A STUDY ON THE EFFECTS OF UNIAXIAL CYCLIC TENSILE LOADING ON HUMAN BONE MARROW DERIVED-MESENCHYMAL STROMAL CELLS *IN VITRO*

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Title of Thesis ("this Work"): A Study On The Effects Of Uniaxial Cyclic Tensile Loading On Human Bone Marrow Derived-Mesenchymal Stromal Cells *In Vitro*

Field of Study: Tissue Engineering

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ABSTRACT

Mesenchymal stromal cells (MSCs), being multipotent cells, have the ability to undergo both self-renewal and multi-lineage differentiation. Although many techniques to drive cellular response using chemical or hormonal cues have been described, the use of mechanical loading as a method to harness the potential of MSCs has not been fully realized. In tendons especially, mechanical stimulation modulates cellular proliferation and tenogenic expression/differentiation thereby regulating tissue homeostasis. Similar to MSCs, the exact mechanisms involved in the process of converting mechanical signals into cellular differentiation have yet to be elucidated. For this reason, the present thesis comprising of several key studies was conducted to assist us in getting us closer to better understand the possible mechanisms underpinning these aforementioned laboratory and clinical observations. In the present study we hypothesized that uniaxial cyclic loading is expected to improve cellular and direct tenogenesis differentiation through the activation of ion channels. The studies were conducted with the following aims: 1) to demonstrate that cells proliferation can be regulated through mechanical loading, 2) to determine the parameters that will lead to superior tenogenic differentiation of human MSCs (hMSCs), 3) to determine the role of ion channels, specifically epithelium sodium channel (ENaC) and stretch-activated calcium channel (SACC), in regulating the observations made in aims 1 and 2. To achieve the first aim of this study, hMSCs were isolated, expanded, and subjected to cyclical uniaxial stretching of 4%, 8% or 12% strain at 0.5 Hz or 1 Hz for 6, 24, 48 or 72 hours. This was compared to unstrained hMSC cultures. Morphology and alignment of the cells was documented, whilst cell viability and proliferation were assessed using live/dead cell staining and alamarBlue assay. The result demonstrates that strained cells appear to be realigned perpendicular to the direction of tensile loading in contrast to unstrained cells, which were arranged randomly. The highest cell proliferation was observed when 4%

strain+1 Hz was applied ($p < 0.05$). To obtain aim 2, hMSCs differentiation was analysed using cells topography, immunostaining, immunofluorescent staining, biochemical assays and mesenchymal cell gene expression markers. At 8% and 12% strain (1 Hz), an increase in collagen I, collagen III, elastin, fibronectin, and N-cadherin production were observed; but not for collagen II and glycosaminoglycans. Tenogenic genes expression were only highly expressed when subjected to 8% and 12% ($p < 0.05$), although in the former it was higher. The osteoblastic, chondrogenic and adipogenic marker genes appeared to be down-regulated. Lastly, the tenogenic differentiation of hMSCs was examined in the presence and absence of ENaC and SACC by adding benzamil and gadolinium, respectively. The results show that by inhibiting these two mechanosensitive channels, mechanical stretching retards biochemical signalling queues and that stretch-induced tenogenic differentiation process is aborted. In conclusion, the observations of our studies suggests that MSCs are sensitive to mechanical stimulation specifically to tenogenesis response, and can be regulated by altering certain parameters such as ion channel sensitivity, duration of stretching and, rate and amounts of strains.

ABSTRAK

Sel-sel stem mesenkima (MSCs), sebagai sel pelbagai potensi, mempunyai keupayaan untuk menjalani proses pembezaan kepada beberapa keturunan dan pembaharuan kendiri. Walaupun banyak teknik untuk mendorong tindak balas selular yang menggunakan isyarat-isyarat bahan kimia atau hormon telah diterangkan, penggunaan pemuatan mekanikal sebagai satu kaedah untuk merealisasikan potensi MSCs masih belum difahami sepenuhnya. Dalam tendon khususnya, rangsangan mekanikal memodulatkan percambahan selular dan ekspresi/pembezaan tenogenik, dan dengan itu tisu homeostasis dapat dikawalselia. Sama seperti MSCs, mekanisma-mekanisma tepat yang terlibat dalam penukaran isyarat mekanikal ke dalam pembezaan selular masih belum dijelaskan. Oleh sebab itu, tesis ini yang terdiri daripada beberapa kajian pokok, telah dijalankan untuk membantu kami untuk lebih memahami mekanisma-mekanisma yang mungkin menyokong pemerhatian-pemerhatian makmal dan klinikal yang dinyatakan di atas. Dalam kajian ini, kami menghipotesis bahawa rangsangan berkitar ekapaksi dijangka menambahbaikkan pembezaan dan menghalajukan pembezaan tenogenesis melalui pengaktifan saluran-saluran ion. Kajian-kajian telah dijalankan dengan matlamat-matlamat berikut: 1) untuk menunjukkan bahawa pembiakan sel-sel boleh dikawalselia melalui rangsangan mekanikal, 2) untuk menentukan faktor-faktor pembolehubah yang akan membawa kepada pembezaan tenogenik yang terbaik daripada MSCs manusia (hMSCs), 3) untuk menentukan peranan saluran-saluran ion, khususnya saluran natrium epitelium (ENaC) dan saluran kalsium regangan-diaktifkan (SACC), dalam mengawalselia pemerhatian-pemerhatian yang dibuat dalam matlamat 1 dan 2. Untuk mencapai matlamat pertama kajian ini, hMSCs diasingkan, diperkembangkan, dan dikenakan regangan berkitar ekapaksi dengan tegangan 4%, 8% atau 12% pada 0.5 Hz atau 1 Hz untuk 6, 24, 48 atau 72 jam. Sel-sel hMSCs ini akan dibandingkan dengan kultur-kultur hMSC yang tidak ditegang.

Morfologi dan penjajaran sel-sel didokumenkan, manakala kelangsungan hidup dan percambahan sel disiasat dengan menggunakan pewarnaan hidup/mati sel dan asai alamarBlue. Keputusan menunjukkan bahawa sel-sel ditegang muncul diselaraskan semula berserenjang dengan arah pemuatan tegangan, berbeza dengan sel-sel tidak ditegang yang disusun secara rawak. Percambahan sel tertinggi dapat diperhatikan apabila tegangan 4%+1 Hz diaplikasikan ($p < 0.05$). Untuk mendapatkan matlamat 2, pembezaan hMSCs kemudiannya dianalisa dengan sel topografi, pewarnaan immuno, pewarnaan immuno berpendarfluor, asai-asai biokimia dan pengekspresan penanda-penanda gen sel mesenkima. Pada tegangan 8% dan 12% (1 Hz), terdapat peningkatan dalam pengeluaran kolagen I, kolagen III, elastin, fibronektin, dan N-cadherin; tetapi tidak untuk kolagen II, dan glikosaminoglikan. Ekspresi-ekspresi gen-gen tenogenik hanya diekspresikan pada kadar tinggi apabila dikenakan tegangan 8% dan 12% ($p < 0.05$), walaupun tegangan 8% adalah lebih tinggi. Gen-gen penanda osteoblastik, kondrogenik dan adipogenik kelihatan dikawalselia kurang aktif. Akhir sekali, pembezaan tenogenik daripada hMSCs diperiksa dalam kehadiran dan ketiadaan ENaC dan SACC, masing-masing dengan menambah benzamil dan gadolinium. Keputusan-keputusan kajian menunjukkan bahawa dengan membantut kedua-dua saluran mechanosensitif ini, regangan mekanikal akan melambatkan aturan isyarat biokimia dan proses pembezaan tenogenik dengan induksi regangan akan terhenti. Kesimpulannya, pemerhatian-pemerhatian daripada kajian kami mencadangkan bahawa MSCs adalah sensitif terhadap rangsangan mekanikal, khususnya terhadap tindakbalas tenogenesis, ini boleh dikawalselia dengan mengubah pembolehubah-pembolehubah tertentu seperti kepekaan saluran-saluran ion, tempoh regangan, serta kadar dan jumlah tegangan.

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LIST OF SYMBOLS AND ABBREVIATIONS

A_{260}	:	Absorbance of ultraviolet at 260 nm
A_{280}	:	Absorbance of ultraviolet at 280 nm
AB	:	AlamarBlue
AFM	:	Atomic force microscope
ALP	:	Alkaline phosphatase
AM	:	Acetoxymethyl ester
ANOVA	:	Analysis of variance
APC	:	Allophycocyanin
ATP	:	Adenosine triphosphate
BMMSCs	:	Bone marrow derived MSCs
^oC	:	Degree celsius
CCD	:	Charge-coupled device
CD	:	Cluster of differentiation
CO_2	:	Carbon dioxide
DCN	:	Decorin
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
Ca^{2+}	:	Calcium
CFU-F	:	Colony-forming-unit fibroblastic
COL1	:	Collagen type I
COL2	:	Collagen type II
COL3	:	Collagen type III
COMP	:	Cartilage oligomeric matrix protein
CPD	:	Critical point dry

e.g.	:	Exempli gratia (for example)
ECM	:	Extracellular matrix
EDTA	:	Ethylenediaminetetraacetic acid
EGTA	:	Ethylene glycol tetraacetic acid
ENaC	:	Epithelial sodium channel
ERK	:	Extracellular-signal-regulated kinases
EthD-1	:	Ethidium homodimer-1
FACS	:	Fluorescence-activated cell sorting
FAK	:	Focal adhesion kinase
FBS	:	Fetal bovine serum
FITC	:	Fluorescein isothiocyanate
FSC/SSC	:	forward scatter/side scatter
g	:	Gram
g	:	Gravity
G_0/G_1	:	Gap 0/gap 1
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
Gd^{3+}	:	Gadolinium
GDF	:	Growth differentiation factor
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
h	:	Hour
H^+	:	Hydrogen
hBMMSCs	:	Human bone marrow derived MSCs
HBV	:	Hepatitis B virus
HCV	:	Hepatitis C virus
HIV	:	Human immunodeficiency virus
HLA-DR	:	Human Leukocyte Antigen - antigen D Related

Hz	:	Hertz
i.e.	:	Id est (that is)
IGF-1	:	Insulin-like growth factor 1
IL	:	Interleukin
ISCT	:	International Society for Cellular Therapy
JNK	:	c-Jun N-terminal kinase
kD	:	Kilodalton
kV	:	Kilovolt
K^+	:	Potassium
L	:	Litre
LG-DMEM	:	Dulbecco's modified eagle low glucose
log	:	Logarithmic
LSD	:	Least significant difference
m	:	Meter
M	:	Molar
MAPK	:	Mitogen-activated protein kinase
MFI	:	Mean fluorescence intensity
Mg^{2+}	:	Magnesium
MMP3	:	Matrix metallopeptidase 3
mRNA	:	Messenger RNA
MSCs	:	Mesenchymal stromal cells
min	:	Minute
mg	:	Milligram
mL	:	Millilitre
mm	:	Millimeter
mM	:	Millimolar

N	:	Newton
Na^+	:	Sodium
nm	:	Nanometer
OCN	:	Osteocalcin
PBS	:	Phosphate buffered saline
PDL	:	Population doubling level
PE	:	Phycoerythrin
peridinin chlorophyll protein	:	PerCPCY5.5
PGK1	:	Phosphoglycerate kinase 1
PI	:	Propidium iodide
PPAR γ	:	Peroxisome proliferator-activated receptor-gamma
PRR16	:	Proline rich 16
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
ROCK	:	Rho A kinase
ROS	:	Reactive oxygen species
rRNA	:	ribosomal ribonucleic acid
r.p.m.	:	Revolutions per minute
RUNX2	:	Runt-related transcription factor 2
s	:	Second
SACs	:	Stretch-activated ion channels
SACC	:	Stretch-activated calcium channel
SCX	:	Scleraxis
SD	:	Standard deviation
SEK1	:	Stress-activated protein kinase 1
SEM	:	Scanning electron microscopy

sGAG	:	Sulphated glycosaminoglycan
siRNAs	:	Small interfering RNAs
SOX9	:	SRY (sex determining region Y)-box 9
SPSS	:	Statistical package for the social sciences
TGFβ1	:	Transforming growth factor beta 1
TNC	:	Tenascin C
TNFα	:	Tumor necrosis factor alpha
TNMD	:	Tenomodulin
VACC	:	Voltage activated calcium channel
w/v	:	Weight in volume
2D	:	Three-dimensional
2.5D	:	Two-and-a-half-dimensional
3D	:	Two-dimensional
α	:	Alpha
&	:	And
β	:	Beta
γ	:	Gamma
δ	:	Delta
%	:	Percent
µg	:	Microgram
µL	:	Microlitre
µm	:	Micrometer
µM	:	Micromolar
TAGLN	:	Transgelin

LIST OF APPENDICES

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

Tissues in and around the shoulder, knee, and ankle have been shown as areas that are most likely to be injured during trauma (Ress et al., 2006). Injuries to these musculoskeletal soft tissues, especially tendons, present a major clinical challenge to orthopaedic practitioners due to the tissue's low cellularity and highly structured extracellular matrix. It is important to note that damage to tendons stimulate a natural healing response that often fails to provide complete regeneration (Goh et al., 2003; Yang et al., 2013). Moreover, because tendons are tissues specifically designed to sustain tensile loading, healing of the tendon needs to result in the complete restoration of its original architecture in order for the organ to be fully functional. It is nevertheless important to note that leaving injured tendons to rest without loading has been shown to result in tissue atrophy. It therefore suggests that cellular response to mechanical signals may play a key role in tissue healing and repair (Massoud, 2013; Woo et al., 2000). This results in a dilemma which arises between the need to load or to not load a tendon during its reparative stage. Clinically, damaged tendons have been subjected to both conservative and surgical repairs. These reports however are somewhat mixed for partial tendon damage and in fact, there have been studies to suggest that repairing tendon without loading will result in poor healing outcomes (Massoud, 2013; Woo et al., 1982). The use of regenerative medicine as a promising alternative treatment have therefore been suggested since it is postulated that repair using this strategy results in early healing of the damaged tendons thus resulting in the ability to allow early tendon loading. Indeed, such approaches although theoretically sound, need to be further investigated in order to provide clues that can be used to provide an early restoration of damaged tendon. One mechanism that is worthwhile investigating would be the role of mechanical loading on cells; since this has been known to influence tendon healing.

The understanding of how mesenchymal stromal cells (MSCs) respond to mechanical signals now become a major focus since these cells not only provide the matrix needed for tendon repair, but also because they are self-renewing multipotent cells, that are expected to result in long term regenerative outcomes. In major labs worldwide, such endeavours have become a major area of research, providing interest in the field commonly known as mechanobiology (Castillo & Jacobs, 2010; Hao et al., 2015; Lim et al., 2010). While some progress has been made in understanding how mechanical signals are sensed by MSCs, the mechanotransduction processes that occur during tensile loading is still not well understood (Ingber, 2006; Hamill & Martinac, 2001; Na et al., 2008). Further, the effects of tensile loading on cellular behaviour, such as proliferation and differentiation have yet to be elucidated (Kearney et al., 2010; Leong et al., 2012). From what limited knowledge that has been mentioned, it does suggest that extracellular molecules, membrane-bound proteins (eg. integrin, ion channels) and intracellular molecules (eg. cytoskeletal components, focal adhesion complexes) are key players in determining how MSCs sense and transmit mechanical signals, leading to extremely complex mechanotransduction pathway activation (Chiquet et al., 2009). Therefore, a well controlled experiment that elicits the effects of the composition of the extracellular environment on MSCs phenotype; in the presence and absence of tensile stimulation can provide fundamental knowledge needed for rationalize the design of future *ex vivo* systems to promote specific differentiation commitment of MSCs. Such processes result in the alterations in the cell cytoskeleton and stretch-activated ion channels that ultimately results in the tenogenic response in MSCs. Knowledge on mechanotransduction mechanisms will aid in a better understanding of the physiological responses of various tissues, such as the effects of training or disuse of tendons and ligaments (Benjamin et al., 2006; Buchanan & Marsh, 2001; Couppé et al., 2008; Hayashi, 1996).

Using these principles, a series of mechanical settings to apply variable levels of loading characteristics were proposed in this thesis to investigate the influence of mechanical stimuli on cells differentiation; in particular the tenogenic differentiation pathway involved in MSCs. In selecting different strains, frequencies, and duration of stretching on MSCs; the present thesis also aims to ameliorate the understanding of modifiable physical factors that influences MSCs behaviour and expression. The influence of these factors which results in the activity of selected ion channels was also investigated in order to prove that the mechanisms involved in this process are regulated through the transportation of intra- and extra-cellular ions. It is finally our hope that in defining all these parameters, our study may provide a platform for future research to be conducted that can lead to the control of multi-potent cell expression and behaviour, and thus lead to better treatment regime for damaged tendons.

1.2 Study Rationale

Considering that previous studies have not looked into the effects of alternate loading such as cyclic tensile loading combined with ion channels regulations on cell behaviour, a study was conducted to determine the role of mechanobiology in controlling human MSCs during cyclic tensile loading and their extracellular expression as the result of tenogenic transformation. Most previous studies emphasized on chondrocytes and osteocytes, but only few studies were conducted on tenocytes differentiated from human bone marrow derived MSCs (hBMMSCs). To the best of our knowledge, this is the first study to determine the relationship of proliferation and differentiation of hBMMSCs towards tenogenic cells using a combination of these parameters.

The findings of this study have wide implications to both research and clinical areas. The studies detailed in this thesis provide a basis for researchers to further understand how the interactions of mechanical cues affect the proliferation and promotion of a

characteristic tendon fibroblast phenotype in hBMMSCs. In addition, ion channels that provide a connection between the extracellular environment and cell physiology, were investigated in order to explore the influence of cell-mediated signalling on the production of major tenogenic extracellular matrix proteins and differentiation. More interestingly, the translation of these findings into its clinical potential will provide interesting inference such that the repair of tendon is dependent on the nature of the tensile loading such as rate, frequency etc. Results from this study further provides the possibility of using these transformed cells for transplantation, and thus to improve cell function *in vivo* by determining their behaviour *in vitro*. As such, we can therefore suggest that the present thesis contributes to the body of knowledge culminating in the birth of paramount concepts that make MSCs a potential source tenogenesis differentiation by mechanical cues for tendon regeneration.

1.3 Hypotheses

The hypothesis of the research presented in this thesis is that when subjected to various cyclical mechanical loading settings, isolated hBMMSCs may undergo a preferential differentiation process towards tenogenesis and, results in changes in cell behaviour including morphology and proliferation changes. Furthermore the study also examines the mechanism leading to the activation of sodium and calcium signalling, and whether this mechanism involving the differentiation pathways.

To summarize, the hypotheses of the studies carried out for the content of the thesis are as follows:

1) Application of a uniaxial cyclical tensile loading at different strain amounts, frequencies, and duration will modulate the alignment of hBMMSCs, and enhance the proliferation of hBMMSCs.

2) hBMMSCs cultured in standard culture growth medium without applying any biochemical reagent and exposed to uniaxial cyclic tensile loading at different rates and for different durations will modulate the production of extracellular matrix (ECM) and eventually lead to a tenogenesis differentiation.

3) Restriction of sodium and calcium supplies which inhibit epithelial sodium channel (ENaC) and stretch-activated calcium channel (SACC) during the application of cyclical tensile loading will influence the ECM production and tenogenesis differentiation of hBMMSCs.

1.4 Aims and Objectives of The Study

The main aim of this thesis is to determine the effects of uniaxial cyclic tensile loading on the induction of tenogenesis in human MSCs and the role of ion channels. This is achieved through the use of human bone marrow derived MSCs, ion channel inhibitors, continuous stretching system and a unidirectional stimulation unit.

In order to accomplish the main aim of this study, the following specific objectives that were set within the parameters of the studies involved in this thesis were defined as follows:

1) To develop a dynamic tensile loading system results in cellular and phenotypic changes to hBMMSCs.
2) To observe the changes in cell alignment and cell proliferation when subjected to tensile loading at different strains and frequencies
3) To determine the differentiation potential of hBMMSCs by quantifying ECM production and gene expression when subjected to various tensile loading regimens
4) To elucidate the role of ENaC and SACC in the tenogenic differentiation potential of hBMMSCs as the consequence of mechanical stretching

1.5 Study Overview

Chapter 1 begins with an introduction on the background of tendon injuries, and the need of tissue engineering. The basis for the investigating the effects of mechanical stimuli on MSCs for tendon tissue engineering will also be discussed. This chapter concludes with the description of the objectives and hypotheses of the current research. Chapter 2 then provides the literature review that gives knowledge on the need to benchmark in MSCs and related mechanobiology studies. The review continues by exploring the conventional control of cell fate, namely MSCs. This is then followed by a discussion on the potential of mechanical stimulation to direct cell behaviour of MSCs for improving cell therapies. Others factors including ion channels involved in controlling cell fate are also discussed. Chapter 3 contains the results of proven identification characteristics of isolated human primary cell as human MSCs. The aim of this thesis is to determine the effects of mechanical stimuli on hBMMSCs, and is presented in Chapter 4 to Chapter 7. Chapter 4 describes the stretching device system, and the results from mechanical stretching of cells; namely i.e. on the cell morphology, cell alignment and cell proliferation. The effects of tensile loading at different strains and frequencies for different durations, was examined. This chapter describes cell behaviour changes during mechanical stretching as the result of different loading regimes. Chapter 5 includes the study of mechanical stimulation on ECM production and cell differentiation, i.e. the tenogenic differentiation potential of hBMMSCs. This part of study then confirms our expectation as to whether the hBMMSCs can be differentiated by mechanical stimulation, and thus potentially be used in tendon repair or tissue engineering. These two chapters then provide information that the differentiation and proliferation follows different pathways. Next, by manipulating sodium and calcium supplies, we examined whether disturbances in sodium and calcium signalling might impact on the cellular responses to tensile loading. These are described

in Chapters 6 and 7. Here, the chapters implicate the role of ion channels of the cells, ENaC and SACC, with ECM production and tenogenesis differentiation of the cells. Lastly, Chapter 8 contains our conclusions of our studies based on the collective results obtain from the various studies conducted, and probes the implications of this study especially in the potential of inferring the findings to clinical applications.

CHAPTER 2: LITERATURE REVIEW

2.1 Stem cells

Stem cells were first mentioned in the early 1960s, following the results of several experiments which reported the existence of cells that have superior differentiation potential to those of somatic cells. Based on the work by Ernest A. McCulloch and James E. Till, bone marrow cells injected into irradiated mice were found to result in nodules developing in the spleens, proportionate to the number of bone marrow cells injected. From the results observed, they concluded that each nodule arose from a single marrow cell (Becker et al., 1963). Later studies then made it more apparent that stem cells are defined as unspecialized precursor cells which have self-renewal ability even after undergoing extensive proliferation (Choumerianou et al., 2008; Hwang et al., 2008). They are able to do this by maintaining their stemness, and can only undergo terminal differentiation into specialized cell lineages when subjected to the appropriate stimuli (Ding & Schultz, 2004; Morrison et al, 1997). To take advantage of this finding, present day researchers are now more focused on developing technologies that will harness these two properties to expand cells and direct these cells toward forming specific cell types. According to their ability to differentiate, stem cells can be classified in a hierarchical order, as shown in Figure 2.1.

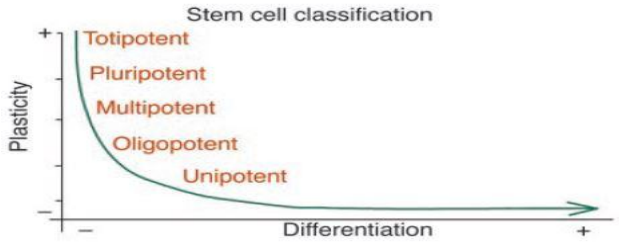

Figure 2.1: Classification of stem cells based on their developmental potential Totipotent suggests that cells are able to differentiate into all embryonic and extra-embryonic cell types; whilst pluripotent indicates ability to give rise to all cell types of the embryo proper (cells derived from any of the three germ layers). Downstream, multipotent refers to cells that are able to give rise to a subset of cell lineages; oligopotent, able to contribute a restricted subset of cell lineages; and unipotent referring to cells that are able to become only one mature cell type. (Reproduced from Trucco, 2005)

Generally, stem cells are classified into three categories: embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPS). ESCs were first isolated via *in vitro* fertilization of pre-implantation blastocysts (Thomson et al., 1998). These cells have high telomerase activity and pluripotent differentiation potential, where they can differentiate into all of the embryonic germ layers. ESCs help to replenish differentiated cells that cannot proliferate by themselves. As embryos grow, ESCs give rise to multipotent cells that can be isolated from a variety of adult tissues (Hwang et al, 2008), also known as ASCs. However, their potential to differentiate into different cell types appears to be more limited than the other stem cell types (Lee & Hui, 2006).

The first identification of ASCs was in the early 1900s, which are now known as adult hematopoietic stem cells, are mainly found in the blood. Over the years, ASCs have also been discovered in bone marrow (Brehm et al., 2002). These cells are easier to obtain than ESCs and have the potential to provide tissue replacement or regeneration (Lee & Hui, 2006). iPS can be obtained by genetically reprogramming somatic differentiated cells into a dedifferentiated state, and thus have similarity to ESCs capability (Yu et al., 2007). This method is to gain autologous pluripotent stem cells sourced not only from embryonic origins, but also from adult tissues. However, the use of ESCs and iPS cells may pose to be a greater challenge than using ASCs as there may be ethical, legal and political concerns, not to mention the critical safety issues (eg. inserting oncogenes and insertional mutagenesis) that first need to be addressed (Lo & Parham, 2009; Zhong et al., 2011). Therefore, the use of ASCs has been widely encouraged as it has the advantage of avoiding possible immunologic responses that occurs when performing allogenic cell transplantations (Bongso et al., 2008). As there are potential benefits to be gained from researching these cells, stem cells have been

researched very extensively as preferred cells for potential clinical applications by many researchers (Choumerianou et al., 2008).

2.1.1 Mesenchymal stromal cells

In contrast to hematopoietic stem cells, cultured mesenchymal stromal cells can be identified based on their ability to adhere to plastic, which means they can be easily cultured *in vitro*. Mesenchymal stem cells or mesenchymal stromal cells (MSCs) (also called as bone marrow stromal stem cells, multipotent adult progenitor cells, mesenchymal adult stem cells, or tissue stem cells) are adult stem cells and have been widely described in literature. According to Taylor et al (2006), the word 'stroma' is defined as 'anything spread or laid out for sitting on'. These cells can be isolated from bone marrow (Pittenger et al., 1999), adipose tissue (Cao et al., 2005), peripheral blood (Zvaifler et al., 2000), umbilical cord blood (Huang et al., 2007), amniotic fluid (Kim et al., 2007), and even synovial membrane (Harvanová et al., 2011). However, bone marrow is considered the original and most significant source of MSCs. There is a subpopulation of multipotent cells in bone marrow stroma to generate mesenchymal tissue, which develops from the mesodermal layer of the embryo. Hence, this subpopulation of cells is termed mesenchymal stem cells (Caplan, 1991).

Non-hematopoietic stem cells in the bone marrow were first described by Cohnheim 150 years ago, suggesting that bone marrow is also a source of fibroblasts that contribute to the process of bone healing and wound repair (Prockop, 1997). Bone marrow derived stem cells were first isolated by Friedenstein and his team (Friedensteinet al., 1968). They incubated bone marrow in plastic culture dishes and removed non-adherent cells after 4 hours. They found that adherent cells became spindle-shaped and began to multiply rapidly. The differentiation of the cells into

colonies resembling deposits of bone or cartilage was also successfully achieved by the team (Friedenstein et al., 1987).

2.1.2 Therapeutic potential of MSCs

Due to their multipotent differentiation capacity, there has been a great interest in utilizing these cells for cell therapy and tissue engineering (Chanda et al., 2010; Kim & Cho, 2013; Rosenbaum et al., 2008). Many studies have already revealed the capacity of MSCs to migrate and be engrafted into organs (Chamberlain et al., 2007; Baksh et al., 2004; Bianco et al., 2001). In a previous study, intra-injection of autologous MSCs was shown to result in the repair of the damaged cartilage in caprine model (Nam et al., 2013). MSCs may also be effective in the repair of bone, tendon, cardiovascular systems, lungs, and spinal cord injuries (Awad et al., 1999; Barry & Murphy, 2004). However, care must be taken in the interpretation of these results to ensure that tissue repair is mediated by MSC differentiation and not through cell fusion (Terada et al., 2002).

Despite the clear evidences of MSCs have the potential to generate tissue regeneration, the exact mechanism involved is still poorly understood. Furthermore, the existence of a mixed population in MSCs has been shown to result in a loss of viability after being transplanted into tissues. Rodrigues et al (2010) have speculated that such repair outcomes may be attributed to other factors such as the release of cytokines, interaction with the extracellular matrix, ischemia, or other causes. There are also growing concerns as to the development of unwanted lineage drive of these cells such as those resulting in *in situ* ectopic bone formation in soft tissues (Harris et al., 2004). To overcome such problems, a number of tissue engineering approaches are presently being developed to induce differentiation in a specific lineage, for example, tenogenic

differentiation, prior to its use in patient transplantation. This will be discussed further in Chapter 5.

Another problem associated with the use of MSCs in therapeutic applications is that they represent only a small fraction of the cells present in bone marrow, which will also be further discussed in Chapter 4. Whilst these cells can be propagated *in vitro*, two significant concerns exist. First, *in vitro* expansion is associated with a decrease in proliferation and differentiation capacity (Bonab et al., 2006; Chamberlain et al., 2007). Second, the use of foetal bovine serum to expand cell populations *in vitro* may elicit an immune response when cells are transplanted *in vivo* (Tasso et al., 2009; Taylor et al., 2006). Thus, identification and thorough understanding of factors which regulate the behaviour of these cells will be vital for the successful use of MSCs in therapeutic practices. Common factors that direct MSCs proliferation and differentiation are described in the following subsections.

2.1.3 MSCs differentiation capacity to mesenchymal lineages

As tissue engineering technology develops, there are some new methods to successfully provide tissue replacements. Stem cells are effective due to their ability to undergo differentiation (Moshiri et al., 2013). It has been found that the higher the differentiated state, the more effective the cells are in promoting the healing process. *In vitro* differentiation of MSCs into tissue progenitors prior to transplantation is thus a possible approach. This is to evade the problems of ectopic bone and tumour formation during tissue repair (Lui et al., 2011). Current strategies to direct MSC differentiation include mechanical stimulation, topography of extracellular matrix (ECM), growth and differentiation factors, gene transfection, and co-culture with specific tissues or cells (Figure 2.2). These are generally divided into the effects of physical treatments

(biomechanics and topography) and biochemical factors. In this literature review, current physical strategies will be the focus.

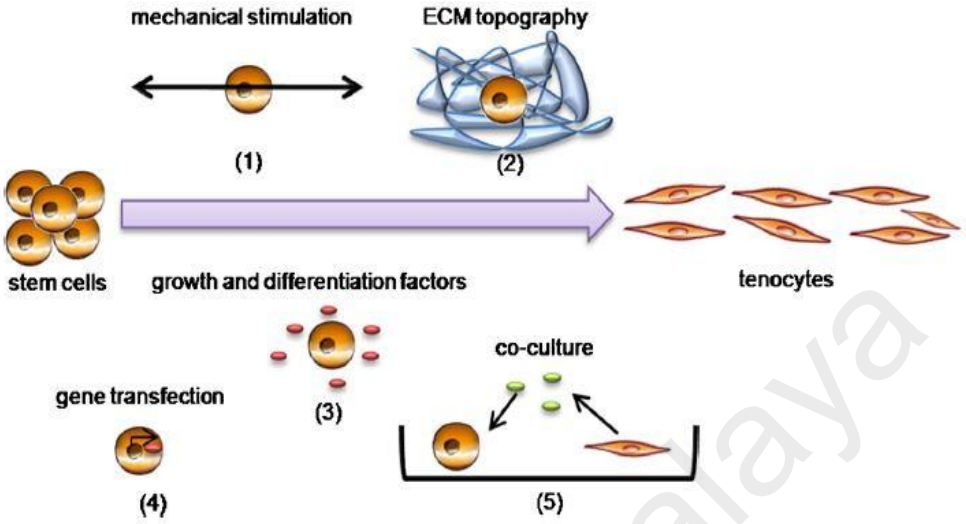

Figure 2.2: Current strategies to direct differentiation of stem cells into tenocyte lineage (1) mechanical stimulation; (2) topography of ECM; (3) growth and differentiation factors; (4) gene transfection; (5) co-culture with tendon tissues or cells. (Reproduced from Chen et al., 2014)

2.1.3.1 Biological and biochemical factors

Cytokines and growth factors are signalling molecules that play an important role during tissue development, homeostasis and repair. For example, during tendon repair, tendon cells will express some factors that activate specific cell signalling pathways, and lead to transcription of the regulatory genes of resident progenitor cells (Ho et al., 2014). The appropriate cells will be stimulated to migrate and proliferate, synthesize collagen, matrix components, or promote angiogenesis as part of the reparative phase in tendon repair (James et al., 2008). This concept was taken to the laboratory by determining the biological significance of growth factors in regenerating tendons *in vitro* and *in vivo* (Chen et al., 2008a; Ho et al., 2014; James et al., 2008; Scott et al., 2007).

In order to induce specific phenotype expression and cell differentiation *in vitro*, various cytokines or growth factors can be added to the culture medium. Differentiating

MSCs in this manner is referred to as chemical stimulation (biochemical cues), where occurred by the reciprocal interactions between the cell, soluble bioactive agents (eg. growth factors, morphogenetic factors, enzymes, cytokines), and ECM (Nava et al., 2012). A number of studies have postulated that tenogenesis differentiation of MSCs can be induced with growth differentiation factor (GDF) family members such as GDF-5 (BMP-14), GDF-6 (BMP-13), and GDF-7 (BMP-12) (Chai et al., 2013; Park et al., 2010; Violini et al., 2009), connective tissue growth factor (CTGF) (Lee et al., 2006), and platelet lysate (Zhang & Wang, 2010b). Besides this, osteogenesis can be induced by treating MSCs with dexamethasone or BMP-2 (Karageorgiou et al., 2004; Ma et al., 2013); adipogenesis of MSCs by using 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin (Fink & Zachar, 2011); while chondrogenic differentiation of MSCs can be prepared by adding transforming growth factor-β3 to serum free medium (Bosnakovski et al., 2006).

In order to obtain the right mixture of growth factors and other chemicals, an in-depth understanding of the molecular aspects of the different phases of tendon formation will be required (Bagnaninchi et al., 2007). It is worth noting that the growth factors and other chemicals provide different roles depending on the dosing and time of exposure. Although the effect of biochemical cues has been widely studied *in vitro*, the regulation of self-renewal and lineage commitments of MSCs, however, is still poorly understood and difficult to mimic. In living systems, the spatial and temporal organization of ECM adhesive ligands *in vivo* is finely tuned, and this seems difficult to be achieved through engineering methods (Guilak et al., 2009; Kjaer, 2004).

2.1.3.2 Directed differentiation using biophysical cues

Besides biochemical factors, mechanical factors actually have the potential to control MSC fate, at least in *in vitro*. Resembling biochemical cues, physical cues also act in

many forms, including dynamic or static deformations of the ECM, matrix elasticity, surface topography of the biomaterial scaffold, and from external forces applied to cells (Figure 2.3). Mechanical forces act on humans at different levels in different parts of body. Cells and tissues in their innate environment are exposed to mechanical loads (eg. tensile, compressive, and shear forces) to influence their development and natural function (Mammoto & Ingber, 2010). Hence, mechanical stimulation is suggested to be a more appropriate method for cell differentiation. For example, ligament and tendon fibroblasts are continuously exposed to mechanical loading under normal physiological conditions (Benjamin et al., 2006). Thus, for functional tendon tissue engineering to occur, chemical stimulation may not be sufficient, since it is clear from biological models that mechanical stimuli are required to improve the effect of tendon repair.

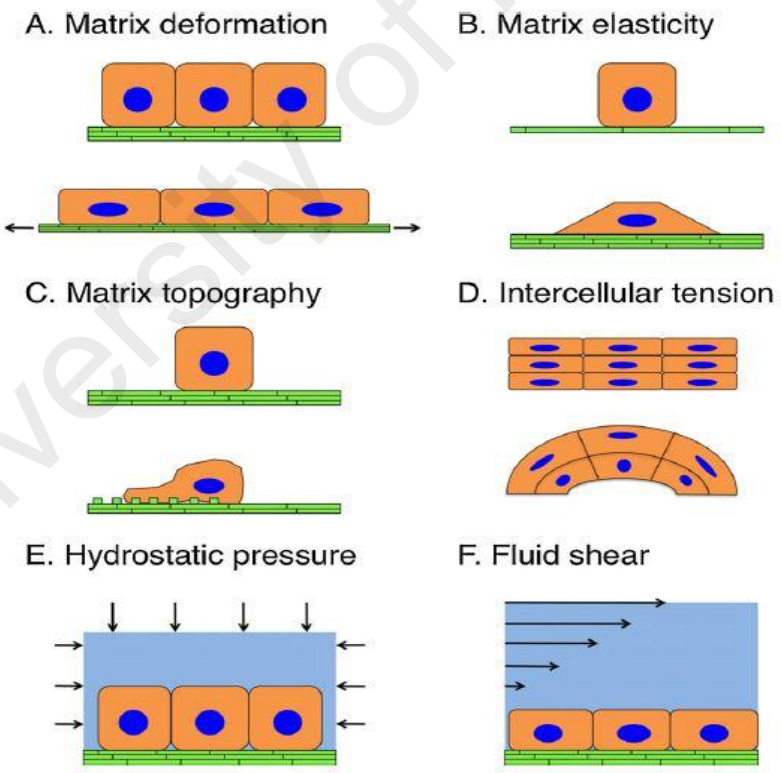

Figure 2.3: Types of biophysical stimuli experienced by cells
Biophysical cues and mechanical forces can be applied on stem cells in different ways. (A) Static or dynamic deformations of the ECM can manipulate attached cells. (B-C) The changes of matrix stiffness or topography can be sensed by the cells. (D) Movement and deformation of multi-cellular structures exerts forces through intercellular adhesion. (E-F) Cells surrounded by blood or interstitial fluids experience hydrostatic pressures and shear stress. (Reproduced from Costa et al., 2012)

2.2 Tendon disease and cell-based therapies

2.2.1 The mechanobiology of tendons

Tendons are dense, regularly-arranged soft connective tissues. Tendons connect muscle to bone, transmitting tensile loads generated by muscles to move and improve joints stability. In the article by Kuo et al (2010), adult tendons have relatively low oxygen and nutrient requirements, low cell density, and poor regenerative capacity. Tendon possesses a structural hierarchy consisting of fascicles, fibrils, subfibrils, microfibrils and tropocollagen, which is designed to resist tensile loads (Figure 2.4).

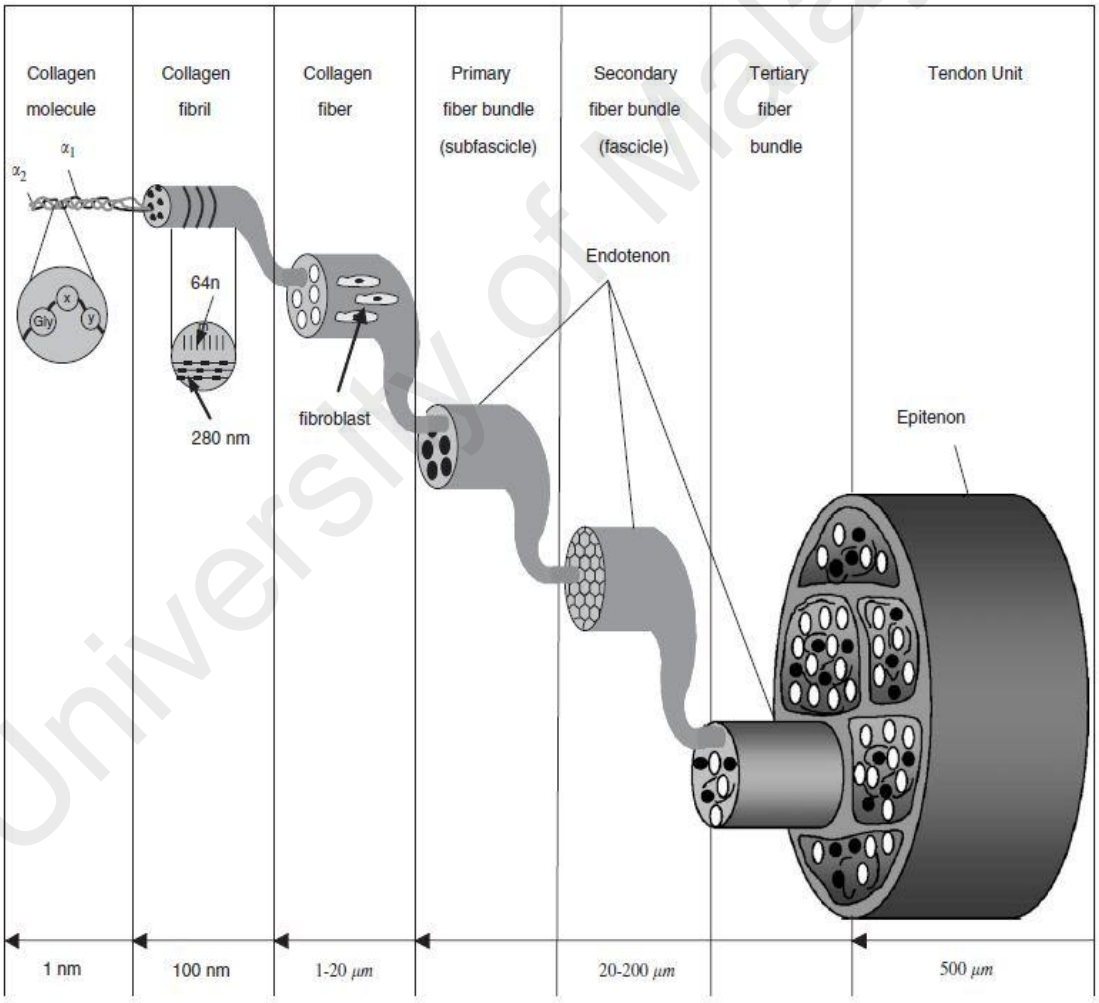

Figure 2.4: A schematic of a multi-unit hierarchical structure of the tendon The tendon is composed of collagen molecules, fibrils, fiber bundles, fascicles and tendon units that in parallel alignment with the tendon's long axis. (Reproduced from Wang, 2006)

Tendons comprise of collagens, proteoglycans, glycoproteins, water and cells. The composition of tendon is described in Table 2.1. Tenocytes and tenoblasts are the two main cell types that coexist in tendon, have a mesenchymal origin. Tenoblasts are immature tendon cells with a spindle shape, which have various cytoplasmic organelles which reflect their metabolic activity than mature cells. Tenoblasts mature into tenocytes which have a fibroblastic morphology, and a much lower nucleus-to-cytoplasm ratio, thus reflecting lower metabolic activity (Hampson et al., 2008; Kannus, 2000). Tenocytes, which are terminally differentiated cells, have a very limited proliferative capacity (Chuen et al., 2004), lead to a relatively low tissue turnover rate. This may explain why tendons seem to possess a poor capacity for natural healing (Louie et al., 1998). In pathological tendon tissue, inflammatory cells, macrophages and non-resident fibroblasts might also be present (Rodrigues et al., 2013).

Table 2.1: Structural composition of tendon (Hampson et al., 2008; Wang, 2006)

Composition	Amount	Site	Function/Remarks
Collagens			
Type I	60% of dry mass of the tendon, 95% of the total collagen.	Self-assemble into highly organised fibrils which form collagen fibres.	Conferring them a high tensile strength and providing mechanical strength to the tendon tissue.
Type III and V	5% of the total collagen.	Type III is mainly located in the endotenon and epitenon.	May results in decreased mechanical strength.
		Type V is intercalated into the core of type I collagen fibrils.	Regulate fibril growth.
Type II, VI, IX, X, and XI	Trace quantities in tendon.	Mainly found at the bone insertion site of fibrocartilage.	Strengthen the connection by reducing stress concentration at the hard tissue interface.

Table 2.1, continued

Composition	Amount	Site	Function/Remarks
Proteoglycans	Small quantities, varies with the site of the tendon and depends on the mechanical loading to which the tendon is exposed.		To enhance the mechanically properties of the tissue.
Aggrecan		Is located on the surface located on the surface of the middle portions of collagen fibrils.	To retain water within the fibrocartilage and resists compression.
Decorin			To facilitate fibrillary slippage during mechanical deformation.
Biglycan, fibromodulin, lumican, versican			Influence fibril diameter, cellular proliferation and migration, and the viscoelasticity of the pericellular matrix.
Glycoproteins		In the ECM of the tendon.	
Tenascin-C		Almost exclusively at the sites subjected to heavy mechanical forces or requiring elastic properties.	Contributes to the mechanical stability of the ECM through its interaction with collagen fibrils.
Fibronectin		Is located on the surface of collagens.	To facilitate wound healing, and play role in cell attachment to prevent cell removal due to gliding friction during tendon movement.
Elastin and microfibrillar proteins	2% of the dry weight of the tendon.	Comprise elastin and microfibrillar proteins.	Contribute to the recovery of the crimp configuration of the collagen fibers after stretching.

Table 2.1, continued

Composition	Amount	Site	Function/Remarks
Cells			
Tenoblasts and tenocytes	90-95% of the cellular elements of the tendon.	Align in rows between collagen fiber bundles.	Synthesizing ECM proteins, producing an organized collagen matrix, and remodelling it during tendon healing.
Endothelial cells, synovial cells and chondrocytes	Remaining 5-10%..		
Myofibroblast-like, contractile cells			Involve in the modulation of the contraction-relaxation of the muscle-tendon complex.

Since tendons play a predominant role in transferring the pull of muscle contraction to the bone, they need to cope with different mechanical strains. The mechanical loads are then transferred to tendon cells through different matrix components and compartments. This mechanical load bearing function is adapted by the tendon-bone junction, known as enthesis (Benjamin et al., 2006). The unique structure of the enthesis has to withstand high stress concentrations (McGonagle et al., 2003). McGonagle et al (2003) have described that these forces may lead to the formation of fibrocartilage at the tendon-bone junction, and thus due to the accumulation of proteoglycans in tendon enthesis, is implicated in enthesopathy. Another junction, called the myotendinous junction, transfers muscular forces to the tendon and enhances muscle growth. At this junction, the collagen fibrils allow tensile forces generated by actin and myosin to be transmitted to collagen fibers (Docheva et al., 2015). However, the myotendinous junction is the weakest point of the muscle-tendon unit (Wang, 2006).

Since the tissue is subjected to varying levels of mechanical loading, it does suggest that mechanobiological may influence the cell behaviour. An understanding of the

mechanobiological phenomena in tissue and cells is vital for the maintenance of tissue homeostasis. Understanding the mechanobiologic factors of how this tissue is shaped by mechanical factors, will no doubt lead to improving healing strategies and surgical interventions.

2.2.2 Disuse and overuse effects on tendons

In vivo, tendons are exposed to numerous types of strain due to everyday movement. The effect of disuse and immobilization of tendons is much slower and less dramatic than of skeletal muscles, due to their slower metabolism and vascularity (Maffulli & King, 1992). Woo et al (1982) stated that immobilization decreases the total weight of the tendon, and eventually reduces its stiffness and tensile strength.

While physiologic loads are required to maintain tendon homeostasis, abnormal loading can lead to tendon injury. Tendon overuse injury, commonly referred to as tendinopathy (Khan et al., 2002), involves repetitive stretching of a tendon and results in the inability of the tendon to endure further tension. Excessive mechanical loading causes tendon inflammation, and thus contributes to the development of tendon degeneration (Wang, 2006). Therefore, knowledge of the mechanobiology of tendon cells can help to understand the pathophysiology of tendon disease, as well as rehabilitation during tendon loading.

2.2.3 Current treatments

To date, Achilles and patellar tendons are the most commonly injured tendons, ranging from calcifying tendinopathy, partial tears, and finally to complete ruptures (Järvinen et al., 2005). Treatment of tendon injuries is challenging. Impaired tendon healing results in long-term pain and discomfort. Although spontaneous healing can occur, this often causes the formation of scar tissue, which affects the functionality of the repaired tissue, its movement and its strength (Sharma & Maffulli, 2005).

There are currently two methods for the management of tendon injury: either conservative (rehabilitation, pain relief) or surgical (percutaneous or open techniques, tendon grafts) (Hampson et al., 2008). Regardless of which of these routes is followed, it may not result in a full gain of function. Conservative management results in prolonged treatment times, possible weakness in the affected area, and also recurrent injury (Bullough et al., 2008). The surgical option is considered only for serious injuries. However, this option has numerous drawbacks. Artificial implants not only have a finite lifetime, their mechanical properties will also degrade over time, and yet increase inflammatory responses, antigenic reactions, and lack of long-term biocompatibility (Beris et al., 2003).

In the last decade, tissue engineering has been introduced to manage tendon and ligament injuries (Moshiri et al., 2013; Shearn et al., 2011). Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes, by affecting the structure and architecture of any viable and nonviable tissue with the aim to increase the effectiveness of the construct in biologic environments" (Moshiri & Oryan, 2012). Basically, tissue engineering is categorized into tissue scaffolds, healing promotive factors, stem cells and gene therapy (Moshiri & Oryan, 2012).

Cells are an important factor in wound healing (Nam et al., 2013). Nowadays, the potential sources of human cells for therapeutic repair are autologous or allogenic tenocytes, and autologous or allogenic adult stem cells (Bullough et al., 2008). There are some issues with the use of autologous or allogenic tendon cells, including donor site morbidity, and the low number of cells obtained from the explanted tissue. There is a decrease in tenocyte density with increase in passage number, and also dedifferentiation and loss of their characteristic tenocyte morphology (Yao et al., 2006).

As mentioned in our earlier section, adult stem cells especially MSCs have been shown to be capable to differentiate into tendon cells and form tendon tissue. The tenogenic MSCs lineage is one of the MSCs lineages that have been of great interest to orthopaedic surgeons, especially for cell based therapy for tendon disorders. However, to date, tenogenic lineage differentiation has not been comprehensively discussed especially biomechanical cues on MSCs, compared to other lineage transition such as ostegenesis and chondrogenesis. More research is required in this field in order to obtain the optimal conditions for the *in vitro* expansion and differentiation of MSCs.

2.3 Biophysical regulation of cell behaviour

Cells that play a role in response the human body when receiving transmitted mechanical forces are called mechanosensitive cells. Examples of such cells include tenocytes in tendons, fibroblasts in ligaments and skin, chondrocytes in articular cartilage, osteocytes in bone, and endothelial cells in blood vessels (Wang & Li, 2010). Mechanical forces have been shown to influence various cellular functions, including cell attachment, spreading, proliferation, migration, differentiation and secretion of ECM proteins (Guilak et al., 2009; Wang & Thampatty, 2008).

2.3.1 Types of mechanical stimuli for cell responses

According to Nava et al (2012), it is actually difficult to determine the biomechanical and biochemical mechanisms *in vivo* that are somehow linked to the cytoskeletal conformational changes that occur. Therefore, biomimetic mechanical stimulation (*in vitro* model systems) has been used to investigate these mechanisms *in vitro* (Wang & Thampatty, 2008) (Figure 2.5), and in general the cellular response appears to match the type of forces experienced *in vivo* (Engler et al., 2006). Mechanical forces can be divided into 3 main groups: compressive, shear and tensile, either in static or dynamic mode. In terms of duration of load application, they can be grouped into interrupted,

intermittent and continuous. Dynamic mechanical force plays important role in the growth and maintenance of functional connective tissues, as they are needed for full differentiation and committal of MSCs within mature tissues (Arnsdorf et al., 2009; Huang et al., 2004; Potier et al., 2010).

It has even been observed that cyclic compression or hydrostatic pressure is more potent in directing chondrogenic lineage differentiation of human MSCs (Henrionnet et al., 2012; Huang et al., 2004). A physiological shear flow on MSCs induces the expression of endothelial cell and smooth muscle cell markers (O'Cearbhaill et al., 2008). It has been also reported that intermittent fluid flow induced shear stress can promote osteogenic differentiation of MSCs (Grellier et al., 2009; Riddle et al., 2006). Tensile force may be uniaxial or equibiaxial. The material is stretched at one direction in uniaxial tensile force; while in equibiaxial load, the material is stretched at all directions (Liu et al., 2012). Compared to equibiaxial stretching applied on dermal fibroblasts, uniaxial stretching is more appropriate for mechanical studies on cells originating from tendons and ligaments, as these cells are aligned with their long axis parallel to the tendon or ligament *in vivo* (Wang, 2006; Yang et al., 2004). Mechanical strain was reported in few studies that can trigger ECM synthesis from cells (Chen et al., 2009; Deng et al., 2009; Lui et al., 2011). Furthermore, mechanical stimulation has been shown to improve cell culture models for gene delivery and toxicology assessment (Harris & Giorgio, 2005; Huh et al., 2010). The MAP kinase pathway was found to be up-regulated in MSCs exposed to cyclic tensile strain, suggesting it as an important mechanotransductive pathway in MSCs differentiation (Ward et al., 2007). Another study of Kearney et al (2010) found ERK and p38 to be involved in cyclic tension mechanotransduction, and stretch-activated cation channels are implicated to mediate collagen I gene expression.

