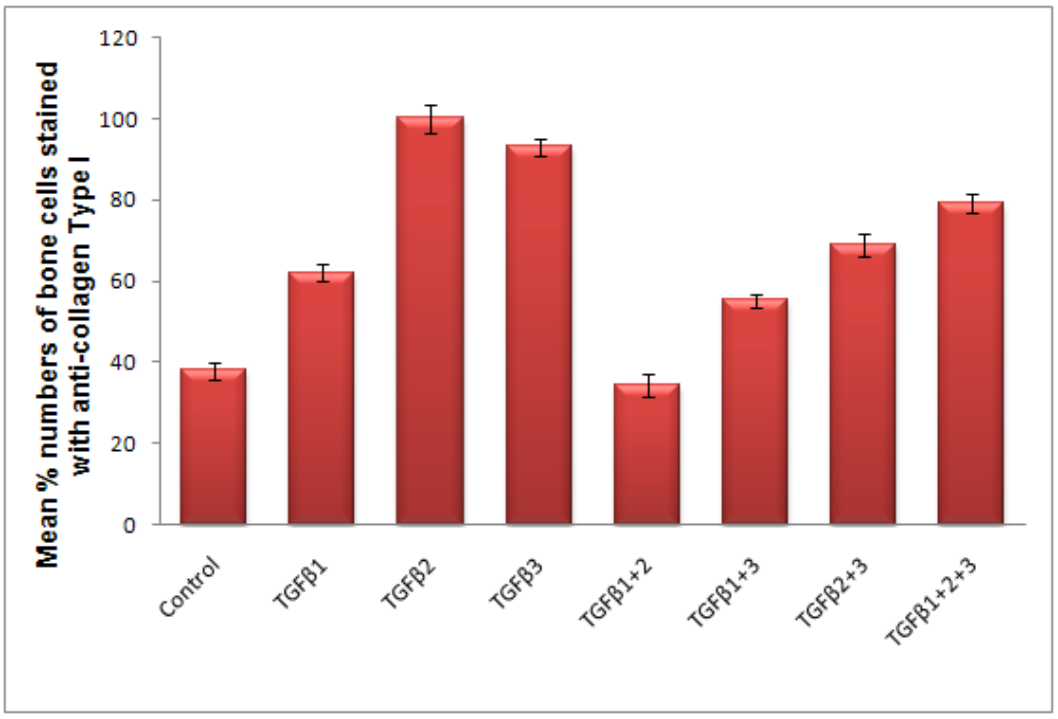


Figure 7.4 *Bar chart showing mean % number of bone cells stained with anti-collagen type I for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control (Error bars represent standard error).*

7.5.1.2 Fibronectin immunostaining

The mean % number of stained cells per treatment group for immunocytochemistry with anti-human fibronectin antibodies did not show normal distribution and were analysed using SPSS via Kruskal-Wallis test and serial Mann Whitney tests (See Tables 7.3 and 7.4). Figure 7.5 shows the immunofluorscence micrographs of human bone cells stained for anti-human fibronectin. It can be seen that bone cells treated with TGF-β3 show good distribution of fibronectin around the periphery of the nucleus with a mean % number of cells stained per micrograph with anti-human fibronectin of 98.41% ± 16.63SD. Staining was brighter compared to the control and although this could be an artefact, it could indicate a high secretion of fibronectin. Bone cells cultured and then

treated with TGF-β1 show evidence of less (weak) staining with a mean % number of cells stained per micrograph with anti-human fibronectin of 53.33% ± 7.69SD while TGF-β2 treated culture shows greater fibronectin production with more strong staining and a mean % number of cells stained per micrograph with anti-human fibronectin of 93.33% ± 11.33SD (see Figures 7.5, 7.6, Tables 7.3 and 7.4). Statistically, there were no significant differences in the mean % number of cells stained per micrograph for anti-human fibronectin between TGF-β2 and TGF-β3 treated cultures (p=0.266), while there are significant differences between cultures treated with TGF-β1 and other two isomers (p<0.001). Cells looked brighter and bigger in the case of TGF-β3, while fewer cells were stained in the case of TGF-β1. However, TGF-β1, TGF-β2 and TGF-β3 treated cells showed higher % number of cells stained as compared to control. Figure 7.5 indicates that the presence of TGF-β3 in any combination was related to more stained cells with a mean % number of cells stained per micrograph with anti-human fibronectin of 72.66% ± 18.44SD for TGF-β(2+3) treated cells and 80.00% ± 11.33SD for TGF-β(1+2+3) treated cells (See Figures 7.5, 7.6, Tables 7.3 and 7.4). In all cultures treated with TGF-β3 combinations including TGF-β(2+3), TGF-β(1+3) and TGF-β(1+2+3) there were some degree of strong staining with higher secretion of fibronectin protein as compared to TGF-β(1+2) in which less fibronectin secretion near the nucleus occurred. TGF-β(1+2) seemed to inhibit fibronectin significantly more than any other treatment, including those cells not exposed to any TGF-β (treatment controls).

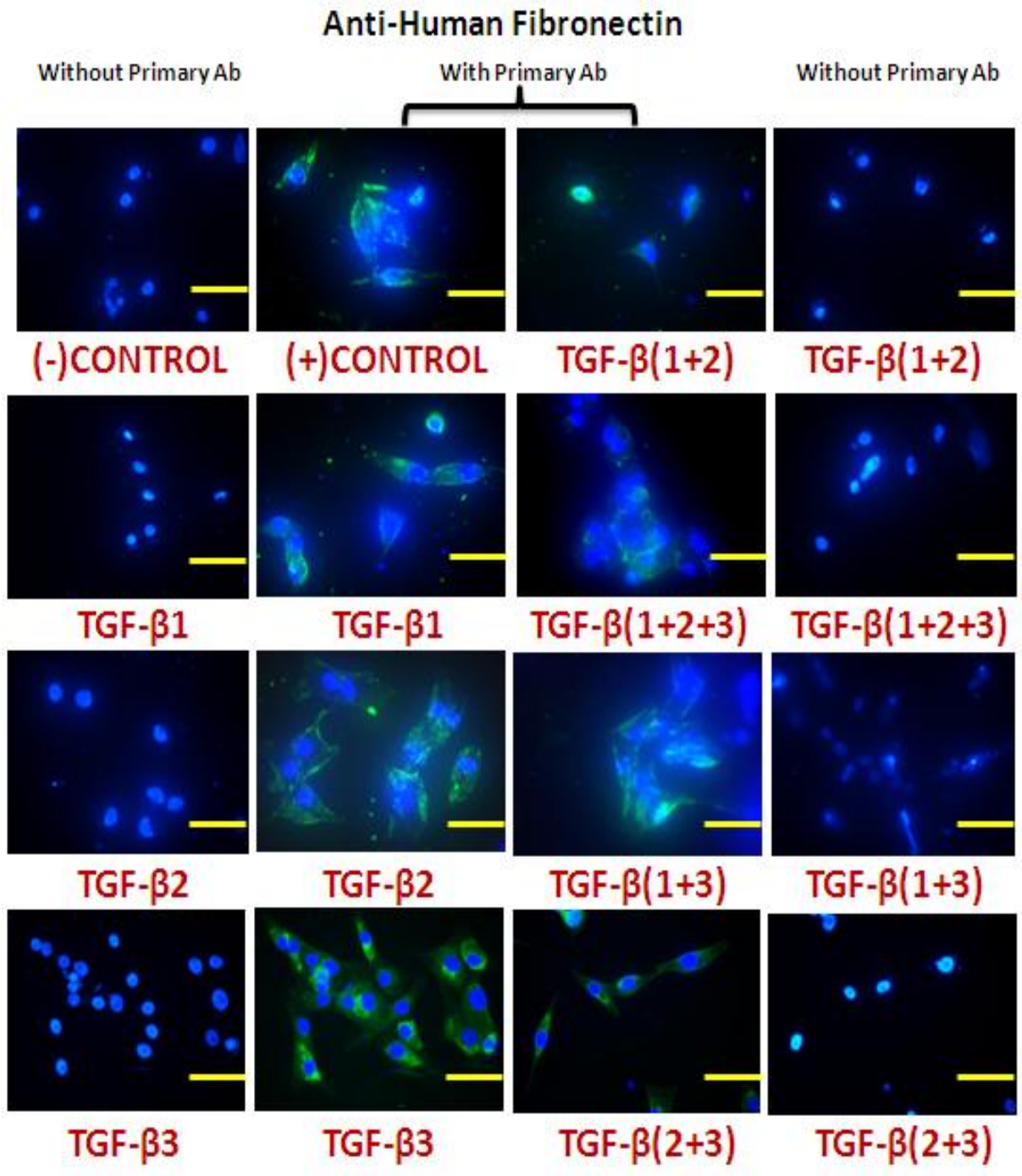


Figure 7.5 *Immunofluorescence micrographs of human bone cell (MG63) treated with different TGF-β isomers and their combinations stained with dapi solution (blue) and anti-human fibronectin (green). Negative control represents absence of both TGF-β and primary antibody while positive control represents absence of TGF-β only with addition of primary antibody. Images in the right and left columns show dapi stained negative control bone cells following immunostaining in which the primary antibody was omitted (Scale bar = 100μm).*

Table 7.3 Descriptive statistics for mean % numbers of bone cells stained with anti-human fibronectin in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control.

Treatment	Mean % numbers of bone cells stained with anti-human fibronectin ± Std. Deviation (SD)	Std. Error (SE)
Control	33.33 ± 7.69	2.43
TGFβ1	53.33 ± 7.69	2.43
TGFβ2	93.33 ± 11.33	3.58
TGFβ3	98.41 ± 16.63	5.26
TGFβ1+2	20.00 ± 6.29	1.98
TGFβ1+3	60.00 ± 17.21	5.44
TGFβ2+3	72.66 ± 18.44	5.83
TGFβ1+2+3	80.00 ± 11.33	3.58

Table 7.4 P value derived from pair wise comparisons of mean % numbers of bone cells stained with anti-human fibronectin in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	*P*-value	Sig diff	Treatment	*P*-value	Sig diff
Control – TGFβ1	0.001	***	**TGFβ2 - TGFβ1+2**	0.001	***
Control – TGFβ2	0.001	***	**TGFβ2 - TGFβ1+3**	0.001	***
Control – TGFβ3	0.001	***	**TGFβ2 - TGFβ2+3**	0.013	*
Control – TGFβ1+2	0.002	**	**TGFβ2 - TGFβ1+2+3**	0.025	*
Control – TGFβ1+3	0.001	***	**TGFβ3 - TGFβ1+2**	0.001	***
Control – TGFβ2+3	0.001	***	**TGFβ3 - TGFβ1+3**	0.001	***
Control – TGFβ1+2+3	0.001	***	**TGFβ3 - TGFβ2+3**	0.005	*
TGFβ1 - TGFβ2	0.001	***	**TGFβ3 - TGFβ1+2+3**	0.001	***
TGFβ1 - TGFβ3	0.001	***	**TGFβ1+2 - TGFβ1+3**	0.001	***
TGFβ1 - TGFβ1+2	0.001	***	**TGFβ1+2 - TGFβ2+3**	0.001	***
TGFβ1 - TGFβ1+3	0.539		**TGFβ1+2 - TGFβ1+2+3**	0.001	***
TGFβ1 - TGFβ2+3	0.012	*	**TGFβ1+3 - TGFβ2+3**	0.148	
TGFβ1 - TGFβ1+2+3	0.001	***	**TGFβ1+3 - TGFβ1+2+3**	0.013	*
TGFβ2 - TGFβ3	0.266		**TGFβ2+3 - TGFβ1+2+3**	0.338	

There are significant differences in the mean % number of cells stained per micrograph between all TGF-β isomers and control (p<0.001). Also, no significant differences were obtained between TGF-β(1+3) and TGF-β(2+3) (p=0.148). Similar result were recorded between TGF-β(2+3) and TGF-β(1+2+3) (p=0.338). No specific staining was observed in the micrographs with absence of primary antibody confirming the specificity of the primary antibody.

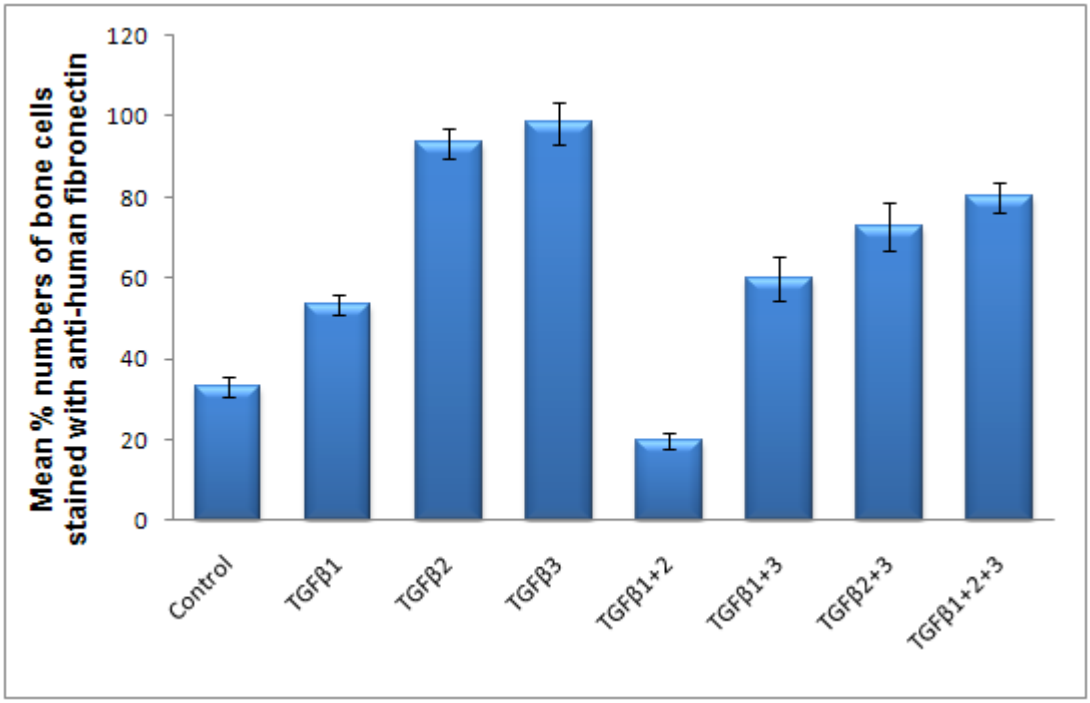


Figure 7.6 *Bar chart showing mean % number of bone cells stained with anti-human fibronectin for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control (Error bars represent standard error).*

7.5.1.3 Integrin immunostaining

The mean % number of stained cells per treatment group after immunostaining with anti integrin-β1 antibodies were analyzed using SPSS via Kruskal–Wallis test as data was

not normally distributed (See Tables 7.5 and 7.6). Figure 7.7 shows the immunofluorescence micrographs of human bone cells stained for β1 integrin. It can be seen that bone cells treated with TGF-β3 show good protein secretion at the cell periphery giving the periphery of the cells bright and sharp edges. The mean % number of cells stained per micrograph with integrin β1 was 97.31% ± 12.65SD which indicate an increased occurrence of focal contact compared to the control treated cultures. Similar results were obtained for TGF-β2 with a mean % number of cells stained with integrin β1 of 89.47% ± 16.40SD, while bone cells treated with TGF-β1 show partial β1 integrin staining concentrated near the nucleus with a mean % number of cells stained per micrograph of 57.89% ± 7.84SD (See Figures 7.7, 7.8, Tables 7.5 and 7.6). Treatment with all three TGF-β isomers seemed to induce higher integrin expression compared to the control in which the mean % number of cells stained per micrograph with integrin β1 was 42.11% ± 7.44SD. No specific staining was observed in the micrographs with absence of primary antibody, confirming the specificity of the primary antibody. There are significant differences recorded in the mean % number of cells stained with β1 integrin between cultures treated with TGF-β1 and the other two TGF-β isomers (p<0.001). Treatment with TGF-β3 combinations including TGF-β(2+3), TGF-β(1+3) and TGF-β(1+2+3) showed some staining with a mean % number of cells stained per micrograph with integrin β1 of 68.42% ± 9.28SD, 57.89% ± 12.65SD and 84.21% ± 11.34SD, respectively. Thus, treatment with TGF-β(2+3), TGF-β(1+3) and TGF-β(1+2+3) induced an increased expression of β1 integrin when compared with the mean % (47.36% ± 9.28SD) of cells stained with anti β1 antibodies after TGF-β(1+2) treatment. However, this increase was only significant in the case of TGF-β(2+3) and TGF-β(1+2+3) (See Figures 7.7, 7.8, Tables 7.5 and 7.6).

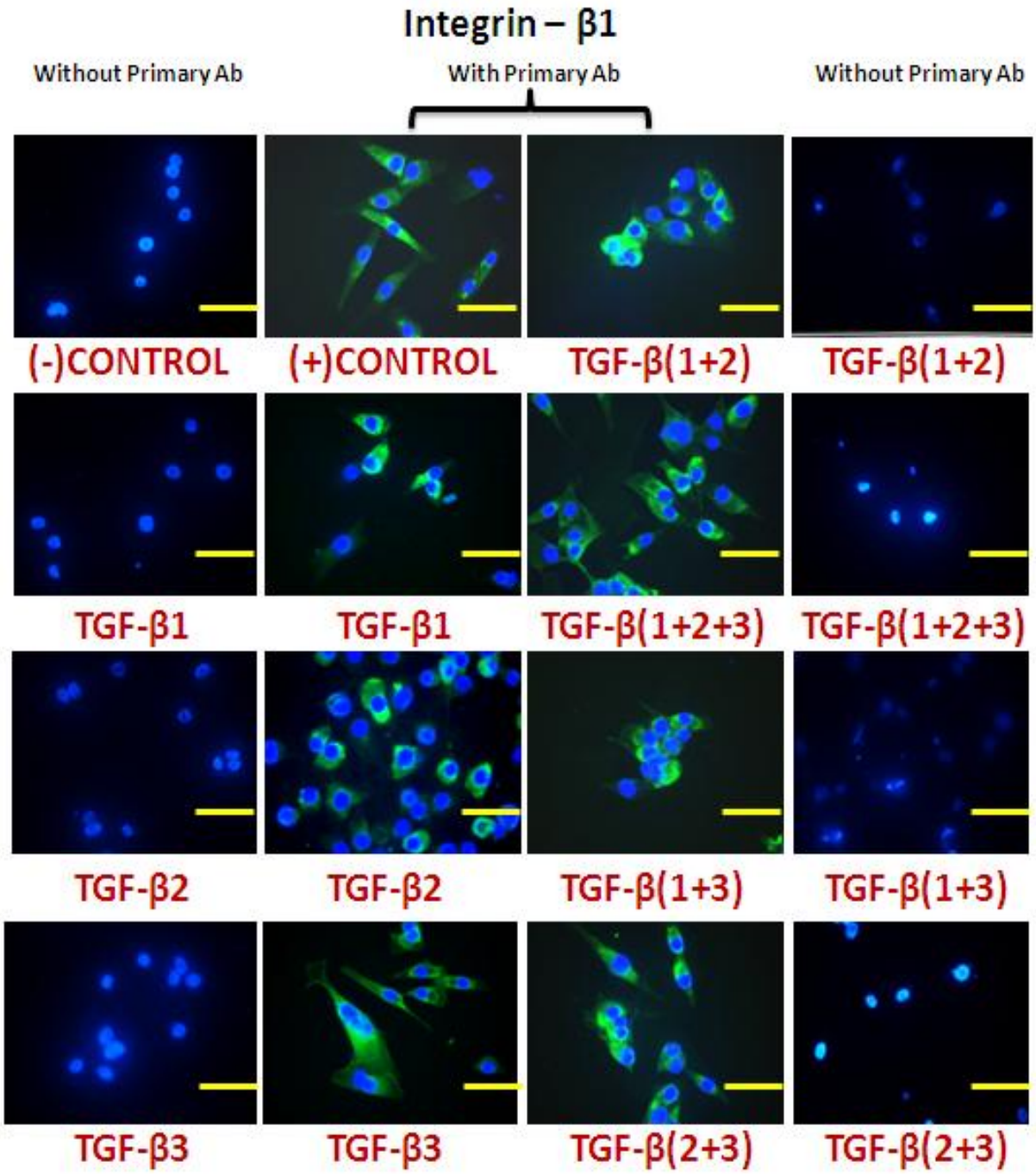


Figure 7.7 *Immunofluorescence micrographs of human bone cell (MG63) treated with different TGF-β isomers and their combinations stained with dapi solution (blue) and Integrin-β1 (green). Negative control represents absence of both TGF-β and primary antibody while positive control represents absence of TGF-β only with addition of primary antibody. Images in the right and left columns show dapi stained negative control bone cells following immunostaining in which the primary antibody was omitted (Scale bar = 100μm).*

Table 7.5 Descriptive statistics for mean % numbers of bone cells stained withβ1 integrin in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control.

