°Stress Conditioning and Heat Shock Protein Manipulation for Bone Tissue Engineering

Eunna Chung

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Marissa Nichole Rylander, Chairman
Linda A. Dahlgren
Joseph W. Freeman
Aaron S. Goldstein
Abby W. Morgan

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ABSTRACT

External stresses surrounding bone can stimulate heat shock proteins (HSPs), which are involved in anti-apoptosis, cell proliferation, and differentiation. *In vitro* stress modulation and HSP induction may be critical factors for enhancing bone regeneration. We investigated whether applying individual or combinatorial stress conditioning (thermal, tensile, and biochemical) and effective HSP modulation could induce *in vitro* responses in preosteoblasts indicating mitogenic/osteogenic/angiogenic/anti-osteoclastic effects. A preosteoblast cell line (MC3T3-E1) was exposed to conditioning protocols utilizing thermal stress applied with a water bath, tensile stress using a Flexcell™ bioreactor, and biochemical stress with the addition of growth factors (GFs) (*i.e.* transforming growth factor-beta 1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2)). Furthermore, the role of HSP70 in osteogenesis under normal conditions and in response to heat was investigated by transfecting preosteoblasts with HSP70 small interfering RNA alone or in combination with thermal stress and measuring cellular response. Heating at 44°C (for 8 minutes) rapidly induced osteocalcin (OCN), osteopontin (OPN), osteoprotegerin (OPG), vascular endothelial growth factors (VEGF), and cyclooxygenase 2 (COX-2) mRNA at 8 hour post-heating (PH). The addition of GFs with heating induced OPG and VEGF genes more than heating or GF addition alone. OPN, OCN, and OPG secretions increased with the addition of GFs. However, matrix metalloproteinase-9 (MMP-9) secretion was inhibited by heating, with more significant declines associates with GF inclusion. Equibiaxial tension (5%, 0.2 Hz, 10 seconds tension/10 seconds rest, 6 days) with GFs enhanced proliferation than tension or GF addition alone. MMP-9 secretion decreased in response to tension alone or more with GFs. Tension (1-5%, 24 hours) with GFs induced prostaglandin E synthase 2 (PGES-2), OPG, and VEGF genes more than tension or GFs alone. Combinatorial conditioning with thermal stress (44°C, 8 minutes) and tension (3%, 0.2 Hz, 10 seconds tension/10 seconds rest, 4 hours for HSP

gene and 24 hours for VEGF secretion and MMP-9 gene) induced HSP27 and HSP70, secretion of VEGF (protein), and suppression of MMP-9 (gene) more than heating or tension alone. HSP70 silencing followed by heating (44°C, 8 minutes) enhanced expression of HSP27. Mitogenic activity was inhibited by heating with more significant decrease occurring by heating and HSP70 silencing. At 10 hours PH, TGF-β1, MMP-9, and ALP mRNA decreased in response to heating and HSP70 silencing. At 48 hours PH, heating following HSP70-silencing induced VEGF secretion significantly. In conclusion, effective application of individual or combinatorial conditioning utilizing heating, tension, and GFs could be beneficial as a bone healing-strategy by rapidly inducing stress proteins (HSPs), angiogenic factor (*e.g.* VEGF), anti-osteoclastogenic cytokines (*e.g.* OPG), and bone matrix proteins (*e.g.* OPN and OCN) with anti-resorptive activity by inhibiting MMP-9.

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Attribution

Marissa Nichole Rylander, a co-author in the main research chapters (chapter 3-6), supervised the hypothesis, aims, methodologies, and interpretation of studies and assisted to prepare the dissertation (manuscripts) as my advisor faculty.

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Abbreviation

HSP	heat shock protein
BMP-2	bone morphogenic protein-2
TRAP	tartrate-resistant acid phosphatase
TGF-β1	transforming growth factor beta 1
OPN	osteopontin
OCN	osteocalcin
ALP	alkaline phosphatase
OPG	osteoprotegerin
RANK	receptor activator of NF (nuclear factor)-κB
RANKL	receptor activator of NF-κB ligand
VEGF	vascular endothelial growth factor
MMP	matrix metalloproteinase
ON	osteonectin
BSP	bone sialoprotein
PGE	prostaglandin
PGES-2	prostaglandin E synthase 2
COL-1	type I collagen
COX	cyclooxygenase
IGF-I	insulin-like growth factor-I
PDGF	platelet-derived growth factor
FGF	fibroblast growth factor
RT-PCR	reverse transcription polymerization chain reaction
ELISA	enzyme-linked immunosorbent assay
MEM	minimal essential medium
FBS	fetal bovine serum
PS	penicillin-streptomycin
FITC	fluorescein isothiocyanate
DAPI	4’,6-diamidino-2-phenylindole
PDVF	polyvinylidene difluoride

PH	post heating
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
GF	growth factor
FDA	U.S. Food and Drug Administration
EU	European Union
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
WTS	water soluble tetrazolium salts
PKC	protein kinase C
PTK	protein tyrosine kinase

Chapter 1: Introduction

1.1. Background of Bone Physiology

1.1.1. Bone anatomy

Our body is composed of 206 bones systematically connected to each other and located precisely to facilitate specific function [1]. **Bone** can be categorized according to its location or shape such as craniofacial bone, long bone in arms and legs, and spine, etc. Bone is responsible for supporting the weight of the entire body and controls physical movement. Due to this mechanical function, bone is surrounded by a highly dynamic environment that provides mechanical cues such as compression, tension, and fluid shear stress to bone cells. Bone tissue is composed of 5-10% cells surrounded by inorganic minerals (50-70%) and organic matrix (20-40%, mainly type I collagen) with extra lipids (3%) [2]. It includes different cell types (*e.g.* **osteoblasts, bone-lining cells, and osteocytes**) and a variety of extracellular matrix components including collagens, non-collagenous matrix proteins, and minerals (*e.g.* **hydroxyapatite**, $Ca_{10}(PO_4)_6(OH)_2$) [1, 2]. Bone **extracellular matrix** (ECM) can be primarily divided into collagens (I, III, V, and X) and non-collagenous proteins such as osteopontin (OPN), bone sialoprotein (BSP), osteonectin (ON), and osteocalcin (OCN) [1, 2]. Gaps between multiple aligned collagen fibers produce an ideal space for minerals [2]. Extracellular matrix (ECM) in bone controls bone formative responses of osteoblasts and can deposit minerals [3]. As illustrated in Figure 1-1, a long bone has complex structures closely associating with bone marrow, nerves and blood vessels. This long bone tissue such as tibia or femur was composed of two different kinds of bones: compact (cortical) and spongy (trabecular) bone, which are classified according to their morphological characteristics. The outer bone surface is covered with a membrane called the periosteum and the inner surface in contact with bone marrow is layered with endosteum. **Compact bone** consists of multiple subunits of an **osteon** including osteocytes, which interact through a tiny tubule, or canaliculus. Passageways through canaliculi can generate mechanical stress due to fluid flow near bone cells [4]. Blood vessels and nerve tissue reside within the Haversian canal, penetrating the center of the osteon and connecting neighbor osteons. The cooperation between osteoblasts and osteoclasts has a critical role in

controlling bone remodeling through growth factors and signaling molecules such as ephrins, parathyroid hormone-related protein, and receptor activator of NF-κB ligand (RANKL) [4, 5].

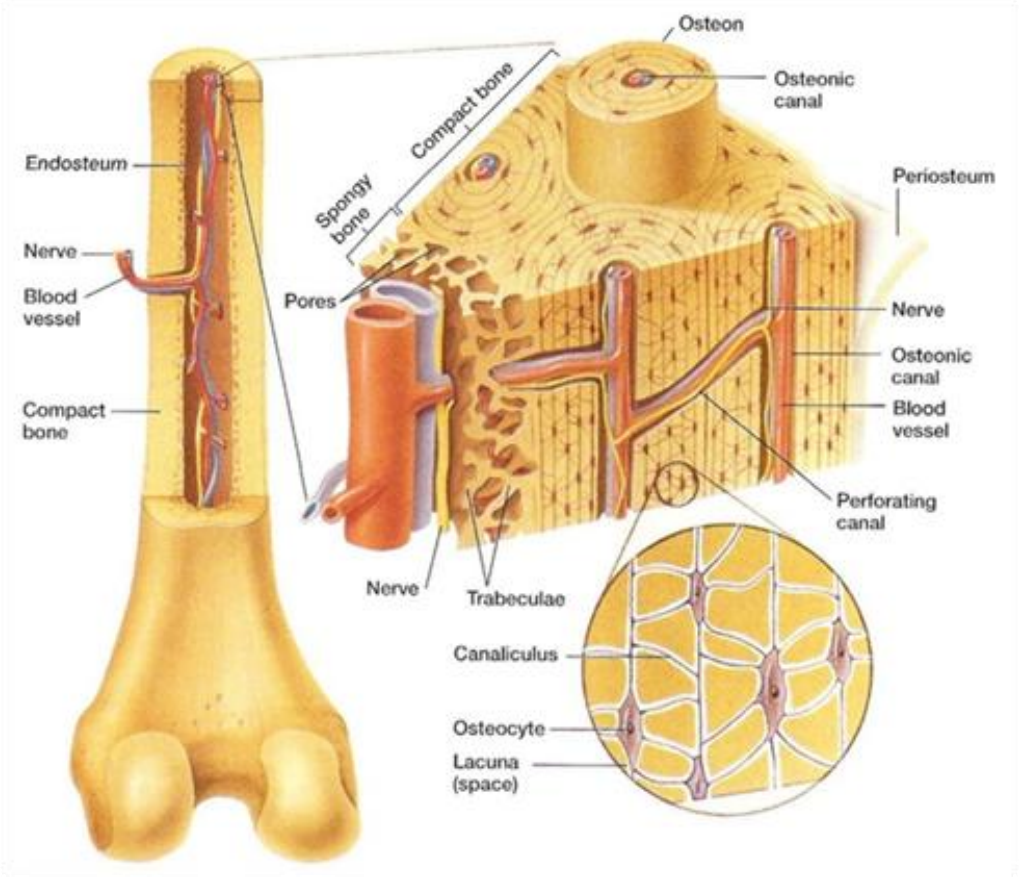

Figure 1-1. Anatomic illustration of long bone [6]. Used with permission of McGraw-Hill. "*Hole's Human Anatomy and Physiology (7th edition)*," David Shier et al., Figure 7.5. Compact bone is composed of osteons cemented together by bone matrix (p.190)

1.1.2. Cells critical for bone physiology

Osteoblasts generate minerals for formation of bone ECM environment and express growth factors for osteogenesis and angiogenesis [3, 7]. The roles of osteoblasts are mediated by many chemical cues such as growth factors, lipid metabolites, steroids, and hormones [8]. Osteoblasts can be observed on the bone surface where bone generation happens [9]. Osteoblasts

mature into osteocytes, which contribute to bone hormone-mediated metabolism and mechano-sensing [4]. **Osteocytes** account for the largest number of cells (95%) in mature bone [10] and are cells differentiated from the developmental lineage of mesenchymal stem cells via osteoblasts [11]. Osteocytes regulate osteoblasts and osteoclasts by transforming mechanical strain-induced shear stress into the cellular cues through integrin and CD44 receptors and secreting mechano-active molecules such as prostaglandins and nitric oxide (NO) [12]. **Bone lining cells** (or resting osteoblasts) are surface-covering long cells, contact bone marrow on one side, and interact with osteocytes, which reside in the osteon unit, on the other side through gap junction [13]. Like osteocytes, bone lining cells are hypothesized as a mechano-sensor and derived from osteoblasts in the surface [13, 14]. Secretion of biochemical signals by bone lining cells in response to mechanical loading initiates bone remodeling [13].

Osteoclasts, which differentiate from hematopoietic cell origin, are critical for bone remodeling as bone-resorbing cells possessing several nuclei [15]. Key molecules in osteoclast-mediated bone resorption are hydrochloric acid (HCL), which is responsible for breaking down hydroxyapatite, and hydrolases (*e.g.* cathepsin K and tartrate-resistant acid phosphatase (TRAP)) that can degrade collagen fibers or other ECM proteins [16]. Three different kinds of bone cells (*i.e.* osteoblasts, osteoclasts, and osteocytes) can communicate with each other by secreting various signaling molecules such as bone morphogenic proteins (BMPs) and NO [16]. The balance between osteoblasts and osteoclasts dynamically controls bone remodeling. Osteoblasts promote bone formation and osteoclasts enhance bone resorption. If the osteoblast-osteoclast balance breaks down, bone diseases result from insufficient mineralization and mechanically weak bones. However, the mechanism of bone remodeling is not clearly understood. Bone formation can be involved in different physiological processes such as fracture healing, distraction osteogenesis, and bone development, etc.

1.1.3. Important events in bone physiology: angiogenesis, osteoblastogenesis, and osteoclastogenesis

Bone regeneration requires blood vessel formation during the fracture healing process as illustrated in Figure 1-2. Bone healing is initiated by inflammation and angiogenesis [17]. Formation of new blood vessels is associated initially with regeneration of spongy bone near the gap of a fracture area [18]. *In vitro* co-culture strategies of bone cells and endothelial cells in a

separate but permeable chamber or scaffold have been used to demonstrate the interaction between osteoblasts and endothelial cells [19]. **Osteoblastogenesis** is the differentiation process into osteoblasts from mesenchymal stem cells [20].

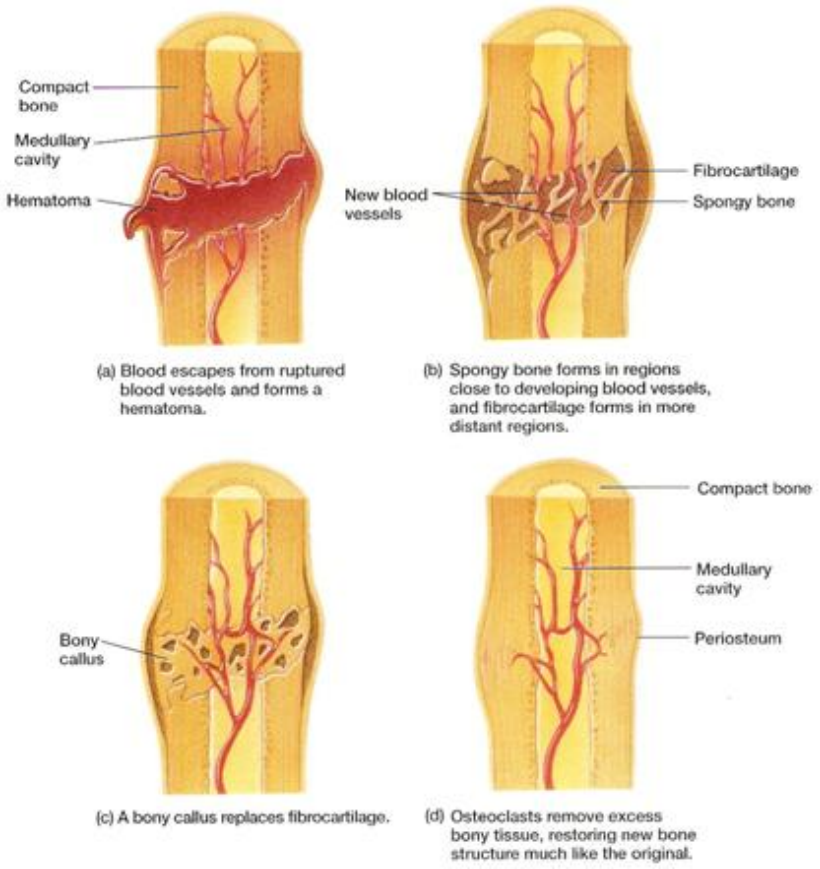

Figure 1-2. Fracture healing [18]. Used with permission of McGraw-Hill. "*Hole's Human Anatomy and Physiology (7th edition),*" David Shier et al., Figure 7B. Major steps in repair of a fracture. (p.199).

In addition to osteoblastogenesis, **osteoclastogenesis** is another important process controlling bone tissue metabolism**.** Osteoclastogenesis is a serial differentiation from hematopoietic stem cells to osteoclasts which occur through commitment, differentiation, multi-nucleation, and maturation process [21, 22]. Since osteoclasts are in charge of removing bone,

mediators of osteoclast differentiation or osteoclast activity can be clinical targets to treat bone diseases with low bone mass density. MMP-9 is one of the osteoclastic markers with cathepsin K and TRAP. Recently, therapeutic approaches for osteoporosis have evolved from inhibition of osteoclast resorptive activity to improvement of bone forming capacity in the bone microenvironment [23].

1.2. Failure of Bone Tissue: Statistical and Pathological Approach

1.2.1. Prevalence of bone failure

Bone loss and skeletal deficiencies arising from traumatic injury, abnormal development, cancer, and degenerative bone diseases significantly influence the health and mobility of millions of Americans and frequently require surgical intervention. Degenerative bone disorders such as osteoporosis (which affects approximately 75 million people in Europe, USA, and Japan [24]) weaken bones, making them so brittle that even mild stresses such as bending over, lifting a vacuum cleaner, or coughing can cause a fracture. Nearly 30-50% of women and 15-30% of men will suffer a fracture related to osteoporosis in their lifetime [25-28] and seniors in the age range of 80-85 showed higher fracture risks of 3.81 fold for non-vertebral and 13.4 for vertebral case compared to people within the 50-59 year old range [29]. The developed countries of EU, Canada, and the U.S.A spend 48 billion dollars annually to treat osteoporosis-caused fractures in the workplace [30].

1.2.2. Cause of bone failure

The factors causing bone failure associated with injury and diseases can be categorized according to the following [31]: severe trauma, inflammation, virus infection (*e.g.* Paget's disease), genetic abnormality, aging, degenerative disease (*e.g.* osteoporosis), and cancer. Major bone diseases are associated with different pathological processes. In terms of osteoporosis, in particular, the underlying cause is an abnormal functionality between proliferation and differentiation of bone cells such as osteoclasts or osteoblasts, inducing an imbalance between bone formation and resorption [32]. Furthermore, since bone disease can be mitigated by controlling osteoclast activity, several targets have been suggested. These are impeding MMP-9,

RANKL/RANK interaction, integrin-mediated mechanism, and cathepsin K protease or stimulating osteoclast apoptosis [31]. In this study, we are focused on osteoporosis to develop a therapeutic approach to inhibit osteoclast activity as well as to enhance osteoblast differentiation or function.

Similar to osteoporosis, Paget's disease, the second most prevalent metabolic bone disease, induces bone failure due to a problematic surplus of osteoclast activity [33]. Candidate causes for this disease include gene modification and viral attack and may possibly be related to abnormal outcomes of bone structure and mechanical properties in Paget's diseases [33]. Osteopetrosis is also related to an abnormal modification of osteoclasts, but an impaired bone-resorptive activity, due to gene mutation, causes an excess of bone mass [34].

1.3. Targets for therapeutic approach and clinical trials for bone diseases

Currently, novel medical research and clinical trials, which treat damaged tissue using artificially engineered substitutes or exogenous delivery of gene, protein, and pharmaceutical molecules, have been investigated.

1.3.1. Cell-based therapy: tissue engineering

Functional tissue regeneration requires the use of a successful combination of cells, scaffolding, stress preconditioning, and bioactive molecules. The slow rate of angiogenesis and limited mass transfer in scaffolds frequently result in diminished cell proliferation [35, 36]. This issue requires harmonious incorporation of neo-blood vessel formation and tissue-engineered bone by microfabrication, microsurgery, growth factors, and co-cultivation with endothelial cells [35].

1.3.1.1. Cell source for bone tissue engineering

Bone tissue engineering takes advantage of the highly regenerative efficiency and availability of various cell sources such as osteoblasts, mesenchymal stem cells, embryonic stem cells, multipotent periosteum cells, skeletal muscle-derived stem cells, prenatal stem cells, genetically modified cells, and human skin fibroblasts to induce bone formation [37]. Among these cell types, osteoblasts and mesenchymal stem cells have been investigated most extensively. **MC3T3-E1** cells, a murine calvaria-originated preosteoblast cells, is one of the

most popular cell lines in bone research [38-40]. It is well-known that the MC3T3-E1 cell line can facilitate mineralization *in vitro* in response to culturing with osteogenic conditioned media for a period of several weeks (after 16 days) [41]. This osteogenesis is controlled by osteo-inductive chemicals (*e.g.* L-ascorbic acid and β-glycerol phosphate) dose-dependently [42]. Moreover, MC3T3-E1 cells have demonstrated proliferation and differentiation in various scaffolds such as polycaprolactone (PCL) electrospun scaffolds [39]. According to Choi *et al.*'s study [41], MC3T3-E1 cells express several bone-related proteins (*e.g.* type I collagen, transforming growth factor-β1, and osteocalcin) with different induction trends depending on the cultivation period. MC3T3-E1 cells undergo a proliferative process with histone gene inductions during days 4-10, a bone matrix production process with type I collagen, TGF-β1, and osteonectin inductions during days 10-16, and a mineralization process with osteocalcin and bone nodule production during days 16-30 *in vitro* [41]. Primary stem cells can be applied as a more appropriate cell source for tissue engineering because they conserve their natural properties of proliferation and differentiation. The isolation of **mesenchymal stem cells (MSCs)** from bone marrow by aspiration autologously is followed by culturing and seeding of cells into a 3D scaffold. These cells can be induced into a specialized tissue or organ such as cartilage, bone, tendon, ligament, and fat [43]. According to a recent review paper [43], MSCs can function as an immuno-supressor, blocking T cell-involved reactions, promoting angiogenesis and proliferation by generating several bioactive factors (*e.g.* granulocyte colony-stimulating factor, interleukin-6, and stem cell factor), as well as have multi-differentiation capacity. In the future, MSCs may be one of the most clinically feasible cell sources. These characteristics of MSCs can not only enable safer application of allogenic sources but, also solve the insufficiency of vasculatures in a tissue-engineered scaffold [43].