Based on the literature review above, it is clearly demonstrates the positive effects of mechanical stimulation on cell phenotype maintenance, stem cell differentiation and cellular drug/gene delivery uptake. In order to develop tenogenesis of MSCs, the system of cyclic tensile stretching on MSCs responses will be used and determined in our study.

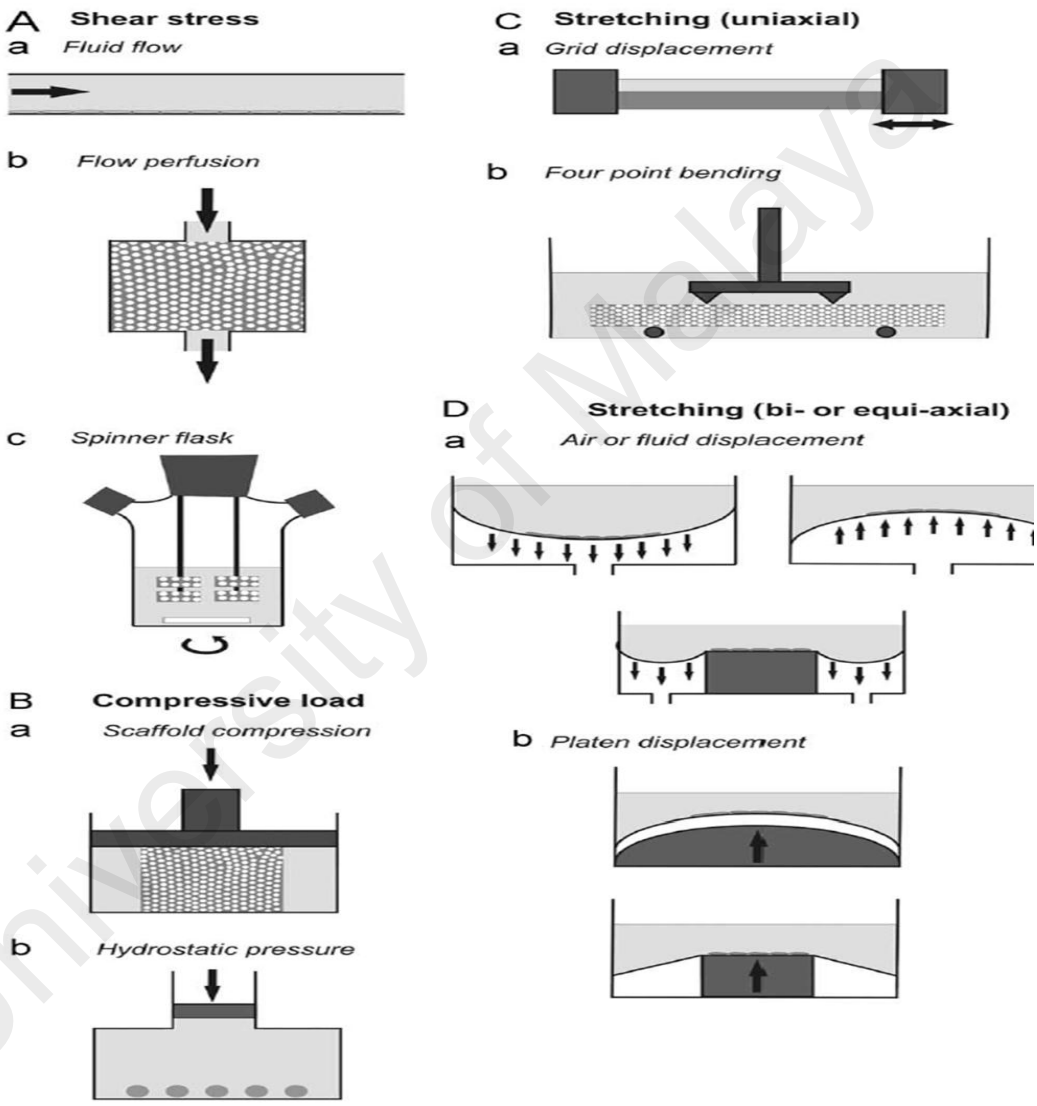

Figure 2.5: Techniques for mechanical stimulation of cells *in vitro*
(A) Fluid-induced shear stresses. (B) Compressive loads. (C) Scaffold stretching (uniaxial). (D) Scaffold stretching (biaxial or equiaxial). (Reproduced from Potier et al., 2010)

The common basic stretching devices involve pneumatically or electromechanically deformed membranes on which cells are seeded on a two-dimensional (2D) surface (Figure 2.6), considered as a purely 2D environment; similar to that used in this study. There are some studies relating to mechanical loading used altered substrate anisotropy. Theses have included topographical features such as pillars, posts, microgrooves, and fibers. These are generally referred to two-and-a-half-dimensional (2.5D) environments (English et al., 2015; Subramony et al., 2014). There have been very few studies that involve limited three-dimensional (3D) (Burk et al., 2016; Qin et al., 2015). It is suggested however, that stretching can be achieved 3D cell-scaffold constructs is accomplished via pneumatic deformation or clamping mechanism (Figure 2.7). It needs to be noted though that a 3D environment is developed by mimicking *in vivo* physiology structure, and thus more complicated. In 2D and 2.5D studies, parameters were studied including the nature of the stretch applied (frequency rate, strain magnitude, uniaxial vs. equiaxial, rest period, etc.), correlation between cell orientation and stretch direction, construct surface topography (pattern, alignment, height, depth, and width of pillars, microgroove or nanogroove), and cell-substrate interaction affect cellular outcomes under stretch (Kurpinski et al., 2006; Wang et al., 2005). In contrast, 3D stretching has been focusing on the engineering of functional tissues (eg cardiac tissue, ligament and tendon, smooth muscle), through stretching cells embedded in 3D scaffolds or constructs (Benhardt & Cosgriff-Hernandez, 2009; Garvin et al., 2003; Liao et al., 2004; Qin et al., 2015; Shimko & Claycomb, 2008). Though very little is known with regards to the difference in cell behavior for 2D versus 3D stretching environment, it is speculated that stretching cells on 3D environment may induce potentially more biomimetic effects than the other two (Nieponice et al., 2007; Zimmermann et al., 2002). It is further suggested that stretching contractile tissue constructs promotes a higher degree of differentiation, greater functionality, and more physiologically relevant

structures (Riehl et al., 2012). However, variations exist in the sophistication of 3D stretching design, including the disintegration of ECM into the construct, and the complication of diffusion rates of soluble factors and nutrients through the matrix (Riehl et al., 2012). A number of 3D stretch-based tissue engineering studies have been reported (Altman et al., 2002; Diederichs et al., 2010; Guan et al., 2011). In addition, the stretching cells in 3D environement increases the difficulty in strain profile calculation (Gladilin et al., 2007; Marquez et al., 2005). Many measurement methods developed for 2D culture can be applied to 3D, but at times the 2D measurements are more appropriate. Thus, it is apparent that the results of 2D stretching may be relevant and applicable when using to translate the results into real live situations that occur in tendons.

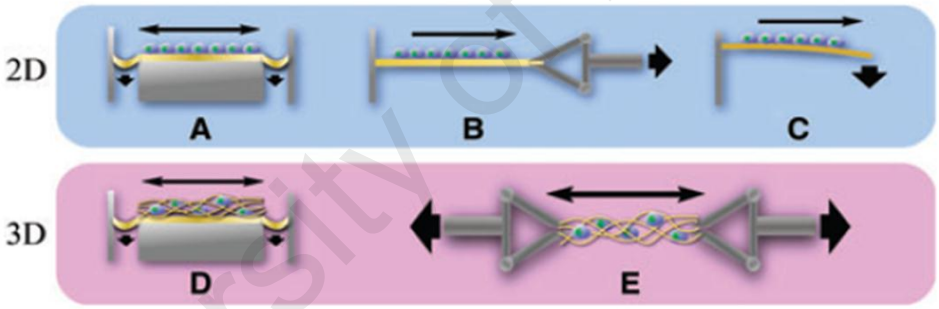

Figure 2.6: Schematic of 2D and 3D stretching devices
Stretching of 2D environment can be obtained via deformation of the membrane (A), clamp arm mechanism (B), and bending (C), while stretching of 3D environment constructs can be achieved via pneumatic deformation (D) and clamping mechanism (E). (Reproduced from Riehl et al., 2012)

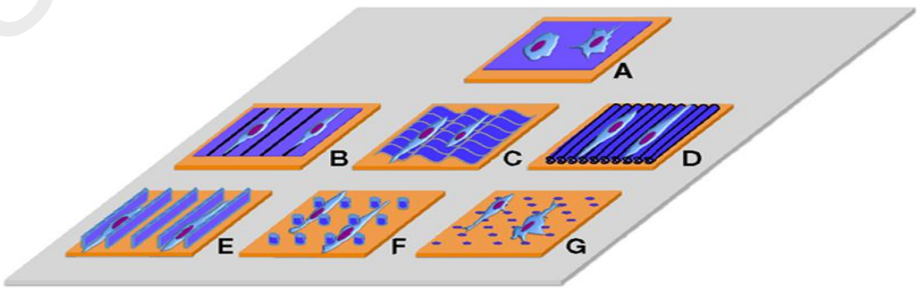

Figure 2.7: Various topography surfaces for 2.5D environment
Traditional surfaces used to culture cells on (A) Flat surface, (B) contact guidance lines, (C) grooves, (D) aligned fibers, (E) 2D confined environments, (F) nano/micro pillars, and (G) nano / micro pits. (Reproduced from Ballester-Beltran et al., 2015)

2.3.2 How do MSCs sense force?

MSCs actively sense and react to their microenvironment surroundings (Kress et al., 2012). Although cells in different tissues detect load in a similar fashion, the outcome however is depending on the cell type and the mechanical loads of the tissue (Banes et al., 1995). Understanding how MSCs sense and respond to these signals is vital in this research area. Both intrinsic and extrinsic mechanical signals are key regulators of MSCs differentiation. In an article by Steward & Kelly (2015), it is said that MSCs have the ability to transmit the 'outside-in' signals to the nucleus via the actin microfilament network and subsequently alter gene expression and protein activity. This response may alter the surrounding matrix of the cell, including integrins at focal adhesions, microcilia and mechanosensitive ion channels (Cigognini et al., 2013), and therefore triggering 'inside-out' signals (Figure 2.8). This "outside-inside-out" signalling in mechanotransduction mechanism will be reviewed in more detail in the following section.

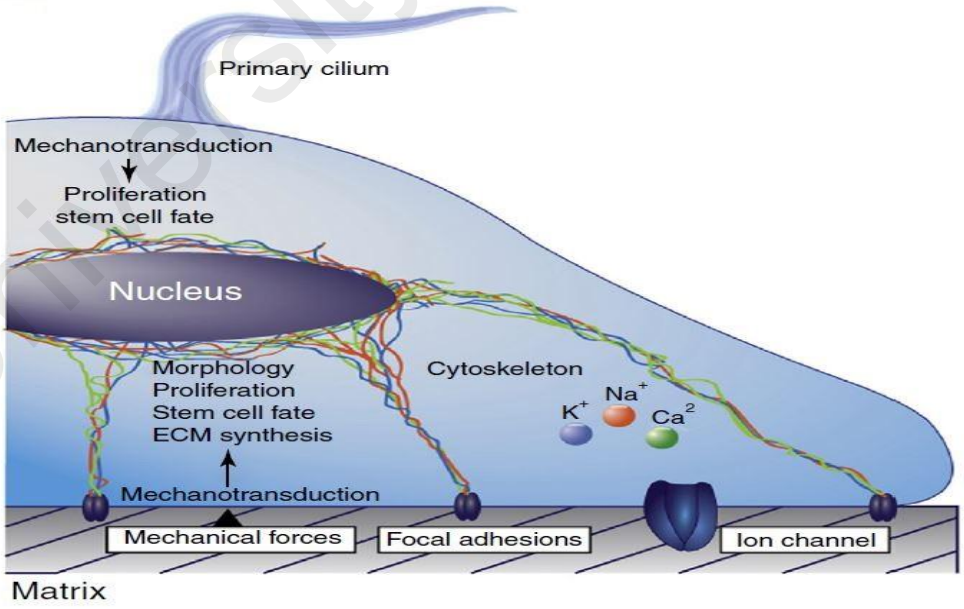

Figure 2.8: The mechanisms of the cell react to mechanical stimuli
Mechanical forces act via integrin receptor proteins, mechanosensitive ion channels or primary cilium, mediating mechanotransduction. Forces are then applied to the nucleus through intracellular mechanisms (e.g. actin filaments or chemocascades), activating protein transcription. Physical external forces are translated via chemistry to a biological response. (Reproduced from Cigognini et al., 2013)

2.4 Molecular mechanisms of mechanotransduction and ion channels modulation

When cells perform their various functions, they respond to signals that regulate internal maintenance. This may involve the activation of pre-existing molecules, the expression of genes and production of proteins, and also the conversion of energy from one form to another (MacQueen et al., 2013). There are some efforts in recent years on investigating mechanobiological response of stem cells. Still, there is limited understanding of how MSCs sense these mechanical signals and translate them into the cell cellular response. Advances in understanding of this mechanism which leads MSCs to associate with the dynamic cellular microenvironment for their renewal, differentiation, and assembly in native tissues need to be reconstructed.

2.4.1 Mechanosensing and mechanotransduction

Mechanobiology is defined as the study of mechanisms by where the cells detect and respond to mechanical stimuli (Lee et al., 2011). How MSCs sense external forces has been described in an earlier section. When MSCs detect mechanical forces, the forces are converted into chemical signals inside the cell to provoke a cascade of cellular and molecular events (MacQueen et al., 2013). Such a process is termed cellular mechanotransduction (Lee et al., 2011). Cells that are able to sense the mechanical signals are described as being mechanosensitive, such as tendon cells. All eukaryotic cells basically are capable of being mechanosensitive (Hampson et al., 2008).

Living cells and tissues were found to use a form of architecture called tensegrity, to organize and mechanically stablize thier cytoskeleton (Ingber, 1997). Generally, external mechanical forces acting on ECM are transmitted into the cell through integrin-mediated adhesions (Harburger & Calderwood, 2009). Integrins contain both a large ECM domain (for binding substrates) and a cytoplasmic domain (MacQueen et al.,

2013). Integrins are the main adhesive receptors and mechanotransducers that link the cytoskeleton to the ECM, presented in Figure 2.9. They are non-covalently linked, heterodimeric molecules containing an α and a β subunit, each of which is a single-pass type I transmembrane protein (Humphries et al., 2006). There are comprising at least 18 α and 8 β subunits in mammals (Takada et al., 2007). To date, approximately 24 integrin homologues have been identified in human cells, each having specific binding affinities for various types of collagen, fibronectin, laminin, vitronectin and other ECM proteins (Docheva et al., 2007). Through contractility-based mechanisms, integrins with other transmembrane molecules, move into the membrane, and then form focal adhesion sites that responsible cell-substrate adhesion (Giancotti & Ruoslahti, 1999). Focal adhesions bind the ECM to provide physical links to cell cytoskeleton, and assemble in response to substrate properties such as rigidity and topography (MacQueen et al., 2013). Therefore, in a tensegrity model, the ECM-integrin-cytoskeleton pathway plays a major role in the mechanosignalling process, where also were indicated in many *in vitro* studies (Bacabac et al., 2010; Chiquet et al., 2009; Hughes-Fulford & Boonstra, 2010; Rubin et al., 2006).

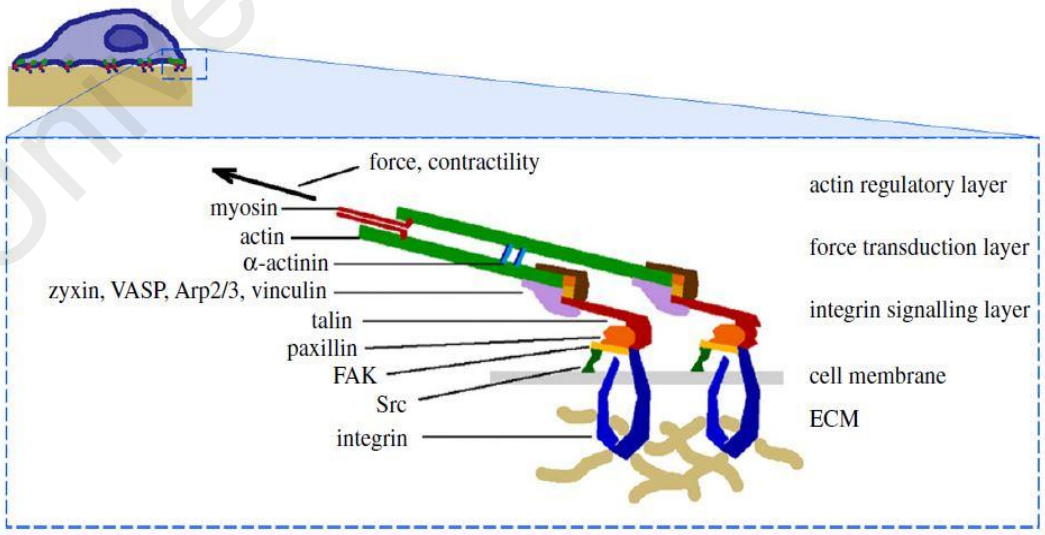

Figure 2.9: Schematic of the integrin focal adhesion complex
The molecules link the ECM with the cell's internal cytoskeleton. (Reproduced from MacQueen et al., 2013)

The interconnected cytoskeleton is composed of microtubules (i.e. α, β-tubulin), intermediate filaments (e.g. vimentin), and microfilaments (e.g. actin) (Geiger et al 2009). Microtubules, approximately 25 nm in diameter, have hollow cylindrical structures, and functions including intracellular transport, the axonal movement of cilia, and mitotic spindle formation. Intermediate filaments, approximately 10 nm in diameter, are stronger than microtubules and microfilaments. They play the role of cell shape maintenance and provide support for cell structures. Microfilaments, being 6 nm in diameter, are fibres made up by the intertwining of 2 chains, and have the function of tension resisting and cell shape maintenance. They are of particular importance in cytokinesis owing to their close relationship with myosin fibers, forming a mechanoresponsive network that transfers forces which can contract, and cause changes in cell shape (Geiger et al., 2009; Pellegrin & Mellor, 2007). It is suggested that the synthesis of both stress fibre and focal adhesion are used for balancing cell-generated forces with ECM mechanical properties and external forces, and thus later affect MSCs response (MacQueen et al., 2013). By activating these systems, a huge variety of cellular responses can be elicited as summarized in Figure 2.10, in response to an applied strain. Stretching may directly open membrane channels such as stretch-activated channels, voltage-activated channels, gap junctions or connexin hemi-channels (Matthews et al., 2006), and activate gene FAK, SRC, or trigger MAPK and NF-kβ pathways (Hynes, 2002; MacQueen et al., 2013).

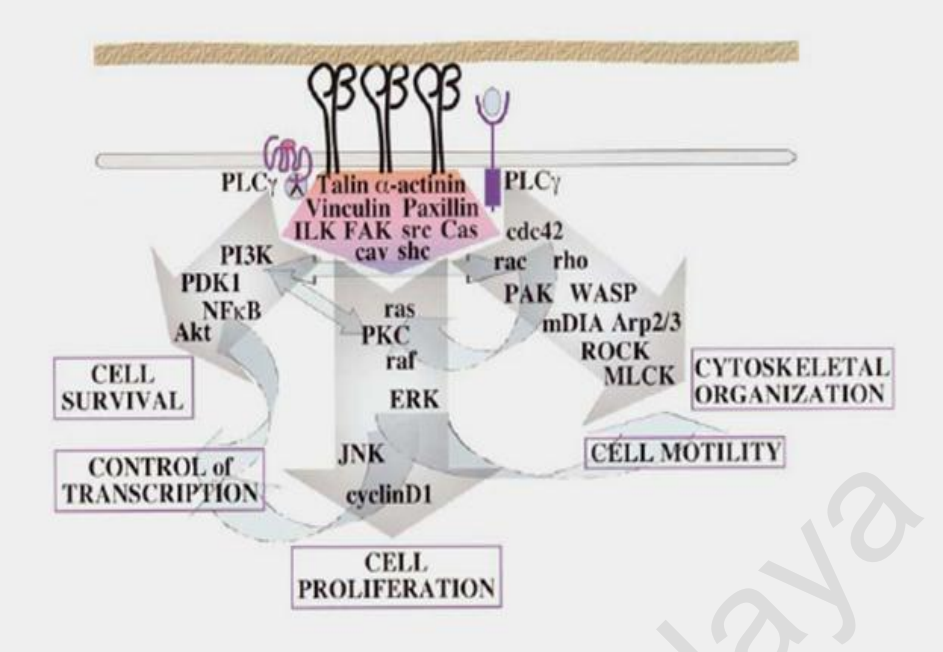

Figure 2.10: Integrin signalling
This activation allow cell signalling to occur thereby activating a series of cell expression. (Reproduced from Hynes, 2002)

Some previous studies have suggested that, besides the cytoskeletal and focal adhesion molecules, ion channels also are involved in the activation of mechanosensing cells (Markin & Martinac, 1991; Sachs, 2010; Sukharev & Corey, 2004; Wang & Li, 2010). Ion channels are embedded within the plasma membrane, and consist of one or more proteins with a central aqueous pore, which is opened by conformational change (Neher & Sakmann, 1992). They are essential components that control ion movement in and out of the cell by specific gating to each ion channel, based on the article by Hille (2001). Basically, ion channels can be voltage, chemically or mechanically induced (Hille, 2001). Mechanosensitive ion channels will be the focus in this study, as they have been involved in cellular mechanotransduction. Araki et al (2008) has indicated that influx of signalling ion (e.g. Ca^{2+}, Na^{+}) can be occurred via mechanically-activated ion channels, and coupled with the release of secondary signalling molecules such as adenosine triphosphate and nitric oxide, to trigger cellular activities.

2.4.2 Stretch-activated ion channels

Stretch-activated ion channels (SACs) exist in all cells. There are many synonyms for SACs, including mechanosensitive channels, mechanical channels, mechanogated channels, pressure-induced, or pressure-sensitive (Sachs, 2010). When the mechanical stress is applied, SACs will change shape between closed and open states (Markin & Martinac, 1991) to allow an influx of extracellular ions like calcium (Ca^{2+}), sodium (Na^{+}), and potassium (K^{+}) across the cell membrane into the cytoplasm, then transducing mechanical signals into activation of intracellular signalling molecules (Sukharev & Corey, 2004; Wang & Li, 2010). Sodium channel (i.e. epithelial sodium channel) and calcium channel (i.e. stretch-activated calcium channel) will be focused in this thesis.

2.4.2.1 Epithelial sodium channel

The epithelial sodium channel (ENaC)/degenerin (ENaC/DEG) gene family was only newly discovered at the beginning of the 1990s. According to Kellenberger and Schild, (2002), the first identified genes from *Caenorhabditis elegans* mechanosensory pathway are degenerin genes *deg-1* and *mec-4*. They revealed that ENaC/DEG genes are present only in animals (metazoa) with specialized organ functions for reproduction, digestion, and coordination. This is different with other channels such as potassium, chloride, or water channels. The ENaC is a member of the ENaC/DEG family of ion channels, and permeable to protons and cations, particularly Na^{+}-ions (Canessa et al., 1993). Some researchers described that the ENaC is representative of a new type of voltage-independent, amiloride-sensitive sodium channel (Simon et al., 2010; Stockand et al., 2008).

Epithelial sodium channels (ENaCs) are widely distributed in different tissues, including kidney, lung, sweat glands, and gastrointestinal tract (McDonald et al., 1994).

The main functions of these channels are sodium reabsorption (maintenance of body salt and water homeostasis), taste reception, touch sensation, and mechanotransduction (Kellenberger & Schild, 2002). In humans, either gain or loss of ENaC function will lead to severe blood pressure and electrolyte disorders (Lifton et al., 2001). Abnormal ENaC activity was reported to cause cystic fibrosis and polycystic kidney disease, and abnormal epithelial cell transport (Staruschenko et al., 2005).

The canonical ENaC consists of three non-identical, but homologous subunits, α-, β-, and γ-ENaC (Figure 2.11), which are each 85 to 95 kD in size in their unmodified state (Bhalla & Hallows, 2008). Each subunit consists four different domains, which are the cytoplasmic N-terminus, the large extracellular loop, the two short hydrophobic segments, and the cytoplasmic C-terminus (Canessa et al., 1994; Firsov et al., 1998; Rossier et al., 1994). The first subunit that was identified in the rat colon was α-ENaC (Canessa et al., 1993), and subsequently the authors found two more subunits, that are β- and γ-ENaC. The β- and γ-ENaC subunits were reported by McDonald et al (1995) share approximately 33-34% amino acid identity with α-ENaC. In other words, the α-ENaC subunit is the pore-forming subunit while β- and γ-subunits modulate channel activity (Caci et al, 2009). In a study of Canessa et al (1994), they discovered that the β- and γ-subunits alone did not exhibit Na^+ current in *Xenopus* oocytes, but the current was more than 100 fold higher when co-expressed together with α-ENaC. They then suggested that the α-subunit contains all of the biophysical and pharmacological properties of ENaC. However, β- and γ-subunits has been suggested to aid in the formation of the channel pore (Schild et al., 1997). The fourth subunit, the so called δ-subunit, has been identified by Waldmann et al (1995) and found mainly expressed in the human brain. Although this subunit shares similarity features with the α-subunit, its physiological role is still unclear. Compared to α-subunit containing channels, human ENaCs containing the δ-subunit display a higher single channel conductance (Wesch et

al., 2012), increased open probability (Haerteis et al., 2009), and a decreased sensitivity to the ENaC inhibitor amiloride (Waldmann et al., 1995). Different groups of researchers have proposed different stochiometry of ENaC, where ENaC is assembled either four (Anantharam & Palmer, 2007; Firsov et al., 1998) or eight to nine subunits (Eskandari et al., 1999; Synder et al., 1998). However, Stockand et al (2008) has suggested ENaC is likely to be a trimer. Subsequently, Stewart et al (2011) stated that the trimer-of-trimers organization would account for earlier reports that ENaC contains eight to nine subunits.

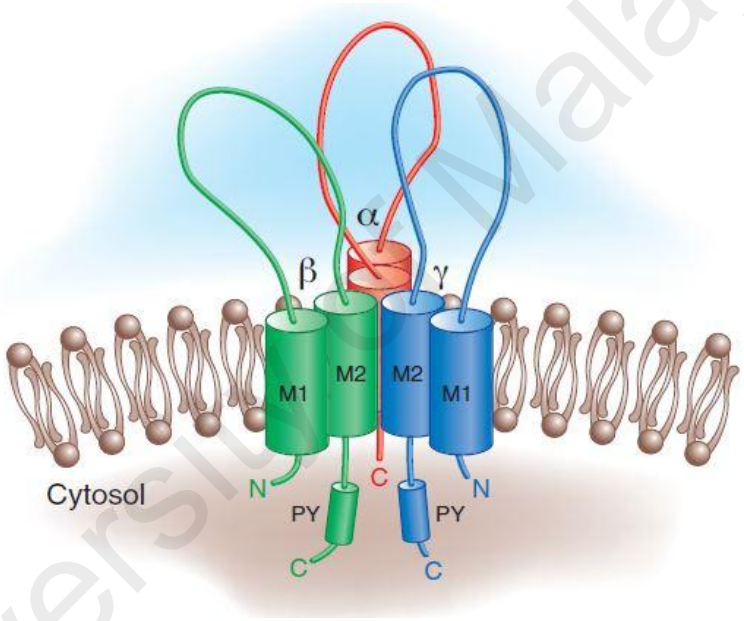

Figure 2.11: ENaC architecture
The tetrameric assembly model of a canonical ENaC consists of α-, β-, and γ-subunits, each subunit has two membrane-spanning domains (M1 and M2) with intracellular N- and C-termini. The β-, and γ-subunits each contain a canonical "PY" motif in their COOH-termini. (Reproduced from Bhalla & Hallows, 2008)

ENaC is regulated by a variety of extrinsic and intrinsic factors, which include the regulation of channel expression/synthesis, intracellular channel trafficking, and single-channel properties such as open probability (P_o) (Garty & Palmer, 1997). A schematic of the regulation of ENaC by various signalling pathways is shown in Figure 2.12.

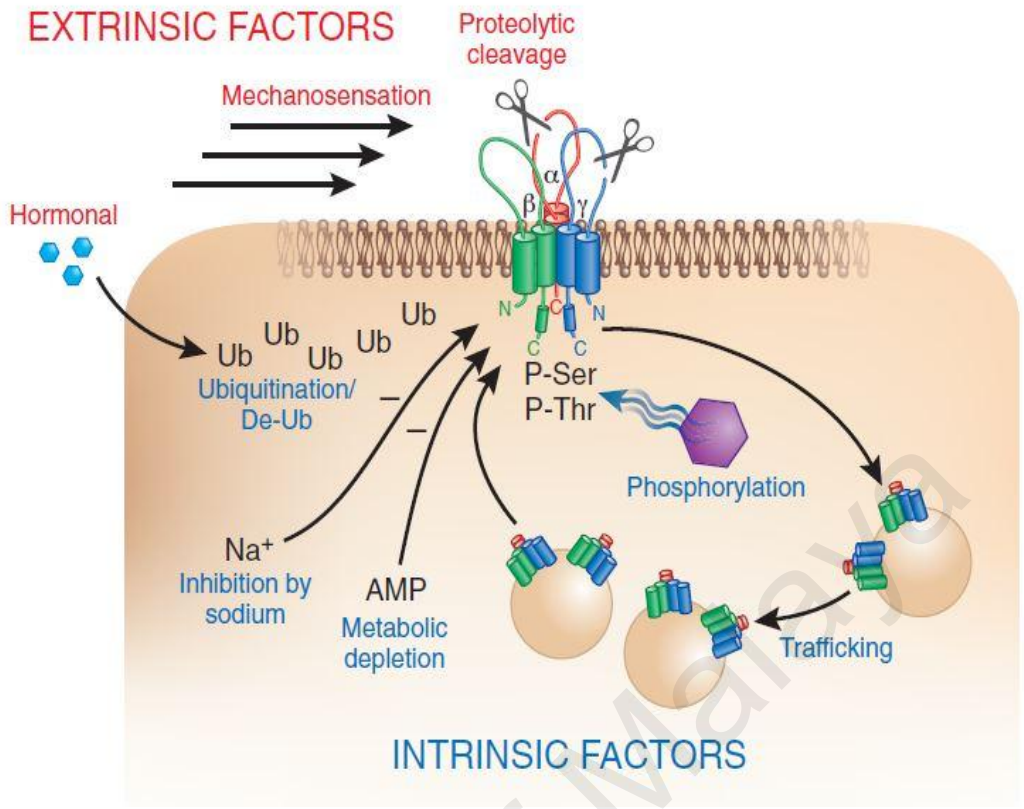

Figure 2.12: Modes of ENaC regulation
ENaC can be regulated by extrinsic and intrinsic mechanisms. Extrinsic regulation of the channel includes mechanical stretch, hormone activation, and/or proteolytic cleavage. Intrinsic regulation may be due to ubiquitination/de-ubiquitination, intracellular trafficking, various kinases, sodium, and metabolic substrates. (Reproduced from Bhalla & Hallows, 2008)

In past, ENaC has been implicated in the mechanosensation and transduction processes in mechanical sensory cells (Bhalla & Hallows, 2008; Kashlan & Kleyman, 2012; Kellenberger & Schild, 2002). In a review article of Schild (2010), it was clearly mentioned that ENaC is localized at the apical membrane of cells, and have relatively short half-life which is less than 1 hour. However, studies showed that ENaC activity can be inhibited by some potent pharmacological blockers (e.g. triamterene, amiloride, benzamil), and thus disrupted mechanical transduction process into cellular (e.g. neuronal, blood vessels, and vascular smooth muscle cells) (Drummond et al., 2001; Drummond et al., 2004; Kellenberger et al., 2003). This indicated that the sodium channels had a functional role in transducing mechanical stimuli into cell responses.

To date, the bulk of the research done on ENaCs has focused on the kidney and airways (Kashlan & Kleyman, 2012), but ENaCs are also present in the MSCs (Kizer et al., 1997). It is also has been reported that this ion channel plays important role in load bearing cells such as chondrocytes and osteoblasts (Kizer et al., 1997; Lewis et al., 2013), however there is lack of information related the role of ENaCs in tenocytes. Recently, it has been proposed that the response of ENaCs is to mechanical forces such as stretch and shear force (Althaus et al., 2007). The cytoskeleton regulates ENaC, as the COOH-terminus of α-ENaC interacts directly with the actin cytoskeleton, and ENaC activity is enhanced by actin-disrupting agents or by addition of short actin filaments *in vitro* (Corey & Garcia-Anoveros, 1996). Based on this principle, it is granted its role in MSCs sensing and responding to changes in cell status. This topic will be studied and discussed further in this thesis.

2.4.2.2 Stretch-activated calcium channel

Calcium signalling is important for all cells. It is difficult to find a physiological process in a cell that is not dependent on calcium. Calcium ion (Ca^{2+}) is a ubiquitous second messenger, as one of the most important biochemical signals, is involved in many cellular processes. There are a number of studies which have revealed that cytosolic or dynamic Ca^{2+} signals can be affected by mechanical stimulation in organelles or subcellular compartments (e.g. mitochondria, focal adhesion sites) (Hung et al., 1997; Kim et al., 2014; Ko et al., 2001; Steward et al., 2014). A similar principle to ENaC has been described in a previous section, in which the incoming signals across the cellular membrane via calcium channels and are transmitted to their target. The translated extracellular signal is carried by the intracellular information carrier, named second messenger. Calcium signalling involves a number of messengers; however, two members are employed commonly throughout all living cells, via ionositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and the ryanodine receptor-mediated Ca^{2+} release (calcium

induced calcium release) (Lipp & Niggli, 1996). Others established include cyclic adenosine 5'-diphosphoribose, nitric oxide, H_2O_2/O_2^-, nicotinic acid adenine dinucleotide phosphate, diacylglycerol, arachidonic acid, and sphingosine (Berridge et al., 2003; Bootman et al., 2001; Munaron et al., 2004).

Intracellular Ca^{2+} can be increased by either the influx of extracellular Ca^{2+} via plasma membrane channels, or by Ca^{2+} release from intracellular stores of the endoplasmic reticulum (Nowycky & Thomas, 2002). Plasma membrane channels permeable to Ca^{2+} include voltage-gated, receptor-gated and mechanogated channels (Berridge et al., 2003; Nowycky & Thomas, 2002). Multiple systems are required to maintain a low intra-cellular calcium concentration. Generally, cells at rest have an intracellular Ca^{2+} concentration of approximately 100 nM while the extracellular Ca^{2+} is above 1 mM. But the level of intracellular Ca^{2+} concentration can increase 10-fold to 1 μM during stimulation (Berridge et al., 2000; Bootman et al., 2001). This elevation is important for triggering various types of events, which are shown in Figure 2.13. Changes in the intracellular Ca^{2+} concentrations can result in cell proliferation, cell differentiation or cell death (Berridge et al., 2000; Bootman et al., 2001; Clapham, 2007; Landsberg & Yuan, 2004; Maeda et al., 2007; Orrenius et al., 2003).

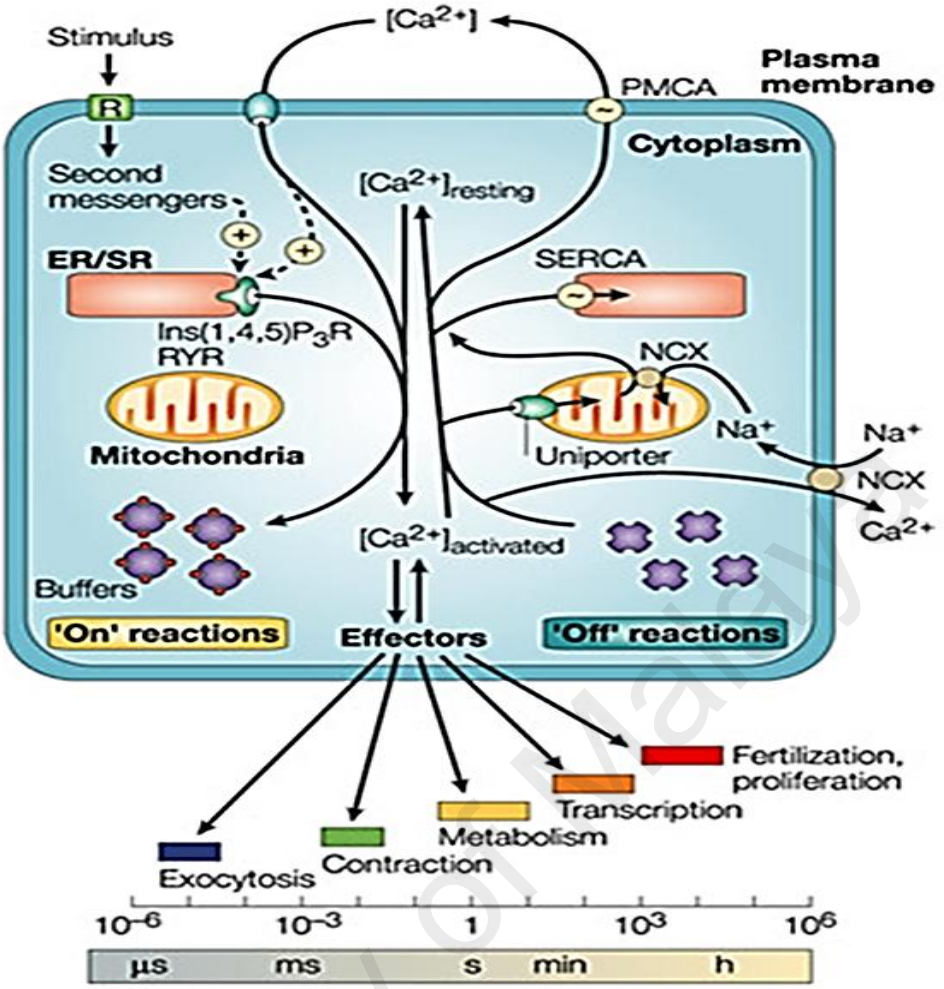

Figure 2.13: Calcium-signalling dynamics and homeostasis
During the 'on' reactions, stimuli induce both the entry of external Ca^{2+} and the formation of second messengers that release internal Ca^{2+} that is stored within the endoplasmic/ sarcoplasmic reticulum (ER/SR). The Ca^{2+} is bound to buffers, whereas a small proportion binds to the effectors that activate various cellular processes. During the 'off' reactions, Ca^{2+} leaves the effectors and buffers and is removed from the cell by various exchangers and pumps. The Na^{+}/Ca^{2+} exchanger (NCX) and the plasma-membrane Ca^{2+}-ATPase (PMCA) extrude Ca^{2+} to the outside, whereas the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps Ca^{2+} back into the ER. During recovery process, mitochondria sequester Ca^{2+} rapidly through a uniporter, and this is then released more slowly back into the cytosol to be dealt with by the SERCA and the PMCA. (Reproduced from Berridge et al., 2008)

As shown in the above figure, the main function of calcium signalling is achieved by the utilization of 4 functional units, described as a 'calcium signalling toolkit' (Berridge et al., 2000), including (i) stimulus; (ii) on mechanisms, (iii) messenger, and (iv) off mechanisms. Basically, the cell response to perceived stimuli activates a combination of 'on' and 'off' mechanisms involving a variety of channels, pumps and exchangers.

According to the article of Berridge et al (2003), the associated families of Ca^{2+} entry channels include voltage-operated calcium channels (VOCCs), receptor-operated calcium channels (ROCCs), stretch-activated calcium channels (SACCs), store-operated calcium channels (SOCCs), and calcium induced calcium influx (CICR).

Of particular interest with regard to mechanosensing are the stretch-activated ion channels, as mentioned in an earlier section. While much highlighting on the intracellular calcium increment by various mechanisms has taken place, very little has been discussed about the effects of mechanical stretching on calcium signalling, especially on stretch-activated calcium channel (SACC). Calcium dyshomeostasis can be documented to be developed via SACC. SACC opens when mechanical forces are applied to the channels. Sun et al (2012a, 2012b) believed that the expression of SACC due to the alteration of action potential from a mechanical stimulus, which is located on mechanoreceptor cells. Mechanical deformations due to tensile strain, fluid flow, compression, and vibration induced an increase in intracellular calcium concentration in each of the cell types tested (Nowycky & Thomas, 2002). A study has compared intracellular calcium release in response to static stretch (equibiaxial) and shear stress in human tenocytes (Wall & Banes, 2005). They found that stretch activated channels were involved in the response to cyclic strain, but not to shear/fluid flow. This shows that mechanically stimulated signalling mechanisms appear to be specific to types of mechanical deformation. The presence of SACC has been demonstrated in migrating fibroblast-like cells, which respond to stretching forces (Lee et al., 1999). SACC thus will be focused in this thesis based on application of tensile loading in this study. This topic will be further discussed in Chapter 7.

2.5 Summary, challenges and future perspectives

In summary, cellular mechanotransduction is a complex phenomenon involving multiple mechanisms. The fact that MSCs respond to mechanical stimulation is well-known but the cellular response to the loading depends upon the mechanical environment factors applied to it, in relation to the cells. There are still many remaining cellular mechanisms to be discovered that can provide the interface between mechanosensation and the activation of biochemical processes, such as gene expression. The fundamental knowledge on this aspect will facilitate clinical translation of cell based and pharmacological therapies. It also provides rationale and foundation for use of mechanical stimulation to improve human health, by designing appropriate instruments, exercise protocols, and rehabilitation regimens. This study will investigate the hypothesis outlined in Chapter 1 using human primary bone marrow derived mesenchymal stromal cells subjected to uniaxial cyclic tensile loading to better understand the conditions which might lead to tendon regeneration and successful tissue engineering.

CHAPTER 3: CHARACTERISATION OF ISOLATED HUMAN BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS

3.1 Introduction

It has been suggested that cells used in tissue engineering must possess the capacity to proliferate, exhibit tendon-like phenotype, deposit the appropriate ECM, and not provoke an immune response *in vivo* in order to obtain superior clinical outcomes. As mentioned previously in our Literature Review section (Chapter 2), MSCs are cells which has been deemed by many as promising cells that encourages tissue regeneration, and are commonly employed in tendon tissue engineering studies (Awad et al., 1999; Bullough et al., 2008; Chong et al., 2007; Ouyang et al., 2004). Several sources of these cells have been identified, which includes bone marrow. Despite sharing similar characteristics, MSCs from different sources differ in their differentiation potential and gene expression profile (Wagner et al., 2005). Among the different types of adult stem cells, stem cells harboured in the bone marrow are considered to have the greatest multi-lineage potential (Mafi et al., 2011) and have been extensively studied for therapeutic purposes. Adult stem cells derived from bone marrow, typically from the iliac crest of the pelvis, are commonly referred to as bone marrow stromal cells (Kuroda et al., 2007).

It has also been demonstrated that MSCs from different species behave differently with their proliferation and differentiation ability (Caplan, 2007; Mafi et al., 2011). It is therefore important that MSCs used for these experiments are of human origin to ensure the relevance of the study. Choosing human MSCs (hMSCs) has also added advantages since commercial kits presently available to detect gene and protein products of human cells are more readily available. Owing to the aberrant cell proliferative ability and lack of cell contact inhibition that exist in cell lines (Ramakrishnan et al., 2013), the use of primary hMSCs was selected for this study. In this chapter, the protocols describing the

isolation and culturing of hMSCs from bone marrow; and the hMSCs stemness expression using flow cytometry will be mentioned. Further analysis to confirm the differentiation potential of hMSCs into mesodermal lineage including chondrogenic, osteogenic, and adipogenic lineages using immunohistochemical analytical methods will also be described.

3.2 Literature Review

It is well established that cell culture techniques used to isolate and expand hMSCs provides us with the ability to increase cell population in large numbers *in vitro*. However, despite this technique being successful, the hMSC cell population are not homogenous since they are entwined with a subset population of other cells, namely that of haematopoietic origin such as macrophage. In the late 1980s, the technique for isolating, purifying and mitotically expanding MSCs from marrow specimens was first published (Haynesworth et al., 1992). It has been described such that the setting up of primary MSC culture is relatively straightforward. Nevertheless the outcomes of hMSC culture are never perfectly replicated. One must expect that variations in outcomes may be led by the wide variation of the nature of donor intrinsically. Thus, when using human primary cultures in this study, fine-tuning techniques to determine the optimal conditions required for the development of maximal cell expansion would still be required.

It is worth noting that MSCs are multipotent cells which can be derived from both marrow and non-marrow tissues. These cells have several particular characteristics, which define them as the same subset of cells, albeit been given different names. The plastic adherent adult stem/progenitor cells from bone marrow were first described as fibroblastoid colony forming units. This name had then undergone a slow evolution to being described as marrow stromal cells. In later years, these cells were further renamed

subsequently as mesenchymal stem cells; referring to the fact that these cells are embryologically derived from the mesodermal layer of the embryos. As further research developed, it became apparent to many that certain cells may not be of stem cell origin but may behave similarly, thus a name which is more reflective of the ambiguous nature of these cells were given, i.e. multipotent mesenchymal stromal cells (MSCs).

The identification of MSCs is mainly via *in vitro* culture experiments. Classically, a subset of bone marrow derived MSCs (BMMSCs) is designated as clonogenic if it is able to generate colonies of fibroblast-like cells from single cells when plated in culture (Bianco et al., 2008). Friedenstein et al (1970) was the first to describe the identity of MSCs when attempting to characterize the stromal cells from rodent bone marrow. They observed a rapidly adherent group of cells on the plastic surface when plated at low density, and observed the formation of distinct colonies from single cell precursor on the plate known as "colony forming unit-fibroblast". This technique of separating the non-adherent hematopoietic cells eventually has become the basis for BMMSCs isolation.

This plastic-adherent method, however, does not necessarily yield a homogeneous BMMSCs population with the same clonogenic and multipotential abilities. Cells in bone marrow may have lower clonogenic ability as BMMSCs are only present in small numbers in bone marrow. It is estimated that there is approximately one MSC per 34,000 nucleated cells in human bone marrow (Wexler et al., 2003). It is also shown that the number of BMMSCs decreases with age of the donor further contributing to the low-abundance of BMMSCs during isolation (Caplan & Bruder, 2001). This phenomenon can also be attributed to the presence of other cell population from epithelial cells and hematopoietic origins. To overcome this, researchers have further modified the plastic adherence method by applying density gradient centrifugation when

isolating BMMSCs (Boyum, 1974; Colter et al., 2000). Using this technique, the mononuclear cells layer can be isolated from the interface layer and at the same time the unwanted cell types present in the marrow aspirate can be separated.

MSCs have the ability to expand many folds in culture while still retaining their growth and multi-lineage potential. Cell cultures have an initial lag phase of three to five days, followed by rapid proliferation with an average initial doubling time ranging from 12 to 24 hours, albeit varying from one donor to another (Spees et al., 2004). At confluence, MSCs enter a stationary phase. Morphologically, MSCs are characterized by a small cell body with a few long and thin cell processes. MSCs can be expanded 500-fold through as many as 50 generations to produce billions of cells (Deans & Moseley, 2000; Devine, 2002). The expanded mesenchymal stem cells do exhibit a finite lifetime and do not display properties of immortalized cells. Colonies derived from a single MSC may vary to some extent in their differentiation capacity and expansion potential. Entry of MSC into senescence is almost undetectable, and they lose their stem cell characteristics and differentiation potential from the sixth passage onwards (Bonab et al., 2006).

There are no specific cell markers to identify MSCs from other cell types. According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, the minimum criteria to define human MSCs are (Dominici et al., 2006):

(1) MSC must be plastic adherent when maintained in standard culture conditions.
(2) MSCs must express CD105, CD73 and CD90, and lack expression of CD14, CD34, CD45 and CD11b, CD79a, CD19 and HLA-DR surface molecules.
(3) MSCs demonstrate multi-potency: must differentiate into osteoblasts, chondrocytes and adipocytes *in vitro*.

Although, there is a great deal of experiments on MSC characterization that have been done, none of these characteristics is specific enough to adequately define MSC as a specific cell type (Nardi & Meirelles, 2006). Therefore, research efforts are now focused on the biological and phenotypic characteristics of this highly useful stem cell type are crucial. Moreover, the identification and maintenance of MSCs in the undifferentiated phenotype depend on efficient methods of isolation as well as optimal conditions for subsequent culture *in vitro*, such as the tissue culture substrate, specific culture media (Sotiropoulou et al., 2006), starting and passaging cell-plating density (Baksh et al., 2004), and supplementation with proliferative and differentiation factors (Chamberlain et al., 2007). Therefore, establishing an optimal cell culture system is of critical importance.

3.3 Materials and Methods

3.3.1 Primary human cell culture

3.3.1.1 Donors selection and human samples procurement

Experiments conducted using human mesenchymal stromal cells from bone marrow were obtained with consent and in accordance to the approval of the University of Malaya Medical Centre Medical Ethics Committee (Reference No: 369.19) (Appendix A) from adult donors (Appendix B) undergoing total joint arthroplasty for osteoarthritis treatment in University Malaya Medical Centre. The information sheets and examples of the consent forms are attached in Appendix C and Appendix D respectively. The inclusion and exclusion criteria of the study are summarized in Table 3.1. Before we start the study, the cell proliferation and differentiation potential for MSCs from both normal donor (trauma) and osteoarthritic pathological patients were optimised. Since there is no impact evidence from our optimization results and other studies showing that donor had osteoarthritis pathology pose any issue to the integrity of the cells, thus bone marrow samples from osteoarthritic patients were used in this study.

Before the sample collection, bone marrow donors were counselled and provided with the necessary information about the scope of this research. Their physicians also subjected them to physical examination in order to determine their health and eligibility to be recruited for our study. Bone marrow specimens were aspirated from the femoral canal of the donors by orthopaedic trained surgeons, and placed into 3 mL EDTA anticoagulant tubes under aseptic conditions. The labelled bone marrow samples were kept on ice throughout the transportation to the laboratory and proceeded for cells isolation immediately.

Table 3.1: Inclusion and exclusion criteria for patient selection in human bone marrow donation

Inclusion criteria
Malaysian patients with either male or non-pregnant female
Age between 40 and 70 years
Mentally intact and psychologically stable
Has sufficiently bulky muscle
Understands the nature of the procedure and provides written informed consent prior to any study procedure
Exclusion criteria
Positive test for HBV, HCV or HIV
Active infections
Suffering from significant cardiac disease, malignant disease or any other disease that may risk the patient or interfere with the ability to interpret the results
Subject unwilling or unable to comply with the requirements of the protocol

3.3.1.2 Primary hMSCs isolation and culture

Human bone marrow specimens were processed using the method as described in our previous publication (Nam et al., 2013). A total of 2 mL aspirated gelatinous bone marrow was added to an equal volume of 1X PBS (pH 7.2) into a 15 mL centrifuge tube, and suspended with 3 mL Pasteur pipette. The diluted marrow suspension was then gently layered on 3 mL density of 1.077 g/mL Ficoll-Paque Premium, where the interface between the marrow suspension and Ficoll-paque should not be disrupted. Density gradient centrifugation at 2,200 rpm for 25 min at room temperature was then immediately performed. The mononuclear cells (Figure 3.1) were collected and washed

with 10 mL LG-DMEM through centrifugation at 1,600 rpm for 10 min. The supernatant was then discarded, and the cell pellet was resuspended with 1 mL of FBS. At last, the cells were cultured in growth medium (LG-DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 1% GlutaMAX-1) and plated on T-75 tissue culture flasks. The flasks were gently rocked to ensure uniform cell distribution. The cells were maintained in humidified incubator at 37ºC with 5% CO_2 in the air. The flasks were checked for adherent cells after 48 h of incubation under an inverted phase contrast microscope. At 5 days after culture, the medium was discarded, followed by washing with 1X PBS to further remove any non-adherent cells. Medium replenishment was carried out every 3 days with 15 mL of fresh and pre-warmed growth medium, until the plate become 80% to 85% confluent with the adherent cell population. This primary population of the cell is considered to be at a passage number zero, and represented as P0 hMSCs. The cells were checked regularly under inverted phase contrast microscope and representative micrographs were taken at different time point using A^Cell software. The confluent cells were prepared for the next passage. The process of hMSCs isolation is summarized in Figure 3.2.

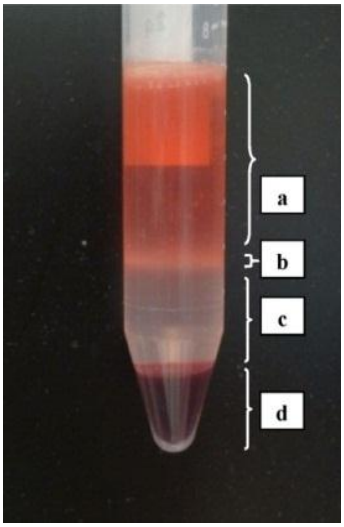

Figure 3.1: Density gradient separation of human bone marrow
Due to their lower density, mononuclear cells (b) were found at the interface between the plasma layer (a) (platelets ad PBS) and the Ficoll-paque layer (c) after being subjected to density gradient centrifugation. Erythrocytes and granulocytes (d) were found at the bottom of the layer.