Treatment	Mean % numbers of bone cells stained with β1 Integrin ± Std. Deviation (SD)	Std. Error (SE)
Control	42.11 ± 7.44	2.35
TGFβ1	57.89 ± 7.84	2.48
TGFβ2	89.47 ± 16.40	5.18
TGFβ3	97.31 ± 12.65	4.00
TGFβ1+2	47.36 ± 9.28	2.93
TGFβ1+3	57.89 ± 12.65	4.00
TGFβ2+3	68.42 ± 9.28	2.93
TGFβ1+2+3	84.21 ± 11.34	3.59

Table 7.6 P value derived from pair wise comparisons of mean % numbers of bone cells stained with β1 integrin in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	*P*-value	Sig diff	Treatment	*P*-value	Sig diff
Control – TGFβ1	0.001	***	**TGFβ2 - TGFβ1+2**	0.001	***
Control – TGFβ2	0.001	***	**TGFβ2 - TGFβ1+3**	0.001	***
Control – TGFβ3	0.001	***	**TGFβ2 - TGFβ2+3**	0.001	***
Control – TGFβ1+2	0.177		**TGFβ2 - TGFβ1+2+3**	0.23	
Control – TGFβ1+3	0.005	**	**TGFβ3 - TGFβ1+2**	0.001	***
Control – TGFβ2+3	0.001	***	**TGFβ3 - TGFβ1+3**	0.001	***
Control – TGFβ1+2+3	0.001	***	**TGFβ3 - TGFβ2+3**	0.001	***
TGFβ1 - TGFβ2	0.001	***	**TGFβ3 - TGFβ1+2+3**	0.008	**
TGFβ1 - TGFβ3	0.001	***	**TGFβ1+2 - TGFβ1+3**	0.076	
TGFβ1 - TGFβ1+2	0.013	*	**TGFβ1+2 - TGFβ2+3**	0.001	***
TGFβ1 - TGFβ1+3	0.756		**TGFβ1+2 - TGFβ1+2+3**	0.001	***
TGFβ1 - TGFβ2+3	0.027	*	**TGFβ1+3 - TGFβ2+3**	0.055	
TGFβ1 - TGFβ1+2+3	0.001	***	**TGFβ1+3 - TGFβ1+2+3**	0.001	***
TGFβ2 - TGFβ3	0.017	*	**TGFβ2+3 - TGFβ1+2+3**	0.005	**

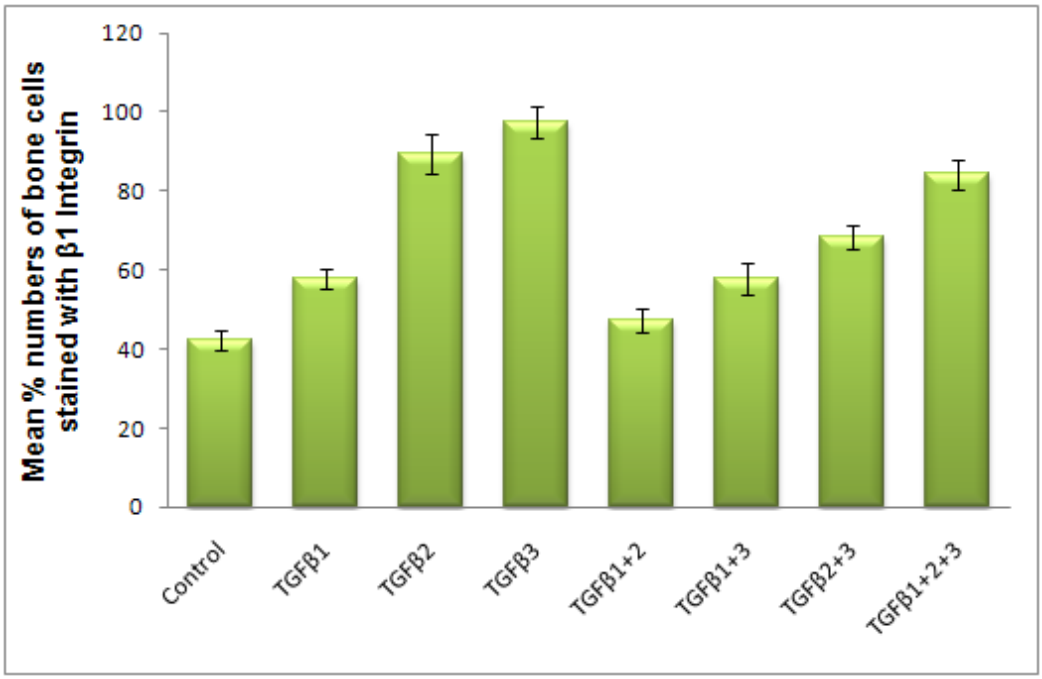


Figure 7.8 *Bar chart showing mean % number of bone cells stained with β1 integrin for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control (Error bars represent standard error).*

The mean % number of stained cells per treatment group after immunostaining with anti integrin α3 antibodies showed normal distribution and were analysed using SPSS via Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test (See Tables 7.7 and 7.8). Figure 7.9 shows the immunofluorscence micrographs of human bone cells stained for α3 integrin. More integrin expression can be seen in the micrographs of cells treated with TGF-β3 and TGF-β(1+2+3) with mean % numbers of cells stained per micrograph with integrin α3 of 98.19% ± 16.14SD and 94.49% ± 11.71SD, respectively. High integrin expression was seen and this was most intense near the periphery of the nucleus (See Figure 7.7). No significant difference in the mean % number of stained cells were recorded between TGF-β3 and TGF-β(1+2+3) (p=0.956). Results for TGF-β2 and TGF-β(2+3) showed some degree of

immunostaining with a mean % number of cells stained per micrograph with integrin α3 of 72.22% ± 8.28SD and 77.78% ± 11.11SD, respectively (See Figures 7.9, 7.10, Tables 7.7 and 7.8). TGF-β treatment control cells and TGF-β(1+2) treated cells showed a similar results with mean % number of cells stained with integrin α3 of 27.77% ± 6.41SD and 33.00% ± 12.83SD, respectively (p=0.956). There are no significant differences recorded between TGF-β2 treated cultures and cultures treated with TGF-β(2+3) and TGF-β(1+3) (p=0.956). No specific staining was observed in the negative immunostaining controls confirming the specificity of the primary antibody and blue dapi staining represents live cells (See Figures 7.9, 7.10, Tables 7.7 and 7.8).

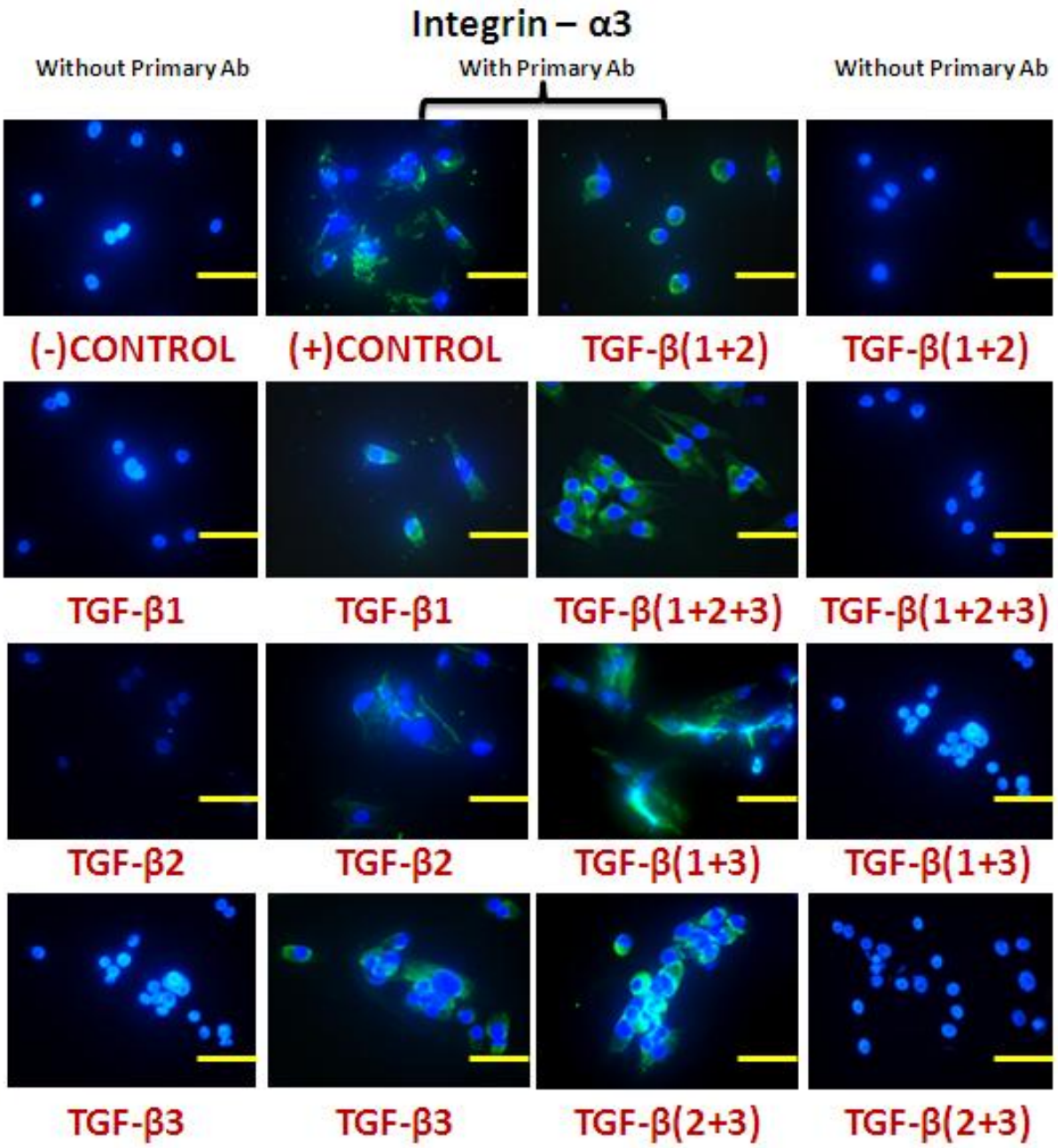


Figure 7.9 *Immunofluorescence micrographs of human bone cell (MG63) treated with different TGF-β isomers and their combinations stained with dapi solution (blue) and Integrin-α3 (green). Negative control represents absence of both TGF-β and primary antibody while positive control represents absence of TGF-β only with addition of primary antibody. Images in the right and left columns show dapi stained negative control bone cells following immunostaining in which the primary antibody was omitted (Scale bar = 100μm).*

Table 7.7 Descriptive statistics for mean % numbers of bone cells stained with α3 integrin in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control.

Treatment	Mean % numbers of bone cells stained with α3 integrin ± Std. Deviation (SD)	Std. Error (SE)
Control	27.77 ± 6.41	2.03
TGFβ1	55.55 ± 10.79	3.40
TGFβ2	72.22 ± 8.28	2.61
TGFβ3	98.19 ± 16.14	5.11
TGFβ1+2	33.00 ± 12.83	4.06
TGFβ1+3	66.66 ± 10.79	3.41
TGFβ2+3	77.78 ± 11.11	3.51
TGFβ1+2+3	94.49 ± 11.71	3.70

Table 7.8 P value derived from pair wise comparisons of mean % numbers of bone cells stained with α3 integrin in culture treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+3), TGF-β(2+3), TGF-β(1+2+3) and control. Pairs that are significantly different from one another are marked by an asterisk (*) whereas * P<0.05, ** P<0.01 and *** P<0.001.

Treatment	*P*-value	Sig diff	Treatment	*P*-value	Sig diff
Control – TGFβ1	0.001	***	**TGFβ2 - TGFβ1+2**	0.001	***
Control – TGFβ2	0.001	***	**TGFβ2 - TGFβ1+3**	0.956	
Control – TGFβ3	0.001	***	**TGFβ2 - TGFβ2+3**	0.956	
Control – TGFβ1+2	0.956		**TGFβ2 - TGFβ1+2+3**	0.001	***
Control – TGFβ1+3	0.001	***	**TGFβ3 - TGFβ1+2**	0.001	***
Control – TGFβ2+3	0.001	***	**TGFβ3 - TGFβ1+3**	0.001	***
Control – TGFβ1+2+3	0.001	***	**TGFβ3 - TGFβ2+3**	0.001	***
TGFβ1 - TGFβ2	0.032	*	**TGFβ3 - TGFβ1+2+3**	0.956	
TGFβ1 - TGFβ3	0.001	***	**TGFβ1+2 - TGFβ1+3**	0.001	***
TGFβ1 - TGFβ1+2	0.001	***	**TGFβ1+2 - TGFβ2+3**	0.001	***
TGFβ1 - TGFβ1+3	0.369		**TGFβ1+2 - TGFβ1+2+3**	0.001	***
TGFβ1 - TGFβ2+3	0.001	***	**TGFβ1+3 - TGFβ2+3**	0.369	
TGFβ1 - TGFβ1+2+3	0.001	***	**TGFβ1+3 - TGFβ1+2+3**	0.001	***
TGFβ2 - TGFβ3	0.001	***	**TGFβ2+3 - TGFβ1+2+3**	0.032	*

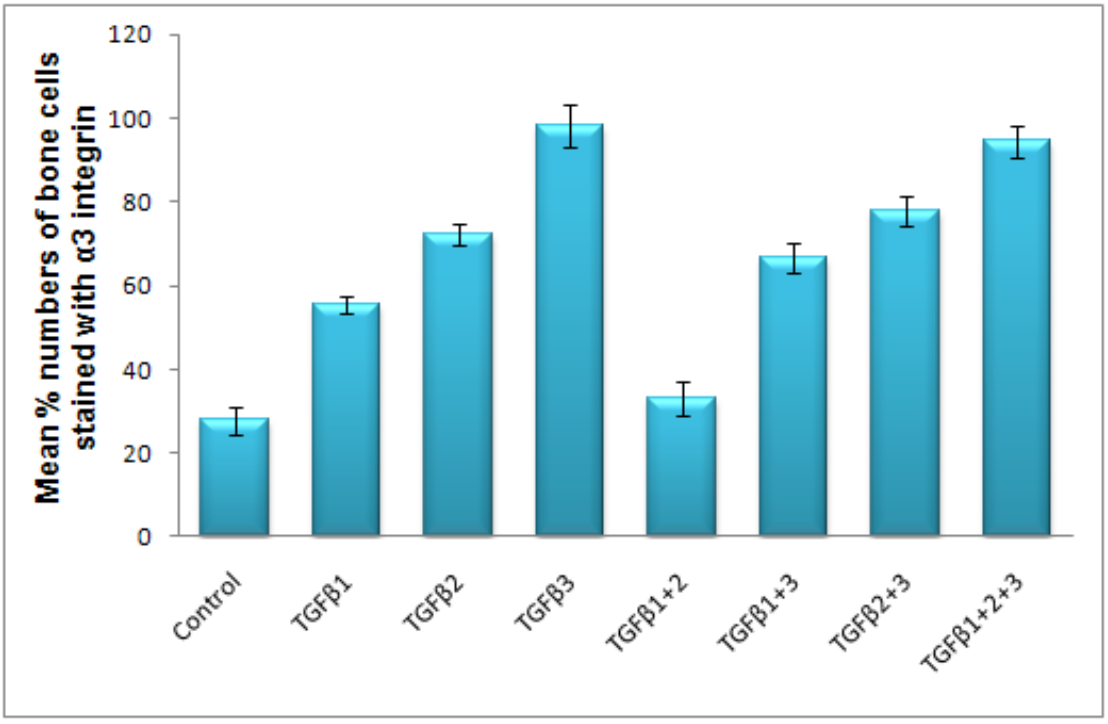


Figure 7.10 *Bar chart showing mean % number of bone cells stained with β1 integrin for cells treated with TGF-β1, TGF-β2, TGF-β3, TGF-β(1+2), TGF-β(1+2+3), TGF-β(1+3), TGF-β(2+3) and un-treated as control (Error bars represent standard error).*

7.5.2 WSPR

7.5.2.1 Micro contact printed fibronectin

The WSPR images, Figures 7.11a-b and 7.12, show cells on micro contact printed and un-treated surfaces. In the case of un-treated substrates, WSPR images revealed that high contrast features were arranged at the periphery of the cell and in concentric circles around the nucleus. Evidence from the previous work suggests that these bright contrast regions represent bands of focal contacts (Abdul Jamil, 2008a, Sefat, 2010a, Sefat, 2010d). Cells on the patterned substrates had a very different morphology. They had high contrast features that mostly occurred around the periphery. No bands like

structures were seen around the nucleus. These features were brightest distal to the edge of the fibronectin guidance pattern.

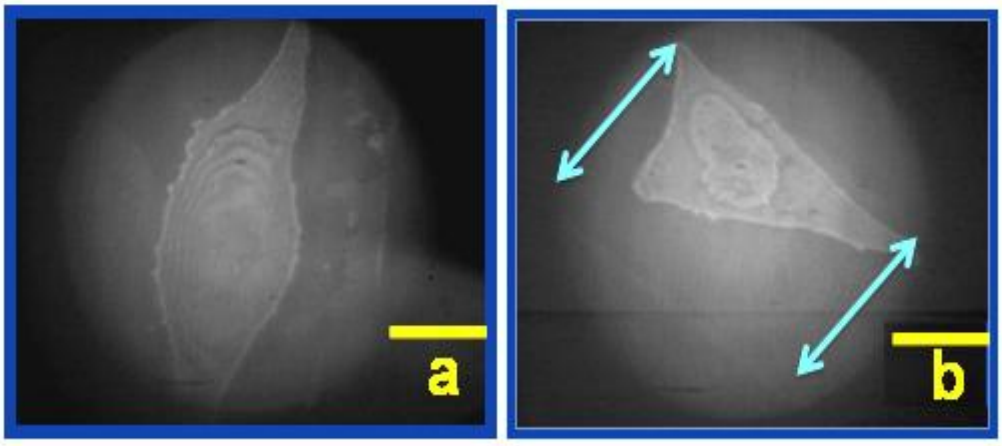


Figure 7.11 *WSPR Images (a) un-guided MG63 bone cell outside the patterned area; (b) guided MG63 bone cell aligned almost 100% to the 50µm fibronectin stamp patterned (gold substrate) (scale bar 25µm) (Sefat, 2010d).*

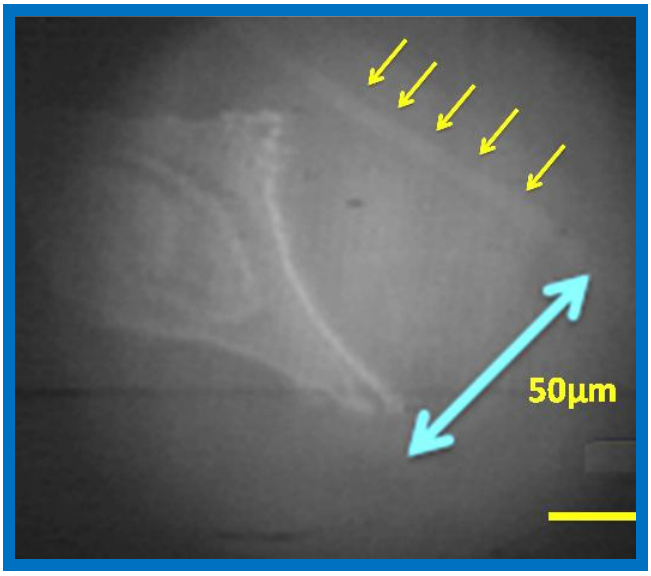


Figure 7.12 *WSPR Image of guided MG63 bone cell aligned almost 100% to the 50µm fibronectin stamp patterned on gold substrate; stamp pattern showed with yellow arrows (scale bar 25µm) (Sefat, 2010d).*

7.5.2.2 TGF-β isomers

Imaging with the WSPR system revealed that bone cells attached to the un-treated surface with high contrast concentrically arranged band like structures as shown in Figures 7.13. The highest contrast bands like components were localized at the cell periphery. Treatment with TGF-β1, TGF-β2 and TGF-β3 also modified the arrangement of the high contrast features within cells imaged with the WSPR. In the case of TGF-β1 treated cells the bright high contrast regions were intense but only arranged around the periphery of the cell. In TGF-β2 and TGF-β3 cells the bright contrast regions were weaker but again mostly localised around the periphery. However in the TGF-β3 treated cells bright spec like features were visible scattered across the underside of the cell.