1.3.1.2. Bioactive molecules

Growth factors have been utilized to enhance tissue regeneration and stem cell differentiation in scaffolds [44, 45] or through direct delivery to cell cultures [46]. Major growth factors related to bone physiology are insulin-like growth factor (IGF), bone morphogenetic protein (BMP), transforming growth factor (TGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) groups [47]. A number of *in vitro* and *in vivo* studies have shown that *in situ* delivery of exogenous bone-relevant growth factors have a significant healing effect on bone tissue [48-50]. Recently, growth factor delivery has been developed and evaluated as

one of the most powerful chemical cues in tissue engineering [45, 51]. Recombinant human (rh) **BMP-2**, a FDA-approved growth factor [52], can induce osteogenesis of mesenchymal cells [53]. MC3T3-E1 cells have shown enhanced ALP and OCN following exogenous delivery of BMP-2 and increased cell proliferation associated with TGF-β1 addition [38]. BMP-2 and BMP-4 expression are critical for bone formation [54]. **BMPs** are produced by osteoprogenitor cells, osteoblasts, and chondrocytes, *etc*., and can provide chemical cues for osteoprogenitor migration, proliferation, and differentiation, and bone matrix production [47]. **TGF-β** is produced from various cell types such as osteoblasts, endothelial cells, chondrocytes, platelets, and macrophages, etc [47]. In bone, TGFs are an initial inducer of differentiation of osteoblasts [47]. However, introduction of osteoclast precursors by TGF prevents the final differentiation of osteoblasts [47]. In addition, the activated signaling pathway by BMPs and TGF-β can interact in several intracellular locations with other signaling pathways such as Wnt, PI3k/Akt, MAPK, protein kinase C (PKC), and protein tyrosine kinase (PTK) [55, 56]. Two growth factors (*i.e.* rhBMP-2 and TGF-β1) have been developed as strategies for tissue repair in prior studies [45, 51]. MC3T3-E1 cells exhibit enhanced osteogenic responses expressing alkaline phosphatase (ALP) and osteocalcin (OCN) following an exogenous delivery of BMP-2 [57], and increased cell proliferation by TGF-β1 treatment [38]. In addition, the synergistic stimulation effect of exogenous growth factors and stress conditioning is not well known.

1.3.1.3. Bioreactor

Bioreactors serve as *in vitro* culture systems, which can modulate automated stress treatment of cells or artificial tissues inducing down-stream signaling to promote mitogenic or functional activity. Bioreactors capable of modulating mechanical stress (*e.g.* tension, compression, and shear stress) have been utilized for cell monolayers and 3D scaffolds containing differentiated cells such as osteoblasts or stem cells to induce their stimulation via mechanotransduction [58]. For monolayer cells or 3D hydrogels, tensile stress can be generated by modulating a flexible silicone surface at the bottom of multiwall plates by negative vacuum pressure using the **Flexcell® Tension System**. This system is a commercial bioreactor, which has been utilized by numerous research groups for tensile stress conditioning [59-61]. Advantages of this bioreactor are that tensile stress can be applied in the uniaxial or equibiaxial (biaxial) direction depending on selection of specific loading posts. This system is computer-controlled and tensile loading can be monitored in real-time. Simple set-up is convenient to modulate the

rest time and cycles of tension loading for short- or long-term study. In addition to cell monolayer on the silicone membrane, 3D scaffolds such as polyurethane nanofibrous scaffold [62], hydrogels (*e.g.* fibrin [63], and collagen gel [64]) can be used in the Flexcell® Tension bioreactor.

1.3.2. Gene or protein therapy: gene modification, drug delivery system (DDS)

A novel technique, **small interfering RNA (siRNA) gene silencing**, has been used to investigate the function of proteins of interest by suppressing the specific gene expression of the target protein [65]. For example, HSP27 silencing was investigated due to its protective role against apoptosis to target chronic lung failure [65]. The small piece (21-25 nucleotides) of double-stranded RNA breaks down specific mRNA, impairing the production of the target protein [66]. The siRNA technique can be used to suppress the expression of problematic proteins involved in disease pathology or the degeneration of tissue [67, 68]. In contrast, we can utilize **transfection** for gene induction of "beneficial" proteins. For example, bone marrow cells infused with FK1012, a drug that is involved in protein dimerization, showed higher proliferative capacity, according to Jin *et al.*'s study [69]. Drug molecules can also be introduced in a diseased area of the body using an artificial shuttle. For example, polymeric carriers combined with anti-cancer **drugs** (*e.g.* Doxorubicin and Paclitaxel) have been investigated to overcome the limitations of targeting and delivering a drug into a "tough" destination such as the nucleus [70, 71].

1.3.3. Combined therapy: gene or protein intervention with tissue engineering

Gene or protein delivery systems to upregulate growth factors of interest have been established. This is a critical methodology in regenerative medicine given that long-term activation of chemical cues is required for *in vivo* tissue regeneration. Adult stem cells transfected with VEGF cDNA showed enhanced blood vessel density in PLGA scaffolds [72]. Furthermore, protein delivery systems for growth factors such as rhBMP-2 [44, 51] and TGF-β1 [45], combined with polymeric carriers, have been commonly used in bone relevant research. In general, bone tissue engineers have preferred gene therapy to promote the expression of growth factor-specific genes over protein-based delivery due to several disadvantages of protein-based delivery such as precise targeting and expensive cost of exogenous growth factors [73].

1.4. Evaluation of Bone Cell Activity for Bone Regeneration

1.4.1. Mitogenic activity: proliferation or cytotoxicity/apoptosis

Numerous studies, which have investigated stress conditioning (*e.g.* thermal stress [74-76] and tensile stress [59, 77-80]) and tissue regeneration [81, 82], measured proliferative or cytotoxic response to varying protocol parameters. Examination of cell survival and growth over time is essential to evaluate whether a novel biomedical strategy may have toxic side effects. To measure the alteration of proliferation or viability, accepted assays include: metabolic assay (*e.g.* 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [79], 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) [81], water soluble tetrazolium salts (WST) [82], and alamarBlue [83]), apoptosis assay (caspase3 [75]), DNA assay (*e.g.* PicoGreen [84], ^{3}H-thymidine [85], and bromodeoxyuridine (BrdU) labeling [76]), crystal violet staining [59], trypan blue staining/direct counting of cell numbers [86], and fluorescence-activated cell sorting (FACS) (*e.g.* propidium iodide fluorescence tagging [87]).

1.4.2. Characterization of critical bone-related molecules or hormone

Identification of osteoblasts is accomplished by examining specific genes or proteins of bone phenotype though molecular biological research methodologies such as PCR, immunocytochemistry, and western blot. Investigation of bone regeneration and osteogenesis has been accomplished through detection of expression of bone relevant proteins (*e.g.* OPN [88], OCN [89], and OPG [90]), growth factors (*e.g.* VEGF [91], BMP-2 [40] and TGF-β1 [41]), and enzymes such as matrix metalloproteinases (MMPs) [92] in osteoblasts. Depending on the localizations and roles of bone marker genes or proteins, the most appropriate characterization method for each protein must be chosen and optimized. For example, proteins released outside the cells (*e.g.* VEGF and OCN) can be detected from culture media by enzyme-linked immunosorbent assay (ELISA). COX-2, a membrane-binding protein, can be analyzed by cell lysate with ELISA and western blot or visualized with immunofluorescence staining.

Bone matrix proteins are one of the most prevalent bone-related proteins for osteogenesis identification. Since **type I collagen** is a major type of bone collagens, numerous research groups have been investigated type I collagen expression by osteoblasts to evaluate the

cell activity producing matrix proteins [93-95]. As a noncollagenous matrix protein, **osteopontin (OPN)** is expressed from diverse cell types such as macrophages [96] and bone cells [88]. In bone, it has been recognized as an important marker related to differentiation of preosteoblast [88] or bone marrow cells [97]. **Osteonectin (ON)**, also known as a secreted protein acidic and rich in cysteine (SPARC), is a matrix glycoprotein with the ability to interact with collagen and calcium [98] and is enhanced under cyclic uniaxial tensile stress in human osteoblast [99]. **Bone sialoprotein (BSP)** and **OCN** are bone-specific matrix proteins that are indicators of bone formation [9, 100]. BSP includes Arg-Gly-Asp (RGD), sequences for cell attachment, polyglutamic acid which seems to be related to hydroxyapatite nucleation, and the sites for modifications such as sulfation, phosphorylation, and glycosylation [101]. BSP also mediates various bone-related biological processes such as association with calcium and MMP-2, mineralization, and angiogenesis [102]. MC3T3-E1 cells cultured on a substrate coated with BSP, in spite of denaturation, showed enhanced proliferation, ALP formation, and mineralization [103]. In contrast, fibronectin induced only cell growth with no effect on ALP activity [103]. From X- ray crystal analysis by Hoang *et al.,* porcine OCN with vitamin-K-dependent γ–carboxylated glutamic acid (Gla), which can interact with hydroxyapatite including 5 calcium ions, is a sphere form consisting of three α-helices residues [104]. OCN has been considered a clinical marker of the bone phenotype [100]. However, it is difficult to measure clinically due to rapid degradation and instability [100]. Furthermore, MC3T3-E1 cells expressed OCN after 4 day osteogenic cultivation in the polymer-based matrix, poly(lactic-co-glycolic acid) (PLGA) which was incorporated with BMP-2 [105].

In addition to matrix proteins, bone-related enzymes such as **alkaline phosphatase (ALP)** and **matrix metalloproteinases (MMPs)**. ALP gets rid of the phosphate group as a common hydrolase enzyme discovered in a wide range of organisms from human to bacteria [106]. Four isozymes are known to exist in human: germ cell, intestinal, tissue non-specific (liver/bone/kidney), and placental ALP [106, 107]. Among these, tissue non-specific alkaline phosphatase (TNAP), which in several cell types such as osteoblasts and odontoblasts, transforms pyrophosphates into inorganic phosphates to require for hydroxyapatite synthesis, inducing mineralization process [108]. MMP is an enzyme that can break down extracellular matrix (ECM) by directing various ECM-mediated cellular or tissue-level modifications such as cell migration, development, and wound healing [109]. This enzyme family exerts critical roles

in bone physiology. **Matrix metalloproteinase 13 (MMP-13)**, which can be expressed from osteoblasts, contributes to bone formation by dissociating collagen in endochondral ossification with MMP-9 [110]. **Matrix metalloproteinase 9 (MMP-9),** known as 92 kDa gelatinase or type IV collagenase is among 20 types of MMP-9 [109], and is an osteoclast-phenotyping protein that can potentially be a promising target to block osteoclast activity in bone microenvironment to cure bone abnormality [31]. Regulation of MMP-9 is very important in bone physiology since it plays a pivotal role in bone remodeling. MC3T3-E1, a mouse preosteoblastic cell line, appears to express several types of MMPs including MMP-2, MMP-9, and MMP-13 [111]. From Blavier *et al.*'s study, down-regulation of MMP following treatment with MMP inhibitors (*i.e.* CI-1 and RP59794) caused a decrease in osteoclast-mediated calcium release and migration resulting in MMP-9 and MMP-13 expression [112]. Furthermore, it is highly expressed in rat osteoporotic bone tissue identified through dot blot hybridization and in situ hybridization [113]. However, bone of osteoporotic women showed low levels of MMP-9 gene [114]. Treatment of receptor activator of NF-κB ligand (RANKL), an important communicative molecule between osteoblast and osteoclast for osteoclastogenesis, showed significant increase of MMP-9 gene in bone marrow-derived osteoclasts through NFATc1 mediation [115]. Possibly, over-expression of MMP-9 by osteoblasts in bone microenvironment could be pathogenic for bone diseases such as osteoporosis, which is resulted primarily from hyperactive osteoclast activity compared to that of osteoblasts. HSP70 transfection impaired MMP-9 elevation in asteocytes experiencing neuro-ischemic situation as well as normal status [116].

Prostaglandin E_2 (PGE_2), a fatty acid-derived hormone [117], and its synthases (*e.g.* cyclooxygenase) are related to diverse physiology (*e.g.* ovulation and bone metabolism) and pathology (*e.g.* cancer and inflammation) in our body [117, 118]. It also has been reported to be induced under bone mechanical stress response such as fluid shear stress [119] and can enhance differentiation in osteoblast [120]. With COX-1, the constitutive type of COX, **cyclooxygenase-2 (COX-2)** contributes to generate prostaglandin G_2 **(**PGG_2), a intermediate form in the PGE_2 synthesis cascade, from arachidonic acid [117]. Similarly to COX-2, **prostaglandin E synthase 2** (PGES-2) (gene name: Ptges2) is the enzyme involved in the process of **PGE_2** from PGH_2 originated from PGG_2 [117]. COX-2 can be indicators to bone metabolic or regenerative processes. A COX-2 inhibitor, a pain-releasing and anti-inflammatory drug, can hamper bone formation [121, 122]. Even though COX-2 can be an important indicator protein for bone-related

research, COX-2 is also known to be upregulated under mechanical stress in chondrocytes [123] and its suppression is targeted to develop an anti-cancer drug due to its increased expression in cancers [124]. In this study, we focus on regulations of two enzymes (COX-2 and PGES-2), involved in the synthesis of PGE_2, in response to external stress.

Osteoprotegerin (OPG) has a critical role in hampering osteoclast differentiation by intervening in the association between receptor activator nuclear factor κB (RANK) and RANK-ligand [125]. Moreover, bone morphogenetic protein-2 (BMP-2) can up-regulate OPG in MC3T3-E1 cells through Wnt/β-catenin [126]. Transforming growth factor (TGF-β1) can increase OPG/OCIF (osteoclastogenesis inhibitory factor) induction in MC3T3-E1 and bone marrow stromal cells [127].

Runx2 is a transcriptional factor that mediates osteoblast differentiation with β-catenin, Osterix and AP-1 [11, 20]. This factor causes multipotent mesenchymal stem cells to differentiate into preosteoblasts in the early process of osteoblastogenic differentiation while hampering other lineages, which are mediated by adipogenic (PRARγ2 and C/EBP) and chondrogenic (Sox5, 6, and 9) transcription factors [11, 20]. BMP-2 activates Runx2 via the collaboration of Smad 1/5 with Smad 4, inducing osteoblastogenic process in contrast to inhibitory aspects of TGF-β [20].

BMP-2 and **TGF-β1** are important osteoinductive growth factors. Evaluation of TGF-β1 as a drug molecule for bone regeneration has been actively performed even though there are contradictory functions of TGF-β1 in controlling osteoclast activity (*e.g.* osteoclastogenesis) depending on targeted cell type and cultivation system [23]. It seems that levels of active TGF-β in osteoclastic process may determine positive and negative cues by TGF-β [128]. Higher concentration of TGF-β than in the initial osteoclast differentiative phase control bone resorption of mature osteoclasts by stimulating OPG production by osteoblasts [128].

VEGF is a key growth factor controlling bone formation as well as angiogenesis by mediating communication between endothelial cells and bone cells [129]. Due to VEGF's role as an angiogenic inducer, numerous tissue engineering experts have investigated enhancing VEGF levels in a osteo-scaffolding system with VEGF gene or protein delivery [35].

1.4.3. Mineralization

Mineralization during osteogenic cultivation of preosteoblast and stem cells has been used as a standard evaluator for *in vitro* bone formation [76, 130]. Depending on cell types (*e.g.* MC3T3-E1 cells, primary osteoblastic, and bone marrow-derived stem cells) and culture conditions, *in vitro* osteogenic cultivation durations and calcium deposition levels can be different. MC3T3-E1 cells undergo the mineralization process by the supply of dexamethasone, and ascorbic acid, and β-glycerol phosphate, *etc* [130]. According to Choi *et al.*[41], these cells can mineralize for days 16-30 with the addition of ascorbic acid and β-glycerol phosphate. In addition, the mineralization of osteoblastic cells can be used to evaluate stress stimulation such as water bath heating, laser irradiation, and mechanical stress treatment. Counting of bone nodule or measurement of calcium content by staining of Alizarin red S and von Kossa have been commonly used in evaluating mineral deposition on cells or tissues [76, 131-133]. Quantification for mineralization can be accomplished using a commercially available kit (*e.g.* Osteogenesis Assay kit, catalog no=ECM815, Millipore) based on Alizarin red S straining by dissolving the stained spots of the cell culture system with acetic acid.

1.5. Stress Conditioning

Stress conditioning protocols can be a powerful therapeutic scheme to induce regenerative response from cells or tissues. Recently, a number of research groups, for the purpose of therapeutic application in bone tissue engineering, have developed chemical treatment with growth factors and cytokines as well as physical devices involving thermal, photochemical, and mechanical stresses (*e.g.* tension, compression, and hydrostatic pressure). The magnitude and duration of stress treatment is a key factor in determining the type and degree of biological impact on cells. Due to inhibitory effects of overdosed stress, determining the optimal conditioning methods and threshold levels of stress without cytotoxicity is a critical research objective. A better understanding of *in vitro* bone tissue formation in response to external stress is important to develop a medical methodology for bone disease treatment and fracture healing. A number of research groups have investigated the response of bone cells and regenerative potential of stress conditioning (*e.g.* mechanical stress, chemical cues) to mimic physiological conditions and enhance bone regeneration through supra-physiological stresses. **Hormesis**

indicates that exposure to low or mild levels of stresses such as heating or toxins can confer positive survival ability upon cells in danger or deterioration such as aging [134]. The previous hormetic research has primarily focused on anti-aging using fibroblasts. For example, repeated mild heat stress (RMHS) (41°C at an incubator, 60 minutes, 4 times, 2 day gap between repetitions), applied to fibroblasts, enhanced collagen synthesis and more rapid shrinking of the collagen gel matrix, suggesting faster wound healing [135]. The advantageous mechanism due to RMHS (41°C for 1 hour twice per one week) is attributed to the repair of impaired proteins by stimulating heat shock proteins though the modulation of heat shock factors [134].

1.5.1. Thermal stress

Utilization of thermal stress requires elaborate stress conditioning protocols because a small difference in temperature elevation or heating duration can induce cellular injury and death. Through thermal induction of heat shock proteins (HSPs), cells can auto-protect themselves and survive otherwise lethal insults. Notorious biological responses such as cell death and impaired of protein synthesis can be triggered by heating at over 45-50°C for shorter time such as 10 minutes, and temperatures of 43-44°C for longer durations than 15 minutes [74, 136-139]. Hojo *et al.* reported that thermal stress was dominated by heating temperatures and durations [74]. Low-level heating (T<41°C, t<30 minutes) has been shown to have stimulatory effects on chondrocyte proliferation and proteoglycan metabolism. However, high levels of thermal stress (T>43 °C, t>15 minutes) impaired chondrocyte activity [74]. The cellular mechanism associated with thermal stress appears to proceed by the commingling of signaling pathways between two HSF-mediated HSPs and MAPK-signaling pathways [140].

1.5.1.1. Heating method: incubation in the incubator, water heating bath, microwave treatment

Using an incubator or a water bath for low-level heating -usually lower than 40-42°C- can induce *in vivo* or *in vitro* positive responses (*e.g.* anti-apoptosis, proliferation, and inductions of extracellular matrix proteins) for tissue regeneration in several cells/tissues related to skin [141], muscle [75], cartilage [74], teeth [142], and bone [76, 143]. In addition, microwave heating at 42.5-44°C stimulated bone formation *in vivo* [144]. Heat-shocked osteoblasts can assist in differentiating bone marrow cell into bone cells [131]. Moreover, heat shock can cause growth factor production from cells [85]. However, severe heat shock (higher than 48°C) can

promote inhibitory effects on stressed cells which varies with cell type [137, 138]. For example, heating osteosarcomic cells (*e.g.* HOS85, SaOS-2, and MG-63 cells) at 42°C for 1 hour inhibited cell proliferation and ALP production [145]. Thermal conditioning to enhance cellular activity (*e.g.* proliferation, migration, and differentiation) has rarely been investigated compared to other stress conditioning strategies such as chemical cues and mechanical stress. Therefore, *in vitro* optimization of thermal stress conditioning should be further demonstrated for bone tissue engineering applications.

1.5.1.2. Laser irradiation

Laser irradiation may provide a slightly different mechanism than water bath heating for bone regeneration by mediating combined thermal and photochemical stress [146]. Bio-stimulation using lasers can enhance cell proliferation and differentiation [132, 147]. However, biological effects associated with laser irradiation showed a big variation depending on laser type, output power, treatment protocols (treatment duration, dose times, and dose gaps) as well as target cell type or tissue. Low intensity laser irradiation (*e.g.* Ga-As-Al, wave length=670 nm, power=15 mW) showed faster induction of bone formation in laser-treated groups compared with the sham-treated rats [148]. Laser-induced thermal stress is correlated with HSP70 expression and can be used as a methodology for wound repair [146]. In laser research, stem cells or progenitor cells have also been studied *in vitro* with diverse laser types. Eduardo *et al*.' s group reported 20 mW of low level laser irradiation showed higher proliferation of stem cells (human dental pulp stem cells, hDPSCs) compared to 40 mW [147]. Furthermore, laser stimulation of bone marrow cells with a polarized He-Ne laser (632.8 nm) enhanced proliferation and osteogenesis from results acquired from diverse bone-related staining (*e.g.* von Kossa and alizarin) and a biochemical assay such as ALP activity assay [132]. To investigate the stimulation effects of laser treatment as an additional treatment for post-surgery, 780 nm laser was used to treat rat bones leading to activation of osteoblasts and more bone marrow cells [149]. In addition, laser-treated osteoblasts showed promoted levels of bone nodules [150], inductions of bone-related proteins [95], and increased ALP activity [151]. However, protocols for conditioning with laser irradiation are not fully optimized. To optimize laser conditioning for wound healing, HSP70 expression was analyzed as an indicator to represent cellular response to treated stress [146]. Furthermore, the bio-stimulating process due to thermal and photochemical stress has not been totally understood.