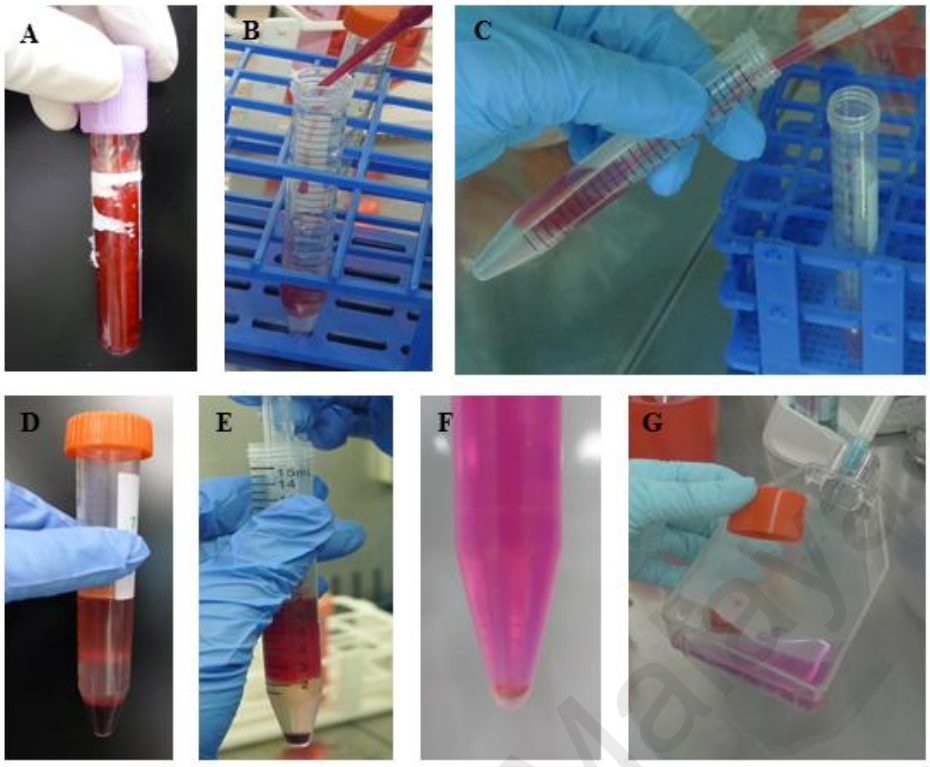

Figure 3.2: Cell extraction from human bone marrow by ficoll gradient separation A: Bone marrow sample was obtained from donors. B-C: Bone marrow was diluted with 1X PBS and carefully layered on the ficoll layer. D: Different layers become apparent after centrifugation. The mononuclear cells are trapped in the layer between ficoll and serum. E: With a sterile pasture pipette, the mononuclear layer was carefully collected minimizing suction of both the ficoll layer between and the serum layer above. F-G: After second centrifugation, the visible cell pellet was collected and cultured on a T-75 flask.

3.3.1.3 Subculture of primary hMSCs

Sub-culturing hMSCs was necessary to obtain the cells required for subsequent experiments and to avoid cell contact inhibition (Nardi & da Silva Meirelles, 2006). Growth medium from flask was discarded completely, and the monolayer cells were then washed with 5 mL of pre-warmed 1X PBS to remove residual serum. Enzymatic removal of the cells was used by adding 3 mL of pre-warmed TrypLE into flask which was then rocked gently so that the entire surface of monolayer was covered with the solution. The flask was then incubated at 37ºC in the CO_2 incubator for only 3 min as prolonged exposure to TrypLE can result in cell damage and decrease cell viability. Ten mL of growth medium was added into the flask to neutralize the effect of TrypLE. Cell

detachment from the flask surface was hastened by tapping at the bottom of the culture flask. A cell scraper was used to further detach the remaining cells that were not initially removed. The flask was inspected under inverted microscope to ensure most of the cells had detached from the surface. The cell suspension was then pipetted up and down with a 5 mL serological pipette to further loosen the cells yielding single cell suspension, and transferred into a 15 mL centrifuge tube for centrifugation at 1,800 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 mL of growth medium to produce a uniform single cell suspension. The viable cells were then counted for further seeding before divided into new T-75 flasks. hMSCs were serially passaged and expanded up to passage-2 for the experiment use.

3.3.1.4 Assessment of cells counting

The viable cell count was estimated by the 'dye exclusion' assay (Freshney, 2005). Total cell count was estimated using an improved version of the Bauer haemocytometer (Boyce et al., 2006) together with 0.4% w/v trypan blue solution, which is a cell stain used to access cell viability through dye-exclusion test. It was important to ensure that both the haemocytometer and its cover slip were cleaned with 70% ethanol before counting the cells. The cover slip was placed in the centre of the haemocytometer covering the counting chamber. After the cells were harvested from flask, the cell pellet was gently re-suspended with 1 mL of growth media. Ten µL of cell suspension was then mixed with equal volume of trypan blue solution using micropipette and was kept for 1 min before applied onto the haemocytometer chamber where 10 µL for each side (Figure 3.3), and viewed under a direct light microscope with 10X objective. Cytoplasm of non-viable cells stained blue due to cell membrane dysfunction (inability of the membrane to actively transport the dye out of the cells), thus used to identify dead cells. Viable (non-stained) cells were then counted within the grid of the nine large squares (each square measuring 1x1 mm). In this study, a total of 10 large squares were

calculated (5 large square for each side of the chamber). Viable cell density was calculated as follows:

$$\text{Number of cells per mL} = \frac{n}{10} \text{ x } 2 \text{ x } 10^4$$

where, n = number of cells counted in the ten large squares

2 = dilution factor (cell suspension to trypan blue, 1: 1)

10^4 = volume (each square of the haemocytometer has a surface area of 1 mm^2 and the depth of the chamber is 0.1 mm. Each square represent a total volume of 0.1 mm^3 or 10^{-4} cm^3).

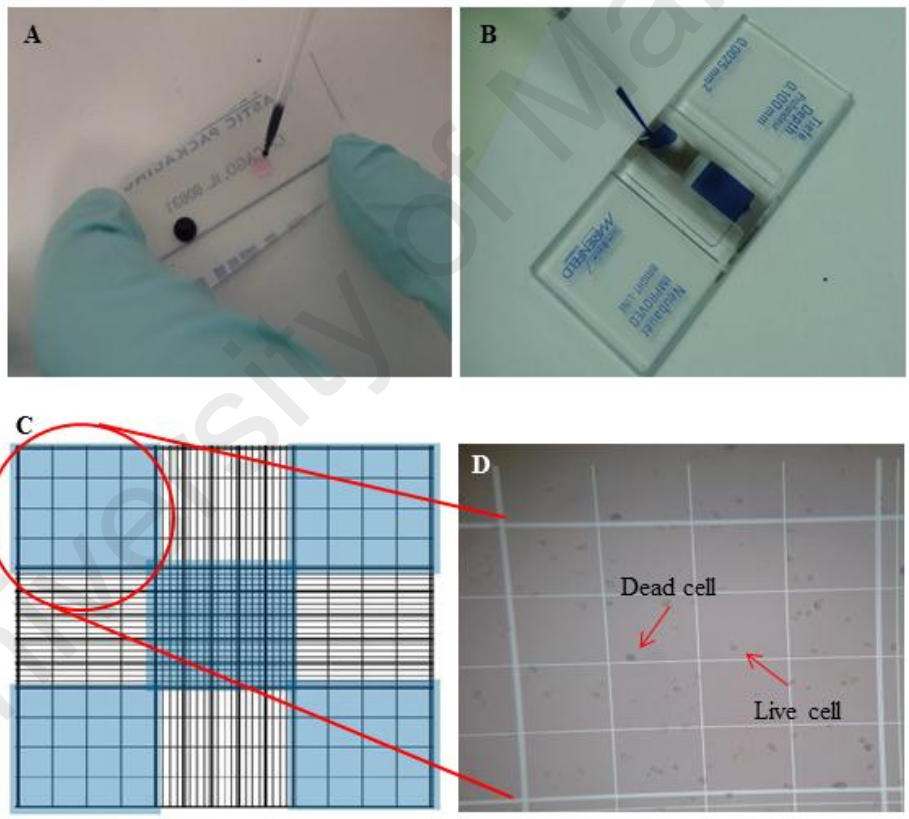

Figure 3.3: Cells counting by haemocytometer
A: A total of 10 µL cell suspension was mixed with 10 µL of trypan blue using micropipette tip. B: The mixture was placed close to the glass cover edge, with the plunger released slowly watching the liquid enters the chamber uniformly, being absorbed by capillarity. C: Example of cell count area (blue highlight) in one of the 9 big squares of a Neubauer chamber. D: Appearance of the haemocytometer grid with viable cells visualised under the microscope.

3.3.1.5 Cryopreservation and thawing of hMSCs

Freezing mix was prepared by adding 10% of dimethylsulfoxide (DMSO) to 90% of FBS. The freezing mix was pre-cooled before adding to the cell pellet by keeping it at 4ºC in a refrigerator. For freezing, the cells were trypsinized and the pellet was collected by centrifugation. The cells were counted and ice-cold freezing mix was added to the cells at a ratio of 1 mL (100 µL DMSO + 900 µL FBS) freezing mix per 1 x 10^6 cells. The cells were re-suspended in the freezing mix and 1 mL each of the cell suspension was transferred to 1.7 mL cryovials. The cryovials were labelled properly describing the type and passage of the cells, and date of cryopreserved. The vials were transferred to a freezing container, known as Mr. Frosty, containing isopropyl alcohol that provides the critical, repeatable -1ºC/min cooling rate required for successful cryopreservation of the cells. The freezing container was kept at room temperature before keeping the vials for efficient freezing. The freezing container with the vials was then kept at -80ºC overnight in a freezer. The next day the frozen vials were transferred to a cryopreservation box and placed in liquid nitrogen (-196ºC) for long term storage.

To thaw the cells, the frozen vial was removed from liquid nitrogen container, and the cap of the vial was slightly loosened to allow the nitrogen gas to escape during thawing. The vial was placed immediately by gently swirling the vial in a 37ºC water bath to prevent the formation of intracellular ice. As the last ice crystal was about to melt, the thawed cell suspension from the vial was then transferred by adding slowly drop wise into a 15 mL centrifuge tube containing 10 mL of warm growth medium. This is to reduce osmotic swelling upon rehydration (DMSO is hypertonic) and improve post-freezer viability. The cells were mixed gently with the medium and centrifuged at 1,800 rpm for 5 min in order to remove DMSO. The cell pellet was resuspended in fresh warm growth medium. The cell number and viability was performed again prior to

being implanted into the culture flasks. The flasks were incubated at 37ºC in a humidified incubator with 5% CO_2 in air.

3.3.2 Characterisation of hMSCs

3.3.2.1 Cell morphology documentation

The cellular morphology of cultured hMSCs at passage 0 (P0), passage 1 (P1), and passage 2 (P2) were captured using an inverted phase contrast microscope fitted with a digital camera. The cells were observed at 4X, 10X, 20X, and 40X objective lenses.

3.3.2.2 Immunophenotyping by flow cytometry

Passage-2 hMSCs at the concentration of $1x10^6$ cells/mL were trypsinized, washed with 1X PBS, and resuspended in 100 µL of FASC sheath buffer. Samples were aspirated up and down for several times to help disaggregate clumps. The cells suspension was transferred into a 5 mL polystyrene round-bottomed tube and stained in the dark on ice with 10 µL fluorescein isothiocyanate- (FITC)- or 10 µL phycoerythrin- (PE)- or 10 µL peridinin chlorophyll protein- (PerCPCY5.5)-, or 5 µL allophycocyanin- (APC)-conjugated anti-marker mAbs (in optimal concentrations). Tested markers included hematopoietic lineage markers (CD14, CD34, CD45, HLA-DR), matrix receptors (CD44, CD105), integrins (CD29), and stromal cell-associated markers (CD73, CD90). After 15 min of incubation, cells were washed using 2 mL of FASC sheath buffer and centrifuged at 300*g* for 5 min to remove the unbound antibodies. The pellet was further resuspended in 500 µL 1X PBS. Non-specific fluorescence emission was detected by incubating cells with fluorescence conjugated isotype control. The cells were analysed using a FACScan flow cytometer. Data acquisition and analysis were performed using FACS DIVA software. At least 10,000 events were captured by the system, and positive expression was defined as a level of fluorescence greater than 99% of the corresponding unstained cell sample. Gating was performed to exclude cell debris

and unwanted aggregates (FSC/SSC dotplot). To set background fluorescence levels, unstained and/or matched isotype controls were used.

3.3.2.3 Induction of tri-lineage mesodermal differentiation

To evaluate the nature of MSCs in these isolated clusters cell; the multipotent capacity of hMSC were tested after *in vitro* culturing using specific supplements by inducing differentiation into osteogenic, chondrogenic and adipogenic phenotypes with triplicate cultures respectively. Samples were compared with cells from the same cell lineage cultured at the same time using non-differentiating supplements which act as control. For each differentiation lineage assay, the StemPro® differentiation medium was prepared by mixing the supplement solution into basal medium for the ratio 1:9 followed by the addition of 1% penicillin-streptomycin solution. During differentiation period, the differentiation medium changes were made every 3 days in the dark. Evaluation of the differentiated cells was then performed using staining method (Figure 3.4).

a) Adipogenic differentiation

Based on the cell numbers obtained using manual cell counting, MSCs at P2 were harvested and seeded on 4-well chamber slides at a density of $1x10^3$ cells/cm^2 in all wells. Once the cells reached 85% confluence, the growth medium were replaced with 1 mL pre-warmed complete adipogenesis differentiation medium. Fourteen days after culture initiation, the cells were rinsed with 1X PBS twice, and fixed with methanol at room temperature for 15 min. The fixed cells were washed twice with 1X PBS, and 60% isopropanol was added for 5 min. The cells were then stained by using freshly prepared Oil Red O solution in 99% isopropanol for 10 min to visualize the presence of lipid droplets. The centre of each well was rinsed down with tap water until the water runs clear. The slides were kept wet to keep the lipid vacuoles from disrupting. Lastly,

the slides were visualized and images of the cells were captured with a light microscope connected with a camera.

b) Osteogenic differentiation

Similar to the adipogenesis differentiation assay, confluent passage-3 cells on 4-well chambers were cultured in osteogenic medium for 21 days. After that, the cells were rinsed with 1X PBS twice, and fixed by methanol for 15 min at room temperature and then subjected to freshly prepared 2% Alizarin Red S solution (pH 4.2) for 3 min to observe the matrix mineralization associated with osteoblasts. After staining, the slides were dipped 20 times in acetone and a mixture of acetone:xylene (1:1 ratio) sequentially. The wells were then cleared in xylene, mounted onto coverslip with mounting reagent, and observed under light microscope.

c) Chondrogenic differentiation

To induce chondrogenic differentiation, pellet culture system was used. The 3-dimensional chondrogenic system utilized approximately $1x10^6$ passage-3 MSC per pellet. Cells were centrifuged in 15 mL conical centrifuge tube at 1,800 rpm for 5 min to create a pellet and cultured in a pre-warmed chondrogenic medium (Figure 3.4C). After centrifugation, the caps on the conical tubes were loosened for gas exchange and the pellets were incubated in a humidified atmosphere at 37ºC with 5% CO_2. After every medium change, the pellet was gently agitated to ensure that the pellet was free floating and spheroid. Twenty-eight days after the initiation of the culture, the cell pellet was gently washed in 1X PBS, but the vigorous washing was avoided as this will dissociate the cell pellet. The cell pellet was then encapsulated into a cylindrical shape containing 4% agarose solution. A needle was used to prick any bubbles formed and to align the cell pellet into the centre of the cylindrical agarose mould. The mould was then quickly cooled in 4ºC for 10 min. The cell pellet encapsulated in the cylindrical agarose mould

was then trimmed using a razor blade to remove excess agarose gel before placing into a histological cassette. The specimen was then fixed in 10% neutral buffered formalin for 1 h at room temperature, and sent for tissue processing overnight (dehydrating in ascending concentrations of ethanol and clearing in xylene). Sample was then embedded in paraffin wax and sectioning at 4 μm using a microtome before mounted on poly-L-lysine-coated glass slides. The sections were then processed for histologic evaluation to observe cartilage matrix by staining with 0.1% Safranin O solution for 5 min. The specimen was rehydrated in 95% alcohol for 5 times followed by twice immersions in absolute alcohol, and mounted onto coverslip with mounting reagent.

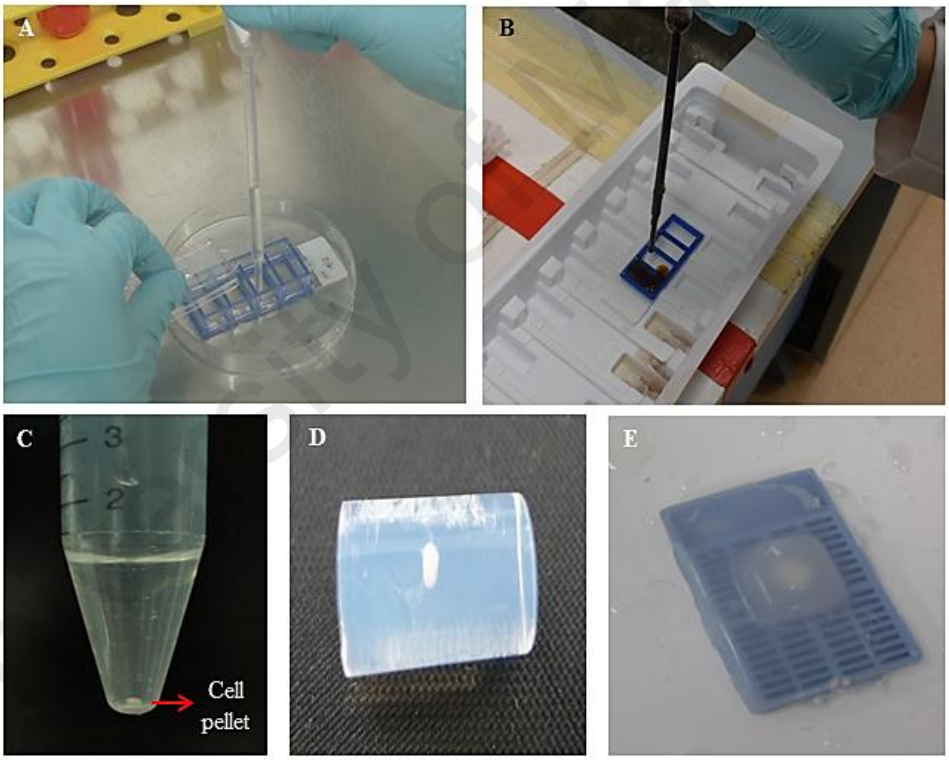

Figure 3.4: Tri-lineage differentiation assay
A: hMSCs were seeded on 4-well chamber, and the differentiation medium changes were made every 3 days. B: The differentiated monolayer cells, osteogenic and adipogenic differentiated hMSCs, were stained with Alizarin Red S and Oil Red O, respectively. C: Pellet culture system was used in chondrogenic differentiation. D: The cell pellet was encapsulated into an agarose cylindrical shape. E: The sample was fixed into a histological cassette before sectioning.

3.4 Results

3.4.1 Identification of human bone marrow derived MSCs

3.4.1.1 Plastic-adherent and hMSCs morphology

Isolated marrow derived mononuclear cells were expanded as monolayer cultures (Figure 3.5). Primary cultures of mononuclear cells appeared morphologically similar to the heterogeneous population obtained. Twenty-four hours post primary culture, a few of oval cells started adhering to flask bottom, with some floating cells. After 3 days of culture, ovoid, fusiform and spindle-like adherent cells, and round unattached hematopoietic cells were observed under microscope. Some rounded mononuclear cells begun to change into fibroblast-like cells, where the changes were evident in the periphery of dense colonies. Following medium changed after 5 days of cell cultures, scattered small MSCs colonies formed and gradually enlarged until the fusion. At day 7, MSCs significantly proliferated and cell density increased, and showed narrow spindle-shape. After 2 weeks of culture, the growth of the spindle-shaped cells with pseudopodium formation became evident. Spindled shape cells with apparent nuclei were also clearly observed. After 3 weeks of culture, MSCs started stacking and showed fingerprint-like orientation when achieving confluence. The morphology of re-plated cells showed more homogeneous cell population and had a relative fast growth speed. Further, they exhibited an elongated fusiform shape resembling fibroblasts. With the increase in passage, nuclear cytoplasm ratio of the MSCs was decreased and cell became bigger. At P2, adherent cells population were of majority spindle or fibroblastic-like cells. Trypan blue exclusion viability test showed at least 96% viable cells at P2. The freeze-thawed cells also showed more than 90% viability. However, a distinct reduction of the growth rate was observed at different passage. To exclude possible reduction of the differentiation capacity of the cells, only P3 cells were used.

The cell morphology and its plastic adherent ability were consistent to MSC properties of previous studies.

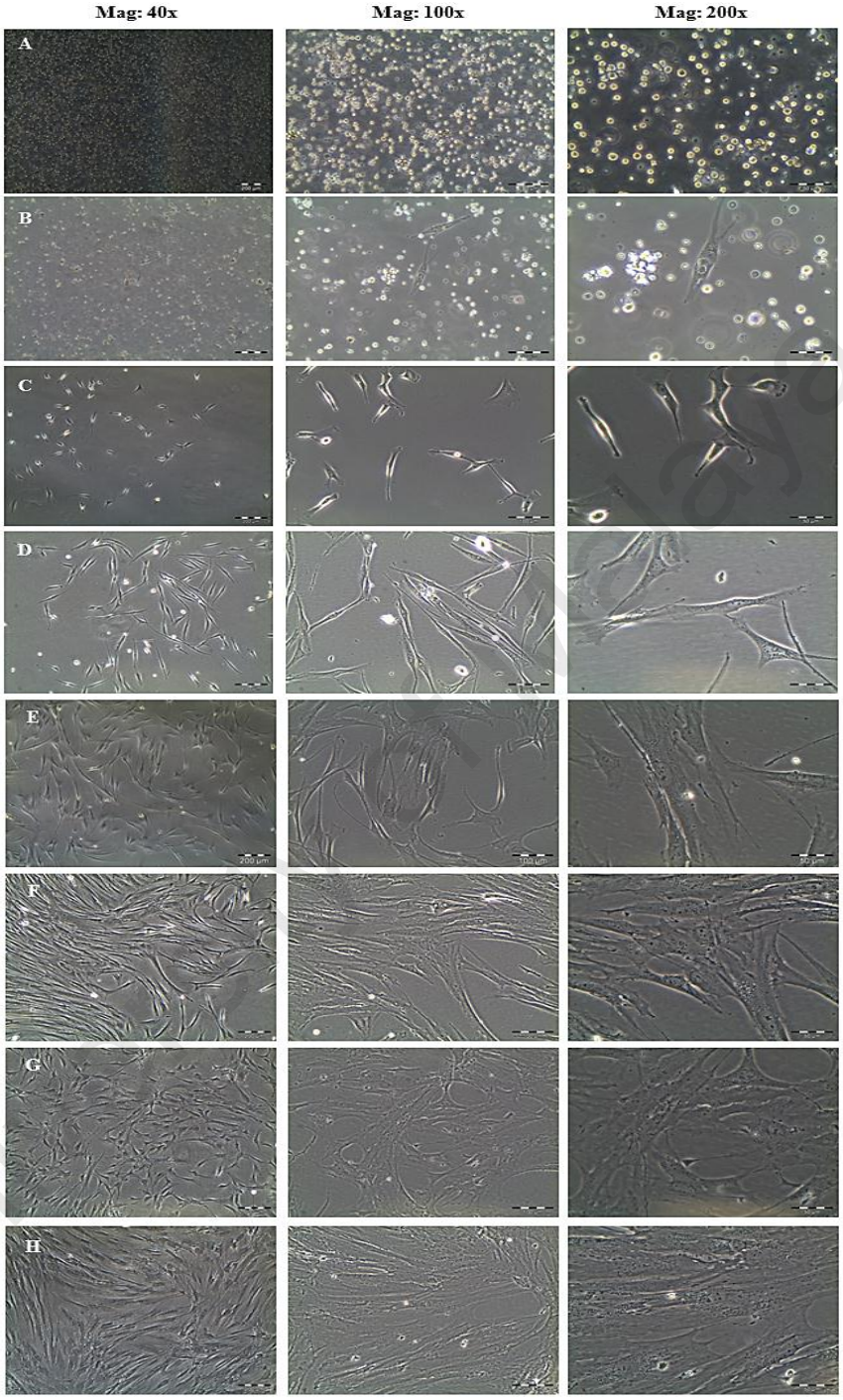

Figure 3.5: Different magnification for photomicrographs of human bone marrow derived MSCs at different duration of culture

A: 24 hours after primary culture, the mononuclear cells showed rounded cells where some of the cells adhered to the plastic surface, with some floating cells. B: Attachment of rounded cells and appearance of fibroblast-like cells. C: At day 5, the primary cultures of the cells contained mainly fibroblastic cells as well as a few small round cells. Colony-forming units of the cells were formed. D: hMSCs at day 7 showed narrow spindle-shape. E: hMSCs morphology at day 14. F: Cells confluence was obtained at day 21. G-H: Passaged-1 and passaged-2 hMSCs morphology, respectively. Mag 40x (200 µm); Mag 100x (100 µm); Mag 200x (50 µm).

3.4.1.2 Flow cytometry analysis

The hMSCs used in this study expressed positive surface markers for CD29, CD44, CD73, CD90, and CD105, as tabulated in Table 3.2. The lack of surface markers CD14, CD34, CD45, and HLA-DR expression indicate that they were not of hematopoietic or leukocytic in origin. In the multi-colour, at least 90% of hMSCs expressed double-positive expression, double-negative or co-expressed positive and negative markers (Figure 3.6).

Table 3.2. Flow-cytometric analysis of passaged-2 hMSCs

Surface Protein	Antigen	% Positive
Positive hMSCs markers		
CD29	beta-1-integrin	96.5
CD73	ecto-50-nucleotidase	96.6
CD44	lymphocyte adhesion molecule	97.1
CD90	Thy-1 (T cell surface glycoprotein)	99.2
CD105	SH-2, endoglin	97.7
Negative hMSCs markers		
CD14	serum lipopolysaccharide binding protein	2.8
CD34	sialomucin-like adhesion molecule	0.2
CD45	leukocyte common antigen	1.3
HLA-DR	major histocompatibility class II antigens	2.7
Combination of the markers		
CD90 and CD105		97.0
CD90 and CD44		96.2
CD34 and CD45		98.6
CD34 and HLA-DR		97.3
CD90 and CD45		96.3
CD14 and CD105		92.3

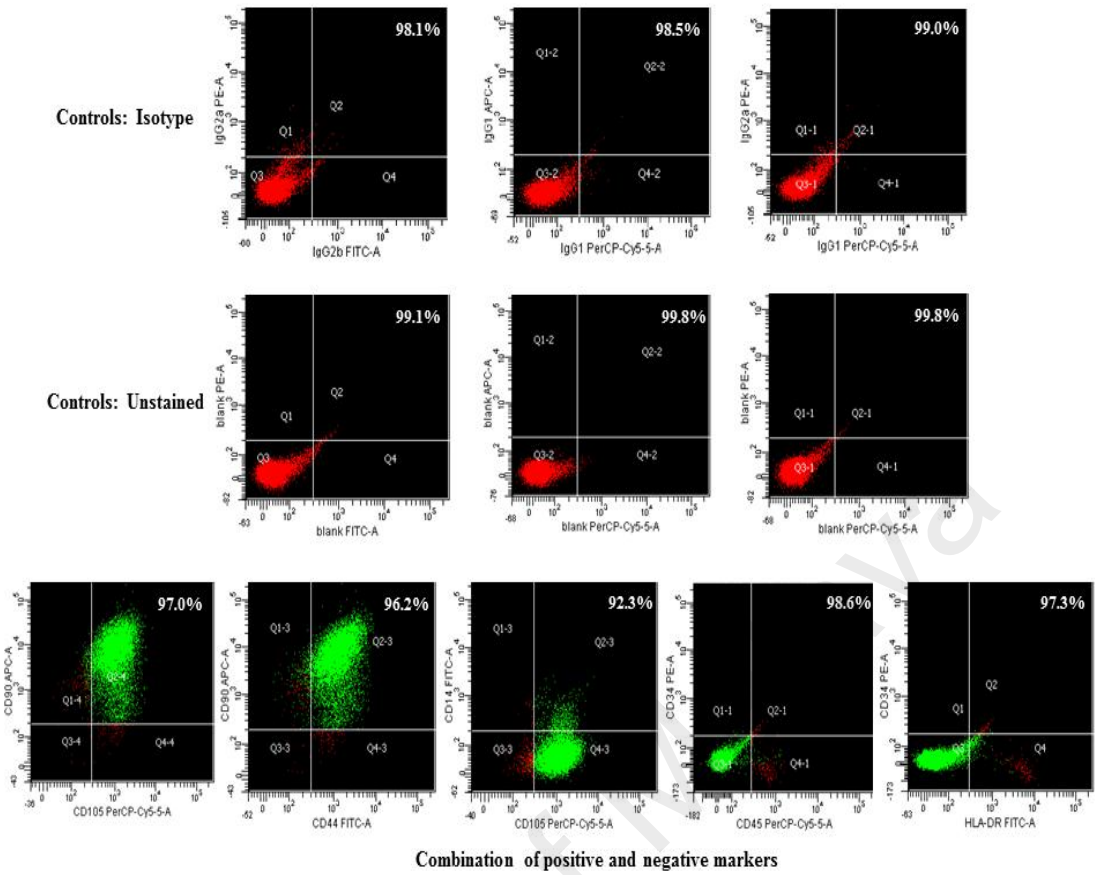

Figure 3.6: Immunophenotypic characterisation of the MSCs surface markers using flow cytometry

Representative dot plots images of immunophenotyping of MSCs are displayed. The y axis indicated the event count, and the x axis indicated the mean fluorescence intensity in a log (10^0 to 10^5) scale. The images of isotype and unstained control are shown above the combination markers stained sample. The results showed that hMSCs expressed at least 90% of double-positive expression, double-negative or co-expressed positive and negative markers.

3.4.1.3 Multi-lineage differentiation

In osteoinductive cultures, after 4 days of incubation in the osteogenic medium, some crystals were seen deposited sparsely on the cells. The number of crystals increased and became very crowded towards day 21, and this had made it difficult to identify the morphology of the cells. The whole monolayer cells appeared brick-red when stained with Alizarin Red S, showing the presence of mineral (Figure 3.7A), indicating that the cells were in the early stages of bone formation. In some areas, nodule-like structures and some sharp edges were observed.

In adipogenic culture, after 4 days of incubation in adipogenic medium, fibroblast-like cells were observed to change into large polygonal cells. Small refractile vesicles believed to be lipids were seen within the cytoplasms of the cells on day 4. These vesicles become enlarged and fused together to form bigger lipid droplets at day 14. The lipid droplets turned into red when stained with Oil Red O (Figure 3.7B).

In the pellet culture system for chondrocyte differentiation, the size of the pellet seemed to have increased during the culture period, probably as a result of matrix production and secretion. After 28 days of incubation in chondrogenic medium, the pellet was fixated and metachromatic nature of the matrix was demonstrated positively by Safranin O staining (Figure 3.7C). Chondrocytes were seen occupying the lacunars and they were separated from each other by the matrix.

These observations suggested that the MSC identity of cells isolated in this study was confirmed with the potential to undergo tri-lineage differentiations, including osteocytic, adipocytic, and chondrocytic mesodermal lineages.

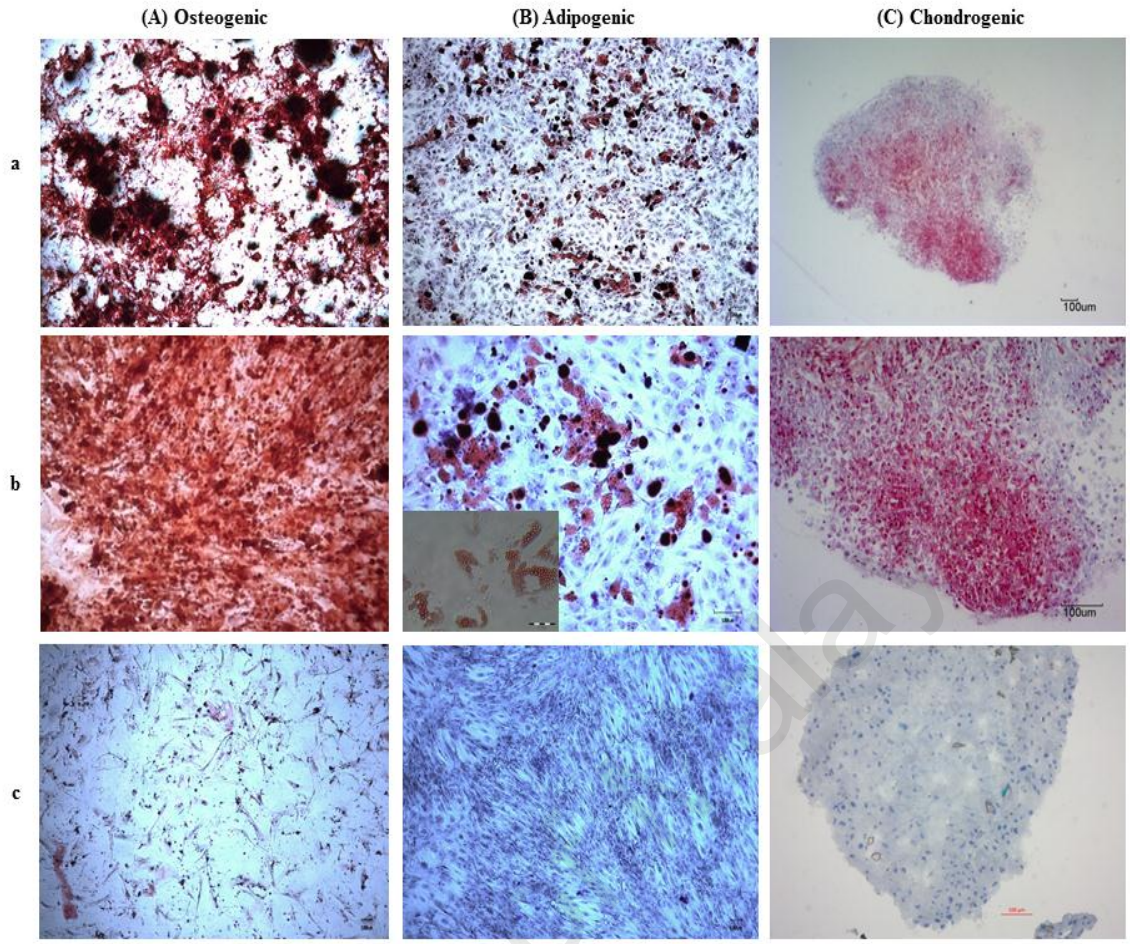

Figure 3.7: Tri-lineage differentiation potential of the hMSCs

A: MSCs were differentiated into the osteogenic lineage. The amorphous calcium phosphate formed by the action of alkaline phosphatase on matrix was shown by positive Alizarin Red S staining indicating early stages of bone formation. The crystals suspected to be hydroxyapatite with sharp edges (Ab). No accumulation of calcium oxalate crystals was observed in non-induced control MSCs culture stained (Ac). B: MSCs were differentiated into the adipogenic lineage. Oil deposition analyzed by Oil Red O staining shows oil droplet accumulation in the adipogenic-induced MSCs (Bb) with the higher magnification shown at left bottom insert. No accumulation of lipid droplets was observed in non-induced control MSCs culture stained with oil-red-O staining for comparison (Bc). C: MSCs were differentiated into the chondrogenic lineage. Chondrocytes were seen occupying the lacunars and was surrounded by territorial matrix which were stained blue, while the interterritorial matrix was stained pink. The presence of glycosaminoglycans or highly sulphated proteoglycans (pinkish colour) in chondrogenic-induced MSCs as revealed by Safranin O staining is illustrated as Cb. No glycosaminoglycan was observed in non-induced control MSCs culture stained (Cc).

3.5 Discussion

Investigators have reported studies relating to MSCs; albeit using different methods of isolation and expansion, and different approaches to characterizing these cells, thus causing difficultly for anyone to make meaningful comparison between the different studies. To address this issue, mesenchymal and tissue stem cell committee of International Society for Cellular Therapy (ISCT) have proposed several defining criterions for MSCs. In a few related investigations that have previously been reported, these important defining characteristics have been ignored (Bosnakovski et al., 2006; Jagodzinski et al., 2004; Saw et al., 2009; Wu et al., 2013b). It was therefore unclear that the cells used by these researchers were MSCs of similar nature. We have previously isolated and identified fibroblast-like cells from the bone marrow of animal models and reported their characteristics (Kwong et al., 2014; Nam et al., 2013). In this thesis, standardized optimized protocols and laboratory techniques were applied on hBMMSCs. Based on the observations made from characterization experiments, it appears that the cells used in this study conformed to the standards set by the ISCT, which thus strongly suggests that human cells used in our experiments were of MSCs or similar mesenchymal origins (Dominici et al., 2006). This presents an important step for our study since the cell used in this study would need to be of a specified cell lineage, in this case of purely of MSC lineage.

Researchers have harvested MSCs using several technique, some rather crudely, resulting in non-homogenous cell population. Some studies used monoclonal antibody magnetic beads and flow cytometer techniques to select MSCs from marrow, but this may affect the cell potential or results in lack of cellular activity when used *in vitro* (Deryugina & Müller-Sieburg, 1993). The use of ficoll gradient centrifugation method was thus proposed in this study in order to harvest mononuclear cells and obtain bone marrow derived hMSCs, similar to that reported in other studies (Ahmadbeigi et al.,

2012; Haack-Sorensen et al., 2008). This method is relatively simple and can easily be used to obtain fairly high counts of fairy pure (homogenous) MSC population. This isolation method has also demonstrated heterogeneity when being cultured; containing other cell types including fibroblasts, hematopoietic progenitor cells, macrophages, endothelial cell and adipocytes (Hubin et al., 2005; Phinney et al., 1999). Therefore, changing growth medium at first 4 days instead of 1 week, was imperative to reduce the adhesion of non-MSCs to the plastic surface of the culture flask. There are numerous references available on the characteristics of hMSCs which one can refer to (Delorme & Charbord, 2007; Mok et al., 2008; Reger et al., 2008). Adherence to plastic surface is one of the basic criteria that have to be demonstrated by the isolation of MSCs from bone marrow. Unattached cells which are presumably haematopoietic progenitor cells were gradually depleted during the change of medium. Adhered MSCs exhibit fibroblastic morphology (Barry & Murphy, 2004; Docheva et al., 2007). In this study, MSCs tend to form single colony-forming-unit fibroblastic (CFU-F) and exhibited fibroblastic morphology which is agreement to the earlier statement. hMSCs at P0 to P3 consisted of elongated fibroblastic-like cells which aligned themselves end to end along their axis upon reaching 80% confluence, consistent with previous studies (Mok et al., 2008; Wagner et al., 2005). Long term *in vitro* expansion of cells may hold several disadvantages which can include culture contamination, telomere shortening, clonal evolution, and carcinogenicity (Kindler et al., 2006; Tolar et al., 2007). The use of earlier passage cells is recommended in order to prevent such complications. In this study, passage-3 hMSCs were used in mechanical stimulation test, since cells of higher passage (P5 and above) exhibited slow proliferation rate with some of the cells forming larger and flatter phenotypic expression, with obvious bipolar or multipolar prominences. Other researchers such as Ma et al. (2005), have also demonstrated similar phenotypic changes in cells obtained from umbilical cord Wharton's Jelly-derived

MSCs. Cells which were initially of spindle shape appear to process bipolar morphology as they undergo from 3rd to the 8th passages, finally appearing as nerve-like cells. It is presumed that in the early passages, MSC cultures contain largely of 'early primitive' cells which may consist of rapidly dividing progeny cells. As passaging continues, many of these MSCs reach their division limit and exhibit slow division rates. As the cultures approach high density, the hMSCs enter a stationary phase and transform from a spindle-like morphology to a spreaded morphologies (Sekiya et al., 2002). Moreover, it has been clearly demonstrated that repeated passages progressively reduce the multi-lineage differentiation ability, introducing a further element of complexity (Muraglia et al., 2000).

The presence of MSCs population was further confirmed using the second set criteria of the stem cell society. The cells that used in the present study was found to express positive to CD29, CD44, CD73, CD90, and CD105 while negative to CD14, CD34, CD45 and HLA-DR, indicating that the cells were not of hematopoietic origin (Deans & Moseley, 2000). These surface markers are considered for the immunophenotyping and identification of MSCs of different sources (Carvalho et al., 2011; Maleki et al., 2014; Sabatini et al., 2005) such as the use of flow cytometry and immunohistochemistry. Dominici et al. (2006) suggested that 95% or more purity of the MSCs population can be speculated from the population of the cells expressing CD105, CD90 and CD73, along with 2% or less for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. Flow-cytometry technique similar to that of their study was performed in our study. Flow-cytometry is widely used owing to its ability to determine the specific percentage of cells of interest observed by the labeled celles using special surface marker. Cultured MSCs are uniformly and strongly positive for CD105, CD90, and CD73, regardless of their passage or time in culture. However, CD105 and CD73 are also expressed on skin fibroblasts and umbilical vein endothelial cells (Chan et al., 2004; Ishii et al., 2005).

This implies that sole demonstration of CD105 and CD73 expression without CD90 on adherent cultured cells is insufficient to prove their MSC identity. In this present study, more than 95.0% of the hMSCs expressed CD73, marker for lymphocyte differentiation, and CD29, the integrin β1 receptor. The expression was 97.1% positive for CD44, the transmembrane hyaluronate receptor for osteopontin, ankyrin, and fibronectin. Moreover, most cells expressed the surface antigen CD105/endoglin, a putative marker for MSCs, and CD90, T cell surface glycoprotein. Our findings on surface antigen expression in hMSCs were consistent with the observation reported by other studies (Kilic et al., 2007; Meligy et al., 2012). We can therefore suggest that P2 culture employed for our study contains homogenous or at least a near-homogenous population of MSCs. The basic of characterizations of MSCs, including plastic adherence, fibroblast-like cell morphology, and the expressions of some surface antigens, seem to indicate that the cells in our study were hMSCs. In this experiment, we also successfully established the protocols for trypsinization, propagation, freezing and thawing of the isolated hMSCs.

The uniqueness of the MSCs is the ability of these cells to commit into multi-lineage phenotypes of mesenchymal origins such as osteogenic, chondrogenic and adipogenic under specific *in vitro* culture condition (Deans & Moseley, 2000). This is also a part of criteria for MSCs where these cells should able to differentiate into aforementioned tri-lineage phenotypes. In this study, culture expanded and osteo-induced cells constituted calcium deposits that were demonstrated by staining of the cells using Alizarin Red S. Different methods have been used for this purpose, including pycrotionine stain (Bilir et al., 2000) and Kossa technique (Gotoh et al., 1995). Other methods to confirm osteogenic lineage include the measurement of alkaline phosphate activity, type-1 collagen development, bone gla protein that can be stimulated with Vitamin D3 and osteocalcin (Yamamoto et al., 1991). While adipogenic and osteogenic differentiation

experiment were performed on monolayer cell cultures, the chondrogenic culture was conducted in the form of a pellet, as well as Safranin O staining, which was mildly different from that of other studies (Wang et al., 2003b; Worster et al., 2001). This is because chondrocytes isolated from human may undergo dedifferentiation when cultured as monolayer, and even experience a decrease in collagen and cartilage protein synthesis. This suggests that chondrogenic induction demands for conditions whereby close contact between progenitor or precursor cells has to occur (Imabayashi et al., 2003).

Lastly, one concern has to be addressed in this study is the age of the MSCs donors. Using MSCs from older patients is a contentious issue since it is generally assumed that cell proliferative and differentiation ability reduces with age. While several publications appear to support this (D'Ippolito et al. 1999, Muschler et al. 2001), others have suggested for this not to be the case (Leskelä et al. 2003, Phinney et al. 1999, Scharstuhl et al 2007). In this study, we had made the assumption that age was not an issue since in all our MSC cultures, cell confluence was reached within 14 days, thus demonstrating that proliferation was not an issue.

3.6 Conclusion

In summary, although a completely pure and homogeneous culture of MSCs is difficult to obtain, our study have demonstrated fairly good isolation results. These cells were also well characterized based on morphology, surface markers expressions and their differentiation potential. Thus this section of our study confirms that the MSCs from adult human bone marrow are valid for the purpose of the subsequent experiments.

CHAPTER 4: THE EFFETCS OF CYCLIC TENSILE LOADING ON MORPHOLOGY AND PROLIFERATION OF HUMAN MESENCHYMAL STROMAL CELLS

4.1 Introduction

In this chapter, we discuss the outcome following our experiment involving the use of uniaxial cyclical tensile strain activity to enhance cell growth and cell proliferation of hMSCs. It has been demonstrated that the rate of MSC proliferation in monolayer environment is slow and possess a problem which needs to be addressed in view of the need to obtain sufficient number of cells within a reasonable time frame for various purpose, including for therapy. *In vitro* growth and activities of MSCs are supported and enhanced by biochemical cues, such as those which are found in cell culture medium. These include growth factors, cytokines, and hormones. However, it is recently reported that mechanical forces also play a central role in the physiological process involving a wide variety of tissues (Kaspar et al., 2002; Shalaw et al., 2006; Song et al., 2007). Cyclic uniaxial strain applied on elastic substrates causes changes in cell behaviour, orientation and alignment. The application of mechanical force also influences cell growth and cell proliferation. Hence, the aim of this study is to investigate the effects of uniaxial cyclic stretching on the morphology and proliferation of human bone marrow derived MSCs. Further interest into this also involves the investigation of the effects of continuous cyclic tensile strain on alignment of hMSCs. To the best of our knowledge, this is the first study to determine the relationship of the outcome parameters using a combination of strain and frequency stretching rates. The results of this study may have wider implications for future research as well as clinical applications since the role of specific mechanical loading characteristics may provide the optimal and desired response from undifferentiated cells, which may include progenitor cells and MSCs. These optimized cells hypothetically may have the capacity to promote tissue repair,

which can either be obtained intrinsically from existing tissues or even be implanted from external sources such as bone marrow.

4.2 Literature Review

Molecular and mechanical mechanisms that control the development and maintenance of musculoskeletal tissues have been progressively uncovered in recent years. It is a well-established fact that mechanical loading plays an important role in maintaining the function of human tissues, especially that which involves the musculoskeletal system (Shwartz et al., 2013). When tissues are not mechanically loaded for prolonged periods, disuse atrophy and in some cases tissue degeneration inevitably occur resulting in the complete loss of their physiological function (Mueller & Maluf, 2002; Nakano et al., 2012). An extreme but good example that demonstrates this effect is in astronauts that are subjected to zero-gravity environment for prolonged periods. It is reported that many, if not all these astronauts will suffer from severe osteopenia, muscle atrophy and tendon weakening when subjected to long-term weightlessness environment. To overcome this problem, astronauts are required to frequently and strictly undergo cyclical exercises to reverse the atrophic effects of weightlessness in space (Lester et al., 2013; Tesch et al., 2013).

Although mechanical loading is generally accepted as the requirement for tissue homeostasis, it is not unreasonable to expect that the types and characteristics of mechanical loadings would not be universally appropriate for all tissues. It has been described in the literature that the application of mechanical loading onto specific cell types have demonstrated different responses, and it has generally been shown that the type of responses also depends greatly on the type of mechanical stimuli perceived (Altman et al., 2002; Boo et al., 2011; Hsieh et al., 2000; Kulik & Alvarado 1993). In more robust studies, many researchers have attributed these changes to a complex and

hierarchical process by which mechanical signals are transmitted into the cells, which then directly changes the gene or protein expressions of these cells (Calderwood et al., 2000; Ingber, 2006). This process, known as mechanotransduction, has also been implicated in the changes observed in many other facets of cell behaviour, which includes cell proliferation and cellular differentiation (Lim et al., 2010; Wang & Chen, 2013).

There have been reports that this phenomenon can be exploited if appropriate apparatus that has the ability to induce cell deformation in a controlled environment are used (Brown, 2000). With this, cell behaviour can be altered and, to a certain extent be tightly regulated (Kim et al., 2009a). This has enormous implications when applied to multipotent cells, which inherently have a high degree of self-renewal capacity as well as the ability to undergo multi-lineage transformation (Kelly & Jacobs, 2010; Kreja et al., 2012). It has been suggested that if multipotent cells such as human bone marrow derived MSCs are subjected to the appropriate mechanical loading, cellular pre-programming can be expected. It is further postulated that by implanting these cells into damaged tissues, good tissue repair outcomes are more likely to occur owing to the physiological loading nature of the environment that the cells are introduced to (Lee & Hui, 2006; Zhang et al., 2009). Heretofore, several studies have demonstrated that the implantation of MSCs may result in superior outcome, such as those in tendon repair (Awad et al., 1999; Kryger et al., 2007). It has been deemed that mechanical stimulation, as a regulatory process, plays an important role in cell proliferation (Ingber, 1997). This process, which results in chemico-biological cell response have been previously elucidated and demonstrate (Kaspar et al., 2002; Kostenuik et al., 1997; Xu et al., 1998; Zeichen et al., 2000) and yet, the appropriate mechanical stimulation, which induces and promotes hMSCs proliferation, remains largely unknown. The present

study was thus conducted to determine the optimal settings that may results in best cellular proliferation.

4.3 Materials and Methods

4.3.1 Mechanical system setup

A commercial instrument (Model ST-140, STREX Co., Ltd, Cupertino, USA) was used to conduct experiments to determine the effects of cyclic uniaxial strained on hMSCs. The cell stretching system consisted of a control unit, a strain unit, and rectangular, and elastic silicone chambers (Appendix EB). The chambers were used in the strain unit driven by an eccentric motor that allowed variation in magnitude and frequency of the applied strain. The chambers were 51 mm long, 35 mm wide, 10 mm high, and the wells had a 32 mm x 32 mm cell culture surface. New chambers were autoclaved at 121ºC before the cells were seeded.

4.3.2 Silicone surface coating and cells seeding

Autoclaved transparent elastic silicone chambers that have a potential growth area of 10 cm^2 were coated with 0.02% collagen type I overnight to compensate for the difficulties in cell adhesion and growth due to the hydrophobic nature of the silicone culture flasks. A total of 10^5 hMSCs was seeded into each silicone chamber of every flask. After 48 h of culture, the medium were replaced with medium containing 1% FBS and kept for 24 h. This was done in order to achieve a synchronized condition (by arresting the cells at the G_0/G_1 stage of their cell cycle progression) at the beginning of each experiment. To re-enter the cell cycle, the next step involved the replacement of the cell culture medium with a standard growth medium containing 10% FBS that has no additional growth factors. The silicone chambers were then mounted to a mechanical stretch device, in which hMSCs were exposed to cyclic uniaxial stretch generated by stretching the elastic chambers in one direction (Figure 4.1). Different frequencies (0.5

Hz, 1 Hz) and strains (4%, 8%, 12%) were applied to the hMSCs in the culture environment. Specimens were collected over different time points (6 h, 24 h, 48 h, 72 h) throughout the experiments. The frequencies and strain magnitudes were chosen based on our preliminary optimisation data (Appendix F) and that which was previously published (Couppé et al., 2008; Lee et al., 2011; Wang & Thampatty, 2006). Control cells were maintained in quiescent culture conditions but were cultured in a similar silicone chamber culture flask used during the stretching experiments in order to ensure that a valid comparison can be made.

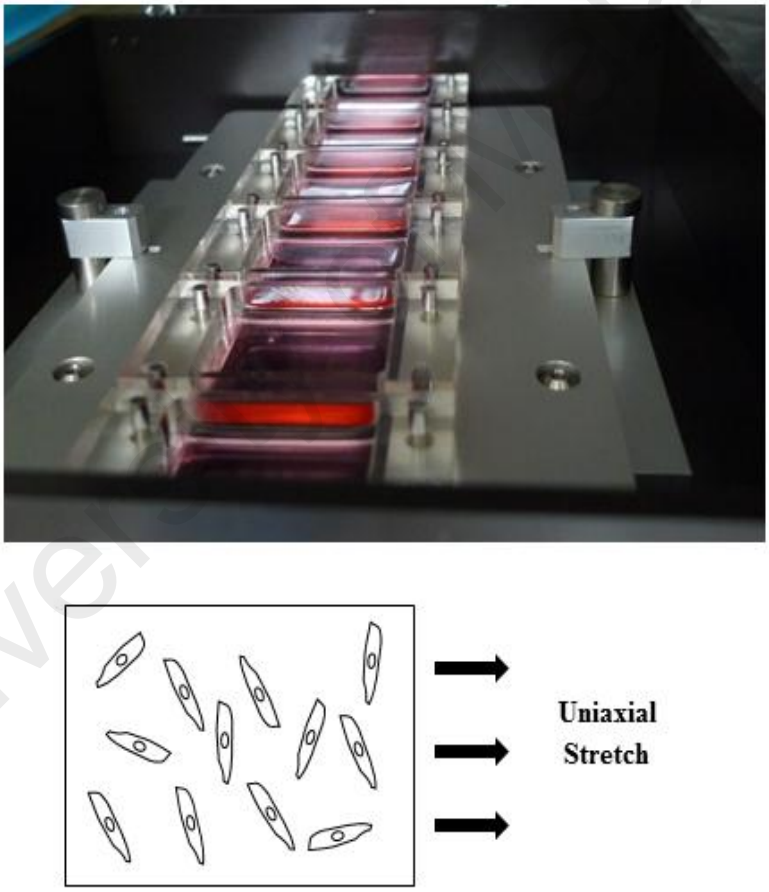

Figure 4.1. System for cell culture with mechanical cyclic uniaxial stretching hMSCs were exposed to cyclic uniaxial stretch generated by stretching the elastic chambers in one direction. For control sample, no mechanical stimulation was applied.

