Low Frequency Stimulation of Cells in Dynamic Culture Modulates Differentiation Pathways

By

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To my parents, who always want the best for me!

Abstract

Living cells, depending on their physiological functions, are subjected to a variety of mechanical stimulation. The magnitude and frequency of such mechanical stimulation varies dramatically in different organs. Oscillatory mechanical stimulation at relatively high frequancies, as occurs in walking, respiration and circulation, is one of the most extensively studied schemes. However, the stimulation at extremely low frequencies is rarely examined. This research investigates the effects of relatively low frequency mechanical stimulation in molecular scale, on different cell types. Throughout the work presented in this document, the emphasis was on the stem cells differentiation, and primary cells dedifferentiation. The results suggested that performing extremely slow activities, namely low frequency movements, significantly affects the differentiation pathways of stem cells. In addition, it was found that slow movement of surface culture area enhances phenotypical characteristics of primary cells.

Résumé

Toutes les cellules vivantes, selon leur fonctions physiologiques, sont soumises à différentes stimulations mécaniques. L'ampleur et la fréquence de ces stimulations mécaniques varies considérablement d'un organe à un autre. Les stimulations oscillantes dues notamment à la marche, la respiration et la circulation sanguine sont largement étudiées. Par contre, les travaux concernant les stimulations a très faibles fréquences sont rare. Cette recherche examine les effets sur différents types de molécules, des stimulations mécaniques à relativement basse fréquence, à l'échelle moléculaire. Tout au long du travail présenté ici, l'accent a été mis sur la différenciation des cellules souches et la dedifférenciation des cellules primaires. Les résultats suggèrent que la pratique d'activités extrêmement lentes, à savoir les mouvements à basse fréquence, affectent, de manière significative, le mécanisme de différentiation des cellules souches. En outre, il a été constaté que les mouvements lents à la surface des cultures améliorent les caractéristiques phénotypiques des cellules primaires.

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Contribution of Authors

In this section the roles of each author in the preparation of the manuscripts presented in this thesis is described.

Article 1 - Low Frequency Mechanical Stimulation Inhibits Adipogenic Differentiation of C3H10T1/2 Mesenchymal Stem Cells

Contributions:

Ghazaleh Khayat – designed and performed the experiments, analyzed and interpreted qPCR data and protein expression, and helped in writing the manuscript and replying to reviewers.

Derek H. Rosenzweig – assisted in designing the experiments.

Thomas M. Quinn – contributed to the experimental design and revised the manuscript.

Article 2 - Low Frequency Mechanical Stimulation Modulates Osteogenic Differentiation of C2C12 Cells

Contributions:

Ghazaleh Khayat – designed and performed the experiments, analyzed and interpreted qPCR data and protein expression, and helped in writing the manuscript and replying to reviewers.

Derek H. Rosenzweig – assisted in interpretation of data and writing the manuscript.

Zohreh Khavandgar – performed some qPCR experiments.

Jingjing Li – helped in performing Alp histology and analyzing qPCR data.

Monzur Murshed – helped in writing the manuscript, developed the study and interpreted the results.

Thomas M. Quinn – contributed to the experimental design and revised the manuscript.

Article 3 - Culture of primary bovine chondrocytes on a continuously expanding surface inhibits dedifferentiation

Contributions:

Derek H. Rosenzweig – designed and performed the experiments, analyzed the data, wrote the manuscript and responded to the reviewers.

Mourad Matmati - designed and performed complementary experiments.

Ghazaleh Khayat – cultured the primary cartilage cells on regular culture dishes, performed qPCR and analyzed the results.

Sidharth Chaudhry - performed some immunofluorescence experiments.

Boris Hinz – assisted in interpretation of results and provided some biological ingredients for the experiments.

Thomas M. Quinn – contributed to the experimental design and revised the manuscript.

Publications

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2. Ghazaleh Khayat, Derek H. Rosenzweig, Zohreh Khavandgar, Jingjing Li,Monzur Murshed and Thomas M. Quinn, "Low Frequency Mechanical Stimulation Modulates Osteogenic Differentiation of C2C12 Cells", *ISRN Stem Cells*, 2013, vol. 2013, Article ID 138704, 9 pages, doi:10.1155/2013/138704.
3. Derek H. Rosenzweig, Mourad Matmati, Ghazaleh Khayat, Sidharth Chaudhry, Boris Hinz and Thomas M. Quinn, "Culture of primary bovine chondrocytes on a continuously expanding surface inhibits dedifferentiation", *Tissue Engineering Part A*; 18(23-24)(2012):2466-76.
4. HooiChuan Chin, Ghazaleh Khayat and Thomas M. Quinn, "Improved characterization of cartilage mechanical properties using a combination of stress relaxation and creep", *Journal of Biomechanics*, 44(2011), pp198-201.

1 General introduction

1.1 Introduction

Every year, many people lose their organs due to functionality failure, damage or injury. Whether it is a vital organ such as heart or lung, or a less-crucial tissue such as cartilage, it needs to be replaced. The replacement technique, organ transplantation, could be performed within someone's own body. Alternatively the transplants could be donated by a different person. The main challenges in the organ transplantation technique are long waiting times to receive the transplant, and the immune system rejection. In order to overcome the challenges facing the organ transplantation, scientists and engineers together attempt to open new avenues in 'Tissue Engineering' field to find out the best solutions to help people in need.

To create new implants to rehabilitate the dysfunctional organs, cells with special characteristics, such as the ability to self-renew and create different cell types, are required. Amongst the very different cell types in the body, stem cells possess these characteristics, thus they are suitable candidates to be employed in constructing the implants. However, they should be induced anyhow to differentiate to a new cell type.

Mechanical stimulation is one of the strategies that induce the differentiation process. Human cells are subject to mechanical loading from the very beginning stages of development as an embryo. Respiration and blood circulation are

common examples of mechanical loading on different parts of the body. The magnitude and the frequency of these mechanical stimulations are different depending on the function of each specific organ.

To date, the effects of mechanical stimulation on redirecting the differentiation pathways at frequencies near 1 Hz have been studied. Few studies have investigated the effects of low frequency (less than 0.01 Hz) mechanical stimulation on cell differentiation. Given that physiological frequencies range from 0 (getting up every morning) to 1 Hz (walking), it is of significant interest to evaluate the effects of very low frequency mechanical stimulation on the differentiation of stem cells. Of particular interest is to investigate whether low frequency mechanical stimulation has the potential to change the differentiation pathways. As a result, knowledge of these potential interactions will provide a better understanding for improvement of tissue engineering methods.

1.2 Objectives

The research question is: does low frequency mechanical stimulation affect the adipogenic and osteogenic differentiation pathways? The underlying hypothesis of this thesis is that low frequency mechanical stimulation can modulate cell differentiation pathways. To investigate this hypothesis, the biological response of stem cells to low frequency mechanical stimulation was evaluated and compared under static and dynamic conditions. Three different cell types were exposed to different stimulation protocols and the effects of

mechanical loading on their differentiation were investigated. The main objectives were as following:

1- Determining whether low frequency mechanical stimulation in a highly adipogenic culture environment suppresses C3H10T1/2 cells differentiation to adipocytes.
2- Investigating the effects of low frequency mechanical stimulation on C2C12 differentiation and determining whether the combined effect of mechanical stimulation and BMP-2 produces an additive, synergistic or antagonistic induction of osteogenic genes.
3- Examining whether culturing primary bovine chondrocytes on highly expandable surfaces, at very low frequency limits the dedifferentiation effects.

1.3 Thesis outline

The thesis structure is manuscript-based with a literature review (chapter 2), three main chapters (chapters 3 to 5), and conclusion (chapter 6). The scope of each chapter is as following:

The scientific and engineering literature was reviewed in chapter 2 to summarize current challenges and advances in the mechanical stimulation of stem cells.

A study was conducted to investigate the effects of low frequency mechanical stimulations on the adipogenic differentiation of stem cells. To address the effects of low frequency mechanical stimulations, mouse stem cells, C3H10T1/2 cells, were cultured in a strong adipogenic medium and then the culture was subjected to mechanical stimulation. This study is presented in chapter 3.

A series of experiments were conducted to investigate the effects of low frequency mechanical stimulations on the osteogenic differentiation of mouse cell line C2C12. To accomplish this goal, the cells were induced by a strong osteogenic culture medium supplemented with BMP-2 protein and the effect of mechanical stimulation with different experimental protocols was monitored in both gene and protein levels. The corresponding results are presented in chapter 4.

The effect of culturing the primary cartilage cells on expandable surfaces was also investigated in order to eliminate the passaging steps and limit the dedifferentiation of chondrocytes. The results of this study are presented in chapter 5. This dissertation ends by providing a discussion and overall conclusions along with the future perspectives presented in Chapter 6.

2 Literature review

2.1 Tissue engineering

Tissue engineering is an interdisciplinary field, which employs principles of engineering, cell biology, and materials science. It utilizes clinical research toward the creation of a biological construct to improve, restore and maintain the tissue function (Ikada, 2006; Langer and Vacanti, 1993; Lanza, 2009). Within the tissue engineering paradigm, cells are extracted from body tissues. The extracted cells are cultured in porous constructs (scaffolds) in order to mimic the extracellular matrix (ECM) environment in terms of physiological and mechanical properties, as well as, chemical composition. The seeded cells in the scaffolds start communicating with each other through producing enzymes and growth factors leading to production of the molecules to build the tissue of interest. When the tissue is built, the constructs will be implanted in the body (Figure 2.1)(Abarrategi et al., 2008; Appasani, 2011; Ikada, 2006; Tezcaner et al., 2002).

Choosing a cell source is a critical step in tissue engineering. An optimal cell source should yield a sufficient number of cells that maintain their phenotype and biological activities. Cell sources depending on living species are divided into three groups: xenogeneic (animal source), allogeneic (human other than the patient), and autologous (from the same patient). Autologous cells offer attractive characteristics for eliminating immunogenic rejection problems. However, when a surgery is to be performed on short notice, the autologous cells may not be readily available (Norbert, 2011). Moreover, it is difficult to harvest a sufficient number

of such cells especially when the patient is aged or has severe tissue damage. To overcome the problem of an insufficient quantity of the cells, they should be cultured and expanded *in vitro.* Nevertheless, the *in vitro* cell expansion has its own drawbacks: it is time consuming and susceptible to infection. Xenogeneic and allogeneic cell sources are "off-the-shelf available"; nevertheless immunogenic rejection is the main concern (Appasani, 2011; Ikada, 2006; Tezcaner et al., 2002; Norbert, 2011). Moreover, in vitro expansion of primary cells leads to rapid phenotype loss (Athanasiou et al., 2009).

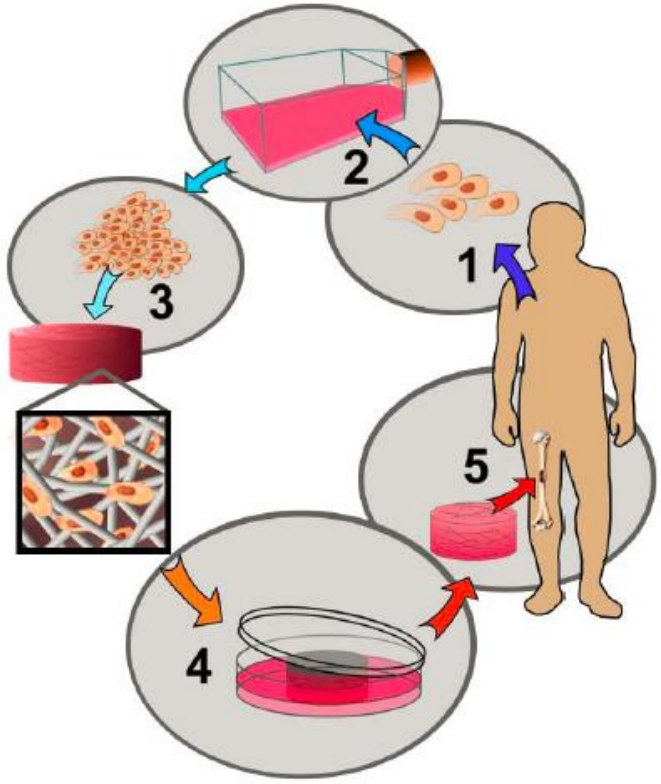

Figure 2.1 Schematic of tissue engineering paradigm: 1) cell isolation form body, 2) cell culture *in vitro,* 3) seeding in the scaffold, 4) culture of cellular scaffold *in vitro*, 5) implantation of construct in the body(George, 2009)

2.2 Stem cells

Stem cells exist in all multi-cellular living systems. They are characterized by two distinguished features: 1) unlimited capacity to self-renew, 2) differentiation potential, which means the acquisition of a terminal phenotype of a cell due to the expression of specific proteins (Figure 2.2). Differentiation plays a fundamental role during developmental stages of an embryo and throughout an adult life (Appasani, 2011; Lanza, 2009). Depending on the source of isolation, stem cells are divided into three different types: embryonic stem cells (ESC), induced pluripotent stem cells (*i*PS) and adult stem cells. ESCs are isolated from the inner cell mass of an embryo. iPSs are the results of transformation of the adult somatic cells through reprogramming which is usually induced by transfection procedure. Adult stem cells can be found in many tissues of the human body and they are in general tissue-specific (i.e., cardiac stem cells and bone marrow stem cells) (Norbert, 2011; Lanza, 2007).

Cells are categorized as totipotent, pluripotent, multipotent and unipotent, depending on their developmental potential. The cells from the very early stage of embryonic development (first cell division) are called blastomeres, which are considered totipotent; these cells have the ability to differentiate to all types of embryonic tissues including trophoblast (outer cells of the embryo, which make the placenta). The inner cell mass, from which the embryo develops, can differentiate into all three germinal layers (ectoderm, mesoderm, and endoderm) except placenta; thus, it is called pluripotent. Multipotent stem cells are the cells that are isolated from adult tissues or organs and can differentiate to a limited

range of differentiated lineages. Unipotent stem cells, also known as committed progenitors, can only generate one specific cell type and have a very limited proliferative capacity (Appasani, 2011; Lanza, 2009; Norbert, 2011).

If a stem cell is isolated from mesenchyme (connective tissue formed in the very early embryo), it is called a mesenchymal stem cell (MSC). MSCs are identified as adherent and elongated cells, which can be harvested from different organs such as bone marrow and adipose tissue; hence, they are promising cell sources due to the ease of isolation, high proliferation rate and multi-potency. MSCs are characterized based on the expression of cell surface markers such as: CD29 (integrin β1), CD44 (receptor for hyaluronic acid and matrix proteins), CD105 (endoglin) and CD166 (cell adhesion molecule) (Deans, 2000; Deans and Moseley, 2000; Norbert, 2011). According to the International Society of Cellular Therapy (ISCT), cells meeting the following criteria are classified as MSCs: ability to adhere to plastic dishes, expression of specific genes such as CD105, CD73 and CD90, lack of surface molecule expression and class II major histo-compatibility complex antigen (HLA-DR) as well as ability to differentiate to osteoblast, adipocyte and chondrocyte *in vitro* (Dominici et al., 2006).

As previously mentioned, one of the main characteristics of stem cells is their ability to differentiate to other cell types. There are different strategies to direct the differentiation process (Norbert, 2011). One of the most common strategies is the addition of soluble molecules such as growth factors to the culture. These soluble factors bind to specific membrane receptors and initiate downstream biochemical processes, which can result in necessary gene activation

and inactivation for specific terminal differentiation (Pittenger et al., 1999; Sekiya et al., 2001).

Mechanical stimulation is another important strategy to induce differentiation. Cells utilize various mechanisms to sense external forces (mechano-sensing) and to translate them to biochemical signals (Riehl et al., 2012). Mechano-sensing occurs through force-induced conformational changes in cellular structures such as ion channels, integrin complexes and cell-cell adhesion molecules (CAMs) (Klein-Nulend et al., 2012). Ion channels connect intracellular to extracellular anchors and control the flow of ions into the membrane; the opening and closing of the ion channels convert the mechanical stimuli to an electrical signal by changing the electrical potential of the membrane (Gillespie and Walker, 2001; Hamill and Martinac, 2001). Integrins are receptors that provide structural connection between the cell and extracellular matrix enabling transmission of mechanical loadings to the cell interior (Han et al., 2004; Shyy and Chien, 2002).

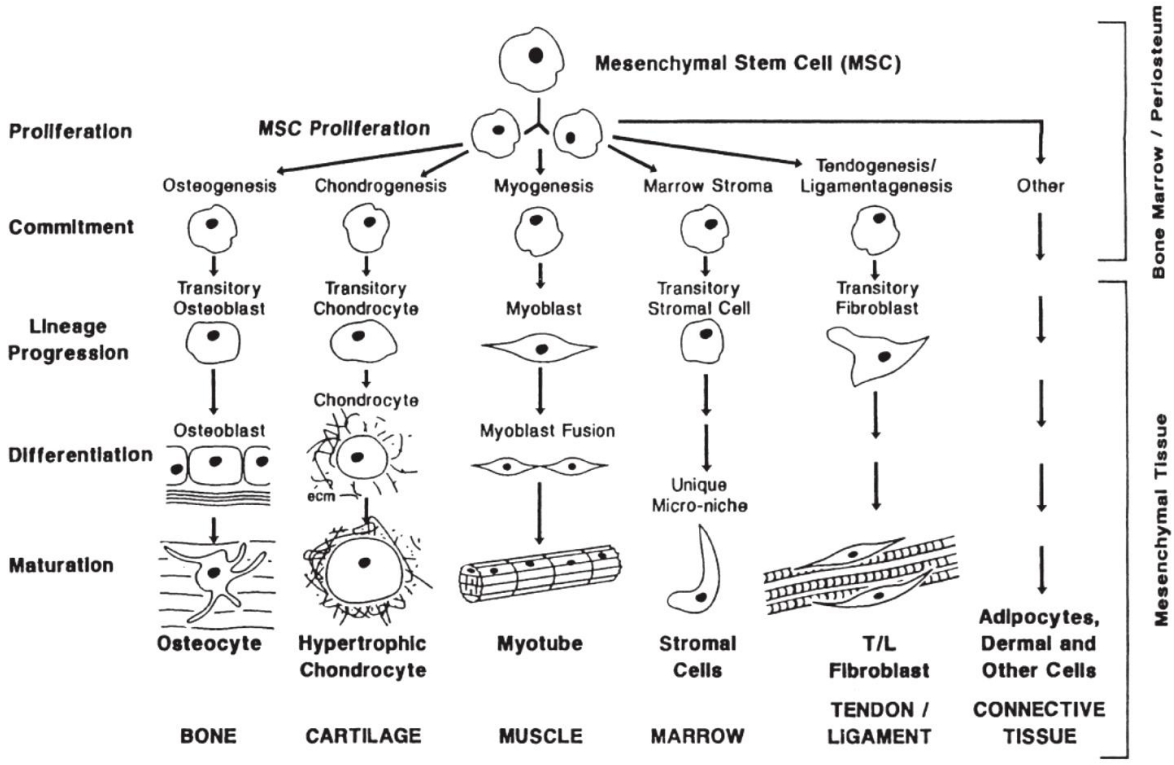

Figure 2.2 Cell differentiation process (Lanza, 2007)

2.2.1 Adipogenesis

The development of fat cells (adipocytes) from preadipocytes, known as adipogenesis, is one of the most studied models of cellular differentiation. Adipocytes derived from mesenchymal stem cells consist of two different phases; the first phase, known as determination, deals with the commitment of stems cells to the adipogenic lineage. Determination leads to the conversion of pluripotent stem cells to pre-adipocytes. Pre-adipocytes are not morphologically distinguishable from their precursors. However, they have lost their potential to differentiate to other cell types. The second phase, called terminal differentiation, deals with the conversion of pre-adipocyte to fully mature adipocytes. In this

process, cells have the characteristics of adipocytes such as lipid transport and synthesis, insulin sensitivity and the secretion of other proteins that are specific to adipocytes. (Otto et al., 2005; Rosen and MacDougald, 2006).

2.2.1.1 Adipogenic transcriptional cascade

Adipogenic differentiation is regulated by specific gene expression being performed by proteins with a DNA-binding domain. Such proteins are called transcription factors. In order to regulate the expression of a gene, transcription factors must bind to either the enhancer or the promoter region of the gene, causing an upregulation or downregulation of that particular gene's expression (Figure 2.3).

Two major transcription factors have been extensively studied in adipogenesis: 1) nuclear receptor peroxisome proliferator-activated γ (PPARγ) and, 2) members of the CCAT-enhancer-binding proteins (C/EBP) family. PPARγ is believed to be a key regulator of adipogenesis and is necessary and sufficient to direct adipogenic differentiation. To date, no factor has been discovered to promote adipogenesis in the absence of PPARγ (MacDougald and Mandrup, 2002; Rosen and MacDougald, 2006; Rosen et al., 2000; Younce, 2009). PPARγ belongs to the nuclear hormone receptor family and controls gene expression, when activated by ligands(Otto et al., 2005). Although the two isoforms of PPARγ (PPARγ1 and PPARγ2) can promote adipogenesis in some cell types, it seems that PPARγ2 is more effective. While PPARγ2 is ubiquitous in adipocytes, PPARγ1 can be found in other cell types (Mueller et al., 2002). Some of family

members of C/EBPs such as C/EBPα, C/EBPβ and C/EBPδ also are expressed in adipogenesis. C/EBPβ and C/EBPδ are known to be early regulators of preadipocyte differentiation (Tang and Lane, 1999). However, they cannot function efficiently in the absence of PPARγ(Linhart et al., 2001; Rosen and MacDougald, 2006; Zuo et al., 2006).

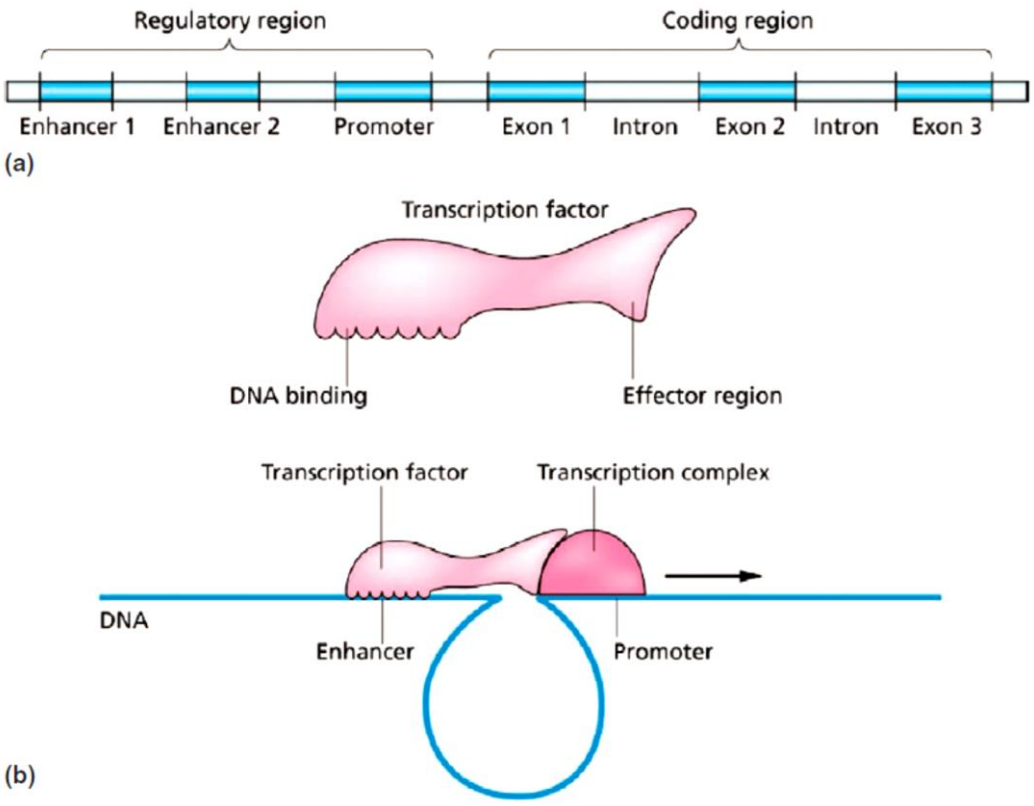

Figure 2.3 a) Structure of a gene b) transcription factor operation (Slack, 2006)

2.2.1.2 Extracellular signaling in adipogenesis

Cells communicate with their surrounding environment by transmitting extracellular signals to the nucleus through a network of interacting proteins. This process is known as cell signaling. Signaling pathways function upstream of transcription factors. The signals from extracellular factors are integrated in the nucleus by transcription factors affecting the expression of PPARγ and C/EBPα in adipogenic differentiation. To date, many extracellular signaling pathways are known to have positive or negative influence on adipogenesis. Amongst them, mitogen-activated protein kinase (MAPK) and bone morphogenetic proteins (BMPs) positively affect adipogenesis (Figure 2.4) (Rosen and MacDougald, 2006).