1.5.2. Mechanical stress

Over 100 years ago, Julius Wolff, stated that bone tissue can adapt to external mechanical loads by functional and structural modification [4]. Similarly, according to Frost, lower mechanical strain than 200 με results in a decrease in bone mass but, higher supraphysiological level than 2500 microstrain (με) (*i.e.* 0.25% magnitude) of tensile stress stimulated bone growth [152, 153]. In addition, this adaptation by NO and prostaglandin is dominated by osteocytes and bone-lining cells which can respond to fluid shear stress through canaliculi, which the interstitial fluid of bone flows into due to mechanical compression [4]. This low-level of shear stress near bone cells can not only deliver necessary nutrients (glucose) but also intracellular signaling cascades sensitive to mechanic signal [4]. The importance of mechanical stress can be demonstrated by investigating the problematic physical conditions of bone without mechanical stress. Lack of mechanical stress for example during space flight can decrease bone formation, serum osteocalcin, parathyroid hormones, and bone strength [154].

1.5.2.1. Tensile stress

Tensile stress can be defined from Hooke's law as shown in Eq. 1 [155]. This equation is usually illustrated as Stress-Strain (S-S) curve graph; X-axis is stretched level (usually %) and Y-axis denotes stress (Pa). To some extent, the correlation between strain and stress is linear and the slope is defined as the Young's modulus. As an indicator of biomechanical analysis, Young's modulus or maximum stress at fracture has been interpreted to determine the tensile property of tissues or materials.

$Equation\ 1$ $\sigma = \frac{F}{A} = E \times \varepsilon$,

where F is the loading force, A is unit surface area, σ is the loading stress, and E is the Young's modulus (E or tensile elastic modulus), and ε is strain or deformation [155].

Tensile stress conditioning in bone research is designed to mimic naturally generated tensile stress, usually lower than unidirectional strain of 0.3% in human and animal bone (*e.g.* ulna of the rooster) due to physical movement or exercise [4, 156]. In fact, 0.2-5% of low-level strain occurs in bone healing processes (*e.g.* distraction osteogenesis and fracture healing) in our body [119]. However, i*n vitro* strategies for tensile stress treatment are dependent on various test conditions such as loading style (stretching 2D flexible monolayer by vacuum pressure or direct-stretching 3D scaffolds), elongation magnitude, cycle number, loading duration, frequency,

matrix materials, and the intervention of a rest phase. Numerous studies have utilized the **Flexcell® tensile bioreactor** (Figure 1-3) for bone research. Depending on the type of loading post below the flexible membrane, the cells attached on the flexible substrates are deformed differently, primarily in the uniaxial and biequiaxial direction. According to Geest *et al.*'s modeling work using strain energy function (Appendix I) and finite element analysis, actual levels of strain treated with a Flexercell™ bioreactor (Flexcell Corp, McKeesport, PA) were generated differently according to the loading post [157]. In biaxial stress with a circular loading post, the radial strain level near the edge of the loading post was greater than those of other areas. In addition, the circumferential strain in the center area was higher even though both directions of biaxial strain showed almost similar levels suggesting adequacy of the "equibiaxial" term for biaxial tensile. On the other hand, in uniaxial tension with a rectangular loading post, X-axial strain remained similar along the X axis, but decreased significantly on the Y axis. In contrast, in

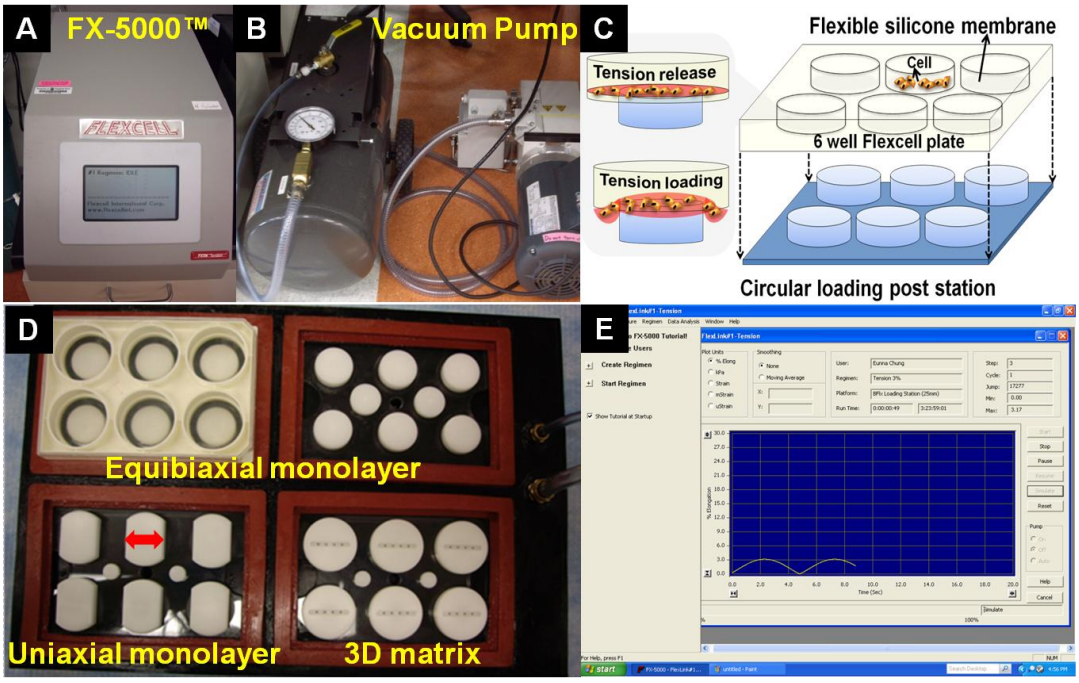

Figure 1-3. Flexcell® bioreactor. FX-5000™ (A), a vacuum pump (B), illustration of tensile stress generated by negative vacuum pressure (C), different loading posts (D), and FX-5000 computer program (E). Photos and illustration by author, 2010

Y-axial strain, there were the modifications of stress type from compression to tension as it was getting farther from the center in both X and Y directions [157]. In addition, Colombo *et al.* confirmed similar strain levels between longitudinal (Y) and horizontal (X) direction using a biaxial Flexcell® bioreactor [158]. Novel equations representing the correlation between biaxial tensile stress and vacuum pressure, which is generated by Flexcell® bioreactor after 24 hour stretching is shown in Eq.2 [158]. The equation was modified depending on the tensile stress loading time, showing tensile strain level was elevated, as the loading time increased [158]. Furthermore, Colombo *et al.* determined the loaded level of biaxial tensile stress variation depending on the size of the circular loading post (25 and 28 mm) and conditioning duration (0, 24, and 48 hours), frequency (0.1, 0.5, and 1 Hz), and magnitude (5, 10, 15, and 20 %) of stretching using Flexcell® bioreactor [158].

$Equation\ 2 \quad \varepsilon_{24h} = -1.64 \times 10^{-04} \times p^3 + 1.02 \times 10^{-02} \times p^2 + 7.62 \times 10^{-02} \times p$

$Equation\ 3 \quad \varepsilon_{flexcell} = -9.49 \times 10^{-06} \times p^3 + 2.27 \times 10^{-03} \times p^2 + 1.01 \times 10^{-01} \times p$

where, *P* is Flexercell pressure output.

Cyclic tensile stress treatment *in vitro* (9% magnitude, 0.2 Hz) induced IL-6, PGE_2 and COX-2 in MC3T3-E1 cells analyzed by RT-PCR and ELISA [159]. According to Liu *et al.*, osteoblast-like SaOs-2 cells were exposed to 5-12.5% (0.5 Hz) for 24 hours to investigate the inductive effect on type I and III collagen genes depending on stress magnitude [160]. Cyclic tensile conditioning protocol can be divided into uniaxial and equibiaxial stretching. Modification of cell shape was different depending on the direction of tensile stress. U937 macrophage-like cells aligned as distinct spindle shapes parallel to the stretching direction in uniaxial tension. In contrast, under biaxial stretching, cells exhibited shape variation throughout the dish and appeared to possess a "starfish"-like cell morphology (center area) [161]. Smooth muscle cells under cyclic tensile stress in Flexcell® bioreactor not only promoted proliferation and migration but also the up-regulation of MMPs (*e.g.* MMP-1, 2, and 3) [162]. In addition, Metzler *et al.* demonstrated that endothelial cells produced different levels of pro-inflammatory proteins (*e.g.* E-selectin, ICAM-1, and VCAM-1) depending on the magnitude of tension (5, 10, and 20% for 24 hours) using a Flexcell® bioreactor [163]. Continuous tensile load (8%, equibiaxial) induced MMP13 and *c-fos* gene in MC3T3-E1 cells following treatment for several

hours through a MEK/p42/p44 pathway [164]. Cycle number and frequency are pivotal determinants in controlling proliferation of bone cells [165]. Furthermore, mRNA of growth factors such as TGF-β1 [80] increased in osteoblastic cells by low-level tensile stress (0.17%). However, equibiaxial strain by Flexcell® bioreactor is difficult to analyze because the cells respond differently depending on the location of the culture substrate [153]. Table 1-1 shows the tensile stress conditioning protocols utilized for this study.

Table 1-1. Flexcell® tensile stress conditioning protocols utilized for the current study.

Tensile stress conditioning factor	Level	
Maximum magnitude (%)	1, 3, 5, 10 (chapter 4) 3 (chapter 5)	
Frequency (Hz)	0.2	
Duration/Cycle number	1 hour	179
	4 hours	719
	24 hours (1 day)	4319
	72 hours (3 days)	12959
	144 hours (6 days)	25919
Mode	Intermittent (10 seconds stress/10 seconds rest)	
Direction	Equibiaxial	
Plate	Type I collagen-coated 6-well BioFlex® plate	

1.5.2.2. Fluid-flow induced shear stress

A number of research groups have been developing effective protocols to utilize fluid shear stress for conditioning of various cell types (*e.g.* Schwann cell [166], chondrocytes [167], and endothelial progenitor cells [168], endothelial cells [169]) in a bioreactor. Fluid-induced shear stress caused different levels of cellular anabolic effects depending on continuity or duration. Especially, shear stress conditioning is thought to have higher impact on bone cell activity than stretching [170]. According to Kapur *et al.*'*s* research, fluid flow stress on osteoblasts activates several different signaling pathways such as ERK, NO, and COX, using inhibitors specific for each pathway mediator [171]. When exposed to oscillatory shear stress, MC3T3-E1 cells express increased levels of PGE_2 in the 3D scaffold [172]. Besides bone cells,

mesenchymal stem cells produced higher levels of OPN, OCN, BSP, and type I collagen as well as growth factors such as BMP-2, BMP-7, VEGF-A, and TGF-β1 in response to shear stress generated using a parallel-plate flow chamber [173]. This flow-induced activation of mesenchymal stem cells may mediate enhanced phosphorylation of MAPK kinases such as ERK and p38 [174].

1.5.2.3. Comparison between tension and fluid flow effect on bone cells

Depending on stress, bone cells respond with different sensitivity in regulating bone related molecules. For example, human primary bone cells induce PGE_2 promotion due to pulsating fluid flow but type I collagen was enhanced when exposed to cyclic tension [119]. It is believed that these two stresses may influence bone cells through the ERK 1 and 2 signaling system in the bone cells as demonstrated by several inhibitors (*e.g.* N-ω-nitro-2-arginine methylester, nifedipine, TMB-8 hydrocholide, and indomethacin) targeting different signaling action [119, 175]. This distinct response represents a variation in the ERK 1 and 2 activation manner of mediators or mechanisms involving calcium, NO, and prostaglandins [175]. Even though Owan *et al.* suggested fluid flow may be a more powerful stress for bone regeneration than tensile stretching, demonstrating osteopontin induction by shear stress [170], more detailed research is required to determine the superiority of shear stress conditioning over tension for bone growth stimulation. In addition to tensile and shear stress, other mechanical stresses such as compression and hydrostatic pressures have been applied as a mechanical stimulator. Table 1-2 below summarizes previous research focused on regulation of bone marker proteins or genes by stress conditioning.

Table 1-2. Literature focused on induction of bone marker proteins or cytokine by stress preconditioning. ↑ and ↓ indicate increased and decreased levels of bone phenotype proteins, respectively. = denotes similar level between stress-treated group and controls. *hFOB1.19: human osteoblastic precursor cell line, **MSC, mesenchymal stem cell, ***BMSC: bone marrow stromal cells.

Bone	Stress conditioning	Cell/Tissue	2D/3D	Regulation	Reference

Marker					
OPN	intermittent hydrostatic pressure (13 kPa)	MC3T3-E1	2D	↑	[88]
	cyclic uniaxial tension, 1%, 1 Hz (cycle/s), duration: 30 minutes/day, 3 weeks	hFOB 1.19*	3D gel	↑	[176]
	Oscillation, inward/compression or outward/distraction duration: 3-16 days	MC3T3-E1	3D gel	↑	[177]
OCN	cyclic uniaxial tension, 1%, 1 Hz, duration: 30 minutes/day, 3 weeks	hFOB 1.19	3D gel	↑	[176]
	Oscillation, inward/compression or outward/distraction duration: 3-16 days	MC3T3-E1	3D gel	↑	[177]
	Superpulsed laser: 3, 7, 10 superpulsed, pulse width=200 nanoseconds, frequency=30 kHz, 5 minutes, 6.7 J/cm^2, minimum peak power=45 W, one time/days (until 5 days), one time/2 days (until 20 days)	Human osteoblast-like MG-63 cells	2D	↑: at 10 days, =: at 4 and 20 days	[95]
ON	cyclic uniaxial tension, 3, 6, 9%, 1 Hz, duration: 8 hours	Human osteoblasts	2D	↑	[99]
ALP	cyclic uniaxial tension, 1%, 1 Hz, duration: 30 minutes/day, 3 weeks	hFOB 1.19	3D gel	↑	[176]
	distraction, 0.5 mm per day, duration: 3-16 days	MC3T3-E1	3D gel	↑	[177]
	oscillation, inward/compression	MC3T3-E1	3D gel	↑	[177]

	or outward/distraction duration: 3-16 days				
	Drynariae Rhizoma	MC3T3-E1	2D	↑	[40]
	Ga-Al-Ar diode laser (output=800 mW, 91 seconds, 3 times/week, 50 mW/cm^2)	Bone marrow cells	2D	=	[178]
	Polarized He-Ne laser (output power=10 mW, 0.5 mW/cm^2, 10 minutes/day, 28 days)	MSC** cell line	3D	↑: initial culture duration, ↓ : late	[132]
	Hyperthermia, 39-45°C, 1 hour exposure, 96 hour post-heating at 37°C	BMSC*** MG-63 cells	2D	↑ ↑	[76]
OPG	cyclic tensile strain 6, 12, 18%, 0.1 Hz, 24 hours	MC3T3-E1	2D	↑	[60]
PGE_2	cyclic biaxial tension, 9%, 0.2 Hz, duration: up to 24 hours	MC3T3-E1	2D	↑	[159]
	intermittent shear stress	BMSC	3D	↑	[174]
	Low fluid shear, 1-63 μPa, 6-12, 24-48 hours	Human osteoblastic cells	2D	↑	[179]
Type I collagen	constant equibiaxial tension, 10%, up to 48 hours	Calvarial osteoblast	2D	↑	[93]
	three-point bending, 0.11%, 0.1, 0.3 Hz, duration: 1, 3, 5 minutes/day, 5 days	human osteoblast-like cells	2D Dentine discs	↓	[180]
	Superpulsed laser: 3, 7, 10 superpulsed, pulse width=200 nanoseconds, frequency=30 kHz, 5 minutes, 6.7 J/cm^2, minimum	Human osteoblast-like MG-63 cells	2D	=: at 4 days, ↑: at 10 and 20	[95]

	peak power=45 W, one time/day (until 5 days), one time/2 days (until 20 days)			days	
Type 3 collagen	cyclic biaxial 5 %, 0.5 Hz, durations: 24 hours 10, 12.5 %	Human osteoblast-like SaOs-2 cells	2D	↑ ↓	[160]
MMP13	constant equibiaxial tension, 8%, duration: up to 4 hours	MC3T3-E1	2D	↑	[164]
VEGF	constant equibiaxial tension, 10%, up to 48 hours	Calvarial osteoblast	2D	↑	[93]
BMP-2	Drynariae Rhizoma	MC3T3-E1	2D	↑	[40]
TGF-β1	cyclic biaxial tension, 0.17%, 1 Hz, duration: up to 24 hours	MC3T3-E1		↑	[80]
	constant equibiaxial tension, 10%, up to 48 hours	Calvarial osteoblast	2D	↑	[93]
	Ga-Al-As, output=84 mW, 1.5 and 3 J/cm^2	Human osteoblast-like (HOB) cells	3D	↑: at 10 days	[181]
FGF-2	constant equibiaxial tension, 10%, up to 48 hours	Calvarial osteoblast	2D	↑	[93]
COX-2	cyclic biaxial tension 9%, 0.1 Hz, 5 s on/off, during 24 hours	MC3T3-E1	2D	↑	[159]
	fluid shear stress, 10 dyne/cm^2, 4-5 hours	MC3T3-E1	2D	↑	[182]
Runx2	10% or 20% compressive strain, 0.5 Hz	Calvarial osteoblast	3D	↑: at 10% ↓: at 20%	[183]

MMP-9	cyclic tension 10%, 48 and 96 hours, 0.5 Hz	Raw264.7	2D	↓	[184]

1.5.3. Cellular mechanism in response to stress

1.5.3.1. Mechanical stress and relevant signaling pathways: Mechanotransduction

Mechanotransduction denotes a signal transition due to external mechanical cues leading to intracellular molecular modulation that dominates cellular activities such as proliferation, migration, differentiation, and deformation. Cell receptors which are sensitive to external mechanical stresses are considered to initiate mechanotransduction [185]. In terms of tensile stress, biological explanation of strain-related response is not totally understood and a possible candidate mechanism is thought to be related to specific ion-channels, intracellular free calcium, and integrin receptors [119]. From these sensors, cells mediate several chains of down-stream signaling pathways related to the following key molecules: G-protein, calcium, MAPK, and nitric oxide (NO) [185]. One of the important signaling cascades controlling mechanotransduction is the **mitogen-activated protein kinase (MAPK) signaling pathway** [185]. This pathway is activated with the phosphorylation of several signaling molecules such as extracellular signal-regulated kinase (ERK), MAP kinase kinase (MEK), c-Jun N-terminal Kinases (JNK), and p38 [185]. MAPK signaling is one of the key signaling pathways that can induce cellular bioactivity by stimulating external stress and environmental factors as shown in Figure 1-4. Each molecule involved in MAPK signaling transduction has a different sensitivity in response to stress and physiological events [186]. To investigate whether stress conditioning effects or bone physiological modification is involved in MAPK signaling pathway, two different research methods can be applied: the gene inhibition by transfection and the utilization of specific inhibitors such as PD98059 (an inhibitor for MEK1/2), SB203580 (against p38) and SP600125 (against JNK) [164]. Mechanical stress such as shear stress can stimulate the phosphorylation of MAPK signaling molecules (*e.g.* ERK1/2, p38, and JNK1) through integrin receptors inducting genes of bone-related proteins such as osteopontin and COX-2 [187]. In addition, growth factors such as TGF-β1 and BMP-2 are involved in MAPK signaling pathway to promote collagen [56].

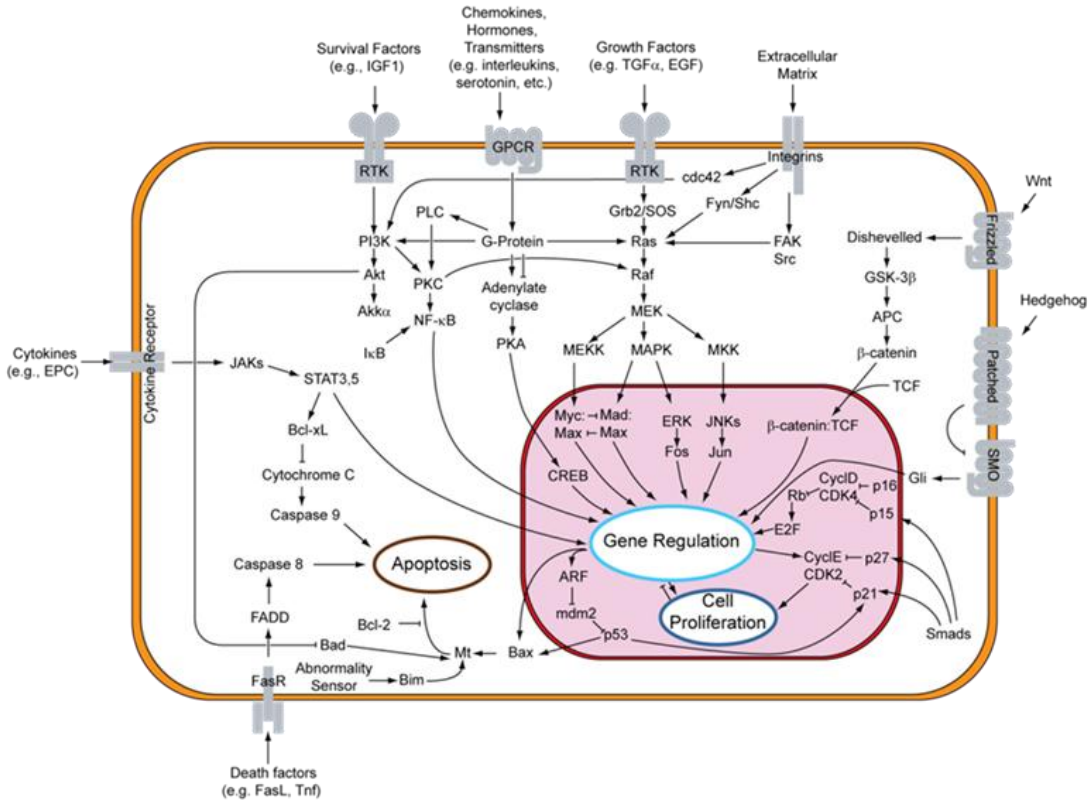

Figure 1-4. External stress and major signaling pathways [188] *http://en.wikipedia.org/wiki/File:Signal_transduction_v1.png*. Available at 2010 August 19. Lisensed under the GFDL by the author; Released under the GNU Free Documentation License. Used under fair use.

