THE EFFECTS OF VIBRATION LOADING ON ADIPOSE STEM CELL VIABILITY, PROLIFERATION AND OSTEOGENIC DIFFERENTIATION

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Master's Thesis
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May 2010

ACKNOWLEDGEMENTS

This study was carried out at Regea - Institute for Regenerative Medicine, University of Tampere. Firstly, I wish to thank our group leader Susanna Miettinen, PhD and my supervisor Suvi Haimi, PhD for giving me the opportunity to explore the fascinating world of adipose stem cells. I could have not wished for a better supervisor, so thank you Suvi for your expertise, support and firm guidance. Secondly, I want to emphasize my gratefulness to all the members of the "Mese group" at Regea for your support and advice. I want to acknowledge Anna-Maija Honkala, Miia Juntunen, Sari Kalliokoski and Minna Salomäki for their excellent technical assistance and never-ending patience. In addition, I have Anna-Maija Koivisto to thank for her invaluable advice on statistical analyses. Special thanks also to Heidi Halonen and other engineers of Biomedical Engineering Department of TUT for technical assistance with the vibration loading device.

Finally, my deepest gratitude goes to my friends, and family; Mom, Dad, my sister Mari and her family, and Jutta, always having time to be there for me. Thank you for all those delightful moments that keep me going. Dear Ile, and our doggies Reukku & Elli, I am so grateful to have you by my side. With you I can take anything.

PRO GRADU- TUTKIELMA

Paikka:	TAMPEREEN YLIOPISTO Lääketieteellinen tiedekunta Lääketieteellisen teknologian instituutti (IMT)
Tekijä:	TIRKKONEN, LAURA ORVOKKI
Otsikko:	Vibraatiostimulaation vaikutukset rasvakudoksen kantasolujen elinkykyyn, lisääntymiseen ja erilaistumiseen luusolujen suuntaan
Sivumäärä:	61
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Tarkastajat:	Professori Markku Kulomaa ja FT Suvi Haimi
Aika:	Toukokuu 2010

TIIVISTELMÄ

Luuston tärkeimpiin ominaisuuksiin kuuluu sen kyky reagoida mekaaniseen kuormitukseen ja säädellä lujuuttaan siihen kohdistuvan rasituksen mukaan. Esimerkiksi vibraatiostimulaation on havaittu edistävän luusolujen metaboliaa ja lisäävän siten luunmuodostusta. Tämän perusteella haluttiin tutkia vibraatiostimulaation vaikutusta rasvakudoksen kantasolujen erilaistamisessa luusolujen suuntaan. Rasvakudoksen kantasolut ovat mesenkymaalista alkuperää olevia monikykyisiä kantasoluja, jotka kykenevät erilaistumaan useiden solutyyppien kuten luu-, rusto- ja lihassolujen suuntaan, kun niitä käsitellään sopivilla indusoivilla tekijöillä. Tutkimuksen tavoitteena oli tutkia vibraatiostimulaation ja luuerilaistusta tukevan soluviljelymediumin yhteisvaikutuksia rasvakudoksen kantasoluihin. Vibraatiostimulaation vaikutuksia ei ole aiemmin tutkittu ihmisen rasvakudoksen kantasoluilla.

Rasvakudoksen kantasoluja stimuloitiin 50 ja 100 Hz:n taajuuksilla sekä 3 g:n kiihtyvyydellä käyttäen erityisesti tähän tarkoitukseen suunniteltua vibraatiolaitetta. Perusmediumin lisäksi soluja viljeltiin luuerilaistusmediumissa, jossa erilaistumista indusoivina komponentteina käytettiin 250 µM askorbiinihappo 2-fosfaattia, 5 nM deksametasonia ja 10 mM β-glyserofosfaattia. Soluja stimuloitiin yhteensä 3 tuntia per päivä kahden viikon ajan, ja vibraatiostimulaation aiheuttamia vaikutuksia rasvakudoksen kantasolujen elinkykyyn, kiinnittymiseen, lisääntymiseen ja erilaistumiseen luusolujen suuntaan arvioitiin. Erilaistumista luusolujen suuntaan tutkittiin mittaamalla alkaalisen fosfataasin aktiivisuutta sekä solunulkoisen matriksin mineralisaatiota.

Vibraatiostimulaatio ei vaikuttanut rasvakudoksen kantasolujen elinkykyyn, mutta solut näyttivät irtoavan helpommin perusmediumissa kuin luuerilaistusmediumissa viljeltyinä. Luuerilaistusmediumin komponentit voivat parantaa solujen kiinnittymistä lisäämällä solunulkoisen matriksin proteiinien tuotantoa. Vibraatiostimulaatio ei aiheuttanut merkitseviä eroja solujen lisääntymiseen, mutta lisäsi merkitsevästi luuerilaistusmediumissa viljeltyjen rasvakudoksen kantasolujen alkaalisen fosfataasin aktiivisuutta 14 päivän kohdalla verrattuna kontrollisoluihin. Vibraatio 100 Hz:llä lisäsi myös mineralisaatiota 14 päivän aikapisteessä luuerilaistusmediumissa viljellyissä kantasoluissa.

Johtopäätöksenä voidaan todeta, että korkeataajuuksinen vibraatiostimulointi yhdistettynä luuerilaistusmediumiin lisäsi rasvakudoksen kantasolujen erilaistumista luusolujen suuntaan. Luuerilaistusolosuhteet todettiin myös optimaalisemmiksi vibraatiostimuloinnille solujen lisääntyneen kiinnittymiskyvyn vuoksi.

MASTER'S THESIS

Place:	UNIVERSITY OF TAMPERE Faculty of Medicine Institute of Medical Technology (IMT)
Author:	TIRKKONEN, LAURA ORVOKKI
Title:	The effects of vibration loading on adipose stem cell viability, proliferation and osteogenic differentiation
Pages:	61
Supervisor:	Suvi Haimi, PhD
Reviewers:	Professor Markku Kulomaa and Dr. Suvi Haimi
Date:	May 2010

ABSTRACT

Bone is a mechanosensitive tissue capable of adapting its mass and architecture according to the prevailing mechanical loading conditions. For example, high frequency vibration has been shown to increase bone formation *in vivo* and to enhance metabolism in human osteoblast-like cells *in vitro*. Therefore it was hypothesized that vibration loading could also stimulate differentiation of adipose stem cells (ASCs) toward osteogenic lineages. ASCs are mesenchymal stem cells (MSCs) capable of differentiating toward osteogenic, adipogenic, myogenic, and chondrogenic lineages *in vitro*, when treated with appropriate inducing factors. ASCs are under extensive research for the applications of bone tissue engineering. In this study, the effects of vibration loading were studied on human ASCs (hASCs) for the first time.

Human ASCs were cultured using either basic medium (BM) or osteogenic medium (OM) consisting of BM supplemented with 250 μM ascorbic acid 2-phosphate, 5 nM dexamethasone and 10 mM β-glycerophosphate. A specifically designed vibration loading device was used to stimulate hASCs with high frequency (50 and 100 Hz) and high magnitude (3 g) vibration for 3 h per day for 2 weeks, and the effects on attachment, viability, proliferation, alkaline phosphatase activity and mineralization were studied. Control cells were cultured similarly without vibration.

Viability of hASCs was not affected by the vibration loading, but it was observed that cells cultured in BM were detaching more easily under vibration than those cultured in OM. Osteogenic supplements may have increased the secretion of extracellular matrix (ECM) proteins enhancing the attachment of the cells. However, no significant differences were detected in proliferation of stimulated hASCs compared to control cells. In contrast, vibration loading increased ALP activity of hASCs significantly at 14 d time point by both frequencies when cultured in OM. Increase in mineralization was also seen in hASCs vibrated with 100 Hz (OM) at 14 d time point when compared to control.

In conclusion, the vibration loading device designed for this study was able to generate controlled vibrational forces to cells cultured on well plates. Osteogenic culturing conditions were found to be more optimal for vibration loading studies than basic conditions due to enhanced attachment of the cells. As a result, it was found that high magnitude, high frequency vibration combined with osteogenic culture conditions enhanced hASC differentiation towards osteogenic lineages.

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1. INTRODUCTION

Bone is a tissue capable of altering its structure and mass according to prevailing mechanical environment. This adaptation process is thought to occur through the mechanosensitive response controlling the bone-forming and -resorbing cells. (Torcasio *et al.*, 2008) It is also widely recognized that physical activity and exercise can positively influence bone architecture (Sievanen & Kannus, 2007; Nikander *et al.*, 2009). However, exercising involving high impacts may not be possible due to age, trauma or musculoskeletal problems. From a clinical point of view, mechanical stimulation such as vibration could be used as a natural method to enhance bone adaptation. For example, high frequency vibration has been shown to increase bone formation *in vivo* (Rubin *et al.*, 2006) and to enhance metabolism in human osteoblast-like cells *in vitro* (Rosenberg *et al.*, 2002). Yet the most optimal parameters of vibration loading, such as magnitude, frequency and duration, in stimulation of bone cells still need to be revealed.

The osteogenic potential of adipose stem cells (ASCs) has stimulated interest in bone tissue engineering (Lendeckel *et al.*, 2004; Haimi *et al.*, 2009; Mesimaki *et al.*, 2009). ASCs are mesenchymal stem cells (MSCs) capable of differentiating toward osteogenic, adipogenic, myogenic, and chondrogenic lineages *in vitro*, when cultured under appropriate conditions (Zuk *et al.*, 2001). The osteogenic capacity of ASCs has been demonstrated in several *in vitro* (Halvorsen *et al.*, 2001; Zuk *et al.*, 2002; de Girolamo *et al.*, 2007) and *in vivo* studies (Lee *et al.*, 2003; Cowan *et al.*, 2004; Hicok *et al.*, 2004; Hattori *et al.*, 2006). Adipose tissue, for example from liposuction, provides an abundant and accessible source of MSCs, *e.g.*, in comparison to bone marrow. ASCs isolated from lipoaspirate can be further expanded or pre-differentiated *in vitro*, and can then be loaded either directly into a bone defect or to a biomaterial scaffold for *in vivo* transplantation. Biomaterial scaffold functions as a carrier during transplantation, and supports cell migration and proliferation during tissue regeneration. ASC-based applications have already been used successfully in clinical practice (Lendeckel *et al.*, 2004; Mesimaki *et al.*, 2009). It is possible that mechanical loading such as vibration could also be used locally to enhance bone formation in the transplant area, if it proves to be efficient osteogenic stimulus.

2. REVIEW OF THE LITERATURE

2.1 PROPERTIES AND FUNCTIONS OF BONE

Bone is a specialized connective tissue consisting of bone cells and mineralized matrix, and it is important to keep in mind that bone as an organ includes several tissues such as bone, cartilage, fibrous tissue, marrow and blood vessels. Bone tissue is substantially strong and rigid when compared to other connective tissues, making it ideally suitable to fulfil its vital role as mechanical support within the body. The properties special to bone – hardness, moderate elasticity, and limited plasticity– make it optimal to protect and support soft tissues and internal organs, and to provide attachment sites for muscles, thereby enabling movement. These mechanical roles require bone to be stiff enough to resist deformation, but flexible enough to absorb energy. In addition to its mechanical role, bone has some fundamental physiological functions. Together with the intestine and kidneys, it contributes to the regulation of calcium homeostasis. During mineralization, calcium ions from the serum are incorporated into the bone matrix, and during bone resorption calcium ions are again released from bone by osteoclasts. Bone marrow also contributes to hematopoiesis. (An & Draughn, 2000; Planell, 2009)

2.1.1 BONE STRUCTURE AND COMPOSITION

BONE MATRIX

The calcified bone matrix consists of organic and inorganic components. The organic bone matrix consists mainly of type I collagen fibrils (almost 90 %), and to a lesser extent of collagen types III, V, X and XII. The remaining 10 % of the organic matrix is made of non-collagenous proteins such as fibronectin, osteopontin, osteocalcin and bone sialoprotein, along with proteoglycans. The organic matrix also contains growth factors and enzymes such as alkaline phosphatase (ALP) and metalloproteinases. (Clarke, 2008)

Collagen is a fibrous protein with a rope-like structure. A collagen fibril is made of three α-polypeptide chains synthesized within osteoblasts. These chains are twisted together into a right-handed coiled coil, a triple-helix pro-collagen molecule. After pro-collagen molecules are secreted from osteoblasts, they assemble into larger

collagen fibrils and further into collagen fibers, which are bundles of fibrils. Collagen gives bone its elasticity and flexibility, whereas bone mineral provides stiffness and rigidity. Without mineral bone tissue would behave as rubber, but without collagen as brittle chalk. Alterations to the structure of collagen in case of aging or genetic abnormalities such as *osteogenesis imperfecta* can affect structural integrity of bone tissue leading to increased risk of fracture. (An & Draughn, 2000; Planell, 2009)

The inorganic mineral substance of bone is mainly in the form of calcium phosphate hydroxyapatite crystals, [$Ca_{10}PO_4OH_2$]. The inorganic matrix functions also as an ion reservoir, storing approximately 99 % of total body calcium and 85 % of phosphorus. (Planell, 2009) Mineralization begins within matrix vesicles budding from the surface of osteoblasts. Matrix vesicles are rich in calcium-binding lipids and proteins, which promote the accumulation of calcium within the vesicles. Calcium and phosphates are transported to the vesicles, and phosphates are also produced by phosphatases within the vesicle. When the accumulation of calcium and phosphate exceed the solubility point for $CaPO_4$, it is deposited in the form of hydroxyapatite (HA). Finally the HA crystals penetrate the vesicle membrane into the extracellular space. However, elongation of the crystals in the extracellular space requires appropriate concentrations of calcium and phosphate also outside the vesicle. Especially in the growth plates of bone the extracellular fluid contains plenty of calcium and inorganic phosphate to support the continuous formation of new HA crystals. (Orimo, 2010) Vitamin D also plays an indirect role in stimulating mineralization as its active derivative, 1,25-$(OH)_2$D, maintains calcium and phosphorus concentrations of serum in adequate levels allowing passive mineralization. (Clarke, 2008)

CELLULAR ELEMENTS

Only a small amount of the total mass of the skeleton is made of the cellular elements of bone. Hematopoietic stem cells (HSCs) and MSCs give rise to bone cells that either mediate resorption (such as osteoclasts) or formation of bone (such as osteoprogenitor cells, osteoblasts, osteocytes and bone lining cells). (Planell, 2009)

Osteoclasts

Osteoclasts are large, multinucleate cells, which are exclusively responsible for the resorption of bone. Osteoclasts develop from HSC-derived mononuclear precursor

cells, which then fuse to form a multinucleated immature osteoclast. Further maturation requires stimulating factors (*e.g.,* RANK-L) from the surrounding cells of mesenchymal origin. The mature osteoclast establishes a microenvironment between itself and the underlying bone by attaching to the bone matrix using integrins. As the osteoclast isolates the bone surface from the general extracellular space, hydrogen ions transported into the compartment acidify the space and dissolve the mineral component of bone matrix. The exposed organic matrix is then subsequently degraded by proteases. (Clarke, 2008; Planell, 2009)

Osteoblasts

Osteoblasts are responsible for the deposition of bone matrix and play therefore an important role in creating skeletal architecture. Osteoblasts are derived from MSCs present in various tissues, for example the bone marrow stroma and periosteum. Commitment of MSCs into the osteoblast lineage requires the canonical Wnt/β-catenin pathway and associated proteins. As a result of Wnt signaling β-catenin accumulates in MSCs, inducing expression of genes such as runt-related transcription factor-2 (Runx2) and osterix, which are needed for the osteoblast development. During this differentiation process osteoblast-specific gene products, such as ALP, are expressed. ALP is a membrane-bound enzyme abundant in early bone formation, which is why ALP activity is often used as a relative marker of osteoblastic differentiation in *in vitro* studies (Lian & Stein, 1995; Coelho & Fernandes, 2000; Ogawa *et al.*, 2004; De Girolamo *et al.*, 2008). ALP regulates phosphate metabolism by hydrolyzing phosphate esters and functioning as a plasma membrane transporter for inorganic phosphates (Liu *et al.*, 2009). The development of the osteoblast phenotype progresses through the highly proliferative, spindle-shaped osteoprogenitor cell to the large cuboidal mature osteoblast. Mature osteoblasts actively synthesize bone matrix by secreting type I collagen and other matrix proteins. Finally the maturation and mineralization of the matrix contributes to the shutdown of proliferation as the osteoblasts become trapped in the matrix. Mineralization of the bone matrix can be detected by histological stainings such as von Kossa and Alizarin Red S, which react with calcium phosphate compounds found in the extracellular matrix (ECM) (Halvorsen *et al.*, 2001). The cells entrapped in the matrix will gradually start developing long cytoplasmic processes as they try to remain in touch

with other cells. Now these cells have started to express E11, an early osteocyte marker, and are considered immature osteocytes. (Planell, 2009) Those osteoblasts that do not get entrapped in the matrix, can become quiescent bone lining cells that form the endosteum on trabecular and endosteal surfaces, and underlie the periosteum (Clarke, 2008).