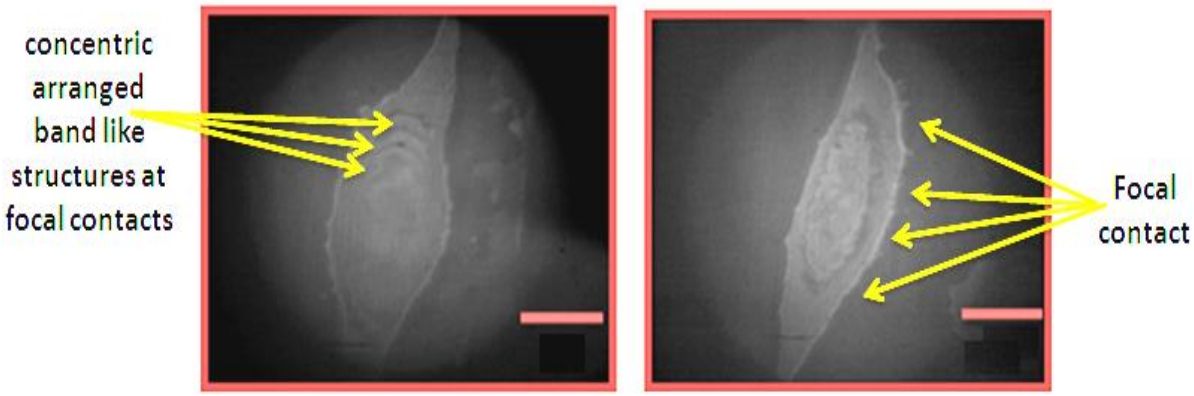


Figure 7.13 *WSPR images show focal contacts and concentric bank like structure of human bone cells on gold substrate (scale bar 25µm).*

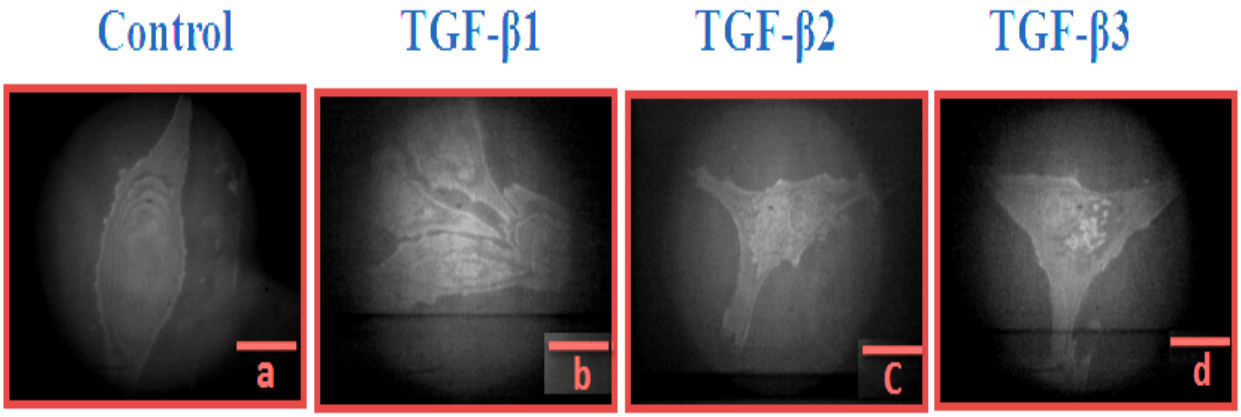


Figure 7.14 *WSPR Images of human bone cell monolayer, a: control (un-treated); b: cell treated with TGF-β1; c: cells treated with TGF-β2;d: cells treated with TGF-β3 (scale bar 25 μm).*

7.6 DISCUSSION

TGF-β1, TGF-β2 and TGF-β3 increased the secretion of collagen type I in comparison to control treated cells, as did most combinations of TGF-β other than TGF-β(1+2). Between all TGF-β isomers, TGF-β2 and TGF-β3 increased collagen type I secretion significantly more than other treatments. TGF-β2 and TGF-β3 also significantly increased fibronectin secretion over all other treatments. There were significant differences between fibronectin secretion between TGF-β1 and TGF-β2 treatments. All TGF-β treatments increased the expression of the β1 integrin subunit, although expression was significantly greater in cells treated with TGF-β3. A large increase in β1 expression was also seen in TGF-β2 and TGF-β(1+2+3) treated cells. The α3 integrin expression was also significantly increased by all TGF-β isoforms, but the expression was greatest in those cells treated with TGF-β3 and TGF-β(1+2+3). In relation to wound repair, it has been found that TGF-β stimulates cell proliferation and bone formation in rat cells (Huang, 2007, Antosz, 1989). TGF-β is detected in cartilage

(Pelton, 1991) and bone (Hering, 2001) and up-regulation of TGF-β occurs during embryonic bone formation (Pelton, 1991) and in adult fracture healing (Wang, 1990). Little is known about the effect of different TGF-β isomers on integrin expression in bone. All three TGF-β isoforms are detected in bone, but TGF-β1 is thought to be the most abundant (Hering, 2001, Linkhartc, 1996). There is also evidence that the expression of all three TGF-β isomers is up-regulated during fracture healing suggesting that their roles are not limited to embryonic bone development, but extend to adult bone remodelling (Cho, 2002). Many contradictory reports have been published on the function of TGF-β-1 in bone and it is necessary to reconcile experimental discrepancies that have been reported for this cytokine (Janssen, 2005, Ehnert, 2010). Several investigations reported the biphasic effects of TGF-β-1 in bone (Shinar, 1990, Yamaguchi, 1995). Recently, however, one group (Kale, 2004a, Kale, 2004b) provided a possible explanation for this in which various concentrations of TGF-β-1 and treatment time result in biphasic effects. Our results in chapter 4 (Sefat, 2009c) showed that TGF-β1 induced a decreased % wound closure compared with the other two isomers. Results in the current chapter also support this finding as culture flasks treated with TGF-β1 showed a smaller mean % number of cells stained for anti-collagen type I, anti-human fibronectin, α3 and β1 integrins when compared to the other two TGF-β isomers. This means that the binding of integrin adhesion receptors to ECM components, such as fibronectin and type I collagen, activates signalling pathways. Integrin-mediated interactions with ECM proteins are central to osteoblastic functions (Garcia, 2005). For instance, Damsky et. al., showed that integrin interactions, in particular α3β1, with fibronectin are essential for osteoblast survival, proliferation, osteoblast-specific gene expression, and matrix mineralisation (Moursi, 1996, Globus,

1998). Integrin interactions with type I collagen are also important. Blocking adhesion with type I collagen-specific peptides or antibodies directed against the collagen-binding integrin α2β1 interferes with the expression of osteoblast specific genes, and matrix mineralisation (Xiao, 1998, Mizuno, 2000). Also, Lemieux and his colleagues implicated the involvement of fibronectin binding integrins including α3β1 and α5β1 as well as glycoprotein in osteoblast proliferation (Lemieux, 2010). This concurs with our finding that antibodies raised against collagen, anti-human fibronectin, α3 and β1 integrins. This suggests that, in combination with growth-factor receptors, focal adhesions activate signalling pathways, such as MAPK and JNK that regulate transcription factor activity and direct cell growth and differentiation (Giancotti, 1999). Many of these integrin-activated signalling cascades are required for osteoblast differentiation (Lai, 2001, Xiao, 2002). To our knowledge, no detailed information on the integrin expression of TGF-β-2 and TGF-β-3 in MG63 bone cell is currently available. However, results from the present study showed that TGF-β-2, TGF-β-3, TGF-β(2+3) and TGF-β(1+2+3) significantly increased the expression of α3 and β1 integrin subunits when compared to the controls. The α3 and β1 integrins were also significantly increased following TGF-β treatments, suggesting that α3β1 is involved in collagen and fibronectin secretion while TGF-β1 showed less protein secretion compared to cultures treated with TGF-β-2, TGF-β-3, TGF-β(2+3) and TGF-β(1+2+3). Additionally it is beneficial to use other technique such as RT-PCR to measure collagen type I.

In this chapter we have also looked at the cell surface interface at submicron lateral resolutions using the WSPR system. This system was used previously to image the

interface between HaCaT cells and the gold layer (Abdul Jamil, 2008a). Imaging in air enabled the visualisation of high resolution and high-contrast submicron features identified by vinculin immunostaining as a component of focal contacts and focal adhesions and, imaging in fluid enabled cell surface interfacial interactions to be tracked by time-lapse video WSPR microscopy. These findings indicated that the cell surface interface, and thus indirectly the cell signalling mechanisms, may be readily interrogated in live cells without the use of labelling techniques (Abdul Jamil, 2008a, Abdul Jamil, 2008b, Abdul Jamil, 2007a, Abdul Jamil, 2007b, Sefat, 2010a). WSPR images revealed that high contrast features associated with the formation of focal contacts with a high prevalence on the edge of the cells distal to the edge of fibronectin patterns. This combined with evidence from the guidance experiments (Sefat, 2010a) indicated that the greatest degree of alignment occurred along the edge of the larger fibronectin protein patterns, which suggests that the cells were in fact aligning to small features, specifically the interface between the fibronectin coated and fibronectin free areas of the culture substrate. WSPR imaging also revealed that MG63 bone cells organised the majority of their focal contacts along the periphery of the cell distal to the edge of the fibronectin pattern and this was quite surprising. It is believed (Britland, 1996, Britland, 1992b) that cells generally cluster their focal contacts along a guidance cue. This change in cell morphology and the distribution of focal contacts associated with the edge of the fibronectin patterns indicates that the interaction between cells and the edge of the pattern induces a reorganisation of focal contacts such that the region of the cell guided by the edge of the fibronectin pattern is relatively loosely coupled to the cell culture substrate, but the region of the cell positioned away from that edge is quite tightly coupled to the fibronectin coated region of the culture substrate. This in turn

suggests that guidance is not necessarily associated with enhanced cell substrate coupling along the guidance cue, but may be more associated with a decreased coupling at the guidance cue. Such an arrangement may influence cytoplasmic streaming and as such modulate cell extension. Verification of this finding is required. The results showed that the edge of the guidance cue (fibronectin pattern) reduced contact between the cell and the underlying substances but cells were guided and oriented in the same direction as the pattern despite the edge related decrease in cell surface attachment (Sefat, 2010a). Bone cell alignment to the edge of the fibronectin pattern is very much in line with the bone cell behaviour *in vivo* in that bone cells are guided by ECM fibres that have dimensions comparable to the edges of our stamp patterns (Hu, 2005). Thus, cells align to the edges of the fibronectin pattern and fibronectin free regions in our cultured substrates indicating that cell guidance *in vivo* may not necessarily be associated with increased attachment to the edge of the fibronectin fibers, but potentially associated with edge-related decreases in cell fibre attachment and increased membrane fluidity. The WSPR images of cells on the patterned surfaces certainly support this view. This could also partially explain the poor guidance to smaller repeat patterns in that cells can span these patterns and are thus subject to multiple guidance cues, which in turn could reduce the guidance effect. On the other hand, TGF-β treated bone cells had a very different peripheral structure only visible via the WSPR images (See figure 7.14a-d). The TGF-β treated cells acquired a high-contrast band like structure at the boarder of the elongated cell along with less concentrically formed band like features across the cell body. This caused the cells to extend more indicating that the interaction between cells and all the different isomers of TGF-β with different rates induces significant changes in cell activity such as cell signalling resulting in up-

regulation of the proteins associated with the cells acquiring a more migratory phenotype.

7.7 CONCLUSIONS

Human bone cells (MG63) were immunostained for collagen type I, fibronectin, β1 and α3 integrin expressions treated with different TGF-β isomers and their combinations. The immunostaining results showed that treatment with TGF-β3 significantly increased collagen type I secretion and β1 and α3 integrin expression as compared to TGF-β1. It was also found that TGF-β1 seemed to decrease collagen type 1, anti-human fibronectin secretion and α3 and β1 integrin expression in comparison to TGF-β2 and TGF-β3. TGF-β2 also showed similar results to that of TGF-β3. All TGF-β isomers showed an increase in collagen type I, anti-human fibronectin secretion and α3 and β1 integrin expression compared to the control. Imaging with WSPR allowed observation of the focal contacts without the need for immunostaining. WSPR images revealed guided cells with high contrast band-like structures at the border of cells distal to the edge of the guided cue to which they aligned and with less concentrically formed band-like features across the cell body for un-guided cells. It is believed that the high contrast features are associated with the formation of focal contacts on the edge of the cells distal to the edge of fibronectin patterns, which suggests that cell guidance is aided by a decrease in cell attachment along a guidance feature.

CHAPTER EIGHT

8. OVERALL DISCUSSION, CONCLUSIONS AND FURTHER FUTURE WORK

The Aim of this thesis was to determine how the different TGF-β isoforms, and extracellular matrix proteins influenced the behaviour of MG63 bone cells in terms of responses to cell guidance on extracellular matrix molecules (Sefat, 2008, Sefat, 2010a), cell surface attachment (Sefat, 2009a, Sefat, 2010c), model wound repair (Sefat, 2009b, Sefat, 2009c, Sefat, 2009d, Sefat, 2010d) and integrin expression. Experiments were also performed to determine if HCl effects on wound closure were dose dependent (Sefat, 2010b). The effect of different TGF-β isomers and their combinations on proliferation rate and cell length of human bone cells were also assessed. Imaging with WSPR allowed observation of the cell surface interactions without the need for immunostaining (Sefat, 2010a, Abdul Jamil, 2008b) and was thus used to assess the effect of TGF-β isomers on cell attachment and guidance.

The results indicated that cells aligned well on the 50μm and 100μm wide fibronectin patterned coverslips compared to those with smaller width. In comparison, cells aligned less readily to collagen and laminin. The results for the trypsinisation assay indicated that treatment with the HCl (TGF-β isomer solvent), TGF-β1, TGF-β2 and TGF-β3 all decreased cell attachment, but this effect was significantly greater in the case of HCl and TGF-β3 (p<0.001). In terms of wound healing, all TGF-β treatments caused

different rate of wound closure *in vitro*. TGF-β3 caused the fastest wound closure over 30hrs followed by TGF-β2 and TGF-β1. Complete wound closure was also achieved after 30hrs in the presence of 50ng/ml HCl and 50ng/ml BSA/HCl, whereas wounds remained open in control cultures over the same time period. Cells treated with combinations of TGF-β isomers behaved similarly *in vitro* but with different rate of wound closure. All TGF-β combinations induced faster wound healing rates than controls. Previous studies indicated that combinations of cytokines such as platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-1) enhanced bone repair (Stefani, 2000), but our results explain that combination of TGF-β isomers did not necessarily further enhance wound closure. Wound closure was dependent on HCl dose with the 80μM and 160μM concentrations inducing increases in wound closure rates that were both significantly greater than those induced by 20μM, 40μM and control treatments ($p<0.001$). Cell morphology changes occurred more significantly in cells treated with TGF-β3. In terms of proliferation rate, TGF-β3 and TGF-β2 alone increased cell numbers compared to TGF-β1 and combinations of TGF-β. Immunostaining indicated that treatment with TGF-β3 significantly promoted the secretion of collagen type I and fibronectin and increased integrin α3 and β1 expression. WSPR images allowed the cell attachment to be examined. The results indicated that the distribution of focal contacts changed depending on treatment. For instance WSPR images revealed that high contrast features associated with the formation of focal contacts occurred with high prevalence on the edge of the cells distal to the edge of fibronectin patterns. In the case of TGF-β1 treated cells the bright high contrast regions were intense but only arranged around the periphery of the cell. In TGF-β2 and TGF-β3 cells the bright contrast regions were weaker but again mostly localised around the

periphery. It follows therefore that TGF-β changed the distribution of focal contact, and hence, without TGF-β treatment focal contact seemed to be in concentric arrays at periphery. Specifically, TGF-β3 focal contact seemed to be scattered across cell surface interface. Thus, TGF-βs reduce cell surface attachment which supports findings in trypsinisation.

The results of the experiment examining guidance of MG63 cells on micro contact printed substrates **(Chapter 2)** showed that cells aligned most readily to fibronectin patterned coverslips, especially to the 50μm width with mean angle of 7.87˚ ± 3.07˚ SD, and 100μm patterns with a mean angle of 6.46˚ ± 5.01˚ SD. In comparison, cells aligned less well to the collagen and laminin patterns, with the best mean angle of alignment of 9.67˚ ± 4.19˚ SD occurring in the 50μm collagen wide pattern and the best alignment on the laminin patterns with a mean angle of 14.36˚ ± 1.57˚ SD occurring on the 50μm stamp patterned substrates. This suggests that fibronectin is an importent protein in the repair of bone. It has also been shown in the literature that fibronectin is an important protein which was upregulated in damaged bone (Tang, 2004). The impact of the ECM proteins on cell morphology and function is also quite well documented. For example, epithelial, osteoblast and chondrogenic cells as well as fibroblasts have been used to examine cell responses to substrate chemistry (Lim, 2007, Khaghani, 2008). To understand how substrates influence morphology it is important to be able to examine cell surface interface. Cells interact with surfaces via lamellipodia and filopodia (Adams, 2002, DeMali, 2003). It has been reported (Dalby, 2007) that cells extend their cell protrusions in all directions on a flat un-patterned surface, but extended

cell protrusions followed guidance features on a micro pattern surface. Consequently, cells elongated and aligned along the pattern direction. The angles of the cell protrusions against the pattern direction were almost three times lower on the MCP plates than those on the un-patterned substrates. We observed clearly via WSPR images **(Chapter 7)** that the leading edges of the cell protrusions extended/elongated along the patterned substrates micro contact printed with fibronectin (Sefat, 2010a). However, the WSPR images also allowed the imaging of the focal contact associated with this guidance. In all cases focal contact accumulated on the edge of the cell parallel to the edge of the pattern indicating that the edge of the cell encountering the pattern was more loosely attached to the substrates than the edge parallel and distal to the edge of the pattern. This suggests that MG63 cells aligned to a substrate via mechanism involving a loose/weak attachment to the edge of a guidance cue and an exploration of that edge via lamellipodia and filopodia. The results from the guidance experiments **(Chapter 2)** indicated that cell alignment is extracellular matrix dependent with guidance greater on fibronectin compared to collagen and laminin (Sefat, 2010a). This indicates that the alignment is in turn dependent on the expression of different integrins and those integrins associated with coupling to fibronectin being most important. Similar findings have been made by others, for example, it has been shown that FGF2 in human osteoblast induces the expression of integrins that couple to fibronectin (Jang, 2002).

The trypsinisation results **(Chapter 3)** suggested that TGF-β2 and TGF-β3 do not stimulate bone-ECM adhesion (Sefat, 2009a, Sefat, 2010c). Bone cells cultured in medium with TGF-β2 and TGF-β3 supplementation separately displayed a faster rate of cell/surface detachment. There is evidence in the literature that many cell types take on

a migratory phenotype during wound repair and this migratory phenotype can be associated with a change in the degree of cell surface attachment (Foppiano, 2007a). Thus, it is possible that TGF-β2 and TGF-β3 could aid this process in bone. In contrast, the degree of cell surface attachment was increased in bone cells cultured with TGF-β1. This finding indicates potentially that inhibition or activation of TGF-β1 may induce bone cell migration and potentially bone repair. This view is supported in the litrature (Davidson, 2005) showing the effects of TGF-β isoforms in damaged cartilage. In this study TGF-β appears to play an important role in repair of cartilage and that lack of TGF-β responsiveness in old mice might be at the root of osteoarthritis (OA) development. There is also evidence in the literature that pH can influence wound repair (Lenghedeit, 1995). This poses a problem in studies examining the effect of TGF-β isoforms. In that TGF-β2 and TGF-β3 are solubilized in HCl solution. Thus, experiments examining the influence of TGF-β isoforms on cell behaviour may have actually identified cell responses that are pH dependent. To examine this possibility cell attachment was examined via trypsinisation studies in which cells incubated in media containing HCl at the concentration that wound be present if the cells were treated with the TGF-β isoforms. The results showed that HCl, BSA and HCl/BSA enhanced the rate of cell detachment in relation to the negative controls, indicating perhaps that TGF-β3 does not act alone in influencing the degree of cell attachment, but instead may function synergistically with signalling pathways that are dependent on the availability of hydrogen ions. Such a mechanism could depend on signalling molecules undergoing a conformational change on binding hydrogen ions. This view is supported by information in the literature that TGF-β in bone is activated by changes in pH/hydrogen ion concentration (Wrana, 1994).