4.3.3 Microscopic evaluation

4.3.3.1 Phase contrast microscopy imaging

To compare the morphology and alignment of the unstrained and strained cells on elastomeric substrate, microscopic images of the cells were obtained using an inverted tissue culture CCD camera-assisted microscope in at least four randomly selected visual fields at 6, 24, 48 and 72 h following mechanical stretching.

4.3.3.2 Laser confocal microscopy imaging

To observe the cellular cytoskeletal arrangements of the stretched and un-stretched cells, the degree of actin filament organization in the hMSCs was evaluated using a fluorescent F-actin marker, i.e. fluorescent phalloidin. The cells were fixed with 4% paraformaldehyde for 30 min. The fixed cells were then permeabilized with -20ºC acetone for 5 min. The specimens were rinsed twice with 1X PBS, and incubated with 1% bovine serum albumin for 30 min to reduce non-specific background staining. Subsequently, each sample was stained with fluorescent phallotoxins for 30 min, washed 3 times with PBS before incubated with Hoechst for 10 min to label the nuclei, and washed again 3 times with PBS. The humidity of stained specimens was maintained with PBS, and the images of cytoskeleton structure were captured as Z-series sections (30 sections) images using a confocal microscopy system. By LAS AF Lite software, the fluorescence images with sequential scanning to avoid fluorescence signal cross-talk; and line averaging (8 lines) was used to enhance the quality of the image.

4.3.3.3 Atomic force microscopy imaging

To determine the surface morphology of unstrained and strained cells, an atomic force microscope (AFM) fitted with a silicon-nitride cantilever was used (Figure 4.2). The walls of the elastic chambers were cut for viewing, and AFM measurement was performed on the 4% paraformaldehyde fixed cells in PBS. The peak force and height

of the silicone surface and the attaching cells were then examined. The AFM measurements were obtained using ScanAsyst-air probes (silicon-nitride cantilever) with nominal spring constant of 0.4 N/m and the nominal tip radius value of 3.5 nm. AFM images were collected from each sample and at random spot surface sampling, at least five areas per sample, and the selected representative images were shown. During the entire experiment process, the cells were adhered to the substrate (slide).

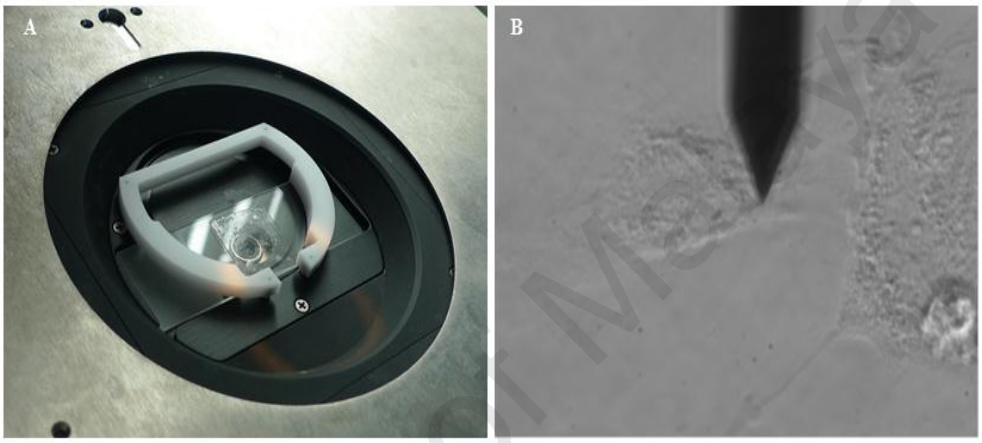

Figure 4.2: The AFM setup
A: The specimen on the slide was mounted on the AFM scanning stage. B: Imaging on the specimen was perfomed with the AFM probe tip.

4.3.3.4 Scanning electron microscopy imaging

For the microstructural analysis, the samples were examined using scanning electron microscopy (SEM). The cells were fixed in 2.5% glutaraldehyde overnight before being further processed. The samples were then rinsed with 0.1 M sodium cacodylate buffer 3 times before being fixed again with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature. Samples were then rinsed with distilled water, and then in a series of dehydration solutions through a graded ethanol series (50, 70, 80, 85, 90, and 95, 100%) for 15 min each (2 times 100%); acetone mixture (pure ethanol + pure acetone; ratio 3:1, 1:1, and 1:3) for 20 min each; and in pure acetone for 20 min each (4 times). Critical point drying was done to remove the alcohol using critical point

dryer Bal-Tec CPD 030. The specimens were mounted on the holder with carbon resin, followed by gold-palladium coating through the use of sputter coater. Sputter coating for SEM is the process of applying an ultra-thin coating of electrically-conducting metal, such as gold (Au), gold/palladium (Au/Pd), platinum (Pt), silver (Ag), chromium (Cr) or iridium (Ir) onto a non-conducting or poorly conducting specimen. Sputter coating prevents charging of the specimen, which would otherwise occur because of the accumulation of static electric fields. It also increases the amount of secondary electrons that can be detected from the surface of the specimen in the SEM and therefore increases the signal to noise ratio. Finally, the cell images were taken by a Jeol JSM-6400 SEM, using beam energies of 10 kV.

4.3.4 Live/Dead® viability/cytotoxicity staining

To investigate whether cyclic stretching results in any untoward effect to the viability and attachment of cells, samples from 1 Hz and 8% strained hMSCs were tested using a live/dead viability/cytotoxicity kit. The cells were incubated in a staining solution containing 2 µM calcein acetoxymethyl ester (calcein AM) and 4 µM ethidium homodimer-1 (EthD-1) for 10 min in the dark. Images of the centre and the two ends of the silicone chamber were captured using a fluorescence NIKON-ECLIPSE-TI-U microscope. Analysis was done using NIS-ELEMENT AR software. At least two different images were obtained at each location. The area (expressed in percentage) that are occupied by live and dead cells in each image was measured and calculated.

4.3.5 alamarBlue® cell proliferation assay

The number of cells in different culture conditions was determined at predetermined time points (6, 24, 48, 72h) directly and indirectly. Direct methods include cell counting using trypan blue dye exclusion method whereas indirect methods which measure metabolic activities include alamarBlue® (AB) colorimetric quantitative analytical

principle. AB is an oxidation-reduction indicator that changes colour from purple to red fluorescence when reduced by cellular metabolic activity. This assay is rapid, sensitive and non-toxic florescence cell viability assay. At 6, 24, 48 and 72 h, the unstrained and strained cells were washed with PBS and 3 ml of a 10% AB solution was added to each sample. After 4 h of incubation (37ºC, 5% CO_2), protected from light, 100 µL of the alamar-containing medium was collected and transferred in 96-wells plate. The absorbance measurement (optical density, which is directly proportional to the cellular / mitochondrial activity) was read on a micro-titer plate reader at 570 nm wavelength while using 600 nm as a reference wavelength. All samples and standards were assayed in octuplicate in the 96-well plates. Signal intensities from pre-prepared samples were plotted to form a standard curve. This was used to infer the values to determine the cell numbers i.e. hMSCs cultured at different cell densities. The calculation to determine the percentage of AB reduction was as recommended by the manufacturer's protocol:

$$\% \text{ AB reduction} = \frac{(\varepsilon_{ox}\lambda_2)(A\lambda_1) - (\varepsilon_{ox}\lambda_1)(A\lambda_2)}{(\varepsilon_{red}\lambda_1)(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(A'\lambda_1)} \times 100$$

In the formula, $\varepsilon\lambda_1$ and $\varepsilon\lambda_2$ are constants representing the molar extinction coefficient of AB at 570 and 600 nm, respectively, in the oxidized (ε_{ox}) and reduced (ε_{red}) forms. $A\lambda_1$ and $A\lambda_2$ represent absorbance of test wells at 570 and 600 nm, respectively. $A'\lambda_1$ and $A'\lambda_2$ represent absorbance of negative control wells at 570 and 600 nm, respectively. The values of % AB reduction were corrected for background values, using medium without cells as the negative control group.

Population doubling level (PDL) was used to describe cell growth in a logarithmic mode, by using the following equation,

$$PDL = \frac{\log (X_1/X_0)}{\log 2}$$

where X_0 is the initial cell number and X_1 is the final cell number.

4.3.6 Cell cycle assay

Cell cycle analysis was performed by DNA content analysis of PI-stained cells after being loaded at 1 Hz, 4% in 6 h or 72 h (variables was chosen based on the cell proliferation results). The experimental pellet cells were collected, fixed and permeabilized with cold absolute methanol for 30 min. The cells were then incubated in 500 µL of PI/RNase staining buffer for 15 min in the dark condition, and placed on ice until flow cytometric analysis. Primary histograms were generated by gating the cells according to the surface and the peak of the fluorescence signal in order to avoid taking doublets into account. These histograms were then submitted to computerized calculations of the cell cycle by using Cellquest software. The percentage of cells for each condition, in each phase was determined. The DNA content was analyzed in at least 10,000 nuclei in each sample at a medium flow rate.

4.3.7 Cell migration assay

Wound assays were produced by scratching the surface of the silicone well, across the region where cells that we cultured as monolayer constructs, using the tip of the pipette as a scrapper. The debris and smooth edge of the scratched area were removed by washing the cells in a single flow using PBS. For strained cells (1 Hz, 4%), images were obtained at the initial time of wounding and at 6, 24, and 48 h thereafter. The unstrained cells were used as control.

4.3.8 Statistical analysis

The assays were carried out with a minimum number of technical triplicates (n) per experimental run, using six independent samples from different donors (N) for each group of the experiments. A summary of the number of samples and replicates used is summarized in Figure 4.3. The data were presented as mean ± 1 standard deviation (SD). One-way Analysis of Variance (ANOVA) test was used for statistical data analysis of the mechanically stimulated cultures and unstimulated controls, followed by Fishers Least Significant Difference (LSD) post hoc test for multiple comparisons. Statistical analyses were performed using SPSS software version 15.0 (SPSS Inc, Chicago, IL, USA), which a probability value of $p < 0.05$ was considered statistically significant.

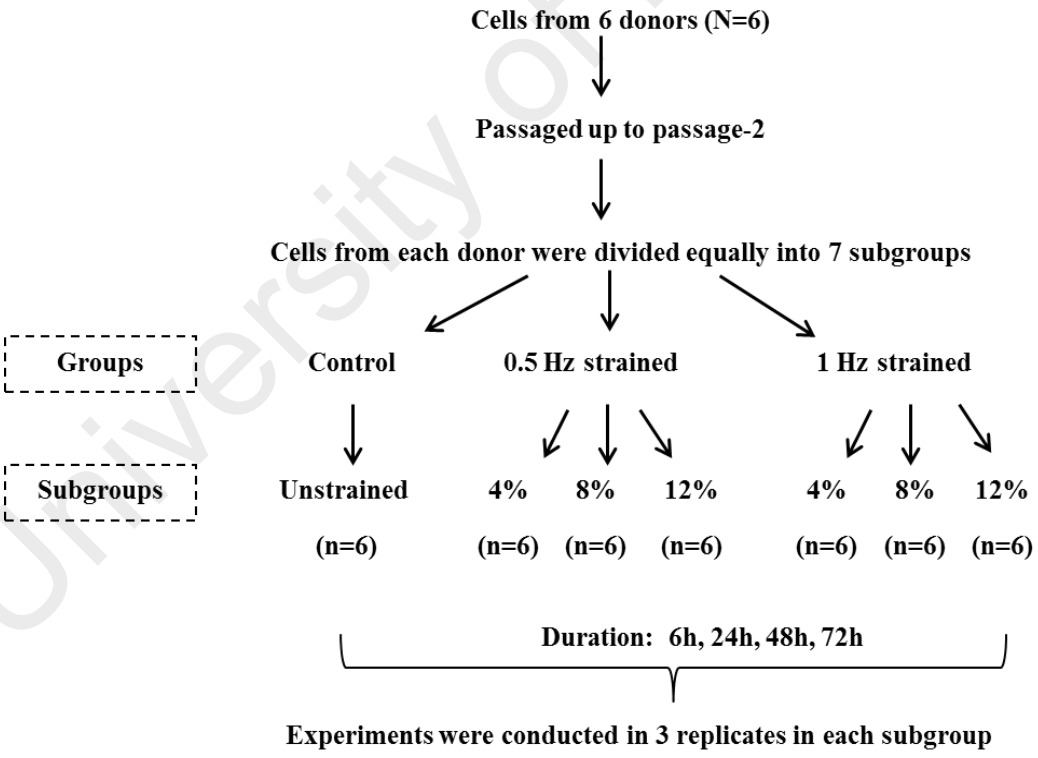

Figure 4.3: An illustration summarizing the experimental groups that were subjected to the different loading regimes used in the present study

4.4 Results

4.4.1 Changes in cell alignment subjected to at different frequencies and strains with time

Cell response appears to be affected by varying the strain magnitude and frequency. Morphological changes in MSCs which results in apparent cellular re-alignment or re-orientation were obvious after being subjected to 24 h and 6 h of stretching, at 8% and 12% respectively (Figure 4.4). However, the unstrained MSCs showed no particular preference in cell orientation. It is found that the number of strained and unstrained cells both increased with time. After being subjected to cyclical uniaxial stretching, MSCs became elongated and were oriented perpendicular to the direction of stretching. At 1 Hz, increase in cell numbers was higher when subjected to 4% strain as compared to 8%. At 12% strain, changes in cell number appeared negligible. In terms of cell re-orientation or alignment, at both 0.5 Hz and 1 Hz, cells preferred to be reorganized in a direction which is in a direction perpendicular to the line of stretching, being more obvious at 1 Hz.

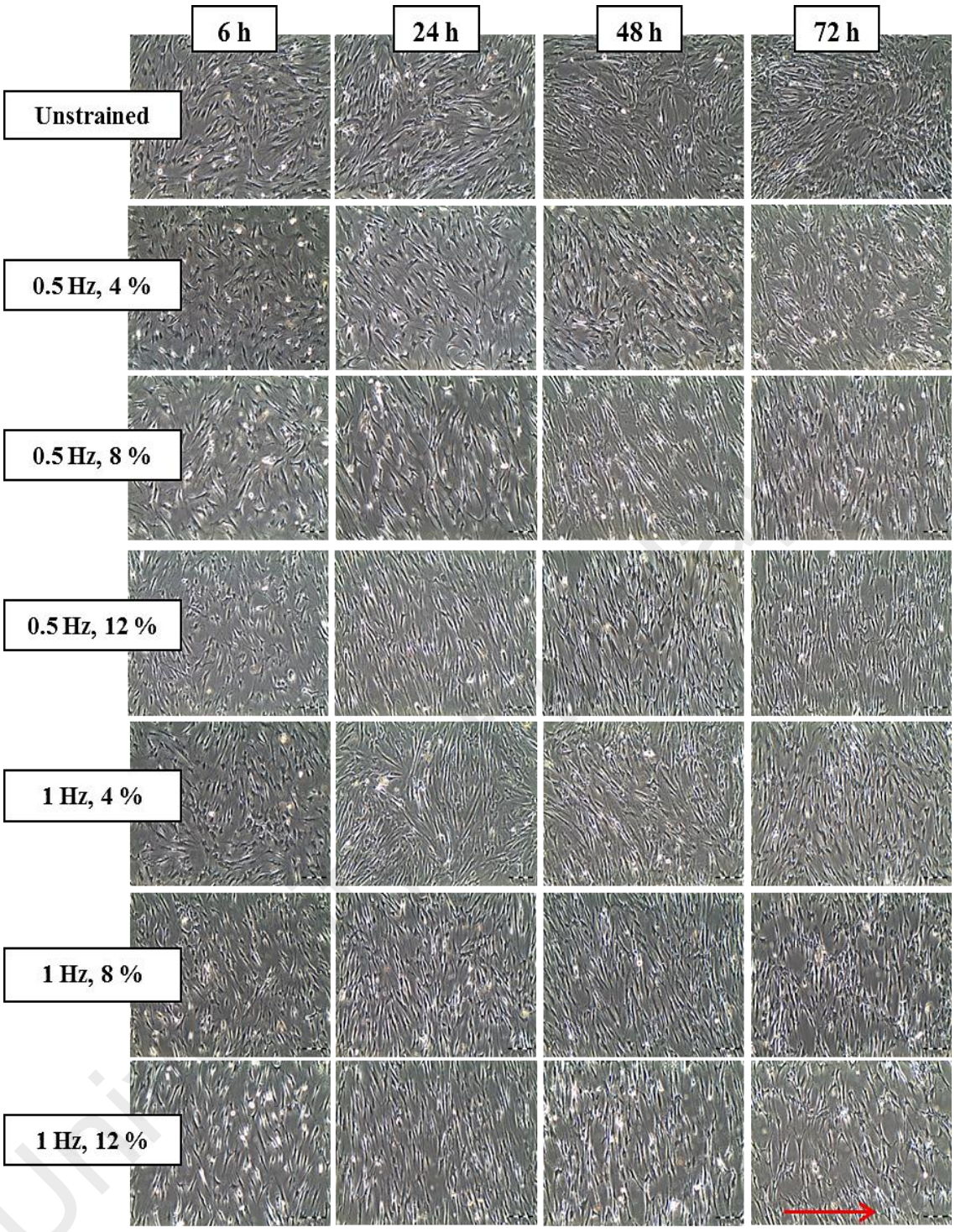

Figure 4.4: Phase-contrast photomicrographs of hMSCs subjected to cyclic uniaxial stretching at different magnitudes, frequency and duration of stretching
The substrate was stretched in the red arrow direction. Scale bar = 200 µm.

4.4.2 Changes in cell cytoskeleton subjected to cyclic loading

During this process of apparent cellular reorientation, the actin cytoskeletal re-organization became apparent as suggested from the images observed in Figure 4.5.

These images also demonstrate that the actin filaments of these cells tend to form thick stress fibers (Figure 4.5b). For the unstrained cells, the distribution of actin filaments in the cells appeared irregular. This was also in contrast to that of strained cells, which appeared to have, a more uniformed and unidirectional pattern of cytoskeleton reorganization. It has suggested from previous studies that these changes may be attributed to the adaptation of actin fibers due to stretching, which aims to minimize the amount of mechanical forces transmitted and therefore of the potential damage to the cell bodies (Choi et al., 2007).

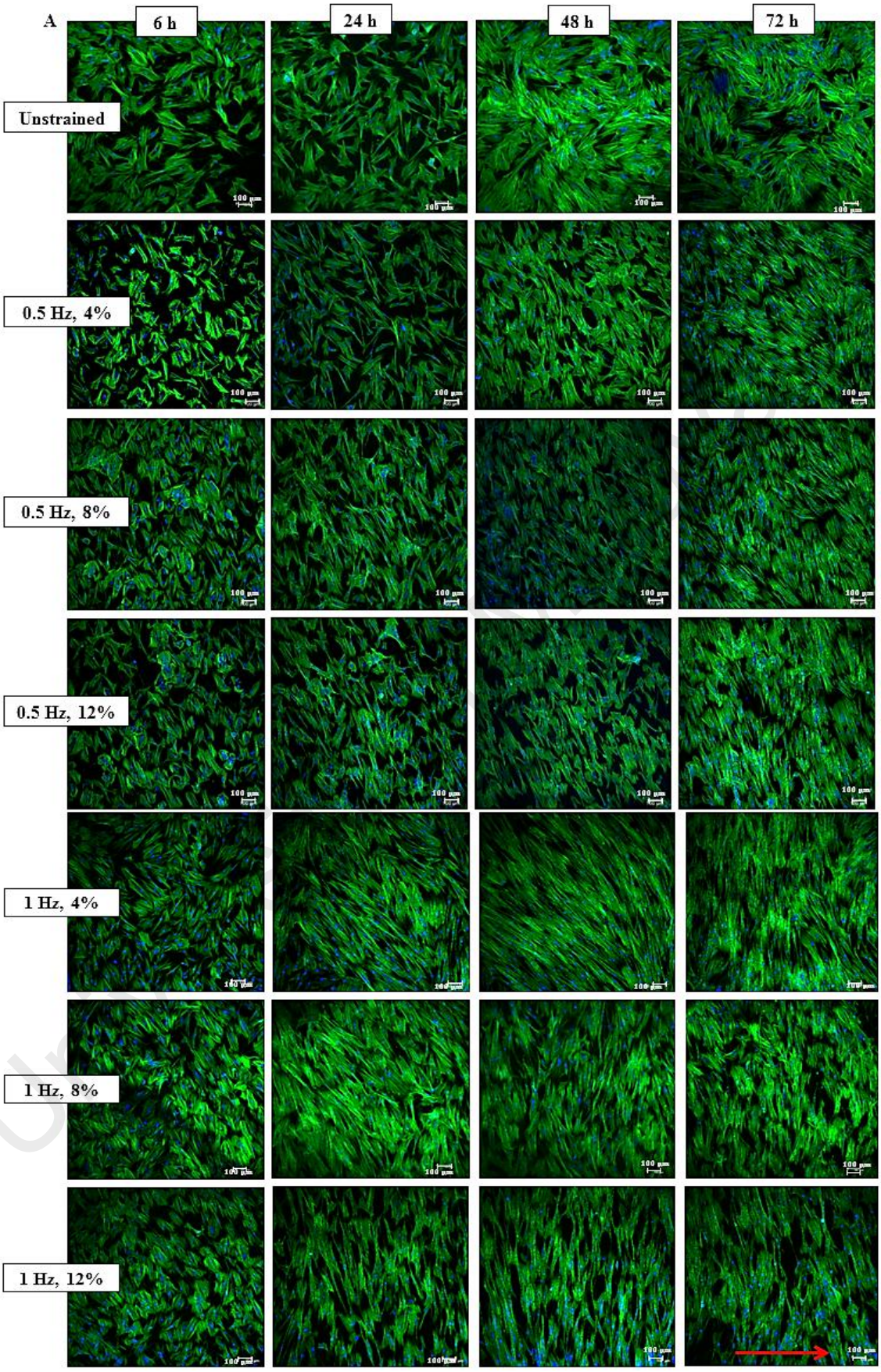

Figure 4.5: Confocal laser scanning micrographs showing actin stress fibers (green) and nuclei (blue) of cells

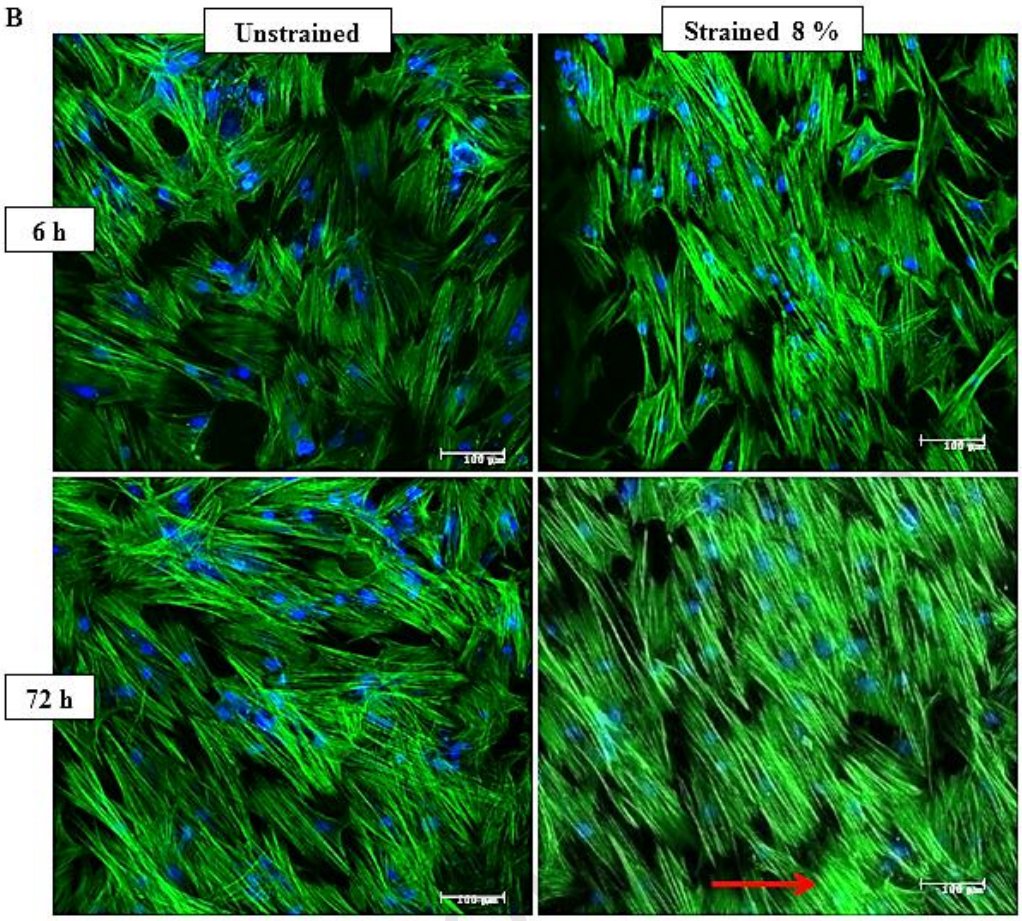

Figure 4.5, continued
A) The distribution of cytoskeleton on unstrained, 4, 8 and 12% strained hMSCs at 0.5 Hz and 1 Hz for 6, 24, 48 and 72 h, respectively. B) Cells at 1 Hz, at higher magnification, the actin stress fibers around the nuclei appeared denser and longer after 6 h, consistent with differentiation, than those in unstrained cells. After 72 h, the actin stress fibers were even denser. The direction of uniaxial strain was in the direction illustrated by the red arrow. Scale bar = 100 µm.

4.4.3 Changes in the topography of cells subjected to cyclic loading

Cells on silicone substrate are generally spread out in a random fashion and had rounded cell bodies with filopodia extensions in multiple directions (Figure 4.6). Using AFM images, we were able to view the changes of the cytoskeletal in greater detail. Images generated from the AFM demonstrated that when cells are subjected to uniaxial cyclic loading, cytoskeletal coarsening and elongation of the actin stress fiber occurs; which appears to be also observed in previous reports (Maloney et al., 2010). Figure 4.7 shows AFM topography images of cells. At the centre of the strained cell, the nucleus can be observed with surrounding coarsed cytoskeleton structures supporting the nucleus.

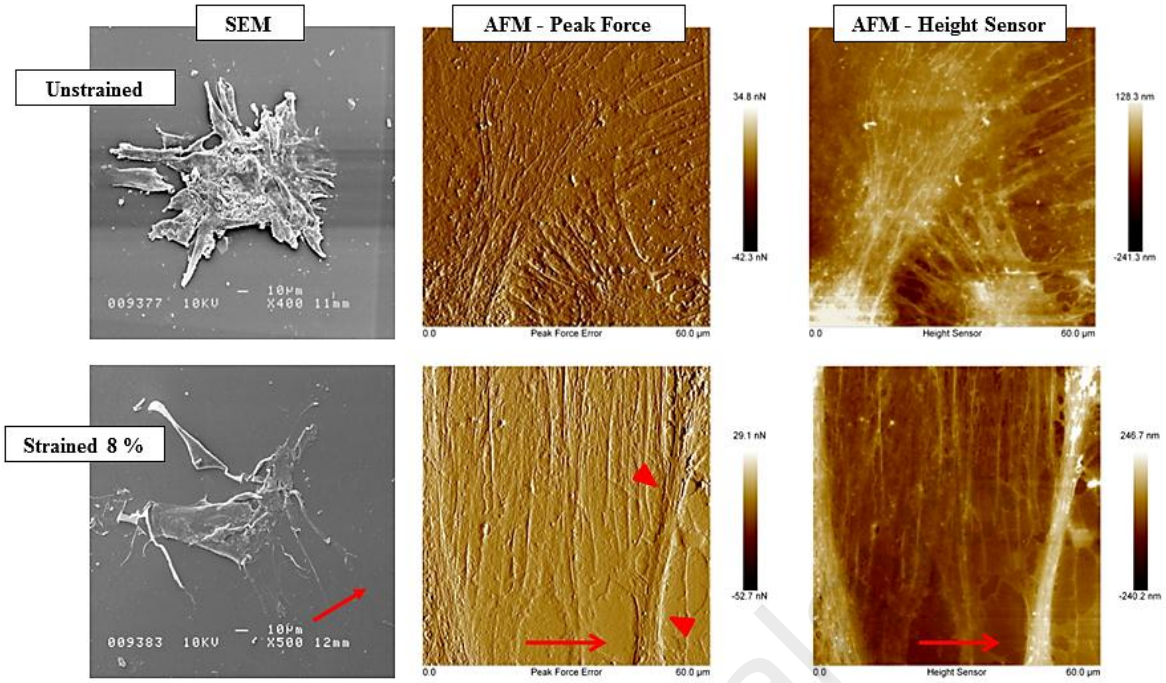

Figure 4.6: The changes in topography of cells after force was applied on it
The attachment and filopodia of unstrained and 8% strained hMSCs on silicone substrate were shown by SEM (scale bar = 10 µm), while the peak force and height measurement of unstrained and 8% strained cells were shown in stress fiber by the aid of AFM (scale bar = 60 µm). The strained cells displayed cytoskeletal coarsening and longer actin stress fiber (pointed by red filled triangle). The direction of uniaxial strain was in the red arrow direction.

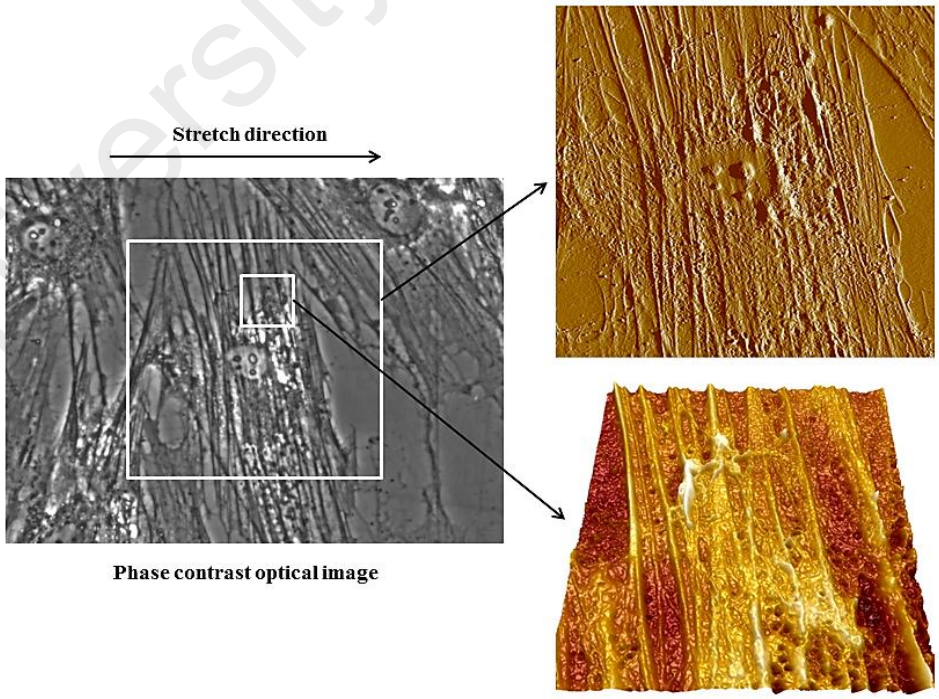

Figure 4.7: The changes of cytoskeleton morphology in different area (nearby nucleus)

4.4.4 Effects of mechanical stretching on hMSCs viability

Assessment at the centre and both ends of the stained silicone chamber revealed that there does not appear to be any differences in the cell morphology as well as the overall impression in cell numbers at areas located within the same culture chamber. Cyclic uniaxial stretching up to 72 h at 1 Hz did not cause extensive cell detachments as observed from the microscopy images (Figure 4.8).

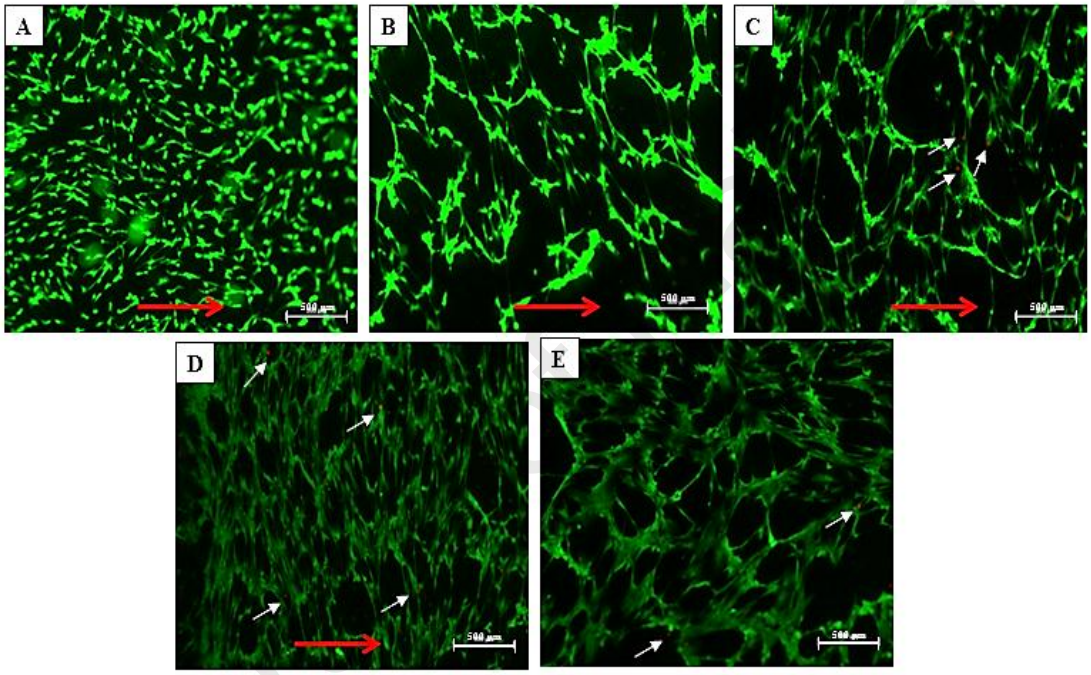

Figure 4.8: Live (green) and dead (red) cells in strained (1 Hz, 8%) and unstrained hMSC-seeded type 1 collagen-coated silicone membrane stained with calcein AM and EthD-1, respectively

White small arrows indicate dead cells. Cyclic uniaxial strained cells at 6 h (A), 24 h (B), 48 h (C) and 72 h (D), while unstrained cells were shown at 72 h (E). The direction of uniaxial strain was in the red arrow direction. Scale bar = 500 µm.

4.4.5 Effects of mechanical stretching on hMSCs proliferation

To infer the increase in cell number over time, a linear plot regression was made to determine the relationship between cell numbers using haemocytometer count with AB reduction. This data were used as a reference value for the succeeding proliferation experiments. The effects of different strain amounts and frequencies on the proliferation rate of hMSCs at different duration of stretching measured from the AB assay only

demonstrates a significant increase in cell proliferation as compared to unstrained cultures when subjected to 4% strain at 1 Hz particularly after 24 h of stretching (Figure 4.9C), with a PDL value of 2 achieved at 24 h. Differences in the hMSCs proliferation between the cultures treated with or without mechanical stimulus at 0.5 Hz were not significant regardless of the strain amounts used (Figure 4.9B), suggesting that proliferation rate of hMSCs is not influenced by stretching at lower frequencies, cell proliferation the physiological rate (1 Hz) is still preferable to hMSCs.

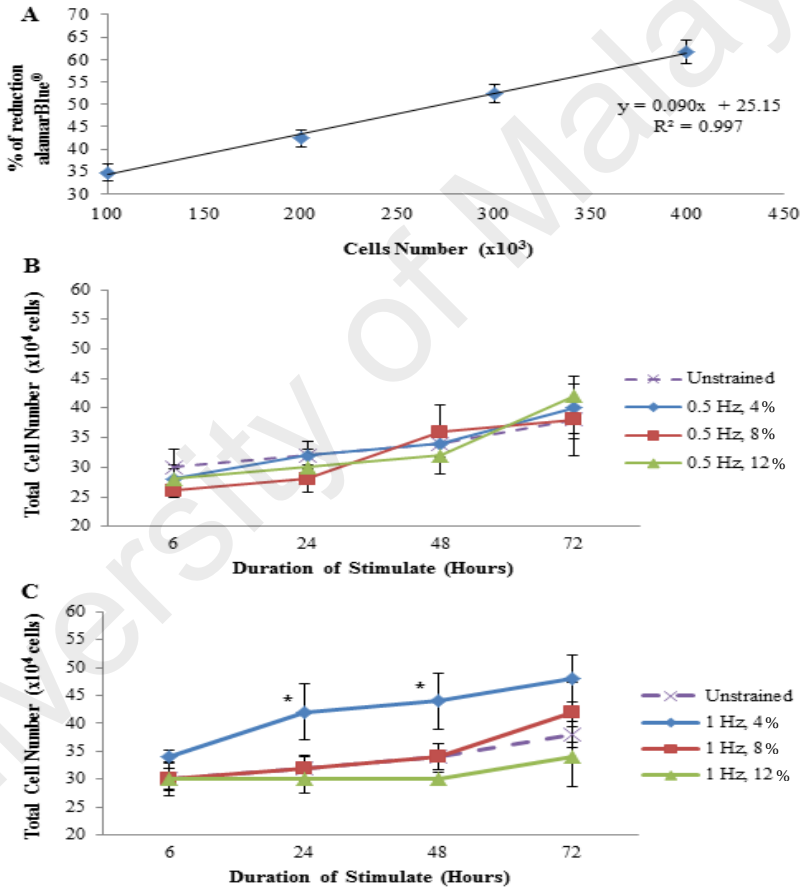

Figure 4.9: Effect of the mechanical stimulate on the growth of hMSCs according to strain amplitudes at 0.5 Hz and 1 Hz

(A) Standard curve showed a correlation between the percentage of AB reduction and cell numbers were carried by measuring the AB absorbance changes and numbers obtained using haemocytometer cell count by Trypan Blue. At 1 Hz (C), compared with unstrained group, a statistically significant increase in hMSCs proliferation was observed at low strain rate (4%) particularly at 24 and 48 h, but not in medium and high strain rate (8 and 12%). There was no significant difference in hMSCs cell proliferation rates at 0.5 Hz (B) with varying percentage of strain. Significance ($p < 0.05$) was represented by asterisk which compared with unstrained. N = 6, n = 3. Error bar = ± 1 SD.

4.4.6 Effect of mechanical stretching on hMSCs cell cycle regulation

To assess the effect of mechanical stretching on hMSCs proliferation at 1 Hz and 4% strained, we analyzed each phase of the cell cycle by flow cytometry. It was found that stretching for 6 h did not change the length of cell cycle phase compared with the non-stretched control (Table 4.1). In contrast, after 72 h strained, showed a significant different in G_0/G_1 and S phase in comparison with non-stretched cells (Figure 4.10). This suggests that the proliferation of hMSCs could be enhanced by longer stretching duration. There were no apoptosis observed following stretching.

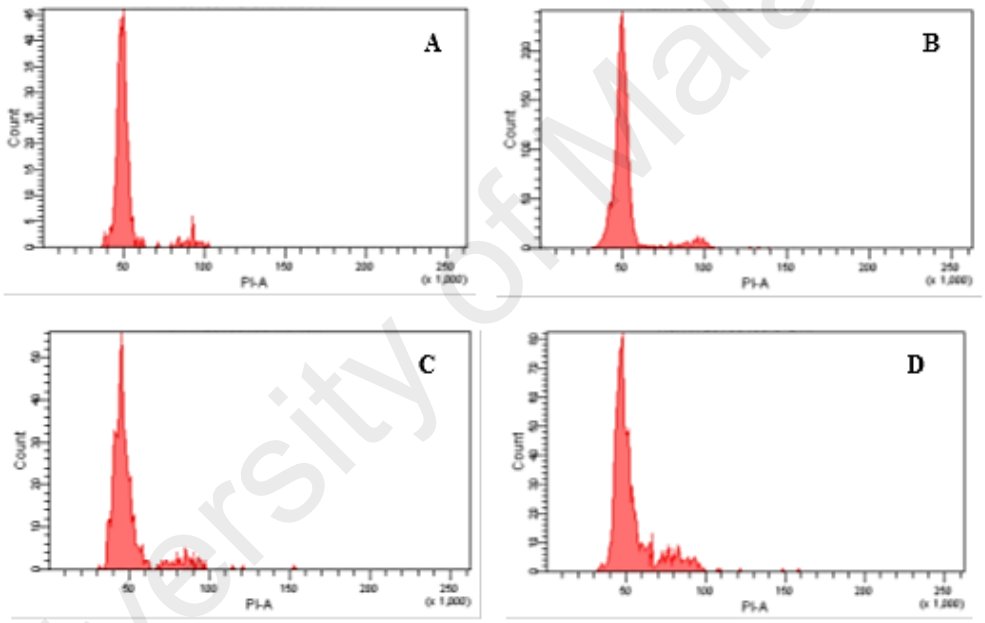

Figure 4.10: The effect of mechanical stretching on hMSCs cell cycle regulation hMSCs were subjected to cyclic stretch (4% strained, 1 Hz) for (B) 6 h, and (D) 72 h, with the control unstrained cells (A) 6 h, and (C) 72 h.

Table 4.1: The percentage of cells in each phase of the cell cycle was analysed by flow cytometry (Significance $p < 0.05$ was represented by asterisk which compared to unstrained; N=3)

Duration (h)	Condition	G_0/G_1 (%)	S (%)	G_2/M (%)	$p < 0.05$
6	Unstrained	91.6	2.0	6.4	
	Strained	90.7	2.4	6.9	
72	Unstrained	87.5	3.6	8.9	*
	Strained	78.5	10.8	10.7	

4.4.7 Mechanical stretch enhances cells migration

Since the group of 1 Hz and 4% strained shows the highest cell proliferation, a scratch test was further used to display the migratory capacity of mechanical strained hMSCs on elastic surface. It demonstrates earlier apposition of the cells subjected to 1 Hz + 4% at 6 h as compared to 24 h for unstrained cells (Figure 4.11).

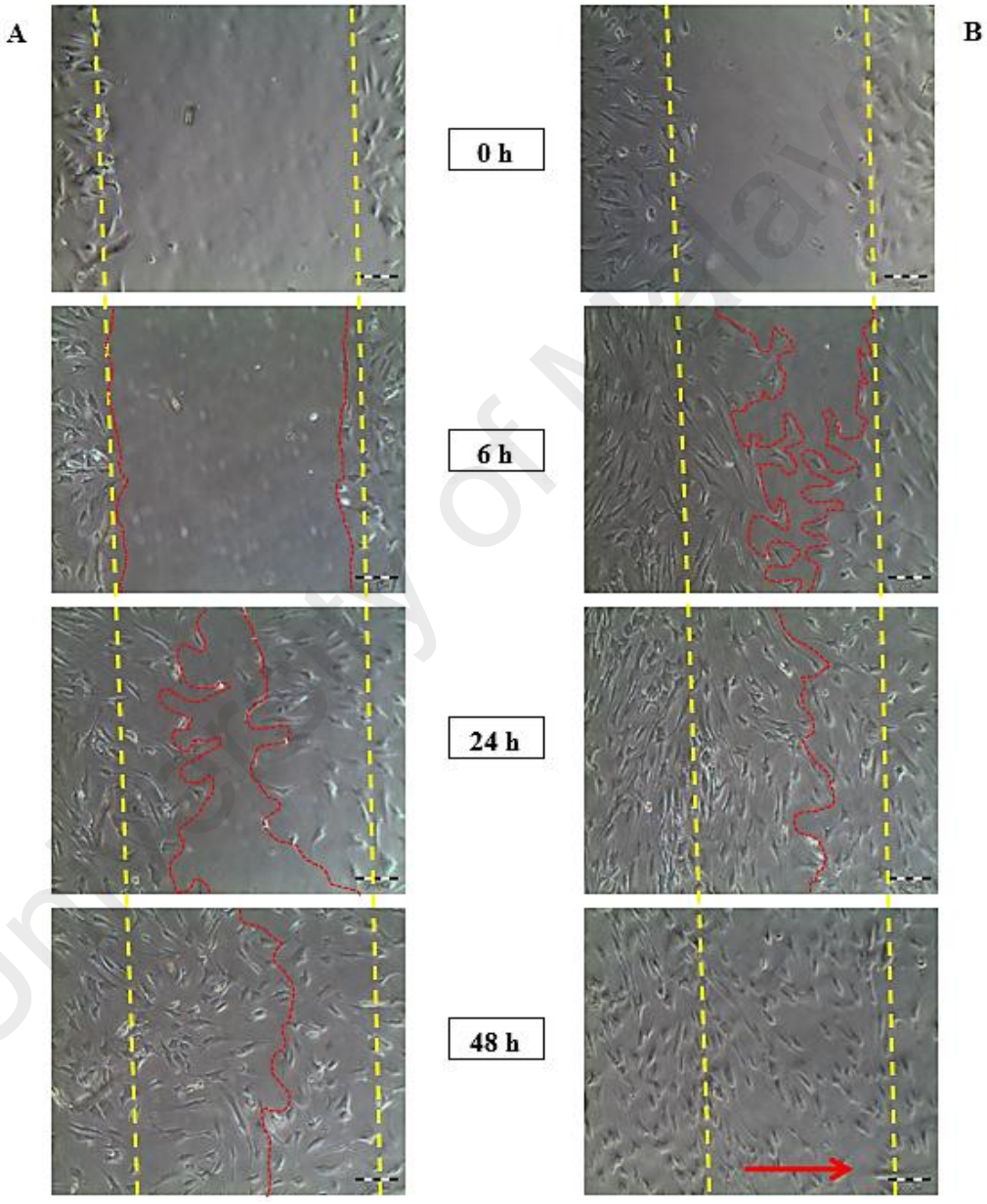

Figure 4.11: The scratch test was used to demonstrate the activity of 1 Hz, 4% strained hMSCs (B) as opposed to unstrained hMSCs (A)
The images taken after 0, 6, 24 and 48 h show that the cell migration of unstrained and strained cells fully crosses the border of the scratch after 48 and 24 h, respectively. The direction of uniaxial strain was in the red arrow direction. The yellow dotted line indicates the area of initial wound area, while red dotted line indicates the area of wound closure.

4.5 Discussion

In the present study, when hMSCs were subjected to uniaxial cyclic loading of various rates and amounts, a link between the rate and/or degree of the forces applied and that of the influence it has on the proliferation of hMSCs became apparent. It also appears that the morphological changes and the alignment of the cells along its longitudinal axis are somewhat related to the direction of stretching. This finding is similar to that reported previously (Chen et al., 2008b; Morita et al., 2013). A successful expansion of hMSCs can lead to future studies by thoroughly investigating the cellular metabolism and characterization of these cells. The choice of using MSCs for this study was obvious especially when several studies have demonstrated that the use of MSCs provided superior tendon repair outcomes (Chong et al., 2007; Ouyang et al., 2004).

The total number of 10,000 cells/cm^2 was seeded in the initial seeding for this study, rather than higher cell density, which was reported in several studies (Chen et al., 2008b; Kuo & Tuan, 2008; Maul et al., 2011; Shalaw et al., 2006). We found that at high cell densities, conditions may not be appropriate for proliferation study to be conducted, i.e. at a point where cell-cell contact or cell contact inhibition has occurred. This may probably reduce the proliferative capacity of BMMSCs cultures and therefore alter the outcome of this experiment. Contact inhibition is a pronounced depression of the mitotic rate in a post-confluence culture that displays a stationary density despite periodic nutrient renewal (Martz & Steinberg, 1972). Furthermore, the cells could also be entering G_0 phase of quiescence, hence cell numbers cease to increase (Stoeber et al., 2001). The surface cells growth area might not be sufficient to support the proliferation, which leads to death of cells, and become in the state of "anoikis" (homelessness) due to lack of nutrients and build-up of toxins.

Although there have been published data with regards to the effect of stretching on multipotent cells (Song et al., 2007; Maul et al., 2011), the present study is unique in that a specific combination of stretch duration, rate and amount results in different cellular outcomes. This was not unexpected since the previous studies do suggest that this is likely to occur given the fact that fibroblast, which is a common cell of mesenchyme origin found in several different tissues such as ligaments, capsules and cartilage, will alter its protein expressions when subjected to different mechanical loading regimes (Li et al., 2011). It is interesting to note that at 0.5 Hz, mechanical loading does not appear to influence hMSCs proliferation significantly, which is in contrast to previous reports. In a study by Lee et al. (2005), human MSCs cell lines subjected to equibiaxial stretching at 0.5 Hz and 10% elongation produced significant proliferation. Nevertheless, the data presented in our study appear to be consistent with at least one other study which investigated the effects of cyclic substrate deformation on the proliferation and osteogenic differentiation of hMSCs (Simmons et al., 2003). Their study showed that the application of equibiaxial cyclic strain (3%, 0.25 Hz) to hMSCs cultured in osteogenic medium inhibited proliferation. It is worth noting however, that the present study utilizes a uniaxial strain model as opposed to a biaxial one such as those reported in these studies. This may explain why a lower frequency may have led to the reduction in cell proliferation in their study since inadequate stress frequency and time will impede the stimulation of cells to proliferate (Horikawa et al., 2000). A study using uniaxial stretching on MSCs demonstrates that both of low frequency (0.1 Hz) and high frequency (1.5 Hz) are detrimental, only 1 Hz is optimal to MSCs proliferation (Song et al., 2008). Similar results are shown on osteoblast-like cells using uniaxial stimulation, combined an appropriate number of cycles with 1 Hz enhanced bone cell proliferation (Kaspar et al., 2002). We found that the best response was observed at 1 Hz, which appears to be similar to that reported by others (Kaspar et al., 2002;

Kurpinski et al., 2006; Song et al., 2008), thus suggesting that this rate should be employed in future studies to elicit maximum cell proliferation rate.

In the present study, mechanotransduction, which is the process by which cellular response is elicited as the result of mechanical loading, appears to be the mechanism driving cell proliferation and differentiation (Wang & Thampatty, 2008). Although many studies have demonstrated the relationship of this process in MSCs, the underlying molecular mechanisms of mechanotransduction have yet to be described in detail (Clause et al., 2010; Hamill & Martinac, 2001). It is thus very likely that future studies will center on the understanding of cell mechanisms (tensegrity and nucleus organization, metabolite use) and on the role of microRNAs and the interrelationship to the epigenome changes of cells, especially when considering that cells move between different environments and have different energy states. It has also been described that within the focal adhesions where integrins are connected and are also attached to the extracellular matrix, focal adhesion kinase (FAK) and Src, both tyrosine kinases, are up- and down-regulated with changes of the surface topography. It is also found to be due to the changes in the mechanical signals the cell receives. These kinases regulate G-proteins involved in cell filopodia and cell contraction, results in changes to the shape of the cells. As a related event, Rho A kinase (ROCK) activities are also altered as shown in previous studies (Dalby et al., 2014). In combination, these resulted in changes to the cell behaviour, leading to increase in cell numbers and cellular differentiation.

The elongation and alignment of cells was obvious and became increasingly stretched thereafter. This may have been a cellular adaptation response to stretching in order to minimize the strain energy required to maintain cellular tensegrity. This reduces the energy required to counter the stretching effects involved during the stretching of actomyosin fibers. Cell reorientation requires the continuous remodelling

of both focal adhesions and actin cytoskeletons (Hayakawa et al., 2001; Na et al., 2007) as explained in the earlier section of this thesis. This orientation is mediated by the activation of the Rho pathway, as inhibition of Rho perturbs the perpendicular orientation of stress fibers (Dalby et al., 2014). Isometric contraction of a cell would result in tension development in the stress fibers, which are anchored at their ends (Kaunas et al., 2005). The actin stress fibers, which are formed in response to cell contraction, need to be depolymerized and repolymerized in a new direction to re-establish a state of equilibrium, following an adaptive response by the cell to the stimuli it perceives (Tondon & Kaunas, 2014; Wang & Grood, 2000). In the absence of polarity, cells move in all directions simultaneously, which resembles spreading. Thus, it is believed that microtubules and filamentous actin are important for establishing and maintaining a cell's polarity. In the present study, these changes were also evident, and as a result of this mechanical stimulation, changes in cell alignment, cellular proliferation and differentiation (in the next chapter) were observed.