1.5.3.2. Thermal stress, heat shock responses, and relevant signaling pathways

Abnormal proteins generated by heat shock and heat shock-activated signaling pathways trigger translocation of heat shock factors (HSFs) into the nucleus following DNA binding [186]. HSF activation produces heat shock proteins, which repair or remove misfolded or damaged proteins [186]. Heat shock, similar to mechanical stresses such as shear stress [174], enhances MAPK signaling pathway related to p38, ERK, and JNK [134, 140]. In particular, p38 signaling pathway descending from apoptosis signal-regulating kinase 1 (ASK1) is involved to induce HSP27 [140].

1.5.3.3. Other bone-relevant signaling pathways

Bone is also related to other signaling cascades associated with stress conditioning and bone physiology. For example, the **Smad** pathway is a down-stream cascade associated with TGF-β and BMP-2 receptors [189]. **Wnt**, a cysteine-rich glycoprotein, pathway is closely related in bone formation including endochondral ossification, fracture healing, and craniofacial bone formation [190].

1.6. Heat Shock Proteins and Relevant Therapeutic Applications

1.6.1. Heat shock proteins

HSP27 categorized as a small molecular weight heat shock protein, has been reported to be related to anti-apoptosis and bone remodeling. HSP27 is up-regulated by estrogen, which contributes to maintaining bone mass and this induction is involved in inhibiting apoptosis as well as stimulating expression by heat shock [191]. Furthermore, HSP27 induced by heat shock can have a role in protecting actin modification at cold temperatures [192]. Enhancement of HSP27 is accomplished though PKC-controlled signaling pathways of p44/p42 and p38 MAP kinase by prostaglandin E2, possibly correlated to Ca^{2+} dynamics of cells [193, 194]. The phosphorylation of HSP27 by p38 and MAPKAP kinase-2 controls actin physiology against "structural disturbance" due to heating and other agonists such as tyrosine kinase [140]. HSP27 is involved in bone physiology by up-regulation of TGF β [195] and estrogen [196], which can increase bone mass, endothelin-1 [197], and prostaglandins [193, 194]. Exercise can also enhance HSP27 expression [198].

HSP47, also called SERPINH1, is a procollagen-binding protein expressed in the endoplasmic reticulum [199] and is involved in the process of collagen biosynthesis such as collagen type I [200, 201]. The correlation between collagen and HSP47 under stress is a pivotal factor in understanding the basic biology to control extracellular matrix formation. TGF-β1 treatment to MC3T3-E1 cells, a preosteoblast cell line, induced elevated HSP47 mRNA expression and type I collagen [94]. HSP47 expression was enhanced in wound healing area [202]. Murine **HSP47** gene deletion model revealed resulted in abnormal collagen production and triple helix formation [203, 204]. Given that close association of HSP47 with type I collagen expression, HSP47 gene manipulation can exert beneficial effects to repair collagen via gene-based therapy targeting for collagen-related bone diseases, *i.e.*, osteogenesis imperfect [205].

HSP70 is one of the most well-known HSPs in the scientific research field. This HSP was upregulated significantly by heat shock in response to elevated heating temperatures (44-50°C) and exhibited variations in induction levels depending on post-heating incubation [136]. There are 8 different types (Hsp70-1a, b, t, Hsp70-3, 5, 6, 9, and HSC70) of HSP70 in humans [206]. They are highly conserved, but have different activation trends or work in different locations [206]. The structure of HSP70 is composed of ATPase domain (44 kDa), peptide binding domain (18 kDa) and 542-640 amino acid resides with 10kDa size from N- to C-terminal [207]. The control of HSP27 and HSP47 expression is very important in determining cellular events happening in skeletal tissues such as bone and cartilage [208]. HSP27 and HSP70 are involved in the apoptosis pathway and operate as an anti-apoptotic molecule [209] against diverse stresses [210, 211]. However, the functions and involvement of HSPs in down-stream cellular signaling pathways are very complex and diverse. They are not well characterized in bone. Contribution of HSP27, HSP70, and HSP90 to control anti-apoptosis and differentiation involves different target proteins, which control apoptosis and differentiation-related mechanisms, and regulates several intracellular or nuclear functions [212]. This may be caused by caspase action mainly in mitochondria and chromatin condensation [212]. Each HSP impedes apoptotic modification in different manners. As an example, HSP27 hinders nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) (having an anti-apoptotic function)-inhibiting molecule (*i.e.* I-κBα) and HSP 70 prevents JNK or Bax action related to mitochondria [212]. Stress kinetics about HSP70 and heating are well described in Kenneth R. Diller's review paper [213]. Even though thermal stress can be differently applied depending on a heating system, HSP70 regulations in heated cells can be analyzed generally according to conditioning factors such as levels of thermal stress, which are determined by heating temperatures and durations, as well as post-heating incubation period at 37°C [213]. In the temperature-dependent manner, HSP70 is known to be induced rapidly, transiently, and significantly in response to thermal stress [213].

Induction of HSPs requires a conserved transcription factor called the **heat shock factor (HSF)** [214]. This protein exists in all organisms and includes similar components including transcriptional activation domain, DNA binding domain, leucine zipper motif oligomerization domain, and repression domain. Each HSF is stimulated through dephosphorylation at serine spots (303 and 307) by trimetric combination with three individual monomers. HSF is activated

by various stresses (*e.g.* oxidative and heat stress), events such as inflammatory and developmental process, but deactivated by cell growth-related regulatory proteins (*e.g.* protein kinase C, glycogen synthase kinase 3, and ERK) and chaperones (HSP70) [214].

HSPs and bone Heat shock proteins are detected in developing bone such as endochondral bone development [215]. In addition, bone in the growth process showed expression of several heat shock proteins, according to *in vivo* analysis of rat tibia using immunohistochemistry [208]. Immunostained osteoblasts in rat tibia visualized high level of HSP27, HSP47, HSP70, and type I collagen [208]. In human mandibular bone developmental process, HSP27 is highly localized in the new bone forming area, especially in osteoblasts not osteocytes [216]. Leonardi *et al.*'s histological study [216] demonstrated strong expression of HSP27 in osteoblasts and dental laminar/epithelium undergoing the development process of craniofacial bones and teeth, respectively, suggesting HSP27 may be a key modulator in controlling bone.

HSP and mechanical stress Insufficient mechanical environment such as long-term flight (microgravity) leads (4 and 5 days) to significant reduction of HSP70 and HSP47 genes as well as TGF-β1 secretion in osteoblasts. [217]. However, correlation between *in vitro* mechanical strain and HSPs in osteoblasts has not yet been explored well. Depending on cell types and physiological situation, tensile stress effects of HSP70 expression seem different. Trabecular meshwork cells and tendon fibroblasts showed increased HSP70 in response to tension [218, 219]. On the other hand, HSP70 expression in gastric mucosal cells undergoing wound healing was diminished by tensile stress [220].

1.6.2. HSP-based study for therapeutic application

The biochemical mechanism and role of intracellular HSPs (*e.g.* small HSPs, HSP70, HSP60, and HSP90) are to serve as molecular chaperones to protect against apoptosis. Using external stresses such as heat shock, HSPs can be induced in the cells. Increased HSP expression can enhance regeneration and cell protection from apoptosis. Therefore, a number of research groups have investigated the effect of endogenous or exogenous up-regulation of HSPs on stress environment (*e.g.* hypothermia [221], nitric oxide [210], and heat shock [142]) and damaged or diseased tissues such as ischemia-reperfusion injury [222] and post-surgery wound. In addition,

HSP incorporation can be applied to improve the efficiency of cell transplantation [75, 223] or tissue grafts [224].

1.6.2.1. Manipulation of HSPs: exogenous delivery

Exogenous delivery of HSPs is an attractive strategy to increase HSP levels in cells while avoiding danger caused by overdose of stress treatment. However, exogenous HSP70 treatment in the mouse calvaria culture system promoted calcium release, and heat stress diminished the resorptive effects of HSP treatments [225]. Therefore, development of effective HSP delivery methodology and demonstration of its effects requires further investigation. Recent methods are listed below: 1) ***Direct injection:*** Exogenous HSP delivery *in vivo* showed better wound healing [226]. Similarly, direct injection of recombinant human HSP70 into a nerve-diseased mouse model showed better cell survival and innervations [227]. 2) ***Upregulation of HSP by transfection or transduction:*** Exogenous delivery of recombinant HSP27 using protein transduction domains enhanced cell survival under hypoxia in a heart injury model [228]. Gene delivery can also be mediated through electroporation. HSP70-specific gene was transferred and localized into the cartilage through electroporation to protect against cell death resulting from arthritis [229]. Similarly, the transfection of HSP70 gene into chondrocytes induced less cell death *in vitro and vivo* [230]. 3) ***Carrier-mediated delivery:*** Long-term delivery of therapeutic proteins or drugs into diseased or damaged tissue requires an artificial carrier such as polymer microsphere to control the release rate of proteins. The fusion proteins of HSP27 with TAT (transcriptional activator) in the PLGA mixture of microspheres and hydrogel were developed to apply the anti-apoptotic effect of HSPs for treatment of myocardial infarction [231].

1.6.2.2. Downregulation of HSPs: inhibition of HSP

HSP inhibition by siRNA techniques can have a positive therapeutic benefit [67]. Pathologically, up-regulation of heat shock proteins is detected in some disease states and a lower level of heat shock proteins can be a good candidate therapy. For example, downregulation of HSP47 or HSP47-homolog protein, gp46, can be applied to inhibit liver fibrosis because the siRNA treatment showed the inhibiting effect on collagen expression [67, 232]. On the other hand, down-regulation of HSP can be used to investigate the original function of HSP by investigating negative effect of HSP inhibition. As an example, Shao *et al.* demonstrated HSP27 anti-apoptotic function using siRNA of HSP27 [65].

Chapter 2: Research Outline: Motivation, Hypothesis, Experimental Outline, and Clinical Perspectives

2.1. Motivation and Objectives

Successful clinical application of engineered tissues is limited by insufficient in-growth/differentiation of seeded cells and scarce neo-blood vessel and extracellular matrix formation in the artificial scaffold system [36]. Bone tissue is exposed to various stresses and chemical signals such as nitrogen oxide, autocrine/paracrine hormones, fluid flow, and mechanical weight-loading and strain, as described in Chapter 1. Bone cells such as osteocytes, osteoblasts, and osteoclasts mediate mechano-sensing and are responsible for generating and removing bone matrix such as minerals. To overcome the aforementioned limitations of engineered tissues, bone tissue engineering has employed stress conditioning protocols utilizing mechanical stress (*e.g.* cyclic tension [176] and fluid flow [173]) and osteoinductive growth factors (GFs), such as transforming growth factors (TGFs) [233] and bone morphogenetic proteins (BMPs) as a promising stimulator for bone regeneration [234]. Prior studies [76] have also suggested low-level heating could be a promising stress conditioning strategy for bone formation. However, the osteogenic effects of thermal stress alone or in combination with mechanical or biochemical cues have not yet been explored. Furthermore, the use of combinatorial conditioning protocols using mechanical stress and growth factors has not been fully explored in bone.

Exposure to stress causes cells to auto-protect by expressing stress proteins, known as heat shock proteins (HSPs), which are also involved in various cellular events such as proliferation and differentiation [212]. Induction of HSPs can be successfully achieved through stress conditioning using thermal, tensile, compression, hydrostatic pressure and shear stress [74-76, 141-143, 235-238]. In addition, growth factors can induce HSP up-regulation [94, 195, 235, 239]. Therefore, HSP regulation by different types of stresses and its role on bone physiology need to be demonstrated to develop a stress conditioning protocol for bone tissue engineering. Taken together, development of effective stress conditioning protocols, growth factor treatment, and methods of HSP modulation have enormous potential for enhancing bone regeneration. Upregulated levels of HSP expression are related to enhanced anti-apoptosis [212], cell

proliferation [240], collagen synthesis [241, 242], and bone development [215]. Despite the beneficial aspect of HSP induction, the role of HSPs on osteogenic and mitogenic processes in bone cells is still elusive.

From this tissue regenerative viewpoint, the ultimate goals of this study are to 1) investigate various stress-induced responses of preosteoblasts following individual or combinatorial stress using heating, tension, and growth factors, 2) clarify the role of HSP in *in vitro* osteogenic responses of preosteoblasts under normal conditions or in response to stress, and 3) develop a stress-modulated and HSP-mediated therapeutic approach for bone tissue engineering. The following are several questions we endeavor to answer through this research:

- Do preosteoblasts express HSPs under stress?
- How do preosteoblasts respond differently to thermal, tensile, and biochemical stress?
- Is individual stress more effective at increasing cell proliferation and induction of HSPs and bone marker expression than combinated stress treatment? If it is, what particular type of individual stress is more effective?
- Does combinatorial stress synergistically upregulate HSPs and bone marker proteins?
- Could growth factors in combination with thermal and mechanical stress further enhance *in vitro* osteogenic process or proliferation of preosteoblasts?
- Is there a correlation between HSP expression and osteoblastic differentiation *in vitro* under external stress?
- Is there a correlation between HSPs and cell proliferation (mitogenic process) under external stress?
- How does combinatorial treatment of thermal and mechanical stress activate MAPK signaling pathway?

2.2. Hypothesis and Specific Aims

In this study, it is hypothesized that applying individual or combinatorial stress conditioning (thermal, tensile, and biochemical) and effective HSP modulation could induce *in vitro* responses in preosteoblasts indicating mitogenic/osteogenic/angiogenic/anti-osteoclastic effects. By using cell-based analysis such as several gene and protein assays (PCR, western blot,

immunofluorescence stain, ELISA, and siRNA gene silencing), this hypothesis will demonstrate the following four specific aims:

I. Determine the effect of **thermal stress conditioning** using water bath heating alone or in combination with **growth factors** on expression of HSPs and bone-related proteins by a monolayer preosteoblast cell line.

II. Determine the effect of **cyclic tensile stress conditioning** in combination with **growth factors** on expression of HSPs and bone-related proteins by a monolayer preosteoblast cell line.

III. Determine the effect of **combinatorial stress conditioning** (tensile and thermal stress) on expression of HSPs and bone-related proteins by a monolayer preosteoblast cell line.

IV. Determine the effect of **HSP manipulation effect** on proliferation and *in vitro* osteogenic response of preosteoblasts under normal or thermal stress environment by HSP70 gene silencing using siRNA.

2.3. Project Outline

The specific aims for this proposal are schematically described in **Figure 2-1**. Various cellular responses following individual or combinatorial conditioning based on thermal and mechanical stress and biochemical cues were investigated as suggested in Specific aims 1-3. We focused on 1) HSP expression, 2) cytotoxic or mitogenic activity, and 3) osteogenic/angiogenic/anti-osteoclastic activity of preosteoblasts in response to *in vitro* stress. In **specific aim 1 described in Chapter 3**, **thermal stress** conditioning strategies using a water bath alone or in combination with **osteoinductive growth factors** (BMP-2 and TGF-β1) were applied to preosteoblastic cells and the effect on cytotoxicity, and the induction of HSPs and bone proteins were measured. This research will be submitted to *Biotechnology and Bioengineering* for the article entitled "Effect of Thermal Stress Conditioning and Growth Factors on Expression of Angiogenic, Bone-related, and Heat Shock Proteins by Preosteoblastic Cells." In **specific aim 2 described in Chapter 4**, the effects of **tensile stress conditioning** were studied using a commercially available tension bioreactor (*i.e*. Flexcell® Tension System) with the aforementioned **growth factors.** This research has been submitted to *Tissue Engineering, Parts A, journal* for the article entitled "Conditioning Effect of Cyclic Tension and Growth Factors on a Preosteoblastic Cell Line for Bone Tissue Engineering Applications." **Specific aim**

3 described in Chapter 5 is focused on investigating the effect of combinatorial conditioning using **thermal and tensile stress** on preosteoblasts. This research will be submitted shortly to *Annals of Biomedical Engineering* for the article entitled "Combined Effects of Heating and Cyclic Tension on the Induction of Heat Shock Proteins and Bone-related Proteins By MC3T3-E1 Cell Line." **Specific aim 4 described in Chapter 6** explored the effect of **HSP70 silencing** on cell proliferation, HSP expression, and regulations of bone-related molecules. This research will be submitted shortly to *Cell Stress and Chaperone* for the article entitled "Effect of HSP70 Small Interfering RNA on Proliferation and Regulation of Bone-related Proteins Under Thermal Stress Preconditioning." Cellular-based research was performed using research techniques through cell viability measurement by mitochondria metabolic activity and DNA assay, gene analysis using quantitative real time RT-PCR, and protein-based assay such as ELISA, western blot, and immunofluorescence staining.

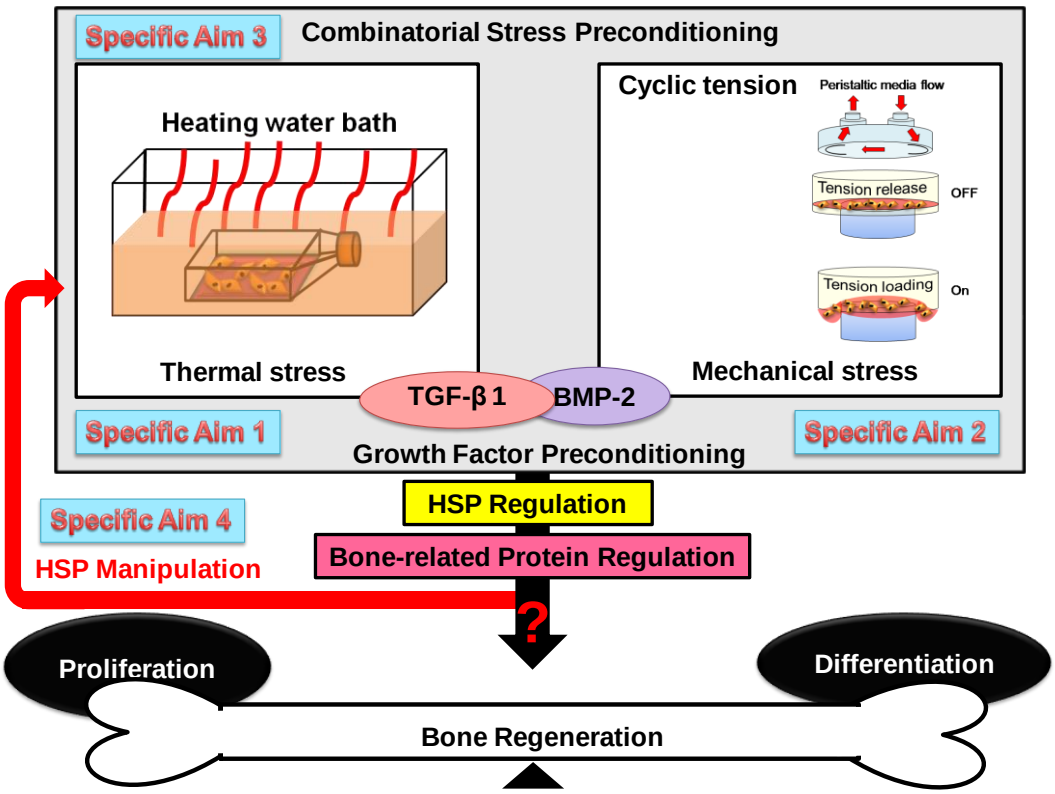

Figure 2-1. Ultimate goal and research outline.

2.4. Clinical Perspectives

As a preliminary study for clinical application targeting bone fracture or diseases such as osteoporosis, this study suggests several promising strategies as shown in **Figure 2-2**. Combinatorial stresses utilizing thermal, mechanical, and biochemical cue can be treated to preosteoblasts or stem cells directly (strategy 1) or in the scaffolds (strategy 2). This cell-seeded scaffold after stress conditioning can be implanted into the diseased (*e.g.* osteoporosis) or fractured bone. In addition, HSP levels in bone cells can be manipulated by penetrating gene or proteins directly through cell membranes before injection into targeted bone spots (strategy 3) or treating using exogenous HSP delivery system or siRNA with polymeric controlled-releasing carriers (strategy 4) such as PLGA microspheres.