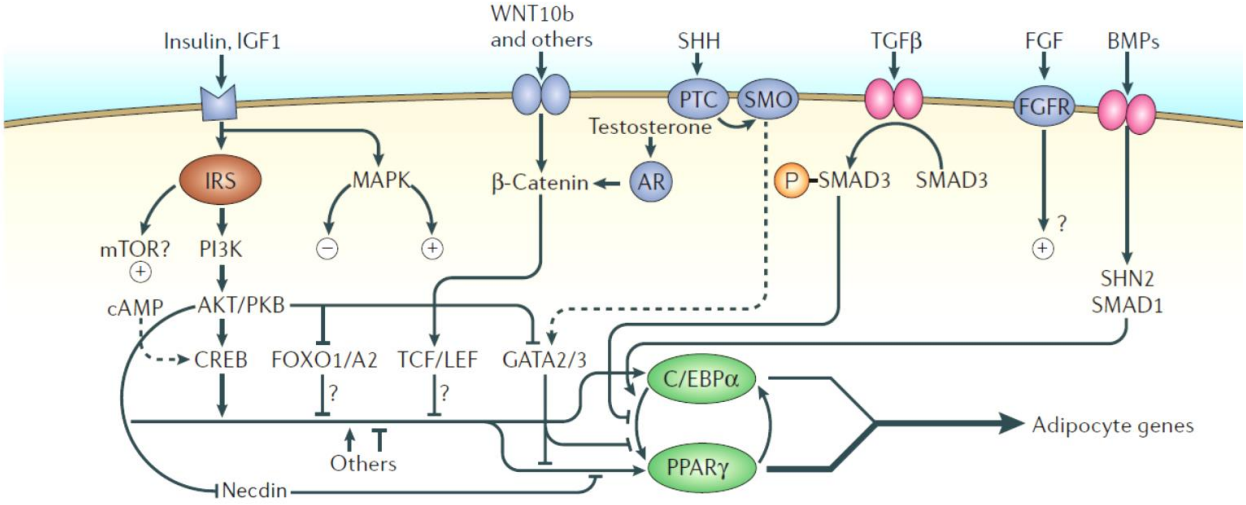

Figure 2.4 Regulation of adipogenesis by extracellular signaling (adopted from (Zuo et al., 2006))

MAPK is a widely studied signaling pathway in adipogenic differentiation. The most known MAPKs are: extracellular signal-regulated

kinases (ERK) 1 and 2, c-Jun amino-terminal kinases (JNKs) 1, 2 & 3 and p38 MAPK (p38) α, β and γ. These MAPKs are regulated by phosphorylation cascade. Since some adipogenic stimuli such as insulin, activate the ERK pathway, the ERK signaling pathway role in adipogenesis has been exclusively investigated (Bost et al., 2005; Seger and Krebs, 1995). However, its role in adipogenesis is contradictory. While ERK activation is a necessary step in starting adipogenic differentiation (Benito et al., 1991), it has been shown that, in mature adipocytes, ERK activation leads to decreased transcriptional activity of PPARγ resulting in its phosphorylation. Therefore, ERK activation inhibits adipogenic differentiation (Camp and Tafuri, 1997; Hu et al., 1996). Although some members of the BMP family can induce adipogenic differentiation in MSCs, the major role of BMPs is in osteogenic differentiation. Therefore, the BMP signaling pathway will be discussed in detail in section 2.2.2.2.

2.2.1.3 Adipogenic inducers

Efficient inducers of adipogenesis include insulin, insulin-like growth factor-1(IGF-1), glucocorticoid (steroid hormones that regulate glucose metabolism) and cyclic adenosine monophosphate (c-AMP). Such inducers are most efficient when used in combination (Spiegelman and Green, 1980; Student et al., 1980).The *in vivo* initiation of adipogenesis is not completely understood; however, *in vitro* adipogenic differentiation has been successfully implemented by using such chemical agents. Experiments revealed that insulin is a major activator of adipogenic differentiation. Insulin increases lipid accumulation in every fat cell, and also leads to an increase in the number of differentiating cells

(Girard et al., 1994). Insulin activates IGF-1 receptors. Both IGF-1 and insulin activate several downstream pathways leading to adipogenic differentiation (Rosen et al., 2000). Glucocorticoids are a group of chemicals exclusively employed to induce adipogenic differentiation. They have been used in the form of Dexamethasone (Dex). Dex activates the glucocorticoid receptors (GR) which are in the same super family of PPARγ (Rosen et al., 2000). Isobutyl methyl xanthine (IBMX) and indomethacin are also known to be *in vitro* adipogenic inducers. Once adipogenesis proceeds, lipid-rich vacuoles accumulate within the cell and gradually are combined and fill the cells. This accumulation can be histologically assayed by oil red O stain (Chamberlain et al., 2007; Pittenger et al., 1999).The Oil red O stain technique is discussed in section 2.6.3.

2.2.1.4 Mechanical stimulation role in adipogenesis

Mechanical stimulation is one of the strategies for regulation of stem cell differentiation. There are lines of evidence demonstrating that mechanical stimulation can inhibit or suppress adipogenic differentiation of stem cells (David et al., 2007; Luu et al., 2009; Sen et al., 2008). It has been shown that mechanical stimulation of stem cells inhibits adipogenic differentiation through downregulation in PPARγ expression. It activates the MAPK signaling pathway and leads to a decrease in PPARγ expression. Hence, it acts as PPARγ antagonist (David et al., 2007)

2.2.1.5 C3H10T1/2 cells represent a useful model for study of adipogenesis

The most commonly used multipotent stem cell line *in vitro* is C3H10T1/2. This cell line is isolated from two weeks old C3H mouse embryo. C3H10T1/2 cells are functionally similar to mesenchymal stem cells in addition, they are similar to fibroblasts in morphology (Reznikoff et al., 1973). When cultured in a standard medium (eagle's minimal essential medium with 10 per cent bovine serum) they proliferate as undifferentiated cells. They respond to many different growth factors such as the BMP family. It has been shown that BMP-2 and BMP-4 can control the C3H10T1/2 cells commitment into osteogenic and adipogenic differentiation, depending on the culture condition (Huang et al., 2009; Tang et al., 2004). They can differentiate along osteogenic, myogenic, and adipogenic lineages.

2.2.2 Osteogenesis

Osteogenesis is a biological process in which stem cells differentiate to bone-making cells. Pre-osteoblasts differentiate to osteocytes by secreting alkaline phosphatase, an early marker of osteogenesis. In the differentiation process, the pre-osteoblasts nuclei become larger, Collagen type I, a bone matrix protein, is secreted, and eventually, pre-osteoblasts turn into mature osteoblasts. (Deng, 2008). Osteoblasts become osteocytes once they are trapped in the matrix they have secreted. Osteocytes are responsible for mineralization and provide mechanical strength to the bones (Franz-Odendaal et al., 2005). In general, osteogenic differentiation consists of three different stages: 1) proliferation, 2) extracellular matrix development and 3) mineralization. In each stage, genes

associated with osteogenesis are upregulated or downregulated asynchronously (Figure 2.5). Specific osteogenic-associated genes include alkaline phosphatase (Alp), collagen type I (Col1), osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP) and parathyroid hormone-1 receptor (PTH1R)(Aubin, 2008).

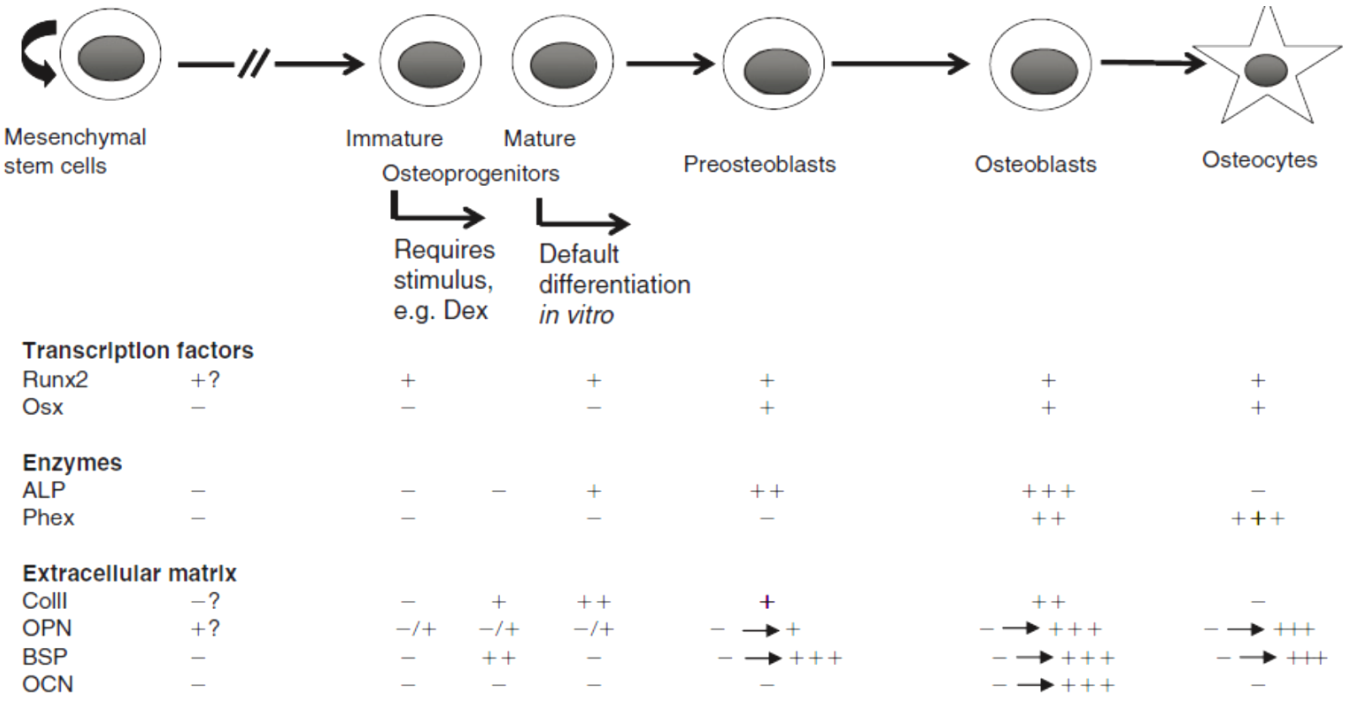

Figure 2.5 Sequential proliferation-differentiation in osteogenesis. (–) no detectable expression; (+/- to +++) from very low to very high expression; (?) intracellular heterogeneous expression (adopted from (Aubin, 2008)).

2.2.2.1 The osteogenic transcription factors

Different transcription factors regulate osteogenic differentiation (Figure 2.6). The main transcription factors include Runx2 and Osterix (Osx). Runx2, one of the runt homology domain transcription factor family members, is the earliest osteogenic differentiation marker, which has a critical role in osteoblast

development. It is expressed in cultures that are treated with BMP-2 (Komori et al, 1997). Osx is another essential transcription factor for bone differentiation, which has three zinc finger motifs (Nakashima et al., 2002). There is evidence showing that Osx is activated downstream of Runx2 by the binding of Runx2 to a responsive element in the promoter of the Osx gene (Nishio et al., 2006). In summary osteogenic differentiation is initiated by Runx2, followed by Osterix and characterized by the expression of Alp, osteocalcin and finally terminated by the mineralization of extracellular matrix (David et al., 2007).

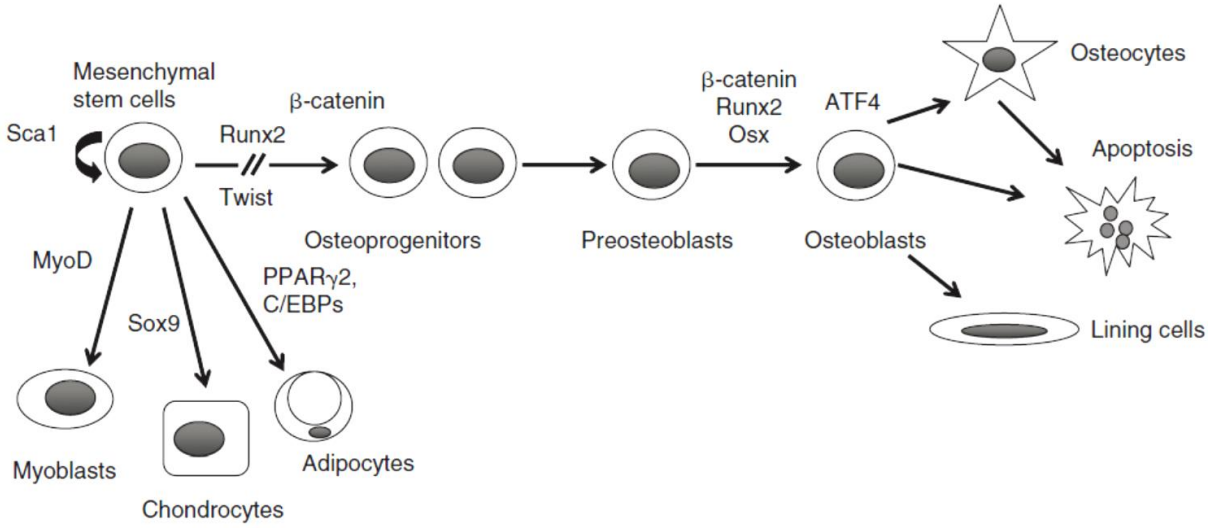

Figure 2.6 Schematic illustration of stem cell commitment to osteogenic lineage with known transcription factors indicated (adopted from (Aubin, 2008))

2.2.2.2 Signaling pathways involved in osteogenic differentiation

Various signalling pathways are involved in the osteogenic differentiation of stem cells. These signalling pathways have been schematically shown in

(Figure 2.7). Amongst the signalling pathways, TGFβ/BMP and Wnt signalling pathways have been subjects of numerous studies.

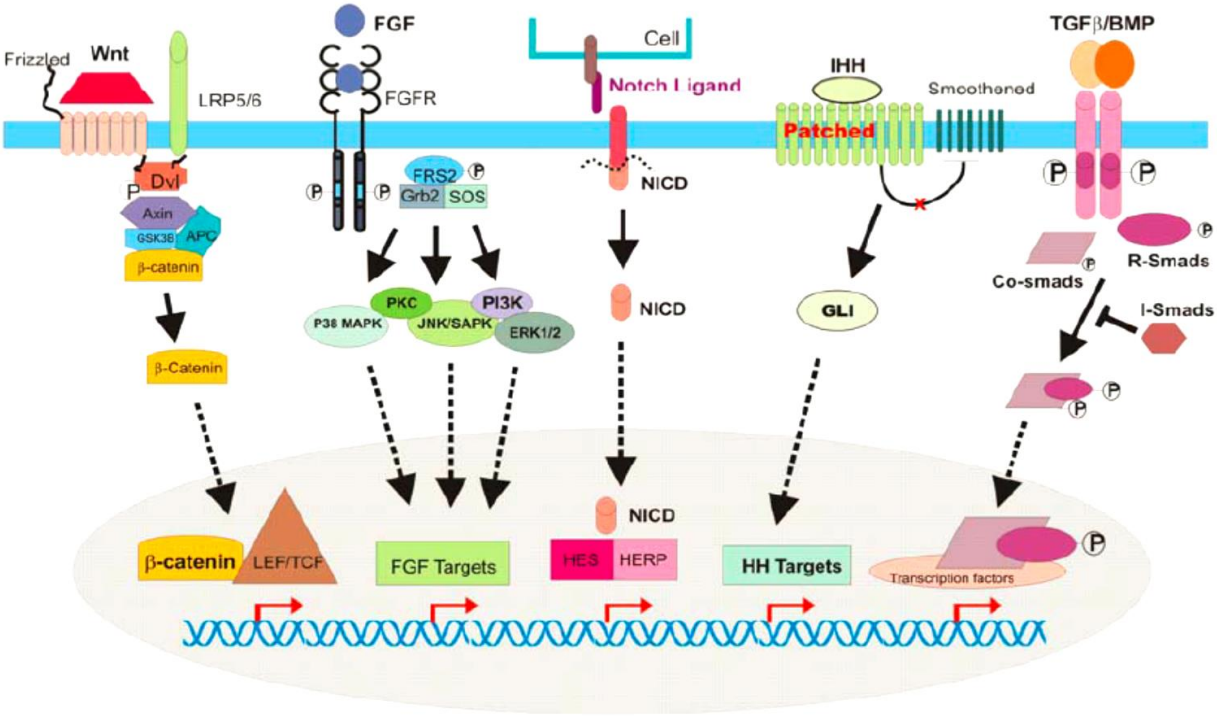

Figure 2.7 Major signaling pathways involved in osteogenic differentiation (adopted from (Deng, 2008))

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGFβ) family of proteins. BMP signaling is initiated by activation of some trans-membrane receptors; These activated receptors phosphorylate SMAD transcription factors (Luu et al., 2007; Xiao et al., 2007). SMADs are intracellular proteins consisting of three different groups: 1) receptor regulated SMADs or R-SMADs (SMAD1, 5 and 8), 2) co-SMAD (SMAD4) and 3) inhibitory SMADs (SMAD6 and 7) (Massagué et al., 2005). Phosphorylated SMADs displace to the cell nucleus and cause the activation of BMP responsive genes. (Deng, 2008).

Wnt signaling pathway involves a series of events downstream of binding the Wnt ligand protein family to the frizzled receptor family. Frizzled receptors activate another protein family called dishevelled (Dsh). Dsh suppress the activity of a downstream protein named glycogen synthase kinase-3 (GSK-3). GSK-3 usually promotes the degradation of the β-catenin intracellular signaling molecule. Nevertheless, when GSK-3 activity is suppressed by Dsh, β-catenin is stabilized and accumulated in the cytoplasm. Once the amount of β-catenin increases in the cytoplasm, it can enter the nucleus and interact with specific transcription factors to promote a specific gene expression (Johnson, 2008). Wnt signaling pathway is categorized to canonical and non-canonical pathways; whereas the former involves β-catenin molecule, the latter functions independently of it (Caverzasio, 2009). It has been shown that canonical Wnt signaling pathway plays an important role in osteoblast differentiation by changing the amount of stabilized β-catenin (Fischer et al., 2002; Gregory et al., 2005).

2.2.2.3 *In vitro* osteogenic inducers

In vitro osteogenic differentiation of stem cells traditionally is achieved by culturing the cells in monolayer on plastic dishes. Once the cells become confluent (attach to surface and cover the area), they differentiate to osteoblasts through the addition of osteogenic supplements. It has been shown that chemicals such as β-glycerophosphate, ascorbic acid, dexamethasone, retinoic acid and 1,25-hydroxy vitamin D_3 induce the differentiation of MSCs to osteoblasts (Buttery et al., 2001; Hwang et al., 2007). After induction, stem cells form aggregates and start expressing alkaline phosphatase, and over time, calcium accumulation

becomes visible. These aggregates can be distinguished when positively stained by alizarin red and von Kossa techniques (Chamberlain et al., 2007).

2.2.2.4 Mechanical stimulation role in osteogenesis

The effect of mechanical stimulation on osteogenic differentiation has been widely studied. It has been shown that mechanical stimulation redirects differentiation pathways of stem cells toward osteogenesis while suppressing other lineages. The mechanical stimulation range from 0.1 to 2 Hz in frequency and between 2% to 10% in amplitude (Fan et al., 2006; Mauney et al., 2004; Sen et al., 2008; Yang et al., 2012). Mechanical loading applied to bone tissue is sensed by bone cells. It is hypothesized that mechanical loadings cause micro-deformation in bone cells and the cells translate these stimulations to biological signals (Burr et al., 1989).

2.2.2.5 C2C12 cells

C2C12 cells represent a useful model for study of osteogenesis. They are a multi-potent immortalized cell line of a mouse skeletal myoblast isolated from a C3H mouse after an injury (Yaffe and Saxel, 1977). They can differentiate to myocytes (muscle cells) if they are cultured in low serum content medium. There are lines of evidence demonstrating their differentiation potential to osteoblasts when treated with BMP-2 (Katagiri et al., 1994; Yamamoto et al., 1997). C2C12 may not be clinically relevant for bone tissue engineering; however, they are a popular cell line to study fundamental mechanisms of redirection to osteogenic differentiation.

2.3 Primary cells

Primary cells are uni-potent cells, which can be harvested from patient tissue. They usually do not have immunogenic rejection problems. However, they have limited proliferation potential. When expanded in culture, they can acquire inappropriate phenotypic characteristics (Langer and Vacanti, 1993; Polak and Bishop, 2006). Primary cells are used in a range of tissue engineering strategies. Examples include cartilage, skin, bone, cardiovascular tissue engineering, etc.

2.3.1 Chondrocyte dedifferentiation and cartilage tissue engineering

Dedifferentiation is a process in which cells lose their specialized morphology, function and biochemistry, initiate division and revert to a less differentiated cell (Hall, 2005). In the case of cartilage tissue engineering, chondrocyte dedifferentiation addresses a significant reduction in cartilage-specific ECM proteins such as collagen type II and aggrecan. Chondrocyte dedifferentiation also involves the production of fibroblast-specific ECM proteins, such as collagen type I during expansion in monolayer culture (Stokes, 2001).

2.3.1.1 Factors causing chondrocyte dedifferentiation

There are some factors associated with the tendency of chondrocyte dedifferentiation to fibroblasts. These factors include plating chondrocytes at low density (Watt, 1988), monolayer culturing of the cells in a flat, rigid, and two-dimensional culture surface (Archer et al., 1990; Darling and Athanasiou, 2005; Responte et al., 2012), treating the cells with some cytokines such as interleukin-1 (IL-1)(Goldring et al., 1988; Goldring et al., 1994) or exposure to degradative

enzymes during passaging (Homicz et al., 2002; Lefebvre et al., 1990), extraction from either cancer or immortalized cells (Mallein-Gerin and Olsen, 1993; Takigawa et al., 1991) and abnormally rapid proliferation (Darling and Athanasiou, 2005; Giovannini et al., 2010).

2.3.1.2 Signalling pathways involved in chondrocyte dedifferentiation

Different cell signalling pathways have been proposed to contribute to chondrocyte dedifferentiation. These pathways include interleukin-1 (IL-1) signalling and the ERK signalling pathway. IL-1 is a super family of cytokines involved in controlling the pro-inflammatory reactions in response to tissue injury. The production of IL-1 is believed to increase with chondrocyte passaging (Lin et al., 2008). It is shown that, IL-1 signalling plays an important role in chondrocyte dedifferentiation (Hong et al., 2011). Other studies revealed that collagen type II is downregulated in the dedifferentiation process through positive ERK activity (Yoon et al., 2002).

2.3.1.3 Biochemical and mechanical factors in blocking chondrocyte dedifferentiation

Different biochemical and mechanical factors have been employed to restore the chondrogenic properties of chondrocytes. Biochemical factors are categorised as hormones and growth factors (Liu et al., 2007). Some members of the BMP family such as BMP-2 and BMP-3 are believed to prevent the dedifferentiation of chondrocyte grown in monolayer cultures (Gründer et al., 2004; Hiraki et al., 1991; Luyten et al., 1992; Sailor et al., 1996). Alternatively, it

is suggested that physiological loading is a prerequisite for maintenance of proper joint functioning (Das et al., 2008). There are lines of evidence indicating that mechanical loading can optimize cartilage function and block the dedifferentiation process in chondrocytes in culture (Salter et al., 2002; Salter et al., 2004).

2.4 Biological responses of cells in monolayer to mechanical stimulation

Mechanical forces are one of the fundamental physiological factors in regulating the structure and function in tissues (Schmidt et al., 1998). Moreover, the mechanical environment of cells has consequential effects on cell behaviour. These effects have been widely studied in order to understand cell interaction with the cellular environment and ultimately, to improve tissue engineering methods.

Several mechanical variables affect tissues or cell responses to loads. These variables include but are not limited to strain and mechanical stress. Mechanical stress measures the extent of the change in the shape of a material due to a load. Strain is the measurement of deformation of a material subjected to a load (Knudson, 2007). Strain rate, number of cycles, strain distribution and resting periods are other mechanical parameters that influence the response to loading (Kaspar et al., 2002; LaMothe et al., 2005; LaMothe and Zernicke, 2004). Strain rate is characterized by strain amplitude and loading frequency (Torcasio et al., 2008).

2.4.1 Mechanical stimulation at gait / respiration / circulation frequencies (1 Hz)

Physiological activities such as walking, breathing and circulation inspire researchers to study the effects of mechanical stimulation with frequencies associated with such activities. (Athanasiou et al., 2009). Since the frequencies of physiological activities are on the order of magnitude of 1 Hz, numerous studies have been conducted at such frequencies (Kurazumi et al., 2011; Li et al., 2012; Maul et al., 2011; Xu et al., 2012). Effects of heart beat frequency on cardiac stem cell growth and differentiation (Kurazumi et al., 2011), effects of mechanical stimulation relevant to the cardiovascular system on MSCs (Maul et al., 2011), and effects of locomotion frequency on bone cells (Robinson et al., 2006) are a few examples of such experiments.