There is a presumption that the realignment of cells may be viewed as cell migration. However, in cell migration, a leading edge and trailing body can be observed, which is distinct from the apparent polarity (or polarities) seen when cells undergo reorientation. When cells are grown as monolayer cultures and subjected to "wounding assay", they respond to the disruption by encroaching into the voided spaces. It has been suggested that an increase in the concentration of growth factors as the result of the "injury" promotes cellular proliferation and migration of these cells (Lampugnani, 1999). The alteration in cytoskeletal protein patterns, binding of growth factors and interactions with ECM proteins and metalloproteinases through specific cells receptors (integrins) have also been suggested as mechanisms responsible for the cell migration into the voided area (Herard et al., 1996). In applying external mechanical force that results in mechanical signal transduction, the intracellular forces generated within the cell initiate

changes to the actomyosin cytoskeleton and focal adhesions complex formation. This results in the leader-cell formation which, in turns creates faster cell migration (Trepat et al., 2009). Therefore, it is not surprising that the wound assay demonstrated faster bridging of cells within the void area when subjected to cyclic stretching as opposed to a non-stretched environment.

In this study, we found that mechanical strain improved the proliferation of hMSCs, albeit when subjected to appropriate loading regime. However, the effect of mechanical stretch on the proliferation of hMSCs and the mechanism involved are still not completely understood. Thus, there is a need for further investigations such as the investigation of several genes markers, signalling pathway, and western blotting to obtain a more complete understanding of the biological mechanisms and pathways underlying the cellular responses to mechanical stimuli in future studies.

4.6 Conclusion

The results of this present study suggest that uniaxial strained MSCs may be an effective and promising approach to enhance the MSCs proliferating capacity by optimising the amount and frequency, and that this may also influence cell reorientation, which is an important behaviour required in cell migration. In short, we found that: (1) hMSCs has a preferential position and undergoes realignment in a perpendicular direction to the line of stretching, being more obvious at 1 Hz and higher strain, (2) uniaxial strains at 1 Hz was the ideal condition to obtain a maximal proliferation response, (3) mechanical stimulation influences the behaviour of hMSCs markedly in longer strain period (at least 24 hours), (4) strains at this frequency and at 4% produced the highest proliferation rate, (5) higher strainss of uniaxial tensile loading does not result in superior proliferation of MSCs but rather promotes hMSCs differentiation and cell expression (Chapter 5).

CHAPTER 5: MECHANICAL STRETCHING INDUCES HUMAN BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS TOWARDS MESENCHYMAL LINEAGE DIFFERENTIATION

5.1 Introduction

The customization of musculoskeletal tissues to mechanical load suggests fairly interesting possible outcomes. It is worth to note that the observations made may be related to several factors, of which many remains to be answers. The obscurity of the underlying mechanism may be due to (i) the type of cells that respond to a particular type of loading, (ii) the processes involved during the translational of mechanical signal into molecular signals, i.e. a process known as mechanotransduction, and (iii) the outcome from the cellular response to the stimulation, e.g. differentiation, proliferation, or simply cell morphological changes.

In order to study these changes and possibly answer the questions above, the present study was therefore conducted. Using uniaxial cyclic stretching at different time points and different strain rates on hMSCs, it is hoped that the experiments may produce results that can be extricated to produce a defining and meaningful explanation for the observed outcomes. It has to be made clear here that the focus of this chapter is to determine whether cyclic mechanical, i.e. tensile loading is able to drive the differentiation of hMSCs towards the mesenchymal lineage including adipogenic, chondrogenic, osteogenic and tenogenic differentiation. This is assessed through the gene expression analyses of the lineage specific markers and extracellular matrix (ECM) production related to mesenchymal lineage- specific markers. Since we hypothesize that the regulation of extracellular matrix remodelling as well as the expression of the differentiation of hMSCs to a particular cell lineage is dependent on the level and duration of tensile forces, the changes in cell morphology and stiffness are

also investigated. By determining the effects of mechanical stretch on hMSCs in a quantitative measure, it may be possible for us to have better understanding on the mechanical characteristics that governs tendon homeostasis thus enabling future potential therapies for tendons and ligaments to be advocated.

5.2 Literature Review

As mentioned in the Literature Review section of this thesis (Chapter 2), bone marrow derived mesenchymal stromal cells, which have the ability to undergo multi-lineage differentiation, have been shown to be therapeutically effective in treating tendon diseases (Ho et al., 2014; Moshiri et al., 2013). Despite demonstrating good efficacy, there have been concerns that undifferentiated cells may possibly progress towards unwanted cell lineages when transplanted into tissues, resulting in patient morbidity (Herberts et al., 2011; Lui et al., 2011). An example for this would be the formation of osteoblastic cells when hMSCs are transplanted into cartilage tissue (Baugé & Boumédiene, 2015). It has been suggested that lineage committed or pre-differentiated hMSCs may provide a better solution to this problem (Wei et al., 2014). Several methods can be employed to direct hMSCs towards a particular lineage. In the past, these have included hormonal, ionic, growth factor and environmental manipulation (Nava et al., 2012). However, one of the mechanisms that can be applied on these cells for differentiation and have not described in detail in the literature is mechanically driven signalling (Li et al., 2011; Shah et al., 2014). It is well known that connective tissues changed in function of their mechanical environment. Mechanical loading plays important role to the development, function, and repair of the major elements of the musculoskeletal system. As such, the prevailing clinical opinion for treating musculoskeletal injuries such as this requires a trans-missive approach which includes the protection of the injured organ from loading, and later followed by a

gradual increase in mechanical loading through a systematic regime that will promote the remodelling process (Hamill & Martinac, 2001; James et al., 2008).

It has been previously suggested that the ability of cells to respond to mechanical stimuli is controlled by a series of mechanosensitive receptors or structures that sense and convert mechanical signals into biochemical signalling events (MacQueen et al., 2013). This process, commonly known as mechanotransduction, translates mechanical cues that are perceived from the environment into intracellular signals. This ultimately regulates the complex processes involved in cell proliferation and differentiation (Castillo & Jacobs, 2010). It has been reported that during this process, the complex interaction of signals generated from the binding of integrins to signalling molecules, the opening of stretch sensitive ion channels, and the resultant cytoskeletal deformation are simultaneously activated (Ramage, 2012; Schwartz, 2010). However, the order of sequence of these events as well as the relationship between an activated pathway and outcome remains to be rationalized (Hao et al., 2015; Wang & Chen, 2013). In particular, the evidence for how physical forces control the MSCs differentiation program is now accruing.

Intracellular tensile forces resulting from cytoskeletal reorganization play an equally important role in regulating cell function (MacQueen et al., 2013; Matthews et al., 2006). At the cellular level, mechanical stimulation affects cell morphology, cytoskeleton structure, cell differentiation and gene expression (Discher et al., 2009). ECM, such as collagen, elastin, metalloproteinases, tenascin and their ligand integrin family, play an important role in mechanical signal transduction. In turn, mechanical stimulation affects the expression and order of ECM (Li et al., 2011). Synthesis and arrangement of ECM are the cellular major features and functions. Mechanical stimulation regulates the secretion and synthesis of ECM at transcriptional and post-

translational levels, and promotes the secretion of growth factors. Under physiological conditions, all cells contact the ECM and receive various forces from the surrounding environment. These native mechanical environments of tissues may facilitate the proliferation and differentiation of cells into specific lineages.

Different mechanical signals, like strain, compression, shear stress, and vibration, can mediate different effects and thus lead to different lineages during differentiation (Kelly & Jacobs, 2010). The uniaxial cyclic stretch *in vivo* is believed to be of primary importance in the development of functional musculoskeletal tissues (Mackey et al., 2008). Although previous works indicate that mechanical stimulation in general guides MSC differentiation in different ways, these studies have predominantly involved cells other than those found responsible for tendon or ligament homeostasis, such as osteoblast, neuron-like cells, and chondrogenic cells (Du et al., 2012; Leong et al., 2012; Li et al., 2010). In addition, there appears to be very few studies investigating the effects of cyclic uniaxial tensile loading, although this stimulus is physiologically relevant to the musculoskeletal system. It is worth noting that this stimulus is probably the single most important signal that regulates the proliferation and functions of both ligament and tendon cells (Benjamin et al., 2006; Hayashi, 1996). However, we also need to be mindful that because of their multipotential ability, it is possible that stimulating stem cells mechanically at an inappropriate manner can result in the cellular differentiation to an undesirable lineage. It is therefore important that the characteristics of the applied mechanical load needs to be established in order to ensure that cellular differentiation will be driven into a specified cell type. However, studies related to this area appears lacking as previous studies have been mainly focused on a narrow range of frequency and strain rates (Hata et al., 2013; Jang et al., 2011; Kuo & Tuan, 2008; Lee et al., 2007) which is in contrary to what occurs during physiological loadings (Couppé et al., 2008).

5.3 Materials and Methods

5.3.1 Cell seeding and application of cyclic uniaxial tensile strain

As described in the methodology section of this thesis i.e. Chapter 3 and Chapter 4, hMSCs were isolated and seeded on the collagen type I coated silicone chamber at the density of $10^4/cm^2$ and allowed to set at 37ºC in complete growth medium for 48 h. The medium was then changed to 1% FBS for 24 h, and proceeded with complete growth medium before being assembled into uniaxial stretching device. The experimental conditions are summarized in previous chapter. Control cells were treated similarly, but were not subjected to cyclic stimulation. The medium and cells were harvested after 6, 24, 48, and 72 h of cyclic loading for downstream analyses; which included biochemical assay, immunostaining, histological staining, topography analysis, and gene expression assay.

5.3.2 Human tenocyte primary culture

Primary native human tenocytes cultures were used as positive controls in the study. It is noted that the aim of this study is to determine the potential of hMSCs differentiation towards tenogenic lineage by mechanical stimulation, using tenocytes as comparison, and thus the effetcs of tensile loading on tenocytes is not investigated in this study. Human primary tenocytes were isolated from hamstring tendons of adult donors, who underwent surgery for joint arthroplasty (Appendix B). The study was carried out following the regulations of the Medical Center Ethics Committee of the University Malaya Medical Centre Medical (Reference No: 369.19). Tendon tissues were harvested to the required size by the operating surgeon and transferred aseptically into containers and immersed with saline solution. Once the tendons were harvested, the cell isolation was immediately performed, using the methods modified from Zhang and Wang (2010a). The tendons were rinsed with 1X PBS 3 times. Epitenon was dissected away from the main tendon substance and discarded. The tendons were minced into

approximately 1 mm^3 in size under a sterile condition. PBS was added to the tissue as minced tendon tissue tends to absorb more fluid. Using pasteur pipette, the minced explants including the surrounding fluid were then transferred into a 15 mL centrifuge tube and added PBS until 10 mL mark on the tube was reached. Subsequently, the mixture was added with 0.4 mg/mL type I collagenase, and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 2 h to allow for the enzymatic digestion process to occur. After digestion, the suspension was washed 3 times with PBS by centrifuged at 1800 rpm for 5 min. The pellet (dissected tissues) appeared as an impacted mass at the bottom of the tube (Figure 5.1). The supernatant was then removed and the pellet was transferred into a T25 tissue culture flask, leaving them to be in contact with the plastic surface for approximately 5 min, before added with DMEM high glucose (4.5 g/L glucose), supplemented with 10% FBS, 1% penicillin-streptomycin 1% GlutaMAX™-I. Cultures were incubated at 37ºC and 5% CO_2 incubator for 24 hours. On the next day, the pellets were then removed from the culture flask to a fresh and newly cell culture flask using pasteur pipette for another 24 hours. The digested tissues were then removed from the cell culture flask and discarded completely. The culture medium was changed at 3-day intervals. The outgrown cells were maintained at 80-85% of confluency for subculture using trypsin digestion. The passaged-3 human tenocyte cultures were used in the subsequent experiments.

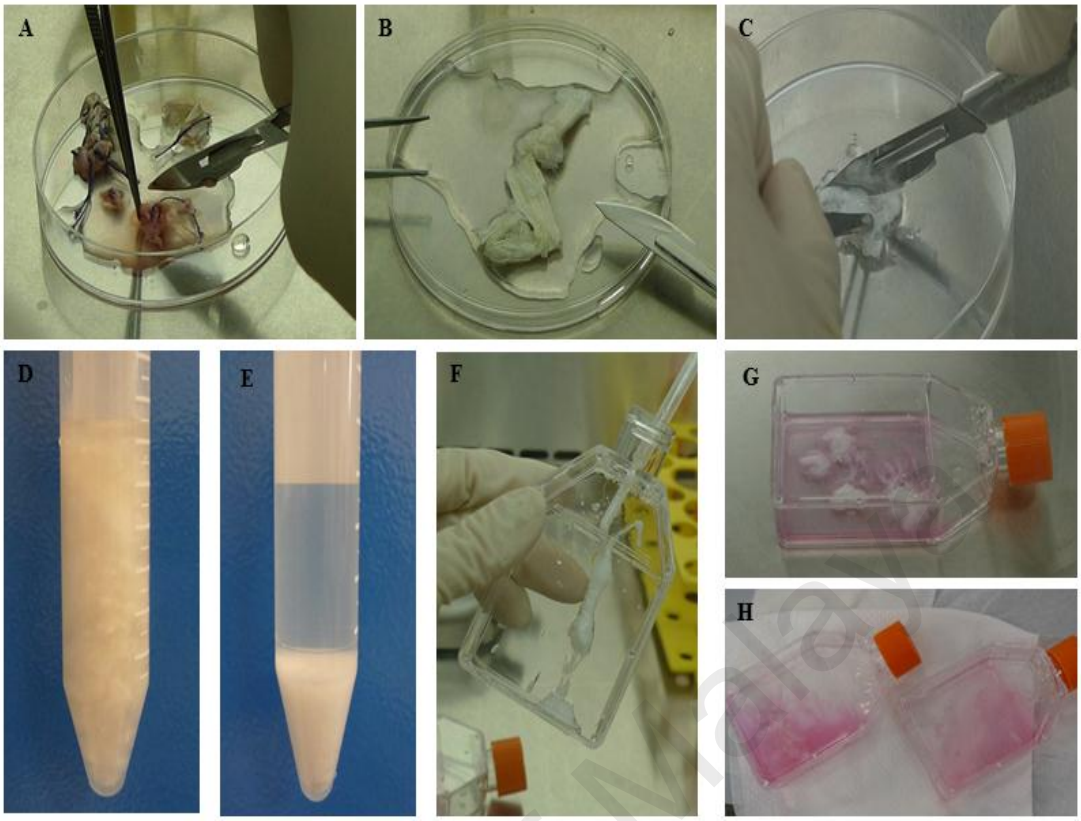

Figure 5.1: Cell extraction from human tendon by enzymatic digestion method
A-C) Tissue processing: A) Human tendons were placed on a petri dish and the string was removed, B) The tissue were washed with PBS until clean, C) Tissues were chopped into small pieces with half of these tissues finely minced.
D-H) Tenoctyes extraction: D) The tissues formed cotton-like structure after enzymatic digestion, E) The pellet was obtained at the bottom of the tube after centrifugation, F-G) The pellets were placed into the T25 flask, H) The remaining visible tissues were transferred into a fresh tissue culture flask.

5.3.3 Quantification of ECM components

The kits used in this study were quantitative dye-binding methods to determine the total quantity of the respective ECM component in the sample which released to medium.

5.3.3.1 Total collagen colorimetric assay

Total extracellular soluble collagen was measured using a Sircol^{TM} collagen assay kit. Briefly, at the end of each time point of the experiment, the culture medium was collected from the experimental cells, mixed with Sircol dye reagent with vigorous agitation for 30 min, and then centrifuged for 10 min at 12,000 rpm to collect the collagen dye complex. The unbound dye solutions were later removed by draining the

tubes carefully. After washing the dye with ice-cold acid-salt wash reagent by centrifugation at 12,000 rpm for 10 min, which was bounded to the collagen pellet, was solubilized using an alkali reagent. As the unbound dye dissolved, the absorbance of the samples was measured at 555 nm wavelength. The collagen content in the medium was calculated based on the standard curve plot, using acid-soluble type-I collagen supplied with the kit as the reference sample. Standard curve shows a correlation between the absorbance and collagen concentration, $R^2 = 0.998$.

5.3.3.2 Collagen type I colorimetric assay

Type I collagen is a fibrillar collagen consisting of two identical α1(I) chains and one α2(I) chain. Type I collagen was further examined since it is the most abundant collagen type in tendon and ligament. An enzyme immunoassay kit was used to measure the levels of type I collagen in strained hMSCs (1 Hz, 8%), following the manufacturer's protocol. In brief, a 96-well micro-titer plate were coated with the capture antibody (concentration: 10 μg/mL) by incubating the plate overnight at 4°C. The plate was then washed 3 times with a washing buffer, incubated with 100 μL experimental cell culture medium per well for 2 h at room temperature, and washed again 3 times with the washing buffer. The wells were then incubated with detection antibody for 2 h at room temperature. Subsequently, the wells were washed 3 times and incubated with streptavidin-conjugated peroxidase for 1 h at room temperature. After washing 3 times, the enzymatic reaction for colorimetric detection was carried out at room temperature for 30 min with 100 μL o-phenylenediamine. Reaction was stopped by adding 50 μL of 2N sulfuric acid. The concentration of collagen type I was obtained by measuring the absorbance at 490 nm on the microplate reader, with 630 nm as a reference. The collagen I content in the medium was calculated based on the standard curve plot, using human type-I collagen supplied with the kit as the reference sample. Standard curve shows a correlation between the absorbance and collagen concentration, $R^2 = 0.957$.

5.3.3.3 Elastin colorimetric assay

Tropoelastin and α-elastin polypeptides of resulting samples were measured using a Fastin^{TM} Elastin assay kit. At each time point of the experiment, the collected culture medium was added with cold elastin precipitating reagent, and left for 15 min to complete precipitation of α-elastin, before centrifuged at 10,000 *g* for 10 min. The liquid content was then drained off carefully, and dye reagent was added to each tube, and vortex to bring the elastin gel precipitate into solution with dye reagent. The elastin and the dye reagent were allowed to interact for 90 min, with gentle mechanical mixing during this period to ensure maximum elastin dye binding. The elastin-dye complex was separated from the remaining soluble unbound dye by centrifuging the tubes for 10,000 *g* for 10 min. The supernatant was then discarded. Any remaining fluid was removed by firm tapping of the inverted tubes onto a paper towel. A dye dissociation reagent was added to each tube, and the dye was released into solution with the aid of vortex mixer. The absorbance of the samples was measured at 513 nm wavelength on the microplate reader. The concentration of elastin present in the test samples was determined by the absorbance value obtained and read from the standard curve, where plotted using α-elastin supplied with the kit as the reference sample.

5.3.3.4 Glycosaminoglycan colorimetric assay

The content of sulphated glycosaminoglycan (sGAG) was determined using a Blyscan^{TM} sGAG assay kit, according to the manufacturer's protocol. Similar to total collagen assay, a total of 100 µL of the experimental cell culture medium was collected and added with dye reagent, followed by gently mechanical shaking for 30 min. During this time period a sulphated glycosaminoglycan-dye complex was formed and precipitated out from the soluble unbound dye. The precipitate was collected via centrifugation at 12,000 rpm for 10 min and then dissolved in dissociation reagent. Unbound dye solution was removed by draining carefully and then dissociation reagent

was added. Subsequently the mixture was vortex, and centrifuged at 12,000 rpm for 5 min to remove foam. Bound dye values were then quantified in a 96-well plate at 656 nm using microplate reader. Values were compared against standard curve of values of chondroitin 4-suphate, which was supplied with the kit. Standard curve shows a correlation between the absorbance and chondroitin concentration, $R^2 = 0.999$.

5.3.4 Immunocytochemical and fluorescent immunostaining for ECM analysis

The ECM expressions on 1 Hz group hMSCs were further examined.

5.3.4.1 Collagen immunostaining

Membranes with hMSCs subjected to the uniaxial straining or in unstrained condition were rinsed by 1X PBS, followed by fixation in methanol for 20 min at room temperature. After rinsed by tris-buffer saline, the cells were incubated with peroxidase block to reduce non-specific background for 5 min. After that, they were incubated with primary antibodies, which including rabbit anti-collagen type I, rabbit anti-collagen type II or rat anti-collagen type III diluted 1:100 for 30 min. The cells were then incubated with streptavidin-peroxidase secondary antibody for 30 min. At last, the collagens in MSCs were visualized by reaction with diaminobenzidine.

5.3.4.2 Fibronectin and N-cadherin immunofluorescence staining

For direct visualization of the adhesion molecules fibronectin matrix and N-cadherin, the 4% paraformaldehyde fixed cells were permeabilized with -20°C acetone, and incubated 1% bovine serum albumin to block non-specific binding of antibodies, before incubated with primary antibody diluted 1:300 for 1 h. The primary antibody was then detected by secondary antibody to rabbit IgG diluted 1:600 for 1 h. Hoechst staining was performed at the end of the staining and examined under laser scanning confocal microscope.

5.3.5 Stimulated cell surface antigen analysis by a fluorescence-activated cell sorter

Antibodies against the human antigen, CD44, CD73, CD90, CD105, were used to characterize the surface antigen expressions of different magnitude loaded hMSCs (1 Hz). Briefly, the loaded cells were resuspended in 100 μL of PBS and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for 15 min at room temperature in the dark. The fluorescence intensity of the cells was evaluated by a flow cytometer, and the data were analysed using the CELLQUEST software. The presence or absence of staining was determined by comparison with the appropriate unstained control.

5.3.6 Histologic assessment of differentiation after mechanical stimulation

The presence of bone-forming nodules was used to determine the osteoblast differentiation in our cell cultures. This was further assessed using Alizarin Red S dye, which stains calcium phosphate deposits. Accumulation of lipid droplets was used to denote adipocyte differentiation, was determined by incubating paraformaldehyde fixed cells with 60% isopropanol, and followed by freshly prepared Oil Red O solution. Unstrained samples were treated as control. Images of samples were then captured using a light microscope.

5.3.7 Atomic force microscopy measurement of Young's modulus

Atomic force microscopy (AFM) images were obtained by scanning the cells surface in air under ambient conditions using AFM operated using the new PeakForce QNM mode. The AFM measurements were obtained using ScanAsyst-air probes, and the spring constant (Nominal 0.4 N/m) and deflection sensitivity has been calibrated, but not the tip radius (the nominal value has been used; 3.5 nm). To increase the stability of cell membrane and to prevent the lateral mobility of receptors, the experimental cells

were incubated with 4% paraformaldehyde for 1 h prior to imaging. AFM images were collected from each sample and at random spot surface sampling (at least five areas per sample). The quantitative mechanical data was obtained by measuring DMT modulus/Pa using Bruker software (NanoScope Analysis). To obtain the Young's Modulus, the retract curve was fit using the Derjaguin-Muller-Toporov model, for that reason called DMT Modulus (Derjaguin et al., 1975).

5.3.8 Multiplex gene expression analysis

Total RNA from unstrained and strained cells was extracted using the RNeasy mini kit according to the manufacturer's instructions (Appendix G). RNA concentration and purity were determined using a spectrophotometer on the setting of A260/280. RNA integrity was assessed by visualization of 18S and 28S rRNA bands on formaldehyde-agarose gels (Figure 5.2). Only samples with high quality were selected for microsphere-based multiplex branched DNA downstream analysis.

The mRNA expression of mesenchymal lineages (Table 5.1) was quantified by the QuantiGene 2.0 Plex assay. Individual bead-based oligonucleotide probe sets specific for each gene examined were developed by a selected licensed manufacturer (the 2.0 plex set 12082). In this assay, each lysate was measured in triplicates. Controls are also included for genomic DNA contamination, RNA quality and general assay performance testing.

Briefly, 20 µL of extracted total RNA for each sample was mixed with 80 µL of mixture containing probe sets with capture beads, lysis buffer, blocking reagent and nuclease free water for each well. After 20 h of hybridization at 54ºC and 600 rpm, hybridizations with bDNA pre-amplifier 2.0, bDNA amplifier 2.0, biotinylated label probe and finally substrate were subsequently carried out according to the manufacturer's instructions. Luminescence of sample was quantified using a Luminex

instrument. The housekeeping gene, *PGK1* (phosphoglycerate kinase 1) was previously validated as the best housekeeping for accurate gene expression analysis related to rat hippocampal neurons (Santos & Duarte, 2008), and human (Willems et al., 2006) and rat embryonic stem cells (Veazey & Golding, 2011) respectively. In a separate experiment, we demonstrated that of the housekeeping genes (*PGK1*, *HPRT1* and *TBP*), *PGK1* appears to be the least affected by mechanical stretching.

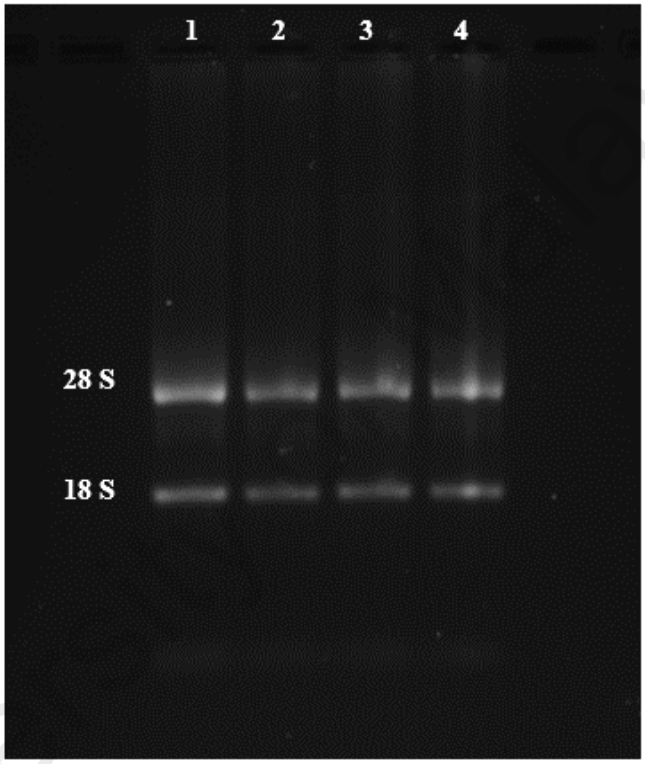

Figure 5.2: Evaluation of RNA integrity by agarose gel analysis
Samples of total RNA (2 μg) were fractionated on a 4% agarose gel in tris-acetate-EDTA buffer and stained with gel loading dye. Lanes 1 - 4 show 4 different samples RNA of high quality.

Table 5.1: The genes of interest were determined in this study

Related marker	Gene name	Abbreviation
	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	*MMP3*
	Proline rich 16	*PRR16*
ECM component	Collagen type I, α1	*COL1*
	Collagen type III, α1	*COL3*
	Decorin	*DCN*
Tendon lineage	Tenascin C	*TNC*
	Scleraxis homolog A	*SCX*
	Tenomodulin	*TNMD*
Bone lineage	Runt-related transcription factor 2	*RUNX2*
	Alkaline phosphatase, liver/bone,kidney	*ALP*
	Osteocalcin	*OCN*
Cartilage lineage	Collagen type II, α1	*COL2*
	Cartilage oligomeric matrix protein	*COMP*
	SRY (sex determining region Y)-box 9	*SOX9*
Fat lineage	Peroxisome proliferative activated receptor, gamma	*PPARG*
Smooth muscle lineage	Transgelin	*TAGLN*
Housekeeping gene	Phosphoglycerate kinase 1	*PGK1*
	Hypoxanthine phosphoribosyltransferase 1	*HPRT1*
	TATA box binding protein	*TBP*

5.3.9 Statistical analysis

The assays were carried out with a minimum number of technical triplicates (n) per experimental run, using six independent samples from different donors (N) for each group of the experiments. Data were presented as mean ± 1 standard deviation. For Young's modulus experiment, Student's t-test (two-sided, unpaired) was carried out to compare the differences in mean values. While the other experiments, statistical significance was analyzed by one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference among groups, the difference was evaluated using least significant difference (LSD). A confidence level of 95% ($p < 0.05$) was chosen for determining statistical significant using the SPSS 15.0 software (SPSS Inc, USA).

5.4 Results

5.4.1 Human tenocytes morphology

The isolated cells appeared to be either stretched, flattened or stellate-like during the early phases of cell culture (Figure 5.3A). When cells were in contact with one another, they exhibited a spindle-like shape, which are elongated, and particularly built reticular colonies in cell chains (Figure 5.3B). The cells proliferated to 80-90% confluence approximately within 14 days. After sub-culture, the cells lose their appearance. They then partially become larger and built wing-like shapes (Figure 5.3C-E).

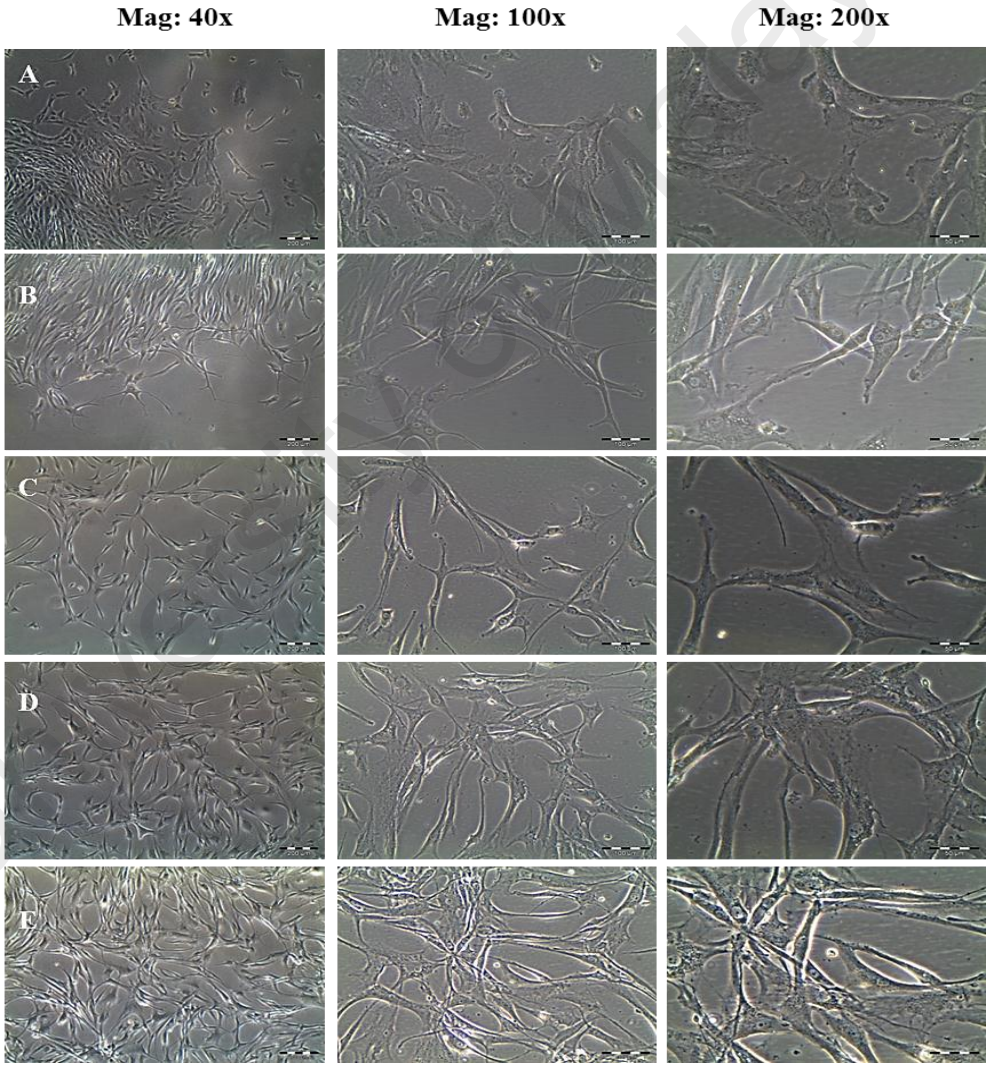

Figure 5.3: Different magnification for photomicrographs of human tenoctyes at different duration of culture

A) The cells showed more stellate-like cells, with some stretched cells during the early phases of cell culture at passage 0. B) Cells appeared to be stretched with long and thin processes, these cells tended to group in close contact, parallel arrangement and grow in high-density colonies. C-E) Passaged-1, passaged-2 and passaged-3 tenocytes morphology, respectively. Mag 40x (200 µm); Mag 100x (100 µm); Mag 200x (50 µm).

5.4.2 Uniaxial tensile loading enhanced collagen and elastin production but not GAG

Collagen is the main component of extracellular matrix produced by cells of mesenchymal origin, and has been found to increase during cell proliferation and differentiation (Choi et al., 2007). At 1 Hz, the results showed that uniaxial stretching increases collagen production (Figure 5.4b), with the exception of the 4% strain group. Higher collagen production was measured as early as 6 h at 12% strain group. For the 8% strained group, the collagen production was enhanced significantly only after 48 h, which is close to the collagen content in tenoctyes (ratio of human tenoctyes/unstrained hMSCs = 1.43, graph not shown). However a significant increase in this protein was not observed when hMSC was subjected to 0.5 Hz cyclic loading (Figure 5.4a).

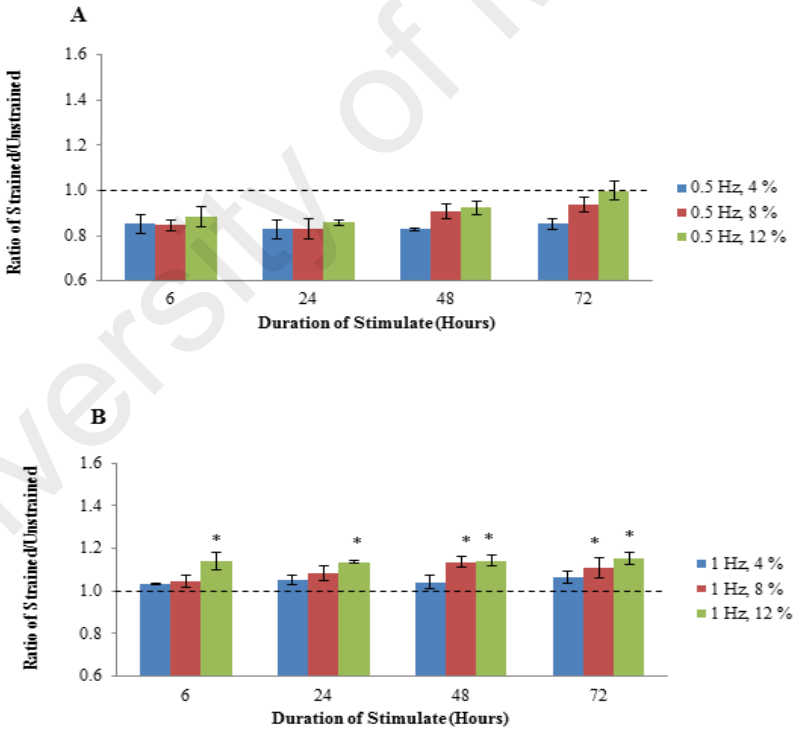

Figure 5.4: Extracellular collagen content analysis of hMSCs cultured with various mechanical stimuli at different duration of stretching
A) There was no increment of collagen content observed in strained hMSCs at 0.5 Hz. However, the collagen content was slightly amplified by increasing the duration of mechanical stimulation. B) At 1 Hz, the total collagen expression in hMSCs was significantly increased at 12% strain compared with unstrained group, while it appeared obviously only after 48 h for 8% strain. Statistical significance ($p < 0.05$) was represented by asterisk which compared to unstrained (indicated as 1). N = 6, n = 3. Error bar = ± 1 SD.

Since collagen type I was reported to be abundant in tendon, ligament and muscle cells, the 8% strained cells at 1 Hz was further tested using ELISA assay. The results showed that collagen type I level in the medium was increased in mechanically stimulated cells as compared with unstrained cells. The content of collagen type I was increased by increasing the duration of the stretching (Figure 5.5).

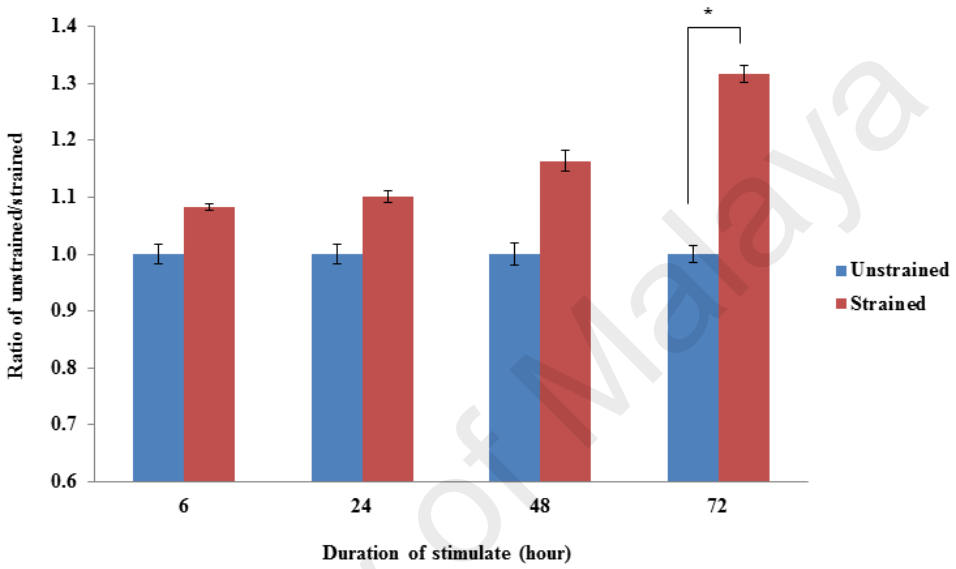

Figure 5.5: The level of collagen type I in the medium was measured by ELISA assay

The ratio of the ECM production was reported by normalizing to the expression amount of corresponding unstrained groups (indicated as 1). Significance $p < 0.05$ was represented by asterisk which compared to unstrained. N = 6, n = 3. Error bar = ± 1 SD.

The results of total collagen production (Figure 5.4) and gene expression (section 5.4.8) show uniaxial stretching has positive effects on hMSCs at 1 Hz group. Thus, the elastin and GAG expression on this group was further examined. Similar to the total collagen production, elastin production appears to be influenced by the strain. Compared to collagen, elastin was only increased after 72 h at higher strain group (Figure 5.6A). However, no enhancement of GAG production was observed in any strained group (Figure 5.6B).

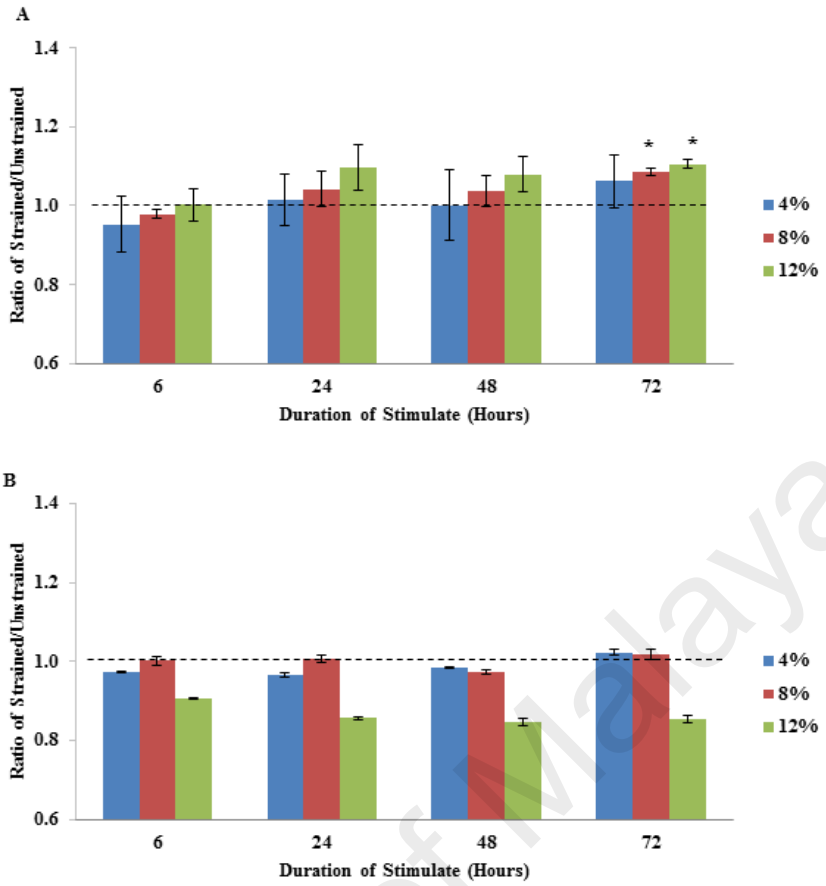

Figure 5.6: Extracellular (A) elastin and (B) GAG analysis of MSCs subjected to various mechanical stimuli for different duration of stimulation at 1 Hz
The ratio of the ECM expression was counted by normalizing to the expression amount of corresponding unstrained groups (indicated as 1). Significance $p < 0.05$ was represented by asterisk which compared to unstrained. N = 6, n = 3. Error bar = ± 1 SD.

5.4.3 Mechanical stimulation promoted collagen type I, collagen type III, fibronectin, and N-cadherin expression

In our study, immunocytochemical assay showed that the uniaxial cyclic straining promoted the synthesis of collagen type I in MSCs. In unstrained control groups, there was only a light brown collagen staining in cytoplasm, while a more intense staining was observed in 72 h strained group for collagen type I (Figure 5.7), accordance with the result of collagen type I by ELISA assay. Overall, collagen I and collagen III staining showed positive protein expression on both unstrained and strained hMSCs, but denser on strained cells, particularly 8% and 12% group. In contrast, expression of collagen II was not observed till the end of stretching experiment. These results are closer to collagen expression of tenocytes, which act as positive control in this study.

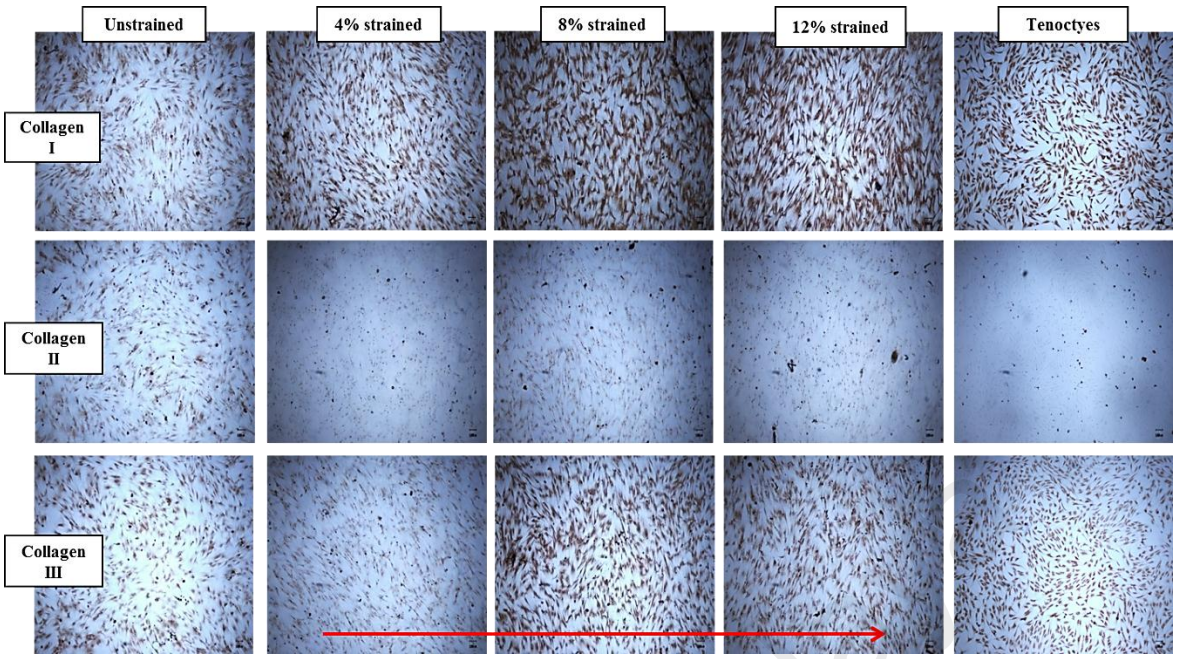

Figure 5.7: Comparison of different collagen staining on various mechanical stimuli hMSCs at 72 h
Compared to unstrained cells, the strained cells show positive expression on collagen I and III; especially on higher strained group, which are closer to expression of tenocytes. The direction of uniaxial strain was in the red arrow direction. Scale bar = 100 µm.

Our results show that for cells under unstimulated condition, fibronectin was arranged as random web-like structure which was mainly distributed at the cell periphery. This peripheral fibronectin staining was increased in stimulated cells as well as increase in fibronectin fibril formation by enhancing the strain magnitude (Figure 5.8A). Furthermore, unstimulated cells appeared to have thin fibronectin fibrils clustered and distributed throughout the entire basal surface of the cell, while cells exposed to 72 h of 8% and 12% uniaxial stretching appeared to form thicker fibronectin fibrils and to have an observable increase in fibronectin fluorescence intensity (Figure 5.8B). To view cell-cell contacts after stretching, we found the expression level of N-cadherin was higher on strained cells (Figure 5.8A). However, this level of expression was decreased under 12% uniaxial stretching.

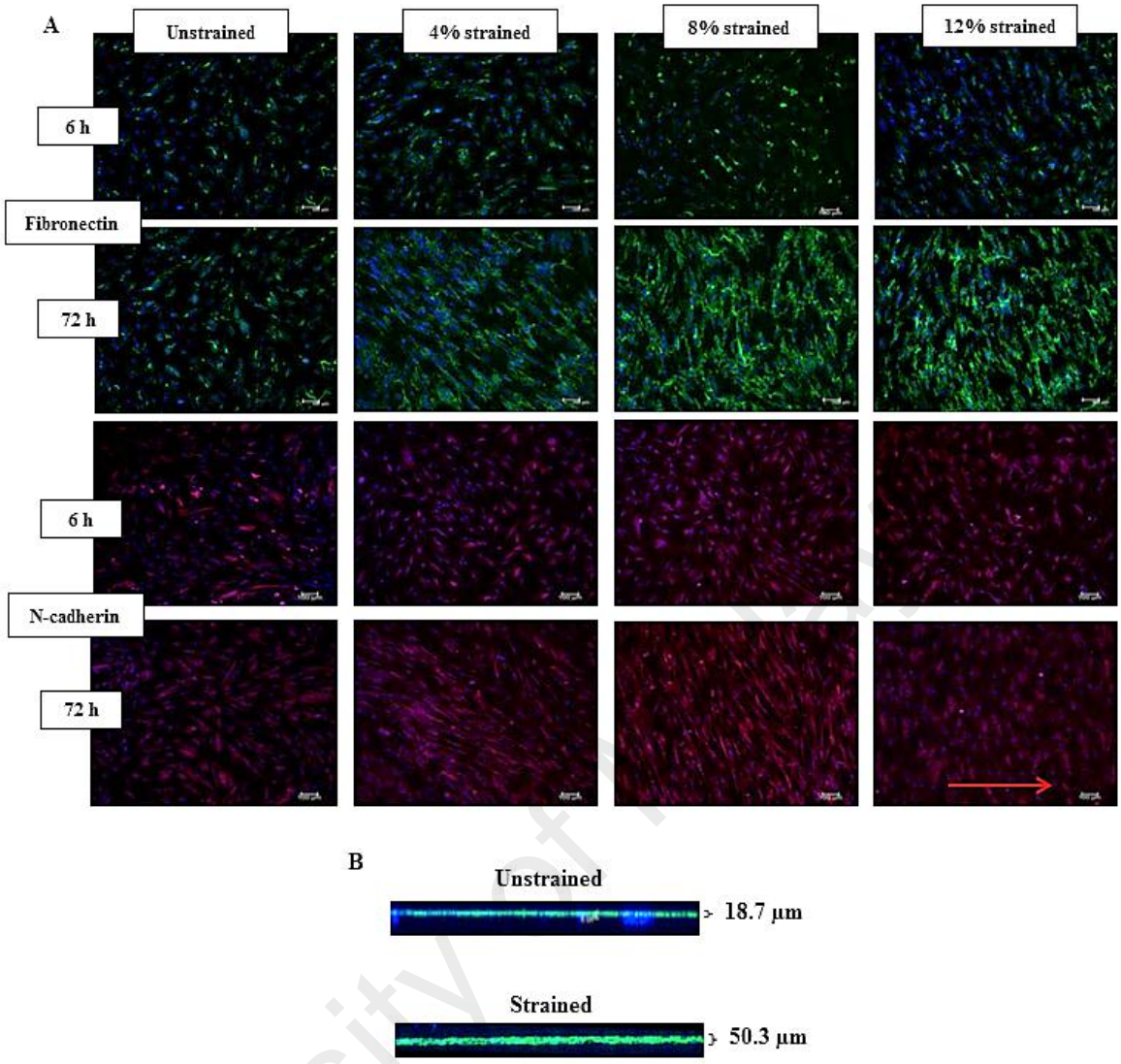

Figure 5.8: Immunofluorescence staining of fibronectin and N-cadherin on various mechanical stimuli hMSCs for 6 h or 72 h

A) The expression of fibronectin and N-cadherin was enhanced by the cyclic stretch and magnitude strain dependent. The direction of uniaxial strain was in the red arrow direction. Scale bar = 100 µm. B) Thicker fibronectin fibrils were formed by cyclic mechanical stimulation.

5.4.4 Mechanical stretch induced the alterations in hMSCs surface antigen expression

The expression of the CD markers was highly expressed in non-stimulated cells on silicone chambers, as similar to hMSCs cultured on culture flasks. Since 1 Hz mechanical strain has produced more pronounced statistical difference in gene expression and ECM production, the CD markers expression at 1 Hz only was analyzed. After 72 h of stretching, no significant difference in the expression of CD markers was

observed in the 4% strain cells compared with unstrained cells. However, low expression were observed for cultures under 8% and 12% stimulation, suggesting that appropriate levels of mechanical stretch induce the alterations in MSCs surface antigen expression (Figure 5.9A). It was noticed that CD44 was significantly decreased at both 8% and 12% strained group, while CD105 and CD90 reduced significantly at 8% and 12% strained group, respectively. Figure 5.9B demonstrates some of the expression of multicolour CD markers at different strain magnitude.

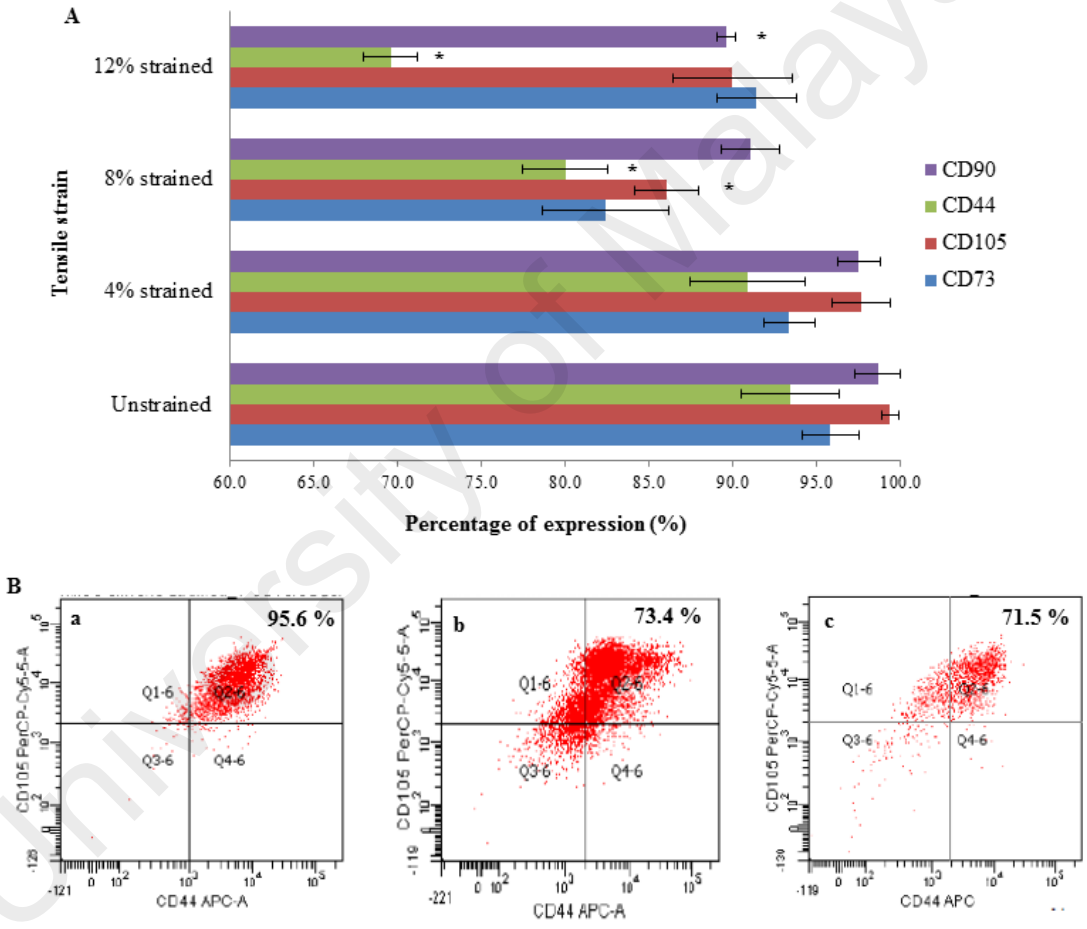

Figure 5.9: Alteration of hMSCs surface antigen expression during mechanical stretching

A) Expression levels of the CD markers of hMSCs cultured in mechanical stretching with different strains. Significance ($p < 0.05$) was represented by asterisk which compared to unstrained. N = 6, n = 3. Error bar = ± 1 SD. B) Percentage of multicolour expression for lymphocyte adhesion molecule CD44 and endoglin CD105. Fluorescent expression intensity and area of CD44 and CD105 in (a) 4%, (b) 8%, and (c) 12% strain magnitude.