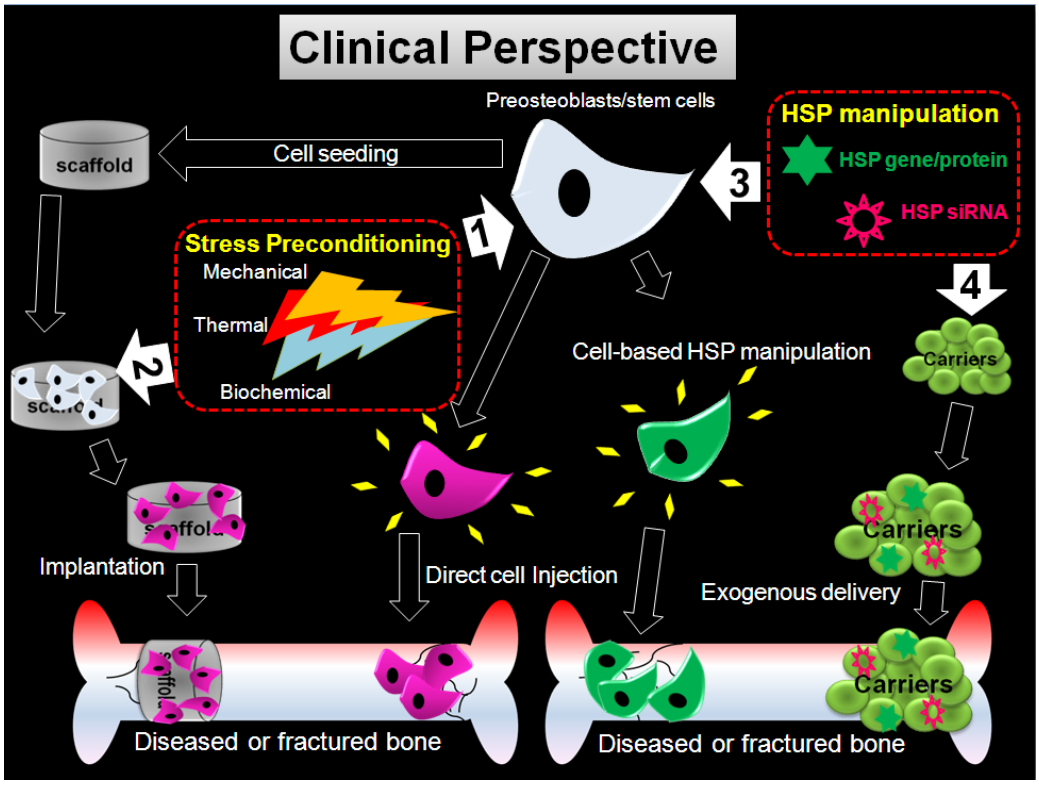

Figure 2-2. Clinical perspectives of stress preconditioning and HSP-based strategies suggested in this study.

To be submitted as Chung, E. et al.

Chapter 3: Effect of Thermal Stress Conditioning and Growth Factors on Expression of Angiogenic, Bone-related, and Heat Shock Proteins by Preosteoblasts

Eunna Chung[1] and Marissa Nichole Rylander[1, 2]*

[1]Virginia Tech –Wake Forest University, School of Biomedical Engineering and Sciences, Virginia Tech, Blacksburg, VA 24061

[2]Department of Mechanical Engineering, Virginia Tech, Blacksburg, VA 24061

3.1. Abstract

Conditioning protocols involving mechanical stresses alone or in combination with chemical cues such as growth factors (GFs) possess significant potential to enhance bone regeneration. However, utilization of thermal stress conditioning for bone tissue engineering has been highly under-investigated. Therefore, a preosteoblast cell line (MC3T3-E1) was exposed to water bath heating (40-44°C, 2-20 minutes) alone or with osteoinductive GFs consisting of bone morphogenetic protein-2 (BMP-2) and transforming growth factor-β1 (TGF-β1) to investigate whether these stimuli could promote induction of bone-related and angiogenic proteins and heat shock proteins (HSPs). Cells remained viable when heating durations were less than 10 minutes at 40-44°C. Increasing heating duration at 44°C showed the increasing trends in gene levels of all HSPs, osteocalcin (OCN), and osteopontin (OPN) at 8 hour post-heating (PH). Heating in combination with GFs caused the greatest gene induction of osteoprotegerin (OPG) (6.9 and 1.56 fold induction compared to sham-treated and GF-treated groups, respectively) and vascular endothelial growth factor (VEGF) (16.0 and 1.61 fold as previously denoted) at 8 hour PH. Both heating and GFs independently suppressed matrix metalloproteinase-9 (MMP-9) genes; however, GFs caused a more significant decrease in MMP-9 at 72 hour PH. Secretion of OCN, OPN, and OPG increased with the addition of GFs, but diminished with heating as measured by ELISA at 72 hour PH. These results suggest that conditioning protocols utilizing heating and GFs alone or in combination can induce HSPs, pivotal bone-related proteins, and angiogenic growth factor,

and downregulate anti-osteoclastic genes, potentially providing a promising therapeutic strategy for treating bone disease such as osteoporosis.

3.2. Introduction

Although the life expectancy for the senior population worldwide has been extended due to medical advances, quality of life can be significantly diminished due to bone disease and degeneration. Approximately 75 million people in Europe, USA, and Japan suffer due to degenerative bone disorders such as osteoporosis, which weakens bones, causing frequent fractures [24]. Bone-weakness associated with osteoporosis primarily results from abnormality of the proliferation and differentiation processes of bone cells such as osteoclasts or osteoblasts, which cause imbalance between bone formation and resorption [32].

Numerous research groups have investigated the cellular response and regenerative potential of stress conditioning (*e.g.* cyclic tension and fluid shear) via supra-physiological loading to develop novel therapeutic strategies for bone repair [59, 173]. For example, cyclic tensile stress conditioning with 12% magnitude and 0.1-0.2 Hz for 24 and 48 hours induced vascular endothelial growth factor (VEGF) and transforming growth factor-beta 1 (TGF-β1) expression in MC3T3-E1 cells [59]. In addition, fluid shear stress treatment (continuous and pulsatile with 0.015-0.074 Hz) can accelerate osteogenic differentiation by elevating the gene expression of several bone-related proteins such as type I collagen, bone sialoprotein (BSP), osteocalcin (OCN), and osteopontin (OPN) by bone marrow stromal cells [173].

Despite the extensive use of conditioning with chemical cues and mechanical stress in the field of tissue engineering, very few studies have investigated thermal stress conditioning as a method for promoting regeneration. Low-level heating in the range of 40-42°C using heating devices (*e.g.* water bath, humidified incubator, heating blanket, and temperature stimulator) can induce positive *in vitro* and *in vivo* responses (*e.g.* anti-apoptosis, proliferation, and induction of extracellular matrix proteins) for tissue regeneration in skin [141], muscle [75], cartilage [74], teeth [142], heart [136, 243], and bone [76, 143]. Conditioned media from heat-shocked osteoblasts in a heated incubator have been shown to induce elevated levels of OCN expression at early stages of differentiation and calcium production by bone marrow cells leading to enhanced differentiation into mature bone cells [131].

The heat-stimulating effects causing bone formation may be mediated by elevated expression of heat shock proteins (HSPs). HSPs are induced by stresses that perturb the cellular environment (*e.g.* hypoxia [244] and hyperthermia [136, 243, 245]). Upregulation of HSPs induce anti-apoptotic effects under cytotoxic environments [191, 231], enhance regeneration in several tissues [246, 247], promote differentiation by regulating caspase activity, and stabilize proteins associated with differentiation [212]. Among HSPs, HSP27, HSP47, and HSP70 can be critical stress proteins involved in bone physiology. HSP27 is involved in bone physiology through upregulation of TGF-β [195] and estrogen [196] which can increase bone mass, endothelin-1 [197], and prostaglandins [193]. HSP47, also called SERPINH1, is a procollagen-binding protein expressed in the endoplasmic reticulum [199] and involved in the biosynthesis of type I collagen [200], one of the primary components of the bone extracellular matrix. TGF-β1 treatment of preosteoblast cells (MC3T3-E1) can induce elevated HSP47 messenger RNA (mRNA) expression and type I collagen [94]. Therefore, control of HSP27 and HSP47 expression is very important in modulating cellular events in skeletal tissue maintenance and formation such as bone and cartilage [208]. In addition, HSP70 may have a prominent role in enhancing bone proliferation in response to elevated temperatures (44-50°C) and has exhibited variation in induction level depending on post-heating duration [136]. HSP27 and HSP70 are involved in the apoptosis pathway and operate as anti-apoptotic molecules [209] in response to diverse stresses [210, 211]. However, the roles of HSPs in bone regeneration are not well-characterized.

Exogenous GF delivery has been documented as a powerful biochemical cue in tissue engineering for reconstituting damaged or diseased bone tissue or accelerating blood vessel formation [38, 47-50, 53]. A number of *in vitro* and *in vivo* studies have shown that *in situ* delivery of exogenous bone-relevant GFs such as like insulin-like growth factor [49], platelet-derived growth factor (PDGF) [50], TGFs [41, 49], and bone morphogenetic proteins (BMPs) [40, 48], and VEGF [91] can induce osteogenesis and healing of bone. Recombinant human BMP-2, a FDA-approved growth factor, can induce osteogenesis of mesenchymal cells into osteoblasts, expressing OCN and BSP, via preosteoblast by various transfection factors such as Runx-2 and Osterix [53]. MC3T3-E1 cells exhibit enhanced alkaline phosphatase (ALP) and OCN following exogenous delivery of BMP-2 [57]. MC3T3-E1 cell proliferation increased following introduction of TGF-β1 [38]. VEGF is produced by several cell types (*e.g.* smooth-

muscle cells, fibroblasts, and osteoblasts) to induce new blood vessel formation, which is one of the most critical factors in bone regeneration [52, 129]. A comprehensive understanding of bone tissue formation *in vitro* following stress conditioning alone or in combination with GFs is critical to developing effective strategies for bone regeneration. However, the synergistic benefit of exogenous GFs and thermal stress conditioning has not been explored.

In vitro differentiative or metabolic responses of bone cells can be detected by measuring induction of bone matrix proteins (*e.g.* OPN [88] and OCN [89]), and regulation of cytokines (*e.g.* osteoprotegerin (OPG)) [60] and enzymes (*e.g.* matrix metalloproteinases (MMPs)) [92]. Matrix proteins such as OPN, OCN, BSP, and type I collagen have been used commonly as indicators to confirm successful osteogenic processes [88, 89, 173]. OPG has a critical role in diminishing osteoclast differentiation by inhibiting the association of receptor activator nuclear factor κB (RANK) and RANK-ligand [125]. In addition, MMP-9 is used to identify osteoclast phenotype or differentiation of osteoclast precursor cells into osteoclasts [248] and is considered to be a therapeutic target to treat bone diseases such as osteoporosis [31]. Our study will utilize these bone-related proteins to characterize diverse responses of preosteoblasts following stress treatment.

We hypothesize that *in vitro* thermal stress conditioning in combination with GFs can promote bone-related molecules, which can be indicators for osteogenesis and angiogenesis, and the stress-protective capacity of MC3T3-E1 preosteoblasts. Therefore, this study measured the induction of HSPs (HSP27, HSP47, and HSP70), angiogenic marker (*e.g.* VEGF), and pivotal bone proteins (*e.g.* OPN, OCN, OPG, type I collagen, ALP, and MMP-9) by MC3T3-E1 cells following a single dose of thermal conditioning utilizing a range of thermal stimulation protocols (40-44°C, 2-20 minutes) and post-heating durations (8, 24, and 72 hours) alone or in combination with two osteoinductive GFs (BMP-2 and TGF-β1).

3.3. Materials and Methods

3.3.1. Cell culture

A murine preosteoblastic cell line, MC3T3-E1 (American Type Culture Collection), was cultured as a monolayer with growth media composed of alpha Minimum Essential Medium (αMEM) (Mediatech), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (PS) in an

incubator at 5% CO_2 and 37°C. For cytotoxicity measurements, cells were seeded into 12 well plates at 5×10^4/well and cultured for 24 hours to allow adhesion before heating. For western blot and PCR analysis, cells were seeded into 25 cm^2 T-flasks at 5×10^5/flask for measurement at 8-24 hour post-heating (PH) and 3×10^5/flask for measurement at 72 hour PH. Cells were cultured for 48 hours prior to heating.

3.3.2. Water bath heating

A constant temperature water bath (ISOTEMP 210, Fisher Scientific) was employed as the mode of thermal conditioning to produce a relatively short thermal time constant to heat experimental specimens similar to methods described in our prior work [136, 249]. Media composed of MEM without L-glutamine was used during the heating process to prevent cell damage caused by the degradation of L-glutamine at high temperatures. Heating media was pre-warmed to the desired temperature and added to a monolayer of MC3T3-E1 cells. Subsequently, the flask was submerged in the water bath that was set at constant temperatures of 40-44°C for heating durations of 2-20 minutes. Following thermal stress, osteogenic media was added to promote osteogenesis during the post-incubation period. Osteogenic media was composed of αMEM including 50 μg/ml L-ascorbic acid, 10 mM β-glycerol phosphate, 1% FBS, and 1% PS with or without the addition of BMP-2 (50 ng/ml) and TGF-β1 (2 ng/ml). The chosen concentrations of GFs were previously shown by Chung, *et al.* to cause differentiation of MC3T3-E1 cells [250]. The osteogenic media for cytotoxicity measurements was supplemented with 10% FBS rather than 1% to permit ideal media conditions for cell proliferation. To permit induction of HSP and manifestation of cellular injury, cells were returned to a 5% CO_2 incubator at 37°C after heating and post-incubated varying durations depending on measurement type: cytotoxicity (24 hours), western blot (24 hours), PCR (8, 24, and 72 hours), and ELISA (72 hours).

3.3.3. Analysis of cytotoxicity

Prior to determining thermal stress conditioning protocols for enhanced osteogenic responses, we identified thermal stress protocols for which cell viability remained high to prevent compromise of cell samples. MC3T3-E1 cells were heated at 40-44°C for 2-20 minutes and post-incubated for 24 hours. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-

(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega). MTS solution mixed with basal αMEM media at a volume ratio of 1:5 was added to cultured cells. After 4 hour incubation at 37°C, the solution was transferred to a 96 well plate and optical density was measured at 490 nm by a microplate reader (SpectraMax M2^e, Molecular Devices).

3.3.4. Western blot

Western blot analysis was used to investigate the correlation between HSP expression and thermal stress conditioning (44°C for 0-8 minutes) at 24 hour PH. Cells were lysed with RIPA buffer (Santa Cruz Biotechnology). Isolated protein was quantified using BCA Protein Assay Kit (Pierce) to permit loading of identical amounts of protein sample in each well of the electrophoresis gel. Proteins were mixed with Laemmli sample buffer (Bio-Rad Laboratories) followed by heating at 95°C for 5 minutes. For electrophoresis, heated protein was loaded in a 10% Criterion Tris-HCl Gel (Bio-Rad Laboratories). Proteins transferred onto the membranes were immunoblotted using primary antibodies for HSP27, HSP47, and HSP70 and secondary antibodies diluted in 5% non-fat dry milk solution in Tween-20/Tris buffered saline (TBS) for blocking. All primary and secondary antibodies were purchased from Santa Cruz Biotechnology except for the HSP70 primary antibody (Stressgen). HSP27 (SC-51956, dilution ratio=1:200), HSP47 (SC-13150, 1:500), HSP70 (SPA-810, 1:1000) were used as mouse monoclonal primary antibodies. HRP-conjugated secondary antibodies (SC-2005 for HSP27 and HSP47, and SC-2969 for HSP70, 1:2000) corresponding with the specific species of each primary antibody were used. After adding a chemiluminescence solution, protein bands were visualized by LAS3000 Image Analyzer (Fujifilm). Quantification of each protein band on western blot images was accomplished by calculating the intensity difference between a band of interest and background per unit are using Multi Gauge V3.0 program (Fujifilm). After imaging, the membranes were stripped for actin normalization by incubating for 30 minutes at 50°C in the stripping solution (7.6 g Tris base, 200 ml 10% SDS, and 7 ml β-mercaptoethanol in the deionized water for 1 L solution, pH 6.8). Actin was detected using goat/rabbit polyclonal anti-actin as a primary antibody (SC-1616, 1:1000) and a secondary antibody (SC-2020, dilution ratio=1:2000).

3.3.5. Quantitative real time RT-PCR

Cells were heated at 44°C for 0, 4, and 8 minutes and gene expressions for all markers shown in Table 1 was measured at 8, 24, and 72 hour PH. RNA was isolated by spin protocol using RNeasy Mini kit (Qiagen) and QIAshredder (Qiagen). Isolation procedures were conducted according to manufacturer's protocols. Isolated RNA was converted to cDNA using a Reverse Transcription System (Promega). RNA samples combined with components of Reverse Transcription System were reacted at 25°C for 10 minutes and 42°C for 45 minutes followed by heating at 99°C for 5 minutes. After reverse transcription, cDNA samples were mixed with Taqman PCR Master Mix (Applied Biosystems) and each specific primer and polymerized in 7300 Real-Time PCR Systems (Applied Biosystems). The PCR reaction was initiated at 50°C for 2 minutes and 95°C for 10 minutes. For each polymerization (total PCR reaction cycles=45), temperature was set at 95°C for 15 seconds and 60°C for 1 minute. Taqman® Gene Expression Assays (Applied Biosystems) used for specific gene detection are listed as a primer and probe in Table 3-1. Relative fold induction (RFI) of each mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method used in Lee *et al.*'s study [251]. Threshold cycle (C_T), derived using SDS v1.2× system software of 7300 Real-Time PCR System, denotes the fractional cycle number at threshold polymerized gene and $-\Delta\Delta C_T$ was derived from (C_T of target gene-C_T of GAPDH)$_{\text{treated group}}$-(C_T of target gene-C_T of GAPDH)$_{\text{control group}}$ [251]. Treated groups denote heated, GF-added alone or in combined treated groups and control groups indicated sham-treated cells without heating and GF addition.

Table 3-1. Primers used for real-time RT-PCR to determine specific gene expression levels of target proteins. Assay ID (TaqMan® gene expression assay, Applied Biosystems), primer sequence, and target size are shown.

Target protein	Gene name	Assay ID	Probe sequence (5'→3')	Size
HSP27	heat shock protein 2	Mm00517908_m1	TCGGAGAAGGCCTCCTGCCAGAAGA	115
HSP47	serine (or cysteine) peptidase inhibitor, clade H, member 1	Mm00438056_m1	TGGTAAACCCTCACAGGTCCTCTGT	76
HSP70	heat shock protein 1B	Mm03038954_s1	GTTAAGGTTTTGTGGTATAACCAGT	141
OPN	secreted phosphoprotein 1	Mm01611440_mH	GAACAGTATCCTGATGCCACAGATG	102
ON	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	Mm01168758_m1	TGTTTCTGGAGGGAGAAGCCCCCCT	81
OCN	bone gamma-carboxyglutamate protein, related sequence 1	Mm00649782_gH	CCTTGGAGCTTCAGTCCCCAGCCCA	89
OPG	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Mm01205928_m1	AGTGTGGAATAGATGTCACCCTGTG	75
BSP	integrin binding sialoprotein	Mm00492555_m1	GGTTTCCAGTCCAGGGAGGCAGTGA	98
ALP	alkaline phosphatase, liver/bone/kidney	Mm01187113_g1	TCCTGGGAGATGGTATGGGCGTCTC	71
Cox-2	prostaglandin-endoperoxide synthase 2	Mm01307330_g1	TGTACTACACCTGAATTTCTGACAA	73
MMP-9	matrix metallopeptidase 9	Mm00600164_g1	TCTTCAAGGACGGTTGGTACTGGAA	72
Col1	collagen, type I, alpha 1	Mm00801666_g1	CGATGGATTCCCGTTCGAGTACGGA	89
VEGF	vascular endothelial growth factor A	Mm00437308_m1	CAAAGCCAGAAAATCACTGTGAGCC	66
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1	GTGAACGGATTTGGCCGTATTGGGC	107

3.3.6. Measurement of released proteins

Protein secretion was analyzed using enzyme-linked immunosorbent assay (ELISA). Conditioned media from cell monolayers was isolated at 72 hour PH following thermal stress (44°C, 0-8 minutes). An ELISA assay was conducted using the manufacturer's protocol. OPG, VEGF, OPN, and MMP-9 were measured using Quantikine® ELISA (R&D Systems). OCN secretion data were acquired by Mouse Osteocalcin EIA kit (Biomedical Technologies Inc.). Culture supernatant was added to a 96 well microplate coated with specific antibodies for each secreted protein. The optical absorbance of the solution was measured at 450 nm by a microplate reader and compared to a standard curve. The concentration of secreted proteins in the culture supernatant was quantified from standard curve estimation.

3.3.7. Statistical analysis

All data and graphs are presented as mean ± standard deviation (SD). Experimental groups with a minimum of three repetitions (3 or 4) were utilized and analyzed independently. One-way ANOVA with a Tukey multiple comparison test was used for cytotoxicity data analysis. Two-way ANOVA with Tukey multiple comparison test was used to analyze experiments involving thermal stress conditioning in combination with GFs to demonstrate the statistical significant mean differences and the interaction between both thermal stress and GFs ($p<0.05$).

3.4. Results

3.4.1. Cytotoxicity of thermal stress conditioning on a preosteoblast cell line, MC3T3-E1

MTS data (Figure 3-1) showed significant cytotoxicity after heating for durations longer than 10 minutes at 40-44°C. Compared to control groups, a single dose of water bath heating did not influence preosteoblast viability significantly for heating durations less than 8 minutes at temperatures of 40°C (Figure 3-1A), 42°C (Figure 3-1B), and 44°C (Figure 3-1C). In contrast, cells heated at 44°C for 2-8 minutes and at 42°C for 8 minutes showed comparable levels of metabolic activity to control groups. In order to maximize gene and protein induction while maintaining high cell viability, we utilized the highest temperature (44°C) for heating durations of 0-8 minutes for thermal induction of HSPs and bone-related proteins.