2.4.2 Mechanical stimulation at high frequencies

Frequencies much higher than physiological activities have been applied to cell cultures in order to investigate cell responses to mechanical loading. High frequency mechanical stimulations, known as vibration, mainly are studied in bone tissues (Dumas et al., 2010; Flieger et al., 1998; Oxlund et al., 2003). It is hypothesized that high frequency mechanical loadings with low magnitude (20 Hz, 0.01% strain) has the same effect on the cells as the locomotion frequency with higher magnitude (1 Hz, 0.1% strain)(Weinbaum et al., 1994). It has been shown that loading applied at higher frequencies (10-50 Hz) was more effective in enhancing bone tissue formation than loading at normal locomotion frequencies

(1 Hz) (Ozcivici et al., 2010; Rubin et al., 2003; Rubin et al., 2001); for example, it has been shown that mechanical stimulation at 50 and 100 Hz frequencies lead to the osteogenic differentiation of human adipose stem cells (Tirkkonen et al., 2011).

2.4.3 Low frequency mechanical stimulation

Although physiological activities range from 0 to 1 Hz, very few studies have considered low frequency mechanical stimulation effects on the body at a cellular level. It is hypothesized that low frequency mechanical stimulation on the order of magnitude of 0.01 Hz has the same effect on cells as the physiological frequencies; however, the mechanism of action may be different. In addition, such low frequencies together with varying growth factor concentrations could have a significant role on stem cell differentiation during development (Henderson and Carter, 2002; Nilsson et al., 2007).

2.5 Techniques for applying mechanical stimulation on cells in monolayer culture

In order to study cell responses to the mechanical loading, several novel techniques and experimental apparatus were developed, which are discussed in the following paragraphs.

2.5.1 Hydrostatic Pressurization

One of the most frequently used techniques of compressing tissue, cells or explants is hydrostatic pressurization (Bourret and Rodan, 1976; Brighton et al.,

1996; Brown, 2000). This system is easy to use and provides homogenized stimulus. It also has the ability to deliver and transduce loading inputs. (Brown, 2000). Nevertheless, this technique cannot be employed for low magnitude and high frequency loads (Tanck et al., 1999).

2.5.2 Longitudinal Stretch

Longitudinal stretch uses controlled uniaxial distension of deformable substrates. It can control the duty cycle parameter and quantify input. Moreover, it is easy to use. Nevertheless, its disadvantage is the heterogeneity of the local strain (Brown, 2000).

2.5.3 Substrate Bending

Substrate bending delivers longitudinal strains to a culture surface. They are supplied with a rectangular silicone dish in which bending will apply. They are designed to deliver low strain levels corresponding to a range that bones encounter *in vivo* (Brown, 2000; Neidlinger-Wilke et al., 1994).

2.5.4 Flexercell: Out-of-plane circular substrate distension

In this broad class of mechano-stimulatory cell culture systems, strains are imposed by displacement of an elastic membrane due to a rigid circular platen moving perpendicular to the membrane surface. It has been commercialized in 1987 under the name Flexercell (McKeesport, PA, USA). To date, it is the most utilized cell culture mechano-stimulus system (Banes et al., 1985; Brown, 2000; Hasegawa et al., 1985).

2.5.5 A Novel System: The Cellerator

In this system, a motorized mechanical device (the Cellerator; Cytomec GmbH, Spiez, Switzerland) with a highly elastic culture dish, made of high-extension polydimethylsiloxane (PDMS) is used. The original conception of this device was to provide a continuously increasing culture surface at very low frequencies (<0.0001 Hz), which would reduce the need for cell passaging to overcome contact inhibition. However, this apparatus can equally be used for mechanostimulation experiments. This apparatus provides the condition for cells to proliferate faster and longer, and reduces the number of enzymatic cell passaging, which has unwanted effects on cell characteristics (Majd et al., 2009; Wipff et al., 2009). In our experiments, we will use this system to apply dynamic stimulation to the desired cell types. The special advantages over other systems are (1) a wider range of stretching amplitudes, and (2) direct associations to applications in long-term cell culture for tissue engineering and real-time monitoring of the culture by the microscope.

2.6 Techniques for quantification of cell function

2.6.1 Polymerase chain reaction technique

Polymerase chain reaction (PCR) is an extensively utilized technique in molecular biology. In this technique, one piece of RNA is amplified to millions of copies of a particular, short DNA sequence. PCR consists of cycles of heating and cooling reactions. During the reactions, the sample DNA is initially denatured in order to unwind and separate the DNA double helix into single strands. This step

is typically achieved by heating the DNA sample at a temperature of 94°C from 30 seconds to 5 minutes. The second step is called annealing in which the hybridisation of specific primers to each strand is achieved by lowering the temperature of the reaction mix to the annealing temperature. The annealing temperature is normally set between 40°C and 65°C. Finally, the temperature is raised to approximately 72°C, (an optimal temperature for thermo-stable DNA polymerase mediated DNA strand replication), and the whole cycle is repeated between 35 to 40 times (Giulietti et al., 2001; Heid et al., 1996; Orita et al., 1989).

Whereas PCR is a qualitative method, quantitative polymerase chain reaction (qPCR) is a more precise technique. It quantifies any changes in the expression of genes of interest in response to any stimuli. qPCR technique follows the principles of PCR procedure plus the insertion of a florescent dye in the procedure. The florescent dye binds to double strand DNA and as the reaction proceeds, the intensity of florescent increases and can be detected at the end of each cycle (VanGuilder et al, 2008).

2.6.2 Protein expression analysis by Western blot

Western blot is an analytical technique in molecular biology, which utilizes gel electrophoresis to separate denatured or native proteins by length of a polypeptide. Protein separation is performed based on the molecular weight. Gel electrophoresis typically employs polyacrylamide gels and sodium dodecyl sulfate (SDS) buffer. The proteins are transferred to nitrocellulose membrane and

subsequently stained with primary and secondary antibodies following a blocking step (Kaufmann et al., 1987).

2.6.3 Protein expression and organization analysis by histological staining

Histology is a technique in molecular biology to detect certain components in a cell body or a tissue by employing a specific stain. There are different protocols for histological staining of cells. One of the most popular forms of staining in osteogenesis is alkaline phosphatase (ALP) staining. In this staining a special solution is used to detect the presence of alkaline phosphatase, an enzyme abundant in osteoblasts, by binding to the ALP causing purple coloration. In adipogenesis, one of the most popular forms of histological staining is oil red O staining. Oil red O is a dye used to detect lipids in cells and tissues. It binds to triglycerides and makes the lipids to change their color to red.

2.7 Summary

Cells respond to mechanical stimuli as well as chemical ones. In order to mimic the mechanical stimulations applied to tissues and cells, different apparatus have been developed. These mechanical loading apparatus encompass stimulations from tissues to the cells. It is shown that mechanical stimulation at common physiological frequencies i.e. 1 Hz, affects the differentiation pathway of stem cells. Adipogenic and osteogenic differentiation are two competent pathways; where one pathway is suppressed, the other one is enhanced. Both lineages are known to be highly affected by mechanical activities in a wide range. In our experiments, we have applied mechanical stimulation at frequencies as low

as 0.01 Hz to investigate their effects on lineage selection. In order to apply such frequencies we have used Cellerator, a device that has the ability to exert frequencies as low as 1000 times less than common frequencies and can expand up to 100 times its initial surface area. We hypothesize that doing any kind of mechanical activities regardless of their frequencies causes a significant change in the expression of genes and proteins in favor of osteogenic differentiation and cause suppression in adipogenic differentiation. In addition, we have a hypothesis that culturing chondrocyte in an expandable surface limits their dedifferentiation process and enhances chondrogenic phenotype. In order to verify our hypothesis, a series of experiments have been conducted and the results are presented in the following chapters.

3 Low Frequency Mechanical Stimulation Inhibits Adipogenic Differentiation of C3H10T1/2 Mesenchymal Stem Cells

Preface

Mechanical stimulation affects the differentiation pathways of stem cells. It has been shown that mechanical stimulations suppress the adipogenic lineage of mesenchymal stem cells in expense of osteogenic differentiation pathway. However, the effect of low frequency stimulation has rarely been examined. In the following chapter, the effects of low frequency mechanical stimulation on the adipogenic differentiation of C3H10T1/2 cells are investigated. It was shown that applying low frequency mechanical stimulation on the stem cells which are cultured in a highly potent adipogenic medium, can suppress differentiation to adipocytes.

Low Frequency Mechanical Stimulation Inhibits Adipogenic Differentiation of C3H10T1/2 Mesenchymal Stem Cells

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Running Title: Low frequency stimulation of C3H10T1/2 cells

3.1 Abstract

Oscillatory mechanical stimulation at relatively high frequencies (0.1 Hz) has been shown to inhibit adipogenic and promote osteogenic differentiation of mesenchymal stem cells. However, for physiological interpretations and ease of implementation it is of interest to know whether different rates of mechanical stimulation can produce similar results. We hypothesized that relatively low frequency mechanical stimulation (0.01 Hz) can inhibit adipogenic differentiation of C3H10T1/2 mouse mesenchymal stem cells, even in a potent adipogenic differentiation medium. C3H10T1/2 cells were cultured in adipogenic medium under control (non-mechanically stimulated) conditions and under oscillatory surface stretch with 10% amplitude and 0.01 Hz frequency for 6 hours per day for up to 5 days. Cell population was assessed by counting and adipogenic differentiation was assessed by real-time quantitative PCR (qPCR) analysis of peroxisome proliferator-activated receptor gamma (PPARγ) and fatty acid binding protein 4 (FABP4) after 3 and 5 days. Involvement of the ERK signalling pathway was assessed by Western blot. Low frequency mechanical stimulation significantly decreased expression of PPARγ after 3 days and FABP4 after 3 and 5 days versus non-stimulated culture. ERK signalling was decreased in mechanically-stimulated culture, indicating a role in the inhibition of adipogenic differentiation.

Application of this study: Low frequency mechanical stimulation may provide a technically simple means for control of mesenchymal stem cell

differentiation in cell-based therapies, particularly for inhibition of differentiation toward undesired adipogenic lineages.

3.2 Introduction

Mesenchymal stem cells (MSCs) are pluripotent cells which are of interest for studying events pertinent to development and tissue engineering because of their ability to differentiate along myo-, adipo-, osteo- and chondrogenic lineages (Caplan and Bruder, 2001; Pittenger et al., 1999). In order to direct differentiation of stem cells to specific lineages, a variety of different cytokines and chemokines may be used. Adipogenic differentiation is induced in expanded MSC cultures by treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin (Fève, 2005; Fink and Zachar, 2011; Nagai et al., 2007; Pittenger et al., 1999). MSCs differentiating along this lineage are characterized by decreased proliferation rate, lipid sequestration in fat bodies and increased expression of peroxisome proliferator-activated receptor gamma (PPARγ) and fatty acid binding protein 4 (FABP4). Since adipogenic differentiation of MSCs occurs at the expense of osteogenic and chondrogenic lineage specificity, it is of importance to understand the control of adipogenesis for tissue engineering applications.

Mechanical stimulation can modulate the effects of cytokine stimulation of mesenchymal stem cells, and may play a decisive role in influencing both lineage selection and progress toward terminally differentiated phenotypes (Rui et al., 2011; Shi et al., 2011). Recent studies have demonstrated that lineage selection in differentiating MSCs can be strongly influenced by oscillatory stretch of a

silicone rubber culture surface at 2% amplitude and a frequency of 0.17 Hz (Sen et al., 2008). Interestingly, mechanical stimulation promotes osteocyte lineage selection at the expense of adipogenic differentiation under these conditions, suggesting relationships between diseases such as osteoporosis and obesity and their modulation by physical exercise (Sen et al., 2008). To date, mechanotransduction experiments have emphasized a relatively limited range of frequencies (0.1 – 1 Hz) of oscillatory stimulation of cells in culture (David et al., 2007; Tanabe et al., 2008; Tanabe and Nakayama, 2004; Turner et al., 2008). However, mechanical activity in vivo covers a much wider range of frequencies (from diurnal variations of loading at roughly 0.0001 Hz to kilohertz frequency components associated with step changes in loading). Furthermore, gene-level responses can be rapid and sustained after only a single cycle of mechanical stimulation (Turner et al., 2008), indicating that a very wide range of mechanical inputs might meaningfully be applied for directed differentiation of MSCs. It is therefore important to more fully characterize the frequency-dependent effects of oscillatory mechanical stimulation on MSC differentiation in order to elucidate mechanotransduction mechanisms and pathways, and to identify the range of mechanical conditions which might be used to direct MSC differentiation in clinical applications.

Recent work has also highlighted the benefits of culture of MSCs on high-extension surfaces for reasons other than application of mechanical stimulation (Majd et al., 2009). Culture on a continuously expanding surface allows MSCs to be maintained at high density as they grow, but with significantly reduced

exposure to degradative enzymes due to decreased regularity of passaging (Majd et al., 2011; Majd et al., 2009). Under these conditions MSCs proliferate more quickly than in standard cultures while maintain a pluripotent MSC character and avoid the emergence of undesired fibrotic phenotypes expressing large amounts of alpha-smooth muscle actin (Majd et al., 2011). It is therefore possible that continuous expansion culture could be combined with oscillatory mechanical stimulation to provide culture conditions under which pluripotent MSCs can proliferate rapidly and then be directed along desired differentiation pathways. This could be achieved largely through relatively simple and inexpensive manipulation of culture surface extension and with decreased need for cytokine stimulation.

We hypothesized that relatively low frequency mechanical stimulation, which can readily be applied using hardware for continuous expansion cultures, can influence lineage selection in differentiating MSCs. Specifically, we investigated the inhibition of adipogenesis by oscillatory culture surface stretch, which was a prominent feature in previous studies using higher frequency mechanical stimulation. The C3H10T1/2 cell line was used, which represents pluripotent MSCs which can be differentiated into multiple lineages including adipocytes (Yamaguchi, 1995). Mechanical stimulation was applied in the presence of strongly adipogenic culture medium, and the potency of low frequency mechanical stimulation in competition with chemical cytokines was assessed.

3.3 Materials and Method

3.3.1 Cell culture and adipogenic differentiation medium

The cell line C3H10T1/2 (ATCC CCL-226; American Type Culture Collection, Location) was maintained on 10 cm diameter petri dishes in growth medium consisting of Dulbecco's modified Eagle medium (DMEM; Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO2 in air until at least passage 5. Cells from passages 5 to 15 were seeded on modified (as below) high extension silicone rubber (HESR) culture dishes and maintained for an attachment period of two days. Seeding was performed using 105 cells on HESR dishes expanded to 20 cm2. On the first day following the attachment period (experimental day 1), medium was switched to an adipogenic differentiation medium (DMEM, 5 µg/ml insulin, 0.1 µM dexamethasone and 50 µM indomethacin, 1% pen/strep). Cells were then maintained in adipogenic medium for 3 to 5 days. A time zero control was established using 105 cells seeded on two wells of a 6-well culture dish (total surface area 19.6 cm2) for the same attachment period; time zero control cells were never exposed to adipogenic medium.

3.3.2 Preparation of silicone rubber culture surfaces

High extension silicone rubber culture dishes (Cytomec GmbH, Spiez, Switzerland) were modified to promote cell adhesion. First, with HESR dishes in their unstretched state (8 cm^2 culture surface area), culture surfaces were coated

with 30% sulphuric acid for 15 min then washed thoroughly with deionized water. Then surfaces were silanized with 1% (3-aminopropyl) triethoxysilane for 2 hours at 70°C and washed thoroughly again. Surfaces were then functionalized with 5% (wt/vol) glutaraldehye for another 15 min and washed. Next, surfaces were sterilized by rinsing with 70% ethanol, washed with phosphate buffered saline (PBS), mounted in a mechanical device (below), expanded to 20 cm^2 culture surface area and coated with protein by incubation with 50 µg/mL collagen type I in PBS overnight. On the next day, HESR surfaces were washed again with PBS prior to cell seeding (Majd et al., 2009).

3.3.3 Mechanical stimulation

Mechanical stimulation was applied using an iris-like device (Cytomec) within which HESR culture dishes were mounted (Majd et al., 2009) (

Figure 3.1). Approximately uniform expansion and contraction of the culture surface was applied by opening or closing of the iris-like device. Pilot studies indicated that between 15 and 25 cm^2 nominal area of the HESR dishes, strain was approximately uniform over the full culture surface (Supplemental Figure S1). For mechanically stimulated cultures, a daily regimen of 6 hours of culture surface stretching and contraction was applied at the beginning of each experimental day (

Figure 3.1). Oscillatory mechanical stimulation was applied about a mean surface area of 20 cm^2 with amplitude of 10% (±2 cm^2) at a frequency of 0.01 Hz. This regimen was maintained for either 3 or 5 days; unstimulated control cultures

were maintained on HESR culture dishes mounted in the iris-like device and held to a fixed surface area of 20 cm^2.

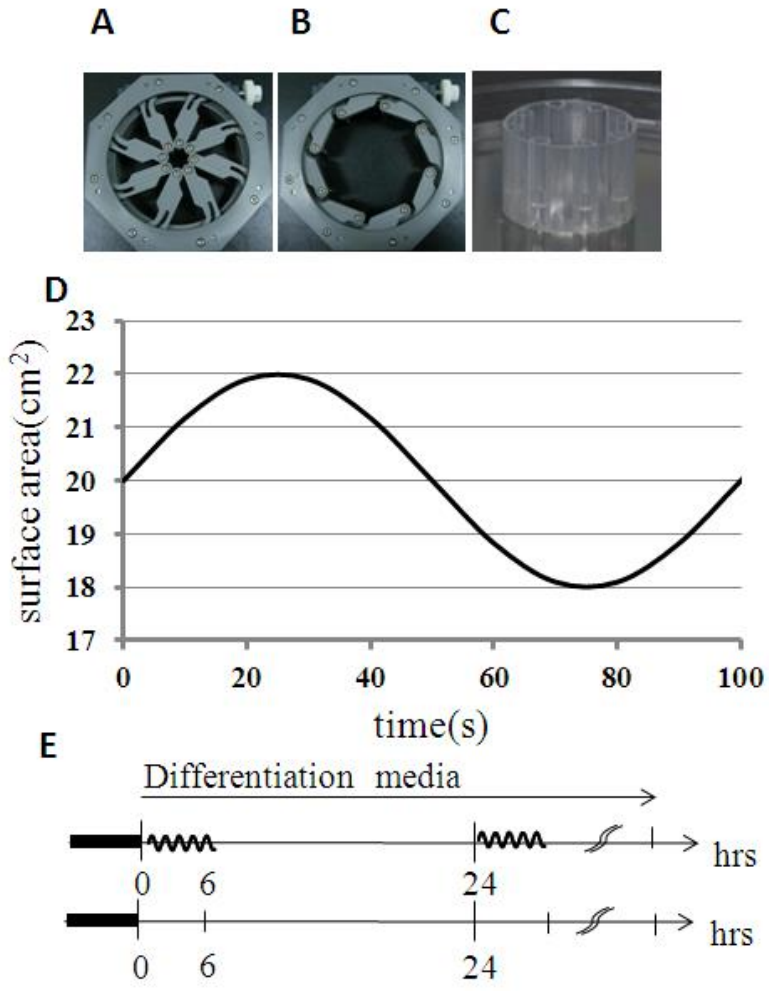


Figure 3.1 Experiments were conducted using an iris-like mechanical device which can stretch a high extension silicone rubber culture surface from 8 to 113 cm2. A) Iris-like device in the fully closed position at 8 cm2. B) Iris-like device in the fully open position at 113 cm2. C) Close-up of the HESR petri dish prior to mounting in the iris-like device. D) Dynamic stretch was applied to cultures at a frequency of 0.01 Hz and amplitude of 10% about a mean culture surface area of 20 cm2. E) Mechanical stimulation was applied to C3H10T1/2 cells in adipogenic medium on HESR dishes on experimental days 1-5. Unstimulated controls were maintained under identical conditions.

3.3.4 Reverse transcription and quantitative real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription was performed using 500 ng of total RNA in 20 μL volume per reaction using the qScript cDNA synthesis kit following the manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD). cDNA was diluted 1:10 and 1 μL of each cDNA sample was loaded per reaction (in duplicate) using PerfeCTa SYBR Green FastMix (Quanta Biosciences). Standard recommended PCR protocols were performed (50°C for 2 min, 94°C for 10 min, 95°C for 30 sec, 60°C for 1 min, with steps 3 and 4 repeated for 40 cycles) using the ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems Inc, Foster City, CA). The average cycle count for each target gene was normalized to HPRT to give the average delta count (ΔCt) using RQ SDS manager software (Applied Biosystems). Then for each target gene the average ΔCt reading from each experimental cDNA was subtracted from the average ΔCt from the comparative HPRT endogenous control (ΔΔCt). The average fold change in gene expression of samples after induction by adipogenic medium and application of mechanical stimulation compared to time zero samples was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). PCR primers for the transcription factors PPARγ and FABP4, and for Adiponectin and HPRT were generated exactly as described elsewhere (Li et al., 2011a; Makarenkova et al., 2009).

3.3.5 Western blotting

C3H10T1/2 cells were lysed in lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 1 mM β-glycerophosphate supplemented with complete EDTA-free protease inhibitor cocktail). 5 µg of total protein was run on a 10% (for 58 kDa PPARγ) or 12% (for 15 kDa FABP4) SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA for 2 hours and probed with antibodies against PPARγ (1:400, Abcam, Cambridge, MA) or FABP4 (1:700, Abcam), and voltage-dependent ion channel (VDAC; 1:1000, Cell Signalling, Danvers MA), followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10000, Cell Signalling). For MAP kinase analysis, blots generated from the same protein samples were probed with antibodies against p42/p44 (1:2000, Cell Signalling) for total ERK1/2 and phospho-p42/p44 (1:1000, Cell Signalling) for active ERK1/2, followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10000, Cell Signalling). Membranes were then washed three times in TBST for 15 minutes. Western blots were developed using Super Signal West Pico Substrate (Thermo Scientific, Rockford, IL) and BioMax MR film (Eastman Kodak). For ERK signal densitometry, films were scanned at 300 dpi, and ImageJ (NIH software) was used to analyze pixel density of bands from 3 independent experiments.

3.3.6 Histology and quantification of final cell numbers

Oil red O (Sigma, Oakville, ON) stock was prepared by dissolving 0.5 g of the dye in 100 mL isopropanol. To prepare working solution, 30 mL of the stock stain were diluted with 20 mL of deionized water and allowed to stand for 10 minutes at room temperature; the mixture was then filtered and covered immediately.

C3H10T1/2 cells were washed with deionized water and fixed with 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) for 5 min. Formaldehyde was then removed by washing with PBS for 1 min. Cells were rinsed with 60% isopropanol and incubated immediately with freshly prepared Oil Red O working solution for 15 min. After incubation with the stain, cells were washed with 60% isopropanol again and oil droplets were observed. Images were captured using a Zeiss Axiovert 40C microscope equipped with a Canon Powershot A640 digital camera attached to a Zeiss MC80DX 1.0× tube adapter.

After 3 and 5 days of culture on HESR dishes, culture medium was removed, culture surfaces were washed with PBS, 1 mL of 0.25 % trypsin-EDTA (Invitrogen) was added to the culture surface and incubated for 10 minutes, and cells were removed from the culture surface. Completeness of cell detachment was verified under a cell culture microscope. The resulting number of cells in suspension was quantified using a hemocytometer yielding total cell populations after 3 and 5 days.

3.3.7 Statistical analysis

All experiments were performed at least five times. Statistical significance of differences between mechanically stimulated cultures and unstimulated controls was evaluated using two-tailed unequal variance t-tests ($p<0.05$).

3.4 Results

3.4.1 C3H10T1/2 cells undergo adipogenic differentiation in defined medium

C3H10T1/2 cells cultured in adipogenic medium for 3 and 5 days specifically differentiated to an adipocyte phenotype. Cytoplasmic triglyceride droplets were evident as of day 3 and continued to increase in number through day 5 (Figure 3.2A). Oil red O staining revealed fat bodies throughout the culture dish after 3 days (Figure 3.2B) and 5 days (not shown). Since certain constituents of the adipogenic medium may also induce osteogenic differentiation (Pittenger et al., 1999) , alkaline phosphatase activity was also assayed but found to be minimal, indicating that C3H10T1/2 cells differentiated specifically to an adipocyte phenotype (Figure 3.2B). qPCR analysis suggested 2, 20 and 5 fold increases in PPARγ, FABP4 and adiponectin respectively after 3 days in adipogenic medium; these differences were all statistically significant. At 5 days, significant increases in PPARγ, FABP4 and adiponectin were still evident (Figure 3.2C).