5.4.5 Mechanical stretching did not induce osteogenesis and adipogenesis

Mineralization of hMSCs was observed in the osteogenic medium-induced differentiation of hMSCs (21 days) after stained with Alizarin Red S staining (Figure 5.10A), but this was not observed in mechanical induction of hMSCs at 8% strained for 72 h., with only slight brown stained around nucleus of the cells (Figure 5.10B). The effect of tensile loading on adipogenic differentiation of hMSCs was studied using Oil Red O staining of the lipid droplets. The lipid droplet formation under adipogenic differentiation was found in adipogenic medium induction hMSCs (14 days) (Figure 5.10C), whereas the mechanical stimulated hMSCs showed no lipid droplet (8% strained, 72 h) (Figure 5.10D).

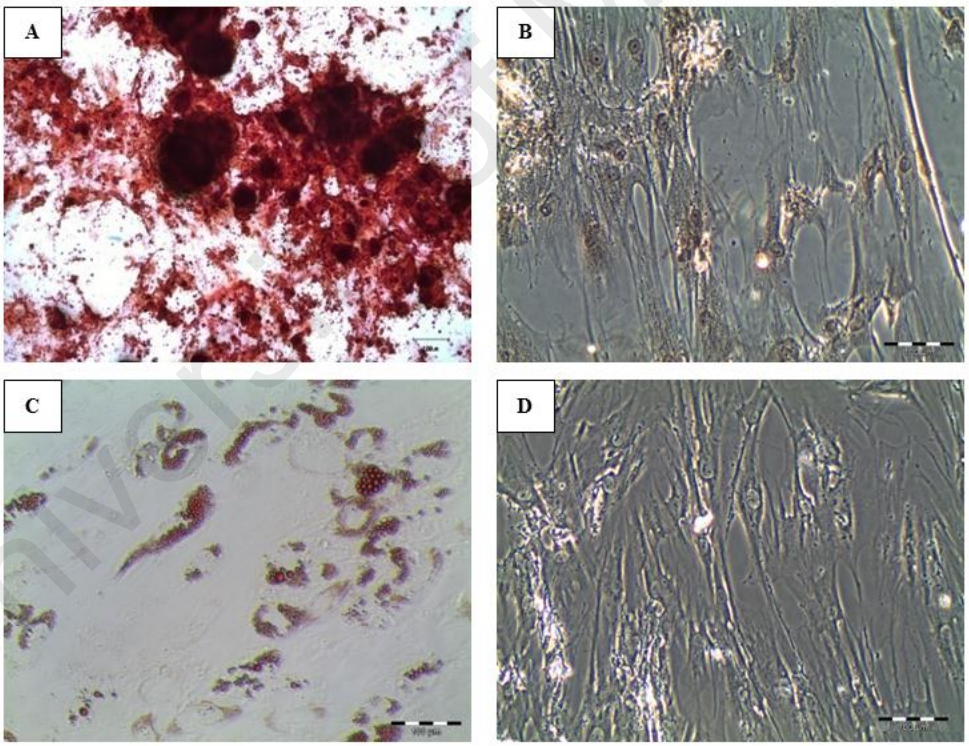

Figure 5.10: Representative images of Alizarin Red and Oil Red O stained hMSCs A) positive staining on osteogenic medium cultured hMSCs, B) negative result on mechanical stimulated hMSCs, C) positive staining on adipogenic medium cultured hMSCs, D) negative result on strained hMSCs.

5.4.6 Topography changes was found in mechanically stimulated cells

Topographical analysis of the unstrained and strained hMSCs at 1 Hz was studied using the atomic force microscopy. Topographical images were obtained in both height and deflection channel (Figure 5.11A). Results of AFM analysis revealed that strained cells appeared elongated spindle-like morphology with microfilament bundles running parallel to their long axes, while unstrained cells appeared large and flat. Height image showed larger height scale for strained cells than unstrained cells. This was apparently related to the thicker actin stress fibers of the strained cells than the unstrained hMSCs, which could be visualized in detail in deflection channel. In unstrained cells, deflection image revealed the fine cytoskeletal structure (presumably actin) just under the cell membrane at detail. The fine cytoskeleton structure began integrating when mechanical stimulation was applied on the cells. The cytoskeleton of the stimulate cells became more pronounced. This effect was much evident with the higher magnitude strain to hMSCs, where compatible with tenocytes.

To show the comparison between strained hMSCs and unstrained hMSCs in different amount, the elasticity measurements (Young's modulus) were performed in the area of the cells cytoskeleton around nucleus. Figure 5.11B shows the average Young's modulus of fixed unstrained and strained hMSCs from 3 independent cultures with 5 different spots of area. The Young's modulus values for strained hMSCs groups were greater than that of unstrained hMSCs group, with significantly increased in 8% and 12% strained group. These results demonstrated that as the strain rate increased, the Young's modulus, and therefore stiffness of the cytoskeleton of hMSCs increased. The unstrained hMSCs is supple compared to strained hMSCs, especially 8% strained group, performing that the mechanical property of undifferentiated hMSCs differs from likely-differentiating (72 h) hMSCs.

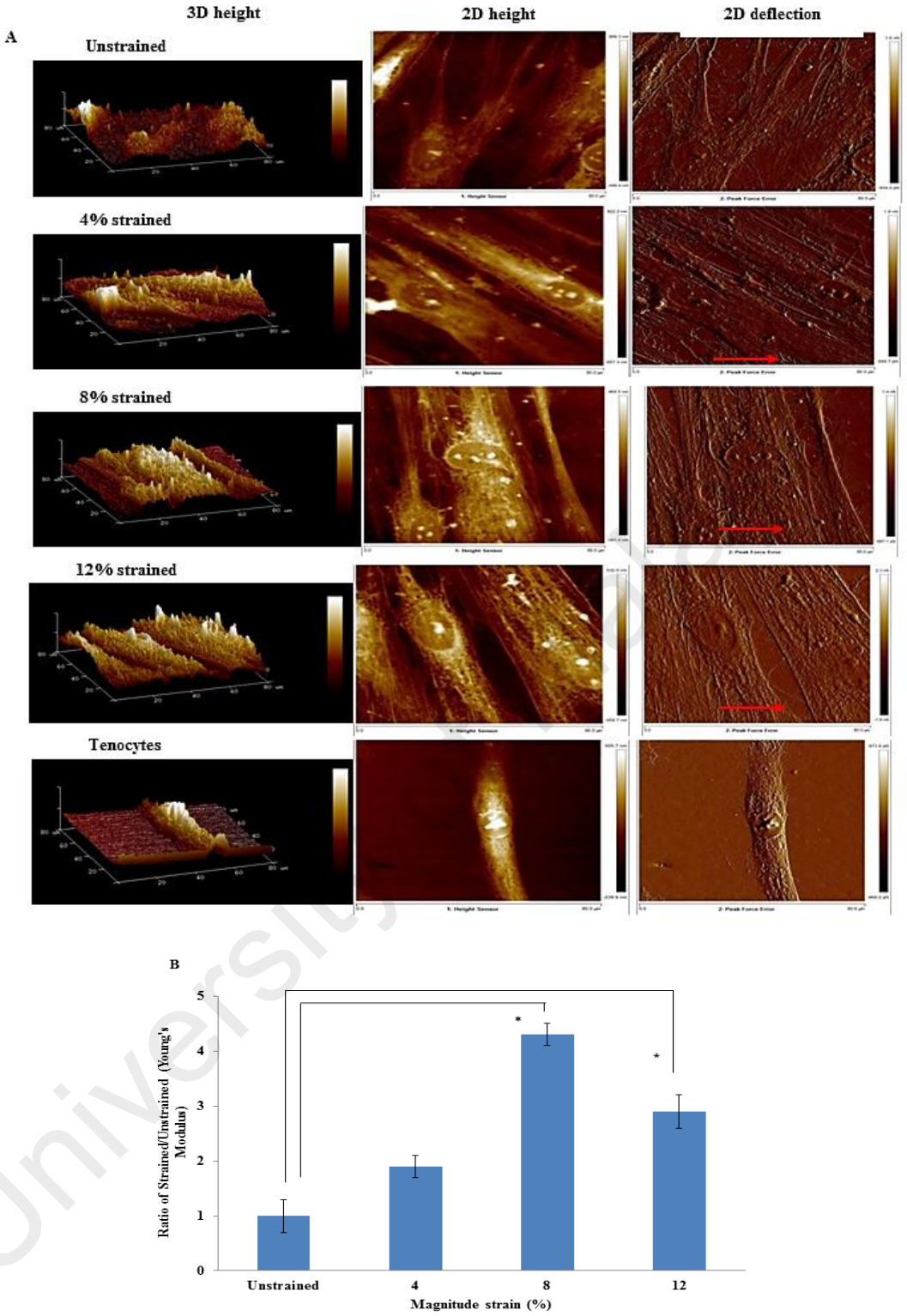

Figure 5.11: The comparison of cell surface topography between the unstrained hMSCs, strained hMSCs, and tenocytes, visualized using AFM

A) Representative AFM height and deflection scans of unstrained hMSCs, 4%, 8%, and 12% strained hMSCs, and tenocytes. In height images, brighter colour indicates higher distance off of substrate. In deflection images, the detailed structure of presumably the stress fiber could be observed with AFM in different cells group. The direction of uniaxial strain was in the red arrow direction. Scale bar = 80 µm. B) Young's modulus on cytoskeleton of the hMSCs subjected to 4%, 8%, or 12% cyclic stretching for 72 h as indicated. Data shown represent mean ± SEM. Significance $p < 0.05$ was represented by asterisk which was compared to unstrained group. N = 3, n = 5, error bar = ± 1 SD.

5.4.7 Mechanical stimulation influences the expression of *PRR16* and *MMP3*

The mRNA expression of *PRR16*, an indicator of the stem cell differentiation (Chen et al., 2008b), upon mechanical stretching was shown in Figure 5.12A-B. At 1 Hz, uniaxial 4% stretching inhibited the gene expression, and significant down-regulation of the *PRR16* gene was noted for both 8% and 12% stretched groups. This effect was more obvious after the cells had stretched for longer period. These results were convoy with the decreasing of CD markers expression after mechanical stimulation. Contrary to the down-regulation of *PRR16*, the mRNA expression of *MMP3* was up-regulated in higher magnitude strain, particularly in 8% stretching group (Figure 5.12D). Interestingly, the exhibitory effect on *MMP3* mRNA expression by mechanical stretching was not obvious for 12% stretching group after 48 h. Taken together, these results suggest that mechanical stimulation active matrix remodelling activity accompanied increased matrix accumulation as detected using histochemical staining. This suggests that *MMP3* may regulate the amounts of biologically active cellular components that participate in MSC differentiation (Mannello et al., 2006). At 0.5 Hz, the results show less remarkable compared with 1 Hz group.

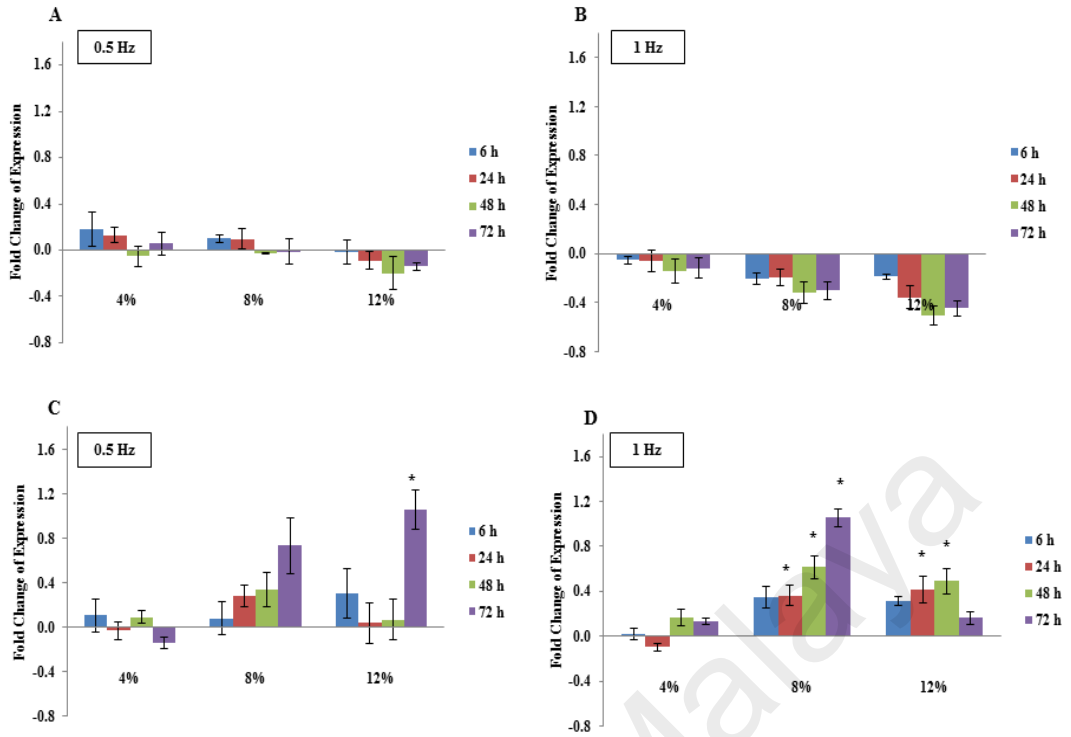

Figure 5.12: mRNA expression of (A-B) *PPR16* and (C-D) *MMP3* in hMSCs subjected to different strains and duration of mechanical stimulation, performed at 0.5 Hz and 1 Hz, respectively

Fold changes of expression were counted by normalizing to the relative expression amount of corresponding control groups (unstrained groups). Statistical significance ($p < 0.05$) was represented by asterisk which was compared to unstrained (indicated as 0). N = 6, n = 3. Error bar = ± 1 SD.

5.4.8 High mechanical strain up-regulated genes for macromolecular components of ECM and induced differentiation markers for tendon-like cell

The differentiation of hMSCs towards tendon-like cells was further examined by measuring the expression levels of selected genes, i.e. *DCN*, *COL1*, *COL3*, *TNC*, *SCX*, and *TNMD*, which was all normalized to the housekeeping gene *PGK1* (Figure 5.13). Unlike cells that are subjected to low frequency stretching, i.e. 0.5 Hz, cells subjected to 1 Hz expressed higher levels of tenogenic gene expression, which are closer to the gene expressions in tenoctyes (*DCN*=1.50, *Col1*=1.59, *Col3*=1.37, *TNC*=2.22, *SCX*=2.65, *TNMD*=1.80; normalized by *PGK1*; graph not shown). In fact at 0.5 Hz, except for *COL1*, all other genes were not highly expressed. This may be due to the fact that *COL1*

is a non-specific marker for tenogenic differentiation and is also produced by several cells types of mesenchyme origin. Most remarkable of these was the fact that *TNMD*, an important marker for tenocytes proliferation and tendon maturation (Docheva et al., 2005), was significantly down-regulated in the 0.5 Hz group, indicating that the hMSCs were less likely to be induced into tendon-like cells when subjected to this rate of stretching.

Despite having mentioned that 1 Hz produces superior tenogenic gene expression, this significant increase was only observed in cells subjected to strains of 8% or more, with most evident in the 8% group after 24 h. uniaxial strain regulated matrix remodelling by increasing *COL1* and *COL3* expression in a stretching magnitude dependent manner (Figure 5.13B). A significant increase was induced by 8% and 12% stretching, but this up-regulation was not significant for the 4% stretched groups. The expression of *COL3* showed a pattern similar to that of *COL1*, but the level of increase was slightly faster than that of *COL1* for 8% strain (at 24 h). Compared to the collagen groups, the level of *DCN* expression was slightly higher, and significantly upregulated in 8% and 12% after 24 h and 48 h, respectively. The tenogenic gene expressions appear to be dependent on specific threshold of strain levels, of which a higher strain level will not necessarily result in higher levels of gene expressions. This was evident from the data obtained from the cells subjected to uniaxial strain at 12%, which resulted in lesser increase of *DCN*, *TNC*, *SCX*, and *TNMD*. Nevertheless, the results of the present experiments also suggests that strains of 12% may have a role in providing superior tenogenic expressions at lower levels of cyclic loading, since amongst the groups subjected to 0.5 Hz, it is cells that are subjected to 12% strains that had demonstrated a significant increase in selected tenogenic gene expressions. Also worth noting is that after 2 days, the gene expression levels of *SCX* returned to its pre-stretched levels, and

were comparable to that of the unstrained control group, suggesting that the increase observed in this experiment was transient.

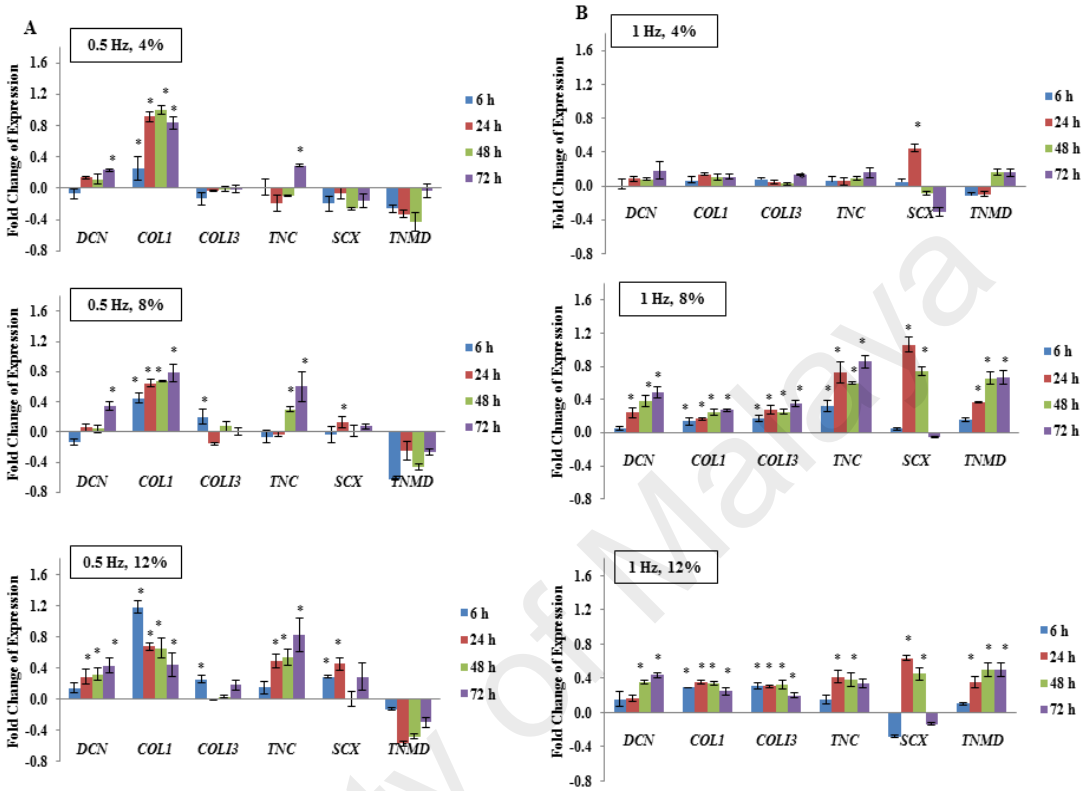

Figure 5.13: Effect of uniaxial stretching on mRNA expression of tenogenic markers in hMSCs, which were performed in (A) 0.5 Hz and (B) 1 Hz, in different strains and duration of mechanical stimulation

Fold changes of expression were counted by normalizing the data to the relative expression amount of corresponding control groups (unstrained groups). Statistical significance ($p < 0.05$) was represented by asterisk which was compared to unstrained (indicated as 0). N = 6, n = 3. Error bar = ± 1 SD.

5.4.9 Uniaxial mechanical strain did not induce osteogenic, chondrogenic, and adipogenic differentiation markers

To determine the responses of hMSCs to uniaxial mechanical strain and whether the differentiation markers other than tendon-like cells markers were induced by mechanical strain, other non-related genes also were investigated. The results indicate that low mechanical loading transiently up-regulate genes of osteoblast lineage (*RUNX2*, *ALP*, and *OCN*) (Figure 5.14). *RUNX2* is crucial for the generation of a mineralized tissue (Deng et al., 2008) and its gene expression was usually noticed

during the early phases of osteogenic differentiation. *ALP* is the effector protein, which is responsible for the mineralization of the ECM (Marom et al., 2005). *OCN* is used as late marker of osteogenic differentiation (Aubin, 2001). The 4% strain group at 1 Hz showed significant increase in the expression of *RUNX2* and *ALP* in early time point (6 h). Similar pattern of gene expression was also observed in 0.5 Hz in 4% and 12% at 6 h, however the expression was not significant compared to the unstrained control. Higher magnitude strain has shown to down-regulated the 3 tested genes of osteogenic differentiation, suggesting low mechanical strain enhanced osteoblastic differentiation transiently.

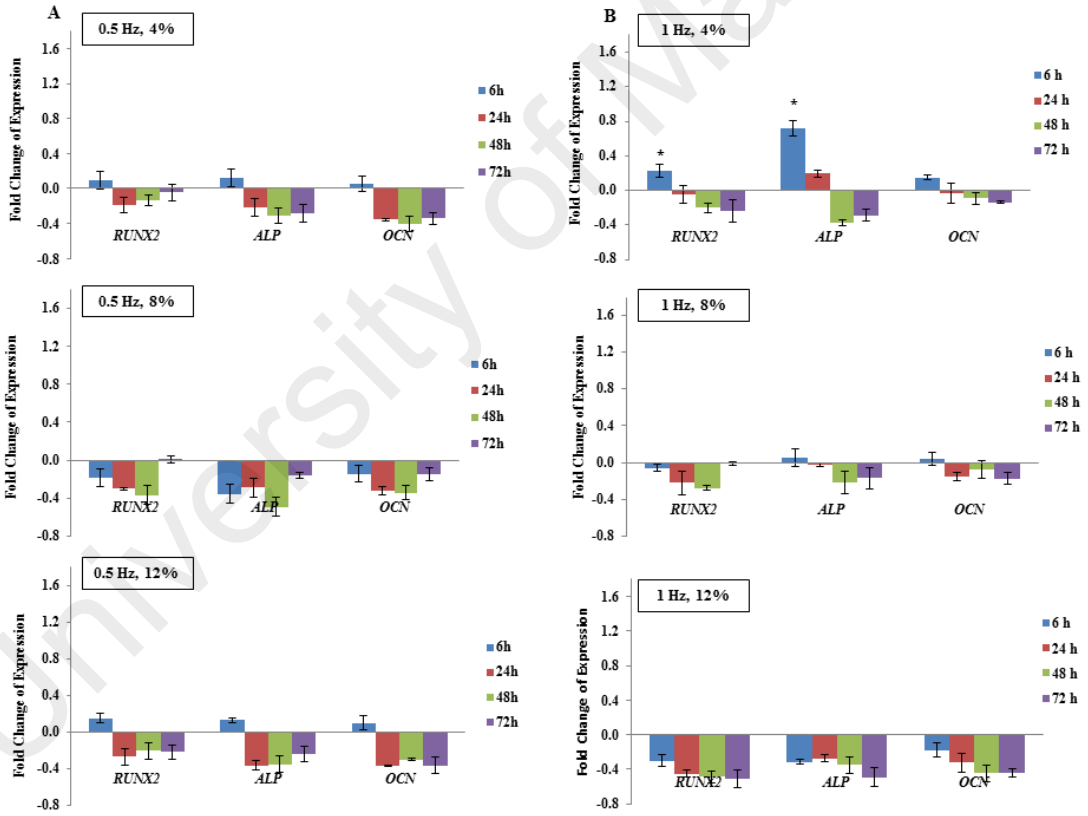

Figure 5.14: Effect of uniaxial stretching on mRNA expression of osteogenic markers in hMSCs, which were performed in (A) 0.5 Hz and (B) 1 Hz, in different strains and duration of mechanical stimulation

Expressions in fold changes were calculated by normalizing to the relative expression amount of corresponding genes in unstrained control groups. Statistical significance ($p < 0.05$) was represented by asterisk which compared to unstrained (indicated as 0). N = 6, n = 3. Error bar = ± 1 SD.

In consistent with the immunostaining on observations, uniaxial strain did not increase *COL2* (Figure 5.15) and *PPARG* (Figure 5.16A-B), which represents the initiation of chondrogenesis and adipogenesis, respectively. Expression of chondrogenic differentiation markers such as *SOX9* and *COMP* were found to be expressed in a magnitude and time dependent manner (Figure 5.15). *SOX9* gene was down-regulated in all the stretching conditions except for a non-significant increase in 12% stretching in short term (6 h). Contrary to *SOX9*, significant up-regulation of *COMP* was observed only in 12% strain group for 72 h. This may due to the fact that *COMP* is a non-specific gene for chondrogenic differentiation and that can be also found in tendon cells (Li et al., 2010). However, the functional significance of these changes in gene expression needs further investigation. Overall, low frequency (0.5 Hz) has not significantly influenced the expression of chondrogenic and adipogenic lineage markers.

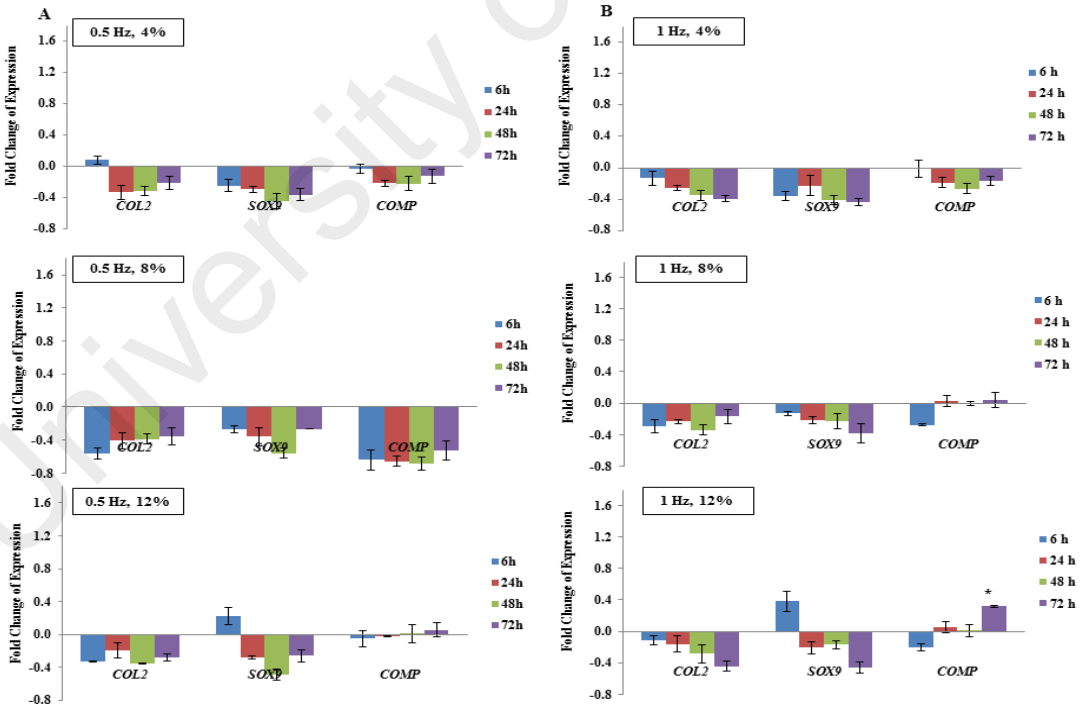

Figure 5.15: Effect of uniaxial stretching on mRNA expression of chondrogenic markers in hMSCs, which were performed in (A) 0.5 Hz and (B) 1 Hz, in different strains and duration of mechanical stimulate

Fold changes of expression were counted by normalizing to the relative expression amount of corresponding control groups (unstrained groups). Statistical significance ($p < 0.05$) was represented by asterisk which was compared to unstrained (indicated as 0). N = 6, n = 3. Error bar = ± 1 SD.

Uniaxial cyclic stimulation has also increased the smooth muscle cell marker, *TAGLN*, transiently by 12% strain (Figure 5.16C-D). Neither low nor high strain did not induce a significant change in this gene expression after 6 h. Similar results were shown that all these genes were not significant upregulated at lower frequency (0.5 Hz). With all these results, suggesting that uniaxial cyclic mechanical strain did not directly promote the differentiation of hMSCs into osteoblast, chondrocytic, adipogenetic, or smooth muscle cells.

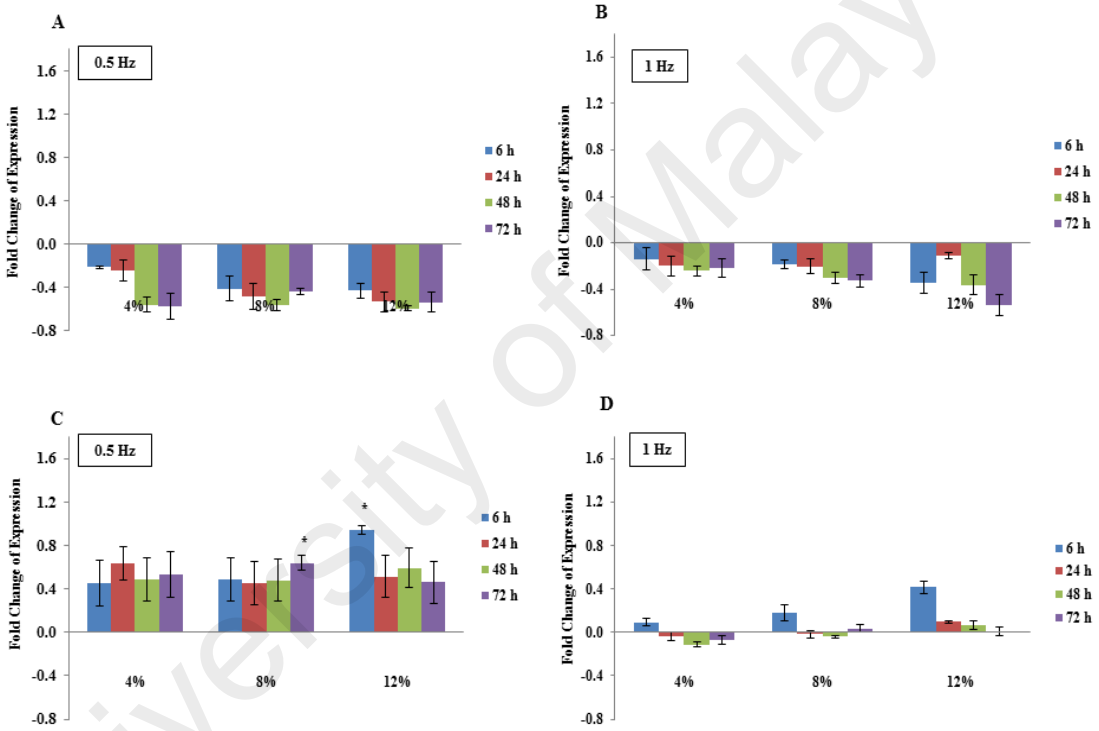

Figure 5.16: Effect of uniaxial stretching on mRNA expression of adipogenic and smooth muscle cell lineage markers in hMSCs, which were performed in (A) 0.5 Hz and (B) 1 Hz for *PPARG*, (C) 0.5 Hz and (D) 1 Hz for *TAGLN*, in different strains and duration of mechanical stimulate

Fold changes of expression were counted by normalizing to the relative expression amount of corresponding control groups (unstrained groups). Statistical significance ($p < 0.05$) was represented by asterisk which was compared to unstrained (indicated as 0). N = 6, n = 3. Error bar = ± 1 SD.

5.5 Discussion

The present study demonstrates that the amount of strain and rates exerts certain influence on the tenogenic differentiation of hMSCs. It also demonstrated that this occurs only when hMSCs are subjected to 1 Hz loading rates and, does not result in chondrogenic, adipogenic or osteogenic differentiation. It has become apparent that cyclic loading at the regime applied during this study drives the cells towards a more distinctive tendon-like cell phenotype. Up-to-date literature survey has indicated that these observations have not been previously reported. Another novelty of this study is that specific combinations of strain amounts and rate of tensile loading provides specific hMSCs differentiation responses. In this case, although 1 Hz is needed to produce significant cellular differentiation response, varying strain rates results in different outcomes; with 4% strain resulting in the highest proliferation (as reported in Chapter 4) whereas 8% strain produces the maximal tenogenic expression (*DCN*, *COL1*, *COL3*, *TNC*, *SCX*, and *TNMD*).

We chose to use a uniaxial cyclic stretching model as the mechanical stimulation for cellular differentiation for several reasons. First, this model is reasonably well characterized, and thus experiments performed using this model would allow us to compare our data with those from previously reported results. Second, the usage of silicone chambers is easily repeatable to achieve a large number of replicate constructs. Third, there are readily available methods of applying mechanical stimulation to collagen coated silicone chambers. The Strex Strain Unit used with silicone chambers has been demonstrated to study behaviour of cells (Morita et al., 2013; Xu et al., 2012). Finally, we chose collagen type I coating for our silicone construct because collagen type I is the primary matrix component of mature tendon and ligament (Wang, 2006). It would be of interest to note that for tendon or bone tissue-engineering applications, ECM protein coatings can facilitate MSCs attachment and encourage cell growth.

Cooke et al (2008) showed that surfaces coated with motifs from collagen I can mimic surfaces coated with the corresponding whole molecules. A recent study showed that contact with type I collagen promotes the differentiation of MSCs (Salasznyk et al., 2004). In our present study, differences in cell behaviours between the experimental and control groups should be attributed to mechanical stretching because unstrained cells in the control group were also cultured on silicone chamber with a type I collagen coating. Additionally, to minimize these cofounding effects, we used a sine wave signal for the mechanical stimulation of cells which is said to reduce unwanted additional strain magnitude (Kaspar et al., 2002).

The findings of the present study demonstrated significant differences to previous published data in several areas however; there were also results of similar outcomes. For example, in terms of changes to cell morphology as the result of cyclic loading, the present study corroborates previous findings that cell orientation is altered when subjected to cyclic loading (Jang et al., 2011; Neidlinger-Wilke et al., 2001; Park et al., 2004) (refer to Chapter 4). Based on our observations, it is likely that the actin stress fibers, which are a major cytoskeletal constituent, may be responsible for the proliferation and differentiation of hMSCs (Titushkin & Cho, 2009; Yourek et al., 2007). In the previous chapter (Chapter 4), the AFM and confocal fluorescence microscopic analyses demonstrates these changes occurring in the actin stress fibers which, if based on previous findings, suggests that the change in Young's modulus was ascribable to the development of the cellular cytoskeleton during the differentiation process (Morita et al., 2013).

Another similarity to previous studies is the fact that hMSCs subjected to tensile cyclic loading results in the apparent increase in the synthesis of collagen type I and III; and potentially other tenogenic protein expressions (Chen et al., 2008b; Farng et al.,

2008; Xu et al., 2011). However, whilst our study did not demonstrate any chondrogenic, osteogenic or adipogenic expressions; these changes have been reported in others (Friedl et al., 2009; McMahon et al., 2008; Sen et al., 2008; Tirkkonen et al., 2011). We hypothesized that these differences may be attributable to the different loading types, magnitude, rates and even of the device used to create the mechanical strained environments employed in each of these studies; since it has been shown that different types of mechanical signals will produce different outcomes, i.e. resulting in the differentiation of hMSCs towards a specific lineage (Costa et al., 2012). For example, low amplitude or low frequency mechanical loading have been shown to promote osteogenic (1 Hz, 3%, 48 h) (Chen et al., 2008b), myogenic (1 Hz, 4%, 24 h) (Ge et al., 2009), and neuronal (0.5 Hz, 0.5%, 8 h) (Leong et al., 2012) differentiation of hMSCs. In addition, the action of cyclic compression appears to be a major contributing factor required for MSCs to undergo chondrogenesis (Grad et al., 2011). Apparently, loading cells in a uniaxial and biaxial manner will also result in different outcomes. In another study using similar rate and magnitudes to ours but employing biaxial loading, MSCs tend to differentiate towards osteogenic lineage (Jagodzinski et al., 2004). Thus, it is not unexpected that uniaxial cyclic stretch is believed to be of paramount importance in the development of functional musculoskeletal tissues (Mackey et al., 2008); especially for the differentiation of MSCs into tendon / ligament fibroblasts. It has been established that different timing of mechanical signals will produce different outcomes, i.e. resulting in the differentiation of hMSCs towards a specific lineage (Kelly et al., 2010). Cyclic uniaxial stretching could induce differentiation of hMSCs, also coincidentally with down-regulation of *PRR16*. Down-regulation of *PRR16* mRNA expression could be detected when MSCs were induced to undergo tenogenic differentiation. Our results were supported by the study of Chen et al (2008). Noted that while the down-regulation of *PRR16* occurred, mRNA expression of *MMP3* were up-

regulated. This can be explained in that mechanical stretching helped to digest cell matrix compared to unstrained cells. This can lead to spatially directed turnover of the ECM, probably followed by adaptations of the matrix composition and architecture. Thus, mechanically induced ECM remodelling may be concomitant with the differentiation of MSCs into specific cell lineages and enables the stretched cells to better adapt to the mechanical deformation forces (Mudera et al., 2000). However, further research is needed to elucidate the involvement of stretch-induced *MMP3* expression in the differentiation tendency of MSCs.

It has been shown that the ECM synthesis would be enhanced either by growth factors or by mechanical stresses, depending of the type of tissue. Cyclic stretch causes change in MSCs phenotype. The link to mechanical stimulation in cellular behaviour such as in MSCs is important due to the intertwining effect of ECM and mechanics behaviors that have been shown (Chiquet, 1999; Reilly & Engler, 2009). Collagen and GAG are the main components of the ECM that are involved in both cell proliferation and differentiation. Elastin is the main component of elastic fibers in the ECM and produces flexibility of tissues and organs (Katsuta et al., 2008). Decorin also has been implicated as a mediator of lateral fibril growth (Reed & Iozzo, 2002) which correlate with size and density of collagen fibrils (Watanabe et al., 2005). Fibronectin is also one of ECM protein that has been found to be important in numerous cellular processes (Yamada, 1983), which constitutes fibrillary structure at extracellular space and is bound to the cell associated matrixes such as collagen I (Lee et al., 2005). Any perturbation of fibronectin's normal interactions, and thus MSCs function, has a diversity of implications due to the importance of the ECM (Larsen et al., 2006). It is known that collagen type I is the primary matrix component of mature tendon/ligament, other matrix molecules including collagen types III, XII, and XIV; elastin; and proteoglycans contain smaller but significant. In our experiments, we have nevertheless

documented a clear increase in the synthesis of collagen types I and III, at immunostaining expression, and both gene transcriptional and translational levels. These results are consistent with previous studies (Park et al., 2004; Shalaw et al., 2006). Similar with the study of Steward et al (2011), whose worked with fibroblast and Mourgeon et al (1999) in fetal lung cells, mechanical stretching induced fibronectin fibril formation increase with higher magnitude strains. This finding supports the proposed importance of mechanical forces in fibronectin modulation. In contrast, Lee et al (2005) stated that the cyclic stretch disrupted the cell-peripheral staining of fibronectin, probably due to their matrix remodeling during the cell reorientation. The study of Choi et al (2007) supported our results that mechanical stimulation enhanced collagen synthesis but not GAG, showed that the cells did not differentiated to chondrocytes. This is also supported by the increase in gene expression. N-cadherin, a homotypic transmembrane adhesion protein, is the predominant cadherin expressed by mesenchymal cells and is found in muscle and connective tissues (El et al., 2007). Our results of N-cadherin expression further confirm that the tensile loading has the ability to mediate the cell-substrate and cell-cell interactions, and thus differentiation of the cells. Ko et al (2001) indicated cadherin-mediated intercellular junctions that are important in tissue remodeling and differentiation, they found that mechanical forces applied to adherens junctions activate stretch-sensitive calcium-permeable channels and increase actin polymerization. Similar to our results, Lefort et al (2011) reported that N-cadherin cell-cell adhesion complexes are regulated by fibronectin matrix assembly.

The use of total collagen as a phenotypic expression marker of hMSCs is appropriate since collagen is the most abundant protein in tendon ECM and provides structural support for adherent cells. In this study, secreted collagen and not deposited collagen i.e. in cells and extracellular matrix, were measured mainly because of the study design which required us to measure the production of this protein from cells without having to

sacrifice the cells at different time points. The other rationale is because it has been demonstrated previously that 90% of newly synthesized collagen is normally present in the medium (Myllyharju & Kivirikko, 2004). When collagen producing cells such as MSCs and fibroblasts are cultured, both pro-collagen and collagen (mature type) are secreted into the medium. It is at this stage and not at the post-modification protein that the collagen content was measured (Lareu et al., 2007). In contrast, levels determined in the cell layer provide an index of the ability of the cells to undergo post-modification process and not just the ability to produce collagen. A similar concept was used in a previous study which unsurprisingly also demonstrated that strained cells produced higher amounts of collagen (Yang et al., 2004).

Collagen molecules, or tropocollagen, form a triple-helical structure and can spontaneous assemble into fibrils (Brodsky & Baum, 2008). Entrapped cells can remodel collagen by remodelling the orientation of collagen fibers. They generate contractile forces which exceed the mechanical resistance of the matrix and lead to the inward compaction of the fibrillar network (Tranquillo, 1999). Although our study showed that increasing strain at 1 Hz resulted in the increase in collagen production, which was similar to many studies (Howard et al., 1998; Shalaw et al., 2006; Yang et al., 2004), Choi et al. (2007) showed that when subjected to low strain (5%) and in very low frequency (0.03 Hz), an increase in collagen production of hMSCs can be observed. This suggests that extremes in strain magnitude may verily influence the expression of collagen protein from hMSCs.

It is interesting to note that at 0.5 Hz, cells did not undergo significant proliferation (Chapter 4) or cellular differentiation (Chapter 5). This suggests that cyclic loading rates below 1 Hz may not be sufficient to stimulate the appropriate cellular phenotypic responses but rather of cell proliferation. In previous studies that look into the effects of

mechanical stimulation on MSCs using low loading frequencies (0.1 - 0.5 Hz), it is shown that cyclic stretching results in osteogenic lineage differentiation. This is in contrast to what has been observed in our study. It has been suggested that at these rates, cells do not perceive the mechanical cues as stretching, but rather of compressive forces (Pitsillides et al., 2003; Ursekar et al., 2014). However the studies did not specifically attempt to prove this hypothesis in their experiments (Huang et al., 2009; Lohberger et al., 2014; Wu et al., 2013a). One aspect that needs to be considered is that the differences observed between our study and that of previous reports, may have been related to the Flexcell system used in their studies. In contrast to the Strex machine used in our study, this device employs a suction mechanism at the centre of the elastomeric cell culture surface to create the stretching effect. It may be the case that the radial stretching effect of the Flexcell system could have produced compounding compressive forces to the attached cells thus resulting in the osteogenic lineage differentiation. This however remains speculative and would require further supportive findings in future studies.

The question of whether cells subjected to higher strains but at lower frequency respond in a similar way to cells subjected to lower strains but at higher frequency remains to be addressed. Our results demonstrate that the increase in strain does not double the increase in protein or of its tenogenic expression. It has been suggested that strain rate as the result of increasing strain levels e.g. from 4% to 8% produces an increase in signaling due to the acceleration involved, e.g. 0-4% over 1 second vs. 0-8% in 1 second (Mosley & Lanyon, 1998; Zaritsky et al., 2014). From the results of our study, this does not appear to be the case. Similarly, the results from the 48 h culture at 0.5 Hz and the 24 h cultures from 1 Hz did not show similar levels of protein or gene expressions. This is true even when comparing 0.5 Hz + 8% and 1 Hz + 4% cultures. When considering that proliferation is only significantly increased when subjected to 1

Hz + 4%, it is suffice to say that cell response is specific to a selected combination of strain and frequency cyclic loading; and that at the present time, there are no simple known correlations between them.

The clinical implication of the study is apparent and may lead to several potential applications. Although further studies are required, it is now possible to extrapolate the data obtained from our study to be applied into patients. In fact, this is not new since many studies have demonstrated that mechanical loading is beneficial to the musculoskeletal system (Dook et al., 1997). This particularly applies to tendon that has been shown to undergo tissue reparative process when subjected to stretching exercises (Buchanan & Marsh, 2001; Couppé et al., 2008). What is new here in this study is the fact that only a certain combination of strain and cycling loading rates may be beneficial for multipotent cells such as hMSCs, while other combinations may not be or in fact quite the opposite, may even result in detrimental outcomes. Once the optimal combination has been established, such as that which is observed in the present study, stretching will elicit anabolic responses from the tendon cells. This in turn increases the production of type I collagen in the peritendinous tissues as demonstrated previously (Olesen et al., 2007).

Tendons, being viscoelastic tissues that are stiffer than most other soft tissues, allows the transfer of large tensile forces to occur without causing tissue or cell damage (Wang, 2006). Indeed, although resistant to tensile forces, tenocytes are still subjected to high mechanical stresses enclave within a highly mechanoactive environment (Engler et al., 2006). However, to study the mechanical processes underpinning the cellular response within an *in vivo* environment would be technically unmanageable hence; a model such as the one employed in the present study may be more realistic, appropriate and informative. We recognize the limitations of a system that do not truly mimic the *in vivo*

environment, however these have been considered during our analyses and that we have not overstated the findings of the present study. We also recognized that although the present study was well designed, several limitations were unavoidable and thus need to be highlighted here. Firstly, it needs to be reminded that as with any *in vitro* studies, the present study does not take into account the complexities of surrounding tissues and thus translating the findings into clinical applications would need to be done with caution. Secondly, the present study assumes that the effect of the stretching occurs in a uniform manner, which in reality may not be the case. When certain areas within the substrate are subjected to a phenomenon known as differential stretching was suggested in previous studies (Brown, 2000; Lazopoulos & Stamenović, 2006; Pitsillides et al., 2003). Limited by the size of the silicone chamber and the maximal rate of which cells can proliferate, the present study could only be conducted up to 72 hours. There is a downside to this, since it is possible that certain gene expressions such as osteogenic expressions may not have been detected. In previous studies, it appears that culturing MSCs up to 14 days may be needed for these changes to be observed. Hence, it may be the case that the tests from our experiment may have shown false negative results. It needs to be reminded however, these changes may be better applied for static cultures and probably not applicable to our stretching cultures (Taira et al., 2007; Yamamoto et al., 2014). Results from other studies seem to suggest that this is the case (Kearney et al., 2010; Qi et al., 2008; Wu et al., 2013a). Notwithstanding these limitations, the findings of the present study are still valid and useful owing to the robust study design employed. It is however hoped that future studies can be conducted using more advanced techniques that are not subjected to the limitations mentioned above.

5.6 Conclusion

Cells subjected to 1 Hz cyclic uniaxial stretching demonstrated significant cell differentiation at higher strain with maximal tenogenic expression observed when

stretched at 8% strain, but the differentiation not towards other mesenchyme lineages. No significant increase in MSC differentiation gene expression was observed when subjected to 0.5 Hz loading. There is no dose-response observed as the result of increased strain magnitude, and it is more likely to be the case that a specific combination of rate and strain magnitude will elicit specific cell responses.

CHAPTER 6: EXPRESSION AND ROLE OF EPITHELIAL SODIUM CHANNEL MECHANOTRANSDUCED HUMAN BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS

6.1 Introduction

Epithelial sodium channel (ENaC), also known as amiloride-sensitive sodium channel has been widely reported to play an essential role in regulating cell physiology. They also act as mechano-sensors in a variety of cell types including the load bearing cells such as chondrocytes and osteoblasts (Kizer et al., 1997; Lewis et al., 2013). Although several studies have suggested the mechanosensitive role of ENaC in cells of mesenchymal lineage (Mow et al., 1999; Mobasheri et al., 2002), its expressional changes and involvement during mechanical strain mediated differentiation of MSCs have not been demonstrated yet. This chapter will investigate the role of ENaC in hMSCs that are subjected to uniaxial mechanical stretching.

6.2 Literature Review

Epithelial sodium channel (ENaC) is a key component of the transepithelial sodium (Na^+) transport in the epithelia of distal renal tubules, lung, distal colon, airways and secretory ducts of many exocrine glands (Duc et al., 1994; Horisbergera & Chraïbi, 2004). ENaC is composed of α, β, and γ subunits (Schild et al., 1997). In humans an additional δ subunit exists but little is known about its function (Waldmann et al., 1995). In addition to transepithelial Na^+ transport, it is suggested that ENaC may play a role in epithelial differentiation (Brouard et al., 1999). Deletion of α subunit of ENaC in keratinocytes and epidermis from mice models resulted in epithelial hyperplasia, abnormal nuclei, pre-mature secretion of lipids, aggregated keratohyaline granules and abnormal expression of the epithelial differentiation markers which includes K1, K6, and involucrin (Mauro et al., 2002). Other than the epithelial cells, ENaC were also

found in non-epithelial tissues that are normally exposed to mechanical stimuli such as the nerve endings of foot pad (Drummond et al., 2000), the nerve endings innervating the aortic arch and carotid sinus (Drummond et al., 1998), vascular smooth muscle cells (Drummond et al., 2004) and load bearing cells such as chondrocytes (Lewis et al., 2013) and osteoblasts (Kizer et al., 1997). Several studies have reported that ion channels residing in the plasma membrane of chondrocytes and osteoblasts are involved in the transduction of mechanical signals (Davidson et al., 1990; Mobasheri and Martín-Vasallo, 1999; Mow et al., 1999; Mobasheri et al., 2002). The existence of ENaC in such load bearing cells suggests that there is a mechanoactive role for ENaC. However in these tissues, the role of ENaC is poorly understood and it is suggested that this ion channel function as transmembrane adhesion molecules that associate directly with cytoskeletal microtubules as well as with ECM components like collagen type IV (Liu et al., 1996). As proposed by Shakibaei and Mobasheri (2003), ECM macromolecules (collagen type II), β1-integrins, ENaC and voltage activated calcium channel (VACC) acts as putative mechanosensitive ion channels that regulates sub-cellular signal transduction pathways through the perception of physical contact and stresses from the ECM. This process, known as mechanotransduction, is said to be mediated through sodium currents and thus can be controlled through sodium channels (Mobasheri et al., 2005).

As described in Chapter 2, besides applying strategies to control MSCs fate using biochemical factors, there is increasing evidence that mechanical factors are potent enough to control their fate *in vitro*. It has been shown that MSCs undergo tenogenic differentiation when subjected to stretching (Popov et al., 2005; Xu et al., 2012) including our study (Nam et al., 2015). It is suggested that this process is controlled through the regulation of sodium channels although; this assumption was based on studies that studied tenocytes and not progenitor cells (Magra et al., 2007; Scherb et al.,

2009; Massoud, 2013). Studies looking at ENaC role in progenitor cells such as MSCs appear to be lacking.

Hence, the present study was conducted to investigate the expression of ENaC during mechanical stretching and its possible role during differentiation of human mesenchymal stromal cells.

6.3 Materials and Methods

6.3.1 Optimization of benzamil concentration

A stock solution of 10 mM of benzamil was prepared in 100% methanol. To optimize the concentration of benzamil which served as ENaC blocker to be used in this study, benzamil at various concentrations (1 µM, 10 µM, 25 µM, 50 µM, and 100 µM) were diluted with culture medium immediately before treating hMSCs.

6.3.2 Cells seeding and application of mechanical stretching

hMSCs were harvested at passage 2, counted with an overall viability of more than 90% was observed using trypan blue exclusion test. A total of $1x10^5$ hMSCs were plated on each collagen type I-coated silicone chamber. After 48 h of culture, the concentration of FBS in culture medium was reduced to 1% for 24 h in order to align most cells into the G0 phase of the cell cycle, and changed to growth medium with or without 10 µM benzamil, before assembling into uniaxial strain device. Uniaxial cyclic stretching at a frequency of 1 Hz and a magnitude of 8% was applied (this variable was chosen since the results from Chapter 4 reveal 1 Hz and 8% show the highest tenogenic expression). Cells in the control group also were cultured on silicone chamber and maintained in the same incubator but without stretching. The cells were harvested after 6, 24, 48, and 72 h of cyclic loading for downstream experiments, including microscopy of cells, immunostaining (72 h), and gene expression assay.

6.3.3 Collagen immnuhistochemistry

The collagen staining was performed according to the manufacturer's recommendation. The methanol fixed unstrained and strained cells were blocked using hydrogen peroxidase to reduce non-specific background "noise" for 5 min. Primary antibodies i.e. rabbit anti-collagen type I or rat anti-collagen type III diluted 1:100 was applied to each specimen and incubated for 30 min. Subsequently, the specimens were incubated with streptavidin-peroxidase secondary antibody for 30 min. For signal detection, 3,3'-diaminobenzidine tetrahydrochloride chromogen substrate were applied for 5 min and examined under light microscopy.