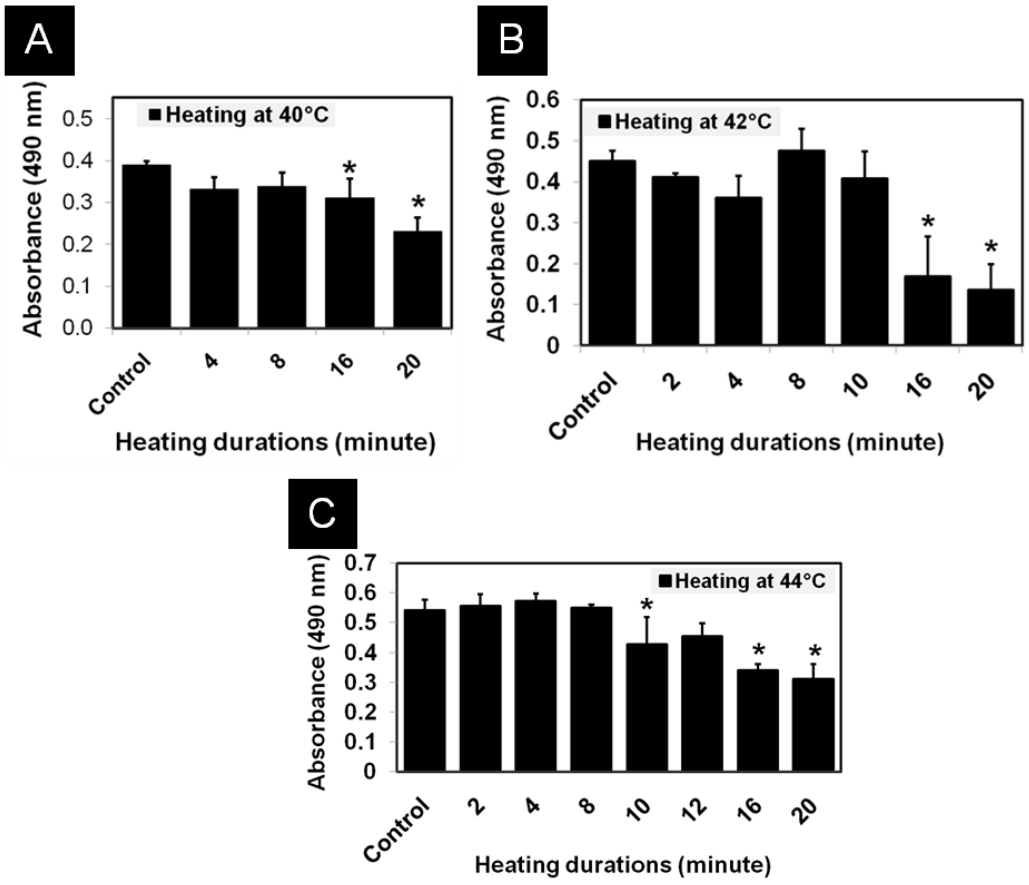

Figure 3-1. Cytotoxicity of MC3T3-E1 cells following a single-dose of water bath heating for varying heating durations and temperatures. MTS assay was performed at 24 hour PH at 40°C (A), 42°C (B), and 44°C (C). Absorbance at 490 nm was used as relative quantification data for cytotoxicity (mean±SD). * represents statistical significant difference between control group (unheated) and heated groups ($p<0.05$).

3.4.2. Expression of HSPs (HSP27, HSP47, HSP70) in response to thermal stress conditioning and GF treatment

The effect of heating duration (0-8 minutes) at 44°C on protein levels of HSPs by MC3T3-E1 cells were measured using western blot analysis (Figure 3-2A) at 24 hour PH. HSP27 expression was observed only in the groups exposed to 6 and 8 minutes of heating (Figure 3-2A) whereas non-heated groups or groups exposed to heating durations shorter than 6 minutes exhibited no visible HSP27 protein bands. HSP47 expression was the most prominent of

all HSPs and the expression level remained unchanged in response to thermal preconditioning. With increasing heating time, HSP70 expression was induced more significantly with maximum expression associated with a duration of 8 minutes.

Next, we investigated the impact of thermal stress conditioning (44°C, duration=0, 4, 8 minutes) alone or in combination with GFs on HSP gene expression by measuring mRNA levels of HSP27, HSP47, and HSP70 using RT-PCR (Figure 3-2C-H) at 8, 24, and 72 hour PH. HSPs were induced more significantly for longer heating durations at 8 hour PH. However, the thermal effect on HSP induction decreased as post-incubation duration increased and GF addition induced varying responses for each HSP. At 8 hour PH, gene expression of all HSPs showed induced trends following heating but the addition of GFs following 8 minutes of heating showed diminished trends of HSP expression. HSPs in increasing order of fold induction are HSP27 (without GFs, 2.4 RFI compared to control; with GFs, 1.6 RFI), HSP47 (without GFs, 4.4 RFI; with GFs, 3.0 RFI), and HSP70 (without GFs, 63.0 RFI; with GFs, 35.6 RFI) (Figure 3-2C, D) at 8 hour PH. At 24 hour PH, HSP70 levels following heating alone or in combination with GFs decreased compared to those observed at 8 hour PH, and HSP27 and HSP47 resumed their basal levels of gene expression (Figure 3-2E, F). However, HSP47 mRNA was induced with GF addition: 7.5 RFI in non-heated groups and 2.3 and 1.1 RFI following 4 and 8 minutes heating, respectively (Figure 3-2E). At 72 hour PH, GFs suppressed HSP70 significantly and showed similar expression levels of HSP27 and HSP47 mRNA to those in cells without GF addition (Figure 3-2G, H).

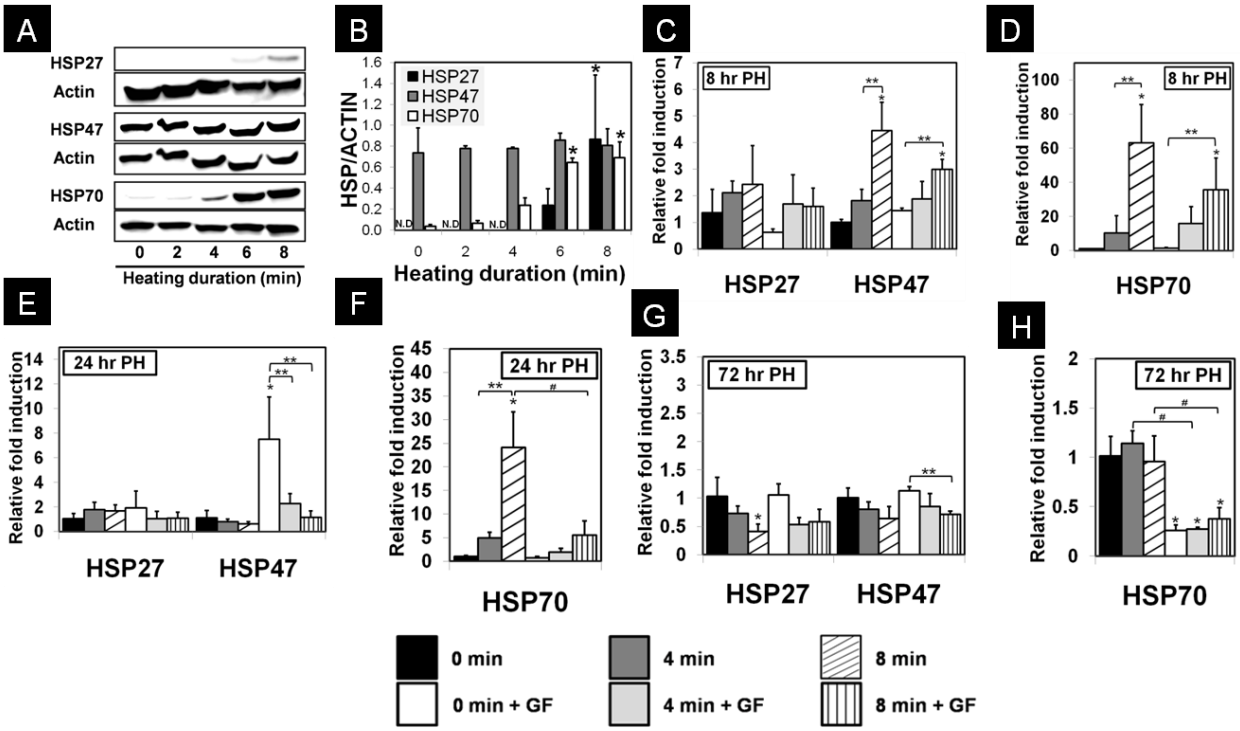

Figure 3-2. Expression of HSP27, HSP47, and HSP70 following heating (44°C for 0-8 minutes) alone or in combination with GFs (BMP-2 and TGF-β1) for varying PH duration (8, 24, and 72 hours). Western blot analysis of HSP expression following heating alone with varying heating duration (0, 2, 4, 6, and 8 minutes) (PH=24 hours) (A) and corresponding western blot quantification of HSP expression (n=3) (B). Gene expression following thermal conditioning (44°C, 0, 4, and 8 minutes) and post-culturing with GFs (BMP-2 and TGF-β1) for 8, 24, and 72 hours (C-H). HSP27, HSP47, and HSP70 mRNA expression at 8 hours (C, D), 24 hours (E, F), and 72 hours (G, H) PH. *, **, and # represent statistical significance between control (non-heated without GFs) and stressed groups, between groups heated for 4 and 8 minutes, and between each heated group in the absence or presence of GFs (*e.g.* 4 minutes and 4 minutes with GFs), respectively ($p<0.05$). N.D. denotes no detection in western blot analysis.

3.4.3. Gene expression of bone-related proteins in response to thermal stress conditioning and GF treatment

Gene expression of bone-related proteins was also measured at 8, 24, and 72 hours PH following thermal conditioning with 44^{o}C for 0-8 minutes using identical methods as described for HSPs (Figure 3-3). OPN, OCN, and OPG genes exhibited more significant induction with longer heating duration at 8 hour PH. However, the addition of GFs caused a decrease in gene levels of OCN and OPN but greater induction of OPG compared to heating alone (Figure 3-3A). Specifically, OPN expression showed increasing trends of 1.8 RFI following 8 minute heating without GFs compared to control, respectively. However, growth factor inclusion in combination with 4 and 8 minute heating suppressed OPN with 0.5 and 0.8 RFI, respectively. OCN expression increased 1.94 and 3.80 RFI following 4 and 8 minute heating without GFs respectively. However, the addition of GFs to samples heated for 4 and 8 minutes suppressed gene expression with 1.33 and 2 RFI compared to the control. OPG expression increased 5.6 and 6.9 RFI for 4 and 8 minute heating respectively in combination with GFs as compared to 1.2 and 2.1 RFI for heating alone for 4 and 8 minutes respectively (Figure 3-3A). However, OPN and OPG expression did not show a significant difference following heating and GF addition at 72 hour PH (Figure 3-3E). MMP-9 increased at 8 hour PH following GF addition for both heated and sham-treated cells (Figure 3-3A). However, MMP-9 decreased more significantly following longer heating durations (8 minutes) without GF addition at 24 and 72 hour PH (Figure 3-3C, E). GFs inhibited MMP-9 more significantly and there was no heating effect on MMP-9 following GF treatment at 72 hour (Figure 3-3E). ALP and BSP expression decreased following heating for both GF-treated and non GF-treated cells for all PH durations (Figure 3-3B, D, and F). However, GFs induced ALP for both heated and sham-treated cells but suppressed BSP at 8 and 24 hour PH. Type I collagen mRNA exhibited no significant difference following thermal conditioning and GFs for all three PH periods (Figure 3-3B, D, F). Osteonectin (ON) expression of GF-treated cells decreased following thermal stress at 24 and 72 hour PH (Figure 3-3C, E). COX-2 mRNA expression increased with longer heating duration without GFs: 2.7 and 9.8 RFI with 4 and 8 minute heating, respectively (Figure 3-4). However, for 8 minute heating and increasing PH duration, the heat-stimulating benefit on COX-2 expression diminished (9.8, 3.3, and 1.6 RFI at 8, 24, and 72 hours, respectively). In addition, GFs caused stimulation of COX-2 mRNA to be more pronounced for all PH durations (Figure 3-4).

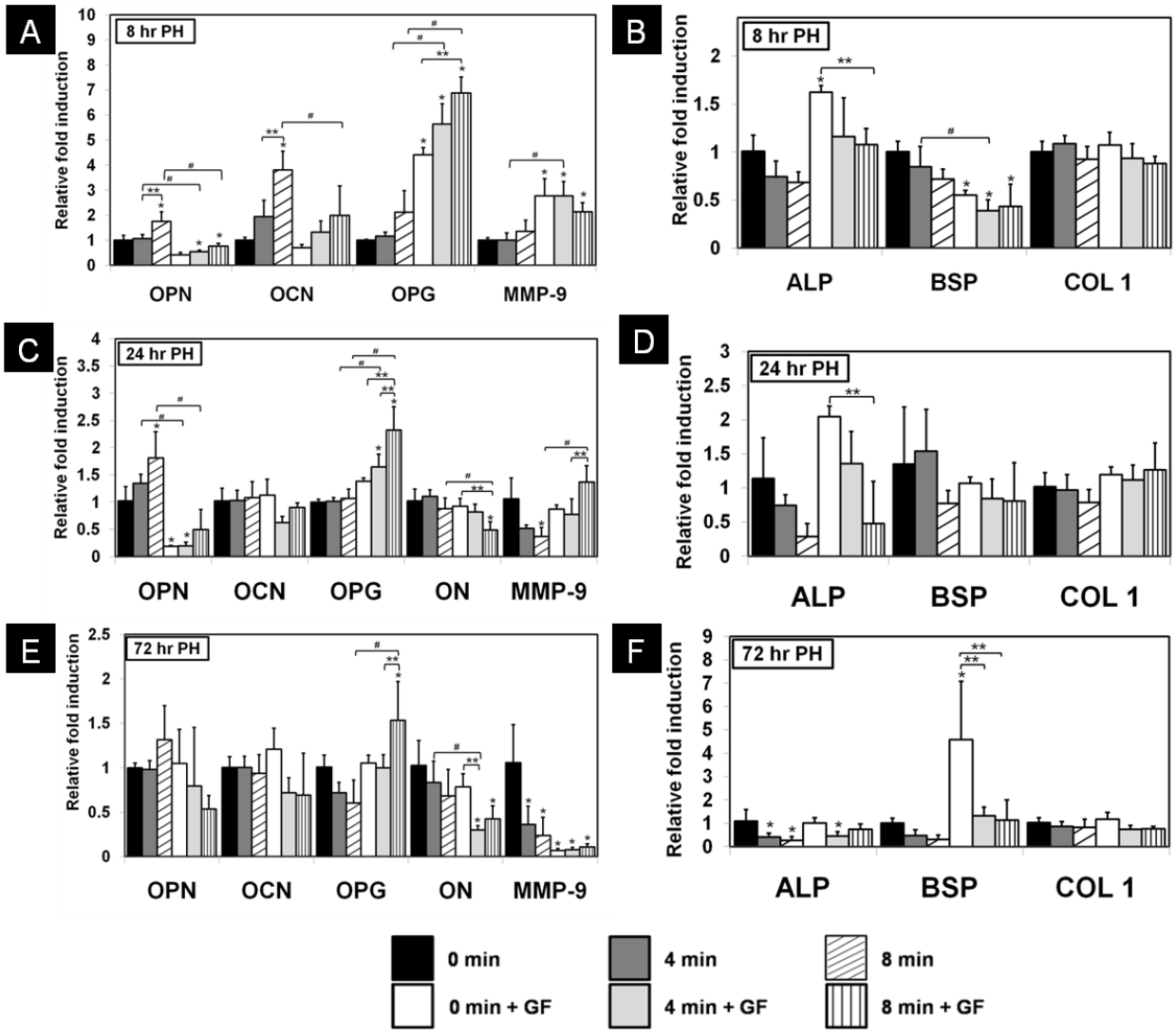

Figure 3-3. Expression of bone-related mRNA following thermal conditioning (44°C, 0, 4, and 8 minutes) and post-culturing with GFs (BMP-2 and TGF-β1) for 8, 24, and 72 hours. OPN, OCN, OPG, and MMP-9 mRNA at 8 hour PH (A); ALP, BSP, and type I collagen (COL 1) mRNA at 8 hour PH (B), 24 hour PH (D), and 72 hour PH (F); OPN, OCN, OPG, ON, and MMP-9 mRNA at 24 PH (C) and 72 hour PH (E). *, **, and # represent statistical significance between control (non-heated without GFs) and stressed groups, between groups heated for 4 and 8 minutes, and between each heated group in the absence or presence of GFs (e.g. 4 minutes and 4 minutes with GFs), respectively ($p<0.05$).

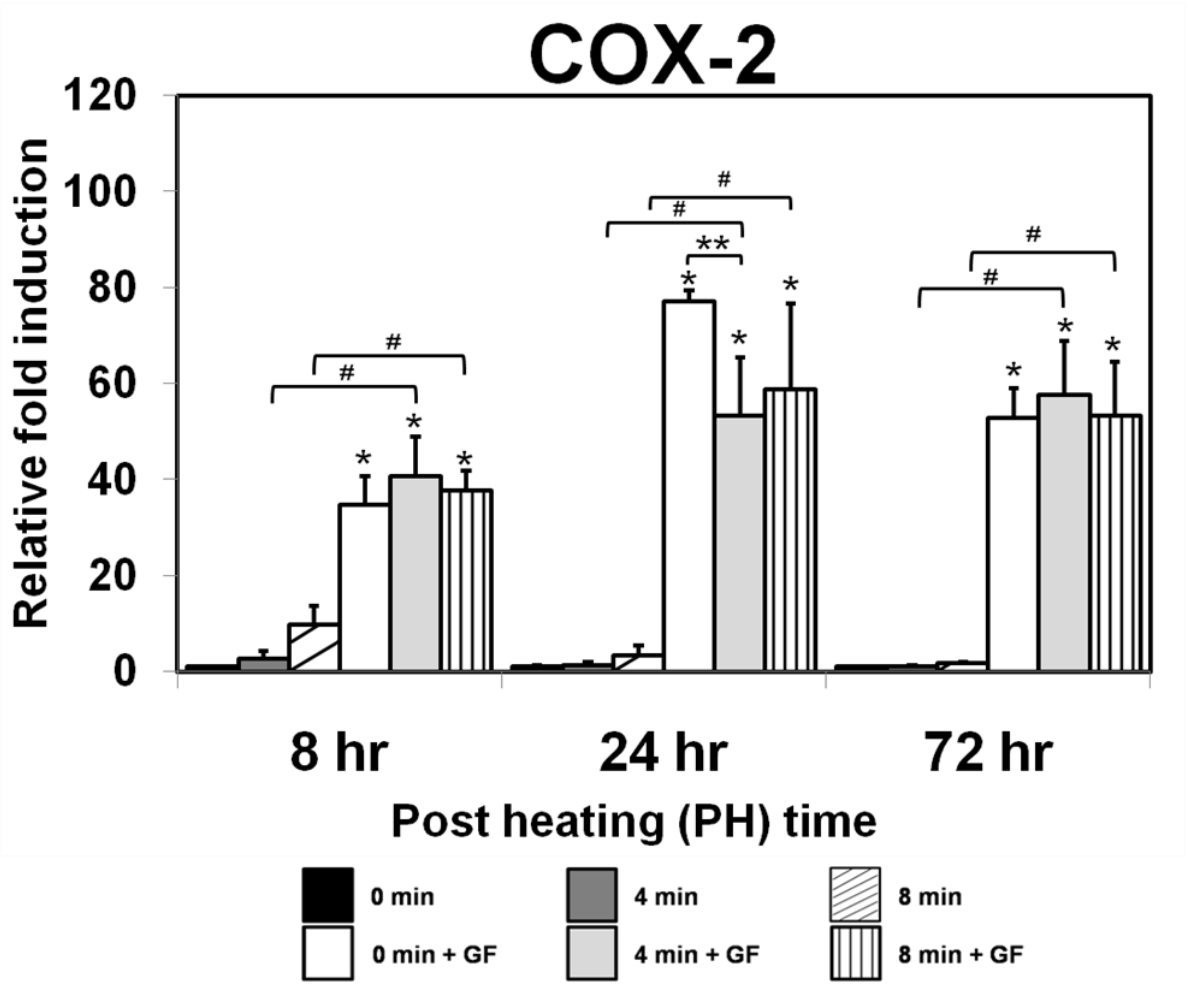

Figure 3-4. COX-2 mRNA expression following thermal conditioning (44°C, 0, 4, and 8 minutes) and post-culturing with GFs (BMP-2 and TGF-β1) for 8, 24, and 72 hours. *, **, and # represent statistical significance between control (non-heated without GFs) and stressed groups, between groups heated for 4 and 8 minutes, and between each heated group in the absence or presence of GFs (*e.g.* 4 minutes and 4 minutes with GFs), respectively ($p<0.05$).

3.4.4. Induction of VEGF in response to thermal stress conditioning and GF treatment

Thermal stress at 44^{o}C in combination with GFs increased the expression of VEGF gene and protein more than heating or GF addition as shown in Figure 3-5. GF addition induced VEGF mRNA more significantly than heating for all PH durations. The combination of 8 minute heating with GFs caused the greatest VEGF mRNA induction at 8 hour PH (16.0 RFI) (Figure

3-5A) whereas heating alone induced only a 5.3 RFI of VEGF mRNA expression (Figure 3-5A). At 24 hour PH, the level of VEGF declined for samples heated alone or in combination with GFs. At 72 hour PH, the heated samples returned to their basal level, but samples treated with GFs still maintained elevated expression. The combination of heating (4 and 8 minutes) and GFs increased the greatest level of VEGF protein secreted in the culture media among test groups at 72 hour post heating (Figure 3-5B). Without GF addition, secreted VEGF into the culture medium was not detectable and heating for durations of 4 and 8 minutes with GFs enhanced the release of VEGF concentration by 9.0 and 6.2 RFI respectively compared to non-heated groups with GFs (Figure 3-5B).