Osteocytes

The most abundant cells in bone are osteocytes, formed from the entrapped osteoblasts described earlier. Osteocytes are dispersed throughout the bone matrix, residing in tunnels (lacunae) with an interconnected network of thin ducts called canaliculi (Figure 1, B). Osteocytes communicate with each other and their surroundings through extensions of their plasma membrane. Gap junctions of neighbouring cells facilitate transport of nutrients and signals. Intercellular communication is also managed by the extracellular fluid, which flows through the lacuna-canalicular system mediating both chemical and mechanical signals. Because of their interconnectivity and vast number, osteocytes are thought to act as mechanosensors, instructing both osteoclasts and osteoblasts where and when bone needs to be resorbed or formed. (Clarke, 2008; Planell, 2009)

TYPES OF BONE

Bone types can be divided into cortical (dense or compact) and trabecular (spongy or cancellous) bone (Figure 1). These two bone tissue types are similar in matrix composition, but they differ in their structure and function as well as in their distribution within bones.

Cortical bone has a great compressive strength, and it makes up around 80 % of the total skeletal tissue mass. Although cortical bone seems solid, it contains microscopic pores making up about 10 % of the total cortical bone volume. Porosity allows vascular and neural supply as well as exchange of metabolites. The degree of porosity varies according to different factors such as location of the bone, age, and physical activity. Increase in cortical porosity can predispose to bone fractures as it reduces the bone strength. (Clarke, 2008; Planell, 2009)

In contrast to cortical bone, trabecular bone has high porosity, around 50-90 % of total trabecular bone volume. The name of this bone type derives from its orderly arranged network of tubular elements called trabeculae, which give trabecular bone a

sponge-like appearance. Due to its high porosity, trabecular bone has only one-tenth of the compressive force of cortical bone. However, trabecular bone provides large surface area for red bone marrow, blood vessels and connective tissue. It also contributes to internal support, giving bone its ability to absorb energy and distribute load evenly, especially in the joint areas. (An & Draughn, 2000; Planell, 2009)

Both cortical and trabecular bone are composed of osteons, which are the structural units of bone (Figure 1). Cortical osteons, also called Haversian systems (Figure 1, A), are cylindrical in shape and form a branching network, housing blood vessels and nerves. Haversian systems have an appearance of a tree trunk, because they are formed from concentric layers of bone tissue. Trabecular osteons (Figure 1, C) are referred as packets. They are saucer shaped and consist of layers of lamellae. (Clarke, 2008)

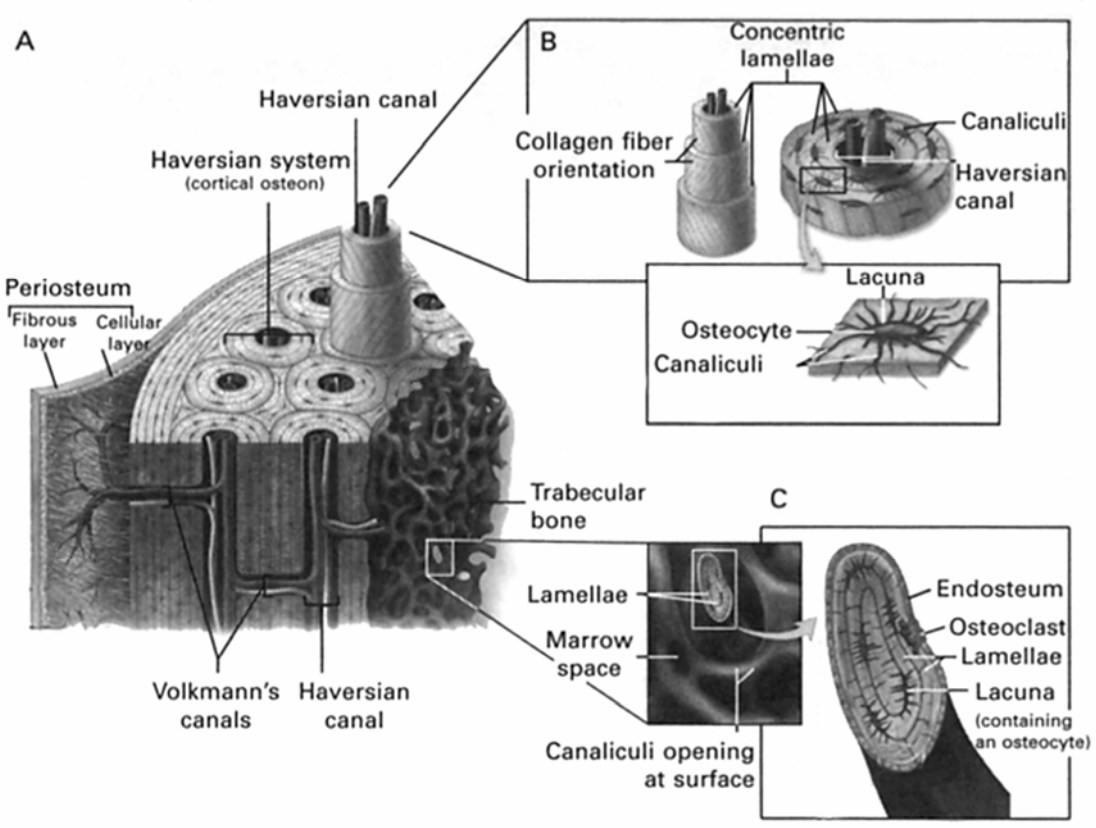

Figure 1. Structure of bone. A) Schematic diagram of bone microstructure describing cortical and trabecular bone. B) Cortical bone osteon also known as Haversian system. C) Trabecular bone osteons or packets shown in cross-section. (Adapted from Planell, 2009)

Microscopically both trabecular and cortical bone contain two different forms of bone tissue, woven and lamellar bone. Woven bone is characterized by irregularly oriented collagen fibrils, giving it enhanced flexibility, but reduced stiffness. The bone formed during embryogenesis is woven bone, but after birth it is gradually substituted by lamellar bone. In adults woven bone can occur in pathological conditions, such as callus formation in bone fractures or bone tumours. Remodelling of immature woven bone results in highly organized structure of mature lamellar bone. (An & Draughn, 2000; Clarke, 2008; Planell, 2009) The external surface of cortical bone is covered by a fibrous connective tissue known as the periosteum, which contains also MSCs. The membranous structure covering the inner surface of cortical bone, trabecular bone, and the blood vessel canals of bone (*i.e.* Volkman's canals) is called endosteum. (Clarke, 2008)

2.1.2 BONE FORMATION, MODELING AND REMODELING

Bone is a dynamic tissue capable of mediating changes in its structure and mass in order to adapt to prevailing conditions. Changes in bone tissue are achieved through diverse processes of growth, repair, modeling and remodeling, each having their own functions and mechanisms. (Planell, 2009)

BONE FORMATION

Bone formation occurs through two distinct developmental processes: intramembranous (*e.g.* in flat bones of the skull) and endochondral ossification (*e.g.* in long bones of limbs). Intramembranous bone formation begins with the condensation of MSCs, which then differentiate into osteoblasts and form ossification centers. This direct bone matrix deposition forms plates, which expand during development. Endochondral ossification also starts with the condensation of MSCs, but in this case they give rise to chondrocytes and perichondrial cells. The chondrocytes produce a cartilaginous template rich in collagen type II. The chondrocytes exit the cell cycle and undergo hypertrophy, finally entering apoptosis. The hypertrophic chondrocytes secrete vascular endothelial growth factor and apoptotic cells create cavities within the space, both enabling the in-growth of blood vessels. The blood vessels deliver hematopoietic cells, osteoprogenitor cells and other cells to the cavity, where hematopoietic cells will later form the bone marrow and osteoblasts derived from

osteoprogenitor cells begin secretion and mineralization of type I collagen-rich ECM. Chondrocyte death is therefore accompanied by blood vessel in-growth and bone matrix production resulting in ossification of the cartilage template. (Shapiro, 2008)

MODELING

Modeling and remodeling are two distinct biological mechanisms affecting bone turnover at tissue level. Modeling is the process adjusting bone size and shape in response to bone growth, physiologic influences or mechanical forces. During bone modeling bone formation and resorption are not tightly coupled. Instead, bone tissue can be selectively removed from an existing bone surface in order to alter the bone geometry without the following bone formation in the same area, and vice versa. This is achieved by the spatially independent actions of osteoblasts and osteoclasts. Therefore bones may widen or even change axis by removal or addition of bone in appropriate surfaces. For example, radial growth of bone is achieved by periosteal bone formation, which simultaneously increases bending resistance. But in order to keep bone mass and motility ideal, periosteal bone formation is often accompanied by endosteal bone resorption, which leads to increase in the medullary area without significant loss of bone strength. Bone modeling is frequent during growth, but it declines to a low level once skeletal maturity is reached. However, modeling may be increased in adults by certain stimuli, *e.g.*, mechanical loading, parathyroid hormone, anabolic agents or withdrawal of estrogen. (Clarke, 2008; Planell, 2009)

REMODELING

Remodeling, in turn, is the lifelong renewal of skeleton and is the main process of bone turnover after completion of skeletal growth. It is needed to maintain bone mineral homeostasis and strength, for example by preventing accumulation of bone microdamage. In remodeling discrete, measureable packets of old bone are constantly being removed and replaced by newly synthesized bone. The process of remodeling is carried out by a tightly coupled group composed of osteoclasts and osteoblasts, which removes and replaces bone in a temporally and spatially coordinated way, without affecting the macroscopic bone shape or density. These groups of osteoclasts and osteoblasts are often called as basic multicellular units (BMUs), which always follow the functional sequence of activation, resorption and formation (Figure 2).

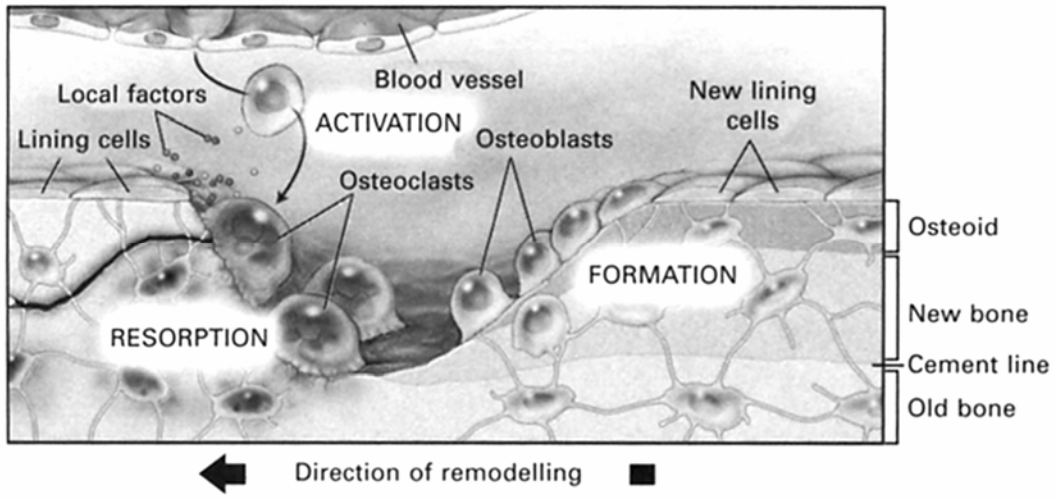

Figure 2. Remodeling of bone is accomplished by a basic multicellular unit (BMU). Activated osteoclast precursors differentiate at the site of remodelling and form an advancing front of actively resorbing osteoclasts. The following osteoblasts deposit osteoid (unmineralized bone matrix), which matures into new bone after mineralization. (Adapted from Planell, 2009)

Resorption is much faster process than formation: an area of bone can be resorbed by osteoclasts in 2-4 weeks, but it takes about three months for osteoblasts to rebuild it. The activation stage involves recruitment and differentiation of mononuclear osteoclast precursors into multinucleate osteoclasts. The precise way of osteoclast recruitment is not known, although signals such as mechanical loading, microdamage and systemic hormones are possible triggers. Remodeling occurring in response to mechanical loading or microdamage replaces specific packets of bone, and is referred as targeted remodeling, whereas remodeling in response to systemic hormones is more random and is therefore referred as non-targeted. Remodeling in response to hormones is often intended to release minerals such as calcium and phosphorus from bone. Approximately 30 % of remodeling is targeted and 70 % non-targeted. (Burr, 2002) Resorption can exceed the formation, and as a consequence, bone loss will occur, or vice versa, formation can exceed resoption and bone will be gained. If the remodeling cycle is in balance, the amount of bone resoption and formation is the same. As can be expected, pharmaceutical agents have been designed in an attempt to fight bone loss. Positive net gain of bone can be achieved by either inhibiting bone resorption (*e.g.* with bisphosphonate therapies) or by enhancing osteoblast activity (*e.g.* by parathyroid hormone). (Clarke, 2008; Planell, 2009)

2.1.3 REGENERATION OF BONE

BONE HEALING

Bone is one of the few organs capable of fully repair itself to a normal pre-injury structure and function when given the optimal conditions. The remarkable repair capacity of bone tissue is achieved by a complex interplay of activated factors and responding cells. Bone healing or repair occurs by different specific mechanisms depending on the biophysical environment. Primary fracture healing (contact repair) takes place under rigid stabilization of the fracture, in an artificial and usually surgical situation, where fixation devices such as screws or plates rigidly hold the fracture ends in contact. The process of primary bone repair with no interfragmentary gap occurs within the cortex by osteoprogenitor cells within Haversian canals as well as osteoclasts creating space for vessels (Shapiro, 2008). Direct bone healing (gap repair) occurs in situations where the interfragmentary gap is greater than 0.1 mm, such that repair can still occur without cartilaginous callus formation, but the bone formed originates from marrow cells (Shapiro, 2008).