6.3.4 N-cadherin and fibronectin immunostaining

hMSCs were fixed with 4% paraformaldehyde in PBS, followed by permeabilization with -20ºC acetone, and incubated with 1% bovine serum albumin to block non-specific binding of antibodies. For N-cadherin and fibronectin staining, the specimens were incubated with respective primary antibodies diluted 1:300 for 1 h, and with appropriate FITC secondary antibodies diluted 1:600 for 1 h. Nuclei were stained by Hoechst in blue. The fluorescently stained samples were imaged by using a laser scanning confocal microscopy system.

6.3.5 RNA isolation and multiplex gene expression assay

To determine the correlation between the tenogenic effects of hMSCs by mechanical stimulation and ENaC blocking activity, we employed multiplex gene expression assay. Total RNA was extracted from unstrained and strained hMSCs (with blocker and without blocker) using the RNeasy mini kit. RNA concentration and purity was assessed using a NanoDrop Spectrophotometer, and RNA integrity was assessed with a BioAnalyzer. Only samples with high quality were selected for microsphere-based multiplex branched DNA downstream analysis. The mRNA expression of mesenchymal

lineages (Table 6.1) was quantified by the QuantiGene 2.0 Plex assay (2.0 plex set 12082, Panomics/Affymetrix Inc., Fremont, CA, USA). The housekeeping gene was *PGK1* (phosphoglycerate kinase 1).

Table 6.1. The genes of interest were determined in this study

Related marker	Gene name	Abbreviation
ENaC subunit	Sodium channel, nonvoltage-gated 1, alpha	*SCNN1A*
	Sodium channel, nonvoltage-gated 1, beta (Liddle syndrome)	*SCNN1B*
	Sodium channel, nonvoltage-gated 1, delta	*SCNN1D*
	Sodium channel, nonvoltage-gated 1, gamma	*SCNN1G*
ECM component	Collagen type I, α1	*COL1*
	Collagen type III, α1	*COL3*
	Decorin	*DCN*
Tendon lineage	Tenascin C	*TNC*
	Scleraxis homolog A	*SCX*
	Tenomodulin	*TNMD*
Bone lineage	Runt-related transcription factor 2	*RUNX2*
	Alkaline phosphatase, liver/bone,kidney	*ALP*
	Osteocalcin	*OCN*
Cartilage lineage	Collagen type II, α1	*COL2*
	Cartilage oligomeric matrix protein	*COMP*
	SRY (sex determining region Y)-box 9	*SOX9*
Fat lineage	Peroxisome proliferative activated receptor, gamma	*PPARG*
Smooth muscle lineage	Transgelin	*TAGLN*
Housekeeping gene	Phosphoglycerate kinase 1	*PGK1*

6.3.6 Statistical analysis

The assays were carried in triplicates (n) per experimental run, using six independent samples from different donors (N) for each group of the experiments. Data were presented as mean ± 1 standard deviation. Statistical significance was analysed by one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference among groups, the difference was evaluated using least significant difference (LSD). A confidence level of 95% ($p < 0.05$) was chosen for determining statistical significant.

6.4 Results

6.4.1 Baseline expression of ENaC subunits in hMSCs

Semi-quantitative PCR was performed to identify the presence of α, β, γ, and δ subunits of ENaC and it was found that all four subunits are expressed in hMSCs (Figure 6.1A). Upon subjecting to stretching at 1 Hz + 8%, the expression of α subunit appears to be increased over time. However, no difference in the gene expression was observed for β, γ, and δ subunits (Figure 6.1C). On using different strain magnitude, it was observed that 8% strained cells expressed higher α subunit as compared to 4% and 12% strain, which coincidentally has resulted in down regulation of α subunit (Figure 6.1B).

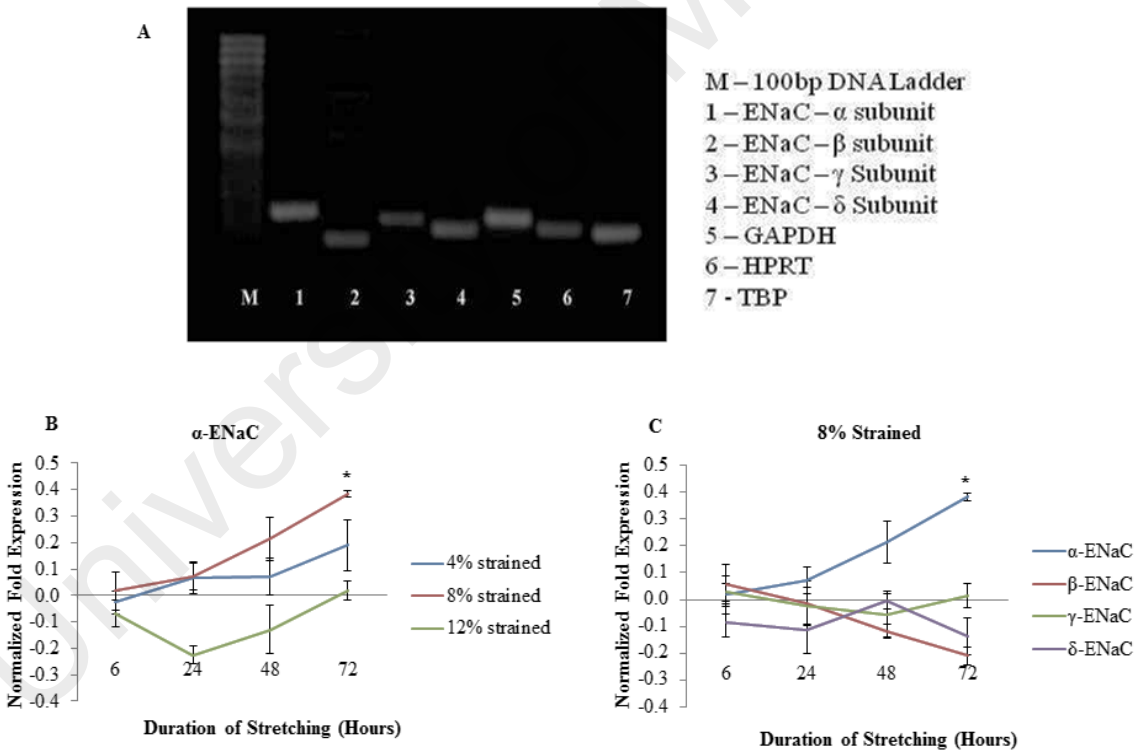

Figure 6.1: The expression of the α-, β-, γ-, and δ-ENaC mRNA in unstrained and strained hMSCs

(A) Analysis of RT-PCR products from hMSCs indicating the presence of the ENaC subunits. (B) mRNA expression of hMSCs show α-ENaC expressed being highest in 8% strained as compared to 4% and 12% strains, (C) Expression of ENaC subunits at 8% strained at 1 Hz. Fold changes of expression were counted by normalizing to the relative expression amount of corresponding control groups (unstrained groups). N = 6, n = 3. Error bar = ± 1 SD.

6.4.2 Morphology of ENaC inhibited-hMSCs after mechanical stimulation

Unstrained hMSCs were treated with different concentration of benzamil (1, 10, 25, 50 and 100 µM) to identify the optimal concentration of benzamil that can be used in the study without causing morphological changes or cell detachment from the silicone chamber (Figure 6.2A). Cells treated at the concentration of 1 µM and 10 µM showed normal fibroblastic appearance of MSCs with similar cell number to that of untreated wells. Cells treated with concentrations above 10 µM have shown apparent changes in the fibroblastic morphology and reduced cell number. These changes occurring at higher concentrations may be due to cell death and/or cell detachment. Based on these results, 10 µM concentration of benzamil was used for further experiments. The morphology of ENaC inhibited-hMSCs showed no significant difference with non-ENaC inhibited hMSCs at the same time point (Figure 6.2B).

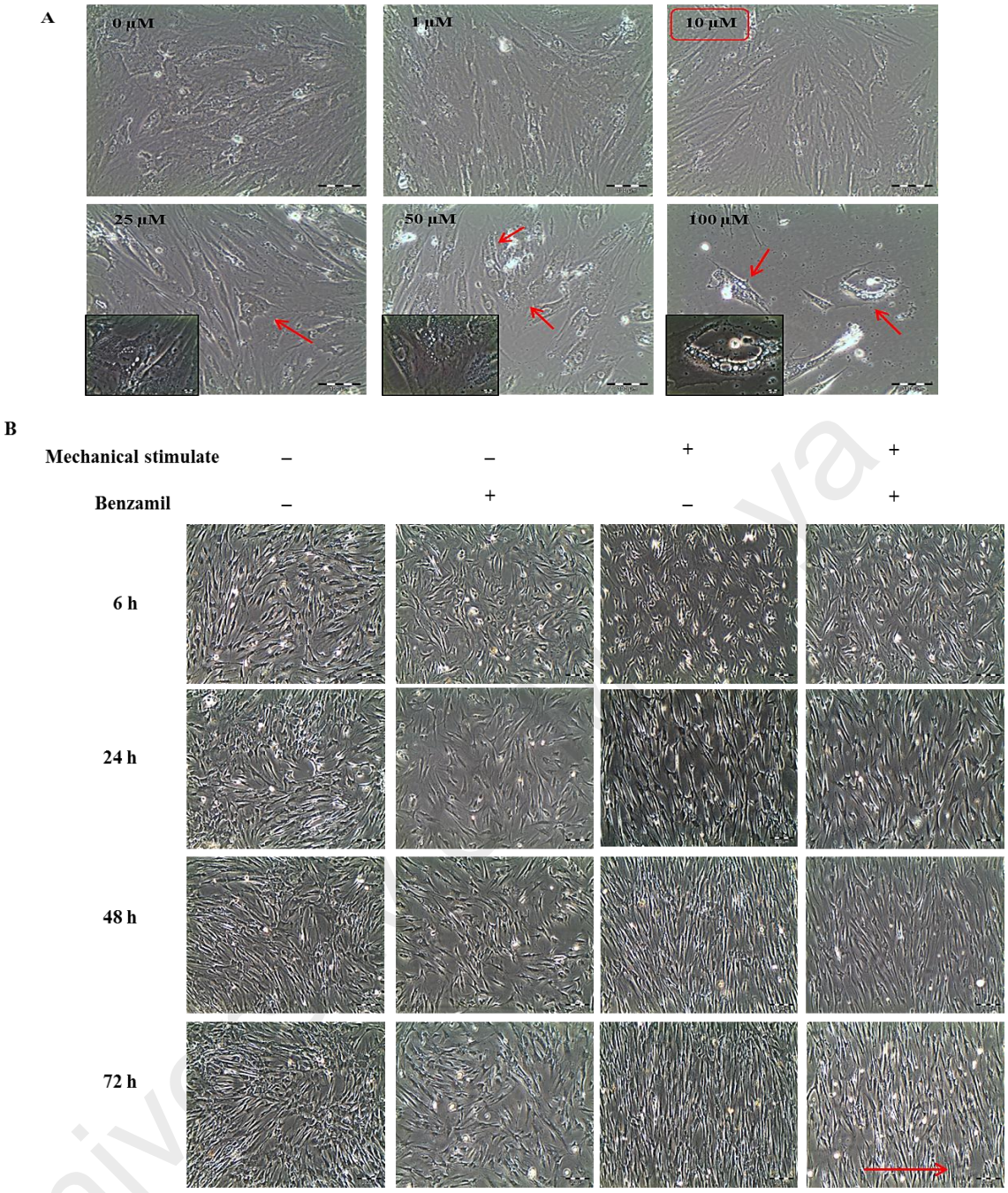

Figure 6.2: Morphology of hMSCs after treated with benzamil
(A) Morphological changes of hMSCs cell culture after 72 hours incubation with benzamil. By increasing benzamil concentration, expression of small vesicles (most likely apoptotic bodies, indicated by red arrows) can be observed. Scale bar = 100 µm; scale bar in higher magnification image at left bottom insert = 20 µm. (B) The unstrained cells and strained cells at 1 Hz, 8%, at different duration of stretching exposure, with or without using 10 µM benzamil, respectively. The direction of uniaxial strain was in the red arrow direction. Scale bar = 200 µm.

6.4.3 Changes in ECM production during stretching and blocking ENaC

Figure 6.3 shows the immunostaining images of collagen I, collagen III, fibronectin and N-cadherin on both unstrained and strained cells treated with or without benzamil. Expression of collagen I and collagen III was found to be decreased in both unstrained

and strained cells treated with benzamil compared to cells without benzamil treatment, apparently in collagen III. The expression of fibronectin and N-cadherin was increased in strained cells as compared to unstrained cells. However, no significant difference in expression was observed between both these conditions when treating with benzamil. Within the group of strained cells, ENaC inhibition has resulted in significantly lower production of ECM fibronectin and N-cadherin as compared cells not exposed to benzamil. Compared the benzamil treated hMSCs group, although N-cadherin expression of ENaC in the benzamil treated hMSCs was slightly increased due to mechanical stretching, other ECM proteins such as collagen I, collagen III and fibronectin remained the same. This may have been due to the fact that the inhibition of ENaC could have resulted in reduced interaction with the ECM components. The results suggest that the production of ECM may be directly correlated with the presence of active ENaC in hMSCs.

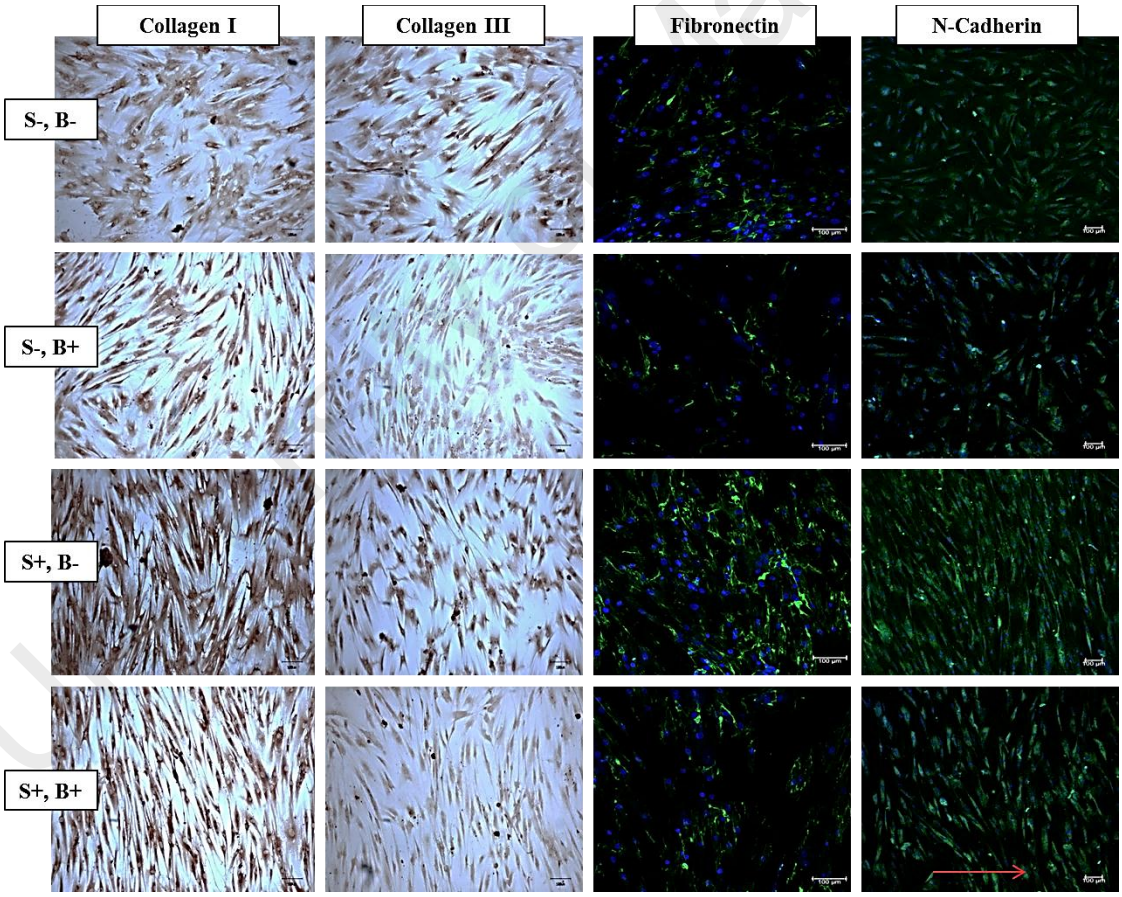

Figure 6.3: Immunostaining and immunofluorescence images of unstained and strained hMSCs cultured with or without benzamil (72 h)

The cells were stained with immunostaining antibody collagen I and collagen III. Immunofluorescence of cells was assessed for fibronectin and N-cadherin proteins using specific antibody dyes. Each cell was stained with Hoechst (blue) to reveal the nucleus, and the images were merged with the corresponding fibronectin or N-cadherin fluoresce images (green). S-: no mechanical stimulation; S+: cyclic stretching applied; B-: no benzamil; B+: with ENaC inhibitor, benzamil. The direction of uniaxial strain was in the red arrow direction. Scale bar = 100 µm.

6.4.4 Correlation between α-ENaC and hMSCs differentiation during mechanical stretching

Table 6.2 indicates the correlation between α-ENaC and hMSCs differentiation as the result of mechanical stretching. Regression analysis showed that there is strong positive correlation between α-ENaC expression with tenogenic markers, except for *SCX*. There are also strong negative association between α-ENaC with *SOX9* and *PPARG*. When these results were combined, we can conclude that the increase of α-ENaC expression will enhance tenogenic markers expression, and at the same time cause a decrease in chondrogenic (*SOX9*) and adipogenic expression, which was comparable with the gene expression results.

Table 6.2. Regression analysis of the relationship of α-ENaC with different cell lineage genes after mechanical strained for mean of different time points

Cell Lineage	Gene	Positive or Negative Correlation (R^2)
Tenogenic	*COL1*	+ 0.8762
	COL3	+ 0.8761
	DCN	+ 0.9557
	TNC	+ 0.7843
	SCX	- 0.0253
	TNMD	+ 0.8318
Osteogenic	*RUNX2*	+ 0.0280
	ALP	- 0.6625
	OCN	- 0.6532
Chondrogenic	*COL2*	+ 0.2760
	SOX9	- 0.9598
	COMP	+ 0.6549
Adipogenic	*PPARG*	- 0.8355
Smooth muscle cell	*TAGLN*	- 0.3345

6.4.5 Influence of ENaC inhibition on tenogenic differentiation

Since previous results (Chapter 5) have demonstrated that cyclic uniaxial stretching may mediate tenogenic differentiation of hMSCs, only tenogenic gene markers were investigated in this study. We evaluated whether inhibiting ENaC can influence the ability of tenogenic differentiation of hMSCs by mechanical stimulation. Although the

cells are mechanically stimulated, blocking ENaC has resulted in the decreased expression of tenogenic markers (Figure 6.4). Expression of ECM components such as *DCN*, *COL1* and *COL3* was increased at the earlier time points i.e. 6 h. However, the expression was decreased over time when benzamil was introduced into the culture medium. No consistent change in the expression of *DCN* was apparent between these 4 time points. In contrast, the *COL1* and *COL3* were slightly increased in the initial time points (6 h and 24 h) as compared to strained cells without inhibited ENaC. However this increase was only transient, and that the levels return to the original levels after 48 h. The collagen results are in consistent with the immunostaining outcome results observed of collagen I and collagen III expression (Figure 6.3) (72 h). α-ENaC gene (*SCNN1A*) was down-regulated after benzamil was added, similar with the expression of specific tenogenic genes markers including *TNC*, *SCX*, and *TNMD*. This suggests that ENaC may have an important role in tenogenic differentiation pathways.

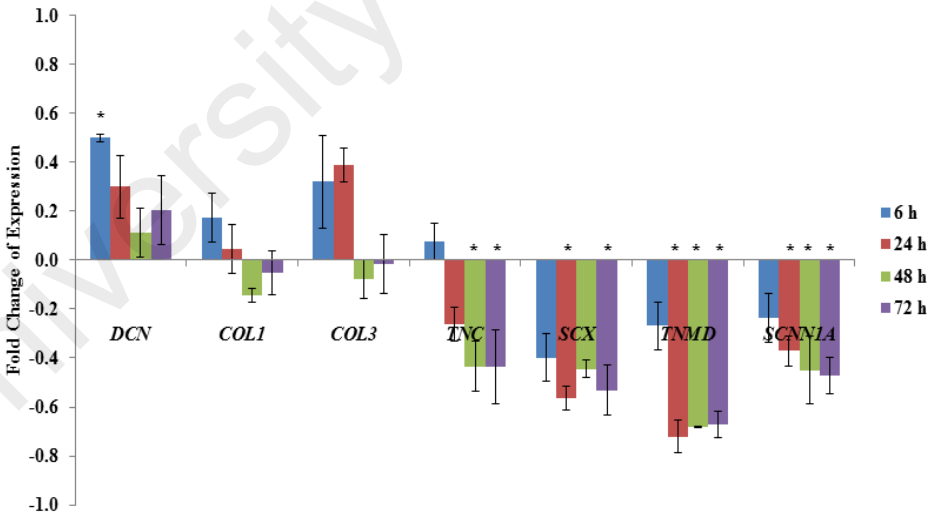

Figure 6.4: Effect of the ENaC blocker, benzamil on tenogenic lineage genes expression subjected to cyclic tensile loading (1 Hz + 8%) at different duration of stretching

The expression level of each gene was normalized against the housekeeping gene. The fold changes in the gene expression were presented as ratio of strained group treated with benzamil to strained group without benzamil. Statistical significance ($p < 0.05$) is indicated by asterisk which was compared to the strained group without treatment (indicated as 0). N = 6, n = 3. Error bar = ± 1 SD.

6.5 Discussion

The ion channel ENaC have been shown to be a potent mechanotransducers in a range of cells types (Lazrak et al., 2000; Roux et al., 2005; Fronius et al., 2010; Simon et al., 2010). In several studies, the role of ENaC especially that relating to terminally differentiated cells appear to be valid (Althaus et al., 2007; Wang et al., 2008; Guan et al., 2009), although studies relating to its effects on specific cellular functions have yet to be widely described. To the best of our knowledge, there has not been any study that has demonstrated the expressional changes of ENaC during mechanical stimulation mediated differentiation in hMSCs. This study is most likely the first to provide evidence of the involvement of ENaC as the potential mechanotransduction mediator that facilitates the processes that underpins the mechanical strain mediated tenogenic differentiation of hMSCs.

We found that all four subunits of ENaC (α, β, γ, δ) were expressed in hMSCs. Similar observation was also reported in other human tissue or cells (Bangel-Ruland et al., 2010; Barrett-Jolley et al., 2010; Kruger et al., 2012). Indeed, ENaC subunits are found in non-epithelial tissues and mediate mechanosensation in cells. For example, β- and γ-ENaC play an important role in mechanotransduction involving both neurons innervating the aortic arch and vascular smooth muscle (Drummond et al., 1998; Drummond et al., 2004). During stretching, only α-ENaC, which was considered as the functional subunit, has been increased and there was no increase in the expression of the other subunits. The results from our study appear to be supported by previous studies. Members of the ENaC family form heteromultimers, with α, β and γ subunits forming the complete channel. The stoichiometry is considered to be 2α/1β/1γ for some authors (Firsov et al., 1998) while others have suggested an association of 3α/3β/3γ subunits (Snyder et al., 1998). Although it has been reported that all ENaC subunits contributes to the formation of functional channel (Schild et al., 1997), the existence of homomeric

channels of α-ENaC alone with distinct properties has also been reported (Canessa et al., 1993; Krueger et al., 2012). Heteromeric channel formation during the expression of all subunits is preferred, however reports also indicate that the α-ENaC alone has the capacity to form homomeric channels in the absence of other subunits (Benos & Stanton, 1999; Ishikawa et al., 1998). In Xenopus oocytes, the expression of α-ENaC alone has been shown to produce small amount of amiloride-sensitive currents suggesting the functionality of homomeric α-EnaC being more important than the others (Canessa et al., 1994). In a study expressing recombinant α-ENaC in stretch-activated cation channels null cells in human primary osteoblast, the expression of non-selective cation channels activity became eminent. These channels were also found to be permeable to calcium ions (Kizer et al., 1997).

We found that during mechanical stimulation, the expression of the functional α-ENaC is increased along with the increase in tenogenic differentiation markers. Our results provide first hand evidence in support of an apparent change in ENaC subunit stoichiometry as a result of differentiation, which also suggest a specific role for α-ENaC in tenogenesis. We speculated that large extracellular loops of ENaC function as antennae that are deflected by mechanical loading, transducing flow stimuli to the channel gating region. Finally, the cytoskeleton regulates ENaC, as the COOH-terminus of α-ENaC interacts directly with the actin cytoskeleton (Mazzochi et al., 2006), and ENaC activity is enhanced by actin-disrupting agents or by addition of short actin filaments *in vitro* (Berdiev et al., 1996). We can therefore interpret that the involvement of other subunits may not be of any significance to the tenogenic expressions, such as that which was observed in this study. Using ENaC inhibitor benzamil in combination with mechanical stimulation, this condition was reversed with reduced tenogenic and ECM component marker expressions observed. These observations suggest that the inhibition of ENaC blunts the tenogenic differentiation of hMSCs, although as observed

in the other arm of the study, the mechanical stimulus exerts a positive role in the tenogenic differentiation of these cells. Our findings was compatible with the study of Park et al. (2009), where they stated that amiloride, benzamil and dimethyl amiloride hydrochloride do not promote osteoblastic differentiation and enhance BMP signalling. However, the findings of Wang et al. (2008) do not suggests that ENaC is involved in MSC differentiation, in that ENaC channel blockers did not reduce myogenic responses in their experiments. It is not surprising that ECM like collagen may not be affected by ENaC inhibition, since sodium Na^+ concentration may have also been influenced. This results in the reduced production of active collagenase, which in turn increases collagen accumulation. It is however unfortunate that due to the limitation of the study design, we are unable to pinpoint as to whether the use of benzamil results in specific α subunit blockage. This would have provided further supportive evidence that the α subunit is truly responsible for tenogenic differentiation.

Our studies, as with that of others, have demonstrated that hMSCs increase their cell density and alter their cells orientation when subjected to mechanical stretching (Nam et al., 2011; Nam et al., 2015). Based on these observations, it is our hypothesis that changes in cell orientation is related to cytoskeletal reorganization, which is dependent on ionic channels. It is therefore surprising for us to observe in this study that when benzamil is introduced in cell cultures, cell morphology and cell growth ability remained unchanged. This suggests that ENaC may not be involved in the control of the steady-state cell volume. Nevertheless, we cannot exclude the fact that ENaC might be involved in transient changes in cell volume. It is widely accepted that ENaC activity is strongly regulated by cytoskeleton elements as discussed previously. Cell cytoskeletons could be deformed by several methods, such as adenosine triphosphate (ATP) stimulation and EGTA. A significant increase in ENaC activity in cells treated with dexamethasone demonstrated that elements of cytoskeleton are important in the

regulation of ENaC activity (Tang et al., 2009). It also explains that ENaC activity is commonly observed in the excised membrane recording. Their study showed that ENaC channels are in high density and distributed in the cells containing splitting pattern, suggesting that ENaC may be involved in cell differentiation and proliferation.

There are other sodium channel blockers that could have been used for this experiment, such as amiloride or phenamiloride. Whilst all of these channel blockers appear to be have an effect with varying efficacy, the specificity of the types of channels have not been previously described. In fact, amiloride, phenamiloride, DMA, MIBA, and EIPA have been shown to block several types of ion transporters, including stretch-activated channel, transducer channel, H-type Na^+ channel, L-type Ca^{2+} channel, and Na^+/Ca^{2+} exchanger (Cox, 1997). In the present study the use of benzamil, a specific inhibitor of ENaC, was chosen instead of amiloride. This is due to the fact that it has been suggested, albeit to a lower conviction, that benzamil is more effective in limiting the adverse effect of ENaC blockage on cell viability (Wesch et al., 2012). Furthermore, amiloride have been shown to interfere with several cellular pathway processes, including inhibiting the Na^+/H^+ exchanger mechanisms (Brouard et al., 1999). Benzamil on the other hand, is more stable and has a very high affinity for the Na^+ channel without affecting other major channels; including K^+ channels (Benos et al., 1992; Simchowitz et al., 1992). It has been suggested that the ionic channel block using benzamil at 1 µmol 1^{-1} results in a complete halt of cellular function and can only be partially reversed. Hence in minimal amounts, the effect of ENaC blocking can be better appreciated without the need to change the volume of the culture media. In previous studies, it has been demonstrated that this enhanced blocking effect of benzamil appears to result from the benzene ring at the guanidino end of the molecule (Figure 6.5A). Guan et al. (2009) reported that 10 µmol/L of amiloride superfused on the inner cortical surface markedly attenuated pressure-mediated afferent arteriolar vasoconstriction.

Benzamil, however, is a benzyl-substituted amiloride analogue that is more potent than amiloride and that it imposes a longer acting inhibition of ENaC than amiloride (Garty & Palmer, 1997). A moderate dose of benzamil (10 μM) was chosen to treat hMSCs for several days, to avoid as much as possible the non-specific (or toxic) effects, where the ENaC of the cells were inhibited but do not result in apoptosis. Yet, this concentration is different in different cell types. Carr et al. (2001) found that the number of action potential mechanical activation of neurons was effectively decreased in the presence of the high dose of benzamil (100 μM) which only occurred at concentrations much greater than that is required to block ENaC channels (< 10 μM) (Alvarez de la Rosa et al., 2000; Kleyman & Cragoe, 1988; McNicholas & Canessa, 1997). In our study, 10 μM of benzamil was deemed sufficient to halt the tenogenic differentiation process in cells. This was also found in other studies such as that of Garty and Palmer (1997), where they reported that at this concentration, benzamil blunted the pressure-mediated afferent arteriolar responses rather effectively. Our findings suggest that there is a role for ENaC in the tenogenic differentiation signalling cascade that is consistent with the observations in myogenic signalling (Jernigan & Drummond, 2005; Oyabe et al., 2000).

There are some studies that demonstrated that inhibiting certain genes expression using siRNAs (small interfering RNAs) approach can also result in similar effects. Whilst the use of siRNAs is an option it has several issues which need to be addressed, for example the variability and incompleteness of knockdowns and the potential existence of non-specificity of the reagents used. In addition, whereas classical genetic screens can identify alleles that uncover regulatory mechanisms, siRNA is purely a loss-of-function technique targeting the mature message (Boutros & Ahringer, 2008). By using siRNAs to target specific genes, the cells need to be easily transfectable, while primary cells are far difficult to transfect in comparison to cancer cell lines. Unmodified siRNA are easily degraded by RNases and assays usually are of short duration due to

transient inhibition only. Besides, the transcripts with high turnover are sometimes difficult to silence. Thus the use of this technique may not be of the best choice if we consider the down sides.

It has been suggested that there are three possibilities as to how ENaC channels can be activated or blocked: (1) by controlling the bilayer tension or curvature directly activating a channel; (2) by controlling the release of another molecule from a cell that in turn activates the channel just like in the case of the present study where benzamil works by preventing sodium from moving intracellularly and competitively inhibiting sodium movements; and (3) by activating a tethering mechanism in which the ion channel binds either to the cytoskeleton or the extracellular matrix. It has also been suggested that ENaC may be responsible for other functions in MSCs, just like those of degenerins (Golestaneh et al., 2001).

In our study, it was mostly unstrained hMSCs, which express low levels of ENaC, that were assayed, as opposed to the differentiated cells expressing high levels of α-ENaC, the latter having lost the capacity to proliferate as a consequence of path differentiation. Alternatively, it is possible that sodium channels are not permanently inserted in the cell membrane of non-polarized epithelial cells such as those of epidermal keratinocytes as suggested by Brouard and his colleagues (1999). This helps to prevent a rapid increase in the intracellular sodium level which would lead to cell swelling and death. It is also possible that the ENaC is addressed to the cell membrane during differentiation by mechanical loading, leading to a transient rise in the intracellular sodium concentration. A rise in cell sodium could activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family, and the stress-activated protein kinase 1 (SEK1) (Kuroki et al., 1997). SEK1 can phosphorylate and activate JNK, which in turn

phosphorylates c-Jun thus leading to an increased transcriptional activity. Therefore the alteration in cell sodium concentration could trigger a cascade of transduction signals ultimately interfering with tenocyte-specific transcription factors. In contrast, if the ENaC of the cells is inhibited, there will be no balance between the extracellular and intracellular sodium concentrations, and thus will influence the signalling pathways, and affect tenogenesis expression (Figure 6.5B).

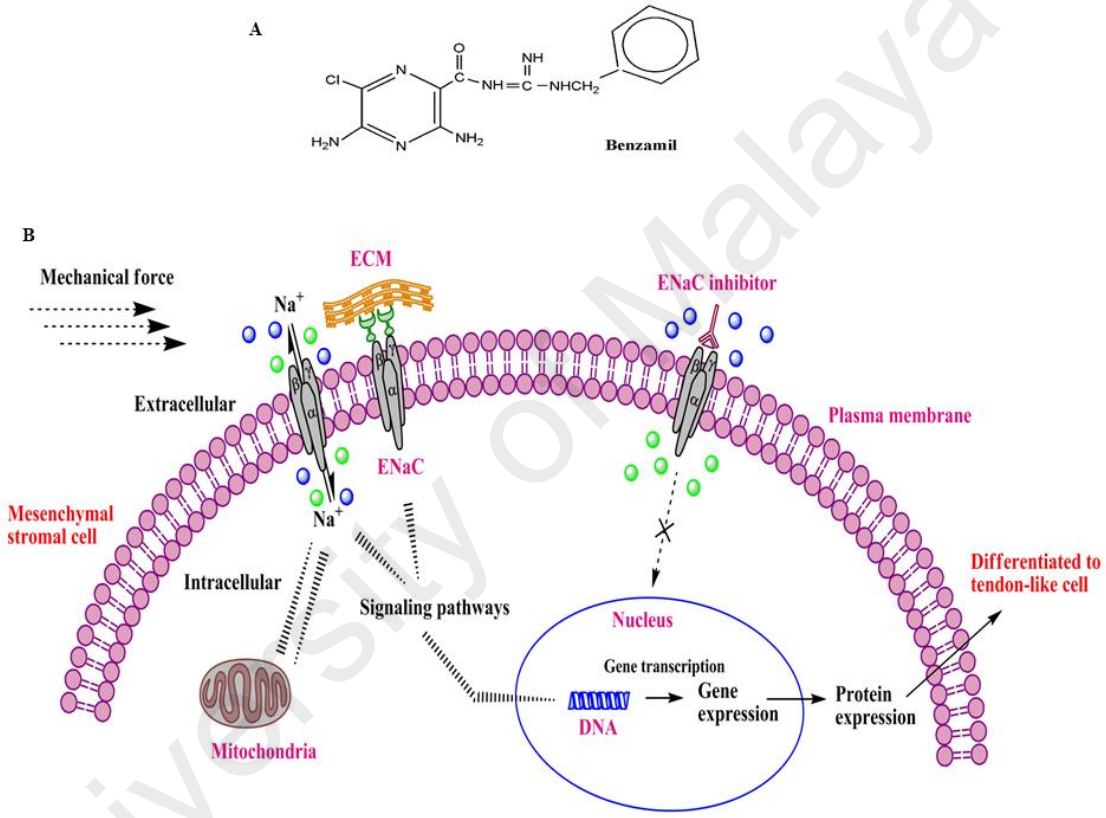

Figure 6.5: A postulated scenario of the mechanism for the regulation of the hMSCs tenogenic differentiation signalling pathways by ion channel ENaC
(B) ENaC leads to up-regulation of the tenogenic gene markers, which in turn activates the tenogenic differentiation signalling pathway. By benzamil (A) which is ENaC inhibitor, caused inhibiting this pathway, and thus influence the cells differentiation.

Investigators have reported that several pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, TNFα and TGFβ1, have been shown to have opposing effects on sodium transport and alveolar fluid clearance. For examples, TNFα decreases the expression of ENaC mRNA and protein levels (Dagenais et al., 2004). Similarly, IL-1β and TGFβ1

decreases the expression of α-ENaC mRNA and protein levels (Frank et al., 2003; Roux et al., 2005). The potential mechanism for the therapeutic effects may be the immunomodulatory properties of MSCs which participates in down-regulation of inflammatory reaction (Dushianthan et al., 2011). There are some studies that have reported that alveolar fluid transport in rat lung was partly improved by the secretion of keratinocyte growth factor, which up-regulates α-ENaC gene expression and Na, K, ATPase activity (Liang et al., 2013; Wang et al., 1999; Ware & Matthay, 2002). Since MSCs could be responsible for inhibiting cytokine expression, further research in this field would be required; with focus being on elucidating the basic mechanisms responsible for the beneficial effects to hMSCs.

Although the current study is robust in its design and provides us with a useful insight to the role of α-ENaC in hMSC differentiation, there were limitations which were unavoidable but are worth highlighting so as to be used to improve future studies. To directly investigate true elevation of cell ENaC activity, the strained and unstrained hMSCs (either blocked by benzamil or not), should be subjected to whole-cell patch clamp recordings to analyse benzamil-sensitive currents. We hope that by using this technique, we are able to demonstrate that the ENaC/degenerin family of proteins (Kizer et al., 1997) is capable of mediating both transepithelial sodium transport and therefore are responsible for the process of mechanotransduction. Secondly, the present study also did not study the relationship of other ion movements which, as many would concur, is dynamic and may also have a role in the tenogenic differentiation processes.

6.6 Conclusion

In short we found that: (1) α, β, γ and δ subunits are expressed in the ENaC in hMSCs and the expression of functional α subunit is higher during stretching at 1 Hz and 8% strain, (2) there is positive correlation observed between the ENaC expression

and tenogenic marker expressions, and (3) the production of ECM staining and the expression of tenogenic marker were reduced after ENaC was inhibited. The findings of the present study thus suggest that α-ENaC may have the role being the main mechanosensitive ion channels that influences tenogenic differentiation of hMSCs. It also further suggests that this ion channel may have a role in tendon regeneration and repair.

CHAPTER 7: THE ROLE OF STRETCH-ACTIVATED CALCIUM CHANNEL ON THE TENOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STROMAL CELLS *IN VITRO*

7.1 Introduction

The role for mechanical stimulation in the control of cell behaviour has been proposed to include the cell proliferation, differentiation and even cell death. It is further suggested that mechanical conditioning of MSCs should be an area of interest as the manipulation of mechanical signalling cues, which enables MSC behaviour to be directed toward a specific cell lineage, can potentially useful for tissue engineering applications. In controlling cell signalling, several reports have suggested that calcium as a signalling molecule is of the upmost importance owing to the fact that there are many facets of cell behaviour that involves calcium dependent cell signalling communication and interactions (Berridge et al., 2003; Bootman et al., 2001; Wall & Banes, 2005). Although previous studies have reported on the role of calcium signalling in many facets of cell physiology and in many cell types, the mechanisms related to the process of mechanotransduction and its relationship with signalling have not been described or suggested extensively. Whilst it may deemed less likely that a minute change in the external mechanical cues can be perceived and transmitted at cellular levels, and therefore to be involved in intracellular calcium dynamics, this phenomenon does occur and is said to involve in general cell pathways and thus be involved in almost all the function of a cell. It is therefore important that the mechanisms involved in the calcium signalling control process be clearly established in tenocytes and progenitor cells, such as MSCs, since the dynamics that occurs in physiological biological conditions where cells are subjected to persistent tensile loading can provide clues for future potential tissue regeneration. In order to explore the possibility of a role for calcium signalling in the management of cellular responses to tensile loading, this

chapter describes a study where we investigated the involvement of extracellular-intracellular calcium movement through stretch-activated calcium channel (SACC) of hMSCs by disrupting its function, and the observe the effects on MSC differentiation and death. It is worth noting that whilst our argument that calcium signalling controls many facets of cell behaviour, in this study we have focused only on cell differentiation and cell death since it is our intention to establish the rate and level of strain that would provide the optimal level of cell response, in line with the studies described in Chapters 4 to 6. In addition, since calcium signalling is a broad topic involving many potential pathways, it cannot be investigated extensively in a short study such as this, hence the focus of this study is limited to the effects of SACC blocker on MSCs under stretching conditions.

7.2 Literature Review

Calcium ion (Ca^{2+}) as one of the most important biochemical signals, plays a pivotal role in regulating a wide range of cellular processes, including muscle contraction, differentiation, proliferation, gene expression, and apoptosis (Clapham, 2007; Orrenius et al., 2003). Ca^{2+} signalling is complex and involves a variety of channels, receptors and secondary messengers. The modulation of Ca^{2+} signalling behaviour in space, time, and amplitude enables cells to activate cellular processes in response to external stimuli (Berridge et al., 2000).

It is believed that MSCs sense mechanical force through different mechanisms but are influenced, or even influences calcium movements' intra- and extra-cellularly (Thompson et al., 2012). Of several notable calcium regulations and mechanisms, stretch-activated ion channels (SACs) has been mentioned as the most like one to be involved extensively in processes involving mechanical stimuli (Wang et al, 2003a). Mechanical stimulation of cells may create calcium signals by causing the entry of the

calcium ion across the plasma membrane or it can be released from intracellular stores (Kim et al., 2014). Stretch-activated calcium channels (SACCs) have been shown to be involved quite extensively in the mechanotransduction process, demonstrating varied responses as different types of mechanical loading are exerted to cells and tissues. This has been reported in many mesenchymal derived cells such as chondrocytes and MSCs (McMahon et al., 2008; Mobasheri et al., 2002; Wright et al., 1997). Despite cells of similar lineage i.e. mesenchymal in origin, different cells utilize different types of calcium channel types, of which these are related to the main of function of the cell. For example, unlike osteoblasts, Ca^{2+} secretion by MSCs does not occur via L-type Ca^{2+} channels but rather, it is mediated by inositol 1,4,5-trisphosphate receptors (InsP3Rs). Further, its entry is controlled by store-operated Ca^{2+} channels and not through the efflux of calcium (Kawano et al., 2002). In general, ion movement in and out of cells response to applied mechanical loading in an almost similar manner despite the differences in their destined functions. It is usually the case that with mechanical stimulation such as stretching, SACs open to allow ions like Ca^{2+}, Na^{+}, K^{+}, and Mg^{2+} to pass through, transducing mechanical signals into activation of intracellular signalling molecules (Hamill & Martinac, 2001; Ruknudin et al., 1993; Wall & Banes, 2005). However, whilst these signals may regulate the process of cell proliferation and differentiation through specific pathways such as focal-adhesion kinases (FAK) or mitogen-activation phosphorylation kinases (MAPK) pathways, in aberrant or extreme mechanical loading conditions, the increase in intracellular Ca^{2+} can trigger the activation of the calpain family of regulatory proteases that help to drive signalling transduction pathways forward to initiate a downstream pro-apoptotic signalling process (McKayed et al., 2012).

Previous studies demonstrated that intracellular calcium signalling is closely interconnected with the mechanical properties of a cell (Eijkelkamp et al., 2013; Kim et

al., 2009b; Kim et al., 2014). Further, several molecules or structural cell proteins such as integrins, the cytoskeleton, and Ca^{2+} channels interact with one another to form a concerted effort for cell responses as the result of calcium signalling cues. Recent reports have indicated that integrin binding and cytoskeletal organization regulate the mechanotransduction process as the result of mechanical stimuli such as stretching, and in several studies have been implicated Ca^{2+} signalling as the most likely candidate ion that is involved in mediating this process (Banes et al., 2001; Wall & Banes, 2005). Ca^{2+} oscillations have been observed in MSCs and are considered as both an indicator and a regulator for MSC differentiation (Kawano et al., 2003; Sun et al., 2007). However, there are no studies that have yet reported the influence of physiological cyclic uniaxial stretching on hMSCs involving Ca^{2+} signalling.

It is nevertheless suggested from the fairly limited literature available today that physiological mechanical loading may be necessary for the development and health of tendon through the control of tenocyte metabolism (James et al., 2008; Mackey et al., 2004). It is further suggested that mechanical forces are converted into biochemical signals in not only tenocytes but also tendon progenitor cells, which are MSCs-like, to regulate aid in the regeneration processes. However, in real-life and physiological biological conditions, mechanical stimuli such as stretching occurs in a cyclic and in many instances, continuous motions. This results in a dynamic flow of ions across the cell membrane and of the phenotypic expression. One can expect that the observed changes may be of the net effect from the ionic exchanges. It is therefore not unexpected that changes in cyclic mechanical loading are expected to activate stretch-sensitive channels in MSCs, and modulate tenogenic differentiation in physiological conditions, albeit under non-apoptosis circumstances. To demonstrate this in a controlled environment, we examined the effects of gadolinium (Gd^{3+}), the most potent blocker of stretch-activated calcium channels known today (Clotfelter & Gendelman,

2014; Hamill & McBride, 1996; Kearney et al., 2008; Perkins et al., 2005) on mechanically strained human bone marrow derived MSCs. It is hoped that our study may help to further our understanding of the fate of hMSCs differentiation through the regulation of calcium movement across cell membrane as the result of mechanical stretching.

7.3 Materials and Methods

7.3.1 Optimization of gadolinium concentration

A stock solution 10 mM of gadolinium was prepared in distilled water. To optimize the concentration of gadolinium which served as SACC blocker to be used in this study, the blocker at various concentrations (2 µM, 10 µM, 20 µM, 50 µM, 80 µM and 100 µM) was diluted with culture medium immediately before treatment was started on hMSCs.

The optimum concentration was chosen and continued for the following experiments. In this case, 20 µM gadolinium was used to block Ca^{2+} entry through SAC of the cells. From the earlier results show that tenogenic differentiation of hMSCs was driven at 8% strain at 1 Hz, thus uniaxial cyclic stretching at these parameters was applied for calcium inhibition experiment.

7.3.2 Cells seeding and application of mechanical stretching

A total of 1 x 10^5 passaged-2 hMSCs per well were plated onto 10 cm^2 collagen type I-coated silicone chamber. The cell seeding and stretching application procedure were similar to ENaC study described in Chapter 6. In this work, the device was programmed to approximate sinusoidal waveforms to 8% strain amplitude at a frequency of 1 Hz. This variable was based on our previous findings where we demonstrated that it was beneficial for cells differentiation, which resulted in enhanced collagen synthesis or tenogenesis gene expression. Cells in the control group were also cultured on silicone

chamber and maintained in the same incubator but without mechanical stimulation. The cells were harvested after 6, 24, 48, and 72 h of cyclic loading for downstream experiments, including microscopy of cells, immunostaining, and gene expression using multiplex assay method.

7.3.3 Collagen immnuhistochemistry

Cells from the experimental groups and corresponding control group were fixed on the STREX chamber stretchable substrate in methanol for 15 min at room temperature. Hydrogen peroxidase was then used to block endogenous peroxidase activity and reduce non-specific background-. Primary antibodies i.e. rabbit anti-collagen type I or rat anti-collagen type III diluted at 1:100 was applied to each specimen and incubated for 1 h. This was followed by the incubation with streptavidin-peroxidase secondary antibody for 30 min. Finally, the samples were developed with 3,3'-diaminobenzidine tetrahydrochloride chromogen substrate for 30 min and examined under light microscopy. All staining procedures were performed in a humidified chamber at room temperature.

7.3.4 N-cadherin and fibronectin immunofluorescence

The cells were fixed with 3.7% paraformaldehyde followed by permeabilization with cold acetone. The cells were then blocked with 1% bovine serum albumin for 30 min, and incubated with N-cadherin or fibronectin antibody at 1:300 dilutions in PBS for 1 h at room temperature. The cells were washed in PBS, incubated with FITC secondary antibodies at a concentration of 1:600 dilution for 1 h in the dark, and counterstained for nuclei with Hoechst for 10 min. Stained cells were analysed under a laser scanning confocal microscope.

7.3.5 RNA extraction and multiplex gene expression assay

To determine the correlation between the tenogenic differentiation potential of hMSCs by mechanical stimulation and Ca^{2+} blocking activity, we used multiplex gene expression assay. The cells were lysed using lysis buffer with 1% β mercaptoethanol. Total ribonucleic acid (RNA) was extracted according to the manufacturers' instructions using the RNeasy mini kit and stored at -80°C until further processing. Quantity and quality of RNA were analyzed using a spectrophotometer, and a BioAnalyzer. Only samples with high quality were selected for microsphere-based multiplex branched DNA downstream analysis. The quantitation of mRNA expression was carried out according to the manufacturer's instructions using the QuantiGene 2.0 Plex assay (2.0 plex set 12082, Panomics/Affymetrix Inc., Fremont, CA, USA). Individual bead-based oligonucleotide probe sets specific for each gene (Table 7.1) examined were developed by a selected licensed manufacturer (Panomics/Affymetrix Inc., Fremont, CA, USA). In determining the optimal housekeeping gene, we compared *PGK1* (phosphoglycerate kinase 1), *HPRT1* (hypoxanthine phosphoribosyltransferase 1), and *TBP* (TATA box binding protein), and observed that *PGK1* was the most stable housekeeping gene in our experiments (Nam et al., 2015).

In order to examine the inhibition of Ca^{2+} by gadolinium affect apoptosis or inflammation of the cells during differentiation by mechanical stretching, same protocol was carried out by using the QuantiGene 2.0 Plex assay (2.0 plex set 12314, Panomics/Affymetrix Inc., Fremont, CA, USA) (Table 7.2). Values for mRNA transcript levels were normalized to corresponding *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) values, compared to *TBP* and *ACTB* (actin beta).

Table 7.1. The tenogenic differentiation genes of interest were determined in this study.

Related marker	Gene name	Abbreviation
ECM component	Collagen type I, α1	*COL1*
	Collagen type III, α1	*COL3*
	Decorin	*DCN*
Tendon lineage	Tenascin C	*TNC*
	Scleraxis homolog A	*SCX*
	Tenomodulin	*TNMD*
Housekeeping gene	Phosphoglycerate kinase 1	*PGK1*

Table 7.2. The apoptosis genes of interest were determined in this study.

Related marker	Gene name	Abbreviation
Extrinsic pathway	Caspase 3, apoptosis-related cysteine protease	*CASP3*
	Caspase 9, apoptosis-related cysteine protease	*CASP9*
	Fas (TNF receptor superfamily, member 6)	*FAS*
	Tumor necrosis factor (ligand) superfamily, member 6	*FASLG*
Intrinsic pathway (pre-apoptotic)	BCL2-associated X protein	*BAX*
Intrinsic pathway (anti-apoptotic)	B-cell CLL/lymphoma 2	*BCL2*
Others	Calpain 1, (mu/l) large subunit	*CAPN1*
	Calpain 6	*CAPN6*
	Inositol 1,4,5-triphosphate receptor, type 1	*ITPR1*
	Catalase	*CAT*
	Cytochrome b-245, beta polypeptide (chronic granulomatous disease)	*CYBB*
	NADPH oxidase 4	*NOX4*
	Cytochrome c-1	*CYC1*
	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	*NFKB1*
	Apoptosis-inducing factor, mitochondrion-associated, 1	*AIFM1*
	Poly (ADP-ribose) polymerase family, member 1	*PARP1*
	Poly (ADP-ribose) polymerase family, member 2	*PARP2*
	Poly (ADP-ribose) polymerase family, member 4	*PARP4*
	Mitogen-activated protein kinase 1	*MAPK1*

Table 7.2, continued.

Related marker	Gene name	Abbreviation
	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult)	*SOD1*
	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	*NFATC1*
	Chromosome 6 open reading frame 4	*TRAF3IP2*
	TNF receptor-associated factor 6	*TRAF6*
	Glutathione peroxidase 1	*GPX1*
	Protein tyrosine kinase 2	*PTK2*
	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)	*CASK*
Housekeeping gene	Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*

7.3.6 Statistical analysis

For every experiment group there was a corresponding non-stimulated control group i.e. control group, cells which were otherwise treated identically and harvested at the same time points. Experiments were performed in six biological (N=6) replicates and technical triplicates (n=3). Data were presented as mean ± 1 standard deviation. For nuclei count experiment, Student's t-test (two-sided, unpaired) was carried out to compare the differences in mean values. While the other experiments, statistical significance was analyzed by one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference among groups, the difference was evaluated using least significant difference (LSD). A p value of < 0.05 was considered statistically significant for all statistical tests.

7.4 Results

7.4.1 Appropriate gadolinium concentration as SACC inhibitor

Unstrained hMSCs were treated with different concentration of gadolinium (2, 10, 20, 50, 80 and 100 μM) to identify the optimal concentration of gadolinium without inducing morphological changes or cell detachment in the silicon chamber (Figure 7.1).

Cells treated at the concentration of 2 µM and 20 µM showed normal appearance of MSCs with similar cell number to that of untreated wells. Cells treated with concentration above 20 µM has showed changes in the fibroblastic morphology and reduced cell number, obviously at higher concentration of 80 µM and 100 µM, where the cell death and cell detachment was detected. Similar for the result of live/dead cells experiment, the amount of dead cells (red colour) increased by increasing the SACC blocker concentration (Figure 7.2). Based on these results, 20 µM concentration of gadolinium was then used for the following experiments.