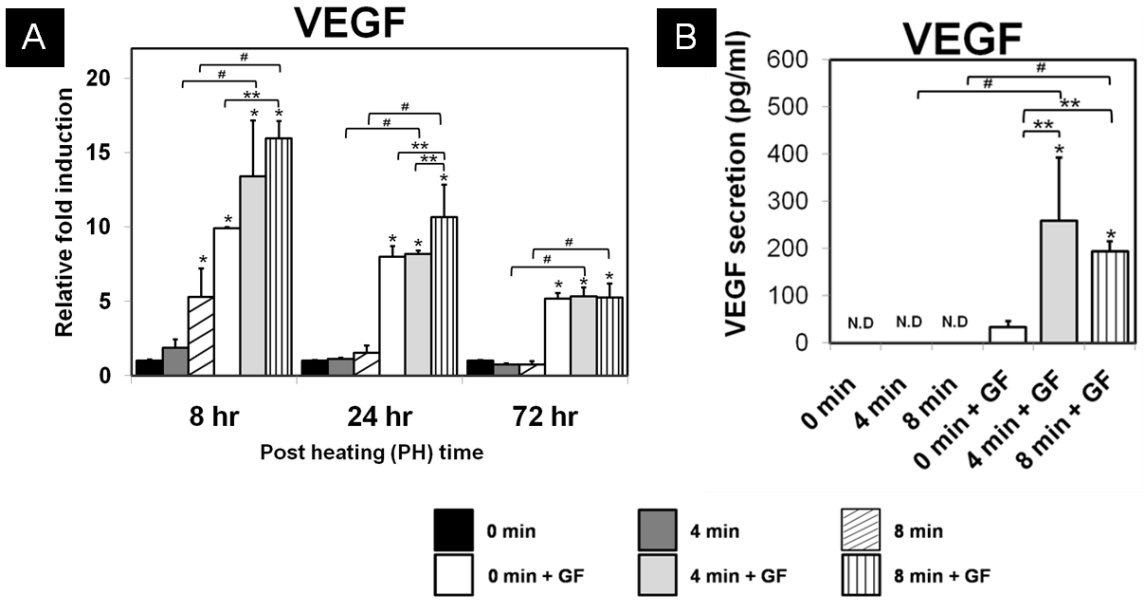

Figure 3-5. VEGF gene and protein expression following thermal conditioning (44°C, 0, 4, and 8 minutes) and post-culturing with GFs (BMP-2 and TGF-β1). VEGF mRNA expression (A) at different PH durations (8, 24, and 72 hours). Released VEGF in the culture media during 72 hour PH with GF addition analyzed by ELISA (B). *, **, and # represent statistical significance between control (non-heated without GFs) and stressed groups, between groups heated for 4 and 8 minutes, and between each heated group in the absence or presence of GFs (e.g. 4 minutes and 4 minutes with GFs), respectively ($p<0.05$).

3.4.5. Protein release in response to thermal stress conditioning and GF treatment

ELISA was performed to investigate the secretion of bone marker proteins into the culture media at 72 hour PH after heating (44^{o}C, duration=0, 4, and 8 minutes). OPG, OPN, MMP-9, and OCN concentrations were investigated since they showed the most significant variation between the test groups in the relative gene fold-induction data described in previous sections. Significantly, higher concentrations of OPG, OPN, and OCN were observed in all GF-treated cells (Figure 3-6A-C). GF-treated cells without heating showed 5.4 fold higher OPN secretion than control groups (neither GFs nor heating) (Figure 3-6A). Similarly, a 10.7 fold higher OPG level was secreted in GF-treated cells without heating compared to control groups. As shown in Figure 3-6A and C, OPN and OPG without GFs showed a slight increase when heated even though the OPN and OPG levels were much lower than those of GF-treated groups. OPG and OPN showed decreased secretion with combined heating and GF addition (Figure 3-6A and C). Moreover, OCN secretion in cells without GFs was too low to be detected by an ELISA assay (Figure 3-6B). On the other hand, sham-treated and groups heated for 4 and 8 minutes without GFs showed inhibited MMP-9 release in a heating duration-dependent manner (621.2, 545.4, and 441.3 pg/ml, respectively). However, MMP-9 in all GF-groups was suppressed so significantly that it was not detectable by ELISA (Figure 3-6D).

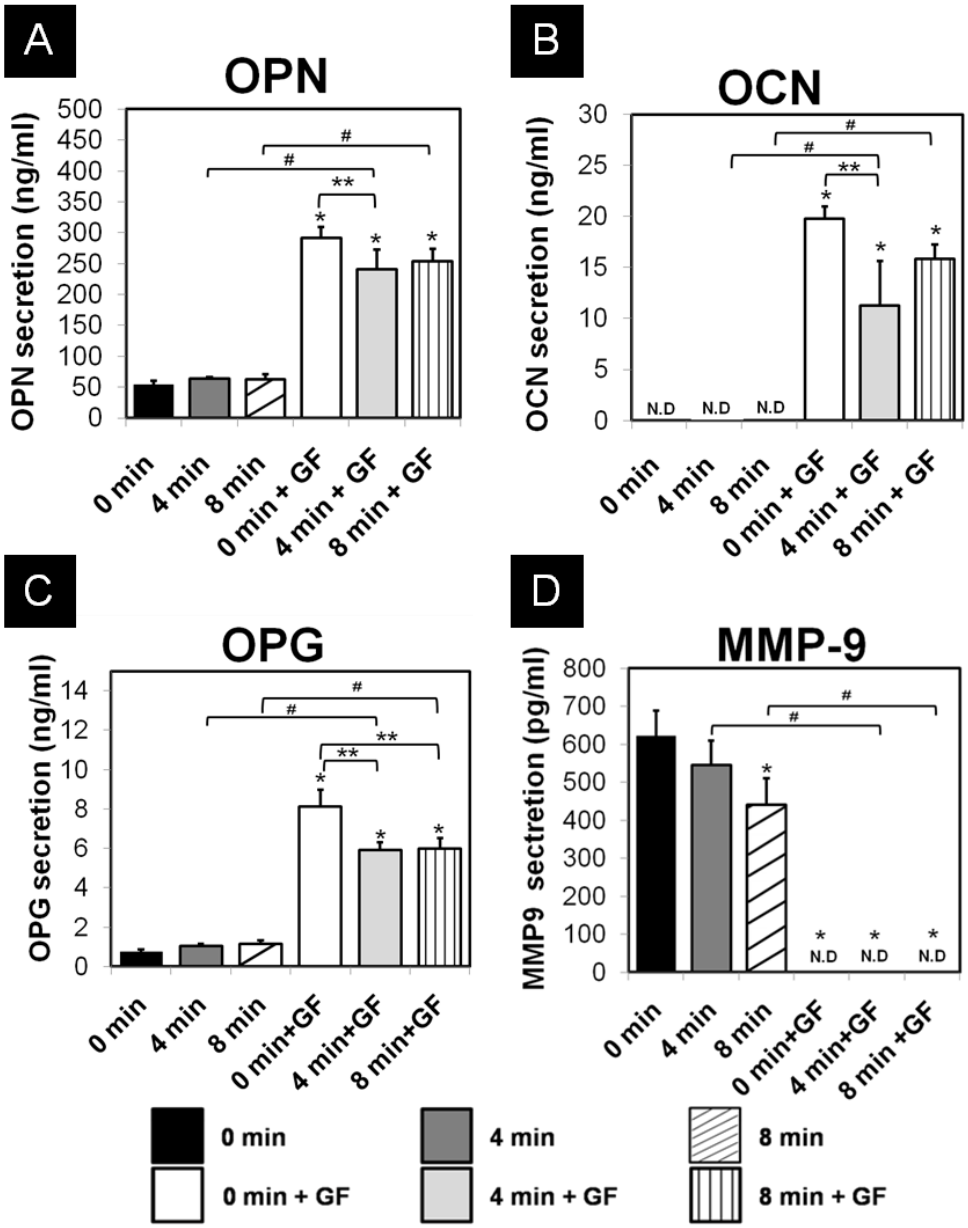

Figure 3-6. Secretion of bone-related proteins following thermal conditioning (44°C, 0, 4, and 8 minutes) and post-culturing with GFs (BMP-2 and TGF-β1) for 72 hours. The concentrations of secreted OPN (A), OCN (B), OPG (C), and MMP-9 (D) were quantified using ELISA. *, **, and # represent statistical significance between control (non-heated without GFs) and stressed groups, between groups heated for 4 and 8 minutes, and between each heated group in the absence or presence of GFs (*e.g.* 4 minutes and 4 minutes with GFs), respectively ($p<0.05$).

3.5. Discussion

The effect of a single-dose of thermal stress and GF treatment on a preosteoblast cell line (MC3T3-E1) was explored by measuring gene and protein expression of critical bone-related proteins, angiogenic marker, and stress proteins (*i.e.* HSPs) to develop feasible conditioning strategies for bone tissue regeneration.

Thermal Conditioning Protocols: Thermal stress conditioning using supra-physiological levels of heating (*e.g.* heat stimulator and heating blanket) and laser irradiation has been previously investigated therapeutically using *in vivo* animal experiments [75, 141, 143, 144] and clinical studies in skin [252], jaw [253], and periodontal therapy [254]. Even though it is very difficult to exactly compare the result of each study due to the variation in cellular response following different heating protocols, utilization of low-level heating is preferred for skeletal cells [74, 76] as well as skin wound healing [141] because this can induce increased metabolic activity and cellular proliferation without cytotoxicity [74, 76, 131]. Shui *et al*. demonstrated heating by a water bath at 39-41°C for 1 hour caused increased cell proliferation and ALP production [76]. Hojo *et al*. reported water bath heating at 41°C or lower for 15-30 minutes enhanced cell proliferation and improved proteoglycan metabolism [74]. Furthermore, the use of conditioned culture media acquired from heat-treated osteoblasts (incubator heating at 42°C for 1 hour) enhanced osteocalcin secretion and mineralization in bone marrow mesenchymal cells [131]. Leon *et al.* showed temperatures in the range of *ca.* 42.5-44°C can generate new osteo-tissue following treatment with 915 MHz microwave for 45 minutes [144].

Cellular Proliferation: Although these prior studies suggested the feasibility of thermal stress conditioning for targeted tissue healing, there is no detailed investigation of cellular response to thermal stress using preosteoblasts to allow optimization of thermal stress conditioning in combination with GFs. As a prerequisite for identifying thermal stress conditioning protocols to enhance beneficial responses for bone healing, we first determined protocols that did not adversely affect cell viability. To accomplish this, we characterized the threshold heating temperature and duration necessary for cytotoxicity. We concluded heating temperature of 40-44°C with durations shorter than 10 minutes were conducive to sustained viability (Figure 3-1). These results are comparable to studies by Rylander *et al.* in which cancerous (PC3) and normal (RWPE-1) prostate cells were heated with a water bath for 44 to

60^{o}C for 1 to 30 minutes [249]. Compared to incubator-based heating studies [131, 142, 221], the thermal response time for our water bath heating is much shorter, causing the onset of injury and protein induction to occur earlier. Other studies using water bath heating have utilized lower temperatures (39-41^{o}C) with longer heating durations (15 minutes-1 hour) and have demonstrated beneficial cellular effects [74, 76]. Since our results showed heating at 44°C for durations shorter than 10 minutes caused minimal cytotoxicity, we focused on measuring the expression of HSPs and bone proteins in preosteoblast cells for these conditions to determine thermal stress conditioning protocols beneficial for bone tissue engineering.

Heat Shock Proteins: Measurement of HSP induction was a critical aspect of this study due to the important role of HSPs in bone physiology [208] and matrix protein production [200] and their documented thermally induced expression kinetics [243, 249]. In particular, osteoblasts of rat tibia near the bone-forming area are known to express HSP27, HSP47, and HSP70 highly with type I collagen [208]. Our thermal conditioning protocols exhibited transient elevations in expression of three HSPs (HSP27, HSP47, and HSP70) for longer heating durations (Figure 3-2). HSP70 exhibited the greatest increase in expression following thermal stress compared to the other HSPs. Both HSP70 and HSP27 expression was induced more significantly with increasing heating duration. HSP47 exhibited a high level of basal expression which was unaffected by thermal stress. Comparable HSP70 expression levels and trends were observed in studies by Rylander *et al.* in which endothelial cells were heated with a water bath (44-46°C for 0-10 minutes) and HSP expression was measured at 16-18 hour PH [136]. Similarly, Wang *et al.* reported heating using a incubator set at 42°C for 1.6 hour induced transient induction of HSP70 with maxim expression for a post-heating duration of 16 hours [243]. Other studies have also shown increased HSP70 levels (*ca.* 1.7 fold) compared to control by chondrocytes heated at 43°C for 1 hour with an incubator [223]. Thermally induced HSP27 and HSP70 expression kinetics observed in this study are similar to prior studies by Rylander *et al.* in which the temporal HSP expression profiles in cancerous (PC3) and normal (RWPE-1) prostate cells were measured following thermal stress [249]. HSP27 upregulation in MC3T3-E1 cells may offer protection to external stresses while also modulating several important molecules relevant in bone physiology. MC3T3-E1 cells have been previously shown to regulate HSP27-related transcripts by estrogen pretreatment under thermal stress [196] and promote HSP27 by

prostaglandin D_2, whose action is mediated by protein kinase C and mitogen-activated protein kinase (MAPK) signaling pathway (*e.g.* p44/42 and p38) [193].

Our results have also measured the combined effect of water bath heating and osteoinductive GFs on expression of HSP genes and protein by bone cells *in vitro*. PCR analysis revealed that heating in combination GFs caused varying responses for each HSP (HSP27, HSP47, and HSP70) which differed as a function of PH duration. GF treatment inhibited induction of HSP27, HSP47, and HSP70 at 8 hour PH following thermal stress (Figure 3-2C-D). However, the addition of GFs increased levels of HSP47 genes in both non-heated and heated cells at 24 hour PH. The varying response of each HSP to GFs may be caused by the difference of their inherent function in cells. HSP47, which can be enhanced by TGF-β1 [94], operates in collagen-secreting cells serving as procollagen-binding molecular chaperone in the collagen synthesis process [200]. However, HSP27 and HSP70 are related to intracellular apoptosis-related mechanisms [212]. Our results demonstrate that a single dose of thermal stress for a few minutes can cause substantial induction in HSP expression. For example, our results show that heating for 8 minutes can induce a 63 fold increase in HSP70 (HSP1B) mRNA at 8 hour PH with lower levels of HSP27 (2.4 RFI) and HSP47 (4.4 RFI) induction. Prior studies using cyclic tension (6 hours, 15% magnitude, 1 cycle/s) showed only an 8.1 fold induction in HSP70 (HSP1B) and a decrease in HSP27 mRNA in trabecular meshwork cells [218]. Therefore, in the viewpoint of HSP inductions, thermal stress may provide a more powerful conditioning method for inducing HSPs requiring only a single thermal dose for a matter of minutes rather than repeated conditioning as is used by other strategies such as stretching [218] or hydrostatic pressure [255].

Bone-related Proteins: *In vitro* responses of preosteoblasts following combined thermal stress conditioning and GFs was determined by measuring several bone-relevant proteins: matrix proteins (*e.g.* OCN, OPN, BSP, and type I collagen), GFs (*e.g.* VEGF), cytokines (*e.g.* OPG), and enzymes (*e.g.* MMP-9 and ALP) (Figure 3-3, 4, 5, and 6). This broad screening of bone-related proteins is critical to determine whether thermal stress conditioning alone or in combination with GFs may potentially cause beneficial or deleterious effects associated with osteogenesis, angiogenesis, osteoclatogenesis, and production of matrix proteins and GFs in bone tissue. The induction or suppression of the aforementioned bone markers regulate bone physiological processes and bone regeneration. For example, the interaction between bone cells

[16] and endothelial cells [19] is controlled by secretion of bioactive molecules such as BMP-2 or mediation through ligands such as the receptor activator of NF-κB ligand (RANKL), which is related to OPG. According to Shea *et al.*'s study [89], elevated levels of OCN mRNA accompanied with high mineralization caused bone-like morphology in MC3T3-E1-seeded scaffolds exhibiting specialized cell localization. Therefore, induction of OCN mRNA by thermal stress preconditioning, as shown in our study, could potentially accelerate *in vitro* osteogenesis. Similarly, our study demonstrated the capability of thermal stress conditioning to upregulate OPN mRNA. Prior studies have shown OPN gene induction by mechanical stress to contribute to bone regeneration thereby offering another benefit to our thermal stress approach [88]. COX-2, which operates as an enzyme critical for prostaglandin production in bone necessary for bone repair [121, 122], and was shown in this study to be transiently induced more significantly with longer thermal stress duration (8 minutes).

Inhibition of bone resorptive proteins such as MMP-9 and promotion of anti-osteoclastogenic proteins like OPG in bone microenvironment could be utilized to mitigate bone over-resorption which cause bone diseases such as osteoporosis [31]. In addition to its inhibitory role in osteoclastogenesis, Grundt *et al.* suggested OPG can up-regulate the level of ALP directly in a dose-dependent manner [90]. Greater induction of OPG genes following combined thermal conditioning and GFs than individual stress as shown in our study could not only inhibit bone disease but also potentially promote ALP expression. Moreover, BMP-2 can up-regulate osteoprotegerin (OPG) in MC3T3-E1 cells by means of Wnt/β-catenin [126]. TGF-β1 can increase OPG/OCIF (osteoclastogenesis inhibitory factor) induction in MC3T3-E1 cells and bone marrow stromal cells [127]. The combined effects of thermal stress and GFs on OPG induction (Figure 3-3A, C, and E) was demonstrated for the first time although the effects of GF stimulation on OPG expression has been previously described [126, 127]. In addition, we showed that GFs and heating alone can downregulate MMP-9, a marker for osteoclastogenesis. ELISA-analyzed protein levels found that GF addition promoted OPN, OCN, and OPG protein expression and inhibited MMP-9 significantly. Therefore, our results suggest that combinatorial heating and GF addition could significantly inhibit osteoclast differentiation or bone resorptive process in bone microenvironment.

Production of VEGF is critical to stimulation of blood vessel formation necessary for bone regeneration. Our results showed the combination of thermal stress conditioning and GFs

could upregulate greater level of VEGF than individual stress implying these methods may be conducive to enhancing angiogenesis in bone healing. Without GFs, thermal stress induced rapid induction of VEGF after short PH periods of several hours.

The thermal conditioning protocols utilized in this study hindered the induction of ON, ALP, and BSP genes (Figure 3-3). These results should be investigated further to develop more optimal protocols for inducing these proteins by using more mild-heating conditioning or long-term analysis of bone-related proteins. Since mild heating at 42°C or lower for 30-60 minutes has been reported in studies to promote levels of ALP for PH periods of 4 days or longer than 1 week, it is difficult to compare the results to our study due to the differences in heating methodology [76, 142]. In general, since we utilized only a single dose of thermal stress followed by analysis at a limited number of timepoints during short-term PH, the enhancement of bone-related proteins and HSPs may be transient and fail to induce sustained upregulation and appropriate timepoints for analysis may not be considered. This problem could be solved through a long-term stress conditioning strategy using a 3D scaffold in an automated thermal conditioning bioreactor to give bone cells sufficient time for *in vitro* bone formation and mineralization.

In conclusion, this study revealed for the first time that a preosteoblast cell line (MC3T3-E1) exposed to elevated temperature (44°C) for varying heating duration (0-8 minutes) and PH period (8, 24, and 72 hours) alone or in combination with two exogenous osteoinductive GFs (BMP-2 and TGF-β1) induced mRNA levels of HSPs (*e.g.* HSP27, HSP47, and HSP70), bone-related proteins (*e.g.* OPN, OCN, and OPG) and an angiogenesis promoter (VEGF), and suppressed an osteoclastic marker (MMP-9). Thermal stress without GFs induced OPN, OCN, COX-2, and HSP mRNA and suppressed BSP and ALP mRNA with more pronounced induction/suppression occurring with longer heating duration. The combination of thermal stress conditioning and GFs showed the most increased expressions of VEGF and OPG mRNA among test groups and suppressed MMP-9 the most. For other bone markers and HSPs, the combined effect of thermal conditioning and GFs caused varying effects depending on heating and PH duration. Based on this study, thermal stress conditioning alone or in combination with exogenous GFs can potentially serve as a new strategy to inhibit the imbalance between osteoclasts and osteoblasts often associated with bone disease such as osteoporosis, and induce bone formation and enhance angiogenesis critical for bone regeneration.