In secondary bone healing fracture repair occurs without stabilization, so that there is initially macromotion and then micromotion between the fragments, as in the case of cast immobilization or traction. Fracture trauma involves bleeding from the torn periosteum and surrounding soft tissue, eventually leading to the formation of a fibrous blood clot, which serves as a medium for cell ingrowth between the bone ends. Cells involved in clot formation and inflammation secrete several growth factors and cytokines acting as signals for other inflammatory cells and MSCs, essential for initiating the correct repair response. This endochondral bone repair involves the recruitment, proliferation and differentiation of MSCs into cartilaginous “callus”, which stabilizes the fracture site. From now on the repair process repeats the same mechanism for bone formation used at the growth plate: chondrocyte hypertrophy, cartilage matrix calcification, invasion of hypertrophic chondrocyte lacunae by osteoprogenitor cells and blood vessels, and finally bone tissue formation by osteoblasts. Finally secondary fracture healing is completed by a remodeling phase. (Shapiro, 2008; Planell, 2009)

CLINICAL BONE REPAIR

Although bone has a remarkable ability to heal itself, some defects do not succeed in healing or are so large that without treatment they will become non-unions, which may lead to morbidity and functional disability. Therefore bone needs to be replaced by an implant or tissue transplant such as bone graft. Bone autografts have been the "gold standard" for repair of skeletal defects for years, but some limitations exist to their use (Khan *et al.*, 2000). Autograft bone is obtained from a different anatomic site of the patient, which guarantees immunological compatibility, excellent osteoinductivity and -conductivity. The problems associated with autografts include donor site morbidity, limited amount of the graft and a need for multiple procedures. Allograft bone can be used when large amount of bone is needed, since it is usually obtained from tissue banks that harvest the bone from cadavers. Allograft bone is also provided in several different forms (powder, chips, cubes *etc*.). Although allograft bone has some advantages over autograft bone, allografts often require processing and sterilization, and therefore lack some of the osteogenic and biomechanical properties of autograft bone. There is also a risk of immunologic response and disease transmission concerning allografts. (Aho *et al.*, 1998; Barbour & King, 2003)

During the last three decades, bone tissue engineering has emerged as a new discipline trying to answer to the enormous increase in demand for bone substitutes, as well as combat some of those limitations related to the use of bone grafts. Medical implants are man-made devices that can be either permanent (*e.g.* metals and ceramics) or temporary, in which case the purpose of the biodegradable implant is only to provide a scaffold for the regeneration of the tissue and to gradually degrade as the new tissue forms. However, permanent implants merely replace the structure or biomechanics of the damaged tissue, but do not allow complete regeneration of the structure or function of the tissue. Material scientists are now able to manufacture biomimetic, bioresponsive and biodegradable materials that are able to meet the requirements for tissue formation *in vitro* and *in vivo*. Biodegradable or –resorbable polymers, ceramics and composites can be used as substrates for cell adhesion, proliferation and mineralization. However, this may not be sufficient to induce complete tissue regeneration, especially under pathological conditions. Instead, incorporation of cells (progenitor or differentiated), drugs (antibiotics) or biomolecules (growth factors, genes, surface proteins) might be needed to enhance the formation of new tissue and integration into the surrounding tissue. Furthermore,

current biodegradable materials are not suitable for load-bearing applications. Regardless, development of biodegradable biomaterials holds a great promise in the field of bone tissue engineering. The potential for regenerating a functional tissue rather than replacing it with a permanent implant has also increased the need for understanding the mechanisms of bone healing. In addition to operative methods, it would be highly important to develop non-operative interventions to enhance bone formation. (Planell, 2009)

2.2 MECHANICAL ADAPTATION OF BONE

The skeleton must be able to bear the mechanical loads and impacts that occur from physical activity. In order to do this, bone cells have to be able to "assess" the suitability of the existing structure in relation to their prevailing loading environment, and adapt or maintain it accordingly (Saxon & Lanyon, 2008). It is widely accepted that load-bearing exercise increases bone mass, and in contrast, lack of physical activity predisposes to bone loss. However, less is known about the exact character of the mechanical stimulus needed to induce bone formation when concerning amount, frequency and duration of the applied load. (Torcasio *et al.*, 2008)

Already in 1892 a German anatomist and surgeon Julius Wolff suggested that the form of bone is related to mechanical stress by a mathematical law, and although partly outdated, the general concept of bone adaptation to its mechanical environment referred as "Wolff's law" still holds its ground and is supported by abundant scientific evidence (Ruff *et al.*, 2006). As a refinement of the Wolff's law, Harold Frost described the mechanostat model in the 1960's. According to the Frost's mechanostat theory, the sensor and effector systems formed by bone cells adjust the skeletal strength by sensing the strains caused by mechanical loading and then effecting changes in skeletal mass accordingly. (Frost, 2003)

The sensitivity of bone to functional loading may enable new potential therapies, if mechanical stimuli could be used to inhibit or even reverse bone loss caused by diseases such as osteoporosis (Rubin *et al.*, 2001; Rubin *et al.*, 2002). Specific loading could also be used to improve the osseointegration of bone-anchored implants, since reduced loading at the bone-implant interface can initiate significant bone resorption, which may finally lead to implant failure (Huiskes *et al.*, 1992). Loading could be used to create a mechanical environment that stimulates the

differentiation of peri-implant tissue towards bone, improving the implant osseointegration (Torcasio *et al.*, 2008). Also animal studies have suggested the importance of mechanical loading for the early healing response around implants (Prendergast *et al.*, 1997; Duyck *et al.*, 2006; Vandamme *et al.*, 2007).

2.2.1 MECHANOTRANSDUCTION IN BONE

The intercellular communication system of bone, mediated by the lacunar-canalicular system, enables bone to observe and control the biophysical mechanisms essential for the development and maintenance of bone tissue. For example, mechanical loading can create pressure gradients within the fluid-filled lacunar-canalicular network and extracellular fluid, leading to the deformation of bone cell matrix, which is further transmitted as a biochemical signal inside the cell. (Shapiro, 2008; Torcasio *et al.*, 2008)

A four-stage theory has been suggested to mediate mechanotransduction in cells by which mechanical stimulus is converted into chemical activity. The four stages involved in mechanotransduction are mechanocoupling, biochemical coupling, signal transmission and effector response. Mechanocoupling is the conversion of physiological loads applied to tissues into local mechanical signals experienced by bone cells. This is followed by biochemical coupling where cells sense load using mechanoresponsive structures and transform it into a biochemical response, leading to signal transmission within and between cells. Finally, downstream signalling activates or modulates factors that influence nuclear transcription, promoting cellular outcomes in osteoblasts and osteoclasts, and leading to build-up, remodelling or resorption of bone matrix. (Scott *et al.*, 2008; Shapiro, 2008)

In osteoblasts, signalling is converted into biochemical response by at least four independent, although interacting mechanisms including integrins, G-proteins, the cytoskeleton and ion channels. Mechanical stimulation may be transmitted by integrins, which are activated by deformation of their extracellular binding partners such as collagen and osteopontin. Activated integrins could mediate activation of an associated G-protein leading to G-protein-mediated opening of a calcium channel. The initial flux of extracellular calcium into the cell could also result from the opening of voltage-gated calcium channels. The following increase in intracellular calcium can activate phospholipase C to generate inositol triphosphate, a well-known

second messenger, which further releases calcium into the cell by opening calcium channels in endoplasmic reticulum. Cytoplasmic calcium can in turn bind to and activate several intracellular regulatory proteins. In addition to the signalling role of integrins in mechanotransduction, they also play an important role in transmitting forces to the cytoskeleton mechanically. (Scott *et al.*, 2008)

The effector responses of bone cells that are most often studied include synthesis and mineralization of the ECM. The major components studied in mineralization experiments are the components of the organic matrix (type I collagen, osteopontin and osteocalcin) and associated mineral HA, but also the expression and activity of ALP, an enzyme whose activity relates closely to the onset of mineralization. In general, it has been shown that mechanical stimulus enhances synthesis and mineralization of the bone ECM. (Scott *et al.*, 2008)

2.2.2 PARAMETERS OF MECHANICAL LOADING

It has been suggested that certain fundamental rules govern bone adaptation. Firstly, dynamic, rather than static loading drives this adaptation process. Secondly, only a short duration of mechanical loading is needed to initiate an adaptive response. And thirdly, bone cells are less responsive to routine loading signals, because they accommodate to a customary mechanical loading environment. (Turner, 1998)

One of the main determinants influencing the bone adaptation to mechanical loading is the level of internal strain within the bone tissue. Strain is defined as the change in length divided by the original length of the bone. It is used to describe the deformation in size, which bone experiences in loading. Strain is a unitless value, but since it is very small for bone, it is usually expressed in terms of microstrain (με). For example, 1000 με means 0.1 % deformation, such as 0.1 mm deformation in a 10 cm long bone. Everyday mechanical strains typically range from 400 to 1500 με, but activities involving high impact loads result in higher strains. Microdamage can be caused by extremely high strain, but also by moderate strain if it is highly repetitive (mechanical overuse). Strain larger than 10 000 με generated from a single load typically leads to a complete bone fracture, although susceptibility to fractures is influenced by multiple factors such as age, hormones, nutrition and diseases. (Planell, 2009)

Several mechanical parameters that affect the anabolic response of bone to loading have been identified, including strain rate (Turner, 1998; Torcasio *et al.*, 2008), number of cycles (Kaspar *et al.*, 2002), strain distribution (Lanyon, 1996), local strain gradients (Judex *et al.*, 1997), and resting periods (Srinivasan *et al.*, 2002; LaMothe & Zernicke, 2004). Furthermore, strain rate can be divided into strain magnitude and loading frequency, suggesting that low magnitude high frequency mechanical stimuli could be as effective as high magnitude low frequency stimuli (Rubin *et al.*, 2002; Bacabac *et al.*, 2006). Normal daily activities often produce low amplitude high-frequency loading in bone (Fritton *et al.*, 2000).

Loading frequency and strain magnitude are features of dynamic loading and important determinants of bone adaptation. The traditional view is that strain magnitude is the primary signal for bone adaptation. The Frost's mechanostat theory defines a level of the minimum effective strain (MES), which is needed to maintain the bone tissue (200-2000 με) (Frost, 1990). Bone loss is predicted for strains below 50-200 με, whereas bone gain is predicted for strains above 1500-2500 με. Thus, increasing the strain magnitude until a certain point can promote osteogenesis. (Planell, 2009) However, greater adaptation is achieved by dynamic loading than if the same strain magnitudes were held statically, indicating that bone adaptation is not dependent on strain magnitude alone. Since the strain rate is the product of the strain magnitude and loading frequency, the cellular accommodation theory is also using the loading frequency to predict the adaptive response to a mechanical stimulus. Loading frequency refers to the number of loading cycles per second (1/sec = Hz). It has been postulated that loading frequency must be 0.5 Hz or more in order to affect cortical bone formation, and that beyond this threshold cortical bone formation increases relative to increasing loading frequency until a certain point, but the exact nature of these limits is still uncertain due to widely differing parameters and results obtained from different experiments. (Scott *et al.*, 2008; Planell, 2009)

Other features of loading stimulus are the duration of load and length of rest between the loading periods. Extended duration of skeletal loading does not usually result in proportionally increased bone mass, because the bone formation response tends to saturate as the mechanosensitive cells adjust to the prevailing environment (Burr *et al.*, 2002). Bone cells also need a period of rest to be able to resensitize between loading periods, in order to be responsive again during the subsequent

loading periods. Depending on the nature of the loading stimulus, this resensitization may occur during seconds or hours. (Robling *et al.*, 2002)

The adaptive response of bone is also highly site-specific, in other words, only those regions that experience sufficient strain stimulus undergo adaptation. The site-specific deposition of new bone is functionally essential as it confines the increase of mass and strength only on those sites needed most, yet keeping the overall weight of the bone under control. In addition to site-specificity, loading should be done in the same direction as the bone is loaded in natural activity. (Planell, 2009)

2.2.3 VIBRATION LOADING

Vibration of cells can be generated through either ultrasound or mechanical energy. In a review by Scott and co-workers it was pointed out that both ultrasound and mechanical vibration experiments resulted in a similar variety of responses as those conducted by fluid flow or subtrate strain. It was also noted that both mechanical and ultrasound vibration can induce similar levels of ECM stimulation as induced by fluid flow or substrate strain. (Scott *et al.*, 2008) Mechanical vibration is usually conducted by a vibrating plate or platform, used both in studies done *in vitro* and *in vivo*, generating either vertical or horizontal oscillation. Technically, vibration can be defined by specifying two of the following three variables; frequency (Hz), magnitude of the induced peak acceleration (a, expressed usually in reference to the acceleration of the Earth, $1g = 9.81\ m/s^2$), and the total displacement produced by the vibrating actuator (D, total peak-to-peak displacement of the oscillating plate, μm-cm). Acceleration and frequency are easily measured and verified with an accelerometer attached to the plate or platform used in the experiment. (Judex & Rubin, 2010) The effects of vibration stimulation have been studied in several *in vitro* experiments, but the widely varying parameters have generated some controversy about the types and magnitudes of loading to which different bone cells actually respond. Variation in the results of different studies is affected by the vast range of frequencies (0.5-100 Hz), accelerations (0-10 g) and/or displacements used. Differences were also found in the duration (from minutes to several hours) and pattern of the stimulation (wave form and resting periods). Finally, it is also uncertain how well the *in vitro* findings correlate with the *in vivo* situation.

IN VITRO STUDIES

Most of the vibration loading studies *in vitro* seem to be performed on cultured osteoblasts, although osteocytes have been regarded as the mechanosensory cells of bone. This may be affected by the fact that both osteoblasts and osteocytes respond to mechanical loading (Scott *et al.*, 2008). In addition, protocols for isolation and culture as well as cell lines, have been established later for osteocytes than osteoblasts (Bonewald, 1999; van der Plas & Nijweide, 2005).

A recent study by Patel and co-workers showed that cultured pre-osteoblasts could sense and respond to low magnitude and high frequency (LMHF) vibration. The pre-osteoblast cells were exposed to a random positioning machine used to simulate disuse or microgravity conditions (0 g), and intervened by applying LMHF (0.1-0.4 g at 30 Hz) for 10-60 min per day. Treatment with LMHF was able to prevent the decreased bone formation caused by the random positioning machine as determined by ALP activity, mineralization and osteogenic gene expression. (Patel *et al.*, 2009)

Rosenberg and coworkers tested the effects of sine shaped vibration with frequencies ranging from 20 to 60 Hz with displacement amplitude of 25 μm. They showed that 60 Hz vibration increased ALP activity of the human osteoblast-like cells and caused either no change or a slight decrease in DNA synthesis, whereas 20 Hz vibration had no effect on ALP activity, but stimulated DNA synthesis. (Rosenberg *et al.*, 2002)

Compressive forces were used to generate vibration in studies by Tanaka *et al.* and Dumas *et al.* to study the effects on cultured osteoblasts or osteoblast-like cells. Both studies suggest that osteoblasts are sensitive to low amplitude and high or relative high frequency vibration (25-50 Hz). (Tanaka *et al.*, 2003; Dumas *et al.*, 2009) In a study by Prè and co-workers different frequencies (10, 20, 30, 40 and 60 Hz) with low amplitude were tested on osteosarcoma cells with a vibrating bioreactor. The results suggest that the optimal frequency would be 30 Hz. (Pre *et al.*, 2009) However, continuous vibration was used during the 30 min stimulation period per day, in comparison to dynamic or alternating stimulation used in the other *in vitro* studies described here. It is, in any case, well-known that dynamic mechanical stimulation is more effective in generating adapting process in bone (Turner, 1998).