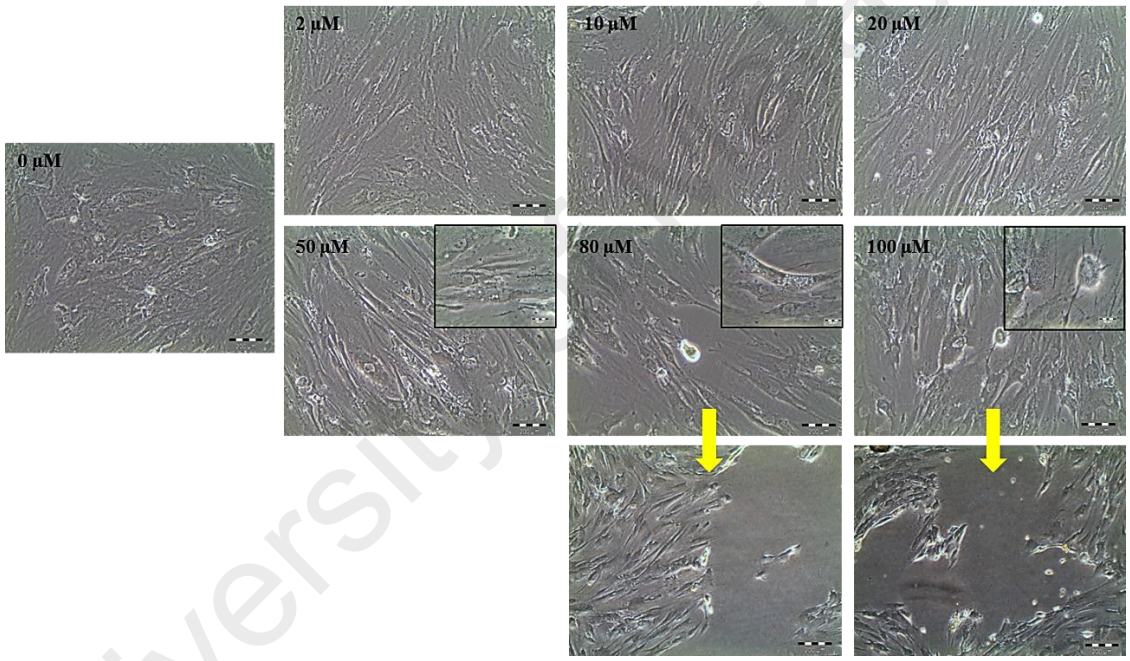

Figure 7.1: Effects of different gadolinium concentration on hMSCs
Morphological changes of hMSCs cell culture after 72 hours incubation of gadolinium. By increasing the gadolinium concentration, the cells were resulted in the expression of small vesicles (probably apoptotic bodies), and thus caused the cells detached (the yellow arrow). Scale bar = 100 µm; scale bar in higher magnification image at left bottom insert = 20 µm.

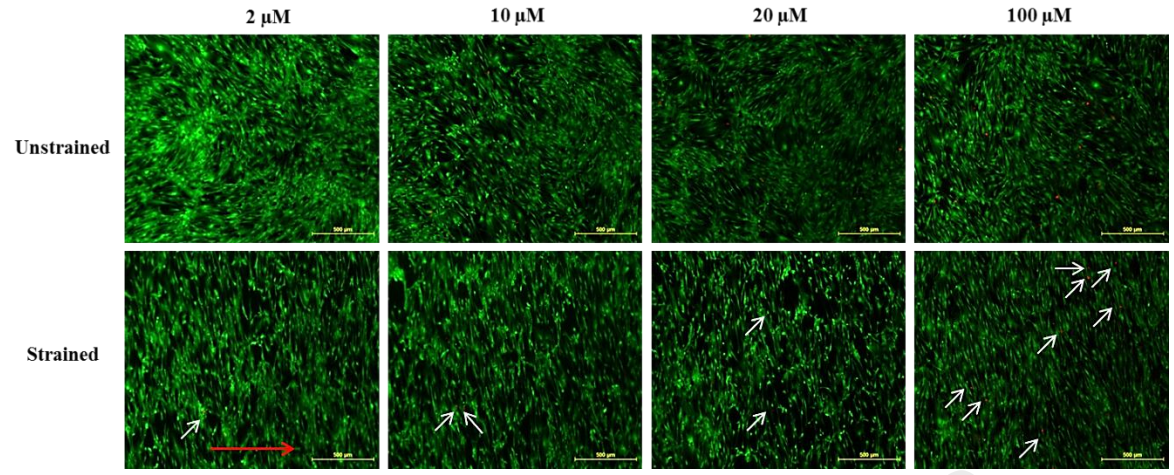

Figure 7.2: Live (green) and dead (red) cells on hMSC treated with different concentration of gadolinium

White small arrows indicate dead cells. The dead cells increased by increasing the SACC blocker concentration. The direction of uniaxial strain was in the red arrow direction. Scale bar = 500 µm.

7.4.2 Morphology of SACC inhibited-hMSCs after mechanical stimulation

The morphology of SACC inhibited hMSCs showed no significant difference with non-SACC inhibited hMSCs at the same time point (Figure 7.3). However the strained cells treated with SACC blocker showed some changes in the morphology and cell number.

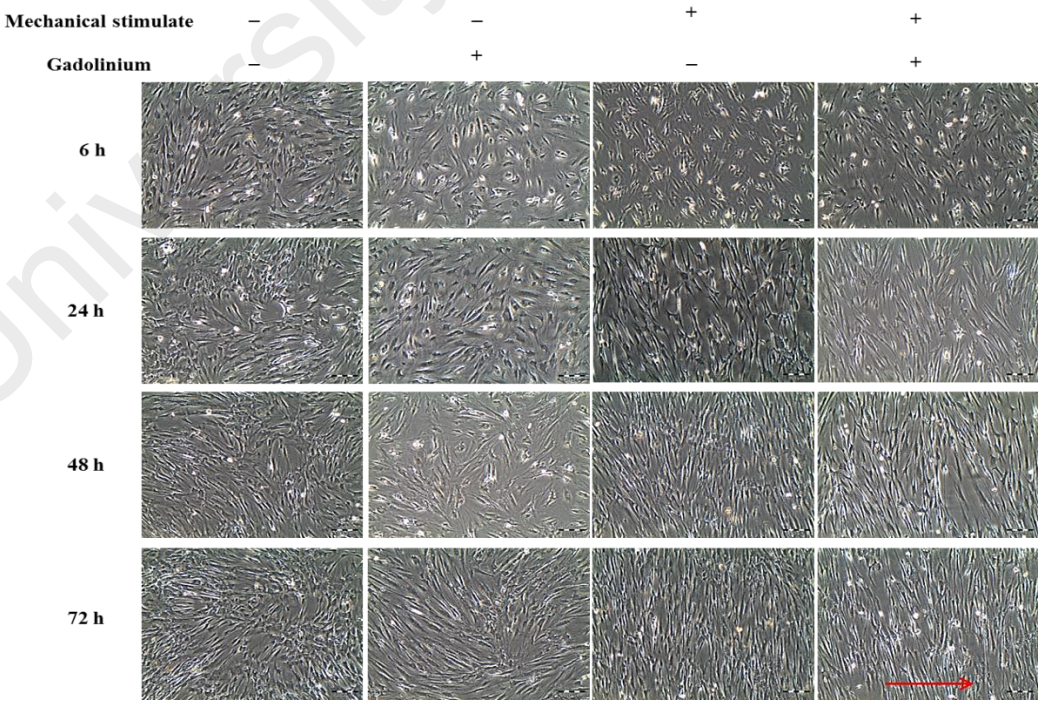

Figure 7.3: Morphology of hMSCs after treated with gadolinium

The unstrained cells and strained cells at 1 Hz, 8%, at different duration of stretching exposure, with or without using 20 µM gadolinium, respectively. The direction of uniaxial strain was in the red arrow direction. Scale bar = 200 µm.

7.4.3 Changes in ECM production during stretching and blocking SACC

Figure 7.4 shows the immunostaining of collagen I, collagen III, fibronectin and N-cadherin on both unstrained and strained cells treated with or without gadolinium. Expression of collagen I and collagen III was found to be decreased in both unstrained and strained cells treated with gadolinium compared to cells without gadolinium treatment. As stated in previous chapter, without SACC blocker, the expression of fibronectin and N-cadherin was increased in strained cells compared to unstrained. However, the intensity of FITC positive cells were relatively less between the strained and unstrained groups, when compared to the gadolinium treated cells. Within the group of strained cells, using gadolinium resulted in the reduction in the production of ECM fibronectin and N-cadherin. These results suggest that the ECM production correlates with the presence of SACC in hMSCs. It also suggests that the inhibition of SACC may have resulted in inadequate cell-matrix interaction due the disruption of ECM formation.

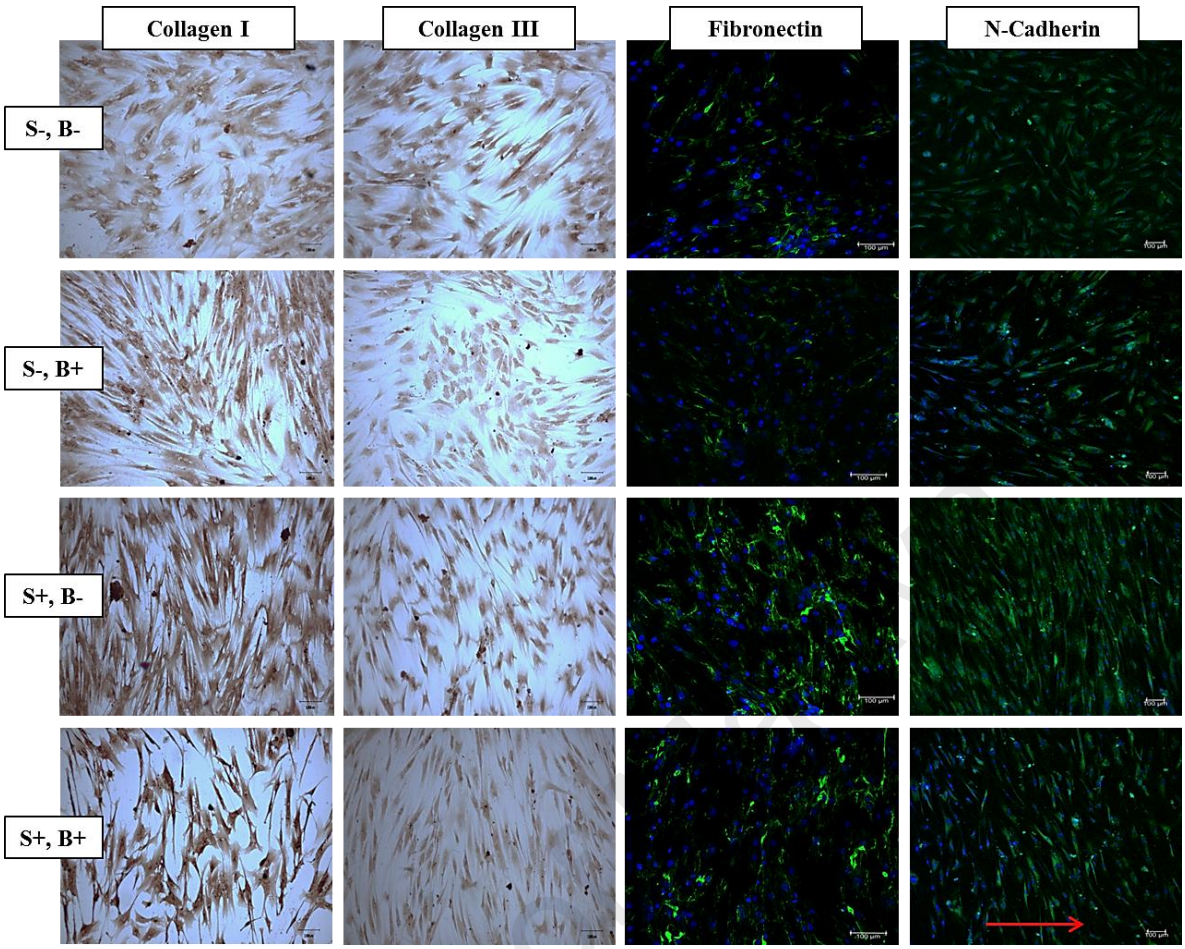

Figure 7.4: Immunostaining and immunofluorescence images of unstained and strained hMSCs cultured with or without gadolinium

The cells were stained with immunostaining antibody collagen I and collagen III. Immunofluorescence was assessed on antibody fibronectin and N-cadherin. Cells were stained with Hoechst (blue) to reveal the nucleus, and the images were merged with the corresponding fibronectin or N-cadherin (green). S-: no mechanical stimulation; S+: cyclic stretching applied; B-: no gadolinium; B+: with SACC inhibitor, gadolinium. The direction of uniaxial strain was in the red arrow direction. Scale bar = 100 µm.

7.4.4 Influence of SACC inhibition on tenogenic differentiation

Influence of ion channels including ENaC and SACC were investigated in this study, as mentioned in Chapter 6 and Chapter 7, respectively, followed by the results from Chapter 5 where shows tenogenic differentiation of hMSCs. We evaluated as to whether inhibiting SACC can influence the ability of mechanical stimulation induced differentiation of hMSCs. Although the cells were mechanically stimulated, blocking SACC has resulted in the decreased expression of tenogenic markers (Figure 7.5). *DCN* and *COL3* was down-regulated after 6 h, significantly for *COL3*, and the level of expression of both ECM genes was lesser folds compared by inhibiting ENaC (Chapter

6). Interestingly, inhibiting ENaC and SACC showed different levels of *COL1* expression. Although similar pattern was shown where the expression level was decreased over time, but the fold change of the expression was shown higher in gadolinium group than benzamil group, significantly in the initial duration (6 h and 24 h). *DCN* and *TNC* were slightly increased at 6 h, compared to strained cells without SACC blocker, however this increase was declined gradually at later time points. The other two specific tenogenic genes markers, *SCX* and *TNMD*, were down-regulated significantly after 24 h. This demonstrated that SACC might play important role in differentiation pathway especially tenogenic lineage.

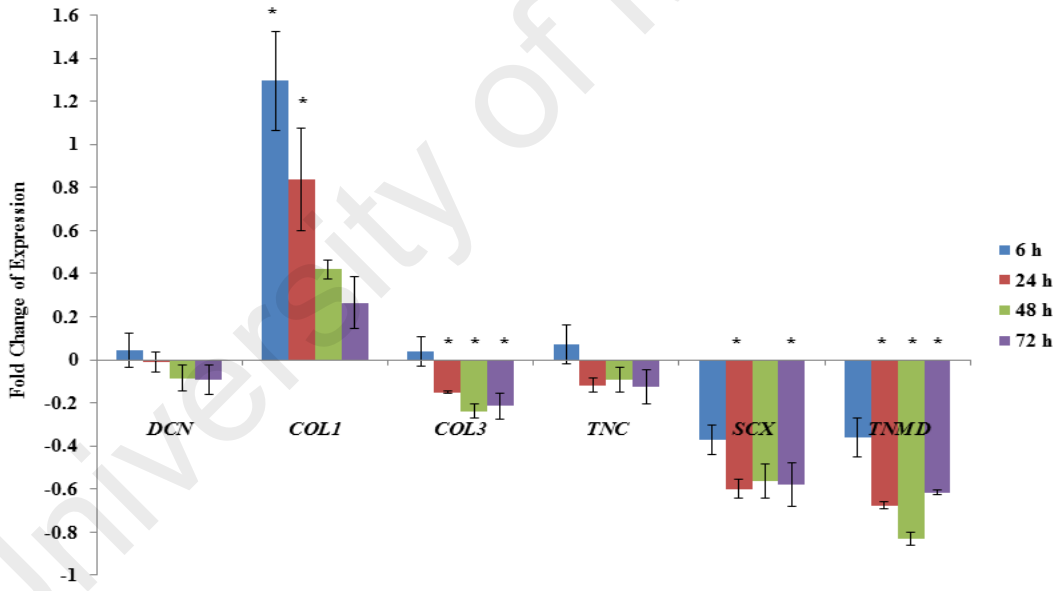

Figure 7.5: Effect of the SACC blocker, Gd^{3+} on tenogenic lineage genes expression subjected to cyclic tensile loading (1 Hz + 8%) at different duration of stretching
The expression level of each gene was normalized with the level of housekeeping gene. The value of fold change was presented as ratio of strained group treated with gadolinium to strained group without gadolinium. Statistical significance ($p < 0.05$) was represented by asterisk which was compared to strained group without treatment (indicated as 0). N = 6, n = 3. Error bar = ± 1 SD.

7.4.5 SACC inhibition did not induce apoptosis of hMSCs

When cells stained with Hoechst, it showed significant reduction (approximately 16%) of cell number in the strained cells in combination with SACC blocker (Figure 7.6). Based on the results of Hoechst staining, we anticipated whether the cell death could be due to apoptosis here, we studied the possibility of the SACC inhibited cells subjected to stretching was driven to apoptotic. Figure 7.7 shows by blocking SACC is not inducing apoptosis of hMSCs.

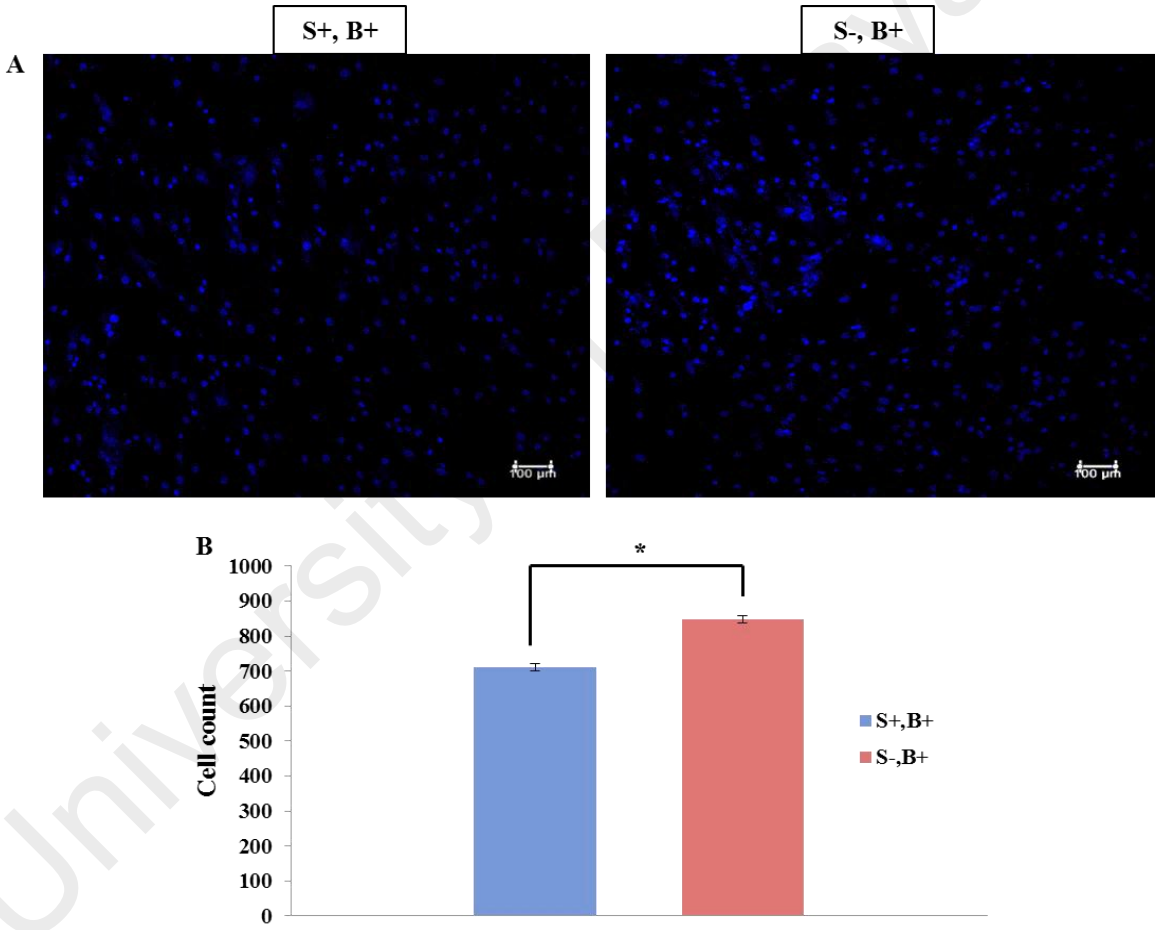

Figure 7.6: Effect of gadolinium on strain-induced decrease in DNA counts
The cells were fixed and DNA stained by Hoechst (blue). (A) The nuclei of the cells were visualized using confocal microscope and counted. Scale bar = 100 µm. (B) In the presence of Gd^{3+}, 8% + 1 Hz for 72 h significantly decreased DNA count. The result was expressed as a mean ± 1 SD for five randomly selected fields in 3 independent experiments. Statistical significance ($p < 0.05$) was represented by asterisk which was compared to unstrained group with gadolinium treated. S-: no mechanical stimulation; S+: cyclic stretching applied; B+: with SACC inhibitor, gadolinium.

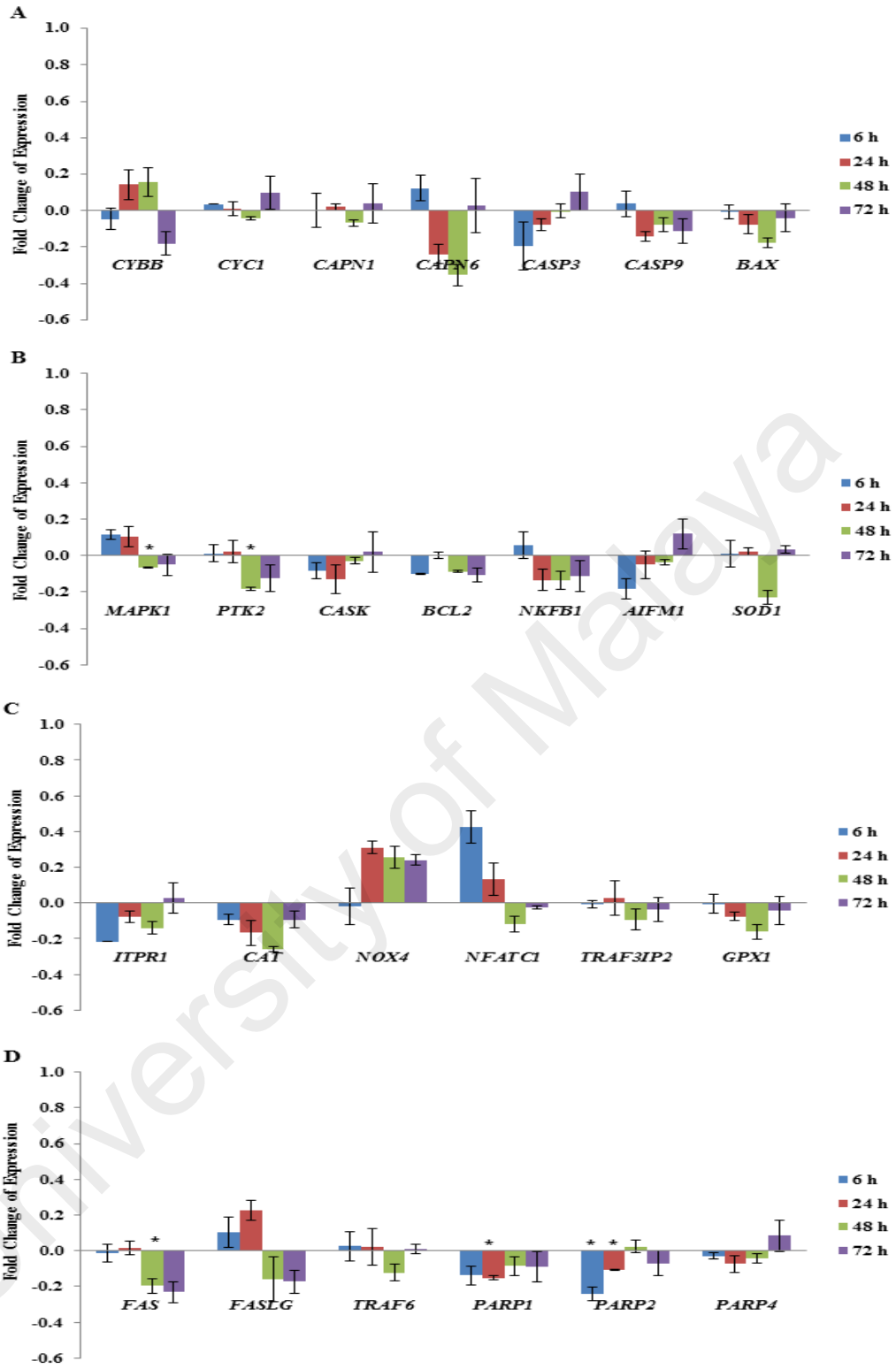

Figure 7.7: mRNA expression of apoptosis genes subjected to cyclic tensile loading at 1 Hz and 8% at different duration of stretching

The expression level of each gene was normalized with the level of housekeeping gene. The value of fold change was presented as ratio of strained group to unstrained group, where both groups treated with gadolinium. Statistical significance ($p < 0.05$) was represented by asterisk which compared to unstrained group (indicated as 0). N = 6, n = 3. Error bar = ± SEM.

7.5 Discussion

The present study used an *in vitro* mechanical stimulation model system in which isolated hMSCs are cultured on type-I collagen coated silicone membrane to determine if tenogenic differentiation are influenced by SACC. As mentioned in the previous chapter, some studies have employed this model to investigate hMSCs mechanotransduction in influencing cell deformation in selected microenvironment changes such pH and osmotic changes (Cigognini et al., 2013; Costa et al., 2012; Ge et al., 2009). Cyclic tensile loading on hMCSs produces an up-regulation of collagen synthesis. In addition, this also results in the transformation of stromal cells into tenogenic-like cells, which is dependent on both the applied frequency and amount of strain subjected to these cells (Nam et al., 2015). However, despite these observations, the essential fundamental understanding underpinning the regulation of the mechanotransduction pathways remain unclear. In our attempt to prove our hypothesis that stretch activated channel may be involved in this process, we investigated the effect of the cyclic uniaxial mechanical stimulation on SACC by using gadolinium ion to block the channel, i.e. because gadolinium is a commonly used antagonist of SACC (Yang & Sachs, 1989). The calcium influx by the mechanical potential appeared to be sensitive to the presence of the gadolinium ion. As predicted, Gd^{3+} being a SACC blocker, reduces the differentiation responses of hMSCs. Our study shows for the first time that Gd^{3+} counteracts the tenogenic differentiation effect of hMSCs by uniaxial cyclic mechanical stretching. Nevertheless, it was interesting to note that cell morphology and expression of genes related to apoptosis was not altered significantly. These results confirm that applied uniaxial cyclic load (1 Hz + 8%) with channel blocker does not induce cell death but instead delayed the differentiation process which indirectly indicates that SACC plays a role in the differentiation process of hMSC into tenogenic like cells.

Mechanotransduction, the transmission and conversion of a mechanical stimulus into a biological response, through a variety of mechanisms of which in the present study suggests that it is channel dependent. There are three main stages of mechanotransduction: mechanocoupling, cell-to-cell communication, and effector cell response. Mechanocoupling is the process by which the applied load is transmitted through the tissues and cells, resulting in different types of cellular deformation. This deformation in cell morphology is then translated into a biochemical response which can include the opening of ion channels such as SACC, and the activation of transmembrane signalling proteins such as integrins and G-protein coupled receptors (Wall & Banes, 2005). The mechanically stimulated cell may also spread the signal to adjacent cells (cell-to-cell communication, involving the passage of calcium ions via gap junction, for instance) thereby amplifying the response (Banes et al., 1999). In response to elevated intracellular calcium and other signals, enzyme activity is initiated within the cell (e.g. activation of MAPK family members) leading to gene transcription and production of protein which can include newly synthesized extracellular matrix, or autocrine/paracrine substances like TGFβ or IGF-I which further amplify the adaptive responses (Maeda et al., 2011). This then suggests that ionic changes, in this case calcium being a widely accepted second messenger, may be heavily involved in the process. It is interesting to note however that Liu et al (2009) reported that greater extracellular Ca^{2+} concentrations did not change cell proliferation but significantly inhibited MSCs differentiation. The possible reason could be the essential role of Ca^{2+} in maintaining the growth and functions of living cells.

A study of Coirault et al. (1999) demonstrated that effects of Gd^{3+} were not due to inhibition of voltage-gated Ca^{2+} channels. A selective stretch-activated channel blocker would allow the relative contribution of stretch- and voltage-activated channels to tension development to be quantified more precisely. At present, there have been no

such blocker is currently available that has a similar function to gadolinium, and as such, gadolinium is presently the only prescribed blocker of stretch-activated channels (Hamill & McBride, 1996; Khatib et al., 2004; Wedhas et al., 2005). In previous studies, cation channels are blocked using Gd^{3+}, in concentrations of 10-100 μM, depends on cell types (Boudreault & Grygorczyk, 2002; Coirault et al., 1999; Kearney et al., 2008; Pingguan-Murphy et al., 2005; Salameh & Dhein, 2013). Incubation of cells with high concentrations of Gd^{3+} blocks electrical stimulation-induced concentration of intracellular calcium increases, as well as inhibiting the influx and efflux of calcium (Khatib et al., 2004). The study further revealed that electrical stimulation-induced mechanical deformation of cells may also be prevented by blocking SACCs and other Gd^{3+}-sensitive pathways. In terms of proliferation and differentiation, a previous study noted that the inhibition of ERK1/2 phosphorylation by mechanical stretching was dependent on the Gd^{3+} concentrations (Yano et al., 2004). A moderate dose of Gd^{3+} (20 μM) was chosen in our study to treat hMSCs to avoid non-specific (or toxic) effect where possible. It is important to recognize that gadolinium ions have a high affinity to bind free bicarbonate and phosphate ions present in several physiological solutions (Caldwell et al., 1998). However, in culture medium (including DMEM used in this study), the phosphate and bicarbonate ions exist as protonated anions that have very low affinity for gadolinium ions. Nevertheless, the possibility that some gadolinium ions are neutralized in the culture medium due to their binding to phosphate and bicarbonate anions cannot be ruled out (Wedhas et al., 2005). Based on our previous literature, the SACC inhibition was assumed to be occurred with 20 μM of gadolinium. A study of Pingguan-Murphy et al (2005) showed that isolated articular chondrocytes in unstimulated agarose constructs exhibited spontaneous characteristic of Ca^{2+} transients, where they explained that this may be associated with a number of

factors including the presence of growth factors in the serum, endogenous release of low levels of ATP, and the generation of oxygen-free radicals caused by laser illumination.

As mentioned previously, one of the earliest effected signalling mechanisms is the fluctuation in intracellular calcium. Rise in intracellular calcium can trigger the activation of the calpain family of regulatory proteases that help to drive signalling transduction pathways forward and have been linked to the initiation of downstream pro-apoptotic signalling. We studied *CAPN1* and *CAPN6*, and the results did not show any significant increase. In fact, it appeared to be down-regulated at 24 h and 48 h for *CAPN6*. Whilst there may be an apparent increase in gene expression, the increment at 72 h is insignificant, and could be due to cytoskeletal remodelling and signal transduction that occurs due to the mechanical stimulation process (Lebart & Benyamin, 2006). Amongst the many downstream intracellular death-signal transmitting pathways the MAPK signalling cascade is the most studied and elucidated, and have been shown to control cell differentiation and survival (Seger & Krebs, 1995). *MAPK1*, also known as *ERK2* (extracellular signal-regulated kinases 2), appears to be down-regulated in long periods of culture conditions (48 h and 72 h) in our study, indicating that cell differentiation can be regulated by mechanical stimulation. Despite the diversity in pro-apoptotic signalling cascades, most cell death pathways ultimately converge with the activation of caspases (Blatt & Glick, 2001). Caspases are specialized proteases that are essential for the physical execution of apoptosis. Apoptosis is usually accompanied by the activation of caspase-3, which is one of the most extensively studied caspases with numerous mechanisms of activation (Jänicke et al., 1998), which is involves activation of caspase-9 (Cain, 2003). Once activated, caspase-3 is directly involved in the sustained induction of DNA damage and the disruption of DNA repair mechanisms. Although *CASP3* increased at 72 h, the increase was non-significant, which suggest that blocking of SACC and with uniaxial stretching does not induced apoptosis mediated

cell death. Expression of the pro-apoptotic Bax protein and mitochondrial dysfunction also mediate apoptosis following mechanical stress (Mayr et al., 2002). This gene expression was down-regulated when mechanical stretching was applied on SACC-inhibited MSCs, demonstrating that mechanical stretching at 8% + 1 Hz did not induce MSCs cell death. Indeed, there is a significant decrease in nuclei count for SACC-inhibited strained cells. However, since there is no apoptosis occurs in this group, we assume that the decrease in nuclei count was due to partial oxidative stress. This is confirmed by the gene expression analysis revealed down-regulation of *GPX1* and *SOD1*, suggest that reduced these 2 genes may result in excessive reactive oxygen species (ROS) production in SACC-inhibited cells under cyclic stretch. *GPX1* is ubiquitously expressed in many tissues, where it protects cells from oxidative stress. The SOD1 enzyme is an important constituent in apoptotic signalling and oxidative stress (Danial & Korsmeyer, 2004). SOD1 inhibits apoptosis by interacting with BCL-2 proteins or the mitochondria itself (Rosen et al., 1993), where was also shown in our gene expression analysis. Study of Tan et al (2015) revealed that decreased osteogenesis of adult MSCs by ROS under cyclic stretch by down-regulation of *SOD1*, and proposed manipulation of the cells with antioxidant would improve their osteogenic ability. Blocking SACCs with gadolinium does not appear to protect cells against strain induced apoptosis, indicating that these channels are not involved in the up-regulation of apoptotic pathways in hMSCs. Similar findings were mentioned elsewhere (Kearney et al., 2010).

When reviewing similar studies we note that other than stretching, which was used in the present study, other loading modalities such as tension, compression, fluid flow, and osmotic pressure (Matthews et al., 2006; Mobasheri et al., 2002; Pingguan-Murphy et al., 2006; Riddle et al., 2006), appears to be dependent on Ca^{2+} signalling as well. This indicates that mechanotransduction for tensile loading in hMSCs may be necessary.

This was evidenced by the lack of a response to the mechanical load when intracellular calcium was chelated. SACCs play a key role in the mechanoresponse of MSCs to tension, compression, and fluid flow (McMahon et al., 2008; Mobasheri et al., 2002); yet, in the current study, their inhibition did not fully suppress the mechanoresponse of the MSCs. These results suggest that the mechanotransductive pathways utilised by MSCs in response to uniaxial tensile loading are distinctive from those used to sense and respond to other loading modalities.

Cells, including MSCs and tenocytes appear to share elements of a load-sensing mechanism similar to osteoblasts and osteocytes; stretch-activated potassium and calcium channels, internal calcium release, interstitial ATP release, and gap junction signalling. All of these appear to play a role in the proliferative response to membrane deformation, substrate deformation, or fluid shear (Scott et al., 2007). A key requirement to triggering the intracellular calcium response in cells under tensile loading is that the calcium must be included in the flow medium, otherwise, the flow by itself does not activate the cells (Hung et al., 1996; Huo et al, 2010). Calcium efflux which occurs locally into cells can potentially alter the osmotic pressure around the hMSCs. Such changes in osmotic pressure might also be responsible for activating the cell by SACC (Hung et al., 1996; Miyauchi et al., 2000; Sun et al., 2012b). The osmotic effects may come into play by the opening of the SACC to allow the influx of extracellular calcium into the cytoplasm of the cells. The total amplitude of intracellular calcium response elicited by the Ca^{2+} supplemented medium however cannot be explained solely by the increase in the osmotic pressure. In another study, it was demonstrated that gadolinium did not affect the intracellular response in osteoblast (Sun et al., 2012a), which further suggests that SACC is not essential in the mechanisms by which osteoblasts detect the elevation of concentration of calcium. Again, this suggests that differences in cell types may lead to different responses.

Results show that supplementing gadolinium ion into cell cultures did not induce the tenogenic differentiation of strained hMSCs. Treatment with this blocker had no effect on unstrained cells, but prevented the increase in signalling associated with cyclic stretching, and eventually influences the cell response. This finding has clear implications on the relationships between calcium with its role in actomyosin contraction of stress fibres, and calcium signalling during tensile loading at 1 Hz + 8% strain. Previous studies in which gadolinium inhibited mechanically induced calcium signalling in mechanically stimulated cells appears to have been observed previously (Caldwell et al., 1998; Mobasheri et al., 2002; Park et al., 2002). These results thereby support the hypothesis that the calcium ion induction by the mechanical stimulation was performed via SACC, and this event is necessary to induce the cell differentiation through mechanical stimulation in our hMSCs.

7.6 Conclusion

Our results show that treatment with SACC blocker followed by uniaxial stretching partially affected tenogenic differentiation and induced cell death. However the cell death was not apoptosis mediated and could be related to decline in the antioxidant markers. This study confirms that tenogenic differentiation of bone marrow stromal cells could be dependent on stretch-activated channels.

Responses to mechanical stimulation involve multiple and redundant pathways, all of which may be related to SACC and may be calcium-signalling dependent. Despite the complexities of intracellular mechanosensitive signalling pathways, there is a possible common mechanism for mechanotransduction through mechanogated ion channels. Mechanical stretch causes Ca^{2+} and other ion influx through the MSCs, which leads to the activation of signalling cascades which in turn results in the activation of several kinases and phosphatase. The activation of these intermediate signalling molecules

leads to the activation of transcription factors that cause increased expression of mechanosensitive genes. Furthermore, an increased in Ca^{2+} influx in response to mechanical stretch could lead to the activation of these transcription factors via Ca^{2+}-responsive proteins, such as protein kinase C. These immediate signalling events to load may be useful in regulating the downstream effects of mechanical stimulation on connective tissues.

The finding that demonstrate changes in hMSCs which are subjected to the regime employed in our experiments are vital to provide further understanding of the potential mechanotransduction mechanisms involved *in vivo*. In practical applications, the importance of mechanical stimulation in normal homeostasis of tissues becomes better understood, such as the response to wounding and in cell-cell communication during passive or active motion during convalescence from injury. The manipulation of the repair process and its application in patients may be integral in advancing therapeutic approaches and motion therapy regimes in the future.

CHAPTER 8: CONCLUSION

8.1 Concluding Remarks

Over the past two decades, studies on molecular and cellular mechanisms that underline the biological responses evoked by physical stimuli have made great progress (Altman et al., 2002; Benas et al., 1999; Maul et al., 2011; Mourgeon et al., 1999; Popov et al., 2005; Steward et al., 2011; Zeichen et al., 2000). However, despite the many advances in this fascinating field of study, many problems still remain unsolved, and their solution will require further exploration and better understanding.

The overall goal of this doctorate thesis was to improve the understanding of the role of biomechanical stimulation and the different potential ions, on hMSC phenotypic expression, behaviour, proliferation and its differentiation toward a tendon fibroblast phenotype. Specifically in this thesis, the effects of cyclic tensile stimuli were studied in two-dimensional (2D) environment that provided controlled presentation of biological moieties. The studies that were incorporated into this thesis provided insights into the contributions of the biomechanical environment to hMSC differentiation toward a tendon fibroblast phenotype. Together, the studies aimed to elucidate the complex interplay between several signalling pathways involved in the tenogenic response of hMSCs to uniaxial cyclic stretching. We can concluded based on the findings of these studies that mechanical stimulation plays a pivotal role in the proliferation and differentiation of human mesenchymal stromal cells. Specifically, uniaxial cyclic stretching stimulation showed a strong potential to increase MSC tenogenesis as demonstrated in our experiments. In the pilot optimization phase of this study, a custom made uniaxial cyclic stretching device (Appendix EA) was designed to apply physiological mechanical loading to progenitor cells leading to the formation of tendon-like cells. Due to the limitations of the custom made device, a commercial uniaxial

tensile loading system (Appendix EB) was then procured for the study on the effects of mechanical stimulation on hMSCs. The system was tested and observed to sustain a population of hMSCs, both viability and proliferative potential, while maintaining sterility. It is worth noting that hMSCs morphology and alignment of the cells are influenced by the mechanical stimulation, and is dependent on the high strain and length of the duration of mechanical stimulation. hMSCs proliferation was also found to be enhanced by mechanical stimulation at low strain levels, as presented in Chapter 4. In Chapter 5, uniaxial cyclic stretching was found to regulate the lineage commitment of hMSCs. At particular strain rate and durations, mechanical stretching (at specific strain rates) enhances the matrix synthesis and propensity towards tenogenic lineage commitment of hMSCs. It then suggests that a specific combination of rate and strain magnitude will elicit specific cell responses. Lastly, our data demonstrated that both ion channels ENaC and SACC may be significantly involved in this process. We were able to observe that inhibiting ENaC and SACC abrogated the beneficial effects of mechanical stretching of tenogenesis in hMCSs. These findings were presented in Chapter 6 and Chapter 7.

8.2 Implications

The motivation for these studies has been developed from the perceived alteration in MSC proliferation and differentiation following musculoskeletal unloading and in many orthopaedic diseases, particularly in tendon-related disease. The implications of this research extend not only to the field of tissue engineering, but also mechanobiology in the context of native tissue development and pathologies with distinct mechanical profiles. While we have examined mechanical stretching has an effect on cells proliferation, our major results have implications for the differentiation of MSCs, since many of signalling cascades are activated by the actions of cyclic tensile loading. Future research in this area can potentially probe a more systematically adapted and

appropriate mechanical stimulation regimes, for both stretch and fluid flow. Preferably this can be achieved by mimicking the microenvironment and physiology of tendon fibroblast. One may also attempt to isolate the effect of membrane and cell deformation from the shear force provided by the media under cell stretch as an alternative to cell function control.

We have demonstrated that the complex nature of MSC tenogenesis commitment, which is dependent on both insoluble and mechanical environments, can be effective in modulating cellular functions and the production of ECM. It is clear that the development of technologies for delivering appropriate physical stimuli, with strictly controlled to the strain amount, frequency and timing of exposure, is an essential in tissue engineering, and thus as a potential avenue for the on-going fight against tendinopathy and tendon related diseases.

8.3 Limitations to Current Studies

The findings in this thesis elucidated the roles that biomechanical stimulation and ion channel cues play on hMSC proliferation and differentiation toward a tendon fibroblast phenotype. Significant studies are still required to provide further understanding of how these types of factors interact to affect hMSC differentiation responses. Limitations in this study have been identified and minimized where possible. However, due to technical and time constraints as well as the question of the research grant is concerned, these limitations remained within the experiments but will be addressed in future studies. These limitations have been mentioned in selected areas within the discussion segments of each chapter.

8.4 Future Research Work

The results obtained from the present study have provided the incentive to further investigation in other factors that may result in degenerative tendon. While future *in*

vitro research should include larger number of replicates and other important parameters such as the varying strain, frequency, and duration of cyclic tensile loading as described in this study, other complementary studies could be conducted to support the application to the clinical problem. In short, the suggestions to improve this study are listed as below:

a) Prolonging the duration of exposure to tensile loading, and adding other parameters of interest in the study design:

At 1 Hz, while cellular responses were not influenced by short-term (6 hours) cyclic tensile strains, but preferable influencing at longer-term (48 hours and 72 hours), yet it remains unclear whether hMSC responses to longer periods (weeks) of strain. There have been suggestions that longer time such as 14 days can further provoke or enhance the hMSCs to form more mature tendon cell / tenocytes, rather than tendon-like cells. Future study can be involved this parameter, since we hypothesis tensile strain periods would provide a significant advancement in the understanding of the impact of the timing and duration of cyclic tension stimulation on hMSCs. Up-scaling the MSCs expansion or differentiation in controllable mechanical stretching machine with controllable parameters for pH, oxygen level, and feeding regime in the machine could also be considered. However, technical difficulties in this remain a challenge such as the maximal cell growth in a restricted environment resulting in contact inhibition (Hayflick limit).

b) Detailed mechanistic studies involving signalling pathway:

More biochemical and gene expression analysis involving other signalling pathway studies has to be included in order to understand the tenogenic processes influenced by mechanical cues. The specific role of these ion channel factors in inducing tenogenesis was not evaluated. Further studies are therefore necessary to focus on investigating the

signalling pathways involved in tenogenesis *in vitro* in order to further elucidate the role of these mechanical factors at different stages of tenogenesis.

c) Three-dimensional (3D) cultures:

One of the next steps for biomechanical alone or combined of biomechanical and biochemical stimulation is to move to 3D stimulation of MSCs. While the use of monolayer cultures and a 2D environment such as STREX plates in this study is simple and can be more easily controlled, and provided sufficient answers to the hypothesis raised in the present study; the development of a 3D culture system would be invaluable with specific fibres alignment. A 3D scaffold or construct more closely represent the tendon formation. Constructs can also more readily be implanted into infarcted tendon to study the integration of tenogenic differentiated MSCs. This again is technically challenging.

d) Animal and clinical studies:

Tendon healing and regeneration are complicated processes. For the design of new therapeutic modalities it will be necessary to investigate certain aspects of tendon formation in greater detail. It will be important to understand the rules for the axial formation of extracellular matrix in tendon and to transfer these rules into novel therapeutic concepts. The necessity of animal based studies is expected to be done in future in order to better clarify the possible mechanisms and solutions involved in mechanotransduction in patients. For patients undergoing rehabilitation, it would be implausible to purpose an exercise regime which employs shoulder or knee or ankle movements at 1 Hz for 24 hours a day. A more practical and workable time frame is therefore needed of which the next experiments to be focused on answering clinically orientated questions and applications. This would be benefited the development of sensible clinical protocols specifically for disease prevention or patient rehabilitation.

Although the work required for a full understanding on the mechanisms involved in cells proliferation and differentiation are to be further investigated, the present study has provided key insights into the problem and suggested a theory, elements of which was tested *in vitro*. The identified genes which were involved in hMSCs tenogenesis would provide a better understanding of the molecular events in tendon formation. This ultimately may allow advances in future tendon treatment strategies. It is hoped that the knowledge contributed by these studies would bring us closer to better understand the mechanisms involved in the cell mechanism processes, and thus enabling future research to identify the right solution for the prevention or treatment of this disease.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Published Articles

1. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. (2015). The proliferation and tenogenic differentiation potential of bone marrow-derived mesenchymal stromal cell are influenced by specific uniaxial cyclic tensile loading conditions. *Biomechanics and Modeling in Mechanobiology, 14(3)*: 649-663. (doi: 10.1007/s10237-014-0628-y) (ISI-cited publication; IF upon accepted: 3.251)

2. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. (2014). The proliferation and tenogenic differentiation potential of human bone marrow derived-mesenchymal stromal cells by uniaxial cyclic tensile loading. *Journal of TESMA Regenerative Research, 3(2)*: 48-49.

3. **Nam HY**, Karunanithi P, Loo WCP, Naveen SV, Chen HC, Hussin P, Chan L, & Kamarul T. (2013). The effects of staged intra-articular injection of cultured autologous mesenchymal stromal cells on the repair of damaged cartilage: A pilot study in *caprine* model. *Arthritis Research & Therapy, 15(5)*: R129. (doi: 10.1186/ar4309) (ISI-cited publication; IF upon accepted: 4.302)

4. Kwong PJ, **Nam HY**, Wan Khadijah WE, Kamarul T, & Abdullah RB. (2014). Comparison of *in vitro* developmental competence of cloned caprine embryos using donor karyoplasts from adult bone marrow mesenchymal stem cells vs ear fibroblast cells. *Reproduction in Domestic Animals, 49(2)*: 249-253. (doi: 10.1111/rda.12262) (ISI-cited publication; IF upon accepted: 1.177)

5. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. (2011). Dynamic behaviour of human bone marrow derived-mesenchymal stem cells on uniaxial cyclical stretched substrate - A preliminary study. *IFMBE Proceedings, 35*: 815-818. (ISI-cited publication)

Submitted Articles

1. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. Uniaxial cyclic tensile loading promotes tenogenic differentiation of human bone marrow-derived mesenchymal stromal cell *in vitro*.

2. **Nam HY**, Murali MR, Abbas AA, Naveen SV, Pingguan-Murphy B, Ahmad RE & Kamarul T. Expression of epithelial sodium channel during mechanical strain mediated mesenchymal stromal cell differentiation.

3. **Nam HY**, Balaji RHR, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. Fate of tenogenic differentiation potential of human bone marrow stromal cells by uniaxial stretching affected by stretch-activated calcium channel agonist gadolinium.

4. Gopal K, Teo YW, **Nam HY**, Kerudin AA, Yaze I, Simrat S, Hindmarch C, & Kamarul T. Mechanical strain triggers the proliferation and differentiation of bone marrow stromal cells and concurrently elicits apoptosis.

Conference Abstract Papers

1. **Nam HY**, Puvanan K, Loo WCP, Suhaeb AM, Mohamed Zubair MA, Haryanti AMW, Sangeetha VN, & Kamarul T. Staged intra-articular injection of cultured autologous mesenchymal stromal cells provides superior cartilage repair outcomes: A study in caprine model. 4th Asian Cartilage Repair Society Meeting (ACRS) 2016.

2. **Nam HY**, Balaji Raghavendran HR, Pingguan-Murphy B, Abbas AA, Merican AM, & Kamarul T. The role of stretch-activated calcium channel on the tenogenic differentiation of human mesenchymal stromal cells *in vitro*. 46th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2016.

3. **Nam HY**, Puvanan K, Loo WCP, Sangeetha VN, Chen HC, Paisal H, Chan L, & Kamarul T. Staged intra-articular injection of cultured autologous mesenchymal stromal cells on the repair of damaged caprine cartilage. 3rd Asian Cartilage Repair Society Meeting (ACRS) 2015.

4. **Nam HY**, Murali MR, Naveen SV, Pingguan-Murphy B, Abbas AA, Merican AA, Ahmad RE, & Kamarul T. The tenogenic differentiation potential of human bone marrow derived-mesenchymal stromal cells is dependent on epithelial sodium channels. 45th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2015.

5. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. The effects of uniaxial cyclic tensile loading on the proliferation and tenogenic differentiation of untreated bone marrow-derived mesenchymal. 44th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2014.

6. Puvanan K, **Nam HY**, Loo WCP, Sangeetha VN, Chen HC, Paisal H, Chan L, Haryanti A & Kamarul T. Staged intra-articular administration of cultured autologous mesenchymal stromal cells improves cartilage regeneration in full-thickness chondral defect: A pilot study in caprine model. 44th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2014.

7. Ahmad RE, **Nam HY**, Murali MR, Abbas AA, & Kamarul T. Role of epithelium sodium channel (ENaC) in mesenchymal stem cell differentiation via the mechanotransduction process. 27th Scientific Meeting of the Malaysian Society of Pharmacology and Physiology (MSPP) 2013.

8. Kamarul T, **Nam HY**, Karunanithi P, Loo WCP, Naveen SV, Chen HC, Paisal H, Chan L. The effects of staged intra-articular injection of cultured autologous mesenchymal stromal cells on the repair of damaged cartilage. Orthopaedic Research Society (ORS) 2013.

9. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. Effect of uniaxial cyclic tensile loading on the proliferation and differentiation of human bone marrow-derived mesenchymal stromal cells. 1st National Stem Cell Congress 2012.

10. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. A preliminary report on the behaviour of human bone marrow derived mesenchymal stem cells during uniaxial cyclic loading. 42th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2012.

11. Ramli A, Kwong PJ, **Nam HY**, Wan Khadijah WE, & Kamarul. *In vitro* development of caprine embryos cloned with adult bone marrow mesenchymal stem cells. 9th Annual Conference of Asian Reproductive Biotechnology Society (ARBS) 2012.

12. Hussin AR, **Nam HY**, & Kamarul T. Down regulation of cell anti-adhesion genes after uniaxial stretch applied on human bone marrow mesenchymal stem cells. 41th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2011.

13. **Nam HY**, Puvanan K, Hussin P, Chen HC, Wagner Loo CP, Murugarah N, Dashtdar H, Wee AS, Lucy Chan KW, & Kamarul T. *In-vitro* differentiation and characterization study on isolated caprine mesenchymal stem cells: a preliminary study. 14th Scientific Meeting of the Malaysian Society of Transplantation 2011.

14. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. Effects on proliferation of human bone marrow derived-mesenchymal stem cells by uniaxial cyclic stretching 7th Annual Conference of Asian Reproductive Biotechnology Society (ARBS) 2010.

15. **Nam HY**, Abbas AA, Merican AA, & Kamarul T. Proliferation rates of human bone marrow derived-mesenchymal stem cells *in vitro* on elastomeric surfaces. 7th Annual Conference of Asian Reproductive Biotechnology Society (ARBS) 2010.

16. **Nam HY**, Pingguan-Murphy B, & Kamarul T. Dehydration rate under static and cyclic tensile loading by self designed uniaxial mechanical stretch device. 40th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2010.

17. **Nam HY**, Pingguan-Murphy B, Abbas AA, Merican AA, & Kamarul T. The effects of cyclic tensile loading on proliferation of human bone marrow derived-mesenchymal stem cells. 40th The Malaysian Orthopaedic Association (MOA) AGM/ASM 2010.