The effect of TGF-β isomers in wound healing of bone cell monolayer was the next target of this research programme **(Chapter 4)** (Sefat, 2010d, Sefat, 2009b, Sefat, 2009c, Sefat, 2009d). Our studies showed that the bone monolayers treated with TGF-β3 and TGF-β2 demonstrated rapid decreases in wound width during 25hrs of wound healing. This may be related to TGF-β increasing the secretion of ECM molecules at the wound edge, which functions by capturing cells via the integrin ECM interactions at the wound edge, thus helping migration into the wound site (Celeste, 2002). In comparison TGF-β3 and TGF-β2 caused cells to detach faster from surface and thus, helping migration into the wound site. Such a mechanism may be useful in therapeutic procedures by filling a wound site with large number of bone cells which would presumably enable the deposition of ECM across the wound surface enabling cell migration and proliferation into the wound. TGF-β1 had slower healing effect *in vitro* compared to TGF-β3 and TGF-β2 suggesting that TGF-β1 decreases cell migration. TGF-β isomers also influence cell morphology which is well known, for example TGF-β1 in chondrocytes plays a very important role in rapid formation of fibroblast like chondrocyte cells *in vitro* (Chi, 2004). 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 (Nakamura, 2007) or by differential upregulation of specific integrins (Hardiman, 2005). HCl and BSA/HCl treated cells also showed very good wound healing response as compared to the control. However, no clinical research has yet been undertaken on TGF-β3 or HCl, thus this work provided an initial step in evaluating the effects of TGF-β3, HCl and HCl/BSA *in vitro* in relation to bone wound closure and the healing process *in vitro*. Growth factors can also be combined in order

to induce cell responses *in vivo* and it is probable that growth factors seldom act alone **(Chapter 4)**. The results of this work showed that in the case of combined TGF-β isomer treated cells, the rate of wound closure was significantly higher ($P < 0.001$) compared to un-treated controls. However, treatment with TGF-β1, 2 and 3 alone induced significantly faster wound closure rates than did treatment with combination of TGF-βs.

A number of chemical, physical and biological mechanisms have also been indicated in the activation of TGF-β (Lawrence, 1985, Brown, 1990, Zamora, 2007). Exposure to extremes of pH or urea, as well as heat treatment activates TGF-β in vitro (Zamora, 2007, Brown, 1990). Further investigations were carried out **(Chapter 5)** to evaluate the effect of various HCl concentrations in wound healing of bone cell monolayers (Sefat, 2010b). The results showed that HCl accelerated the bridging process, thus, enhanced the rate of wound closure. This response was dose dependent with fastest healing occurring in response to treatment with higher concentrations of HCl (80μM and 160μM) as compared to the control. It could be speculated that some of the hydrogen ions from the HCl bind to the serum proteins and cause a change in surface charge and hence change protein folding and function (Kohn, 2002). In this system, changes in hydrogen ion availability could activate signalling proteins in the serum, which then induce changes in cell behaviour associated with encouraging cell differentiation and proliferation during bone healing. In this work it was also found that different concentrations of HCl did not seem to affect the pH level of the media as monitored by observations of media colour, indicating that HCl was being sequestered away. Unfortunately this finding is inconclusive and requires verification via definitive

measurements of media pH via a highly sensitive pH meter. Therefore, an increase in HCl concentration could influence cell behaviour and this could indicate that bone cells are highly sensitive to tiny fluctuations in hydrogen ion concentration.

In this thesis cell morphology (Cell length) and proliferation rate were also examined in response to different TGF-β isomers **(Chapter 6).** The role of TGF-β induced changes in osteoblast morphology is largely unknown. Our results suggested that cells treated with TGF-β3 and TGF-β(2+3) showed high % cell proliferation followed by TGF-β(1+2+3), TGF-β(1+3) and control. TGF-β1 and TGF-β2 did not seem to enhance cell proliferation. These results also suggested that changes in cell length were more pronounced in cells treated with TGF-β(2+3) and TGF-β(1+3) followed by TGF-β3, TGF-β2 and TGF-β1. As mentioned earlier, regulation of cell shape is an important process that controls tissue morphogenesis, cell migration, differentiation, proliferation, and survival, presumably by regulating expression of genes central to these processes (Boudreau, 1999, Lukashev, 1998, Nakamura, 2007). However, it is possible that TGF-β induced osteoblast elongation contributes to several aspects of TGF-β function in bone (Hartsough, 1997, Nakamura, 2007). At present, it is not clear in which situations TGF-β could facilitate bone resorption *in vivo* by altering osteoblast shape (Karsdal, 2001, Serganova, 2009). One possibility is that active TGF-β is produced during bone remodeling within the basic multicellular unit (BMU) of the osteon. Both osteoblasts and osteoclasts are able to synthesize TGF-β (Oursler, 1994, Oreffo, 1989, Bonewald, 1991, Selvamurugan, 2004). Although osteoblasts are responsible for production of most of the TGF-β entrapped in bone matrix (Bonewald, 1994, Selvamurugan, 2004), the majority of active TGF-β appears to be generated by bone resorbing osteoclasts

either through release of TGF-β stored in bone matrix or by biosynthesis of new TGF-β (Pfeilschifter, 1987). Active TGF-β may then be made accessible to osteoblasts through a process involving transcytosis (Mostov, 1997, Ruan, 2010). The selective activation of TGF-β by osteoclasts may fulfill a function by inducing elongation of bone lining cells (Lucas, 1989, Pfeilschifter, 1990, Ruan, 2010). Thus, the precise role and mechanism of TGF-β with regard to osteoblast elongation remains to be established.

The question now arising is how TGF-β related wound repair may associate with changes in the expression of integrins? To examine this MG63 cells were immunostained for collagen type I, fibronectin, β1 and α3 integrin following treatment with different TGF-β isomers and their combinations **(Chapter 7)**. Previous experiments in this thesis identified differences in the way TGF-β isoforms influence cellular behaviour including collective cell migration during model wound repair, degree of cell detachment during trypsinisation assay, cell morphological changes and cell proliferation. However, little is known about the effect of different TGF-β isomers on integrin expression in bone. There is evidence that the expression of all three TGF-β isomers is up regulated during fracture healing (Cho, 2002). This means binding of integrin adhesion receptors to ECM components, such as fibronectin and type I collagen, activates signalling pathways. Many studies have implicated the involvement of integrin/ECM protein interactions with cell proliferation. For example, osteoblast/fibronectin binding via integrins including α3β1 and α5β1 are thought to influence osteoblast proliferation (Lemieux, 2010, Clëzardin, 2009, Hughes, 1993). This supports the finding of the thesis that suggests TGF-β1, 2 and 3 increases the secretion of collagen and fibronectin and the expression of α3 and β1 integrins.

FURTHER FUTURE WORK

It was quantitavely and qualitatively observed in this work that for fibronectin rich patterns cell function greatly improved for osteoblast-like (human bone cell) monolayers, especially with presence of cytokines (TGF-β3) speeding the rate of wound repair *in vitro*. These results along with the findings of other researchers, (Couchman, 1990) imply that TGF-β3 may enhance wound repair *in vivo*. This could be further investigated by examining the effects of wound closure in a 3D model.

Results in this thesis examined cell responses in a 2D environment. For instance, different TGF-β isoforms, and extracellular matrix proteins influenced the behaviour of MG63 bone cells in terms of responses to cell guidance on extracellular matrix molecules, cell surface attachment, model wound repair and integrin expression in a 2D environment. The effect of different TGF-β isomers and their combinations on proliferation rate and cell length of human bone cells were also assessed in a 2D environment. However, evidence from the literature indicates that bone cells behave differently in a 3D environment and that the 3D culture systems in vitro mimic the *in vivo* situation more closely than the two-dimensional (2D) cultures (Trojania, 2005, Kale, 2000, Ferrera, 2002). Normal human osteoblasts or human osteosarcoma cell lines were mixed and cultured into hydrogel (Trojania, 2005). It was demonstrated that this matrice represents a new system of 3D culture in which the differentiation of human osteogenic cells is promoted. The osteosarcoma cells were grown as viable clonogenic spheroids and acquired a more mature phenotype compared to the same cells grown in a monolayer culture on tissue culture grade plastic (Trojania, 2005). Results of this thesis

clearly contributed to a better understanding of osteoblast-like cell behaviour or cell biology from MG63 osteosarcoma cell line (or type). Future/further work would therefore benefit the experiments undertaken in this thesis being repeated in 3D cell cultures on various scaffolds, or on various orthopaedic implants using our model for monolayer cell interactions.

The overall aim should be to develop a 3D scaffold incorporating the ECM molecules required to enhance bone repair and to determine how cell behaviour in a 3D scaffold is modified by TGF-β 1, 2 and 3.

This future work would involve:

1) Development of a 3D culture system in which bone cells may be grown and in which a model wound could be generated.
2) The identification of which combination of cytokines enhance wound repair in a 3D culture system. The aim here would be to find out if the results acquired in the 2D system are mirrored by similar outcomes in a 3D model wound.

We have also investigated the effect of TGF-β1, -β2 and -β3 against control and different concentrations of HCl to determine which treatments promote model wound repair. Further research should be carried out to concentrate on the specific range of 80µM and 160µM concentrations of HCl which seem to enhance wound closure, more effectively. Also, supplementary experiments should be carried out to investigate and compare the wound closure properties of bone cell monolayers treated with different TGF-β isomers using the same concentration. Observation also concluded that the pH of

the media was not changed significantly due to any colour change of the media. However, very small pH differences have detrimental effects on cell behaviour and measurement of the pH values may be required in order to verify that the application of HCl in this study had no impact on the pH of the culture media. If this were the case it would support our suggestion that HCl is being sequestered away by the media and serum proteins. A micro pH meter (e.g. Micro 500 pH meter –Palintest instruments) could be used to enable this.

The MG63 osteoblast-like cell lines used in this work were derived from osteosarcomas, which share certain important features of the osteoblast phenotype. However, it is not clear if all the characteristics of MG63 cells are identical to those of bone forming osteoblasts in the body. It is possible that MG63 cells have acquired gene mutations that make their responses dissimilar to bone cells *in vivo* (Okabe, 2010). Thus repeat experiments with primary bone cells could be required to confirm the findings of this thesis.

The method of the initial scratching of the monolayer was non-uniform which could affect the healing growth rate of the MG63 bone cells. Uneven scratching due to human error could contribute to a variation in scratch width. Thus the MG63 bone cells were able to bridge more quickly and become confluent and that larger initial scratches took longer to heal. A method of uniform scratching in a 3D system need to be developed to allow for more accurate results.

CHAPTER NINE

9. REFERENCES AND BIBLIOGRAPHY

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APPENDIX 1

JOURNAL AND CONFERENCE PUBLICATIONS

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Imaging via widefield surface plasmon resonance microscope for studying bone cell interactions with micropatterned ECM proteins

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Key words. Bone cell engineering, cell alignment and attachment, extracellular matrix proteins, microcontact printing, widefield surface plasmon microscopy.

Summary

The widefield surface plasmon resonance microscope has recently been used to monitor label free antibody/antigen binding events and focal contacts in HaCaT cells at high special resolutions. Thus the aim of this study was to examine MG63 bone cell attachment and alignment to microcontact printed extracellular matrix proteins. Collagen, fibronectin and laminin were stamp patterned onto glass slides using templates consisting of 5-, 10-, 25-, 50- and 100-μm-wide repeat grating. MG63 bone cells were seeded at 50 000 cells per 25 cm^2 and cell alignment was determined from micrographs taken at time-points 2, 5 and 18 h after cell seeding. Cells on the fibronectin pattern attached and elongated at early stages after seeding. In the case of collagen and laminin, cells did not adhere readily and appeared more rounded until 18 h after seeding. This indicated MG63 cells attach mostly via fibronectin specific integrins. The cells aligned well on the fibronectin-patterned cover slips especially to the 50- and 100-μm-wide patterns, although in this case cells did not position themselves in the middle of each fibronectin-coated region, but instead aligned to the small features associated with the edges of the fibronectin-coated regions. Patterned and unpatterned cells also had quite different morphologies. The unpatterned cells had a more rounded morphology and lengths of 25 to 35 μm, whereas patterned cells elongated in the direction of the pattern and had lengths of 50–70 μm. The widefield surface plasmon resonance imaging indicated that cells on unpatterned surfaces had a rounded morphology in which the focal contacts were evenly distributed around the periphery of the cell. However, MG63 bone cells on fibronectin-patterned substrates organized most of their focal contacts along the periphery of the cell distal to the edge of the fibronectin patterns. This suggests that the interaction between the cell and the edge of the pattern induces a reorganization of focal contacts such that the region of the cell guided by the edge of the fibronectin pattern is relatively loosely coupled to the cell culture substrate, but the region of the cell positioned away from that edge is quite tightly coupled to the fibronectin-coated region of the culture substrate. This in turn suggests that guidance is not necessarily associated with enhanced cell substrate coupling along the guidance cue, but may be more associated with a decreased coupling at the guidance cue. Such an arrangement may influence cytoplasmic streaming and as such modulate cell extension. Verification of this finding is required; as such a response to a guidance cue is quite unexpected because it is believed that cells cluster their focal contacts along a guidance cue.

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Introduction

Microcontact printing (MCP) is a strategy that enables a surface to be readily functionalized with a material such as an extracellular matrix (ECM) protein, in a defined pattern. MCP also allows control of cell adhesion geometry on a surface. Microcontact printed substrates maybe used to examine cell mortality in relation to a wide range of ECM molecules, including fibronectin, laminin and collagen (Kumar *et al.*, 1994).

Fibronectin, laminin and collagen are the main components of ECM. These cell adhesion proteins are ideal candidates for MCP printing as they can direct cellular adhesion, migration, differentiation and network formation *in vitro* (Carter, 1965).

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**

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I. 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.

II. Aims 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.

III. Materials and Methods

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.

Bone cells were cultured in a 12 well petridishes and left for 3 days to become confluent with three different cell dilution. Three wells were labeled as A1-A3 on the left of culture dish was seeded with 1:3 ratio of cell to DMEM

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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.**

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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 scratch markings facilitated orientation while imaging and another wound was later applied at 90 degree angle to the initial scratch and pictures were also taken at the cross points. Thus

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The Effect of Transforming Growth Factor Beta (TGF-β3) and Sanicle on Wound Healing

C.B. Beggs, M.C.T. Denyer, A. Lemmerz, F. Sefat, *Member, IAENG*, C. Wright, and M. Youseffi

Abstract- **There is evidence that both the herb Sanicle and the cytokine TGF- β3 can be beneficial in enhancing wound repair. In this study 3T3 fibroblast cells were cultured and the confluent monolayers were wounded (scarred) using a disposable plastic pipette. Various amounts of TGF-β3 (a growth factor) and Sanicle extract were applied to the cell monolayers. TGF-β3 was applied at concentrations of 50ng/ml, 5ng/ml, 500pg/ml, 50pg/ml and 5pg/ml to five different culture flasks with one additional flask acting as control. Sanicle was applied at concentrations of 100µg/ml, 10µg/ml, 1µg/ml, 100ng/ml and 10ng/ml with one additional flask as a control. The cells were imaged over a period of 20 hours with or without presence of TGF-β3 and Sanicle. The results indicated that although there were no significant increases in the rate of wound closure in relation to application of TGF-β3, there is an indication that TGF-β3 may enhance model wound closure at optimum working concentration between 5ng/ml and 50ng/ml. However, the sanicle extract did not stimulate enhanced repair of the model *in vitro* wound, but instead seemed to promote cell death along the wound margin. These results indicate that sanicle may be used in the care of wounds, but not as a growth promoter, but because it acts as an antibiotic agent, and possibly because it aids wound debridement.**

Index Terms: **3T3 Fibroblast Cell Culture; Growth Factor TGF-β3; Sanicle; Astringent; Anti-Scarring Effect (Wound healing).**

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I. Introduction

Sanicle, *Sanicula europaea* (Umbelliferaea) is a perennial herb found in Northern and Central Europe including the British Isles [1, 2]. Although there appears to be little call for Sanicle in herbal medicine today, the plant was formerly an important medicinal plant sought after especially for its wound healing properties. The name of the plant is derived from the Latin *Sano* meaning "I heal or cure" and in medieval times this was reflected in the popular saying: "Celui qui sanicle a De mire affaire il n'a", which translated means "He that hath sanicle needeth no surgeon". Modern medicine has moved away from herbal remedies in wound repair, and focused more on methods of engineering enhanced wound repair via combinations of implantable constructs and therapeutic use of cell growth factors and cytokine. One such cytokine currently gaining a reputation in promoting scarless healing is TGF-β3 [3-7].

Growth factors, and cytokines, are associated with a number of cell and tissue repair mechanisms functioning as signaling molecules in the inflammatory response and in the modulation of cell proliferation and differentiation. Transforming growth factor beta (TGFß) enhances fibroplasia and the production of ECM components such as collagen, elastin and laminin whilst suppressing the ECM breakdown. It is also apparent that the relative proportions of cytokines can modulate the process of wound repair. For example, it has been reported [3] that foetal wounds heal with little or no scarring and that this is due to variations in the concentration of different TGF- isoforms. In adults, it is evident that TGF-β1 and TGF-β2 predominate in the wound site and function through activation of the immune system which in turn promotes scarring. However, in the embryo TGF-β3 is up-regulated and TGF-β1 and TGF-β2 concentrations are low, indicating that TGF-β3 plays a role in scarless healing. This view has been confirmed in experiments looking at wound healing in adults, where virtually scarless healing can be induced by the introduction of between 5 and 50ng/ml of TGF-β3 into the wound site [3, 4, 5, 6].

Although cell and tissue engineering based systems predominate in the generation of new therapeutic treatments in wound repair there is a growing realisation that these may be augmented by aspects of historical treatments. In the case of sanicle [1, 2, 8], its use in wound healing has ceased and little is known about how sanicle extracts improved wound prognosis. This study aims at comparing the effects of a sanicle extract and TGF-β3 on the repair of an *in vitro* model wound.

The Effect of Different HCl Concentrations on Wound Healing of Bone Cell Monolayer

F. Sefat, *Member, IAENG*, C.B. Beggs, M.C.T. Denyer, G.D. Meakin, and M. Youseffi

Abstract- **Bone repair has been studied in order to understand the complex phases involved that result in full conformity of the original bone. Our body has the unique ability to heal a bone fracture without scarring. Bone repair is modulated by different stimulus including growth factors. Transforming Growth Factor-beta (TGF-β) super-family have been studied with strong evidence in vitro and in vivo that TGF-β has significant effects on bone construction and resorption by regulating the duplication and differentiation of chondrocytes, osteoblasts and osteoclasts. Dosage carriers of TGF-β1, 2 or 3 namely HCl was used in vitro to see the effects it has on closure rates using a model wound in cultured monolayers of MG63 bone cells. The wound healing time was investigated using 6.25µl, 12.5µl, 25µl and 50µl concentrations of HCl compared against control. The model wound was made on fully confluent monolayers of MG63 bone cells with an average wound width of 300µm ± 10-30µm. For each concentration of HCl and control after wounding, the wound width was measured over a 30hr period. The results showed that after the 30hr period, the 25µl and 50µl concentrations of HCl enhanced the speed of wound closure in vitro as compared to the control. The culture treated with 25µl concentration of HCl was fully confluent at 25hrs and the 50µl concentration of HCl showed the highest percentage of wound closure but taking the full 30hrs to become confluent. The 6.25µl and 12.5µl concentrations of HCl had slower closure rates than that of the higher concentrations with confluency not achieved at 30hrs. Results from un-treated flasks (control) showed slowest wound closure compared to treated flasks with HCl.**

Index Terms: **Bone monolayer repair; HCl; Transforming Growth Factor (TGF-β3); Wound Healing.**

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I. Introduction

Bone is unique in nature since it has the ability to heal similarly to its original form without the development of a fibrous scar [1,2]. The regeneration of bone goes under a complex set of four overlapping phases, which are characterized by specific molecular and cellular events [3,4,5]. The four phases include: the haematoma formation/inflammatory phase, the soft callus formation, the hard callus formation and the remodeling process. Bone has the ability to repair itself using different methods depending on the biophysical environment. Ultimitely bone synthesis is achieved through osetoblasts via woven and/or lamellar matrix. Investigations have shown that with an increased ageing population healthcare in the United Kingdom is set to cost over £900 million each year [6,7]. A large percentage of that will go to the 150,000 fractures each year in the United Kingdom due to osteoporosis alone.

There are many factors that have effect on bone repair including age, nutrients, hormones, and growth factors, etc. The transforming growth factor-Beta (TGF-β) superfamily has been accepted as the most popular stimulus with strong evidence in both in vitro and in vivo studies that TGF-β has detrimental effects on bone construction and resorption by regulating the duplication and differentiation of chondrocytes, osteoblasts and osteoclasts [8]. Other groups claimed that bone is formed in vivo by osteoblasts when TGF-β has been injected into the fracture site [9,10].

TGF-β has been identified in the fracture haematoma and surrounding periosteal mesenchymal cells with staining techniques [11]. This is thought to orchestrate the cascade of cellular events resulting in fracture repair. The three isoforms (TGF-β1, -β2, -β3) have been found [12] to express overlapping patterns in vivo and with nearly identical biological activities in vitro. TGF-β1 is more potent throughout adult development, whereas TGF- β3 is more potent in tissues with mesenchymal origin. The three isoforms signal through the same kinase receptor with different binding abilities [12].

In order to understand how TGF-β regulates bone repair and formation it is necessary for in vitro experiments using osteoblast cell lines, which are mainly derived from osteosarcomas, and share phenotype similarities to the osteoblast cell. In-vitro experiments have proven that TGF-β stimulates osteoblast chemotaxis, DNA synthesis, cell division, and promotion of bone matrix proteins [13].

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Proceedings of the Anatomical Society of Great Britain and Ireland

The January 2008 winter meeting of the Anatomical Society of Great Britain and Ireland included a symposium on 'Anatomy and matters forensic'. These are the abstracts of the talks and posters presented at that symposium.