Effects of LMHF have been studied on osteocytes by Lau and co-workers. Osteocytes were found to respond to LMHF (0.3 g, 60 or 90 Hz) by producing soluble factors that inhibit osteoclast formation (Lau *et al.*, 2010). Vibration stimulation has

been tested *in vitro* also on cells derived from other tissues than bone, consistently supporting the idea that vibrational stimuli can increase matrix synthesis. For example in a study by Wolchok and co-workers, human laryngeal fibroblasts were vibrated at frequency (100 Hz) and displacement (0.9 mm) comparable to those measured at vocal fold during speech. Stimulation was sufficient to enhance the expression and accumulation of ECM proteins (fibronectin and collagen type I). (Wolchok *et al.*, 2009) High-frequency vibration (100-300 Hz) has also been shown to stimulate the synthesis of chondrocyte matrix proteins such as chondroitin, collagen type II and proteglycan (Liu *et al.*, 2001; Takeuchi *et al.*, 2006)

It is also possible that physiological loading induces survival signals in human bone cells. For example, osteoblasts exposed to low frequency fluid shear increased the expression of a pro-survival protein Bcl-2, as pro-death protein Bax remained unchanged (Scott *et al.*, 2008). Also, other potentially survival-promoting effects were observed in different studies *e.g.*, increased levels of epidermal growth factor receptor (Ogata, 2003) and insulin-like growth factor (Cillo *et al.*, 2000). This may be significant observation especially in the case of osteoporosis, where excessive osteocyte apoptosis is one characteristic feature. However, due to the highly varying results from different studies, more consistent research in this matter is required. (Cillo *et al.*, 2000; Bakker *et al.*, 2004; Bonewald, 2004)

There is also a question of what can be counted as vibration, since there are several ways to conduct vibrational stimulus to the cells. In addition to the vibrating plates or platforms (Rosenberg *et al.*, 2002; Bacabac *et al.*, 2006; Wolchok *et al.*, 2009), some studies have described cyclic compression and tension as a method to create vibration (Tanaka *et al.*, 2003; Dumas *et al.*, 2009). In a study by Tanaka and co-workers, cells seeded in collagen gels were stimulated inside chambers, where mechanical strain was applied by an actuator causing displacement in the gel (Tanaka *et al.*, 2003).

On the other hand, it has been suggested that bone cells sense their mechanical environment through the interstitial fluid flow that causes deformations in the cell membranes. Since several kinds of mechanical forces can induce fluid flow in the lacunar-canalicular systems of bones *in vivo* as well as in culturing medium *in vitro*, it is possible that different loading regimes have a general pathway for cellular excitation. (Fritton & Weinbaum, 2009) Nevertheless, a general concept of mechanical forces that eventually lead to a specific tissue differentiation has been

delineated: tensile stress leads to intramembranous ossification, constant compression leads to cartilage formation, high shear stresses favour fibrous tissue formation, and intermittent compression favours the actual endochondral ossification (Shapiro, 2008). In other words, as long as the mechanical stimulation is intermittent – as in the case of vibration, where there is mechanical oscillation *i.e.* repetitive variation – stimulation should enhance osteogenic differentiation.

IN VIVO STUDIES

High magnitude and low frequency (HMLF) impacts, such as running or other high-impact exercising, can create peak strain magnitudes in a range of 2000-3500 με, that have long been regarded as beneficial to bone strength (Heinonen *et al.*, 2001; Nikander *et al.*, 2009). Also the opposite stimulus, a low magnitude and high frequency (LMHF) loading experienced in everyday activities as standing, has been shown to be anabolic to bone *in vivo* (Rubin *et al.*, 2001; Rubin *et al.*, 2001; Ward *et al.*, 2004; Dickerson *et al.*, 2008). It has been suggested that these low impact strains are mediated by supportive muscle contractions, creating strains in the frequency range of 10-50 Hz and accelerations <1 g (Rubin *et al.*, 2001). For example, studies done on cortical bone tissue showed that stimulation with 2000 με (high magnitude) at 0.5 Hz frequency maintained bone mass, but increasing the frequency to 1 Hz and finally to 30 Hz would require only 1000 με and 70 με respectively, in order to maintain bone mass (low magnitude, high frequency). (Rubin & Lanyon, 1984; Rubin *et al.*, 2001) The effect of LMHF has been tested in treating musculoskeletal pathologies in several pre-clinical and clinical trials, for example, animals (Rubin *et al.*, 2001), children suffering from muscular dystrophy or cerebral palsy (Gray *et al.*, 1992; Ward *et al.*, 2004), young women with low bone mass (Gilsanz *et al.*, 2006), and post-menopausal osteoporotic women (Rubin *et al.*, 2004). Bone's sensitivity to low magnitude signals could enable non-pharmaceutical applications capable of increasing bone mass, strength or integrity also in those cases, where high impact exercising would be impossible, for instance due to injury, disease or age. Although numerous studies have demonstrated the effectiveness of LMHF loading in bone formation *in vivo*, the underlying molecular mechanisms mediating these low level signals into anabolic responses as well as the responding cells have not been ascertained completely.

Other studies have also reported the osteogenic *in vivo* effects of high magnitude (2-3 g) and high frequency vibration (Flieger *et al.*, 1998; Oxlund *et al.*, 2003). For example, in a study by Oxlund and co-workers, vibration with 45 Hz and 3 g increased periosteal bone formation rate and inhibited the endocortical resorption seen in the ovariectomy rats in comparison to lower frequencies (17 and 30 Hz) and accelerations (0.5 and 1.5 g). (Oxlund *et al.*, 2003)

Many different types of vibration loading mechanisms have been shown to affect bone cell metabolism both *in vitro* and *in vivo*. Due to the variations in experiment parameters, it is difficult to fully compare the results of different studies. It is also remarkable how LMHF, HMLF and in addition other combinations of vibration parameters can all induce effects in either bone cells or skeleton. The fundamental paradox in bone tissue is that tissue-level strains rarely exceed 2000 με, which is too small stimulus to initiate intracellular signalling *in vitro*, whereas *in vitro* strains (> 5000 με) could cause bone tissue damage *in vivo*. Therefore, it has been suggested that some kind of amplification system is present in bone cells *in vivo*, so that lower strain signals might be amplified, and would therefore generate similar cellular response as in the case of larger strains *in vitro*. (Torcasio *et al.*, 2008; Fritton & Weinbaum, 2009) Recent ultrastructural studies have suggested that osteocyte processes are tethered to canalicular walls by transverse elements that span the pericellular space. Small tissue-level strains could be amplified 10 to 100 fold as fluid flow through the lacunar-canalicular system creates tension to the tethering elements, producing larger cellular strains *in vivo*. (You *et al.*, 2001) Also in a review by Scott and co-workers it was suggested that bone cells are able to respond to a broad range of mechanical stimuli, because of the partly overlapping mechanosensory system (Scott *et al.*, 2008). These theories may explain why osteogenic activity can be induced by several methods, frequencies and magnitudes of vibration loading, but the optimal magnitude and frequency range in each of the stimulus type, as well as the most effective combination of different parameters still remain elusive, and would require systematic large-scale studies.

2.3 STEM CELLS

Stem cells are the self-renewing progenitors of body tissues that can generate undifferentiated daughter cells upon cell division, but also have the ability to differentiate into one or more committed descendants. This multipotency in addition to their capacity to self-renew makes stem cells extremely attractive candidates for regenerative medicine. Human stem cells are not only prospective candidates for regenerative applications, but also contribute to the understanding of embryonic development, disease progression and pharmaceutical research. (Wobus & Boheler, 2005; Choumerianou *et al.*, 2008)

Stem cells can be of embryonic, germinal or somatic origin. In addition to the adult tissues, the somatic origin comprises also the less-mature sources such as the umbilical cord blood, placenta, and fetal tissues (Choumerianou *et al.*, 2008). Stem cells derived from different origins differ also in their properties. According to their developmental potential stem cells can be divided into totipotent, pluripotent and multipotent. The fertilized egg, *zygote*, has the ability to generate an entire organism, and is thus defined as totipotent. Totipotency is retained by the *zygote* and the early cell division stages such as blastomeres, up to the eight-cell stage of the morula. The subsequent differentiation generates a blastocyst consisting of outer trophoblast cells and the undifferentiated inner cell mass (ICM). The cells of ICM are defined as pluripotent, since they are no longer able to form an entire organism, but yet retain the ability to develop into all cell types of the embryo proper.

In adult tissues, multipotent stem and progenitor cells present in different tissues and organs regenerate lost or injured cells. Multipotent adult stem cells are able to differentiate into a few cell lineages presumably restricted by the particular tissue or organ. The stem cells of adult tissues can be divided according to the germ layer from which they originate, *i.e.*, endothermal, mesodermal and ectodermal origin. Pulmonary epithelial stem cells, gastrointestinal tract stem cells, pancreatic stem cells, hepatic oval cells and ovarian and testicular stem cells are all endothermal stem cells. Stem cells of mesodermal origin include for example mesenchymal stromal cells present in bone marrow and adipose tissue, hematopoietic stem cells and cardiac stem cells. And finally, examples of ectodermal stem cells are neural, skin and ocular stem cells. (Choumerianou *et al.*, 2008) It is important to notice that stem cells are not involved only in the embryonic development, but play an important role in adult

tissues as well by maintaining the normal homeostasis of the organism. (Rao & Mattson, 2001)

2.3.1 EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS

Although the first embryonic stem cell lines derived from mouse blastocysts were generated already in 1981, it took 17 years to establish the first human embryonic stem cell (hESC) line. Ever since the successful isolation of hESCs in 1998 by James Thomson and co-workers, hESCs have been under extensive research, but also ethical and political debate (Thomson *et al.*, 1998). The policies on hESC research vary enormously between different countries. In Finland hESC lines can be established from unsuitable or excess embryos from *in vitro* fertilization, which would otherwise be discarded, but it is forbidden to create embryos merely for research purposes (Medical Research Act, Laki lääketieteellisestä tutkimuksesta 488/1999).

Human ESCs have several attractive features that make them ideal for the applications of regenerative medicine. They have a capacity to differentiate into any cell type, but hESCs are also able to proliferate in long-term cultures without losing their pluripotency. Although holding a great promise for regenerative medicine, it is necessary to overcome the possible problems related to directed differentiation, immune responses and oncogenic properties, and to fully understand the biology and properties of hESCs before therapeutic applications can be considered. (Zandstra & Nagy, 2001; Wobus & Boheler, 2005; Choumerianou *et al.*, 2008) Properties of hESCs, however, make them very valuable for pharmaceutical development. Cell-based *in vitro* assays for pre-clinical assessment are urgently needed. Many human primary cell types, such as cardiomyocytes and neuronal cells, are nearly inaccessible or rapidly lose their properties under *in vitro* culture conditions. In addition, results from animal-based *in vitro* and *in vivo* tests are not always reliable or clinically relevant due to the low concordance between animal and human data. Thus, *in vitro* models based on physiologically relevant human cells will lead to more predictive and cost-effective assays. The most important cell types differentiated from hESCs for drug development are considered to be hepatocytes, cardiomyocytes and neuronal cells. (Jensen *et al.*, 2009)

Employment of hESCs is hindered by the ethical and legal concerns regarding the use of human embryos. However, reprogramming differentiated human somatic

cells into a pluripotent state would circumvent these issues, and additionally provide a way to generate patient- or disease-specific pluripotent cells. These induced pluripotent stem (iPS) cells can be generated from somatic cells, for example from adult human dermal fibroblasts, by a retrovirus-mediated transfection of four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi *et al.*, 2007). The human iPS cells are very similar to hESCs in many ways including function, morphology, proliferation, gene expression and *in vitro* differentiation. Nevertheless, great challenges still exist in the field of iPS cells, such as the risk of pathogen transfer due to viral vectors and the low efficiency of induction. Although clinical applications of patient-specific iPS cells still have to wait for a while, iPS cells will no doubt provide useful models for diseases and pharmaceutical development. (Takahashi *et al.*, 2007; Cyranoski, 2008)

2.3.2 ADULT STEM CELLS

Although having wider differentiation capacity, ESCs and iPS cells have limited usage either due to ethical, political or safety issues. In order to circumvent the problems related to the use of hESCs and iPS cells, research has turned to adult stem cells, which offer several advantages to regenerative medicine.

HSCs

HSCs located in the bone marrow are multipotent stem cells that give rise to all the blood cell types. HSCs are responsible for the constant renewal of blood cells, since mature blood cells are mostly short-lived. As stem cells, HSCs are able to self-renew as well as differentiate into all blood cell lineages. The first clinical uses of HSCs were the treatment of blood cancers such as leukemia and lymphoma, resulting from the uncontrolled proliferation of white blood cells. In treatment of blood cancer the patient's own cancerous hematopoietic cells are first destroyed by radiation or chemotherapy, and then replaced with a bone marrow transplant or a transplant of HSCs collected from the peripheral circulation of a matched donor. (Bellantuono, 2004; Holowiecki, 2008)

MSCs

MSCs are non-hematopoietic stromal cells of mesodermal origin first observed by Friedensten and coworkers in 1960s (Friedenstein *et al.*, 1968), but the term MSC was not popularized until 1990s (Caplan, 1991). Yet there seemed to be a debate of the true potential of these stromal cells, and whether they could actually be referred as stem cells. As the biomedical interest towards MSCs increased, also several different methods of isolation, expansion and characterization arise, making it increasingly difficult to compare and contrast results of different studies. Finally a uniform nomenclature and criteria for defining these multipotent mesenchymal stromal cells was proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) in two position statement papers published during 2005-2006 (Horwitz *et al.*, 2005; Dominici *et al.*, 2006). It was suggested that the fibroblast-like plastic-adherent cells, regardless of tissue they were isolated, should be referred as multipotent mesenchymal stromal cells, while the term mesenchymal stem cells should be used only for cells that meet the specified criteria. However, it was proposed that the widely used acronym, MSC, could be used for both populations as long as the more accurate designation is clearly expressed in the report. (Horwitz *et al.*, 2005) The ISCT criteria for identifying MSCs is based on adherence, surface antigen expression and differentiation capacity. MSCs are able to regenerate mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma (Zuk *et al.*, 2002; Pountos & Giannoudis, 2005; Pittenger, 2008). *In vitro* MSCs can be induced to differentiate along multiple phenotypic pathways under specific differentiating conditions. According to the criteria of ISCT committee, MSCs must be able to differentiate into osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions. MSCs must also be plastic-adherent under standard culture conditions and express cluster of differentiation (CD) surface markers CD105 (endoglin, SH-2), CD73 (ecto-5'-nucleotidase) and CD90 (Thy-1, T cell surface glycoprotein), as well as lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR. CD45 is a pan-leukocyte marker, CD34 is a marker of primitive hematopoietic progenitors and endothelial cells, CD14 and CD11b are mostly expressed on monocytes and macrophages, the most likely hematopoietic cells found in MSC culture. CD79α and CD19 are markers of B cells

and HLA-DR should be expressed on MSC only when stimulated. (Dominici *et al.*, 2006)

Bone marrow was initially used as a source of MSCs, mainly because MSCs were first found from bone marrow, but since then MSCs have been isolated from alternative sources such as adipose tissue and teeth (particularly third molars) (Morsczeck *et al.*, 2005). Even though BMSCs have been under a lot of research both *in vitro* and *in vivo*, there exist some limitations to their use. The yield of BMSCs from the bone marrow aspirate is quite low and is significantly influenced by the age and sex of the donor. (D'Ippolito *et al.*, 1999; Muschler *et al.*, 2001; Slater *et al.*, 2007) In addition, bone marrow harvesting is considerable painful procedure with possible risks. Since ASCs are easily harvested in large quantities with minimal risks, adipose tissue has emerged as a promising source of multipotent adult stem cells for regenerative medicine (Muschler *et al.*, 2001).