3.4.2 Low frequency mechanical stimulation inhibits adipogenic gene expression in C3H10T1/2 cells

As indicated by qPCR, low frequency mechanical stimulation significantly downregulated expression of PPARγ with respect to unstimulated controls after 3 days. However, this difference was no longer evident after 5 days. Mechanical stimulation caused sustained significant downregulation of FABP4 at both 3 and 5

days. A trend of adiponectin downregulation was observed in response to mechanical stimulation but was not statistically significant (Figure 3.3).

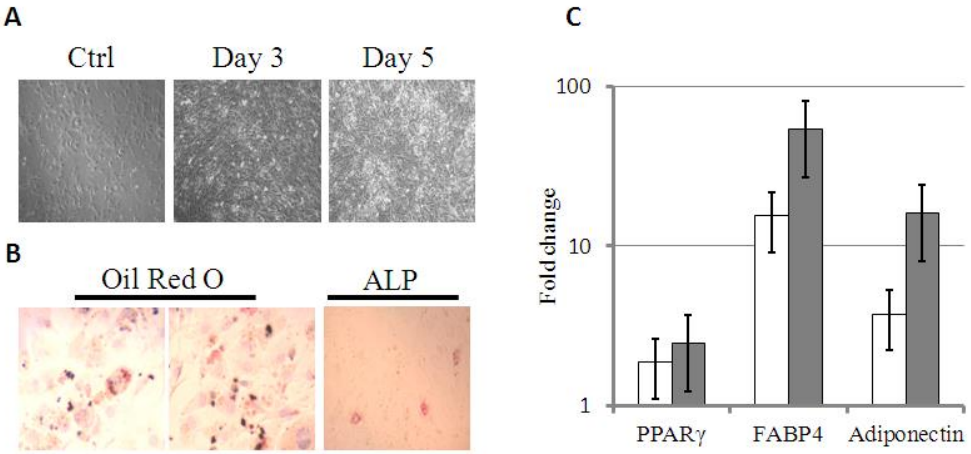


Figure 3.2 C3H10T1/2 cells in adipogenic medium differentiate to an adipocyte phenotype. A) Morphology of C3H10T1/2 cells after 3 and 5 days in differentiation medium. B) Oil red O staining showed fat droplets throughout culture dishes after 3 days, while alkaline phosphatase (ALP) staining was negative. C) RT-qPCR results for fold changes in PPARγ, FABP4 and Adiponectin expression after 3 days (white bars) and 5 days (grey bars), normalized to time zero controls (prior to exposure to adipogenic medium). Mean±SEM, n=3; all the results were statistically significant ($p<0.05$) for 3 and 5 days compared to time zero.

3.4.3 Low frequency mechanical stimulation inhibits adipogenic protein expression in C3H10T1/2 cells

After 3 and 5 days, unstimulated cultures exhibited a steady increase in FABP4 protein level with respect to time zero controls, clearly indicating the progress of adipogenic differentiation (Fig. 4). Mechanically stimulated cultures exhibited a markedly decreased expression of FABP4 after 3 and 5 days. Increases in PPARγ protein level with respect to time zero controls were not

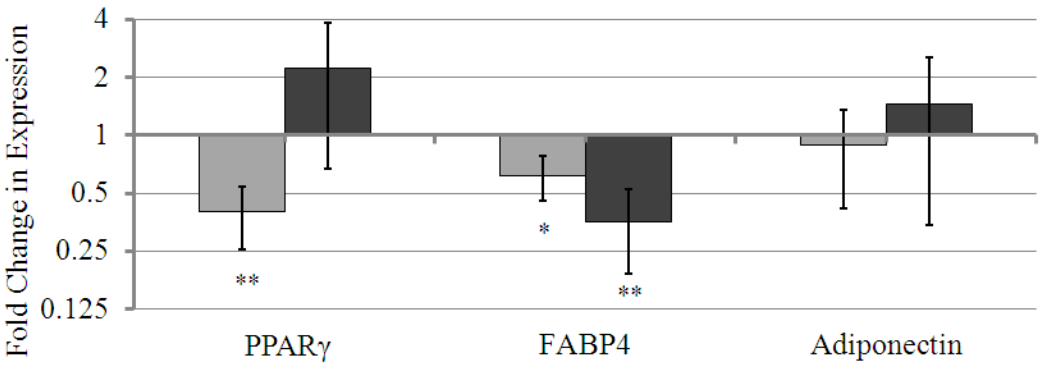


Figure 3.3. Effects of low frequency mechanical stimulation on adipogenesis of C3H10T1/2 cells as evidenced by RT-qPCR. Fold changes in PPARγ , FABP4 and Adiponectin after 3 days (light bars) and 5 days (dark bars) in the presence of mechanical stimulation, normalized to unstimulated controls. Mean±SEM, n=5; * ($p<0.05$) and ** ($p<0.01$) represents a significant difference between mechanically stimulated and unstimulated cultures.

evident; however, downregulation of PPARγ expression due to mechanical stimulation was evident after 3 days (Figure 3.4).

3.4.4 Low frequency mechanical stimulation alters ERK signalling in C3H10T1/2 cells

Recent studies utilizing high frequency mechanical stimulation have indicated a role for MAP kinase signalling in stem cell differentiation. Specifically, ERK was shown to be involved in mechanically stimulated inhibition of adipogenic differentiation (Tanabe et al., 2004). To explore the mechanism by which low frequency mechanical stimulation inhibits adipogenic differentiation, we therefore tested specifically for ERK activity. After 3 days

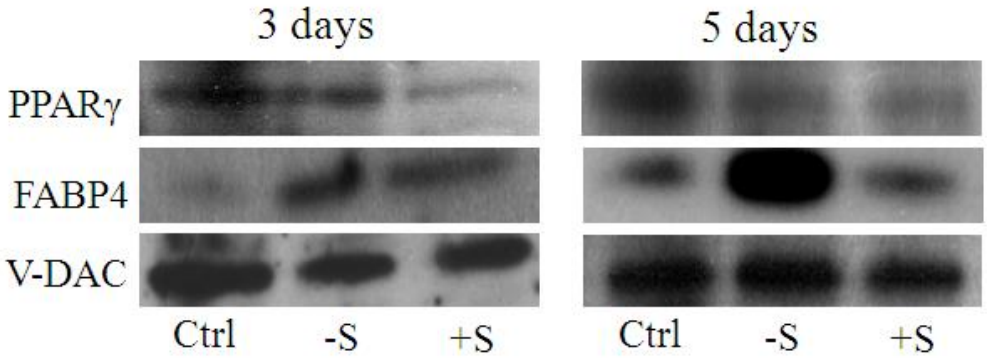


Figure 3.4 Effects of low frequency mechanical stimulation on adipogenesis of C3H10T1/2 cells as evidenced by Western blot. Results are shown for PPARγ and FABP4 after experimental days 3 and 5. V-DAC was used as a loading control. Experimental conditions included time zero controls (Ctrl), cultures which were not mechanically stimulated (-S) and mechanically stimulated cultures (+S).

stimulation, there was a trend for a 35% decrease in p-ERK levels compared with unstimulated cells. However, this was no longer evident after 5 days of stimulation (Figure 3.5).

3.4.5 Low frequency mechanical stimulation enhances proliferation of C3H10T1/2 cells in adipogenic medium

After 3 days, cells cultured without mechanical stimulation exhibited a 4-fold increase in total number compared to the initial number of cells. Cell population did not further increase between days 3 and 5. Mechanical stimulation resulted in a significantly greater 7-fold increase in cell number over the initial number. Similarly, the final cell number did not change significantly between days 3 and 5 (Figure 3.6).

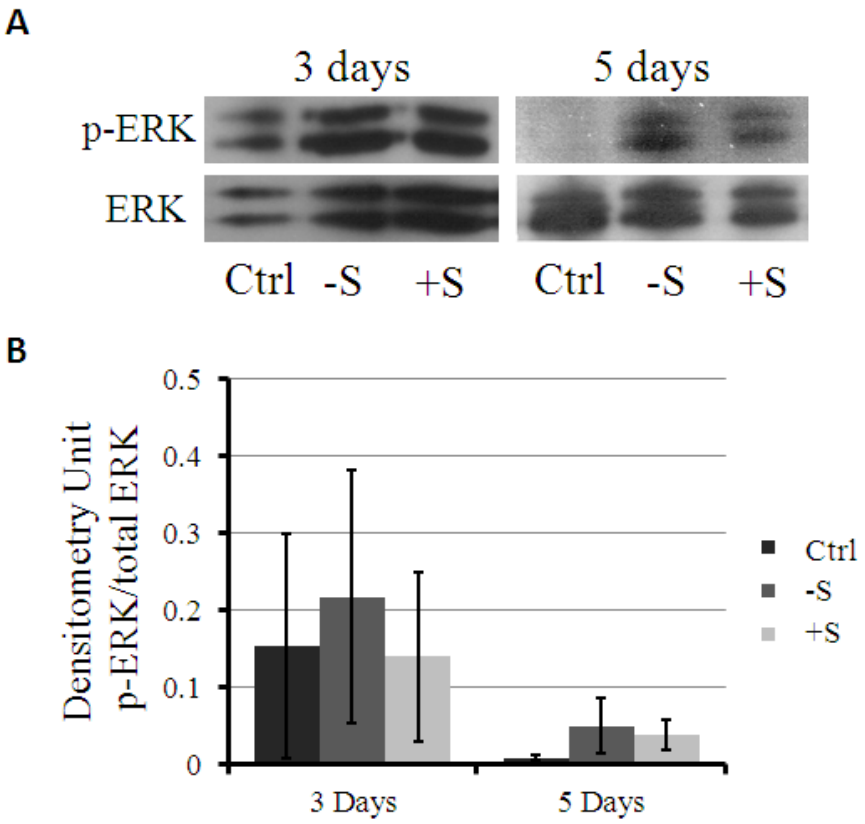


Figure 3.5. Effects of low frequency mechanical stimulation on the ERK signalling pathway of C3H10T1/2 cells cultured in adipogenic medium on HESR dishes, as evidenced by Western blot. Experimental conditions included time zero controls (Ctrl), cultures which were not mechanically stimulated (-S) and mechanically stimulated cultures (+S). A) Representative blot showing phosphorylated ERK (p-ERK) with total ERK used as loading control. B) Densitometry quantification - Mean±SEM, n=3.

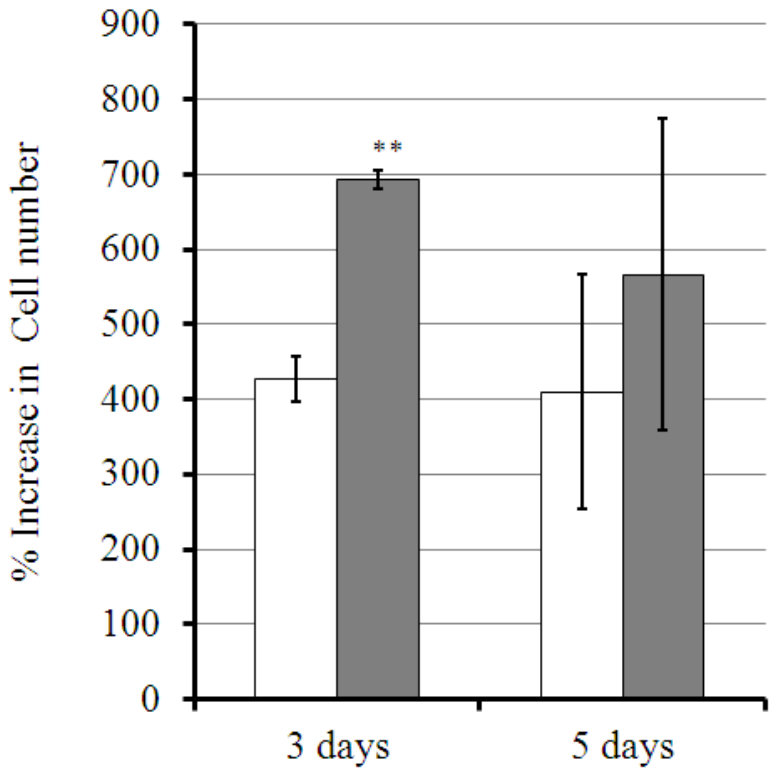


Figure 3.6. Effects of low frequency mechanical stimulation on cell proliferation. C3H10T1/2 cells cultured in adipogenic medium on HESR dishes were counted after 3 and 5 days. Unstimulated control (white bars) and mechanically stimulated culture (grey bars) cell numbers were normalized to the initial seeding population. Mean±SEM, n=3; ** represents a significant difference ($p<0.01$) between mechanically stimulated and unstimulated cultures.

3.5 Discussion

Present results demonstrate that low frequency mechanical stimulation can inhibit adipogenesis of C3H10T1/2 cells through downregulation of the transcription factors PPARγ (Rosen and Spiegelman, 2000) and FABP4 (Lowe et al., 2011) and adipokine (adiponectin), even in a strongly adipogenic medium. These findings are consistent with those of previous studies involving significantly different mechanical conditions, most notably higher oscillatory stimulation frequencies (David et al., 2007; Sen et al., 2008; Tanabe et al., 2004; Tanabe et al., 2008; Tanabe and Nakayama, 2004; Turner et al., 2008). Generally motivated by the fundamental frequencies of locomotion, respiration and circulation, these previous studies have emphasized frequencies between 0.1-1 Hz to show that oscillatory mechanical stimulation can inhibit or induce differentiation of stem cells to desired lineages (Grossi, 2011; Rui et al., 2011; Sarraf et al., 2011; Shi et al., 2011; Solem et al., 2011). Among the lowest frequencies applied for this purpose prior to the present study, Sen et al (Sen et al.) showed that genes specific to adipogenesis can be significantly down-regulated by mechanical stimulation at 0.17 Hz. Present results show that this down-regulation can occur at 0.01 Hz, or nearly 20 times lower frequencies. This indicates that inhibition of adipogenic differentiation by MSCs by oscillatory mechanical stimulation can occur for a much wider range of frequencies than previously believed.

Results also suggest that inhibition of adipogenic differentiation by low frequency mechanical stimulation involves time-dependent interactions between

pertinent factors, and may require an extended period of stimulation to occur. PPARγ is a key regulator for adipogenic differentiation and is expressed early in adipocyte differentiation (Lowe et al., 2011). Three days of low frequency mechanical stimulation was sufficient to significantly down-regulate PPARγ, however, after 5 days of stimulation it seemed to increase compared to controls. Considering that cells lacking PPARγ cannot differentiate into adipocytes (Rosen et al., 2001), downregulation of the transcription factor after 3 days stimulation may be a sufficient step in our observed inhibition of adipogenic differentiation. FABP4 is a transcription factor expressed later in adipogenesis, and a downstream target of PPARγ (Hamm et al., 2001; Wu et al., 1998). We found that FABP4 was significantly down-regulated at both 3 and 5 days post-stimulation further indicating the significance of the initial PPARγ blockade. Adiponectin is a downstream gene in adipogenesis (Davis and Scherer, 2008) and increased PPARγ expression leads to increased adiponectin expression (Sen et al., 2008). We observed a slight reduction in expression after 3 days of stimulation, and an increase after 5 days of stimulation. The initial decrease and secondary increase in adiponectin expression could be attributed to the decline and rise in PPARγ expression observed at 3 and 5 days, respectively. The early (3 days) inhibition of PPARγ, however, was still sufficient to block FAB4 expression through the duration of the experiment resulting in inhibition of adipogenic differentiation: after both 3 and 5 days the morphology of stimulated cells remained more mesenchymal-like and less fat bodies were apparent compared to control unstimulated cells.

In order to determine which signalling pathways are involved in the inhibition of adipogenesis via low frequency mechanical stimulation, we investigated mitogen activated protein kinase (MAPK) signalling. Extracellular related kinase (ERK) signalling is one of the major signalling pathways which occur in cascade with other signalling pathways. The ERK signalling pathway was identified as a strong candidate for this activity since previous work has shown that ERK signalling regulates adipogenic differentiation of pre-adipocytes (Dang and Lowik, 2005; Fève, 2005), and since higher frequency mechanical stimulation of cells inhibits adipogenic differentiation of MSCs through the ERK signalling pathway (Tanabe et al., 2008; Tanabe and Nakayama, 2004). Our findings indicate that inhibition of adipogenesis by low frequency mechanical stimulation also occurs via the ERK signalling pathway, consistent with this previous work. Other reports suggest that inhibition of adipogenesis can involve β-catenin signalling (Sen et al., 2008) and the Wnt signalling pathway; therefore other pathways may be involved in low frequency mechanical stimulation in addition to the ERK signalling pathway.

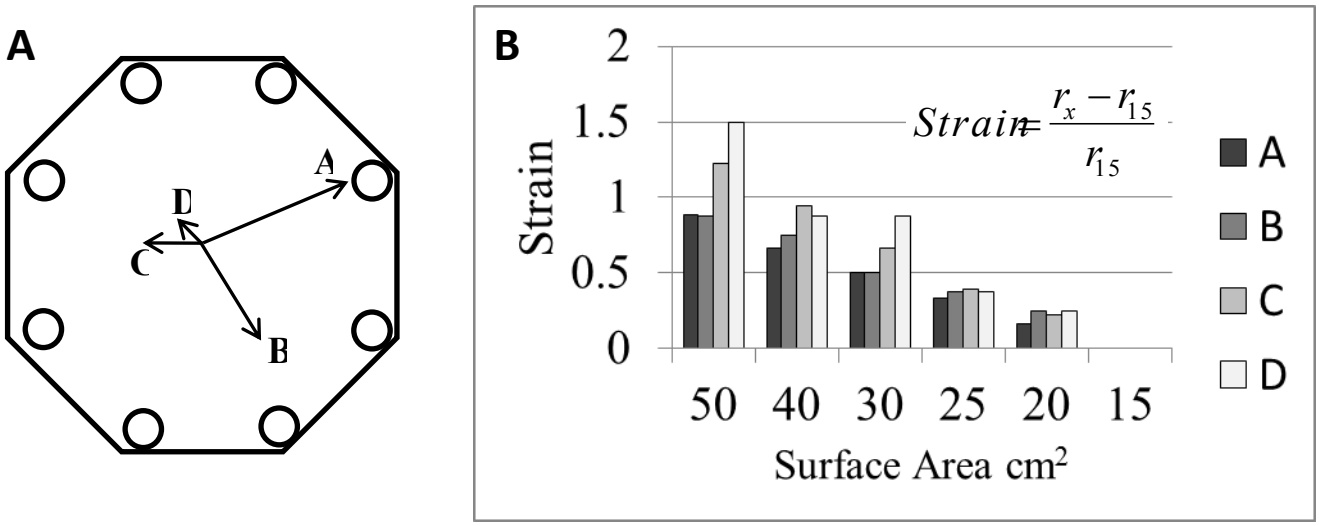
Low frequency mechanical stimulation of C3H10T1/2 cells also affected their population size in strongly adipogenic differentiation medium. After 3 days, mechanical stimulation was associated with a significant increase in cell number by approximately 1.5-fold. This finding was consistent with inhibition of adipogenesis by mechanical stimulation, since more advanced adipogenic differentiation in standard culture was expected to result in decreased rates of proliferation compared to the less differentiated cells in

mechanically stimulated cultures. After 5 days, this difference in population size between the two culture conditions still existed as a trend but was no longer significant due to increased variability among experimental samples. This increased variability may have been associated with alterations in cell behaviours due to confluence under either culture condition. For MSCs differentiating to adipocytes, high cell densities may have interfered with differentiation resulting in increased proliferation. For MSCs with inhibited adipogenic differentiation due to low frequency mechanical stimulation, increased proliferation may have resulted in early confluence and subsequently decreased cell numbers.

Present findings may have important implications for understanding the role of mechanical stimulation in development and in promoting desired cell differentiation for tissue engineering. Mesenchymal stem cells have enormous potential in cell-based therapies for bone and cartilage repair provided that their differentiation pathways can be understood and controlled for desired lineage selection within a mechanically active environment.

3.6 Acknowledgement

This project is supported by the Canada Research Chair and NSERC Discovery Grant programs.



Supplemental Figure S1. Characterization of strain uniformity on HESR culture surface. A) With the octagonal culture surface at a nominal surface area of 15 cm2, 4 points (labelled A, B, C and D) distributed between the center and the mounting posts were marked with a permanent marker. The distance from the center of the culture surface to these points was measured with the culture surface at 15 cm2 (r15) and a range of nominal areas from 20-50 cm2 (rx). B) The mean strain along the line from the center to each point was then calculated as illustrated.

4 Low Frequency Mechanical Stimulation Modulates Osteogenic Differentiation of C2C12 Cells

Preface

In the following chapter, the effect of low frequency mechanical stimulation on the osteogenic differentiation of C2C12 cell line is investigated. There are lines of evidences indicating that mechanical stimulation enhances the osteogenic lineage differentiation. However, the effect of low frequency has rarely been examined. This chapter discusses the sequence of BMP-2 priming and low frequency mechanical stimulation together with its remarkable effect on the osteogenic differentiation of C2C12 cells. It was observed that when there is an initial stage of priming, the low frequency stimulation significantly enhances osteogenesis.

Low Frequency Mechanical Stimulation Modulates

Osteogenic Differentiation of C2C12 Cells

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Running Title: Low frequency stimulation of C2C12 cells

4.1 Abstract

Mechanical stimulation can influence differentiation pathways of stem cells, and may therefore provide improved control of lineage specifications for clinical applications. Oscillatory mechanical stimulation at relatively low frequencies (0.01 Hz) has recently been shown to suppress adipogenic differentiation of mesenchymal stem cells, indicating that the range of effective stimulation frequencies is not limited to those associated with locomotion, circulation, and respiration. We hypothesized that relatively low frequency mechanical stimulation (0.01 Hz) can also promote bone cell differentiation of C2C12 stem cells in combination with BMP-2. Results indicate that low frequency mechanical stimulation can significantly enhance osteogenic gene expression, provided that differentiation is initiated by a priming period involving BMP-2 alone. Subsequent application of low frequency mechanical stimulation appears to act synergistically with continued BMP-2 exposure to promote osteogenic differentiation of C2C12 cells, and can even partially compensate for the removal of BMP-2. These effects are mediated by the ERK and Wnt signalling pathways. Osteogenic induction of C2C12 cells by low frequency mechanical stimulation is therefore critically dependent upon previous exposure to growth factor stimulation, and the timing of superimposed BMP-2 and mechanical stimuli can sensitively influence osteogenesis. These insights may provide a technically simple means for control of stem cell differentiation in cell-based therapies, particularly for enhancement of differentiation toward desired lineages.

4.2 Introduction

Stem cell differentiation to bone is important to growth, development and tissue maintenance; understanding of the cellular processes underlying osteogenesis is necessary for development of cell-based therapies. Growth factor stimulation of stem cells is an initiating event which activates transcription factors that subsequently drive lineage specification. Bone morphogenetic proteins (BMPs) (Urist and Strates, 1971; Wang et al., 2011) comprise a superfamily of growth factors that drive bone cell differentiation and adult bone maintenance. Among the BMP superfamily, BMP-2 is known to have a critical role in inducing osteogenic differentiation of mesenchymal stem cells (Katagiri et al., 1994; Ryoo et al., 2006). Genes which are direct targets of BMP-2 include the transcription factors *RUNX2* and osterix (*OSX*/*SP7*), and the matrix-associated proteins alkaline phosphatase, osteopontin and osteocalcin (Gersbach et al., 2007). It has also been established that the time course and duration of growth factor stimulation can have important effects on differentiation (Shen et al., 2010), and moreover BMP-2 efficacy is dose-dependent (Katagiri et al., 1994).

In addition to growth factor stimulation, mechanical stimulation can also affect osteogenic differentiation of stem cells (Kobayashi et al., 2004; Zhang et al., 2012a). Intracellular signalling pathways induced by mechanical stimulation in neurosensory and vascular cells can induce physiological responses which are much more rapid (Sackin, 1995) than those induced by growth factors which can require minutes to hours (Jones and Kazlauskas, 2000). Furthermore, downstream signalling events overlap between the two types of stimulation, potentially

resulting in interactions between chemical and mechanical cues (Gallea et al., 2001; Sen et al., 2008). Mechanical stimulation utilizing amplitudes and frequencies consistent with the fundamental frequencies of locomotion (0.1-1 Hz) can induce osteogenic differentiation (Kopf et al., 2012; Zhang et al., 2012b). However, stimulation frequencies below this range have not been extensively studied in the context of osteogenesis, though they have been found effective in modulating differentiation of mesenchymal stem cells (Khayat et al., 2012). Since lower frequency mechanical stimulation is often technically easier to apply, study of the effects of low frequency mechanical stimulation may create new avenues for integration of mechanical stimulation into protocols for stem cell differentiation in cell-based therapies.

Since mechanotransduction alone can enhance bone formation (Lau et al., 2010; Reijnders et al., 2007) while BMP-2 is known to effectively drive osteogenesis, our goals were to explore the extent to which the complementary mechanisms interact during during osteogenic differentiation. Specifically, these stimuli were applied either together or in different sequences to elucidate their relative importances as osteogenesis progesses. The murine myoblastic C2C12 cell line, which can differentiate into muscle, adipocyte or osteocyte lineages (Urist and Strates) was used. These cells have recently been shown to be sensitive to the sequence and timing of BMP-2 exposure during osteogenesis (Li et al., 2011b), making them an ideal candidate for testing interactions with mechanical stimulation as well.