Cell Engineering of Skin Substitutes: The Role of Extracellular Matrix (ECM) Protein (Collagen and Fibronectin) and Transforming Growth Factor 3 (TGFβ-3) in Cellular Adhesion

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ABSTRACT: This work examines the role of the extracellular matrix (ECM) proteins, collagen and fibronectin along with transforming growth factor β-3 (TGF β-3) in skin cell engineering and wound repair. The regulation of growth factors in wound repair is known to determine the rate and type of the healing as well as cell infiltration. This gives the rationale to further investigate growth factors in skin substitutes. Our studies investigated the role of TGF β-3 on cell behaviour in combination with culture on extracellular matrix patterns of collagen and fibronectin. Differences in the cell behaviour in vitro, e.g. adhesion, can be attributed to the interaction of different protein-specific integrins during cell–cell and cell–matrix adhesion. Experiments investigating cell guidance found that cells aligned better to collagen stamped patterns than fibronectin patterned surfaces, although fibronectin was more consistent over a range of stamp sizes. This highlights the important role of fibronectin in wound repair, facilitating cell adhesion and migration. It also indicates the potential benefits of fibronectin and ECM proteins in skin replacements, as they provide a platform for cell migration and adhesion. Detachment studies of protein-treated surfaces seeded with keratinocyte cells illustrated that TGF β-3 treated cells detached more readily than cells without TGF β-3. The expressions of these proteins and growth factors are important signalling molecules allowing timely healing and graft integration. The current literature reveals that the use of skin substitutes is still not widespread due to various shortcomings such as high costs; susceptibility to infection and long lead times, which together show the ineffectiveness of skin replacements. It is therefore our conclusion that cell guidance, cell migration and growth factors have a direct impact upon the healing cascade and scarring profiles in skin tissue. A deeper understanding of cellular communication, wound repair, and current skin equivalents may lead to development of better skin substitutes to mimic native tissue and optimize conditions for favourable wound closure.

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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* environment allows the examination of the way in which cells behave in relation to the ECM. Micro-contact printing (MCP) allows the 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. 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 microcontact printed ECM patterns. Collagen, fibronectin and laminin were stamp patterned onto glass slides using templates consisting of 5-, 10-, 25-, 50- and 100-µm-wide repeal grating. MG63 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 18 h). IMAGE J software was used to find the angle between the long axis of cells and different patterns and mean length. Cells on the fibronectin pattern attached and elongated at early stages after seeding. In the case of collagen and laminin, cells did not adhere readily and appeared more rounded until 18hrs after seeding. The cells aligned very well on fibronectin patterned coverslips, especially to the 50 µm, with mean angle of 7.87 ± 3.07 and 100-µm patterns with a mean angle of 6.46 ± 5.01. In comparison the alignment of the cells to the collagen and laminin patterns was poor, with the best mean angle of alignment (mean angle of 9.67 ± 4.19) occurring in the 50-µm collagen wide pattern and the best alignment on the laminin patterns (mean angle of 14.36 ± 1.57) occurring in the 50-µm stamp patterned. Differences in cell length mirrored those of alignment, with cells acquiring the greatest length when showing the greatest degree of alignment. The results indicate that MG63 cells respond most to 50- and 100-µmwide fibronectin patterns, which suggests that the cells attach mostly via fibronectin-specific integrins.

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Alignment of Rat Primary Chondrocyte Cells to Collagen Type-I, Ficronectin and Laminin

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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 characterize 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 100-μm-wide repeal grating of micro contact printed fibronectin, laminin and collagen type-1. Cells were plated on the patterned substrate of concentration of 5000 cells cm−2. At 48 h after seeding, the effect on cell alignment of these patterned proteins was examined. Using IMAGE-J software it was found that chondrocytes aligned to the 50-μm laminin (mean angle = 23.4 and SD 7.02) and showed no alignment to the fibronectin pattern. The highest degree of alignment was observed with chondrocytes grown on the 100-μm-wide collagen type-I pattern (mean angle of alignment 18.9 ± 6.797). In all cases the chondrocyte cells were small (laminin mean size 36.2 μm ± 14.4 SD, collagen mean size 34.03 μm ± 7.2 SD and fibronectin mean size 33.87 μm ± 11.9 SD) and rounded. 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.

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Proceedings of the Anatomical Society of Great Britain and Ireland

As part of the winter meeting of the Anatomical Society of Great Britain and Ireland, at St Anne's College, Oxford, on 6–8th January 2009, a symposium was held on the subject of 'The Art of Anatomy'. The following are abstracts of the papers and poster demonstrations from the meeting.

Investigating Keratinocyte Cell Responses to ECM Proteins Using Microcontact Printing

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ABSTRACT: Following injury to the skin, keratinocytes within the epidermis migrate and proliferate to fill the wound bed, a process known as re-epithelialization. The understanding of keratinocyte- extracellular matrix interactions during wound repair and how these events influence the scarring process remains to be fully elucidated. Studying cellular responses to different ECM proteins will play a central role in the identification of Integrins regulated as part of the response to growth factors such as TGF-β. Microcontact printing has been commonly used for the assembly of protein monolayers onto surfaces. 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. Fibronectin, laminin and collagen type I were patterned onto glass substrates to analyse cell alignment. A variety of stamps were produced with grating repeats 1.8, 5, 10, 25, 50 and 100 lm in width. Following stamp patterning, substrates were incubated with HaCat cells for 24 h at 37 _C. Cells were imaged using a phase contrast microscope and VISICAPTURE software. Cell length, width and angle of alignment to patterns were analysed using IMAGE J software. We demonstrate that HaCaT cell adhesion and alignment can be influenced by the patterning of proteins on glass substrate. For each of the three proteins, fibronectin, laminin and collagen type I the smallest average angle of alignment was seen with the 50 lm 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. The aim of future work will be to identify potential Integrins involved in keratinocyte-ECM interactions and to understand how these integrins are regulated by chemical mediators such as TGF-β.

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Purification of Primary Chondrocyte Cells Extracted from Knee Joint of 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]

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ABSTRACT: Harvesting of pure cells from primary tissue is difficult as isolated tissue contains various cell types. Articular cartilage is highly specialized tissue that functions by forming a smooth surface enabling joint movement. Cartilage is mostly made up of chonrocytes. However, when 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 are roughly about 2 mm in diameter. Thus despite careful isolation from neonatal rat joints, cartilage will be accompanied by a mixture of bone cells and non-cartilaginous connective tissue. Cells derived from this tissue will therefore need to be purified. In this study the 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 min. The unattached cells were then re-plated in a new TC grade cell culture flask. This process was repeated a further six times, after which the final cell suspension was allowed to attach to the surface for 100 min. This resulted in the generation of eight cell cultures, plus one control in which the serial attachment process was not used. All nine cultures were incubated at 37 _C for 4 days and then immunostained with monoclonal Anti S-100 (b-subunit – SIGMA) antibody and monoclonal anti collagen type-I antibody (SIGMA). Immunocytochemical staining 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 min of cell attachment. By the eighth plating the cultures contained almost 100% chondrocytes. These results suggest that chondrocytes can be readily isolated and purified by a prolonged differential adhesion technique with a primary incubation extending for 140 min.

Cell Engineering of Skin substitute: The role of extracellular matrix (ECM) proteins (Collagen and Fibronectin) and Transforming Growth Factor β-3 in cellular adhesion.

C.B. Beggs[1], M.C.T. Denyer[2], F. Sefat[1,2], E.P. Tobin[1], and M. Youseffi[1]

[1]School of Engineering, Design & Technology, [2]Institute of Pharmaceutical Innovation, University of Bradford, BD7 1DP, UK

Abstract: This work examines the role of the ECM proteins, collagen and fibronectin along with transforming growth factor β -3 (TGF β-3) in skin cell engineering and wound repair. The regulation of growth factors in wound repair is known to determine the rate and type of the healing as well as cell infiltration. This gives the rationale to further investigate growth factors in skin substitutes. Our studies investigated the role of TGF β-3 on cell behaviour in combination with culture on extracellular matrix patterns of collagen and fibronectin. Differences in the cell behaviour *'in vitro'* e.g. adhesion, can be attributed to the interaction of different protein-specific integrins during cell-cell and cell-matrix adhesion.Experiments investigating cell guidance found that cells aligned better to collagen stamped patterns than fibronectin patterned surfaces, although fibronectin was more consistent over a range of stamp sizes. This highlights the important role of fibronectin in wound repair, facilitating cell adhesion and migration. It also indicates the potential benefits of fibronectin and ECM proteins in Skin replacements, as they provide a platform for cell migration and adhesion. Detachment studies of protein treated surfaces seeded with keratinocyte cells illustrated that TGF β-3 treated cells detached more readily than cells without TGF β-3. The expression of these proteins and growth factors are important signalling molecules allowing timely healing and graft integration. The current literature reveals that the use of skin substitutes is still not widespread due to various short comings such as high costs; susceptibility to infection and long lead times which altogether show the ineffectiveness of skin replacements.

Introduction

Traditional treatment for the loss or damage of skin tissue due to burns, injures or surgical operations have been limited to reconstructive surgery using autografts or allografts. Each of these conventional treatments have their own potential risks and limitations. It has been confirmed by others that TGF-β3 has anti-scarring effect and acts as a key mediator in cell communication, wound healing, and regulating both inflammation and angiogenesis [1].

Aims and Objectives

The aim of this work was to investigate the effects of substrate patterning with fibronectin and collagen on cell guidance and elongation and to evaluate the role of growth factor TGF-β3 in the attachment and detachment of keratinocyte skin cells and the subsequent impact upon wound healing and scarring.

Materials and Methods

Collagen and fibronectin patterns of size 5, 12.5 and 22μm were stamped onto coverslips. HaCaTs Keratinocyte skin cells were seeded and observed over a period of 48 hrs. TGFβ3 was then applied via the media (2.8ml media + 0.2ml TGF-β3) containing cell suspension to the patterns to study its effect on cell behavior and morphology. Image J software was used to determine the degree of cell alignment and cell elongation. A confocal light microscope was used for the photomicrographs.

Results and Discussion

Cell guidance studies on stamped patterns of collagen and fibronectin showed that the 12.5 and 22μm wide stamps gave the best alignment for fibronectin and collagen, respectively. It is to be noted that cell guidance was a measure of the degree of alignment of cells to the protein pattern. Figure 1 shows the images of collagen stamped cover slips of size 5, 12.5 and 22μm wide, respectively.

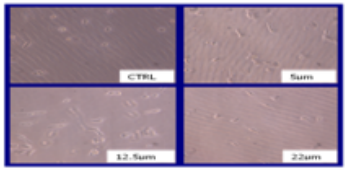

Figure 1. Photomicrographs of stamp patterned coverslips with collagen of various size after 48 hrs of incubation

A clear pattern of alignment can be seen for the 12.5 and 22μm stamped patterns after 48 hrs of incubation. The 22μm wide stamp size gave the best cell alignment (~6±1.2SD) as shown in Figure 2.

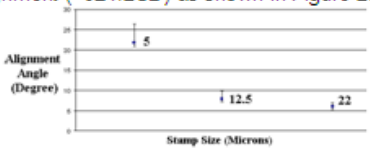

Figure 2. Graph of alignment angle versus stamp size for collagen stamped patterns after 48 hrs of incubation.

This result may be attributed to the large molecular size of the collagen molecule (~300 nm in length) and that the molecules follow the etched ridges to a greater degree for the larger stamp size. All three stamp sizes gave almost the same degree of alignment (~10±3.1SD) for the fibronectin patterns. The bar chart in Figure 3 compares cell length for collagen and fibronectin patterns.

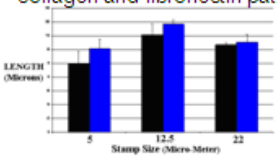

Figure 3. Bar chart of cell length versus stamp size for collagen and fibronectin patterns after 48 hrs of seeding.

Fibronectin performs better causing further elongation of cells. This is in contrast to collagens superiority as a better protein for encouraging cell alignment. It is interesting to note that presence of fibronectin is necessary in wound healing to establish a provisional matrix to allow cells to migrate to the centre of the wound, therefore this result compliments its ability to call changes in cell shape and elongation. The result of cell guidance studies in the presence of TGF-β3 are given in Table 1. The trypsinization assays over a period of 17 minutes indicated that addition of TGF-β3 to the media increased the rate of cell detachment by ~20-30% for both collagen and fibronectin treated media.

	Mean Length (μm)	Standard Deviation	Standard Error
Collagen + TGF-β3	79.247	77.672	17.360
Fibronectin + TGF-β3	84.317	32.268	7.215
	Angle (degrees)	Standard Deviation	Standard Error
Collagen + TGF-β3	13.681	10.980	2.455
Fibronectin + TGF-β3	11.233	10.634	2.378

Table 1. Cell length and cell alignment results with addition of TGF-β3.

Conclusions

It is our understanding that cell guidance, cell migration and growth factors have a direct impact upon the healing cascade and scarring profiles in skin tissue.

- Cells aligned better with marginally higher mean lengths to fibronectin patterns compared to the collagen patterns.
- Addition of TGF-β3 to culture media decreased the detachment time of keratinocyte cells. In addition to reducing the degree of adhesion of cells to ECM proteins, TGF-β3 also modified cell shape and cell thickness.
- Fibronection patterns produced a greater elongation effect on keratinocyte cells compared to collagen. This illustrates the essential role of fibronectin in mediating cell elongation and infiltration in skin.

Reference

Bell E., Ehrlich H.P., Buttle D.J., Nakatsuji T., "Living tissue formed in vitro and accepted as skin equivalent tissue of full thickness, Science (1981 Mar 6) 211:1032-54.

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.

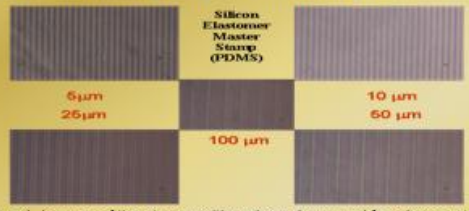

Figure 1. Images of the stamps with various sizes used for micro contact printing of the ECM proteins.

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)

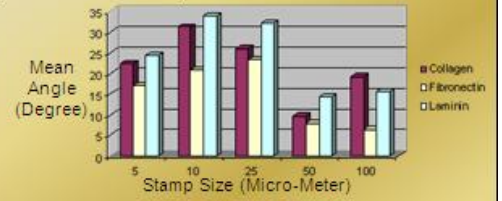

Figure 4. Comparison between three patterned ECM proteins in terms of cell alignment with five different stamp sizes.

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.

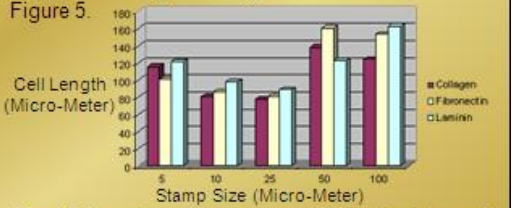

Figure 5. Comparison of three patterned ECM proteins in terms of cell elongation with five different stamp sizes.

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.

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[2] Culture of cells for tissue engineering, R. Ian Freshney, 2006, first edition.

The anti-scarring (wound closure) properties of TGF-β3, BSA/HCl and HCl in cultured human bone cell monolayer

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Abstract: Bone repair can be modulated by different stimulus including growth factors. TGF-β3 is a cytokine known to be associated with 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 properties of TGF-β3, and its dosage carriers, HCl and BSA/HCl. The wound healing response was investigated in culture flasks by creating a wound (with average scratch width of 300μm±10-30μm SD) on confluent monolayers 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. The same method was applied for cell cultured monolayers with no growth factor (control), 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 enhanced the rate of wound repair. After careful observation it was found that after 20 hours 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) induced faster wound healing compared to the control. This indicated that TGF-β3 does have a significant effect on the wound healing process and its healing speed was found to be higher than the control but HCl and BSA/HCl had similar effects.

Introduction

Bone tissue engineering uses the principles of biology and biomedical engineering with the aim of developing a viable tissue substitute that can restore the function of human tissue (Jeffrey O. Hollinger, 2004). Bone repair 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 and stimulating Mesenchymal stem cells (MSC) growth, acting as a chemotactic factor and also enhances bone cells and extracellular matrix (ECM) product secretion (Khan, 2000).

Aims and Objectives

Main objective was to investigate the effect of TGF-β3 on wound closure response on cultured bone monolayers. 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.

Materials and Methods

The wound healing response was investigated in culture flasks by creating a wound (with average scratch width of 300μm±10-30μm SD) on confluent monolayer of MG63 human bone cell. After wounding, cultures were then treated with 50ng/ml TGF-β3 in a concentration of 4mM HCl and 1mg/ml BSA. The same method was applied for cell cultured monolayers with no growth factor (control), with HCl/BSA and HCl only solutions. After wounding, wound width was measured every 5 hours over a 30-hour period (see Figure 1).

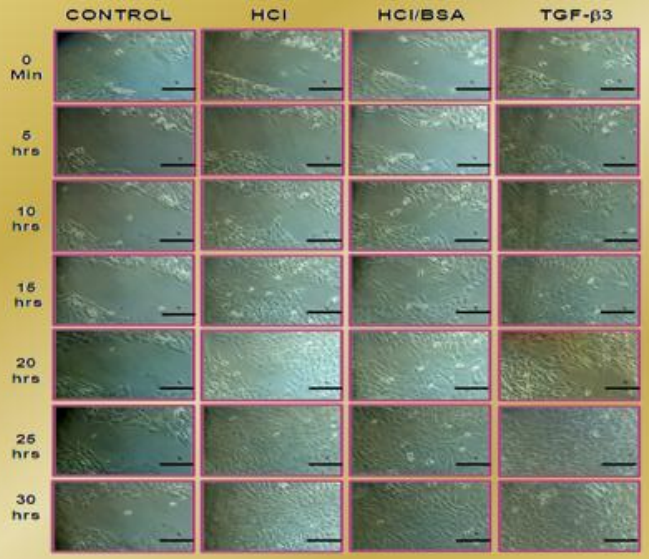

Figure 1. Images of the wound healing process for the bone cell monolayers with 50ng/ml of HCl, BSA/HCl, TGFβ3 and control for the period of 30 hours (Scale bar=100μm).

Image J software was used in order to measure the distance between the wound edges. Six vertical lines at semi-random horizontal distances were drawn and the distances between the intersections of the lines with the wound edges were measured as shown in Figure 2.

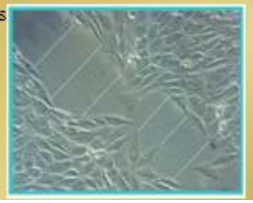

Figure 2. Measurement of wound closure width on cultured MG63 bone cells in the presence of TGF-β3, HCl, BSA/HCl and control, using 'Image J' software.

Results and Discussion

The results showed that TGF-β3 enhanced the rate of wound repair. After careful observation it was found that after 20 hours TGF-β3 (with 15.5% of wound remained open), HCl (with 16% of wound remained open) and BSA/HCl (with 17.7% of wound remained open) induced faster wound healing compared to the control. This indicated that TGF-β3 does have a significant effect on the wound healing process and its healing speed was found to be higher than the control but HCl and BSA/HCl had similar effects as shown in Figure 3.

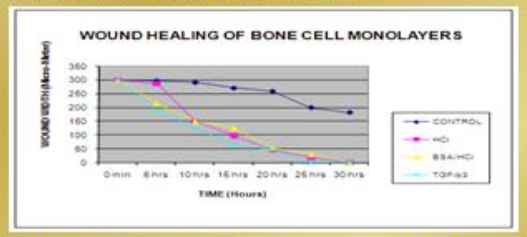

Figure 3. Graph of wound closure width against time for wounded bone cell monolayers with addition of HCl, BSA/HCl, TGFβ3 and control.

Conclusions

TGF-β3 does have a significant effect on the wound healing process and its healing speed was found to be higher than the control but HCl and BSA/HCl had similar effect. In conclusion, TGF-β3, HCl alone and HCl/BSA all enhanced the rate of wound repair indicating that TGF-β3 does not act alone in the wound repair system, but instead functions synergistically with signalling pathways that are dependent on the availability of hydrogen ions. Such a mechanism would depend on signalling molecules undergoing a conformational change on binding hydrogen ions. This is not a new concept, one only has to think of haemoglobin's affinity for oxygen as a prime example, but it is potentially a concept that has been overlooked in the wound repair system.