2.4 ADIPOSE STEM CELLS

Adipose tissue is a mesodermally derived organ composed of adipocytes and several other cells that surround and support them, including a stromal population containing microvascular endothelial cells, smooth muscle cells and ASCs. The stromal population containing ASCs can be enzymatically digested and separated from the buoyant adipocytes by centrifugation. (Gimble & Guilak, 2003) Upon isolation this heterogenous cell population is called stromal vascular fraction (SVF). Although the crude SVF is a heterogenous cell population often expressing also phenotypes of hematopoietic cells, it is suggested that the characteristics of cells derived from SVF change as a function of adhesion and expansion *in vitro* (McIntosh *et al.*, 2006; Mitchell *et al.*, 2006). Subsequent expansion increases the portion of adipose-derived stem cells, which will eventually form a relatively homogenous cell population. Zuk and coworkers originally characterized ASCs isolated from the SVF and found that they were able to differentiate toward several lineages *in vitro* (Zuk *et al.*, 2001).

ASCs isolated in this manner share many of the characteristics of BMSCs including extensive proliferative potential and the ability to undergo multilineage differentiation (Strem *et al.*, 2005). Zuk and co-workers also found that when comparing CD marker profiles of ASCs and BMSCs, both populations express CD29, CD44, CD71, CD73, CD90, and CD105 (Zuk *et al.*, 2001). Yet some differences do exist, for example ASCs express strongly CD49d, which lacks in BMSCs. On the

other hand, ASCs lack the expression of CD106, which is in turn expressed on BMSCs. (Strem *et al.*, 2005) In general there seems to be no difference between growth kinetics, cell senescence, multi-lineage differentiation capacity, or gene transduction efficiency when comparing hASCs and hBMSCs *in vitro* (De Ugarte *et al.*, 2003).

2.4.1 OSTEOGENIC DIFFERENTIATION OF ADIPOSE STEM CELLS

Several studies indicate that ASCs undergo osteogenic differentiation within 2-4 weeks of culturing in similar conditions that are used for the osteogenic differentiation of BMSCs (Halvorsen *et al.*, 2001; Zuk *et al.*, 2001; Zuk *et al.*, 2002). As expected, the osteogenic differentiation is significantly dependent on the medium composition. Various supplements have been used for the osteogenic differentiation of ASCs, including 1,25-dihydroxyvitamin D_3 (Halvorsen *et al.*, 2001) and different bone morphogenetic proteins (BMP)s (Dragoo *et al.*, 2003; Bessa *et al.*, 2009), but L-ascorbic acid 2-phosphate (Asc 2-P), dexamethasone (Dex) and β-glycerophosphate (β-GP) are probably the most commonly used supplements (Zuk *et al.*, 2001; Hattori *et al.*, 2004; Ogawa *et al.*, 2004; De Girolamo *et al.*, 2008). Supplement concentrations vary according to different studies, but traditionally 50 μM Asc 2-P, 100 nM Dex and 10 mM β-GP have been used (Zuk *et al.*, 2001). ASCs cultured in the presence of these supplements are shown to express genes characteristic of osteoblast-like cells, such as ALP, BMPs and their receptors, Runx2, osteocalcin, osteonectin, and osteopontin. Under the same culturing conditions, the ASCs are also able to form mineralized matrix. However, because the osteogenic-inductive media was initially generated for the differentiation of BMSCs, it may not be optimal for the differentiation of ASCs. For example de Girolamo and coworkers suggested that osteogenic medium with higher Asc-2-P concentration (150 μM) and lower Dex (10 nM) would be more effective for osteogenic differentiation of ASCs cultured in FBS. (de Girolamo *et al.*, 2007) In addition to medium supplements, osteogenic differentiation of ASCs can also be induced by osteoconductive material such as HA (De Girolamo *et al.*, 2008; Rada *et al.*, 2009).

2.4.2. ADIPOSE STEM CELLS IN TISSUE ENGINEERING OF BONE

Research involving ASCs in the field of bone tissue engineering is rapidly increasing. Strategies for bone defect repair involve the use of differentiated or undifferentiated, fresh or passaged ASCs, combined with specific culturing media, growth factors and biomaterial scaffolds. A great number of animal experiments using ASCs combined with several types of biomaterial scaffolds have shown the osteogenic capacity of ASCs *in vivo* (Cowan *et al.*, 2004; Hicok *et al.*, 2004; Hattori *et al.*, 2006). It has been proposed that ASCs enhance the healing process by secreting cytokines and growth factors, promoting vessel formation and attracting endogenous progenitor cells (Prockop, 2007).

The initial *in vivo* studies done with ASCs typically involved loading of cells into scaffolds with or without prior osteoinduction, followed by subcutaneous implantation for several weeks, and then characterization of cell differentiation and bone matrix formation. A study by Hicok and coworkers demonstrated that ASCs are able to produce osteoid *in vivo*. Human ASCs were loaded into HA/tricalcium phosphate (β-TCP) scaffolds and implanted subcutaneously into immunodeficient mice. After 6 weeks of implantation osteoid formation was observed in 80 % of the implants loaded with ASCs, whereas no osteoid formation was found in control scaffolds without cells. (Hicok *et al.*, 2004) In a similar study set-up, Hattori and coworkers compared BMSCs and ASCs loaded into atelocollagen (Hattori *et al.*, 2004) and β-TCP scaffolds (Hattori *et al.*, 2006). The cell-seeded β-TCP scaffold constructs were first cultured *in vitro* in osteogenic medium and then implanted for 8 weeks in the back of nude mice. Human ASCs were found to be as effective as BMSCs in regarding their osteogenic potential. In addition, the presence of either of these cells was also shown to enhance the vascularization process. (Hattori *et al.*, 2006) Similarly, pre-differentiated ASCs mixed with an injectable scaffold material induced the formation of vascularized and mineralized woven bone only 4 weeks after subcutaneous implantation into nude mice (Elabd *et al.*, 2007).

More complex animal studies have involved repair of bone defects. Critical-sized bone defects are often used as a model to assess the osteogenic potential of ASCs. By definition, a critical-sized defect will undergo less than 10 % healing over the lifetime of the animal, or in other words, is not able to heal spontaneously. Cowan and coworkers were the first to show that ASCs could regenerate a critical-sized

defect in nude mice without addition of exogenous growth factors or genetic manipulation. Uninduced ASCs loaded on an apatite-coated poly(lactic-co-glycolic acid) scaffolds were effective in healing critical-sized mouse calvarial defects, showing significant intramembraneous bone formation already in two weeks. Furthermore, the rate and extent of bone formation was comparable to that of BMSCs. (Cowan *et al.*, 2004) Some studies have shown preferable results for the use of pre-differentiated over un-differentiated ASCs (Peterson *et al.*, 2005; Dudas *et al.*, 2006; Yoon *et al.*, 2007). Although *in vitro* osteoinduction of ASCs is expensive and time consuming, stimulated ASCs may exhibit better osteoinductive response *in vivo*. For example, Dudas and coworkers found that osteoinduced ASCs loaded onto gelatin foam scaffolds showed greater healing capacity in rabbit calcarial defect treatment than non-induced ASCs (Dudas *et al.*, 2006).

ASC-based treatments have also been successfully used in clinical practice (Lendeckel *et al.*, 2004; Mesimaki *et al.*, 2009). Mesimäki and coworkers described a reconstruction of craniofacial bone defect with a microvascular flap using autologous ASCs, β-TCP granules and BMP-2. A preformed titanium cage was filled with β-TCP granules and autologous ASCs cultured in the presence of BMP-2. The cage was implanted into a pouch inside an abdominal muscle. After 8 months the flap had developed mature bone structures and vasculature, and was transplanted into the maxillary defect area successfully. (Mesimaki *et al.*, 2009) Lendeckel and coworkers described a successful repair of a large, bilateral calvarial defect in a seven year old girl. Autologous ASCs combined with iliac crest bone and fibrin glue were seeded to the calvarial defect. CT-scans taken three months later showed marked ossification throughout the defect. (Lendeckel *et al.*, 2004)

3. AIMS OF THE RESEARCH

It is widely accepted that mechanical signals are essential in maintaining the skeleton, but the exact parameters of mechanical loading affecting bone have not been ascertained completely. Since vibration loading has been proved to have an osteogenic effect on osteoblasts, it was hypothesized that such signals could stimulate the osteogenic differentiation of hASCs as well. After all, osteoblasts are originated from MSCs.

The aim of this study was to test a specifically designed vibration loading device for the stimulation of hASCs. The combined effects of vibration and osteogenic culturing conditions were also studied. Human ASCs cultured in basic or osteogenic culturing conditions were stimulated by high magnitude (3 g) and high frequency (50 and 100 Hz) vibration, and the effects on viability, attachment, proliferation, ALP activity and mineralization were studied.

4. MATERIALS AND METHODS

4.1 CELL CULTURE METHODS

The study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District. The ASCs were isolated from adipose tissue samples collected from four female donors (mean age 56 ±12.6 years) in surgical procedures in Tampere University Hospital.

4.1.1 ADIPOSE STEM CELL ISOLATION AND CELL CULTURE

Mechanical and enzymatic isolation procedures described previously by Zuk and co-workers were used (Zuk *et al.*, 2001). In brief, adipose tissue samples were processed into smaller pieces with scissors and then digested with collagenase type I (1.5 mg/ml; Invitrogen, California, USA) at 37°C gyratory water bath for 45-60 min with intermittent shaking every 15 minutes. After centrifugation and filtration isolated hASCs were maintained and expanded in T-75 cm^2 polystyrene flasks (Nunc, Roskilde, Denmark) in basic medium (BM) consisting of Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12 1:1; Invitrogen), 10 % human serum (HS; PAA Laboratories GmbH, Pasching, Austria), 1 % L-glutamine (GlutaMAX ; Invitrogen) and 1 % antibiotics (100 U/mL penicillin, 0.1 mg/ml streptomycin; Invitrogen). Human ASCs cultured in BM were detached with TrypLe Select (Invitrogen), a recombinant fungal trypsin-like protease. The expanded hASCs were cryo-preserved in liquid nitrogen in freezing solution containing 10% dimethyl sulfoxide (DMSO; Hybri-Max®, Sigma-Aldrich, MO, USA) and HS. The hASCs needed for the experiments were thawed and expanded in the basic medium.

A cell pool consisting of hASCs from four different donors was used at passages 3 or 4. 25,000 cells were plated per well on a 12-well plate in 1 ml of BM. Only 1 ml of medium per well was used instead of usual 2 ml to prevent spilling of medium during vibration loading. Medium was changed every second day. Osteogenic differentiation was started 24 hours after plating by adding osteogenic medium (OM), consisting of BM with osteogenic supplements: 250 μM Asc 2-P, 5 nM Dex and 10 mM β-GP. The osteogenic supplement concentrations were optimized for osteogenic differentiation of hASCs in HS by Mesenchymal stem cell group at Regea (unpublished data).

4.1.2 FLOW CYTOMETRIC SURFACE MARKER EXPRESSION ANALYSIS

Flow cytometry is a laser-based technology used for the characterization of whole cells as well as cellular constituents. It allows both qualitative and quantitative analysis of whole cells or their constituents that have been labelled with a wide range of commercially available dyes and monoclonal antibodies. The underlying principle of flow cytometry is that a single-cell suspension allows cells to flow as single file past a laser beam and as the beam strikes to an individual cell, light is scattered and fluorescence is emitted. Light scattering is directly related to structural and morphological cell features, generating information about the cell size and surface complexity. (Jaroszeski & Radcliff, 1999) Fluorescence mainly occurs if the cells are labelled with a fluorescent probes, such as monoclonal antibodies conjugated to fluorochromes, although cells do contain some endogenous fluorophores generating auto-fluorescence (Monici, 2005). Fluorescent probes of interest are reacted with cells before analysis and the amount of fluorescence emitted as a cell passes the excitation source is proportional to the amount of fluorescent probe bound to the cell or cellular constituent (Jaroszeski & Radcliff, 1999).

After primary culture in T-75 flasks (at passage 1-2), the hASCs were harvested and analyzed by a fluorescence activated cell sorter (FACSAria; BD Biosciences, Erembodegem, Belgium) as described by Lindroos and co-workers (Lindroos *et al.*, 2009). Monoclonal antibodies against CD14, CD19, CD49d-PE, CD90-APC, CD106-PE-Cy5 (BD Biosciences); CD45-FITC (Miltenyi Biotech, Bergisch Gladbach, Germany); CD34-APC, HLA-ABC-PE, HLA-DR-PE (Immunotools GmbH Friesoythe, Germany); and CD105-PE (R&D Systems Inc, MN, USA) were used. A total of 10,000 cells per sample were used, and positive expression was defined as a level of fluorescence greater than 99 % of the corresponding unstained cell sample.

4.1.3 ADIPOSE STEM CELL VIABILITY AND PROLIFERATION

Cell attachment and viability were evaluated qualitatively using Live/dead-staining probes (Molecular Probes, Eugene, OR, USA) at 1, 7 and 14 d. The staining method is based on the probes resulting in different fluorescence reactions on dead and viable cells. CellTracker^{TM} Green (5-chloromethylfluorescein diacetate [CMFDA]; Molecular Probes) is a membrane-permeant dye that freely diffuses into living cells

and reacts with proteins and peptides, resulting in green fluorescence. Ethidium homodimer-1 (EthD-1; Molecular Probes) binds to nucleic acids that are released from the nuclei of dead cells resulting in red fluorescence. Briefly, ASCs were rinsed with DPBS and incubated for 45 min at room temperature with a mixture of 0.5 µM CMFDA and 0.25 µM EthD-1 in Dulbecco's Phosphate Buffered Saline (DPBS; Lonza Biowhittaker, Lonza Group Ltd. Switzerland). The viable cells (green fluorescence) and necrotic cells (red fluorescence) were examined using a fluorescence microscope.

Cell proliferation was studied at 1, 7 and 14 d time points by CyQUANT® Cell Proliferation Assay Kit (CyQUANT; Molecular Probes, Invitrogen), which is based on the amount of total DNA in the sample. The method was performed according to manufacturer's protocol. CyQUANT® GR fluorescent dye exhibits a strong fluorescence enhancement when bound to cellular nucleic acids. At the day of the time point, cells were suspended in 0.1 % Triton-X 100 buffer (Sigma-Aldrich) and frozen at -70°C at least overnight to lyse the cells. After thawing the Triton-cell lysates were collected in Eppendorf tubes, centrifuged quickly, and pipeted as three parallel samples on a 96-microplate (Nunc). CyQUANT® GR dye was added to each well and suspended thoroughly. The fluorescence was measured with microplate reader (Victor 1420 Multilabel Counter; Wallac; Turku, Finland) at 405 nm.

4.1.4 OSTEOGENIC DIFFERENTIATION

ALP activity and mineralization were determined to detect osteogenic differentiation of hASCs. ALP activity was studied at 1, 7 and 14 d of culturing. The quantitative ALP analysis was done using the ALP Kit (Sigma-Aldrich), according to the manufacturer's protocol. In the procedure ALP catalyses the hydrolysis of p-nitrophenyl phosphate in alkaline solution, liberating yellow-colored p-nitrophenol. The rate of yellow p-nitrophenol formation, measured photometrically, is proportional to the catalytic concentration of ALP present in the sample. The ALP analysis was done using the same samples as for CyQUANT. The absorbance was measured with microplate reader (Victor 1420) at 405 nm.