4.3 Materials and Methods

4.3.1 Cell culture

The cell line C2C12 (ATCC CRL-1772; American Type Culture Collection, Manassas, VA) was maintained on 10 cm diameter petri dishes in growth medium consisting of Dulbecco's modified Eagle medium (DMEM; Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded on modified (as below) high extension silicone rubber (HESR) culture dishes and maintained overnight for an attachment period. Seeding was performed using $5x10^5$ cells on HESR dishes expanded to 20 cm^2. After the 24 hour attachment period, cells were typically 60-80% confluent and experiments were initiated.

4.3.2 Preparation of silicone rubber surfaces

High extension silicone rubber culture dishes (Cytomec GmbH, Spiez, Switzerland) were modified to promote cell adhesion as previously described (Khayat et al., 2012). Briefly, unstretched HESR dishes were coated with 30% sulphuric acid for 15 min then washed thoroughly with deionized water. Then surfaces were silanized with 1% (3-aminopropyl) triethoxysilane for 2 hours at 70°C and washed thoroughly again. Surfaces were then functionalized with 5% (wt/vol) glutaraldehye for another 15 min and washed. Next, surfaces were disinfected by rinsing with 70% ethanol, washed with phosphate buffered saline (PBS), mounted in a mechanical device (below), expanded to 20 cm^2 culture

surface area and coated with protein by incubation with 50 µg/mL collagen type I in PBS overnight. On the next day, HESR surfaces were washed again with PBS prior to cell seeding.

4.3.3 Mechanical Stimulation

Mechanical stimulation was applied using an iris-like device (Cytomec) within which HESR culture dishes were mounted (Khayat et al., 2012; Majd et al., 2009) (A-D). Approximately uniform expansion and contraction of the culture surface was applied by opening or closing the iris-like device. During mechanical stimulation, a continuous culture surface stretching and contraction was applied. Oscillatory stimulation occurred about a mean surface area of 20 cm^2 with amplitude of 10% (±2 cm^2) at a frequency of 0.01 Hz. Unstimulated control cultures were maintained on HESR culture dishes mounted in the iris-like device and held to a fixed surface area of 20 cm^2.

4.3.4 Experimental protocols

As an initial control, the osteogenic potential of C2C12 cells was tested under non-mechanically stimulated conditions during culture on tissue culture plastic in osteogenic medium (growth medium supplemented with 200 ng/mL BMP-2 (Invitrogen, Burlington, ON)). 200,000 cells were seeded per well in a 6-well plate. After 24-hour attachment period, cells were harvested from one well as a time zero control. Other wells were then induced with osteogenic medium for 6, 24 and 48 hours after which cells were collected for gene expression and histological analyses (below).

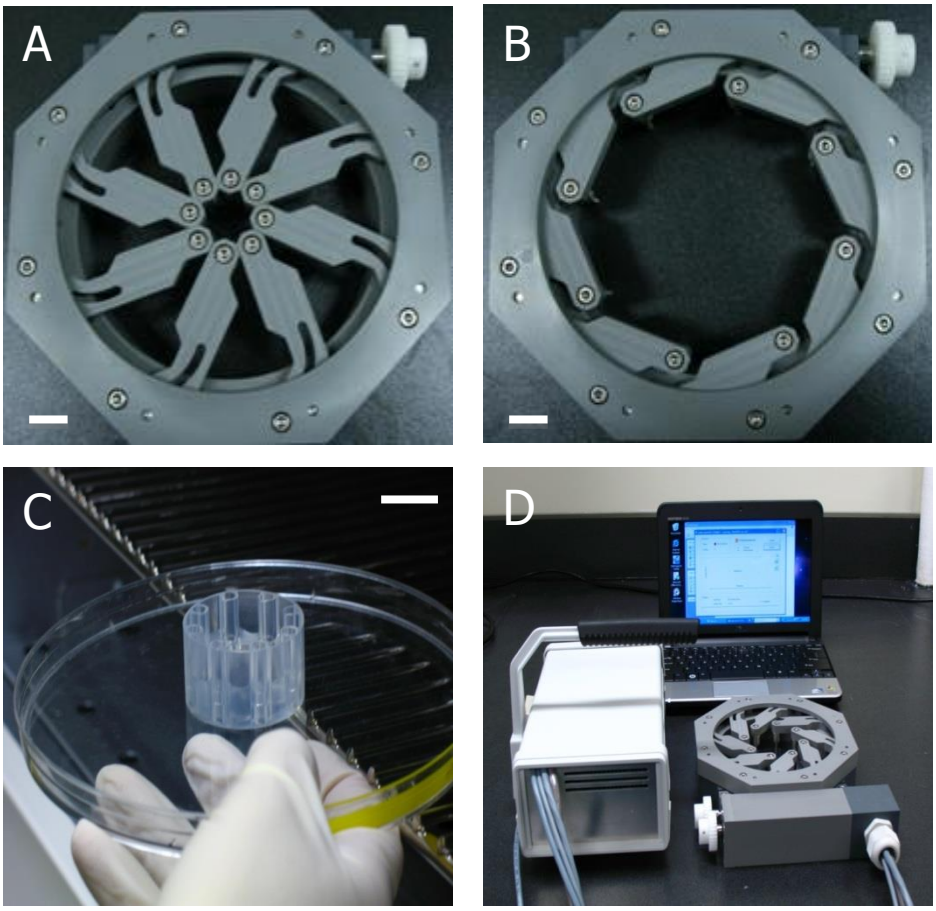

Figure 4.1 Experiments were conducted using an iris-like mechanical device which can stretch a high extension silicone rubber culture surface from 8 to 113 cm^2. A) Iris-like device in the fully closed position at 8 cm^2. Scale bar represents 2 cm. B) Iris-like device in the fully open position at 113 cm^2. C) HESR petri dish prior to mounting in the iris-like device. Scale bar represents 1 cm. D) Mechanical stimulation device connected to the computer. Modified from [Khayat G. , Rosenzweig D., 2012].

Four different experimental protocols (A, B, C, and D) were designed to investigate the combined effects of BMP-2 induction and mechanical stimulation. In experiment A, cells were induced with BMP-2 for 48 hours while mechanical stimulation was only applied for the latter 24 hours. These were compared to controls under BMP-2 induction for 48 hours in the absence of any mechanical stimulation. In experiment B, BMP-2 induction and mechanical stimulation were

applied simultaneously for 48 hours and compared to controls where BMP-2 induction occurred in the absence of any mechanical stimulation (Figure 4.4A). Protocols A and B were designed to investigate how mechanical stimulation could modulate the time course of BMP-2 induction. In experiment C, cells were primed with BMP-2 for 24 hours after which medium was replaced with fresh growth medium and mechanical stimulation was applied for the next 24 hours. These were compared to controls primed with BMP-2 for 24 hours after which medium was replaced with growth medium but no mechanical stimulation was applied (Figure 4.5A). Experiment D was similar to experiment C except the control culture involved BMP-2 induction for 48 hours without any mechanical stimulation (Figure 4.6A). Protocols C and D addressed the question of whether mechanical stimulation could replace BMP-2 induction.

4.3.5 Reverse transcription and quantitative real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription was performed using 500 ng of total RNA in 20 μL volume per reaction using the qScript cDNA synthesis kit following the manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD). cDNA was diluted 1:10 and 1 μL of each cDNA sample was loaded per reaction (in duplicate) using PerfeCTa SYBR Green FastMix (Quanta Biosciences). Standard quantitative real-time PCR protocols were performed using *HPRT1* for normalization as previously described (Khayat et al., 2012). PCR primers to detect the expression of osteogenic markers *Runx2*, *Osx*, *Alpl*,

Spp1, *Col1a1*, *Bglap*, *Ibsp*, *Tnfrsf11b* and *Hprt* were generated exactly as described elsewhere (Li et al., 2011a; Makarenkova et al., 2009).

4.3.6 Western blotting

C2C12 cells were lysed in lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 1 mM β-glycerophosphate supplemented with complete EDTA-free protease inhibitor cocktail). 10 µg of total protein was run on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA for 2 hours. For ERK1/2 analysis, blots were probed with antibodies against p42/p44 (1:2000, Cell Signalling), phospho-p42/p44 (1:1000, Cell Signalling), phospho-p38 (1:1000, Cell Signalling) and phospho-JNK (1:1000, Cell Signalling) followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10000, Cell Signalling). For Wnt signalling, blots were probed with antibodies against β-catenin (1:1000, Abcam) and α-tubulin (1:1000, Abcam, Cambridge, MA), followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000, Cell Signalling). Washed membranes were developed using Super Signal West Pico Substrate (Thermo Scientific, Rockford, IL) and BioMax MR film (Eastman Kodak).

4.3.7 Histology

C2C12 cells were washed with deionized water and fixed with 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) for 2 min. Formaldehyde was then removed by washing with PBS and ALPL assay was immediately performed

according to the manufacturer's instructions (Alkaline Phosphatase Detection Kit ,Millipore). Images were captured using a Zeiss Axiovert 40C microscope equipped with a Canon Powershot A640 digital camera attached to a Zeiss MC80DX 1.0× tube adapter.

4.3.8 Statistical analysis

All experiments were performed at least three times. Statistical significance of differences between mechanically stimulated cultures and unstimulated controls was evaluated using two-tailed unequal variance t-tests ($p<0.05$).

4.4 Results

4.4.1 Osteogenic differentiation of C2C12 cells in the presence of BMP-2 is time sensitive

C2C12 cells cultured in osteogenic medium for 6, 24 and 48 hours specifically differentiated to an osteoblast-like phenotype. qPCR analysis indicated significant changes in *Runx2*, *Osx*, *Alpl*, *Col1a1*, *Spp1* (osteopontin) and *Bglap* (Gla) after 48 hours of induction with BMP-2 (Figure 4.2). While most genes were downregulated compared to time zero controls after 6 hours, there were trends for increases in all of them with time. Strong ALPL activity was clearly visible (purple stain) after 48 hours in BMP-2-treated samples versus untreated C2C12 cells (Figure 4.2).

4.4.2 Low frequency stimulation enhances osteogenesis in C2C12 cells after BMP-2 priming

Experiment A included 24 hours of priming with BMP-2 followed by 24 hours of superimposed mechanical stimulation (Figure 4.3A).As indicated by qPCR (Figure 4.3B), low frequency mechanical stimulation significantly upregulated expression of *Runx2*, *Osx*, *Alp* and *Col1a1* with respect to BMP-2 induced controls at 48 hours. Upregulation of osteopontin also approached significance (p=0.054). Mechanical stimulation caused trends for upregulation of bone Gla protein (encoded by *Bglap1*) and Osteoprotegerin/OPG (encoded by *Tnfrsf11b*) versus controls but these were not statistically significant. This protocol had no obvious effect on gross morphology (Figure 4.3C).

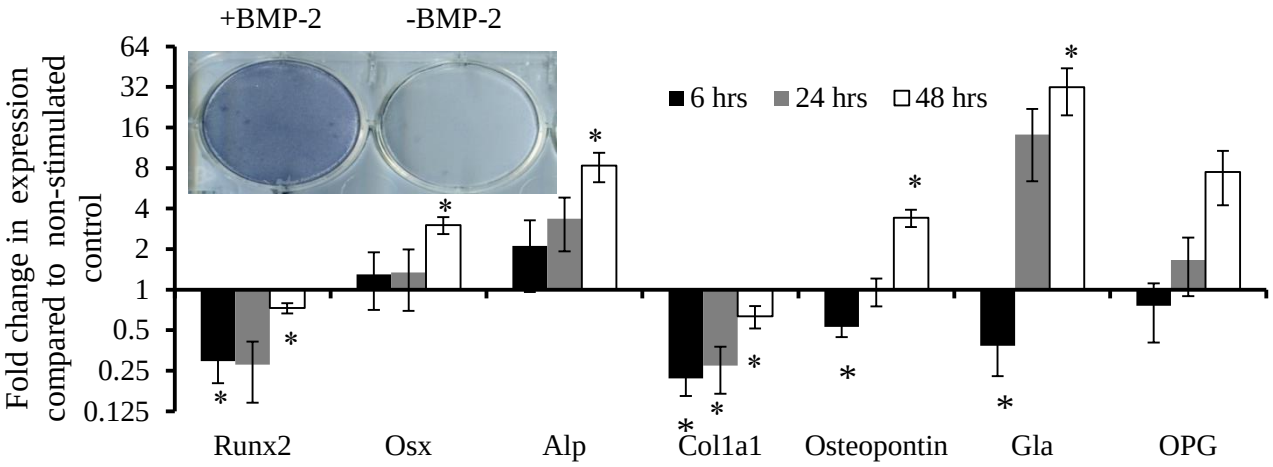

Figure 4.2. Osteogenic medium induces bone-like differentiation of C2C12 cells. RT-qPCR results for fold changes in Runx2, Osx, Alp, Col1a1, Osteopontin, bone Gla protein and OPG expression after 6 hours (black bars), 24 hours (grey bars) and 48 hours (white bars), normalized to time zero controls (prior to exposure to osteogenic medium). Mean±SEM, n=3; * represents a significant ($p<0.05$) difference between BMP-2-treated and untreated cultures. Inset: Cells treated with BMP-2 stained strongly positive for ALP activity (purple) after 48 hours. Scale bar represents 10 mm.

4.4.3 Low frequency stimulation without BMP-2 priming does not enhance osteogenesis

In experiment B, BMP-2 induction and low frequency mechanical stimulation were applied simultaneously for 48 hours (Figure 4.4A). qPCR analysis demonstrated no significant changes in osteogenic gene expression compared to non-mechanically-stimulated controls at 48 hours (Figure 4.4B), although *Runx2* appeared slightly upregulated ($p=0.08$). No changes in cell morphology were observed (not shown).

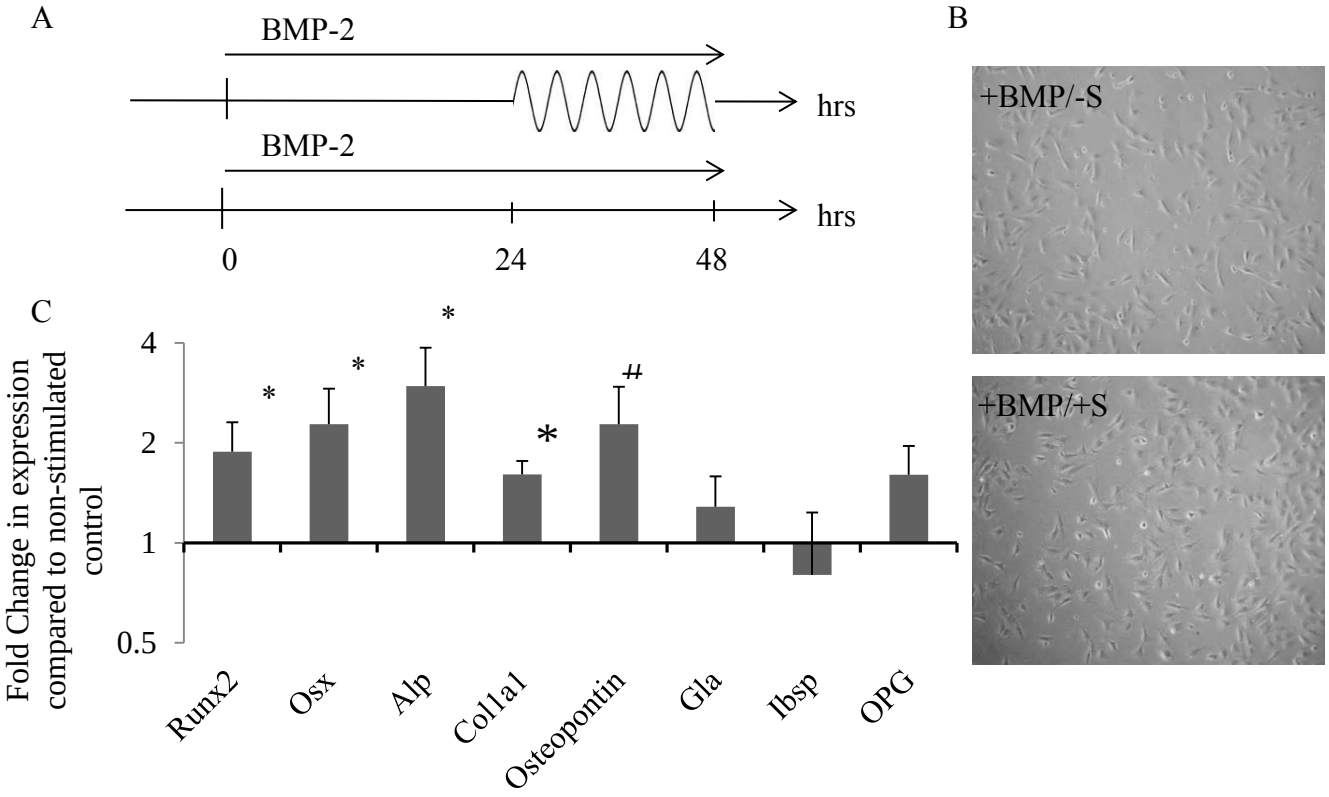

Figure 4.3. A) Experimental protocol A involved BMP-2 induction for 48 hours with mechanical stimulation only for the latter 24 hours. Controls were BMP-2-induced for 48 hours in the absence of any mechanical stimulation. B) Mechanical stimulation in the presence of BMP-2 enhanced osteogenic gene expression versus unstimulated controls. Mean±SEM, n=6; * represents $p<0.05$; # represents $p=0.054$. C) Morphology of C2C12 cells after 48 hours in experimental cultures (top) and controls (bottom). Scale bar represents 200 µm.

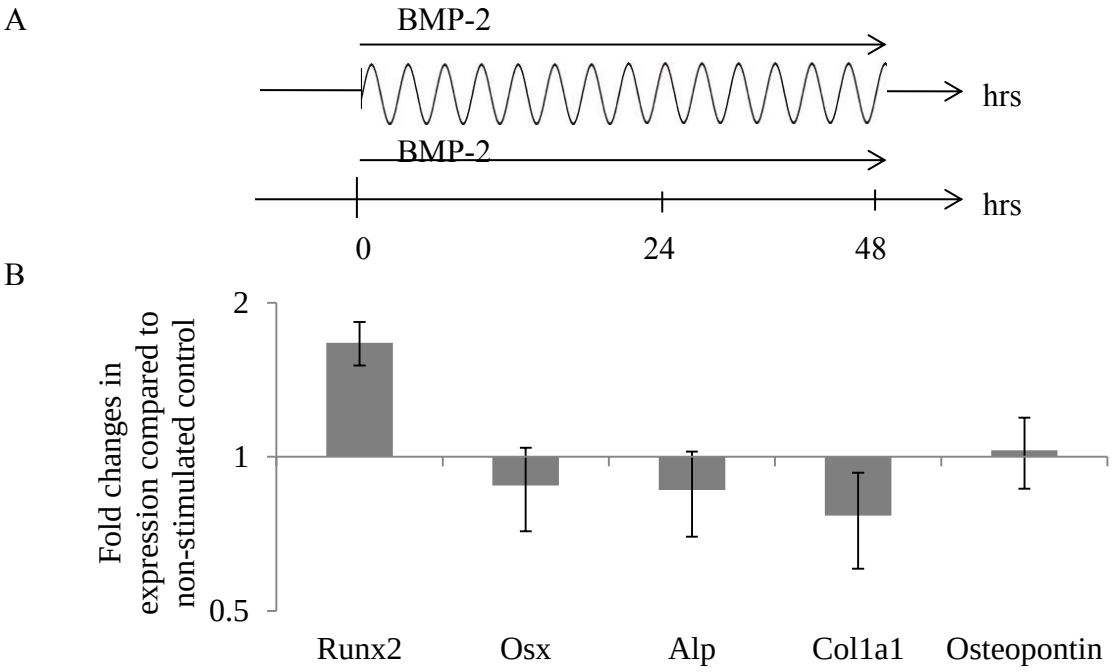

Figure 4.4. A) Experimental protocol B involved 48 hours of simultaneous BMP-2 induction and mechanical stimulation compared to controls with BMP-2 induction only. B) Fold differences in osteogenic gene expression at 48 hours. Mean±SEM, n=3.

4.4.4 Low frequency stimulation is sufficient to modulate osteogenesis after BMP-2 priming

In experiment C, cells were primed for 24 hours with BMP-2, after which low frequency mechanical stimulation was applied for 24 hours in the absence of BMP-2 and compared to controls without BMP-2 (Figure 4.5A). qPCR results indicated significant upregulation of *Col1a1* and strong trends for upregulation of *Osx* and *Alpl* which approached significance (p=0.07 and 0.08) versus non-mechanically-stimulated controls (Figure 4.5B). In contrast, osteopontin (*Spp1*) was significantly downregulated versus controls.

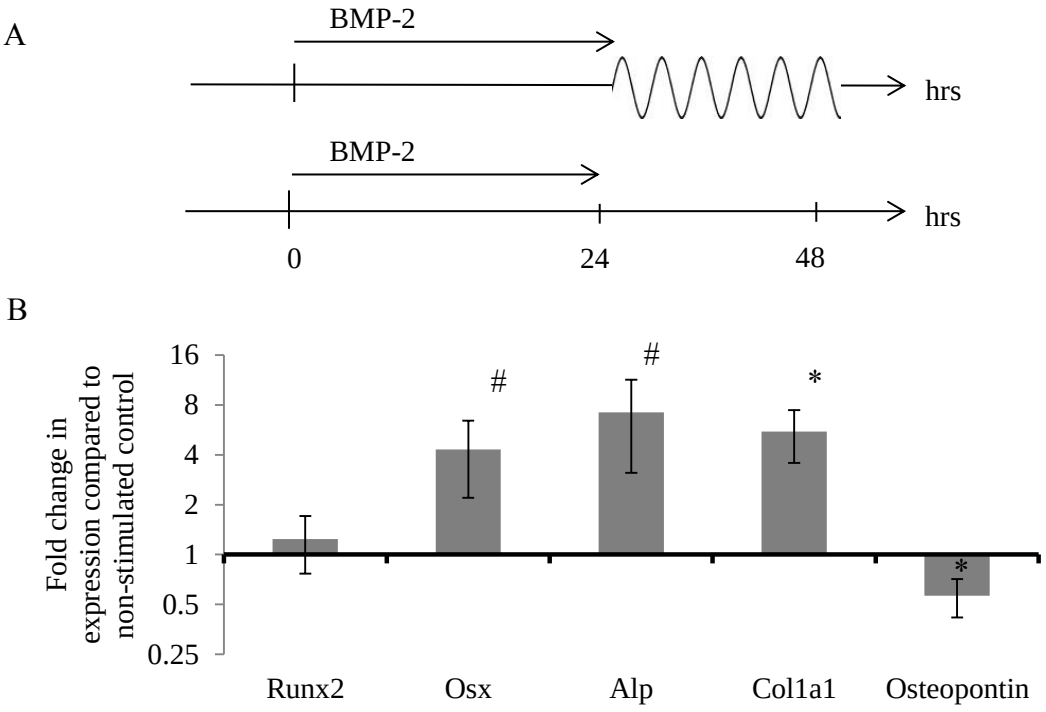

Figure 4.5. A) Protocol C involved 24 hour priming with BMP-2 followed by changing of medium and mechanical stimulation for next 24 hours. Controls were identically treated except for mechanical stimulation. B) Fold differences in osteogenic gene expression at 48 hours. Mean±SEM, n=5; *represents p<0.05; # represents p<0.08.

4.4.5 BMP-2-induced osteogenesis masks the effects of low frequency mechanical stimulation

In experiment D, mechanically stimulated cells from experiment C were compared to controls in which BMP-2 was present for 48 hours (Figure 4.6A). Less osteogenic differentiation was observed in cells exposed to low frequency mechanical stimulation versus those induced with BMP-2 alone for 48 hours (Figure 4.6B). These effects were particularly significant for *Runx2*, *Osx* and *Alpl*.