References

[1] Jeffrey O. Hollinger, Bone tissue engineering, 2004, first edition.
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The effect of transforming growth factor beta (TGF-β3) and BSA/HCl on Trypsinisation of bone cells monolayers

F. Sefat[1,2], M. Youseffi[1] , R.F. Berends[1,3], S.A. Khaghani[1,2] and M.C.T Denyer[1,3]

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Abstract: The *in vitro* processes of bone cell attachment and detachment have been investigated. Cells are not found in isolation and they usually adhere to other cells or surrounding extracellular matrix (ECM) *in vivo* and to a substrate *in vitro*. The effect of TGF-β3 is mediated by a range of signalling pathways. The interaction of bone cells with their surrounding ECM environment influences some physiological function and pathological processes. These interactions are mediated by integrins which are capable of transducing the signals from ECM to the cells resulting in migration, differentiation and specific protein synthesis. Trypsinisation was carried out to investigate the effect of TGF-β3 on cell detachment. Bone cells were cultured in 12 well petridishes and left for 3 days to become confluent with three different cell dilutions. Four wells were seeded with 1:3 ratio of cell to DMEM (as control). Another four wells were also seeded with 1:3 ratio of cell to DMEM with addition of 50µl of BSA/HCl. Last four well were seeded with 1:3 ratio cell and DMEM and addition of 50ng/ml TGF-β3 (HCl (4mM), BSA (1mg/ml) and Distilled water). The 12 well cultured dishes were investigated via imaging techniques using light microscope. Media was aspirated and trypsin (0.5ml) was added to each well separately and images were recorded every 20 second over a period of 15 minutes. Trypsinisation experiments for bone cells cultured with TGF-β3 showed that cells started to detach from the surface ~1 minute after application of trypsin and were completely detached by the fifth minute. Cell detachment was ~85% after 4 minutes and thus faster as compared to the control and BSA/HCl. Trypsinisation results indicated that application of TGF-β3 at 50ng/ml decreased the degree of cell attachment and may also have effects on modification of integrin expression.

Introduction

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 ECM environment in vivo and substrate or a surface in vitro. 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 (Albelda, 1990). These interactions are mediated by integrins. Integrins are capable of transducing the signals from ECM to the cells which results in migration, differentiation and specific protein synthesis.

Aims and Objectives

The aim of this study was to investigate the effect of TGF-β3 on bone cell detachment via Trypsinization process.

Materials and Methods

Trypsinisation was carried out to investigate the effect of TGF-β3 on cell detachment. Bone cells were cultured in 12 well petridishes (see Figure 1) and left for 3 days to become confluent with three different cell dilutions. Four wells were seeded with 1:3 ratio of cell to DMEM (as control). Another four wells were also seeded with 1:3 ratio of cell to DMEM with addition of 50ng/ml BSA/HCl. Last four wells were seeded with 1:3 ratio cell and DMEM and addition of 50ng/ml TGF-β3 (HCl (4mM), BSA (1mg/ml) and Distilled water). The 12 well cultured dishes were investigated via imaging techniques using light microscope (see Figure 2). Media was aspirated and trypsin (0.5ml) was added to each well separately and image recording was carried out every 20 second over a period of 15 minutes.

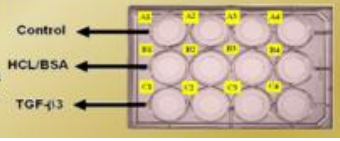

Figure 1. 12well culture dish for trypsinisation with three different cell dilutions.

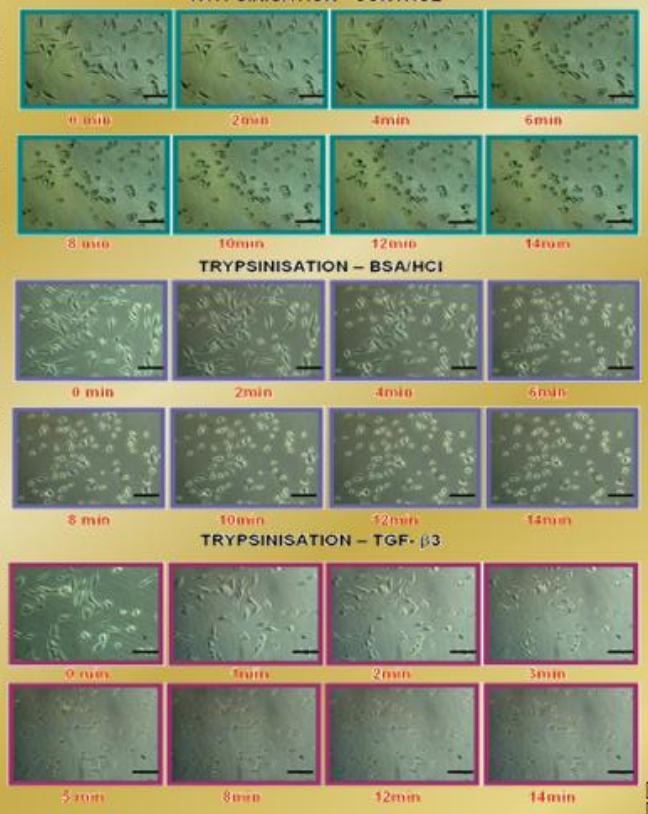

Figure 2. Trypsinisation process in the presence of TGF-β3, BSA/HCl and DMEM; (Scale bar=100 µm).

Results and Discussion

Cell detachment in control was ~43% after 6 minutes. Bone cells in the presence of BSA/HCl started detaching after ~4-5 minutes after applying trypsin. Cell detachment was ~63% after 6 minutes which was faster as compared to the control. Trypsinisation experiments for bone cells cultured with TGF-β3 showed that cells started to detach from the surface ~1 minute after application of trypsin and were completely detached by the fifth minute. Cell detachment was ~85% after 4 minutes and thus faster as compared to the control and BSA/HCl as shown in Figure 3.

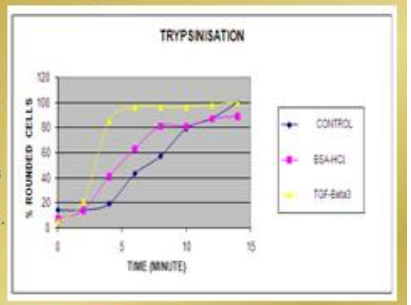

Figure 3. Comparison between percentage rounded cells during trypsinisation process for control, BSA/HCl and TGF-β3 additions.

Conclusions

Trypsinisation results indicated that application of TGF-β3 at 50ng/ml decreased the degree of cell attachment. In conclusion, TGF-β3 and HCl/BSA enhanced the rate of cell detachment in relation to the negative controls, indicating perhaps that TGF-β3 does not act alone in the trypsinisation, but instead functions synergistically with signalling pathways that are dependent on the availability of hydrogen ions or BSA.

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Investigating Keratinocyte cell responses to ECM proteins using Microcontact printing

R.F. Berends[1], M. Youseffi[2], F. Sefat[1,2], S.A. Khaghani[1,2] and, M.C.T. Denyer[1]

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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.

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 (see Figure 1).
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 templates. Cells were seeded at 5.4×10^4/ 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.

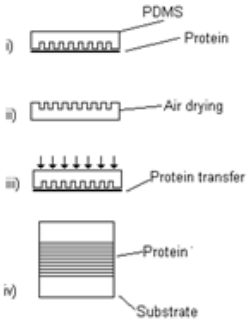

Figure 1. Schematic stamping procedure

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 2 shows examples of cell alignment for each protein. Figures 3 and 4 are bar charts showing angle of alignment and cell length for each protein, respectively. Standard error bars are also shown, for each Figure.

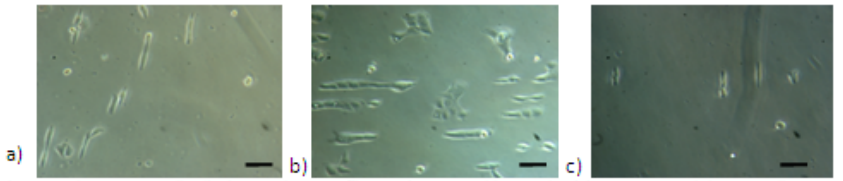

Figure 2. Shows HaCat cells aligning to; a) 100µm fibronectin pattern; b) 50µm laminin pattern; and c) 50µm collagen type I pattern. The scale bar represents a distance of approximately 80µm.

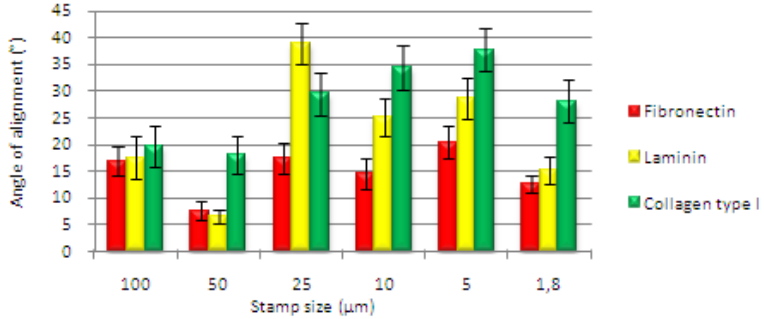

Figure 3. Chart showing angle of alignment for each protein and stamp size with standard error bar.

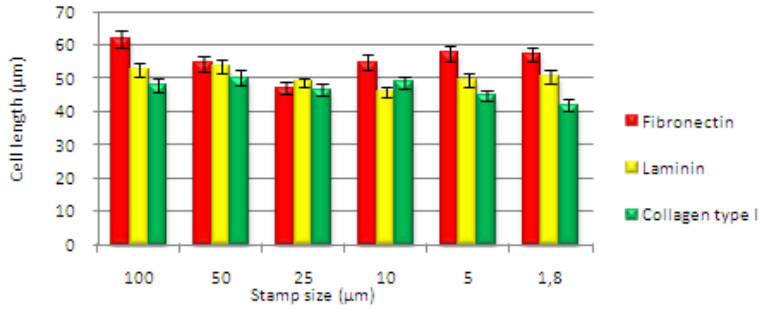

Figure 4. Chart showing cell length for each protein and stamp size with Standard error bars shown.

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.

References

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Effect of TGF-β1 on wound closure properties of cultured human bone cell monolayer

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[1]Institute of Pharmaceutical Innovation (ipi), [2]School of Engineering, Design and Technology-Medical Engineering, [3]School of Life Sciences, University of Bradford, Bradford BD7 1DP, UK

Abstract: Bone repair can be modulated by different stimulus including growth factors. Transforming Growth Factor beta (TGF-β) 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 including bone. TGF-β can be found in three isomers: TGF-β1, TGF-β2 and TGF-β3. The aims of this *in vitro* study were to investigate the effect of TGF-β1 on closure of a model wound in cultured monolayer of the MG63 human bone cells. The wound healing response was investigated in culture flasks by creating a wound on confluent monolayer of MG63 human bone cell. After wounding, cultures were treated with 50ng/ml TGF-β1 and the same method was applied for cell cultured monolayer with no growth factor as control. Wound width was measured every 5 hours over a 30-hour period and the results showed that TGF-β1 enhanced the rate of wound repair significantly as compared to control.

Introduction

Transforming growth factor beta 1 (TGF-β1) is a polypeptide member of the transforming growth factor beta superfamily of cytokines. It is a secreted protein that performs many cellular functions including the control of cell growth, proliferation, differentiation and apoptosis [1, 2]. Many cells produce TGF-β and almost all of them have specific receptors for this peptide. TGF-β isomers (TGF-β1, TGF-β2 and TGF-β3) all function through the same receptor signalling systems. TGF-β1 plays an important role in controlling the immune system and shows different activities on different types of cell [1,2].

Aims and Objectives

The aims of this *in vitro* study were to investigate the effect of TGF-β1 on closure of a model wound in cultured monolayer of the MG63 human bone cells.

Materials and Methods

The wound healing response was investigated in culture flasks by creating a wound with average scratch width of 300μm±10-30μm SD on confluent monolayer of MG63 human bone cell. The wound was inflicted using the tip of a sterilized disposable pipette. After wounding, cultures were then treated with 50ng/ml TGF-β1. The same method was applied for cell cultured monolayer with no growth factor as control. Wound width was measured every 5 hours over a 30-hour period using image J and taking the average of six wound width across the wound bed (see Figure 1).

Results and Discussion

The results showed that TGF-β1 enhanced the rate of wound repair. It was found (see Figure 2) that after 20 hours TGF-β1 (with 29.7% of wound remained open) induced faster wound healing compared to the control (with 86.7% of wound remained open). This indicated that TGF-β1 does have a significant effect on the wound healing process of wounded bone monolayer and its healing speed was found to be higher than the control (see Figure 2).

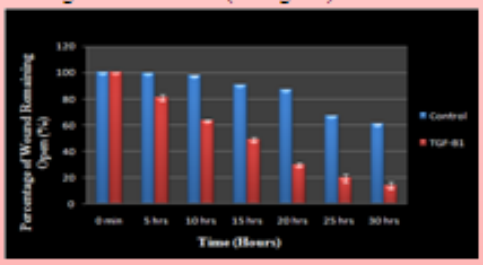

Figure 2. Graph of percentage of wound remaining open against time for wounded bone cell monolayer with addition of TGFβ1 and control.

Conclusions

TGF-β1 enhanced the rate of wound repair indicating that it has a positive effect as compared to control. TGF-β1 is thought to play important roles in inducing bone cells to produce extracellular matrix proteins to cover the wound and in limiting the local inflammatory response. TGF-β1 signalling pathway is involved in many cellular processes and regulates some cellular response including wound closure. However, the effects of TGF-β on the behaviour of the cells involved in primary closure of wounds have not been completely elucidated.

References

[1] **SEFAT, F., YOUSEFFI, M., AND DENYER, M.C.T.**, (2009) The Anti-Scarring (Wound Closure) Properties of TGF-β1, RGA/HCl and HCl in Cultured Human Bone Cell Monolayer. *World congress on Engineering (WCE), International conference of systems biology and bioengineering (ICSBB), July 1-3, 2009, London, UK.* Vol II, ISBN:978-988-18210-1-0, pp1866-1871.
[2] **KHAN, S.N.**, (2000) Bone Growth Factors. *orthop. Clin., 31, 375*

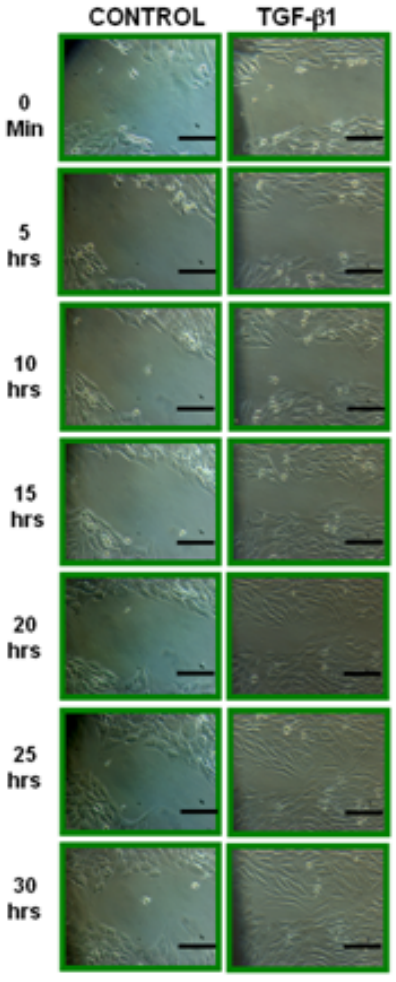

Figure 1. Images of the wound healing process for the bone cell monolayers with 50ng/ml of TGFβ1 and control for the period of 30 hours (Scale bar=100μm).

Effect of TGF-β2, BSA/HCl and HCl on wound closure properties of cultured human bone cell monolayer

F. Sefat[1,2], M. Youseffi[2] and M.C.T. Denyer[1,3]

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Abstract: The aims of this *in vitro* study were to investigate the effect of TGF-β2 on closure of a model wound in cultured monolayer of the MG63 human bone cells. This *in vitro* work examined and compared the wound closure response with and without addition of TGF-β2, and its dosage carriers HCl and BSA/HCl. The wound healing response was investigated in culture flasks by creating a wound on confluent monolayer of MG63 human bone cell. After wounding, cultures were treated with 50ng/ml TGF-β2 at concentration of 4mM HCl and 1mg/ml BSA and Distilled water. The same method was applied for cell cultured monolayer with no growth factor (control), 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-β2 enhanced the rate of wound repair but not as good as its dosage carriers. This indicated that TGF-β2 does have a significant effect on the wound healing process but less than its dosage carriers and its healing speed was found to be higher than the control.

Introduction

The role of growth factors (GFs) in bone repair is broadly documented [1, 2], mainly for transforming growth factor-beta (TGF-β). GFs are usually stored in the extracellular matrix (ECM), but after injury are actively released by ECM, cells and platelets. GFs are the key components of functional bone regeneration and TGF-β2 is a cytokine that may play a role in bone repair.

Aims and Objectives

The aims of this *in vitro* study were to investigate the effect of TGF-β2 and its dosage carriers HCl and BSA/HCl on closure of a model wound in cultured monolayers of the MG63 human bone cells.

Materials and Methods

The wound healing response was investigated in culture flasks by creating a wound (with average scratch width of 300μm±10-30um SD) on confluent monolayer of MG63 human bone cell. The wound was inflicted using a disposable sterilized pipette. After wounding, cultures were treated with 50ng/ml TGF-β2 at concentration of 4mM HCl and 1mg/ml BSA and Distilled water. The same method was applied for cell cultured monolayers with no growth factor (control), with HCl/BSA and HCl only solutions. Wound width was measured every 5 hours over a 30-hour period using image J and average of six wound width was take across the wound bed.

Results and Discussion

It was found (see Figures 1 and 2) that after 20 hours TGF-β2 (with 33% of wound remained open), HCl (with 15.5% of wound remained open) and BSA/HCl (with 17.7% of wound remained open) induced faster wound healing compared to the control (with 86.8% of wound remained open). This indicated that TGF-β2 does have a significant effect on the wound healing process but less than its dosage carriers and its healing speed was found to be higher than the control (See Figure 2).

Conclusions

TGF-β2, HCl alone and HCl/BSA all enhanced the rate of wound repair but also indicating that TGF-β2 does not act alone in the wound repair system and functions synergistically with signalling pathways that are dependent on the availability of hydrogen ions. Such a mechanism would depend on signalling molecules undergoing a conformational change on binding hydrogen ions. This is not a new concept and one only has to think of haemoglobin's affinity for oxygen as a prime example, but it is potentially a concept that has been overlooked in the wound repair system.

References

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(2) **KHAN, S.N.** (2000) Bone Growth Factors. *orthop. Clin., 31, 375.*

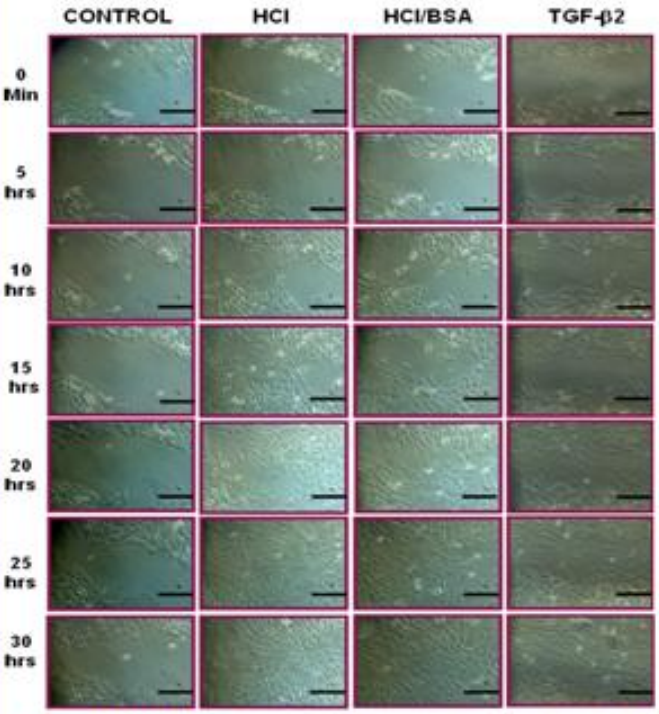

Figure 1. Images of the wound healing process for the bone cell monolayers with 50ng/ml of HCl, BSA/HCl, TGFβ2 and control for the period of 30 hours (Scale bar=100μm).

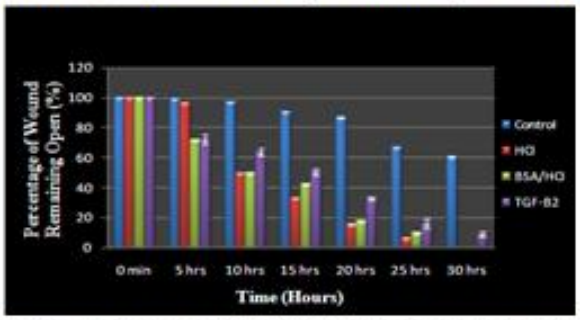

Figure 2. Graph of percentage of wound remaining open against time for wounded bone cell monolayer with addition of HCl, BSA/HCl, TGFβ2 and control.