A quantitative Alizarin Red S method was used at 7 and 14 d to detect calcium compounds deposited in the ECM of the cells. The ability to mineralize the ECM is characteristic of differentiated osteoblasts. In aqueous solution Alizarin Red S and Ca^{2+} ions precipitate to form red deposits. The cultures were briefly rinsed with DPBS

followed by fixation in 4 % paraformaldehyde for 30 min. Fixed cultures were stained with 2 % Alizarin Red S (Sigma-Aldrich), pH 4.2 for 5 min. The dye was extracted with 100 mM cetylpyridinium chloride (Sigma-Aldrich) at gentle shaking for 3 h. The dye intensity was determined by absorbance measurement at 540 nm on a multiplate reader (Victor 1420).

4.2 VIBRATION LOADING

4.2.1 VIBRATION LOADING DEVICE

A specially designed vibration loading device composed of a loudspeaker, amplifier and microcontroller was employed to stimulate hASCs. The vibration loading device was designed and built by engineers of the Biomedical Engineering Department, Tampere University of Technology. Cells cultured on 12-well plates were attached on top of the loudspeaker, where the displacement of the diaphragm generated vertically oriented acceleration to the cells. The magnitude of acceleration, frequency and wave shape were controlled through the amplifier and microcontroller. Acceleration was measured by an accelerometer that was attached to the plates. The device was tested for the vibration loading of cells for the first time at Regea, Institute for Regenerative Medicine. In order to carry out the loading under cell culturing conditions, the device was wiped clean with 70 % ethanol and placed inside a cell culturing incubator (+37^{o}C, 5 % CO_2). Figure 3 shows the vibration loading device assembled inside an incubator.

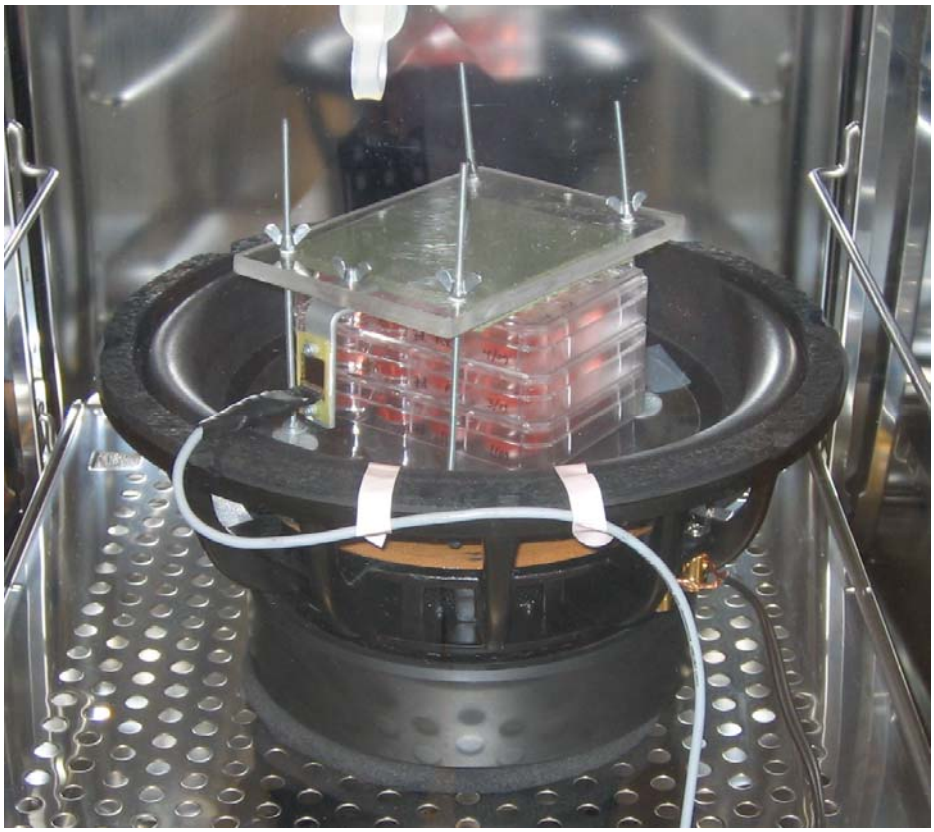

Figure 3. The vibration loading device assembled inside a cell culturing incubator.

4.2.2 VIBRATION LOADING STIMULATION

Vibration loading was started 24 h after addition of OM (*i.e.* 48 h after plating of the cells). Human ASCs were vibrated with acceleration of approximately 3 g, using square wave at either 50 Hz or 100 Hz, for 1.5 h distributed over a 3 h period. The stimulation pattern consisted of 1.0 s of vibration followed by 1.0 s rest period. This was repeated for 3 h, which was followed by a 21 h of rest. The 3 h stimulation period was repeated every day for 1, 7 or 14 d. Control cells were cultured similarly without vibration. Three parallel samples for each condition were used for quantitative analyses and two for qualitative. The experiment was repeated three times. The results of only two repeats were combined in the final results, as the preliminary results were unreliable due to a breakdown of the device at day 9 of the 14 day culture period. Despite the setback, the preliminary experiment was valuable, since it proved that cells can be cultured under rather vigorous vibration and that the parameters chosen were affecting the cells. Preliminary data is shown in Appendix 2.

4.3 STATISTICAL ANALYSIS

Statistical analysis of the results was performed with SPSS version 17. Data was reported in all the quantitative experiments as mean plus standard deviation (SD). Data from two repeats was combined (n=6), and the results were considered significant when $p<0.05$. The effects of different culture conditions on DNA content, ALP activity and mineralization were compared using a one-way analysis of variance (ANOVA) with Tukey *post hoc* test. The effect of culture duration was analyzed using a paired Student's *t*-test for mineralization (7 d and 14 d), and one-way ANOVA for DNA content and ALP activity (1 d, 7 d and 14 d).

5. RESULTS

5.1 FLOW CYTOMETRIC SURFACE MARKER EXPRESSION ANALYSIS

Human ASCs used in the study expressed the surface markers CD49d, CD73, CD90, CD105 and HLA-ABC as shown in Table 1. The expression of CD14, CD19, HLA-DR, the vascular cell adhesion molecule CD106, and the haematopoietic marker CD45 was negative in hASCs, but only moderately negative for CD34. The flow cytometry verified the mesenchymal origin of the used hASCs.

Table 1. Surface marker expression of undifferentiated hASCs cultured in HS (passage 1-2). The results are displayed as mean percentage of the surface marker expression of the four cell lineages used in the study.

Surface Protein	Antigen	Mean	SD	Expression
CD14	Serum lipopolysaccharide binding protein	2.7	1.2	Negative
CD19	B lymphocyte-lineage differentiation antigen	1.1	0.3	Negative
CD34	Sialomucin-like adhesion molecule	19.7	2.0	Moderate expression
CD45	Leukocyte common antigen	2.9	0.9	Negative
CD49d	Integrin α2, VLA-4	62.3	18.2	Positive
CD73	Ecto-5'-nucleotidase	98.1	1.7	Positive
CD90	Thy-1 (T cell surface glycoprotein)	97.6	2.5	Positive
CD105	SH-2, endoglin	98.9	0.9	Positive
CD106	VCAM-1 (vascular cell adhesion molecule)	1.2	0.7	Negative
HLA-ABC	Major histocompatibility class I antigens	73.9	18.4	Positive
HLA-DR	Major histocompatibility class II antigens	1	0.3	Negative

5.2 CELL VIABILITY AND PROLIFERATION

Live/dead staining was done to study the attachment and viability of hASCs. As shown in the Figure 4(A-I) and 5(A-I), the vibrated cells were slightly unhomogenously attached at 1 d and this effect was more pronounced at 100 Hz (G). Vibrated cells also tended to form clusters, especially at 7 d when cultured in BM (E, H). Vibration loading did not greatly affect the viability of hASCs, since only a few dead cells were detected at all times. The amount of viable cells was increasing by time in all conditions. However, it was observed that cells cultured in BM were detaching under vibration more easily than those cultured in OM.

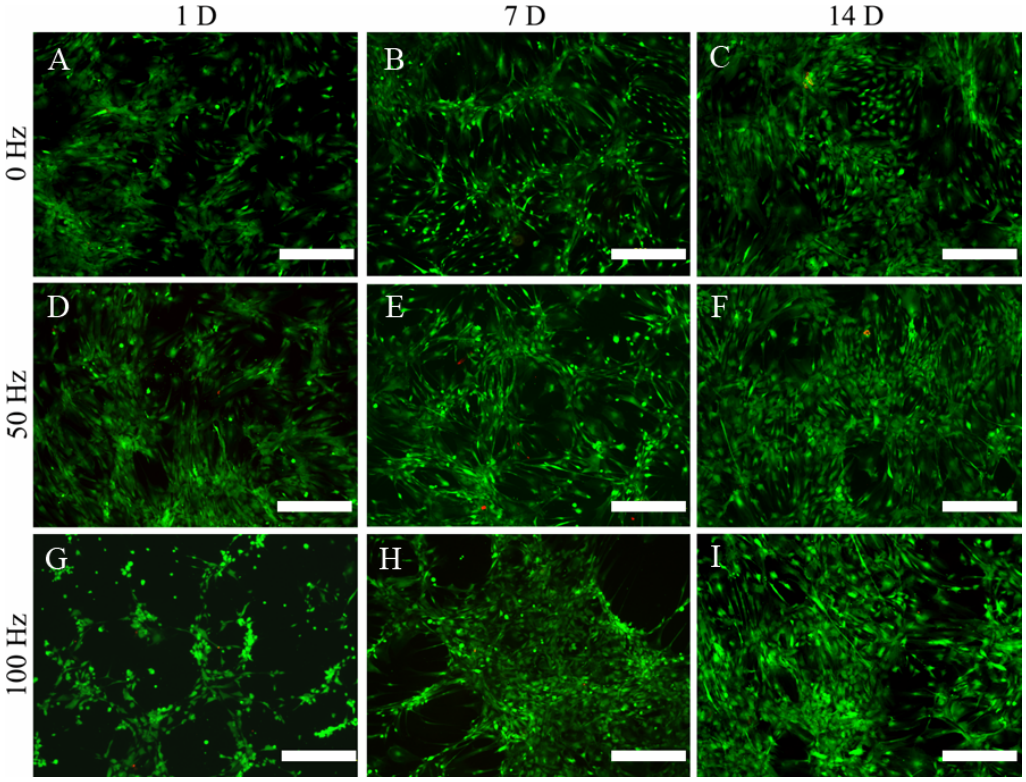

Figure 4. Representative images of viable (green fluorescence) and dead (red fluorescence) hASCs cultured in BM. Unstimulated hASCs (A, B, C), vibration stimulated at 50 Hz (D, E, F) and at 100 Hz (G, H, I) at 1, 7 and 14 d time points. Scale bar 500 μm.

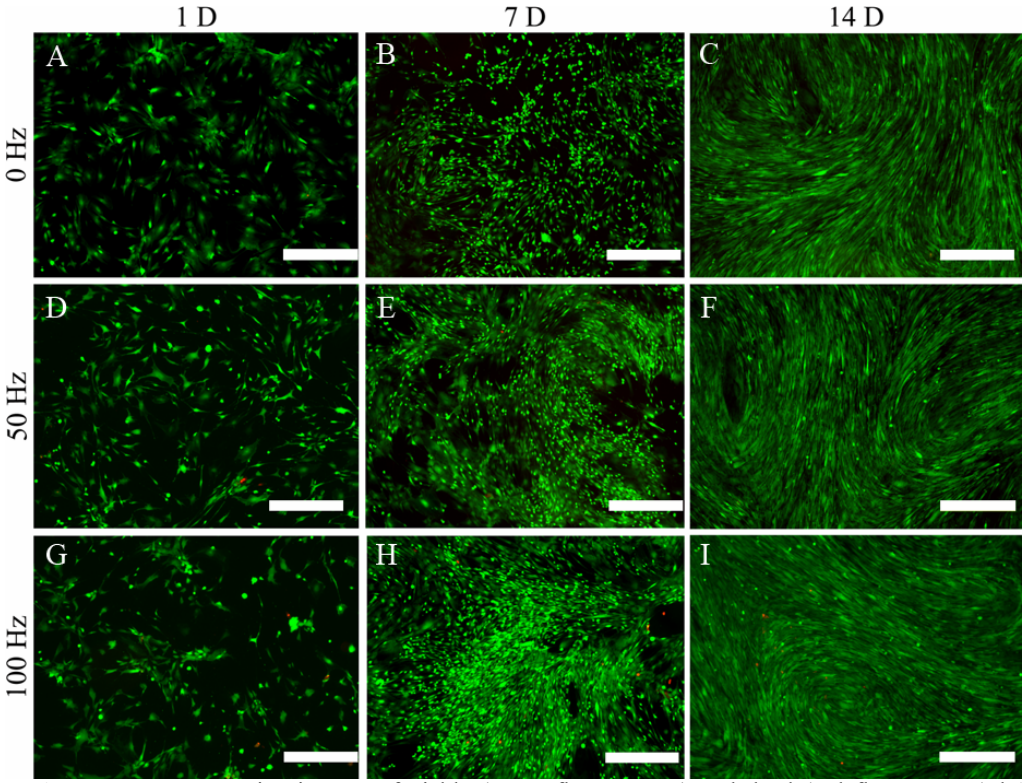

Figure 5. Representative images of viable (green fluorescence) and dead (red fluorescence) hASCs cultured in OM. Unstimulated hASCs (A, B, C), vibration stimulated at 50 Hz (D, E, F) and at 100 Hz (G, H, I) at 1, 7 and 14 d time points. Scale bar 500 μm.

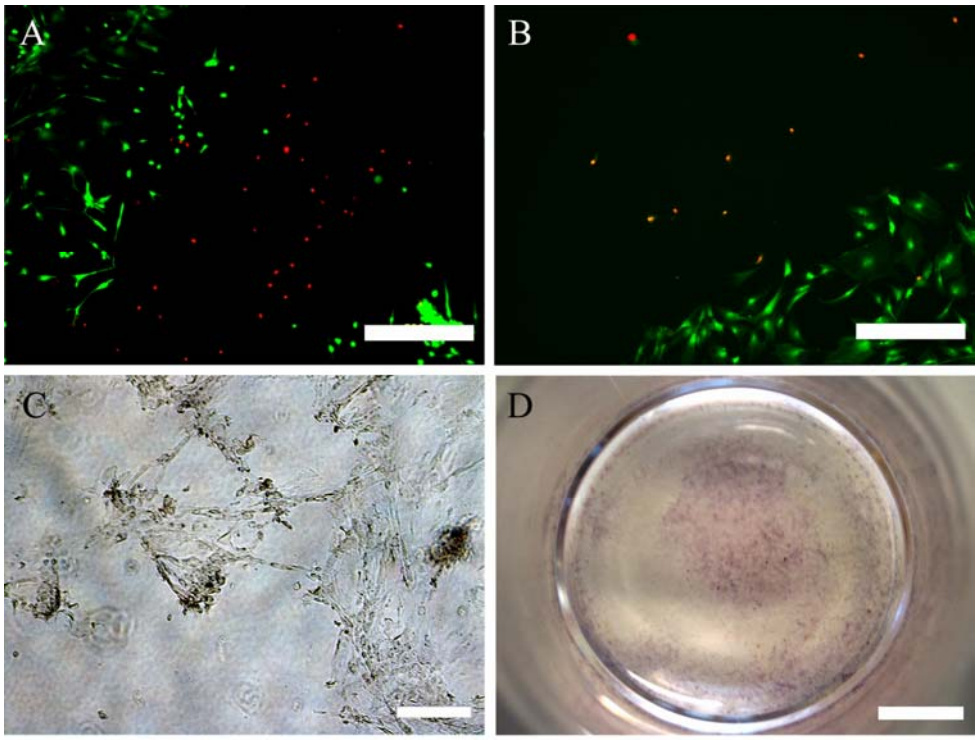

Figure 6. Images of hASCs cultured in BM and stimulated with 50 Hz vibration, showing dead or detached cells. Similar effects were detected in cells stimulated with 100 Hz vibration in BM, but not in any of the stimulated cells cultured in OM. A) Live/dead staining at 1 d, B) Live/dead staining at 7 d, C) light microscope image at 7 d, and D) Alizarin Red staining at 14 d. Scale bar 500 µm (A, B, C) and 5 mm (D).