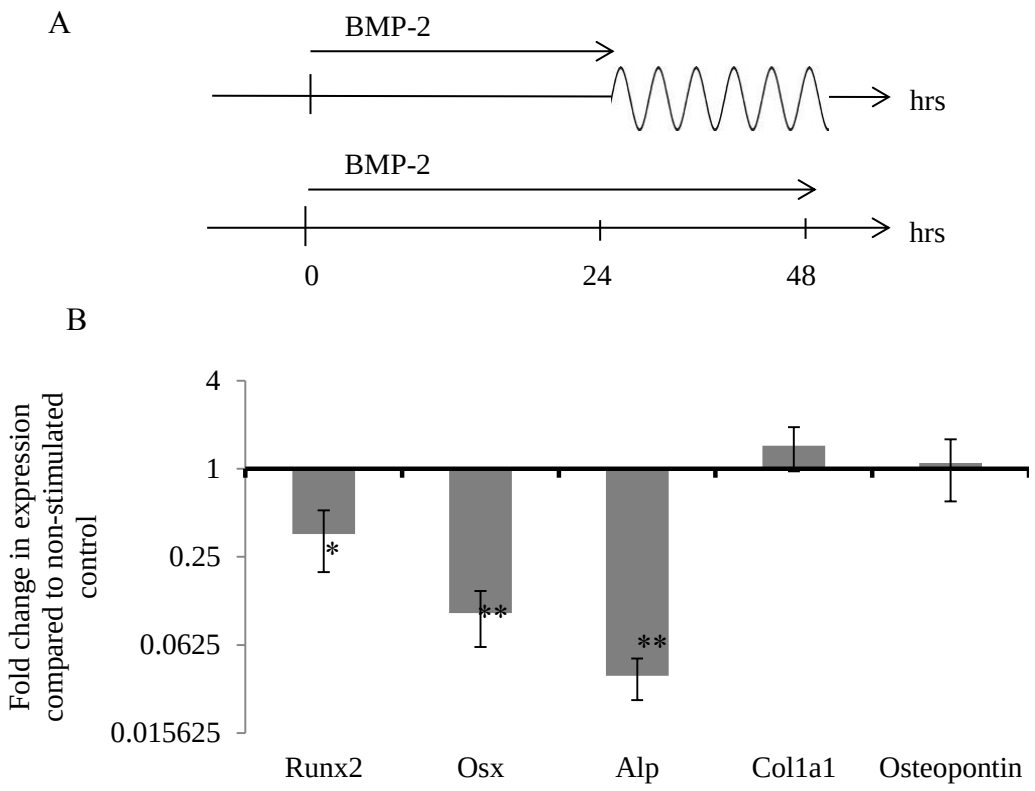

Figure 4.6. A) Protocol D involved 24 hour priming with BMP-2 followed by changing of medium and mechanical stimulation for the next 24 hours. Controls were BMP-2-induced for 48 hours in the absence of any mechanical stimulation. B) Fold differences in osteogenic gene expression at 48 hours. Mean±SEM, n=3; *represents p<0.05; ** represents p<0.01.

4.4.6 Low frequency mechanical stimulation alters MAPK and Wnt signalling in C2C12 cells

In experiment A, Western blot analysis of protein lysates at 48 hours showed that priming with BMP-2 followed by superposition of low frequency mechanical stimulation downregulated ERK and p38 activity (data for p38 not shown) (Figure 4.7). In experiment B, Western blot analysis revealed that

concomitant application of low frequency mechanical stimulation and BMP-2 without priming caused an increase in ERK activity. For experiment C, no changes were observed in MAPK activity between cells primed with BMP-2 then mechanically stimulated without BMP-2 versus non-mechanically-stimulated controls which were primed and then cultured without BMP-2. In experiment D, when mechanical stimulation post-priming was compared to 48 hours of BMP-2 induced controls, an increase in ERK activity was observed with no detectable change in p38 activity (not shown). JNK activity was below detection for all experiments (not shown). When analyzing Wnt signalling, no detectable changes in β-catenin were observed under most experimental conditions, with the exception of a slight increase in experiment D (Figure 4.7).

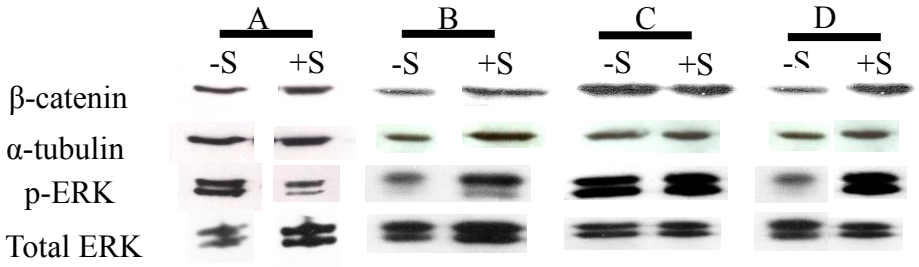

Figure 4.7. Western blot analysis of the effects of low frequency mechanical stimulation on Wnt and MAPK signalling in C2C12 cells undergoing BMP-2-induced osteogenesis.

4.5 Discussion

Previous studies have shown that BMP-2 induces osteogenesis through upregulation of the key transcription factors *RUNX2* (Komori et al., 1997) and *OSX* (osterix) (Niger et al., 2011; Ulsamer et al., 2008). Present results confirm these findings, and moreover demonstrate that low frequency mechanical

stimulation can enhance osteogenic differentiation of C2C12 cells in the presence of BMP-2. BMP-2 alone induces expression of bone marker genes such as *Alpl*, *Spp1* and *Col1a1* (Arosarena et al., 2011), and we have clearly shown here that concomitant low frequency mechanical stimulation has a synergistic effect on C2C12 osteogenic differentiation depending on the timing of these co-stimuli. These findings are consistent with those of previous studies involving significantly different mechanical conditions, notably higher oscillatory stimulation frequencies (Hook et al., 2008; Iwata et al., 2009; Kim et al., 2009). These previous studies employed frequencies between 0.1-1 Hz to show that mechanical stimulation can inhibit or induce stem cell differentiation to desired lineages (Grossi, 2011; Rui et al., 2011; Sarraf et al., 2011; Shi et al., 2011; Solem et al., 2011). Present results show that this up-regulation in bone marker genes can also occur at frequencies as low as 0.01 Hz, which are much lower than fundamental frequencies of locomotion. This indicates that enhancement in osteogenic differentiation of C2C12 cells by oscillatory mechanical stimulation can occur for a wider range of frequencies than previously believed, similar to previous findings for inhibition of adipogenic differentiation (Khayat et al., 2012).

It is known that BMP-2 can induce osteogenic differentiation of stem cells (Katagiri et al., 1990; Wang et al., 1993; Yamaguchi et al., 1991). Moreover, exposure to BMP-2 is necessary for initiation of osteogenic differentiation of C2C12 cells (Li et al., 2011b). Our pilot studies established that low frequency mechanical stimulation in the absence of BMP-2 exposure did not significantly drive osteogenic differentiation of C2C12 cells by itself (data not shown).

Therefore all experiments performed in the present study involved a 24-48 hour exposure to BMP-2 as a "priming" step to provide an environment within which mechanical effects on osteogenesis could be investigated. Interestingly, when mechanical stimulation was started at the same time as BMP-2 induction such that no priming period with BMP-2 alone occurred, no significant changes in osteogenic gene expression were observed (Figure 4). In contrast, synergistic effects on osteogenesis were observed when concomitant BMP-2 and mechanical stimuli were applied following priming by BMP-2 alone (Figure 3). Specifically, the early osteogenic markers *Runx2*, *Osx*, *Col1a1*and *Alpl* (Hassan et al., 2006; Kaivosoja et al., 2011) were significantly upregulated versus non-mechanically stimulated controls. However, no significant changes in the other osteogenic markers *Ibsp*, *Tnfrsf11b* and late osteogenic marker *BGlap1*, were observed. Evidently, osteogenic effects of low frequency mechanical stimulation on C2C12 cells can be critically dependent upon previous exposure to growth factor stimulation, and the timing and sequence of superimposed BMP-2 and mechanical stimuli can sensitively influence osteogenesis.

After initiation of osteogenesis with BMP-2 priming, our latter experiments tested if mechanical stimulation in the absence of BMP-2 could further modulate osteogenesis or match the efficacy of continued BMP-2 exposure. Following 24 hours of BMP-2 priming, low frequency mechanical stimulation significantly upregulated expression of *Osx*, *Alpl* and *Col1a1* versus controls where BMP-2 exposure stopped after priming (Figure 5). Conversely, *Spp1* was slightly downregulated, but overall the effects of mechanical

stimulation alone following BMP-2 priming indicated enhancement of osteogenesis. However, low frequency mechanical stimulation post-priming in the absence of BMP-2 could not match the efficacy of continued BMP-2 exposure in non-mechanically stimulated controls (Figure 6). Specifically, significantly diminished expression of *Runx2*, *Osx* and *Alpl* was observed in mechanically stimulated cells in competition with BMP-2 stimulated controls, though *Col1a1* and *Spp1* were expressed at similar levels in both conditions. Therefore, it appears that low frequency mechanical stimulation can enhance osteogenesis of C2C12 cells when BMP-2 concentration decreases; however, this enhancement is not as potent as continued high concentrations of BMP-2 (Kim et al., 2009; Li et al., 2011b).

Recent studies utilizing high frequency mechanical stimulation have indicated a role for mitogen-activated protein (MAP) kinase signalling in stem cell differentiation (Nakai et al., 2010). Other in vitro studies also implied that β-catenin is required downstream of BMP-2 for osteoblast mineralization (Rawadi et al., 2003). The extracellular-related kinase (ERK) and p38 kinase pathways are strong candidates for this activity since previous work has shown that mechanical stimulation induces a rapid and transient change in both kinase activities in C2C12 and osteoblast-like cells (Rauch and Loughna, 2008; Rubin et al., 2006; Ziros et al., 2002). Also, the Wnt signalling pathway is involved in the response of bone-like cells to mechanical loading (Robinson et al., 2006) and is also active during osteogenic differentiation of C2C12 cells (Day et al., 2005; Hill et al., 2005; Yu et al., 2010). Consistent with these findings, our western blot data

suggest that low frequency mechanical stimulation following BMP-2 priming may act primarily through ERK and β-catenin (Wnt) signalling. However, we were unable to detect differences in p38 and JNK activities under our experimental conditions. Further studies are required to elucidate the differences between modes of mechanical stimulation in terms of the specific intercellular signalling pathways activated during osteogenic differentiation.

Perhaps the most obvious context within which varying growth factor concentrations and low frequency mechanical stimulation act in concert on stem cells is during development (Henderson and Carter, 2002; Nilsson et al., 2007). It is perhaps unwarranted to extrapolate the findings of this in vitro study to more complex processes, however present results suggest a model for interpretation of how these stimuli can contribute in a coordinated fashion during development. For example, during limb bud development, transient growth factor concentration gradients initiate stem cell differentiation and establish basic morphology (Zeller et al., 2009), while subsequent low frequency movement and growth may refine these patterns by modulating cytokine, growth factor, and ECM protein production, thus ultimately promoting terminal differentiation and structure (Henderson and Carter, 2002). These insights may enable future application of present findings for improved control of stem cell differentiation in cell-based therapies.

4.6 Acknowledgement

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5 Culture of primary bovine chondrocytes on a continuously expanding surface inhibits dedifferentiation

Preface

Building a construct in cartilage tissue engineering requires sufficient numbers of chondrocytes. To accomplish this goal chondrocytes need to be expanded *in vitro*. Standard culture methods lead to loss of phenotypic characteristics due to increased passaging numbers. They also result in dedifferentiation to fibroblast-like cells. In order to overcome this problem a novel method for culturing of cells has been proposed in the following chapter. The cells are cultured on a highly expandable surface to minimize the numbers of passaging. The results suggest that using the expandable surfaces enhances the chondrocyte phenotype dramatically.

Culture of primary bovine chondrocytes on a continuously expanding surface inhibits dedifferentiation

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Running Title: Inhibition of chondrocyte dedifferentiation

5.1 Abstract

Expansion of autologous chondrocytes *in vitro* is used to generate adequate populations for cell-based therapies. However, standard culture methods cause loss of chondrocyte phenotype and dedifferentiation to fibroblast-like cells. Here, we use a novel surface expansion culture system in an effort to inhibit chondrocyte dedifferentiation. A highly elastic silicone rubber culture surface was stretched continuously over a 13-day period to 600% of its initial surface area. This maintained cells at high density while limiting contact inhibition and reducing the need for passaging. Gene expression analysis, biochemical assays, and immunofluorescence microscopy of follow-on pellet cultures were used to characterize the results of continuous expansion (CE) culture versus standard (SD) cultures on rigid polystyrene. CE culture yielded cells with a more chondrocyte-like morphology and higher RNA-level expression of the chondrogenic markers collagen type II, aggrecan and COMP. Furthermore, expression of collagen type I RNA and α-smooth muscle actin protein were significantly reduced. Pellet cultures from CE chondrocytes contained more sulphated glycosaminoglycan and collagen type II than pellets from SD culture. Additional control cultures on static (unexpanded) silicone (SS culture) indicated that benefits of CE culture were partially due to features of the culture surface itself and partially due to the reduced passaging which that surface enabled through continuous expansion. Chondrocytes grown in CE culture may therefore be a superior source for cell-based therapies.

5.2 Introduction

Adult articular cartilage exhibits a poor regenerative capacity following injury, and this inability to regenerate is a major factor contributing to development of joint degenerative disease following cartilage injuries (Anderson et al., 2011; Natoli and Athanasiou, 2009; O'Driscoll, 1998; Schulze-Tanzil, 2009). Cell-based therapies such as autologous chondrocyte implantation (ACI) are of interest to enhance the natural regenerative capacity, replace damaged cartilage and inhibit progression of disease. ACI is a stepwise procedure that requires isolation of chondrocytes from a healthy tissue biopsy, population expansion *in vitro* and implantation in a defect (Batty et al., 2011; Brittberg et al., 1994). Chondrocytes must be multiplied in culture to produce cell numbers sufficient for clinical application. For example, assuming roughly constant cell density and tissue thickness, if cells obtained from a cartilage biopsy of surface area 25 mm^2 are used to repair a defect of surface area 10 cm^2, an expansion factor of at least 40 is required; this estimate is roughly consistent with the suggestions of investigators involved in the development of ACI (Brittberg et al., 1994; Gillogly et al., 2006; Vijayan and Bentley, 2011). Therefore *in vitro* expansion of chondrocyte populations is a central feature of leading cell-based strategies for cartilage repair.

During population expansion, moderate chondrocyte densities must be maintained for efficient cell growth (Kolettas et al., 2001). Once chondrocytes become confluent, contact inhibition can lead to loss of phenotype and reduced proliferation (Kolettas et al., 2001; Yu et al., 1997). This limited desirable range

of cell densities means that in standard culture, repeated passaging and reseeding of chondrocytes is required. Passaging of chondrocytes is associated with changes in the chondrocyte phenotype in as few as 2 passages (Darling and Athanasiou, 2005; Lin et al., 2008), or "dedifferentiation" characterized by loss of rounded morphology, decreased cartilage-specific gene expression, increased fibrotic gene expression and rapid proliferation. Ultimately, dedifferentiation reduces the efficiency of cell-based repair methods because it decreases the capacity for implanted chondrocytes to regenerate functional cartilage tissue and necessitates additional protocols for redifferentiation toward the desired phenotype. Many factors promote chondrocyte dedifferentiation, including contact with a flat, rigid, two-dimensional culture surface (Archer et al., 1990; Darling and Athanasiou, 2005), exposure to degradative enzymes during passaging (Homicz et al., 2002; Lefebvre et al., 1990), and abnormally rapid (for chondrocytes) proliferation (Darling and Athanasiou, 2005; Giovannini et al., 2010). Improved understanding and control of these factors may therefore lead to significant simplifications and improvements in procedures required for cell-based cartilage repair.

To minimize and circumvent conditions promoting chondrocyte dedifferentiation during population expansion, we have developed a novel culture technique that facilitates more continuous growth of cells while limiting effects of contact inhibition and reducing the necessity for passaging (Majd et al., 2011; Majd et al., 2009). In this new method, cells are grown on a continuously expanding, elastic dish which allows for increase of the culture surface area as the cell population grows. Relatively high cell densities are maintained which

promote efficient proliferation while confluence (and the need for passaging) is delayed until relatively high cell numbers are attained. This "continuous expansion" culture technique has been used previously to expand human mesenchymal stem cell (hMSC) populations more efficiently than by standard methods while maintaining pluripotent stem cell phenotype and inhibiting an undesired fibrotic phenotype (Majd et al., 2011; Majd et al., 2009). We hypothesized that continuous expansion culture could also be beneficial for population expansion of primary chondrocytes, where maintenance of a chondrogenic phenotype and inhibition of dedifferentiation are desired.

5.3 Materials and Methods

5.3.1 Chondrocyte Isolation

Knee joints from freshly slaughtered skeletally mature cows were obtained from a local slaughterhouse. Articular cartilage was cut from the femoropatellar groove with a scalpel, and chondrocytes were isolated according to established methods (Barbero and Martin, 2007). Approximately 5 g of tissue was washed in sterile phosphate buffered saline (PBS) supplemented with antibiotics and cut into 2 mm pieces using a sterile scalpel. The tissue was transferred to a 50 mL conical tube containing 30 mL of chondrocyte growth medium (high-glucose DMEM; 0.1 mM Nonessential Amino Acids; 10 mM HEPES; 1 mM sodium pyruvate; 10% fetal bovine serum; and 1% penicillin-streptomycin-glycine solution) supplemented with 1.5 mg/mL collagenase type II (Invitrogen/Gibco, Burlington, ON) (sterile filtered). Samples were incubated overnight to allow complete

digestion of extracellular matrix. The digested mixture was passed through a 100 μm filter (BD Biosciences, Mississauga, ON) and centrifuged at 200×g for 5 minutes. The supernatant was removed and pelleted chondrocytes were washed with sterile PBS and centrifuged again at 200×g for 5 minutes. The supernatant was removed, and cells were resuspended in 10 mL of chondrocyte growth medium. Cells were counted using a hemocytometer.

5.3.2 Continuous Expansion and Standard Cultures

Chondrocytes were cultured on several different surfaces. These included (1) high-extension silicone rubber (HESR) dishes (Cytomec GmbH, Spiez, Switzerland) which were continuously expanded, (2) standard polystyrene culture dishes, and (3) polystyrene culture dishes coated with approximately 1 mm of silicone rubber (A-221-05 LSR, Factor II, Lakeside AZ). All silicone rubber culture surfaces were chemically modified to promote cell adhesion as previously described (Majd et al., 2009; Wipff et al., 2009). Briefly, the surface was rinsed with 30% sulphuric acid for 15 minutes and washed copiously with deionized water followed by silanization with 1% (3-aminopropyl) triethoxysilane (Sigma-Aldrich, Oakville, ON) for 2 hours at 70ºC. After another wash with water, the surface was functionalized with 6% (wt/wt) glutaraldehyde and then coated with 2 mL of monomeric rat tail collagen type I (50 μg/mL; Sigma-Aldrich). Standard polystyrene culture surfaces were left unmodified.

5.3.3 Continuous Expansion Surface Manipulation and Modification

To initiate cultures, 10,000 chondrocytes per cm^2 were seeded and subcultured in chondrocyte growth medium. Continuous expansion (CE) cultures were performed on high extension silicone rubber (HESR) dishes which were expanded from 12 cm^2 to 76.8 cm^2 over 10 days following a 3-day initial attachment period. Highly uniform surface expansion was performed using a motorized iris-like device (Cytomec GmbH, Spiez, Switzerland). This 13-day period was defined as one generation, which corresponded to three conventional 1:2 passages in standard (SD) culture on polystyrene (Figure 5.1). The final surface area in CE culture equaled the total surface area of the third passage in SD culture (8 wells at 9.6 cm^2 each). The difference in initial surface area (12 cm^2 in CE culture versus 9 cm^2 in SD culture) was due to limitations of the HESR dishes; the final surface areas were kept equal in order to provide a comparison that could be more easily interpreted in the context of population expansions for cell-based therapies. Confluence at each passage in SD culture was ~80% and 0.25% Trypsin-EDTA solution (Invitrogen/Gibco) was used. Unless otherwise noted, all tissue culture reagents were obtained from Invitrogen/Gibco. For experiments to control for silicone surface chemistry, to reduce unnecessary passaging, and to reduce gross density differences, polystyrene culture dishes (35 mm, 55 mm, and 100 mm) were coated with a silicone elastomer and functionalized with rat tail collagen type I (as above).These “static silicone” (SS) cultures were therefore performed on silicone rubber, but without any mechanical expansion. 10,000 cells per cm^2 were seeded on a 35 mm dish (96,200 cells total).

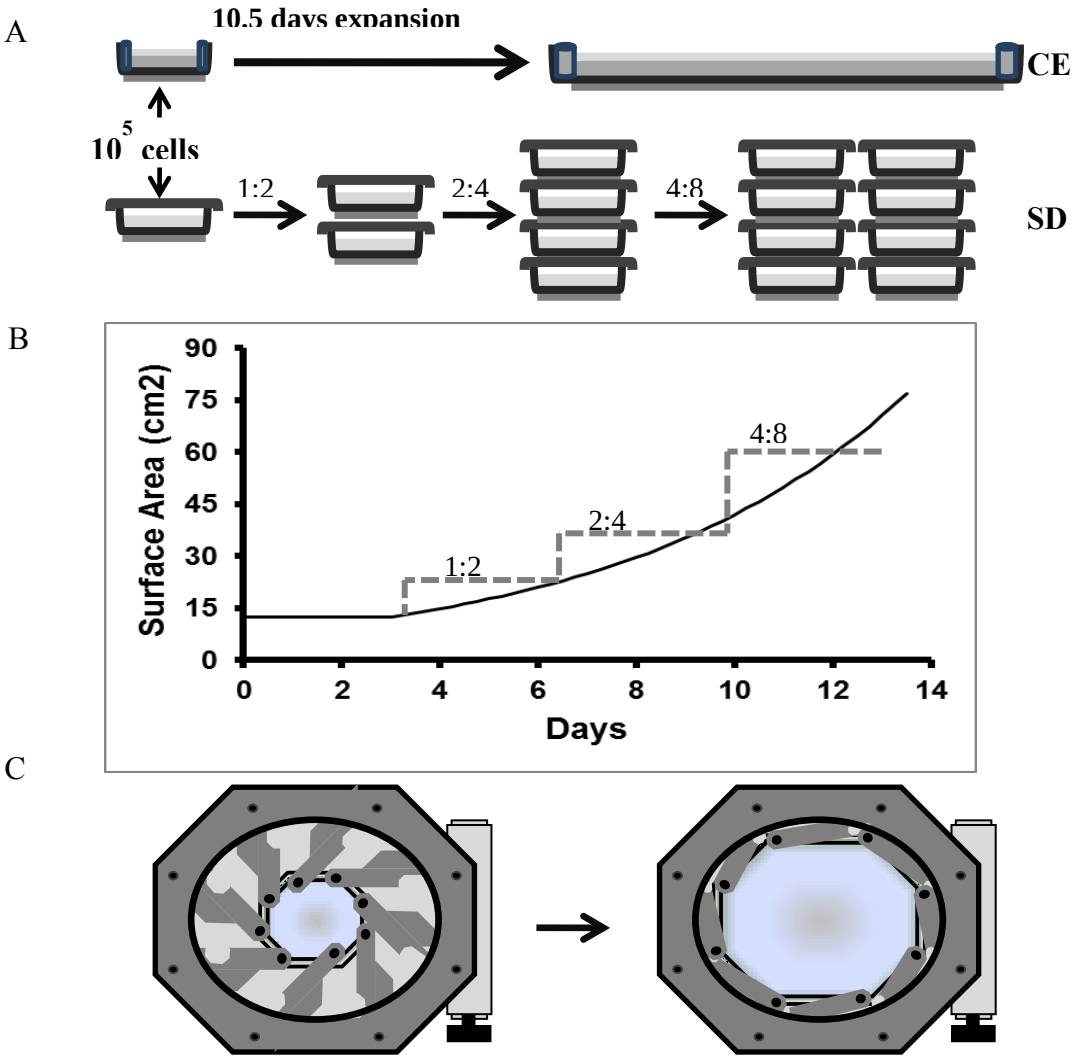

Figure 5.1 Experimental design for continuous expansion culture. A, An equal number of primary articular chondrocytes were seeded on a high extension silicone rubber (HESR) dish for CE culture and one well of a six-well polystyrene plate for SD culture. After a three-day attachment period, the CE culture was slowly but continuously expanded from 12 cm2 to 76.8 cm2 over 10.5 days. During this time, cells SD cultures were passaged 1:2 every three days. The final surface area of the CE dish was equal to eight wells of the SD cultures. B, Surface areas of CE cultureHESR dish (solid line) and SD culture on polystyrene (dotted line) versus time. C, Schematic drawings of the iris-like device on which HESR dishes were mounted, in the closed and open positions.

Initial control experiments (Figure 5.2) were always passaged onto 35 mm throughout P5. After three days of initial attachment and growth (Figure 5.7), the 35 mm dish was trypsinized and passaged (P1) to the 55 mm dish (coated and functionalized in the same way). After 5 days, chondrocytes were trypsinized again and passaged (P2) to the 100 mm dish and cultured for another 5 days. This 13-day expansion protocol was considered one generation in SS culture.