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.**

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

Book Chapter 50

Application of a Novel Widefield Surface Plasmon Resonance Microscope in Cell Imaging and Wound Closure Properties of TGF-β3, BSA/HCl and HCl in Cultured Human Bone Cell Monolayer

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Abstract: A newly developed Widefield Surface Plasmon Resonance (WSPR) Microscope was used to investigate the morphology of MG63 bone cells and their interfacial interactions with ECM proteins. This allowed detailed imaging of cell surface coupling at lateral resolution down to ~500nm. In this work, bone repair was investigated and 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 aim of this study was 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 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 monolayers of MG63 human bone cells. 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 monolayers 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 enhanced the rate of wound repair in a monolayer of MG63 bone cells. It was found 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

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Biological Application of Widefield Surface Plasmon Resonance Microscope to Study Cell/Surface Interactions and the Effect of TGF-β3, HCl and BSA/HCl on Cell Detachment Assay of Bone Cells Monolayer

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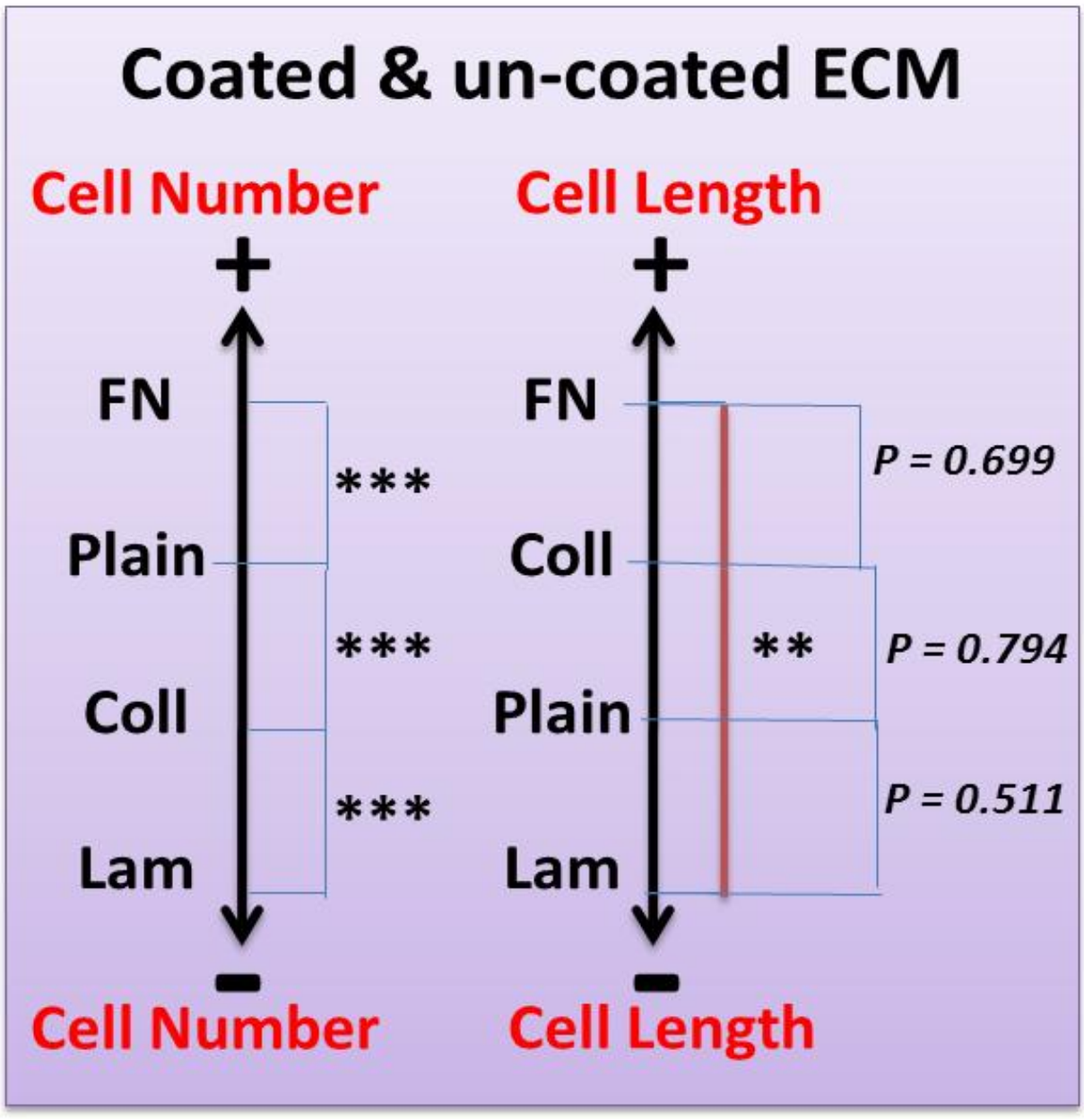
Abstract: Widefield Surface Plasmon Resonance (WSPR) microscope was used to investigate cell surface interactions under two different culture conditions: bone cells cultured on SPR substrate with transforming growth factor β3 (TGF-β3) and without as control. Trypsinisation was carried out in order to 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 one additional flask as control. These results further confirmed that application of TGF-β3, HCl and HCl/BSA decreased the degree of cell attachment on surface of culture flasks. HCl and BSA/HCl were tested as they are the carriers and solvents for TGF-β3. Cell detachment in control was about 43% after 6 minutes, which is slow. Bone cells in the presence of BSA/HCl started detaching from the surface about 4-5 minutes and cell detachment was about 63% after 6 minutes which was faster as compared to the control. Bone cells in the presence of HCl alone started detaching from the surface about 2 minutes (after applying trypsin) and cell detachment was about 69% after 6 minutes which was faster compared to the BSA/HCl and control. Trypsinisation experiments for bone cells cultured with TGF-β3 (50ng/ml) showed that cells started to detach from the surface about 1 minute after application of trypsin and cell detachment was about 85% after 4 minutes which was faster as compared to the control, HCl and BSA/HCl.