Figure 6 presents images of detached or dead hASCs detected in BM-cultured wells at 50 Hz vibration in Live/dead staining (A-B), light microscope (C) and Alizarin Red staining (D). Similar effects were seen in BM-cultured hASCs at 100 Hz vibration, but not in the unstimulated hASCs. Such detachment was not seen in stimulated hASCs cultured in OM. Wells with detached cells were left out of the quantitative analyses. Figure 7 shows the fibroblast-like morphology of the unstimulated, pooled hASCs in light microscope images. The inducing effect of OM in proliferation is clearly shown in both light microscope (Figure 7) and live/dead images when compared to hASCs cultured in BM (Figures 4 and 5).

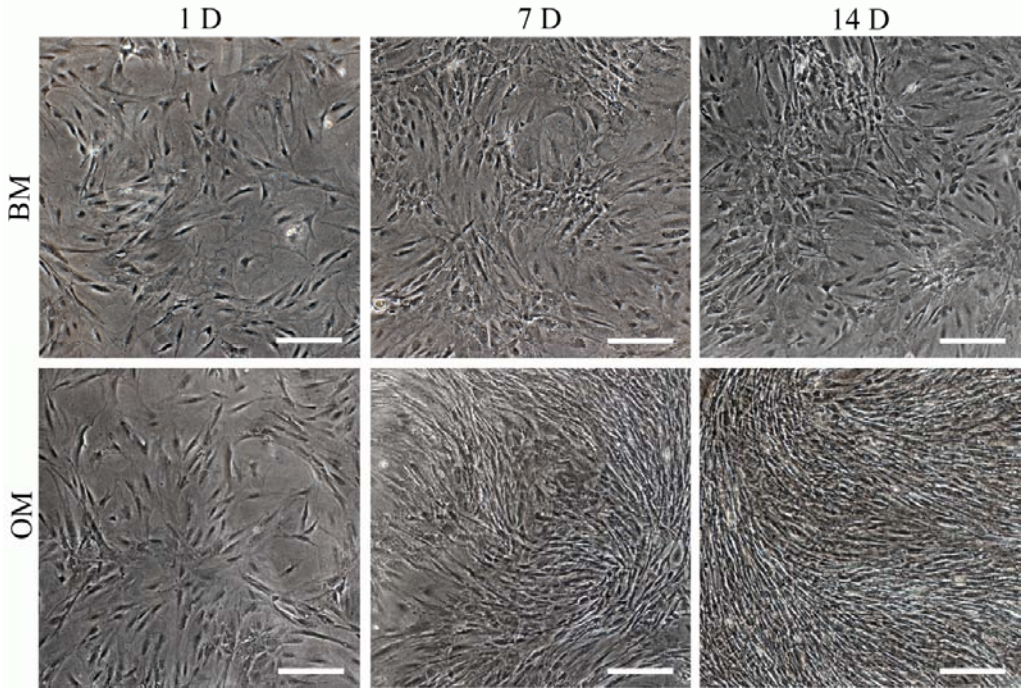

Figure 7. Light microscope images showing the fibroblast-like morphology of pooled, unstimulated hASCs at 1, 7 and 14 d time points in either BM or OM. Scale bar 500 μm.

Proliferation of ASCs in different conditions was evaluated quantitatively by measuring the total DNA content. Culturing in OM increased proliferation significantly ($p<0.05$) at 7 and 14 d time points when compared to hASCs cultured in BM (Figure 8). However, there were no significant differences in cell proliferation between stimulated and control hASCs in either cell culturing conditions (BM or OM) in any of the time points. The number of cells also increased significantly ($p<0.05$) with time, except with 100 Hz stimulation in BM, when comparing 1d and 7d time points.

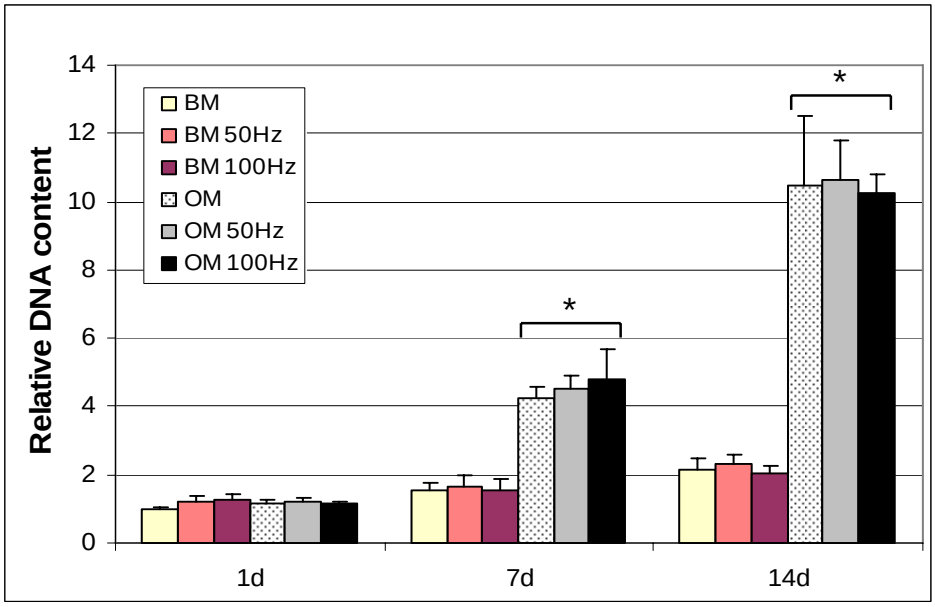

Figure 8. Proliferation of hASCs cultured either in BM or OM, and stimulated with 50 Hz or 100 Hz vibration loading at 1, 7, and 14 d time points. Results are expressed as mean +SD (n= 6) and normalized relative to control culture (BM). *p<0.05 with respect to BM, BM 50 Hz and BM 100 Hz.

5.3 OSTEOGENIC DIFFERENTIATION

5.3.1 ALKALINE PHOSPHATASE ACTIVITY

The quantitative ALP activity assay was done from the same samples as DNA measurement, enabling normalization of ALP levels relative to the DNA content of the sample. The normalized ALP levels varied a lot at 7 d time point in different conditions (Figure 9). At 14 d time point a decrease could be seen in ALP activity in hASCs cultured in BM when compared to control. Conversely, ALP activity was significantly higher in vibration stimulated hASCs cultured in OM at 14 d in contrast to unstimulated (OM) and stimulated hASCs cultured in BM. Both 50 Hz and 100 Hz frequencies were as effective in stimulating ALP activity in OM-cultured hASCs, since no significant effect was seen at 7 or 14 d time points between these two frequencies.

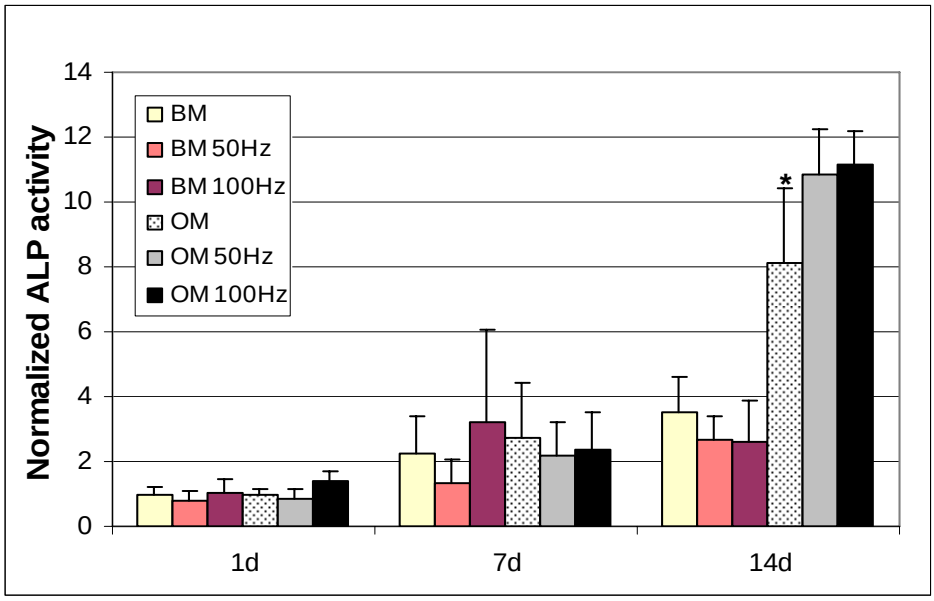

Figure 9. Quantitative ALP activity of hASCs cultured either in BM or OM, and stimulated with 50 Hz or 100 Hz vibration loading at 1, 7, and 14 d time points. Results are expressed as mean +SD (n= 6) and normalized relative to amount of DNA. *p<0.05 with respect to OM 50 Hz and OM 100 Hz.

5.3.2 MINERALIZATION

Mineralization of ECM was measured by quantitative analysis of Alizarin Red staining at 7 and 14 d time points. Figure 10 shows that culturing in OM increased relative mineralization significantly at both time points when compared to hASCs cultured in BM. Mineralization also tended to increase with time, but increase was significant ($p<0.05$) only when hASCs were cultured in OM. No significant differences were detected in relative mineralization between stimulated and unstimulated hASCs in either cell culturing conditions (BM or OM) or time points. However, in absolute mineralization (Appendix 1: Figure 14), a significant difference ($p<0.05$) was indeed found between 100 Hz stimulated and control hASCs in OM at 14 d timepoint.

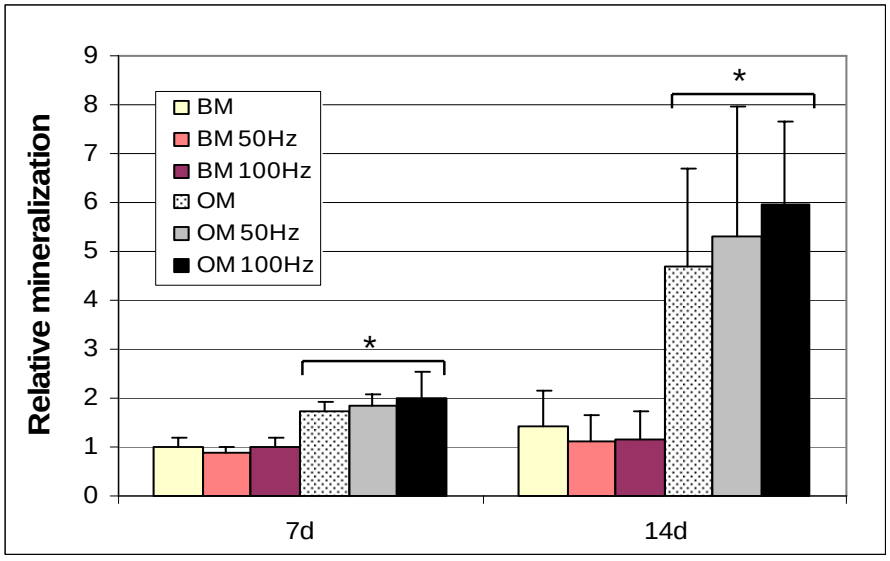

Figure 10. Quantitative mineralization of hASCs cultured either in BM or OM, and stimulated with 50 Hz or 100 Hz vibration loading at 7 and 14 d time points. Results are expressed as mean +SD (n= 6) and normalized relative to control culture (BM). *p<0.05 with respect to BM, BM 50 Hz and BM 100 Hz.

Furthermore, images of the Alizarin Red staining at day 14 showed red staining in OM-cultured hASCs stimulated with 50 and 100 Hz frequencies (detected in both repeats of the study), where as unstimulated ASCs showed only purple staining (Figure 11).

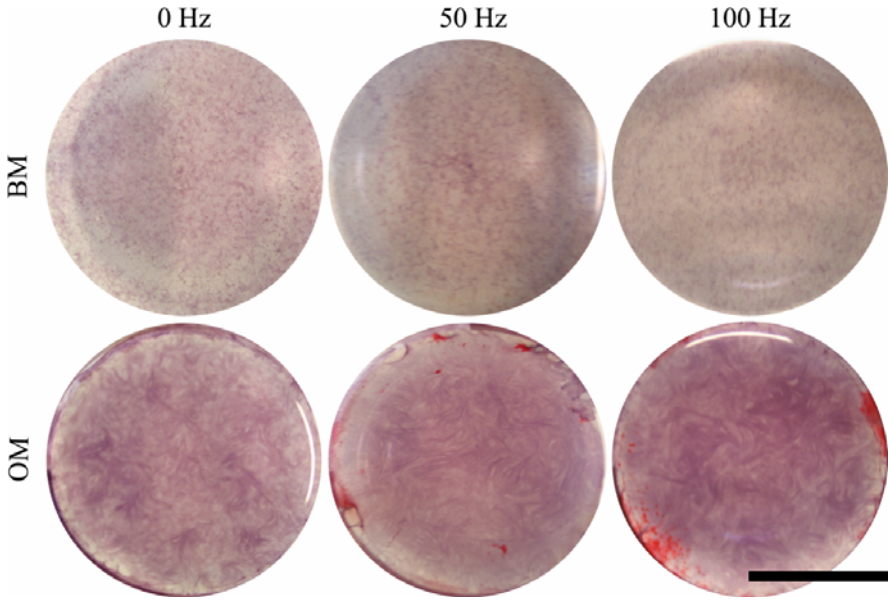

Figure 11. Representative images of Alizarin Red stained hASCs cultured either in BM or OM at 14 d time point. Human ASCs stimulated with 50 Hz and 100 Hz vibration showed red staining. Scale bar 10 mm.

6. DISCUSSION

The aim of this study was to test whether high magnitude and high frequency vibration loading could affect osteogenic differentiation of hASCs. The combined effects of vibration and osteogenic culturing conditions were also studied. The effects of vibration loading on attachment, viability, proliferation, ALP activity and mineralization were analyzed. The mesenchymal origin of the hASCs used in this study was verified by the flow cytometric analysis.

6.1 PARAMETERS OF VIBRATION LOADING

Several studies conducted *in vitro* with osteoblasts have indicated that low magnitude, high frequency vibration has osteogenic effects (Rosenberg *et al.*, 2002; Tanaka *et al.*, 2003; Dumas *et al.*, 2009; Patel *et al.*, 2009). Also high magnitude, high frequency vibration has been studied in both *in vitro* (Wolchok *et al.*, 2009) and *in vivo* (Flieger *et al.*, 1998; Oxlund *et al.*, 2003) studies. Wolchok and co-workers found that HMHF vibration increased ECM production in fibroblasts (Wolchok *et al.*, 2009) Flieger, Oxlund and co-workers in turn demonstrated that HMHF vibration can prevent bone loss in ovariectomized rats (Flieger *et al.*, 1998; Oxlund *et al.*, 2003). Accelerations used in these studies vary from 2 to 3.4 g and frequencies from 45 to 100 Hz. To my knowledge, the present study was the first where vibration loading was tested on hASCs. Since it is still controversial whether magnitude has greater influence on vibration loaded cells than frequency, a high magnitude (3.0 g), high frequency (50-100 Hz) vibration was chosen for this study.