At the end of each generation in CE, SD or SS culture, cells were trypsinized (0.25% Trypsin EDTA solution, Invitrogen), and counted. For a subsequent generation, 10^5 cells were then reseeded within each culture condition. 10^6 cells resulting from each generation were used for redifferentiation experiments, and the remaining cells ($1\text{-}2\text{x}10^6$) were lysed in 1 mL of TRIzol reagent (Invitrogen) and RNA was isolated as described below.

5.3.4 Chondrocyte Redifferentiation

At the end of each generation, $\sim 2\times10^6$ cells were centrifuged at 500×g for 10 minutes in 1.5 mL microfuge tubes to generate a pellet. Chondrocyte growth media was removed and replaced with 500 μl of osteo/chondrogenic differentiation medium (DMEM, 10% FBS, 1.25 mM glutamine, 10 nM dexamethasone, 50 μM/mL ascorbic acid, 1 μM β-glycerophosphate, 5 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1% penicillin/streptomycin solution). This culture procedure and medium was modified from previous studies to avoid rapid cell death (Bernstein et al., 2010; Majd et al., 2009; Zhang et al., 2004). TGFβ was intentionally excluded from this differentiation medium in

order to emphasize effects of CE versus SD culture without the concern of overwhelming growth factor stimulation. Pellets were incubated in centrifuge tubes for six days until they became firm and then transferred to a six-well plate (which helped maintain viability) and incubated for an additional six days to observe chondrocyte outgrowth. Medium was changed every two days. Pellets were then fixed with 4% paraformaldehyde and prepared for cryosectioning.

5.3.5 Reverse Transcription and Quantitative Real-Time PCR

RNA was extracted from chondrocytes using TRIzol Reagent (Invitrogen). Following RNA extraction, 500 ng of total RNA was subject to cDNA synthesis using the qScript cDNA synthesis kit following the manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD). Consequently 1 μl of each cDNA sample was loaded per reaction (in duplicate) using PerfeCTa SYBR Green FastMix (Quanta Biosciences). Standard recommended PCR protocols were performed (50°C for 2 min, 94°C for 10 min, 95°C for 30 sec, 60°C for 1 min, with steps 3 and 4 repeated for 40 cycles) using the ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The average cycle count for each target gene was normalized to GAPDH to give the average delta count (ΔCt) using RQ SDS manager software (Applied Biosystems). Then for each target gene the average ΔCt reading from each experimental cDNA was subtracted from the average ΔCt from the comparative GAPDH endogenous control (ΔΔCt). The average fold change in gene expression of experimental samples compared to controls was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Statistical significance in fold-changes in gene expression was determined using

Student's t-test ($p<0.05$). PCR primers for collagen type II, aggrecan, COMP, Sox9, collagen type I, and GAPDH were generated exactly as described elsewhere (Bosnakovski et al., 2006).

5.3.6 Western Blotting

Chondrocytes were lysed in lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM β-glycerophosphate, supplemented with complete EDTA-free protease inhibitor cocktail). 20 µg of total protein was run on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA for 30 minutes and probed with antibodies against alpha smooth muscle actin (α-SMA; 1:500, Abcam, Cambridge, MA), and α-tubulin (Abcam; 1:2000), followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000, Cell Signalling). Membranes were then washed three times in TBST for 10 minutes. Western blots were developed using Super Signal West Pico Substrate (Thermo-Fisher, Nepean, ON) and Kodak BioMax MR film (Perkin Elmer, Woodbridge, ON).

5.3.7 Immunofluorescence and Histological Analysis

For histological analysis, pellet cultures were fixed in 4% paraformaldehyde and embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). Frozen sections 10 µm thick were cut using a Leica CM3050 S cryomicrotome. Sectioned samples were stained with Alcian Blue for proteoglycan and counterstained with Nuclear Fast Red (Sigma). For

immunofluorescence, samples were blocked in permeabilization buffer for 30 minutes (PBS, 0.1% Triton X-100 and 1% BSA). Permeabilized samples were then incubated with antibodies against phospho-histone H3 (1:250, Sigma), cleaved Caspase 3 (1:250, Sigma), and collagen type II (1:100, Abcam, Cambridge, MA) overnight at 4ºC. Samples were washed 3 times in PBS and then incubated with either Alexa Fluor 488 Goat anti-Mouse IgG (1:250, Invitrogen) or TRITC-conjugated Goat anti-Rabbit IgG (1:250, Sigma) for 1 hour at room temperature. Samples were washed and mounted with Fluoroshield with DAPI (Sigma) and visualized on an Olympus IX81 inverted fluorescence microscope. Morphological images were captured using a Zeiss Axiovert 40C microscope equipped with a Canon Powershot A640 digital camera attached to a Zeiss MC80DX 1.0× tube adapter.

5.3.8 Quantitation of Cell Proliferation and Death

Isolated chondrocytes were seeded (10,000 cells/cm^2) on sterilized 22 mm square uncoated coverslips (glass) or coverslips coated with 200 μl of silicone rubber functionalized with collagen I as described above (SS Culture). Cells were fixed with 4% paraformaldehyde solution and blocked for 30 minutes in permeabilization buffer and probed with antibodies against phospho-histone H3 and cleaved Caspase 3 and immunofluorescence was performed as above. All images were captured using a 10× objective with MAG Biosystems Software 7.5 (Photometrics, Tucson, AZ). Three random positions per slide were captured from

three independent experiments. Positively stained nuclei were counted and plotted as a percent of total nuclei.

5.4 Results

5.4.1 Chondrocyte Growth on Static Silicone Surfaces

To test for effects of modified silicone culture surfaces on cell phenotype in the absence of expansion, chondrocytes were cultured over 5 passages on modified silicone rubber in SS culture and compared to SD culture on polystyrene. Chondrocyte attachment on both SS and SD cultures typically occurred by 3 days after seeding, at which point experiments began (Day 0). Cell morphology appeared similar for the two culture conditions during passaging (Figure 5.2A). RNA-level expression of the cartilage-specific genes collagen type II, aggrecan, and cartilage oligomeric matrix protein (COMP) declined with each passage number for both culture conditions while collagen type I expression increased (Figure 5.2B). Expression of the master regulator of chondrogenesis Sox9 showed no consistent changes during passaging for either culture condition.

To examine influences on proliferation and apoptosis, chondrocytes cultured on SS-coated coverslips or uncoated glass coverslips were subjected to immunohistochemistry for phospho-histone H3 and cleaved caspase-3 (Figure 5.3). A significant reduction in phospho-histone H3 (proliferating cells) was observed on SS compared to the control (Figure 5.3A). However, there was no apparent difference in caspase-3 (actively apoptotic cells) between the two

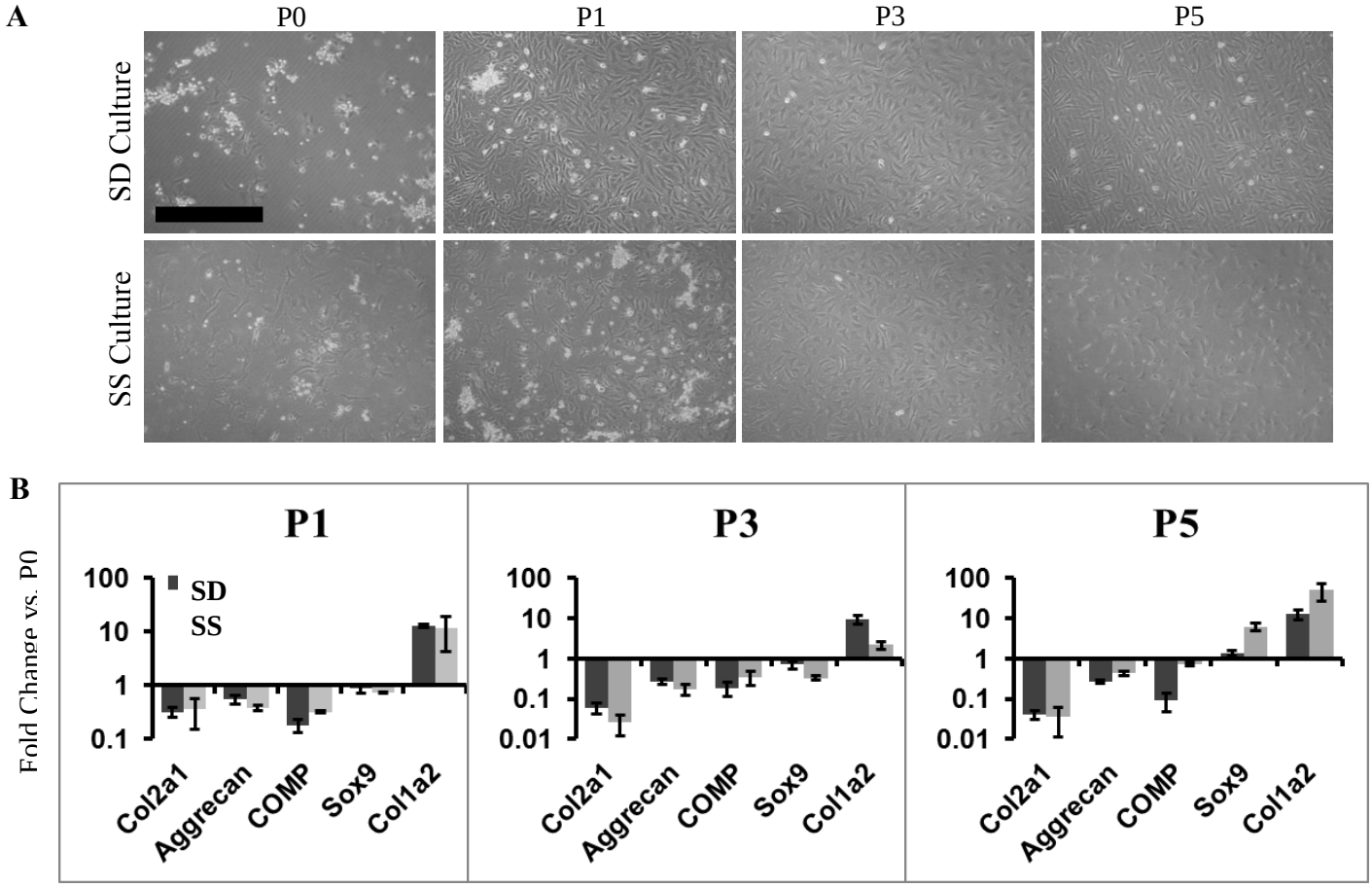

Figure 5.2. Phenotype comparison between articular chondrocytes cultured on polystyrene and a static silicone rubber surface. A, Chondrocytes were cultured for 5 passages on SD and SS surfaces. Images were taken compare gross morphology. Scale bar: 500 μm. B, qPCR revealed similar patterns of dedifferentiation of chondrocytes passaged on SD and SS surfaces. Dark bars represent polystyrene and light bars represent silicone dishes. Mean±SEM (n=3).

culture conditions (Figure 5.3B). When cultured on SS, chondrocyte phenotype and dedifferentiation patterns were therefore similar to SD culture.

5.4.2 Phenotypic Analysis of Continuous Expansion (CE) Culture

Chondrocytes in CE culture had a more rounded and less spindle-like morphology than in SD culture indicating a reduction in fibroblast-like

A

B

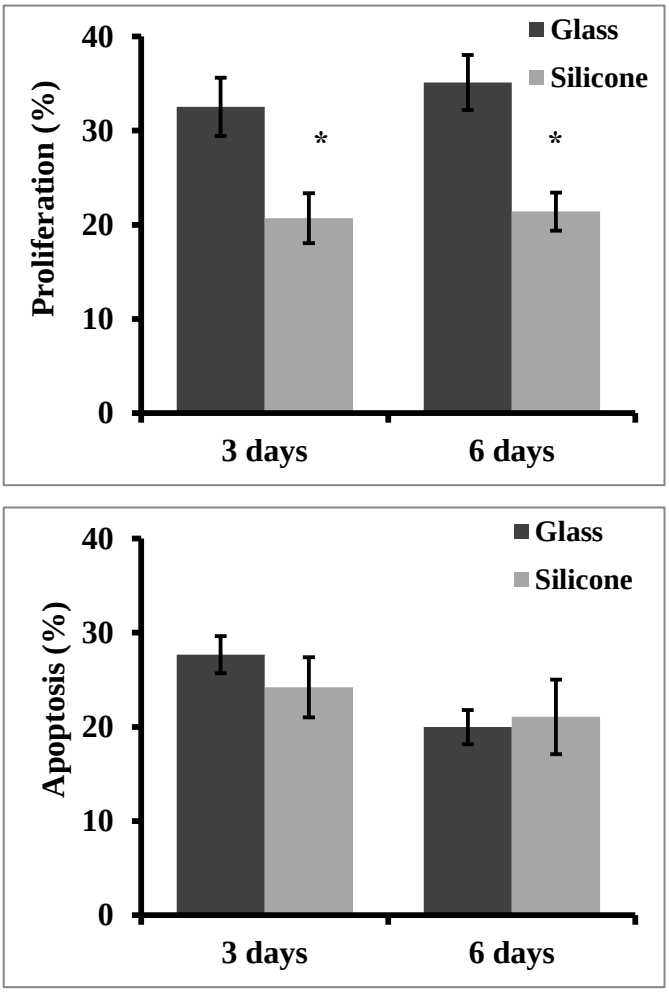

Figure 5.3.

Quantification of chondrocyte proliferation and apoptosis on SS culture surfaces. Primary articular chondrocytes were cultured on glass or silicone-coated coverslips for 3 and 6 days. Fixed cells were probed with antibodies against (A) phospho-histone H3 (proliferation marker) or (B) active caspase-3 (apoptosis marker) and immunofluorescence microscopy was performed. Positively stained cells were counted and compared to total cells (DAPI stained). A, The percentage of proliferating chondrocytes was significantly less on SS surfaces for both 3- and 6-day cultures ($p<0.05$; Student's T-test). B, No significant differences were detected in chondrocyte apoptosis in SS cultures. Dark bars represent chondrocytes grown on glass coverslips and light bars represent SS coverslips. Mean±SEM (n=3).

dedifferentiation (Figure 5.4A). At the end of generation G1, real-time quantitative PCR revealed significantly higher expression of the cartilage-specific genes collagen type II, aggrecan and COMP in CE versus SD culture

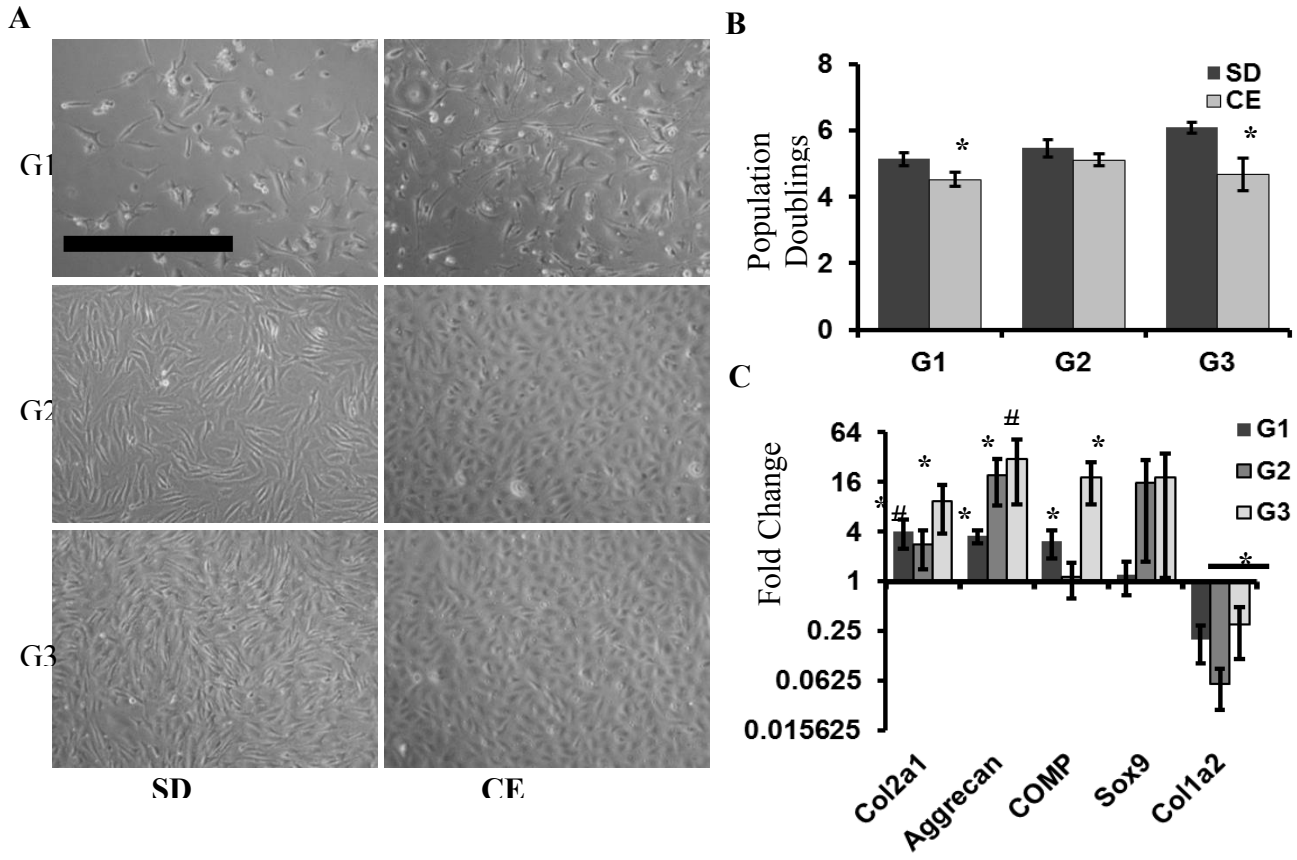

Figure 5.4. Primary articular chondrocytes were grown for 39 days in SD or CE culture. A, At the end of each generation chondrocyte morphology was compared in SD and CE culture. Scale bar: 500 μm. B, Cells were counted at the end of each generation, and doublings were calculated. C, qPCR was performed comparing gene expression in CE culture to SD culture. Error bars represent SEM. Statistical significance was determined by Student's t-test within each generation where (*) indicates $p<0.05$ and (#) indicates $p<0.06$. Twelve, six, and five independent experiments were performed for generations G1, G2 and G3 respectively.

(Figure 5.4C). These trends remained consistent through generations G2 and G3 (Figure 5.4C), and were statistically significant for aggrecan at the end of generation G2 and for collagen type II and COMP at the end of generation G3. In contrast, the fibrotic marker collagen type I was significantly downregulated in CE versus SD culture at the end of all three generations (Figure 5.4C). Cell lysates from CE and SD cultures were subjected to SDS-PAGE and Western blot

probing for the fibrotic marker α-SMA. SD culture lysates revealed a steady induction of α-SMA through the three generations which was inhibited in the CE culture lysates for generations G1 and G2 (Figure 5.5). At the end of each generation, cell counting revealed fewer total chondrocytes in CE culture compared to SD culture (Figure 5.4B).

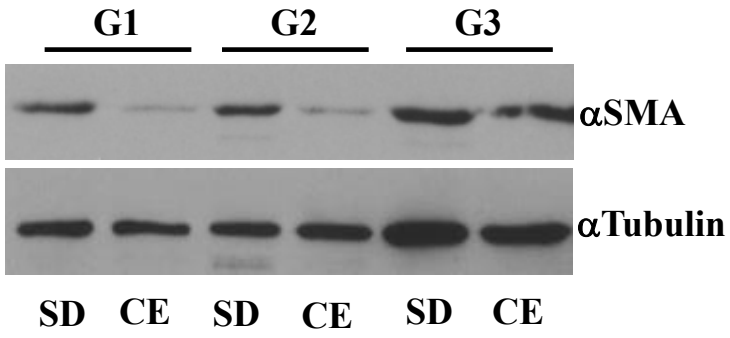

Figure 5.5. Chondrocytes were collected and lysed at the end of each generation of SD and CE culture and 20 µg total protein from each sample was subjected to SDS-PAGE and Western blot analysis. Samples were probed for the fibrotic maker alpha smooth muscle actin (α-SMA). α-tubulin was used a loading control.

5.4.3 Chondrocyte Redifferentiation After Continuous Expansion Culture

To assess the potential of cultured chondrocytes to redifferentiate into cartilage-like tissue, we subjected cells from all three generations of CE and SD cultures to 3D pellet culture in an osteo-chondrogenic medium. After an initial 6 days, pellets were moved to a six-well dish in order to assess chondrocyte outgrowth prior to histological analysis. Pellets derived from SD cultures strongly adhered to the culture dish during the final 6 days of the 12-day pellet culture. In contrast, pellets derived from CE cultures only weakly adhered. Moreover,

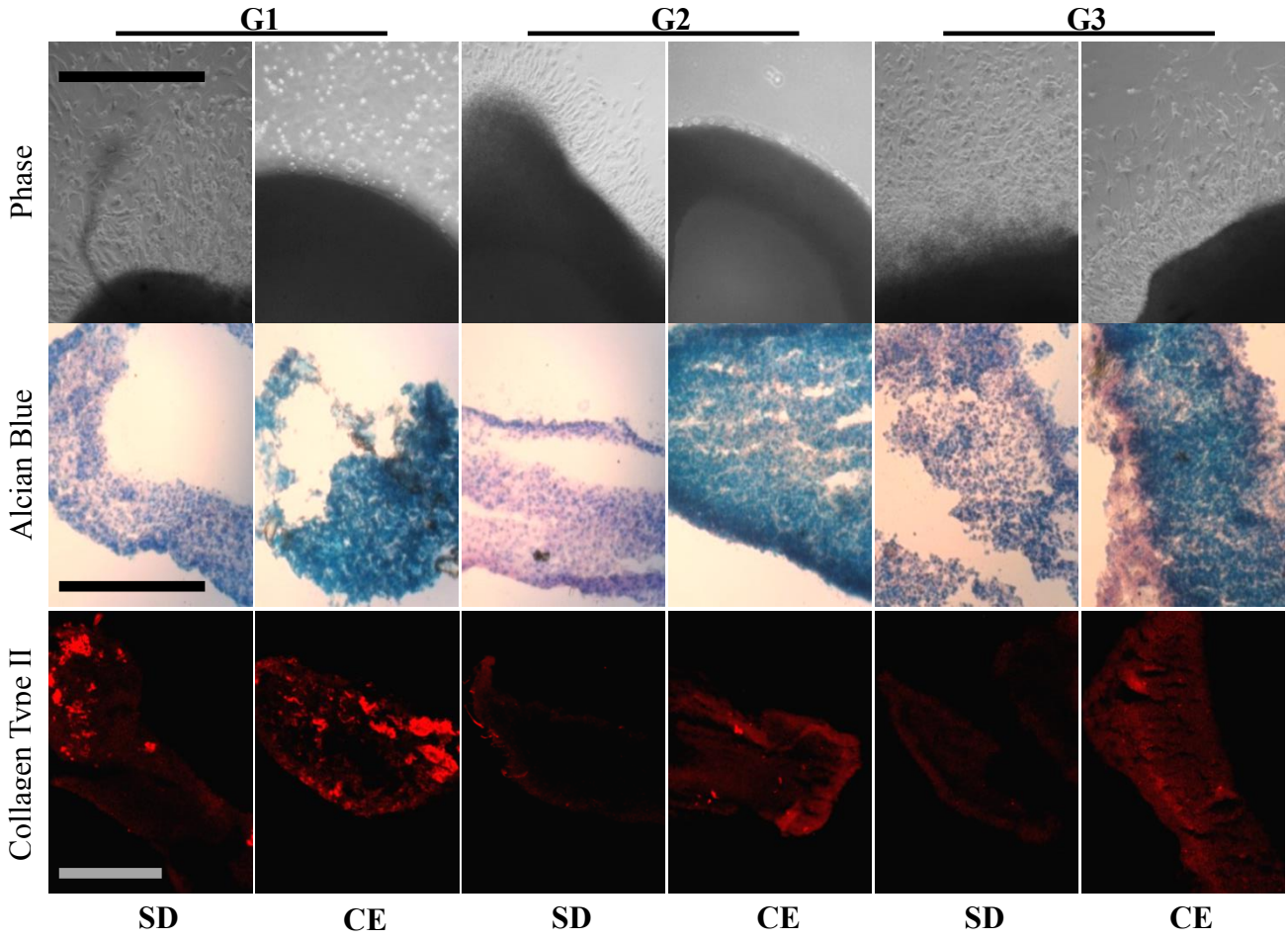

Figure 5.6. Chondrocytes collected at the end of each generation were subjected to redifferentiation in pellet cultures containing an osteo/chondrogenic differentiation medium. Firm pellets were transferred to six-well plates for an outgrowth assay. A, Chondrocyte outgrowth from pellets. B, Alcian blue histological staining on frozen sections from CE pellet cultures compared to SD pellets. C, Immunofluorescence microscopy was performed on SD and CE pellet cultures probing for collagen type II content (red). Scale bar: 500 μm.

chondrocyte migration from adhered pellets was clearly evident for all three generations of SD culture but only for generation G3 of CE culture (Figure 5.6A). CE pellets contained dramatically more sulphated glycosaminoglycans for all three generations compared to SD pellets, as evident by Alcian blue staining. After generation G1, both CE and SD pellets stained strongly for collagen type II immunofluorescence. However, after generations G2 and G3, collagen type II

immunofluorescence was only detectable in CE pellet cultures. CE culture chondrocytes therefore exhibited greater potential for redifferentiation into cartilage-like tissue.