APPENDIX 2

OVERALL RESULTS

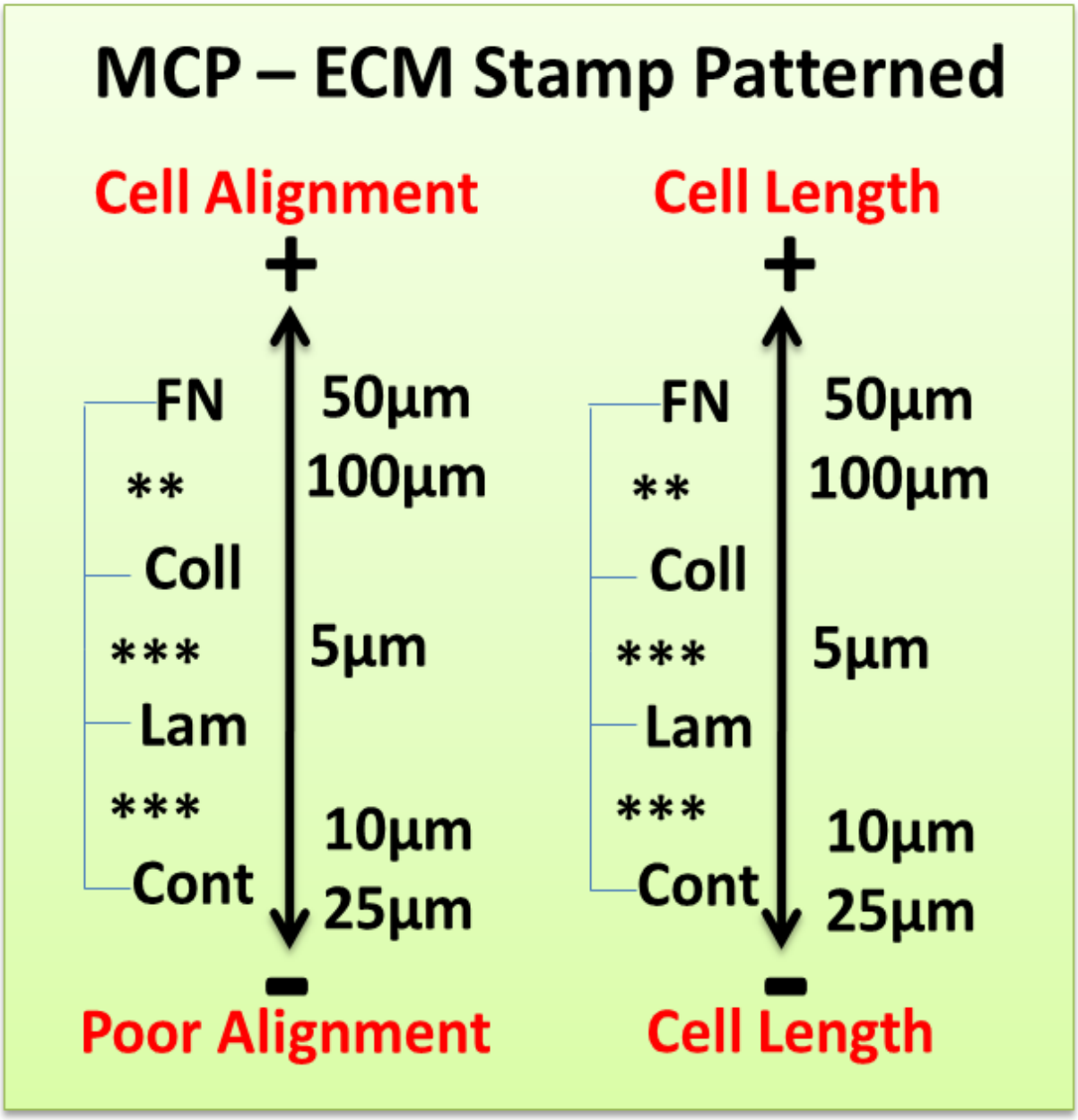
Overall Results

Chapter 2



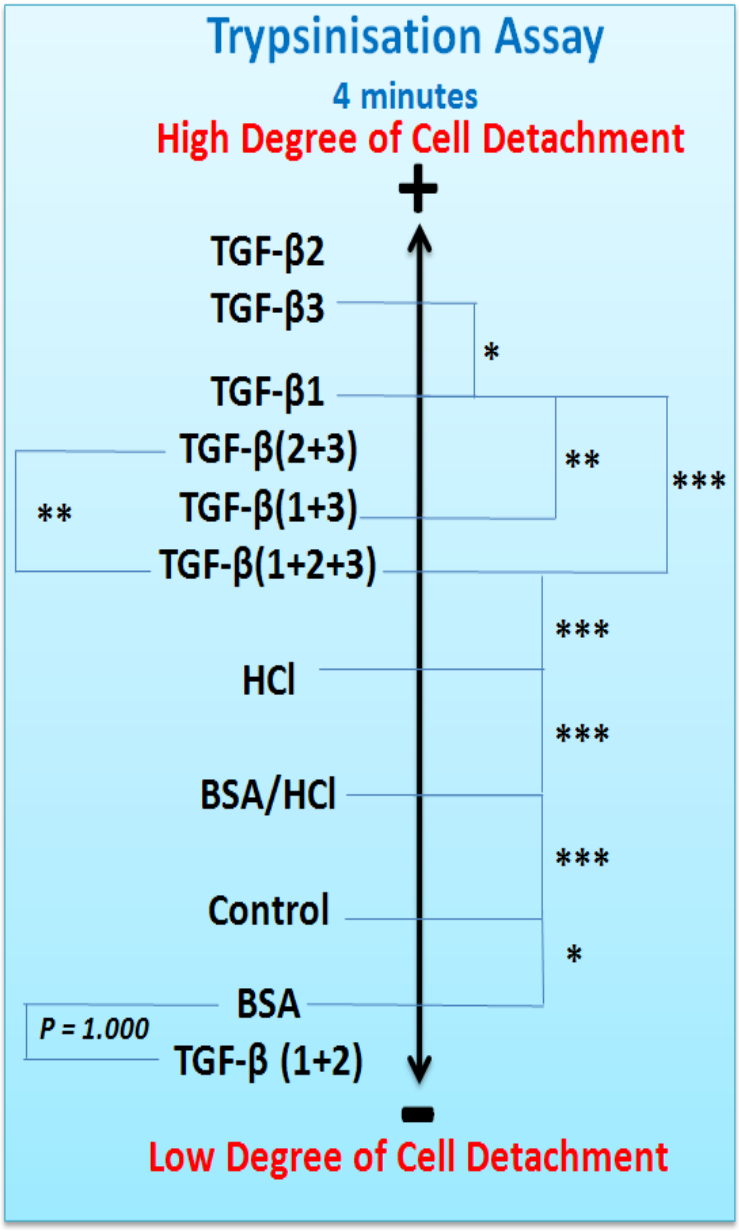
Overall Results

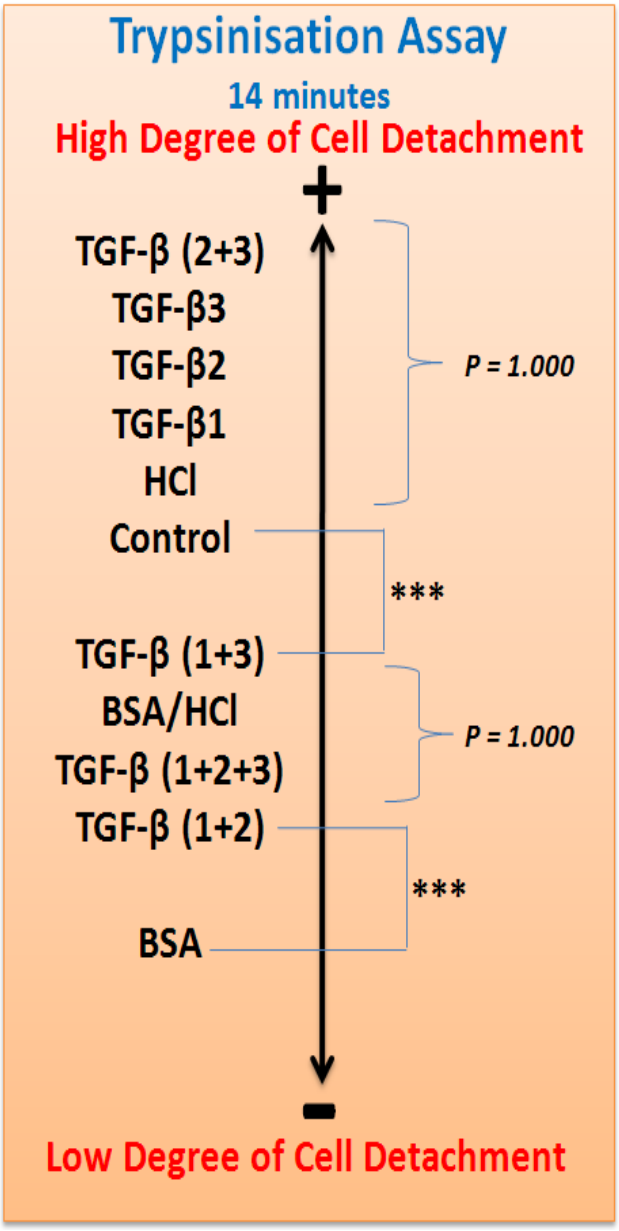
Chapter 2



Overall Results

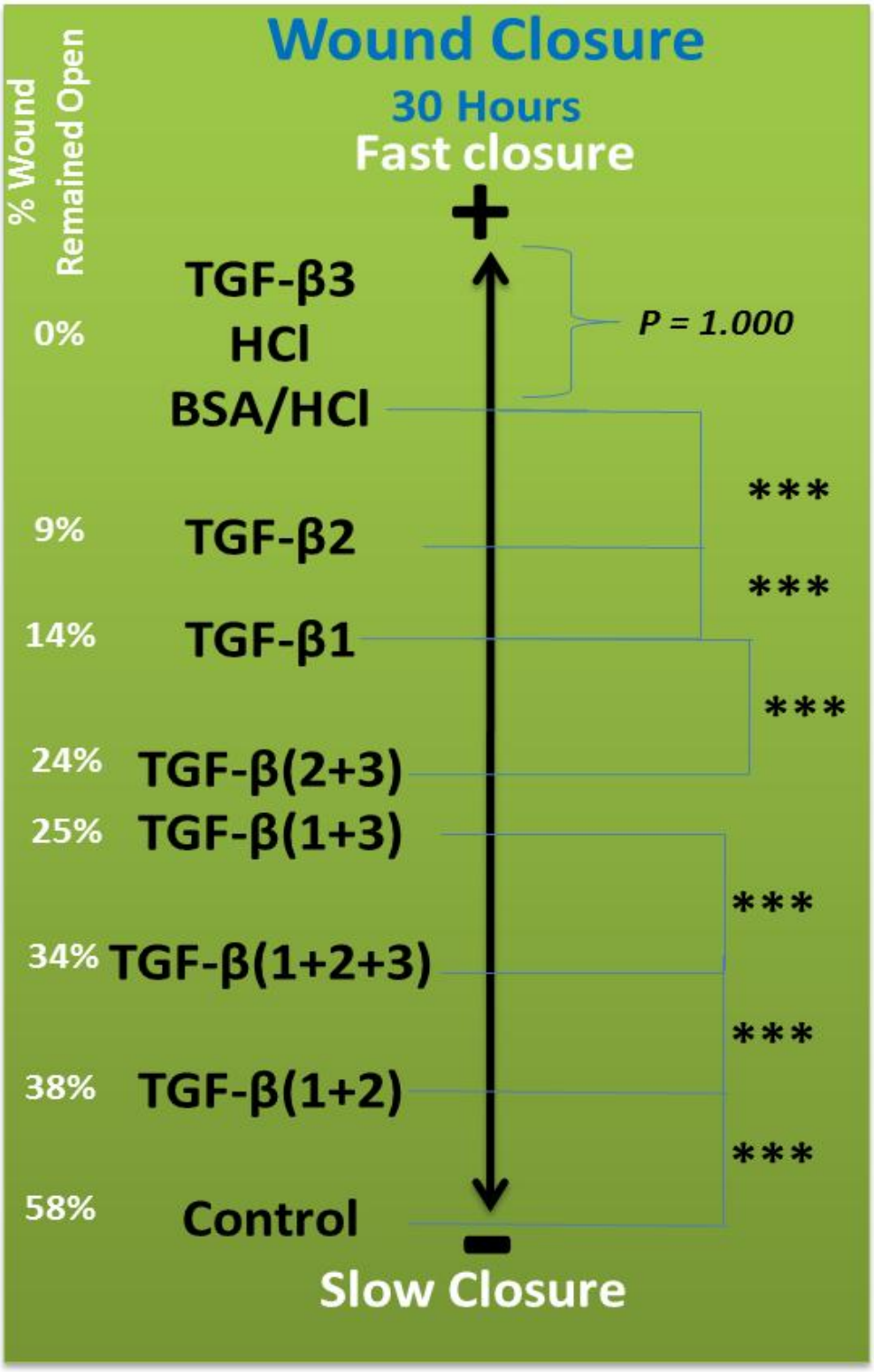
Chapter 3





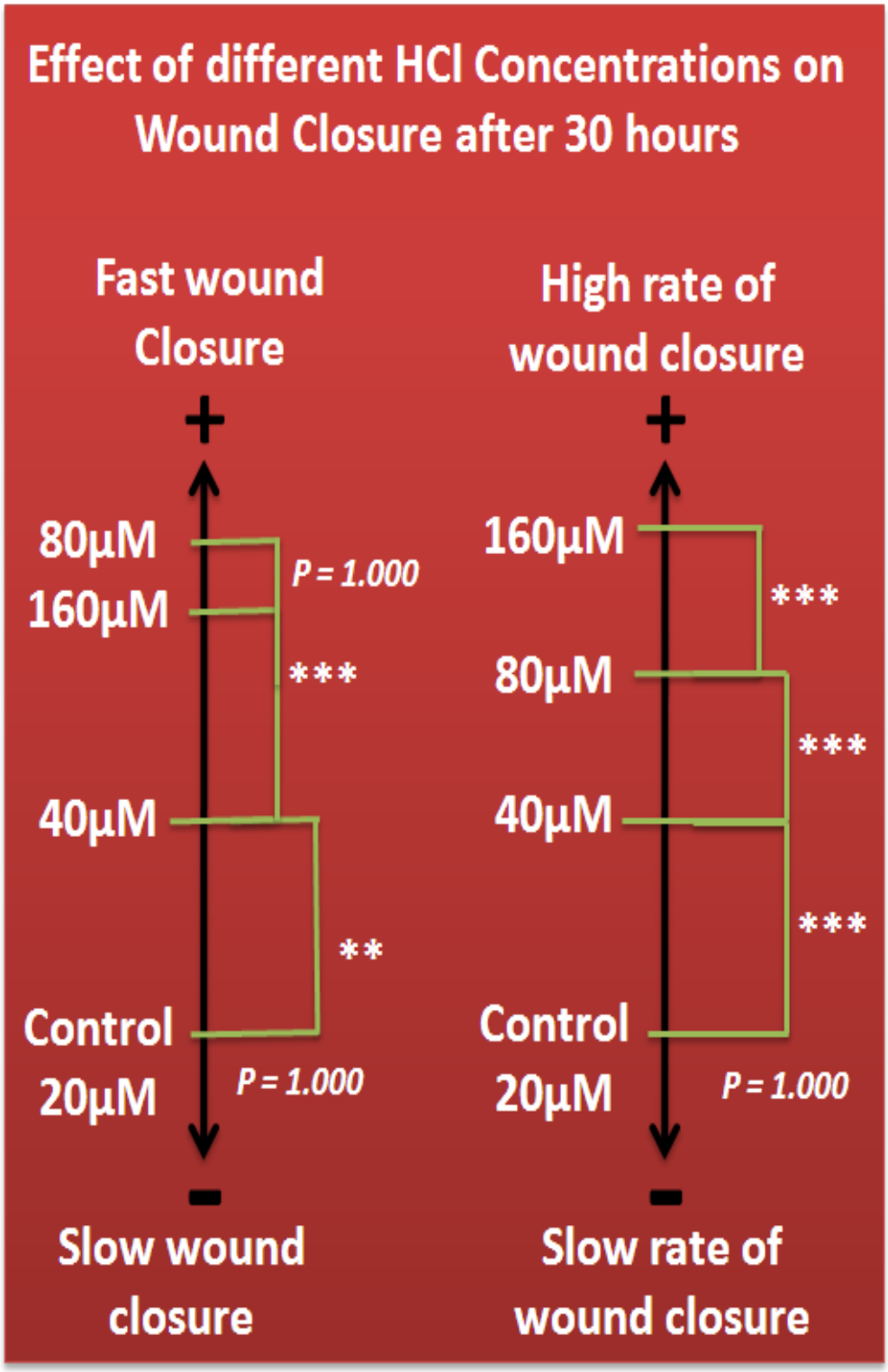
Overall Results

Chapter 4



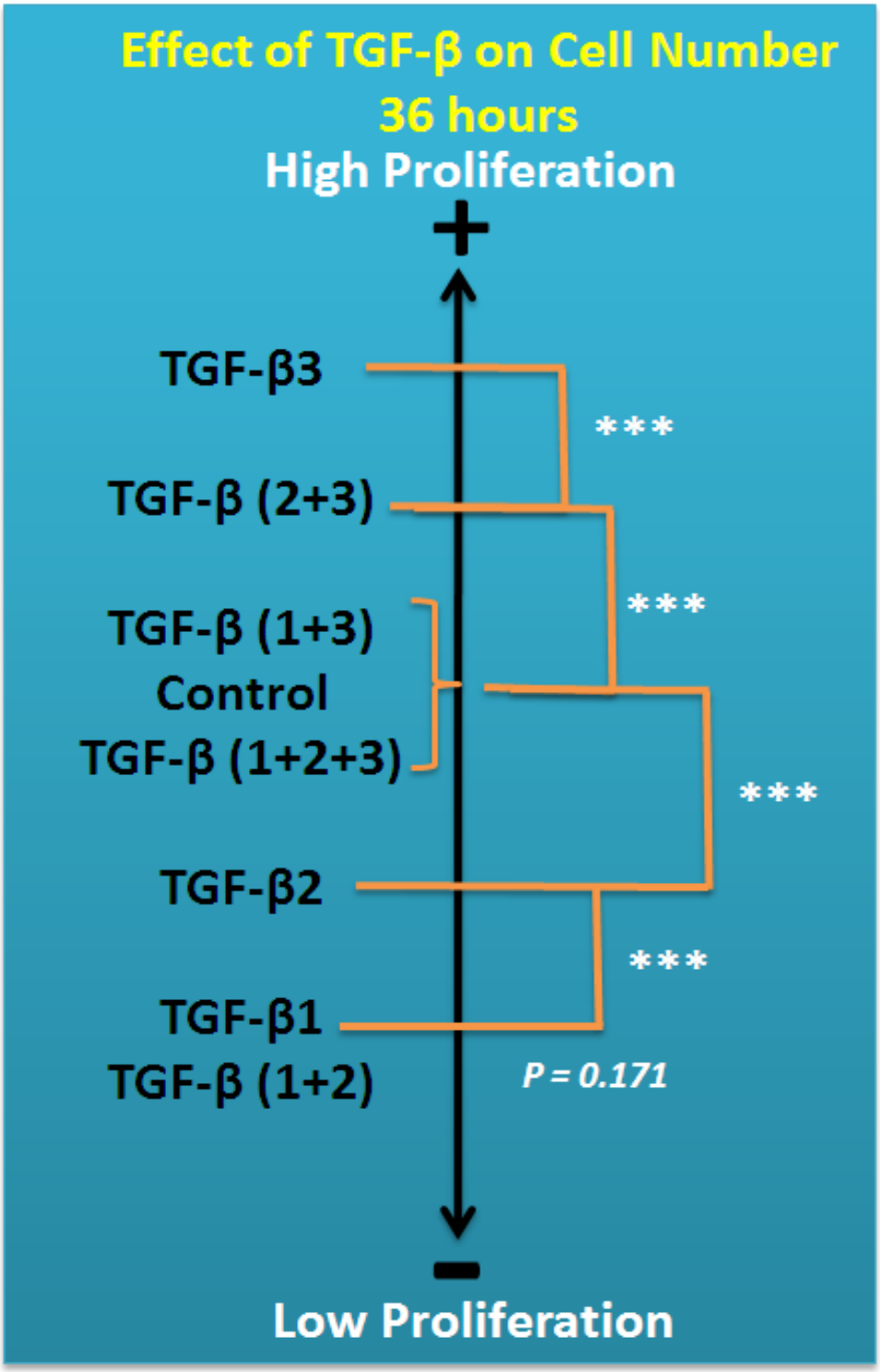
Overall Results

Chapter 5



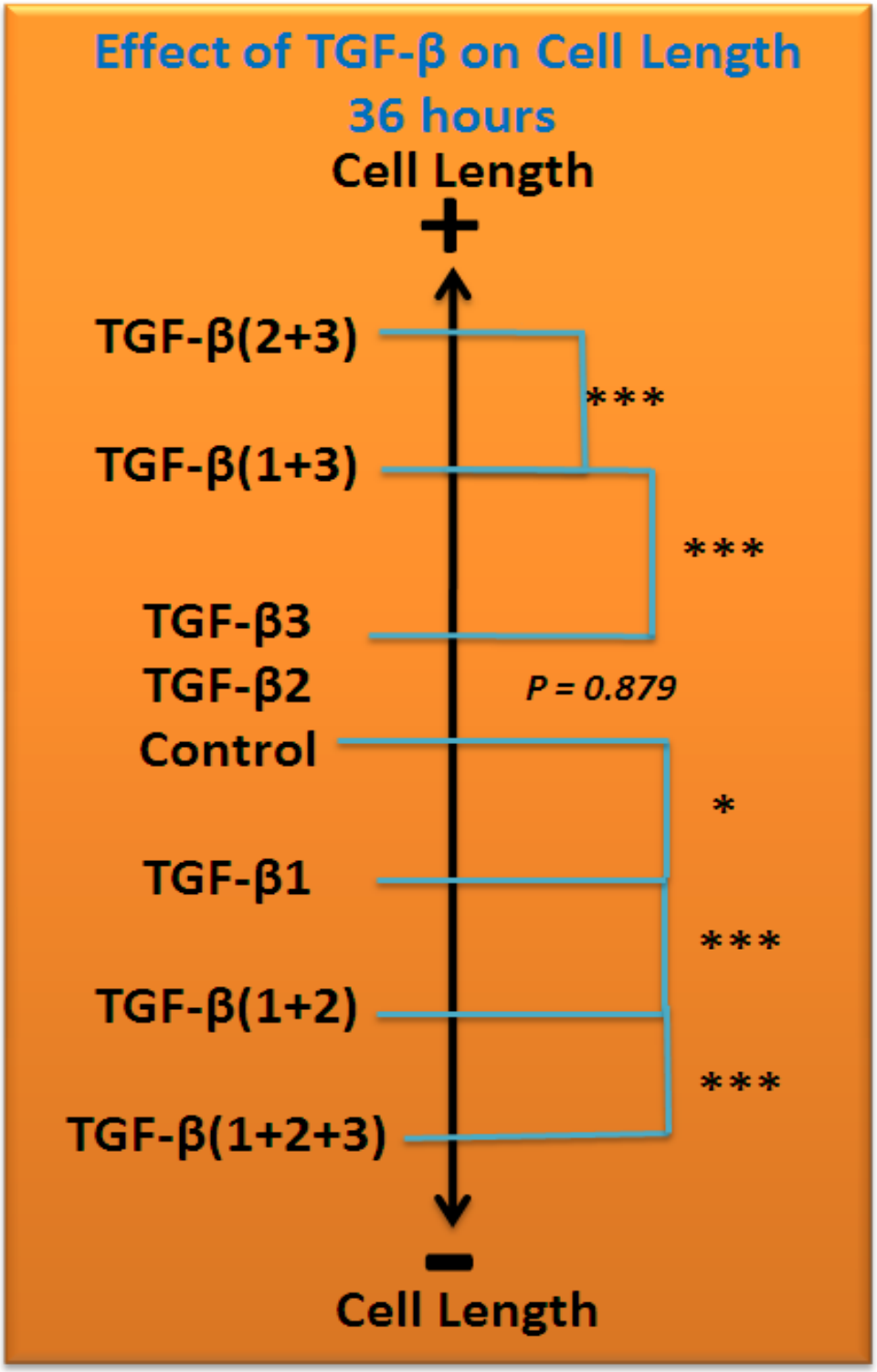
Overall Results

Chapter 6



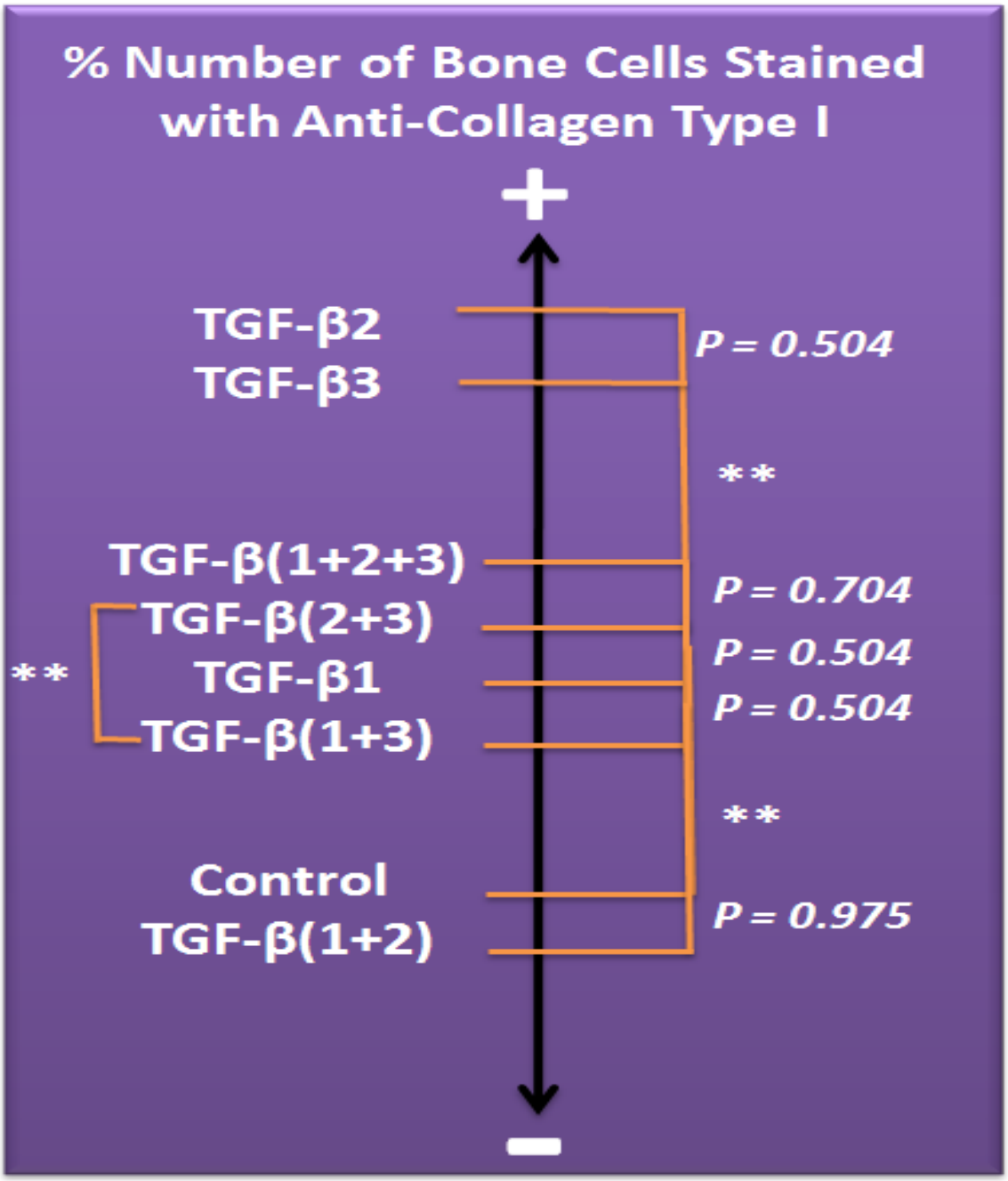
Overall Results

Chapter 6



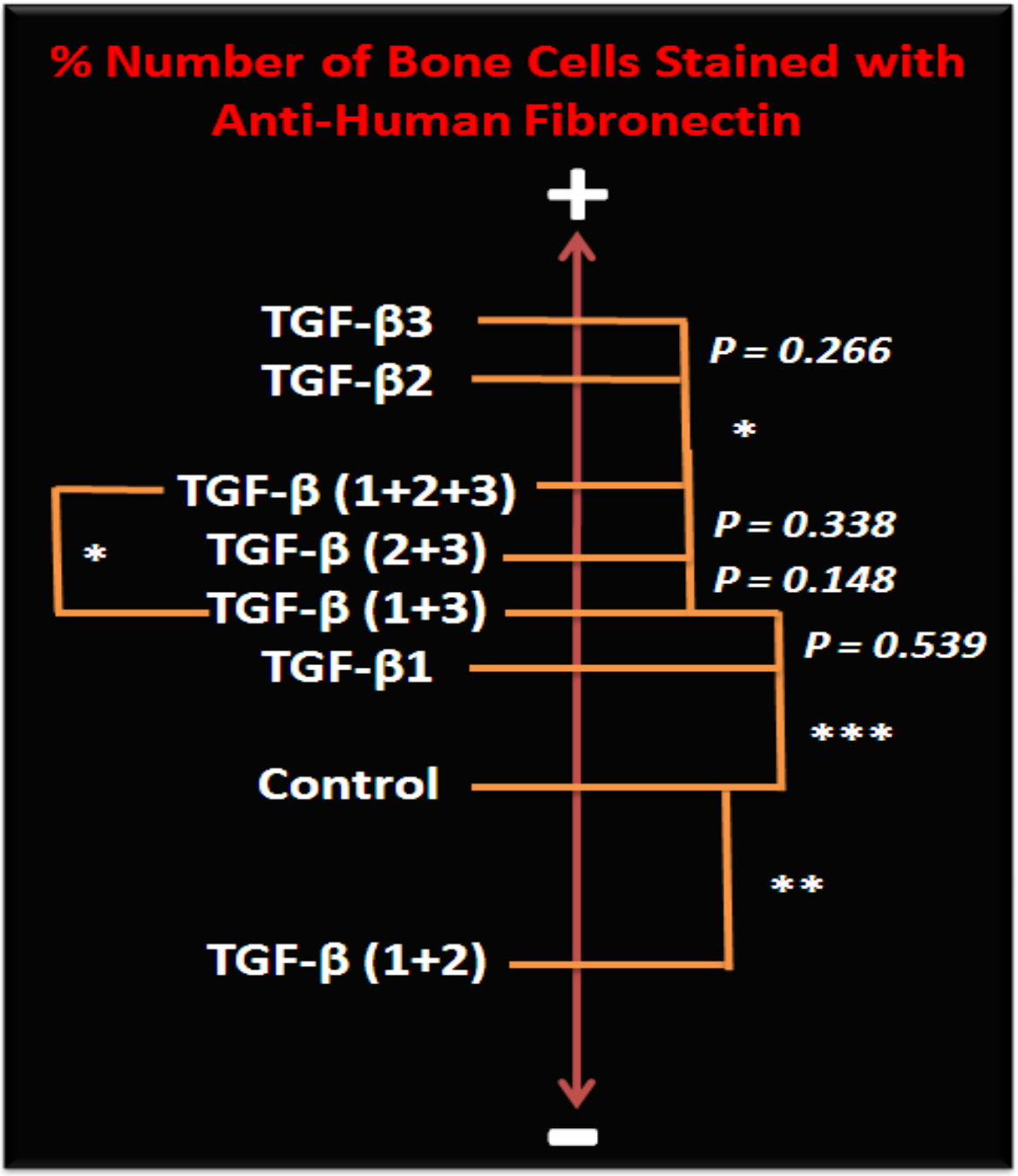
Overall Results

Chapter 7



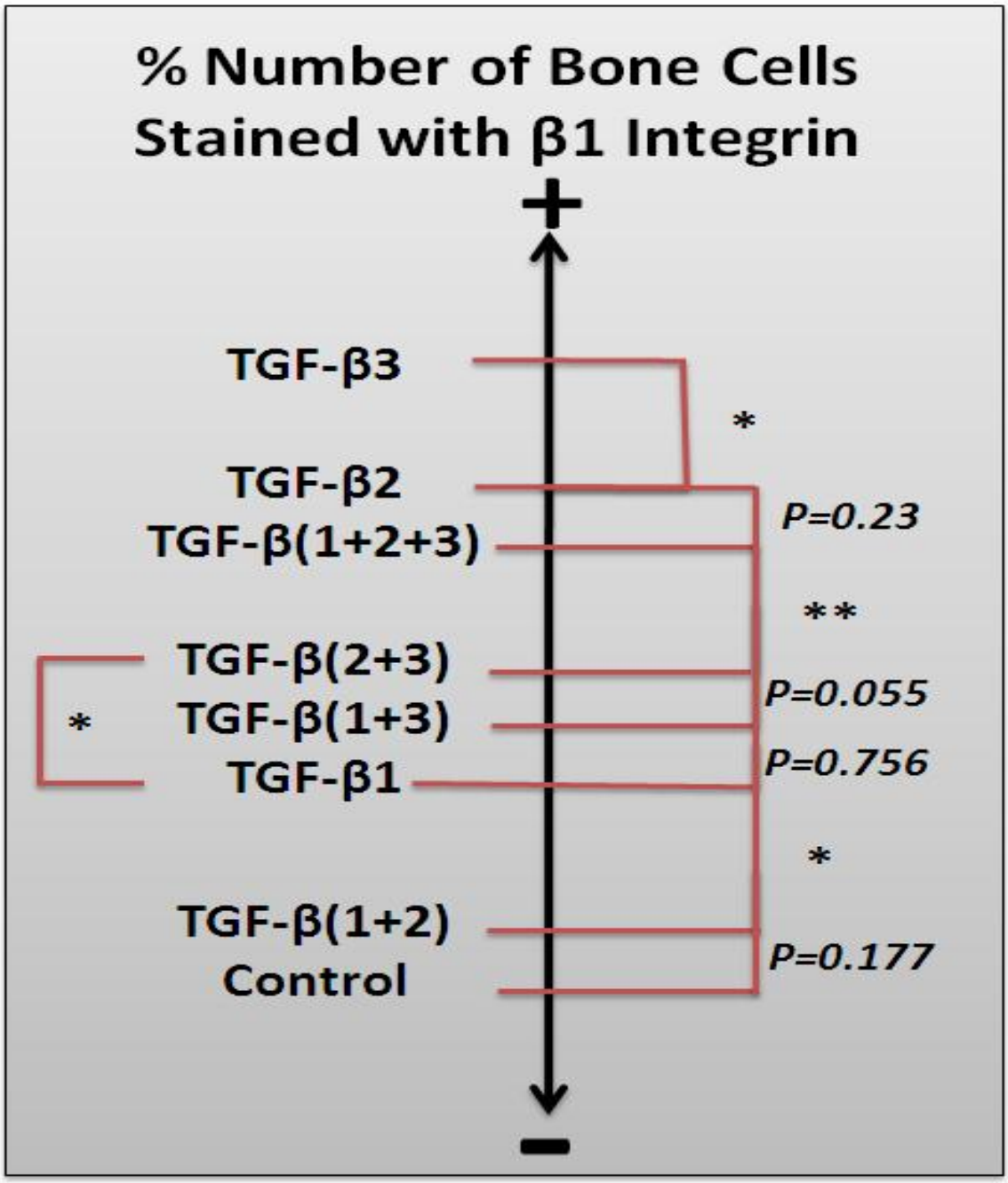
Overall Results

Chapter 7



Overall Results

Chapter 7



Overall Results

Chapter 7