It is well-known that bone adaptation is driven mostly by dynamic, rather than static mechanical stimulation (Turner, 1998). Further, insertion of rest periods within stimulation can prevent saturation of adaptive response (Srinivasan *et al.*, 2002; LaMothe & Zernicke, 2004), and it has been reported that a recovery period of at least 8 h is sufficient to restore mechanosensitivity of saturated bone cells (Robling *et al.*, 2001). Therefore, resting periods were used in this study during the vibration stimulation (stimulation of 1 s was followed by 1 s rest period) and after the stimulation bouts (3 h stimulation bout was followed by 21 h of rest).

6.2 VERIFICATION OF THE MESENCHYMAL ORIGIN OF HUMAN ADIPOSE STEM CELLS

Flow cytometric analysis was used for the characterization of hASCs used in this study. No exact limits have been defined for the expression of different surface markers, but generally expression lower than 2 % is considered strongly negative and higher than 95 % strongly positive (Dominici *et al.*, 2006). The flow cytometric analysis demonstrated that the hASCs used in the study expressed the surface markers CD49d, CD73, CD90, CD105 and HLA-ABC (Table 1). The hASCs were negative for CD14, CD19, HLA-DR, the vascular cell adhesion molecule CD106, and the haematopoietic marker CD45, but only moderately negative for CD34. Moderately elevated expression levels of hematopoietic-associated markers CD34, CD14 and CD45 may be explained by the low passage (1-2) of the hASCs during analysis. It has been reported that positive expression of CD34, CD14 and CD45 may be detected on hASC populations with low passage, but expression declines significantly in successive passages (McIntosh *et al.*, 2006; Mitchell *et al.*, 2006). In addition to flow cytometric analysis, the mesenchymal origin of the used hASCs was verified by their plastic-adherence and fibroblast-like morphology (Figure 7). The osteogenic capacity of the used hASCs was also demonstrated successfully.

6.3 CELL VIABILITY AND PROLIFERATION

Live/dead staining was used to evaluate the viability and attachment of stimulated hASCs in comparison to control cells. Although the high magnitude, high frequency vibration is a rather intense treatment for the cells, viability of hASCs was not greatly affected. Some dead or detached cells were seen occasionally among stimulated cells cultured in BM, but to a lesser extent in OM-cultured, stimulated cells. It seems that osteogenic culture conditions enhance the attachment of the hASCs, possibly due to the increased secretion of ECM proteins. For example, Asc 2-P has been found to stimulate secretion of ECM proteins, such as collagen and glycosaminoglycan, in MSCs (Choi *et al.*, 2008). The OM used in this study was specifically optimized for hASCs cultured in HS, and it contains higher concentration of Asc 2-P (250 μM) compared to other OM described in the literature (Halvorsen *et al.*, 2001; Zuk *et al.*, 2001; Ogawa *et al.*, 2004; de Girolamo *et al.*, 2007). Immunohistochemical or quantitative PCR analyses could be considered in the future to study whether

vibration increases expression and secretion of ECM proteins in combination with OM. Overall, both stimulated and unstimulated hASCs cultured in OM showed significantly higher proliferation, ALP activity and mineralization than BM-cultured cells at 14 d time point. However, no significant differences in proliferation were seen between unstimulated, 50 Hz and 100 Hz vibrated hASCs cultured in OM in any of the time points (Figure 8). Proliferation results of the preliminary experiment were quite similar (Appendix 2: Figure 15).

6.4 OSTEOGENIC DIFFERENTIATION

6.4.1 ALKALINE PHOSPHATASE ACTIVITY

High frequency vibration has been reported to increase ALP activity of bone cells *in vitro* (Rosenberg *et al.*, 2002), and similar results were seen in this study with hASCs. Both 50 and 100 Hz frequencies increased normalized ALP activity of hASCs cultured in OM significantly at 14 d time point. Since there were no significant differences between ALP activities induced by these two frequencies, it is possible that the magnitude of vibration has greater influence on the osteogenic differentiation of hASCs than frequency. Different magnitudes (accelerations) in combination with constant frequency should be tested with hASCs in order to determine the effect of magnitude. However, both 50 and 100 Hz frequencies can be considered as high frequency vibration, which may also contribute to the fact that no significant differences were detected between their effects on ALP activity. In the future experiments it would be interesting to study also low frequency vibration (*e.g.*, 5-10 Hz) in comparison to 50 or 100 Hz stimulation.

The results from the preliminary experiment were included in Appendix 2, since an increasing trend in relative ALP activity was shown at 14 d time point in stimulated, OM-cultured hASCs in contrast to unstimulated cells (Appendix 2: Figure 16). This is interesting, because during preliminary experiment the vibration loading device broke down at day 9, but the stimulatory effect was still detected at day 14. The preliminary results for ALP activity suggest that shorter duration of stimulation could be as effective as or even more effective than longer duration, since cells tend to get saturated with repetitive stimuli. This result is also consistent with the study of Lanyon and co-workers, who showed that bone adaptation is especially sensitive to

unusual strains, rather than repetitious and prolonged strain cycles that are generated during normal activities (Lanyon, 1996).

6.4.2 MINERALIZATION

Patel and co-workers found that LMHF loading (0.1-0.4 g, 30 Hz) can induce mineralization of pre-osteoblasts in a magnitude-dependent manner (Patel *et al.*, 2009). In the present study, a trend of increased relative mineralization in stimulated, OM-cultured hASCs was detected at 14 d time point, although there was no significant difference in comparison to control cells in OM. This trend was more clearly shown in absolute results of mineralization (Appendix 1: Figure 14), showing the results as absorbance. In absolute mineralization, there was a significant difference ($p<0.05$) between 100 Hz stimulated and control hASCs in OM at 14 d timepoint. The difference between relative and absolute results can be explained by a large variation in the mineralization of control cells (unstimulated hASCs cultured in BM) in the two repeats of the experiment. Since the mineralization of control cells was used to normalize all the other results, high control value in the first repeat and low value in the second repeat can cause a great difference in the scales of different repeats. Statistically significant difference was not observed in combined results, because different scales of the two repeats caused large standard deviation (as seen in Figure 10). Therefore, the absolute results of mineralization could be more representative of the actual situation. The absolute results were also supported by the Alizarin Red staining (Figure 11), showing the unusually red staining of the stimulated, OM-cultured hASCs, detected in both successful repeats of the study. Red staining was seen only in the stimulated hASCs in OM and was most intense in hASCs stimulated by 100 Hz vibration. Alizarin Red solution reacts with calcium compounds forming red colour, but according to previous studies of our group hASCs under osteogenic differentiation conditions stain purple rather than red, and the purple colour intensifies as hASCs differentiate towards osteogenic lineages with time. Therefore the intensity of purple staining has been used as an indicator of gradually differentiating hASCs. However, red staining is considered as the actual marker for the mature, mineralized matrix of osteoblasts. In the quantitative analysis the red staining may have been overpowered by the relative majority of purple staining. Nevertheless, the quantitative and qualitative results of mineralization analysis indicated that vibration stimulation enhanced mineralization of hASCs.

Preliminary results of relative mineralization (Appendix 2: Figure 17) suggest that vibration loading for 9 d was not sufficient to maintain stimulation of mineralization until 14 d time point. These results indicate that longer duration of stimulation is needed to enhance mineralization, which is the opposite as with ALP activity. However, ALP activity is considered as an early marker of osteogenic differentiation, and mineralization in turn as a late marker. Therefore enhancement of mineralization may also require longer stimulation period, since it occurs later in the osteogenic differentiation process.

7. CONCLUSIONS

In conclusion, the vibration loading device described in this study was successful in generating controlled vibrational forces to cells cultured on well plates. Osteogenic culturing conditions were found to be more optimal for vibration loading studies done on hASCs than basic conditions due to enhanced cell attachment. The results of this study suggest that high magnitude, high frequency vibration combined with osteogenic culture conditions enhance hASC differentiation towards osteogenic lineage. However, no significant differences were found between the effects caused by 50 Hz and 100 Hz frequencies at high magnitude. Thus, the most optimal parameters of vibration loading inducing osteogenic responses in hASCs should still be determined.

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APPENDIX 1: ABSOLUTE RESULTS

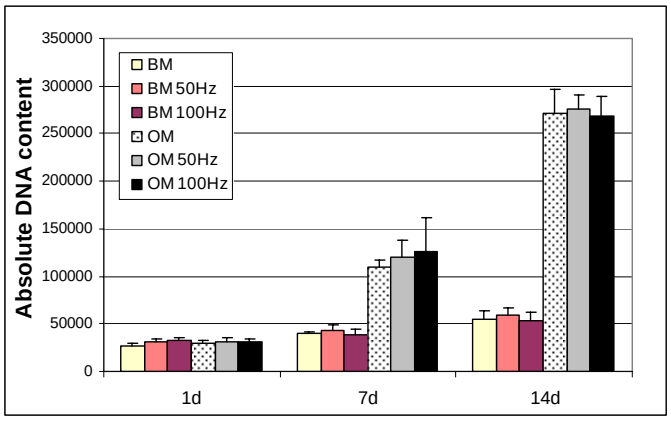

Figure 12. Proliferation of ASCs cultured either in BM or OM, and stimulated with 50 Hz or 100 Hz vibration loading at 1, 7, and 14 d time points. Results are expressed as mean fluorescence +SD (n= 6).

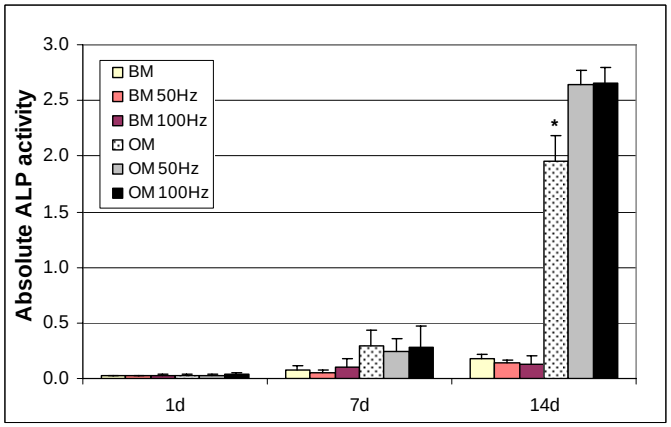

Figure 13. Quantitative ALP activity of ASCs cultured either in BM or OM, and stimulated with 50 Hz or 100 Hz vibration loading at 1, 7, and 14 d time points. Results are expressed as mean absorbance +SD (n= 6). *p<0.05 with respect to OM 50 Hz and OM 100 Hz.

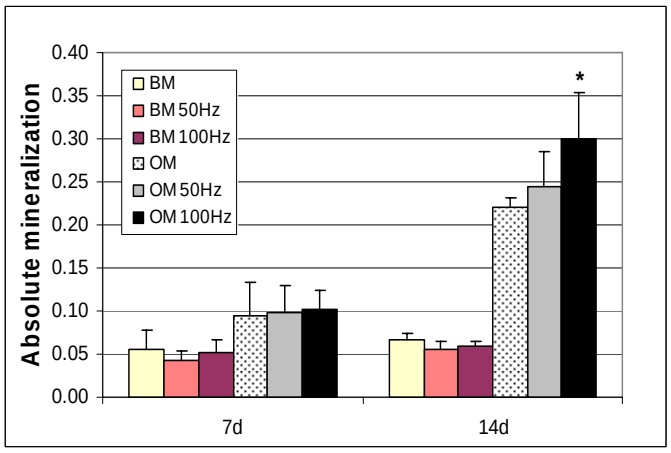

Figure 14. Quantitative Mineralization of ASCs cultured in either BM or OM, stimulated with 50 Hz or 100 Hz vibration loading at 7 and 14 d time points. Results are expressed as mean absorbance +SD (n= 6). *p<0.05 with respect to OM 50 Hz and OM.

APPENDIX 2: PRELIMINARY RESULTS

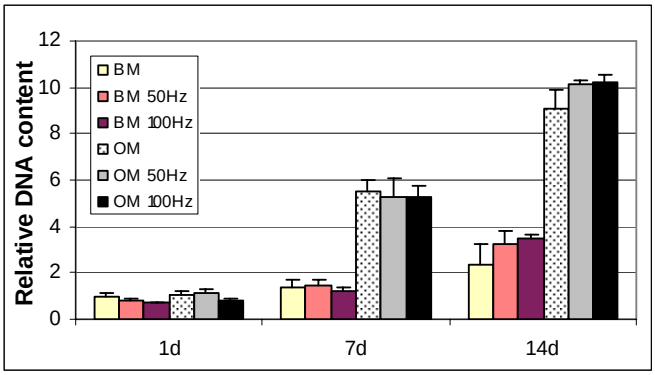

Figure 15. Preliminary results. Relative DNA content of ASCs cultured in BM or OM, and stimulated with 50 Hz or 100 Hz vibration loading at 1, 7, and 14 d time points. Results are expressed as mean +SD (n= 6) and normalized relative to control culture (BM).

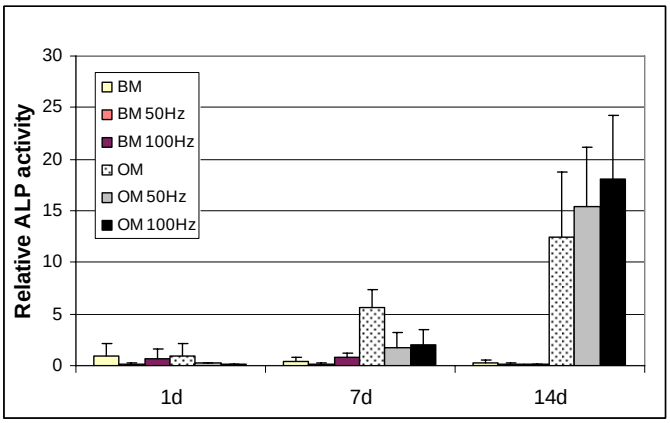

Figure 16. Preliminary results. Quantitative ALP activity of ASCs cultured in either BM or OM, and stimulated with 50 Hz or 100 Hz vibration loading at 1, 7, and 14 d time points. Results are expressed as mean +SD (n= 6) and normalized relative to control culture (BM).

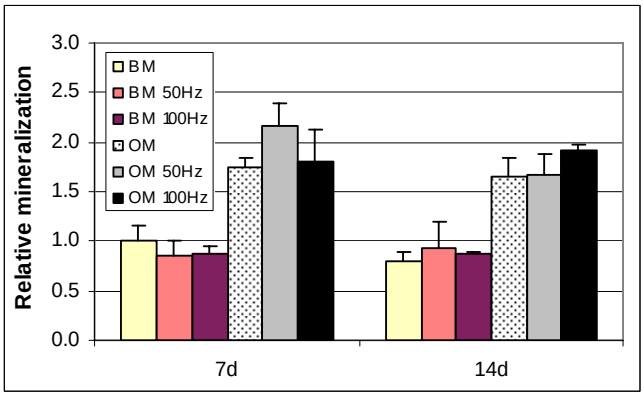

Figure 17. Preliminary results. Mineralization of ASCs cultured in either BM or OM, stimulated with 50 Hz or 100 Hz vibration loading at 7 and 14 d time points. Results are expressed as mean +SD (n= 6) and normalized relative to control culture (BM).