5.4.4 Reduced Passaging Enhances Chondrocyte Phenotype in CE Cultures

To isolate experimental variables that could influence differences between SD and CE culture, such as unnecessary passaging, cell densities, and culture surface stiffnesses, we designed an intermediate control (Figure 5.7A). In this experiment, culture surfaces consisted of silicone-coated polystyrene functionalized with collagen type I on which chondrocytes were passaged to consecutively larger dishes. This SS condition allowed for a growth environment which was similar to that of CE cultured chondrocytes with respect to surface chemistry and mechanical stiffness, but was not mechanically extended. After 2 generations of growth, no differences in chondrocyte morphology or population doublings were observed (Figure 5.7B and C). Real-time quantitative PCR revealed significantly higher expression of collagen type II and aggrecan mRNA in CE versus SS cultures, and there was a trend for reduced collagen type I expression (Figure 5.7D). It is noteworthy that in one experiment collagen type I was below detection limits for the SS culture. There were no differences observed for COMP or Sox9 expression. CE and SS culture chondrocytes from G2 were redifferentiated in pellet cultures as above. CE pellets appeared to contain slightly more sulphated GAG than SS pellets, as evidenced by Alcian stain (Figure 5.8,

left panels). Collagen type II protein content was greatly enhanced in CE pellets versus SS pellets (Figure 5.8, right panels).

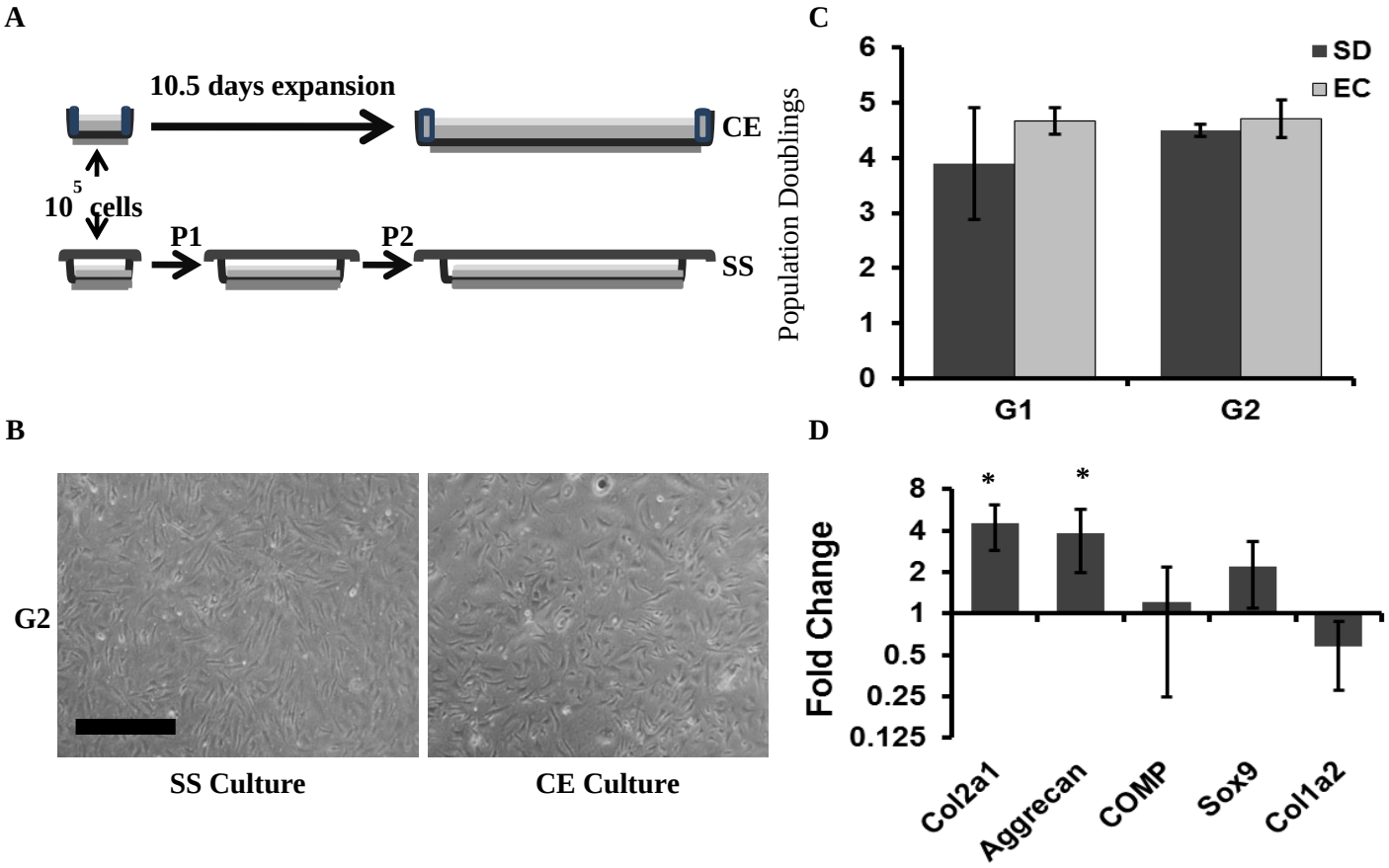

Figure 5.7. Primary articular chondrocytes were grown for 2 generations in functionalized SS or CE culture. A, Controls consist of two passages on consecutively larger functionalized silicone-coated dishes, for each generation. B, Morphology of chondrocytes after 2 generations of SS or CE culture. Scale bar: 250 μm. C, Cells were counted at the end of each generation, and population doublings were determined. D, RNA was extracted after 2 generations and was converted to cDNA. qPCR was performed comparing gene expression in CE culture to SS culture. Error bars represent SEM. Statistical significance was determined by Student's t-test where (*) indicates $p<0.05$, n=3.

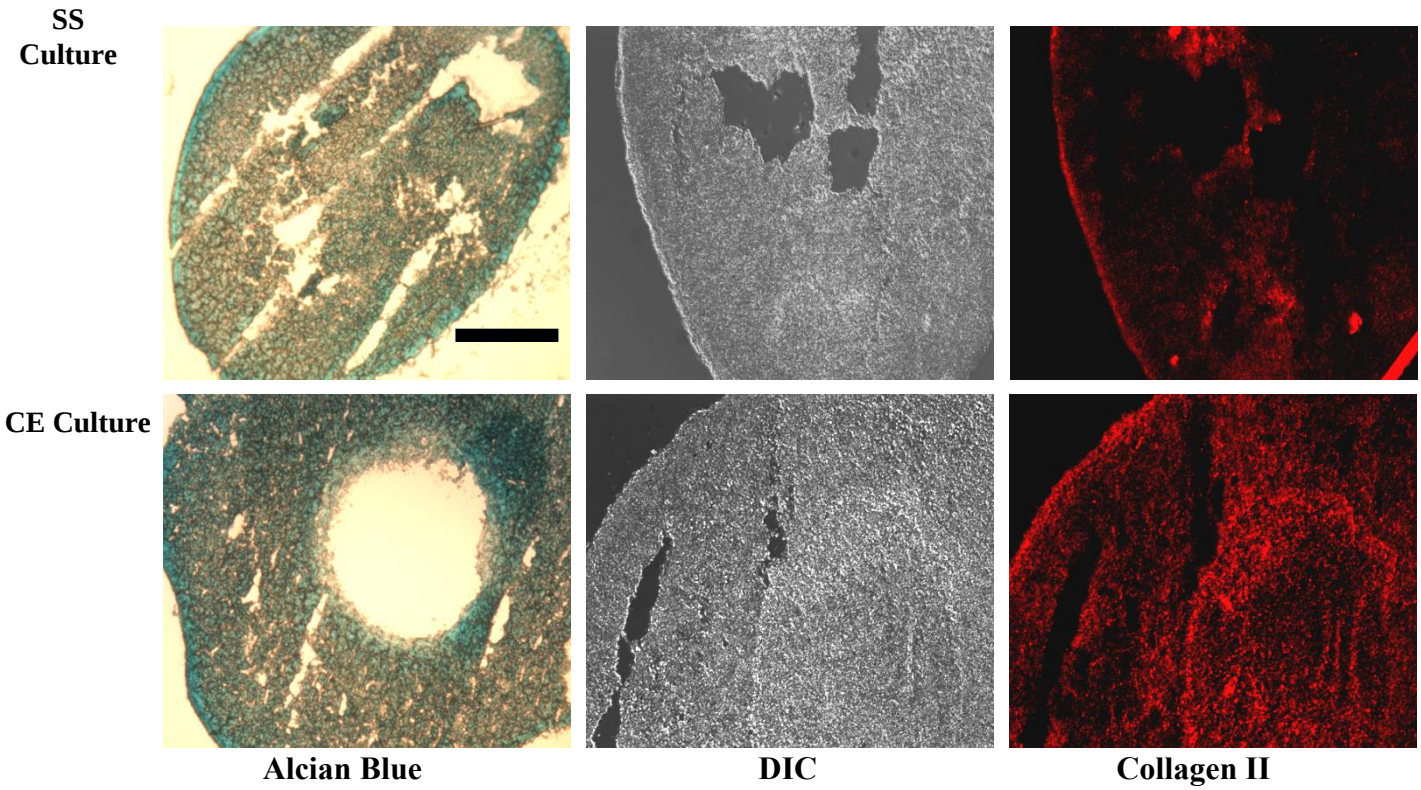

Figure 5.8. Redifferentiation of SS and CE culture chondrocytes. Left panels show Alcian blue histological staining on frozen sections from CE pellet cultures compared to SS pellets. Immunofluorescence microscopy was performed on SS and CE pellet cultures probing for collagen type II content (red – right panels; DIC images in middle). Scale bar: 250 μm.

5.5 Discussion

Continuous expansion culture of primary bovine chondrocytes inhibited the dedifferentiation typically observed with standard culture techniques (Darling and Athanasiou, 2005; Lin et al., 2008). Analyses of cell morphology, cartilage-specific gene expression, fibrotic gene expression, and cell numbers all strongly indicated that CE culture preserves the chondrocyte phenotype during population expansion. SD culture chondrocytes exhibited a spindle-like morphology characteristic of a more fibroblast-like phenotype (Brodkin et al., 2004; Guidry, 1996; Rocker et al., 2006) compared to the more rounded chondrocyte-like appearance evident in CE culture. Consistent with these observations, RNA-level expression of collagen type II, aggrecan and COMP were consistently upregulated in CE versus SD culture while RNA-level expression of collagen type I and protein-level expression of α-SMA were downregulated, indicative of less dedifferentiation to a fibroblast-like phenotype in CE culture. Consistent with improved preservation of the chondrocyte phenotype in CE versus SD culture, significantly fewer chondrocytes were obtained from CE culture at the end of each generation. Considering that chondrocytes do not readily proliferate within adult cartilage under normal physiological conditions while dedifferentiated chondrocytes are fibroblast-like and can rapidly proliferate (Darling and Athanasiou, 2005; Giovannini et al., 2010), this result further supports the conclusion that CE culture helps to maintain the chondrocyte phenotype. Furthermore, since three generations of CE culture achieved an overall expansion of the cell population by approximately 26,250-fold (25-fold, 35-fold and 30-fold

in generations G1, G2 and G3, respectively), it is clear that ample numbers of chondrocytes can nevertheless be generated in CE culture for clinical application in cell-based therapies.

As evidenced by reduced pellet adhesion to culture dishes, cell outgrowth from pellets, and expression of cartilage extracellular matrix proteins, chondrocytes from CE culture were superior to those from SD culture with respect to their ability to redifferentiate toward a mature chondrocyte phenotype. Pellets from SD culture readily attached to culture surfaces and exhibited dramatic outgrowth of chondrocytes, indicating they had transitioned to a more fibroblastic cell type (Kim et al., 2004; Qiu et al., 2000; Singh and Sharma, 2011). In contrast, pellets from CE culture were better able to generate neotissue with more nonadhesive, cartilage-like characteristics. Both SD and CE pellets initially were able to produce GAG and collagen type II, however only CE pellets maintained this production throughout three generations. Taken together, these results indicate that CE culture produces cells which are markedly superior to those obtained from standard culture, with respect to their efficiency at redifferentiating into functional chondrocytes and generating *de novo* cartilage-like tissue.

The aim of the comparison between CE and SD culture was to contrast the continuous expansion culture technique against the current "gold standard" for cell culture: polystyrene dishes. However, the substantial technical differences between these two methods introduced several variables which could have influenced the resulting differences in quality of expanded chondrocyte

populations. Therefore, SS cultures passaged on consecutively larger dishes were employed as an intermediate control. This limited unnecessary passaging and also provided a similar culture surface in terms of chemistry and mechanical stiffness. The only differences between CE and SS cultures were therefore the application of mechanical surface expansion in CE culture, compensated by more frequent enzymatic passaging in SS culture. In two generations, SS cultures were passaged five times compared to once in CE cultures, and CE cultures exhibited significantly superior retention of chondrogenic phenotype versus SS cultures, and also superior redifferentiation capacity for production of cartilage-like neotissue. In light of data showing that passaging of chondrocytes plays a significant role in dedifferentiation (Darling and Athanasiou, 2005; Homicz et al., 2002; Lin et al., 2008), present results indicate that the mechanical expansion aspect of CE culture and its reduction of the need for passaging specifically contribute to enhancement of the phenotype of chondrocytes cultured for cell-based therapies.

A factor by which CE culture inhibits dedifferentiation therefore appears to be associated with reduced passaging and limited exposure to degradative enzymes. Considering that passaging involves repeated nonspecific degradation of cell surface proteins and receptors and the frequent need for wholesale re-establishment of cell-surface attachments, this is perhaps not surprising. Present findings therefore suggest that less disruptive methods for detachment of chondrocytes from a culture surface and from each other may contribute to enhanced phenotype retention during growth, such as the case for endothelial cells

for cornea tissue engineering (Nitschke et al., 2007). Minimization of the need for passaging also has important practical advantages for cell-based therapies. In addition to inhibition of dedifferentiation, CE methods are more automatized and thereby reduce the need for human intervention and handling of cells. These features likely decrease the risk of error and bacterial contamination over long-term cultures.

Physical factors contributing to chondrocyte phenotype preservation in CE culture may also include mechanical signalling. Mechanotransduction through integrin receptors and stretch-activated ion channels is involved in maintenance of chondrogenic phenotypes (Lee et al., 2000; Millward-Sadler et al., 2000; Perkins et al., 2005) and this signalling may play a role in the pro-chondrogenic effects of CE culture. Many reports suggest that dynamic strain or compression can have positive effects on the phenotype of cultured chondrocytes (Holmvall et al., 1995; Wong et al., 2003), particularly regarding proteoglycan synthesis (Chai et al., 2010; Villanueva et al., 2009). It is important to note that these previous studies typically apply dynamic mechanical stimuli at amplitudes and frequencies which are quite different from those involved in CE culture. In contrast to situations involving oscillatory dynamic strain (Chai et al., 2010; Holmvall et al., 1995; Villanueva et al., 2009; Wong et al., 2003), CE culture chondrocytes experience very slow but very high amplitude (over 600%) stretch applied steadily and monotonically over the course of several days. Therefore, mechanotransduction stimuli in CE culture are characterized by a longer time scale than those of cell proliferation and remodelling of cell-substrate attachments. Therefore it is

possible that the nature and relative importance of mechanotransduction are different in CE culture as compared to typical applications of oscillatory dynamic strain. Further studies of the mechanotransduction pathways active during slow, continuous strain may therefore yield important mechanistic insights into how CE culture preserves chondrogenic phenotype compared to conventional passaging. In addition, opportunities remain for optimization of CE culture by superimposed dynamic stimulation of cells during growth.

Because of the ease with which its biochemical surface properties and geometry can be manipulated, silicone rubber is a widely used biomaterial in tissue engineering applications including soft tissue-like compliant surfaces, expansion of stem cells, nanofilms for scaffolds, and growth of chondrocytes (Belanger and Marois, 2001; Castella et al., 2010; Majd et al., 2009; Mhanna et al., 2011; Ni et al., 2009; Wu and Kuo, 2011; Wu et al., 2011). It has been shown that the elastic properties of silicone elastomers can have important influences on cell differentiation (Hinz, 2010; Tenney and Discher, 2009). However, in the absence of continuous expansion, chondrocytes grown on silicone rubber dedifferentiated similarly to standard culture; the only differential effect of silicone rubber was an initial reduction in proliferation, consistent with previous studies (Lynam et al., 2010; Prasad et al., 2010; Sank et al., 1993). Previous studies have also indicated an initial reduction in mesenchymal stem cell proliferation on HESR (Majd et al., 2009) which was subsequently overcome in prolonged CE culture. These findings therefore indicate that culture on a silicone surface does not by itself significantly alter chondrocyte phenotype compared to

effects of standard culture. Future work may explore alternate coatings of the silicone rubber surface which may further support preservation of chondrocyte phenotype and suppression of fibrotic gene expression in CE culture.

The cell signalling mechanisms involved in more advanced chondrocyte dedifferentiation downstream of increased enzyme-mediated passaging (in SD and SS cultures versus CE cultures) remain unclear, but several candidate mechanisms have been identified. Interleukin-1 (IL-1) production increases with chondrocyte passaging (Lin et al., 2008), and IL-1 signalling is directly involved in chondrocyte dedifferentiation (Hong et al., 2011). Other studies have indicated that passaged and dedifferentiated chondrocytes display degradation of the receptor of hyaluronic acid, CD44 (Chow et al., 1998). Disruption of CD44 signalling in chondrocytes results in receptor cleavage, decreased expression of chondrogenic genes, and decreased production of sulphated-GAG (Takahashi et al., 2010): all phenomena associated with dedifferentiation. IL-1 activity and CD44 expression may therefore be of interest for future investigations, to characterize the mechanisms by which CE culture improves maintenance of cartilage phenotype over standard culture methods.

To enhance redifferentiation of chondrocytes in three-dimensional pellet cultures, the growth factor TGF-β is typically added to the chondrogenic medium (Bernstein et al., 2010; Majd et al., 2009; Zhang et al., 2004). To gain a clear indication of the redifferentiation potential of CE culture chondrocytes, we purposely avoided addition of TGF-β so as not to overwhelm the cells with cytokine stimulation. Without TGF-β, CE chondrocytes clearly displayed an

advantage over SD chondrocytes during pellet culture, indicating a potentially cost effective means of chondrogenic maintenance. Also, eliminating the need for TGF-β may also have important clinical implications associated with reducing the possibility of undesired growth factor delivery to patients.

The proposed culture methods (continuous expansion culture) represent a significant departure from standard methods used for chondrocyte expansion. Implementation of these methods therefore requires the introduction of new techniques and technologies at the heart of a laboratory dedicated to chondrocyte expansion. Given the economic and therapeutic importance of the final product, these fundamental changes to decades-old methods of cell culture may be nevertheless warranted. The beneficial effects in terms of significant improvement of the chondrocyte phenotype indicates that disruptive technologies of this nature may provide the improvements necessary for routine clinical implementation of cell-based therapies for cartilage repair.

5.6 Acknowledgement

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analyzed data; DHR and TMQ wrote the paper; MM and BH extensively reviewed and revised the paper.

Conflicts of Interest

One of the authors (TMQ) is a shareholder in the company Cytomec GmbH which makes the iris-like devices for cell culture on high extension surfaces used in this study. There are no further conflicts of interest.

6 Summary

6.1 General conclusion

In this thesis, different ways of applying mechanical stimulation to cell cultures and tissues were investigated. An alternative way of applying mechanical stimulation to different stem cells was tested by studying the effects of low frequency mechanical stimulation on gene and protein levels. In addition, primary cells were cultured in expandable surfaces, and the efficacy of this was verified by testing the effects of slow expansion of a culture surface on maintaining the phenotypical function of primary cells. Through the research presented in this thesis, an understanding was developed with respect to the effects of chemical and mechanical stimulation on the differentiation of the stem cells and their gene regulation. This understanding showed that the timing and sequence of priming with biological growth factors and/or mechanical stimulation plays an important role on the progression of differentiation.

More specifically, a brief introduction to the tissue engineering paradigm and its current challenges was reviewed. Different methods of applying mechanical loading on the cells and tissues were discussed. A novel system for culturing cells and also applying low frequency mechanical stimulation was introduced. The review (Chapter 2) concluded that low frequency stimulation can also change the differentiation pathway of stem cells, a fact that barely has been verified by others.

Consistent with the above conclusion, low frequency mechanical stimulation was applied to stem cells in a highly potent adipogenic medium. The results in both gene and protein levels revealed the fact that even very slow motion movements have significant and consistent effects on the differentiation process, and can suppress its effects. It was shown that low frequency stimulation in the course of three and five days can significantly block differentiation of C3H10T1/2 cells, which is in agreement with the finding of others but in a very different mechanical stimulation environment (high frequency stimulation). This study was presented in Chapter 3.

The research was followed by studying the effects of low frequency mechanical stimulation on osteogenic differentiation of C2C12 cells in the presence of BMP-2. It is shown that although C2C12 cells are muscle progenitors, induction with high concentration of BMP-2 medium can cause osteogenic differentiation. The osteogenesis was enhanced synergistically when low frequency stimulation was superposed on the cultures. The effects of timing and priming with BMP-2 through different experimental protocols were investigated. These results were presented in Chapter 4.

In Chapter 5, the advantage of culturing the primary bovine cartilage cells on expandable surfaces was verified. It was shown when primary cells were cultured in an expandable surface because of a decrease in the number of cell passagings, the cells maintain their chondrogenic phenotype. This is in agreement with the finding of others.

In the approach undertaken in this research, culturing primary cells and stem cells on modified rubber silicone was established. It was shown that this novel culturing surface did not change the cell morphology and had no effects on cell attachment. In addition, culturing cells on expandable surfaces provides adequate surface area, as well as the ability to apply low frequency mechanical stimulation to the cultures at the same time.

6.2 Future work

Classic cell culture surfaces (polystyrene dishes) are typically coated with collagen type I. However, cells *in vivo* are in contact with different proteins, depending on their location in the body. Chondrocytes, as an example, are mainly exposed to collagen type II. Throughout the work presented in this thesis, the expandable culture surfaces were conventionally coated with collagen type I. Therefore, as future research, it is proposed to examine how different coating proteins such as fibronectin, collagen type II, etc. could affect cell proliferation.

In this thesis, it was shown that C2C12 cells express osteogenic-associated genes at early stages of induction with BMP-2, but their actual capability to differentiate to mature osteocytes was not investigated. Therefore, as future work, it is suggested to culture osteoprogenitors and keep them in the culture for a longer period to determine if they would fully differentiate into osteocytes. In addition, it is recommended to culture these cells in 3-D to verify their possible ability to produce bone-like tissues with comparable biomechanical properties.

In this thesis, it was shown that sinusoidal mechanical loading enhances proliferation of cultured cells (Chapter 3). Moreover, it was verified that mechanical loading is a key factor affecting differentiation of stem cells. Therefore, it is suggested to employ a combination of expansion and mechanical stimulation to help sustain a greater cell population during the differentiation process.

Since the expandable culture surface is functionalized and coated by different chemicals, it is not possible to simply measure the exact stress and strain at different spots of the surface. So it is recommended to develop software in order to characterize the exact stresses applied to the cells.

The mechanical loading applied to the culture throughout the experiments presented in this thesis, was in the x-y plane. However, some cells such as chondrocytes *in vivo* are subjected to axial mechanical loading. In order to mimic the *in vivo* cell behavior, it is recommended to compare the axial mechanical stimulation responses of the cells with those of in-plane stimulation.

7 Statement of Original Contribution to Knowledge

A significant original contribution of this research was the demonstration of the effects of low frequency mechanical stimulation on adipogenic and osteogenic differentiation of stem cells. Besides the discovery that low frequency mechanical stimulation can suppress adipogenic differentiation and enhance osteogenic differentiation, another contribution; namely, the effects of slow culture surface expansion for culturing chondrocytes *in vitro*, was made.

Specifically, the following original contributions were made over the course of this research project:

- applying relatively low frequency mechanical stimulation (0.01 Hz), which is within the physiological frequency range;
- blocking the adipogenic differentiation of stem cells in a very strong adipogenic medium by continuous mechanical stimulation at very low frequency;
- enhancing the osteogenic differentiation by superimposing low frequency mechanical stimulation on BMP-2 induced cells; and
- maintaining the chondrogenic phenotype of primary bovine cartilage cells by reducing the passaging number using the mechanical stretching device (Cellerator).

8 Cumulative reference

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