Chapter 4: Conditioning Effect of Cyclic Tension and Growth Factors on a Preosteoblastic Cell Line for Bone Tissue Engineering Applications

Eunna Chung[1] and Marissa Nichole Rylander[1, 2*]

[1]Virginia Tech –Wake Forest University, School of Biomedical Engineering and Sciences, Virginia Tech, Blacksburg, VA 24061

[2]Department of Mechanical Engineering, Virginia Tech, Blacksburg, VA 24061

4.1. Abstract

Bone regeneration can be accelerated by utilizing mechanical stress and growth factors (GFs). However, a limited understanding exists regarding the effects of tension alone or in combination with GFs on the stress response and regulations of bone-related proteins by preosteoblasts. This study investigated MC3T3-E1 cell morphology, proliferation, and expression of heat shock proteins (HSPs) and bone-related proteins following cyclic tension (equibiaxial 1-10% magnitude) alone or with osteoinductive GFs (bone morphogenetic protein-2 and transforming growth factor-β1). Combinatorial conditioning using tensile stress and GFs induced the greatest genes for osteoprotegerin, prostaglandin E synthase 2 (both for 1, 3, and 5% strain), and vascular endothelial growth factor (VEGF) (for all magnitudes) among test groups. Furthermore, cell proliferation and secretion of VEGF and osteopontin (OPN) increased more following 5% strain with GFs than individual stresses of tension or GF addition. The addition of GFs to static-cultured and tensile-loaded cells induced genes of HSP47, cyclooxygenase-2, bone sialoprotein, and type I collagen, and suppressed HSP70 and OPN genes. HSP27 and HSP70 genes were induced by tension, however, GFs with tension exhibited lower levels than tension alone. Matrix metalloproteinase-9 decreased with tension alone or with GFs. Therefore, our results suggest that combinatorial conditioning with tension and GFs may provide a promising method to enhance bone regeneration by initiating angiogenic and anti-osteoclastic effects.

4.2. Introduction

Previous tissue engineering research has explored techniques utilizing scaffolds, cells capable of proliferation and differentiation, bioactive molecules, and stress conditioning to augment cells' healing potential [256, 257]. Bone regeneration in particular requires a highly dynamic environment in which external mechanical stresses (*e.g.* weight-bearing compression, tension, and fluid flow-mediated shear stress) are constantly changing, remodeling is continually occurring, and biochemical cues are triggering cellular responses. Bone formation proceeds with time-dependent modulation of matrix proteins, growth factors (GFs), and various enzymes: alkaline phosphatase (ALP), matrix metalloproteinase (MMP), and cyclooxygenase (COX).

Mechano-signals generated by strain modulation can control diverse cell activities such as proliferation [80, 99], migration [99], and differentiation [258]. *In vitro* modulation of direct stretching or compressive strain has been investigated as a mechanical cue to regenerate skin [259], bone [59], tendon [260], cartilage [261, 262], and blood vessels [263]. Although tensile stress conditioning protocols have variable biological outcomes depending on cycle frequency, duration, and magnitude, it has been reported that tension can regulate bone-related molecules such as collagens [61, 160], osteocalcin (OCN) [61, 99], osteopontin (OPN) [61, 99], MMP-13 [164], osteoprotegerin (OPG) [60], prostaglandin E_2 (PGE_2) [264], COX-2 [159], vascular endothelial growth factor (VEGF) [99, 265], and transforming growth factor-β1 (TGF-β1) [266]. Numerous bioreactor systems (*e.g.* spinner flask, rotating wall vessel, and stretching devices) have been employed to simulate native bone formation *in vitro* [58]. The Flexcell® Tension System is a commercial tensile stress conditioning bioreactor, which has been utilized for investigation of mechanical stimulation of bone cells [59-61]. In addition to cell monolayers, 3D scaffolds, including polyurethane nanofibrous scaffolds [62] and hydrogels (*e.g.* fibrin [63] and collagen gels [64]), can undergo mechanical stress conditioning within this bioreactor.

Other external stimulating factors for bone regeneration are osteoinductive GFs such as TGF, bone morphogenetic protein (BMP), insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) [47]. Diverse delivery methodologies for these GFs have been developed for bone tissue engineering [45, 234] and clinical studies [267-269]. *In vitro* studies using preosteoblasts and stem cells suggest that osteoinductive GFs can induce increased cell proliferation and osteogenic differentiation [47]. For example, exogenous BMP-2 delivery has shown a positive effect in bone regeneration [51] and TGF-β1 can induce higher ALP production and proliferation in bone marrow cells [270]. External stimulation through mechanical and

biochemical cues controls cellular activities through downstream signaling cascades such as the mitogen-activated protein kinase (MAPK) [56, 187, 271, 272] or Smad [189] pathways. However, the combined effects and mechanisms of mechanical stress and GFs on bone cells have not been thoroughly investigated despite numerous researches exploring the individual effects of mechanical stress or GF delivery.

In response to external stress (*e.g.* heat, oxidative stress, and heavy metals [273]) as well as GFs [195], cells can produce elevated heat shock proteins (HSPs). HSP induction or activation can also be enhanced by mechanical stresses including tension [218], compression [238], hydrostatic pressure [255], and shear stress [274]. HSPs such as HSP27 and HSP70 act as molecular chaperones and have pivotal roles in anti-apoptotic protection, proliferation, and differentiation [212, 275]. HSP47 is instrumental in the collagen synthesis process as a procollagen-binding molecular chaperone located in the endoplasmic reticulum [199]. Related to bone physiology, HSP27 and HSP47 can be induced by TGF-β [94, 195] and HSP27 can also be promoted by bone-related molecules such as estrogen or prostaglandin in osteoblasts [191, 194]. In addition, HSPs (*e.g.* HSP27, HSP47, and HSP70) as well as type I collagen are highly expressed in osteoblasts of rat tibia near the bone-forming region [208]. However, prior *in vitro* studies in bone tissue engineering have not demonstrated the correlation between HSPs and mechanical stress conditioning with GFs.

The purpose of this study was to investigate whether combinatorial stress conditioning with tension and GFs could synergistically upregulate three HSPs (HSP27, HSP47, and HSP70), pivotal bone-related proteins (*e.g.* matrix proteins, growth factors, and cytokines), angiogenic marker (VEGF), and promote proliferation of preosteoblasts. We determined the effect of various cyclic tensile stress protocols (1-10% strain) in combination with two osteoinductive GFs (BMP-2 and TGF-β1) on MC3T3-E1 cells using a Flexcell® Tension System. Several assays for morphological modification, proliferation, and gene expression of HSPs and bone-related proteins, as well as protein secretion, were utilized to evaluate the regenerative responses of preosteoblasts in response to the combined effect of tension and GFs.

4.3. Materials and Methods

4.3.1. Cell culture and preparation for stress treatment

A murine preosteoblastic cell line, MC3T3-E1, (American Type Culture Collection), was cultured as a monolayer with growth media composed of alpha Minimum Essential Medium (αMEM) (Mediatech), 10% fetal bovine serum (FBS) (Sigma), and 1% penicillin-streptomycin (PS) (Invitrogen) in a 5% CO_2 incubator at 37°C. Cells were seeded in a 6-well BioFlex® plate (Flexcell International) coated with type I collagen and then cultured further for 16 hours before stress treatment. Cells were seeded at a concentration of 5×10^4 cells/well and cultured in 10% FBS osteogenic media for following studies: cell morphology and measurement of gene expression with PCR following 1% tension, and cell proliferation following tension with GFs after 3 or 6 days of treatment. For PCR (measured following 24 hour stress preconditioning) and ELISA analysis (measured at 3 and 6 days of treatment), cells were plated at a concentration of 2×10^5 cells/well and cultured in 1% FBS osteogenic media during stress preconditioning. Osteogenic media was composed of αMEM, 50 μg/ml L-ascorbic acid, 10 mM β-glycerol phosphate, 1% PS and 1 or 10% FBS. High FBS concentrations and low initial cell seeding were used for the experiments to investigate proliferation and gene/protein expressions following long-term tensile stress but low FBS and higher cell seeding density was used for short-term stress (24 hours).

4.3.2. Stress treatment

The Flexcell® Tension Plus™ System (Flexcell International) was utilized to apply a range of cyclic tensile stress conditioning protocols of 1, 3, 5, and 10% maximum elongation with 0.2 Hz (10 seconds tension on/10 seconds rest) frequency for varying durations depending on analysis. Maximum tensile elongations were represented as a percentage unit here but this percentage unit can be converted into strain (ε) and negative pressure (kPa) according to the vendor-provided conversion chart (Appendix 4). 1, 3, 5, and 10% tensile elongations denote 0.01, 0.03, 0.05, and 0.1 strain (ε), respectively, which were generated by negative pressures of 6.13, 16.74, 25.52, and 42.50 (-kPa).

A circular loading post (diameter=25 mm) was used to apply equibiaxial tension as shown in Figure 4-1. This stretching system generates tensile stress by modulating the flexible bottoms of multi-well plates with negative (vacuum) pressure. Immediately before tensile stress treatment, growth media was replaced with osteogenic media to induce osteogenesis during tensile stress preconditioning. Osteogenic media was composed of Eagle's Minimum Essential Media (αMEM), 50 μg/ml L-ascorbic acid, 10 mM β-glycerol phosphate, 1% Penicillin/Streptomycin and 1 or 10% fetal bovine serum (1 and 10% for short and long-term experiments, respectively). In order to investigate the combinatorial effect of tensile stress and GFs, the cells were cultured during the application of tensile stress in osteogenic media supplemented with BMP-2 (50 ng/ml) and TGF-β1 (2 ng/ml), which were concentrations previously shown to cause differentiation in the MC3T3-E1 cell line as previously reported by Chung, *et al.* [250].

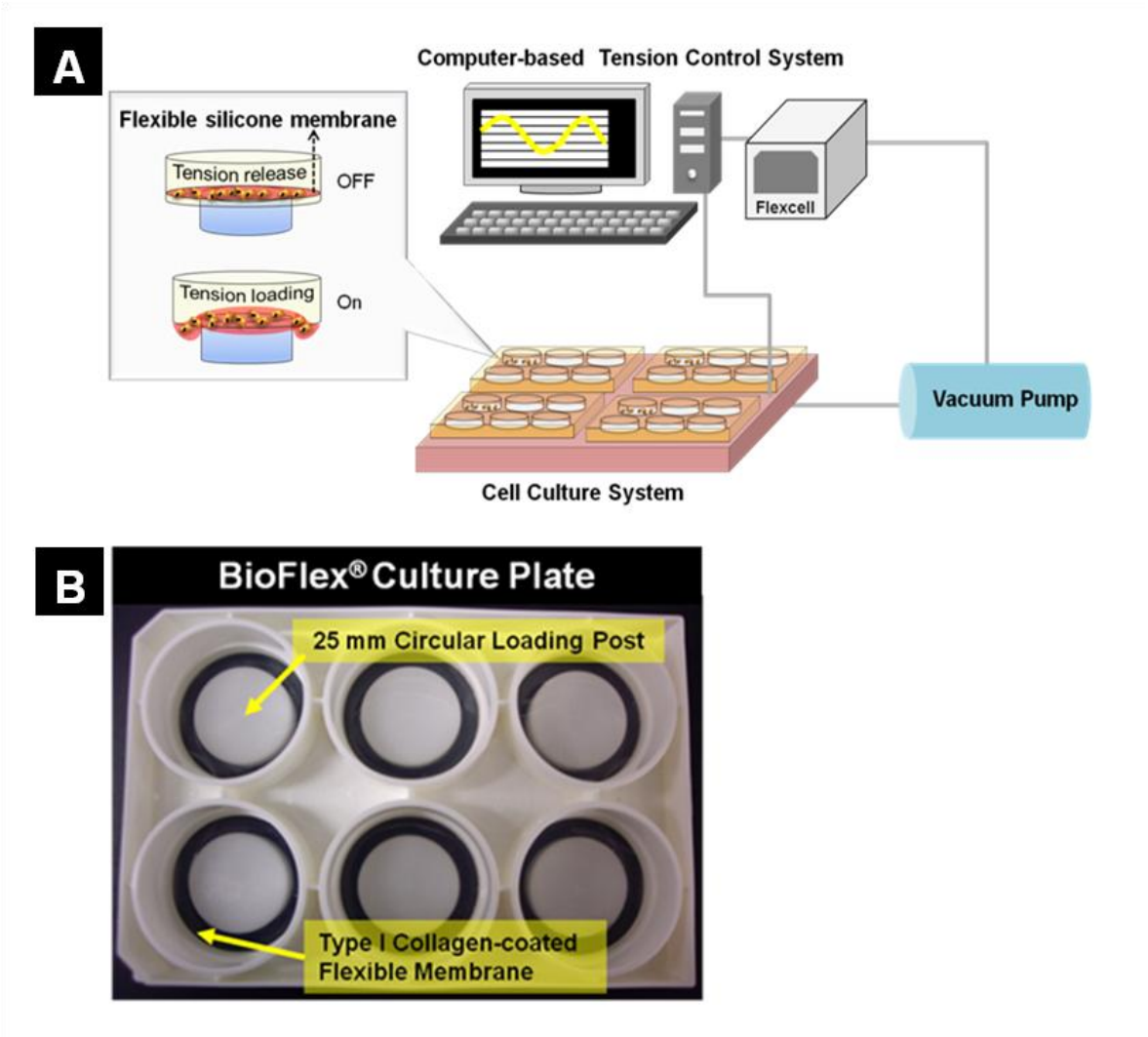

Figure 4-1. Illustration of a computer-controlled Flexcell® tension bioreactor (A) and a 6-well BioFlex® culture plate (B) with a flexible culture substrate coated with type I collagen.

4.3.3. Morphology analysis

Cell morphology following cyclic tension (1% magnitude, 0.2 Hz, 10 seconds tension on/10 seconds rest) for 6 days was visualized by fixing the cells immediately after stress and staining for F-actin, a cellular skeleton protein, using rhodamine phalloidin (Invitrogen). Cells were fixed with 3.7% paraformaldehyde in a phosphate buffered solution (PBS) and permeablized using 0.1% Triton X-100/PBS. For blocking, samples were incubated in 1% bovine serum albumin dissolved in PBS for 30 minutes at room temperature followed by 20

minutes incubation in rhodamine phalloidin solution in the dark. For nucleolus counterstaining, cells were mounted with VECTASHIELD Mounting Medium with DAPI (DAPI: 4',6-diamidino-2-phenylindole) (Vector Laboratories). Stained images were acquired using a fluorescent inverted microscope (CTR6500, Leica Microsystems) (400×).

4.3.4. MTS assay

Proliferation of MC3T3-E1 cells immediately following 5% cyclic tensile stress conditioning alone or in combination with GFs at 3 and 6 days was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega). MTS working solution composed of MTS stock solution and basal culture media (w/o FBS and PS) (volume ratio of MTS and media=1:5) was added to cells. After 4 hour incubation at 37°C, the optical density of the MTS solution was measured at 490 nm by a microplate reader (SpectraMax M2^e, Molecular Devices).

4.3.5. Quantitative real time RT-PCR

Gene expression of markers listed in Table 1 was measured following 24 hour (1 day) short-term and 3 and 6 day long-term stress conditioning alone or in combination with GFs. RNA was isolated by spin protocol using RNeasy Mini kit (Qiagen) and QIAshredder (Qiagen) immediately following stress conditioning according to the manufacturer's protocol. Isolated RNA was converted to cDNA using Reverse Transcription System (Promega) by continuous reactions at 25°C for 10 minutes and 42°C for 45 minutes followed by heating at 99°C for 5 minutes. After reverse transcription, cDNA samples were mixed with Taqman PCR Master Mix (Applied Biosystems) and each specific primer and polymerized in a 7300 Real-Time PCR System (Applied Biosystems). The PCR reaction was performed at 50°C for 2 minutes followed by 95°C for 10 minutes. For each polymerization (total 45 cycles), temperature was set at 95°C for 15 seconds and 60°C for 1 minutes. Taqman® Gene Expression Assay (Applied Biosystems) for specific gene detection was used as a primer and probe as listed in Figure 4-1. Relative fold induction (RFI) of each mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method used in Lee *et al.*'s study [251]. Threshold cycle (C_T), derived using SDS v1.2× system software of 7300

Real-Time PCR System, denotes the fractional cycle number at threshold polymerized gene and -$\Delta\Delta C_T$ was derived from (C_T of target gene-C_T of GAPDH)$_{\text{treated group}}$-(C_T of target gene-C_T of GAPDH)$_{\text{control group}}$ [251]. Treated groups denote tensile-stressed, GF-added alone or in combined treated groups and control groups indicated sham-treated cells without tension and GF addition.

Table 4-1. Specific genes measured with real time RT-PCR with corresponding Assay ID from Applied Biosystems, sequence, and size of marker.

Target protein	Gene name	Assay ID	Sequence (5'→3')	Size
HSP27	heat shock protein 2	Mm00517908_m1	TCGGAGAAGGCCTCCTGCCAGAAGA	115
HSP47	serine (or cysteine) peptidase inhibitor, clade H, member 1	Mm00438056_m1	TGGTAAACCCTCACAGGTCCTCTGT	76
HSP70	heat shock protein 1B	Mm03038954_s1	GTTAAGGTTTTGTGGTATAACCAGT	141
OPN	secreted phosphoprotein 1	Mm01611440_mH	GAACAGTATCCTGATGCCACAGATG	102
OCN	bone gamma-carboxyglutamate protein, related sequence 1	Mm00649782_gH	CCTTGGAGCTTCAGTCCCCAGCCCA	89
OPG	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Mm01205928_m1	AGTGTGGAATAGATGTCACCCTGTG	75
BSP	integrin binding sialoprotein	Mm00492555_m1	GGTTTCCAGTCCAGGGAGGCAGTGA	98
ALP	alkaline phosphatase, liver/bone/kidney	Mm01187113_g1	TCCTGGGAGATGGTATGGGCGTCTC	71
Type I collagen (Col1)	collagen, type I, alpha 1	Mm00801666_g1	CGATGGATTCCCGTTCGAGTACGGA	89
MMP-13	matrix metallopeptidase 13	Mm01168713_m1	CTTTAGAGGGAGAAAATTCTGGGCT	124
MMP-9	matrix metallopeptidase 9	Mm00600164_g1	TCTTCAAGGACGGTTGGTACTGGAA	72
Runx2	runt related transcription factor 2	Mm00501580_m1	GACGAGGCAAGAGTTTCACCTTGAC	129
VEGF	vascular endothelial growth factor A	Mm00437308_m1	CAAAGCCAGAAAATCACTGTGAGCC	66
TGF-β1	transforming growth factor, beta 1	Mm00441724_m1	TGGTGGACCGCAACAACGCCATCTA	99
COX-2	prostaglandin-endoperoxide synthase 2	Mm01307330_g1	TGTACTACACCTGAATTTCTGACAA	73
PGE synthetase 2 (Ptges2, PGE2)	prostaglandin E synthase 2	Mm00460181_m1	CAGGAAGGAGACAGCTTGCAACAGC	73
Angiopoietin 1	angiopoietin 1	Mm00456498_m1	AAAAAACAGTTTACTAGAGCACAAA	118
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1	GTGAACGGATTTGGCCGTATTGGGC	107

4.3.6. Enzyme-linked immunosorbent assay (ELISA)

For the analysis of protein secretion, osteogenic conditioned media was collected at days 3 and 6 following 5% cyclic tension alone or in combination with GFs. Because the osteogenic media was replaced once at days 3 during tensile stress alone or in combination with GFs, the osteogenic media collected at days 6 included the protein secretion during days 3 to 6. OPG, VEGF, OPN, and MMP-9 were measured using Quantikine® ELISA (R&D Systems) and OCN levels were determined by Mouse Osteocalcin EIA kit (Biomedical Technologies) according to the manufacturer's protocol. Cell culture supernatant was added into a 96-well microplate coated with antibodies specific for the previously mentioned markers and incubated further with detection solutions at room temperature. The optical absorbance of the solution was measured at 450 nm by a microplate reader and compared to a standard curve.

4.3.7. Statistical analysis

All data are presented as mean ± standard deviation (SD). Experimental groups with a minimum of three repetitions (3 or 4) were tested and analyzed independently. To demonstrate statistical significance of tension and GF treatment, two-way ANOVA supplemented by a Tukey multiple comparison test was performed to compare the means between each group and determine the interaction term between these two factors. In the case of tests involving tension alone, one-way ANOVA supplemented by a Tukey multiple comparison test was applied. The statistical significance was defined as p value lower than 0.05.

4.4. Results

4.4.1. Morphological analysis of tensile stress conditioning (1% elongation, 0.2 Hz, 10 seconds tension on/10 seconds rest)

F-actin filaments of stretched cells were stained immediately after cyclic stress conditioning after 6 days to determine the effect of cyclic tension on cellular morphology. Static-cultured cells possessed an irregular shape with randomly oriented actin filaments as observed in Figure 4-2A. In contrast, cells located on the edge of the dish exposed to tension exhibited an

elongated and aligned actin morphology (Figure 4-2B). In the center of the dish, stretched cells appeared similar to static-cultured cells (Figure 4-2C).

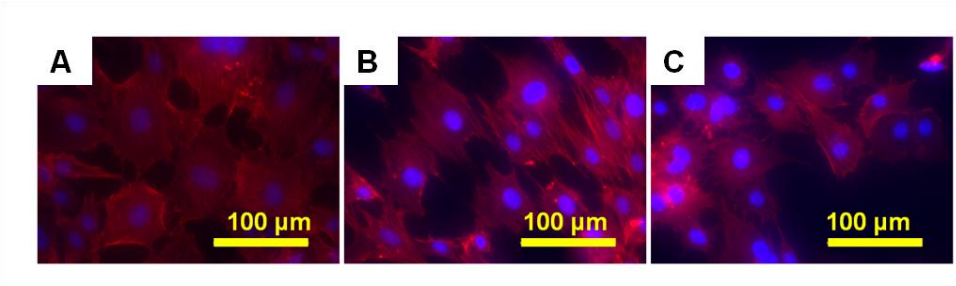

Figure 4-2. Cell morphology (400× magnification) measured following 6 days of cyclic tension (1% elongation). Static cultured cells (A) and stretched cells on the edge (B) and the center (C) of a culture plate (scale bar=100 µm).

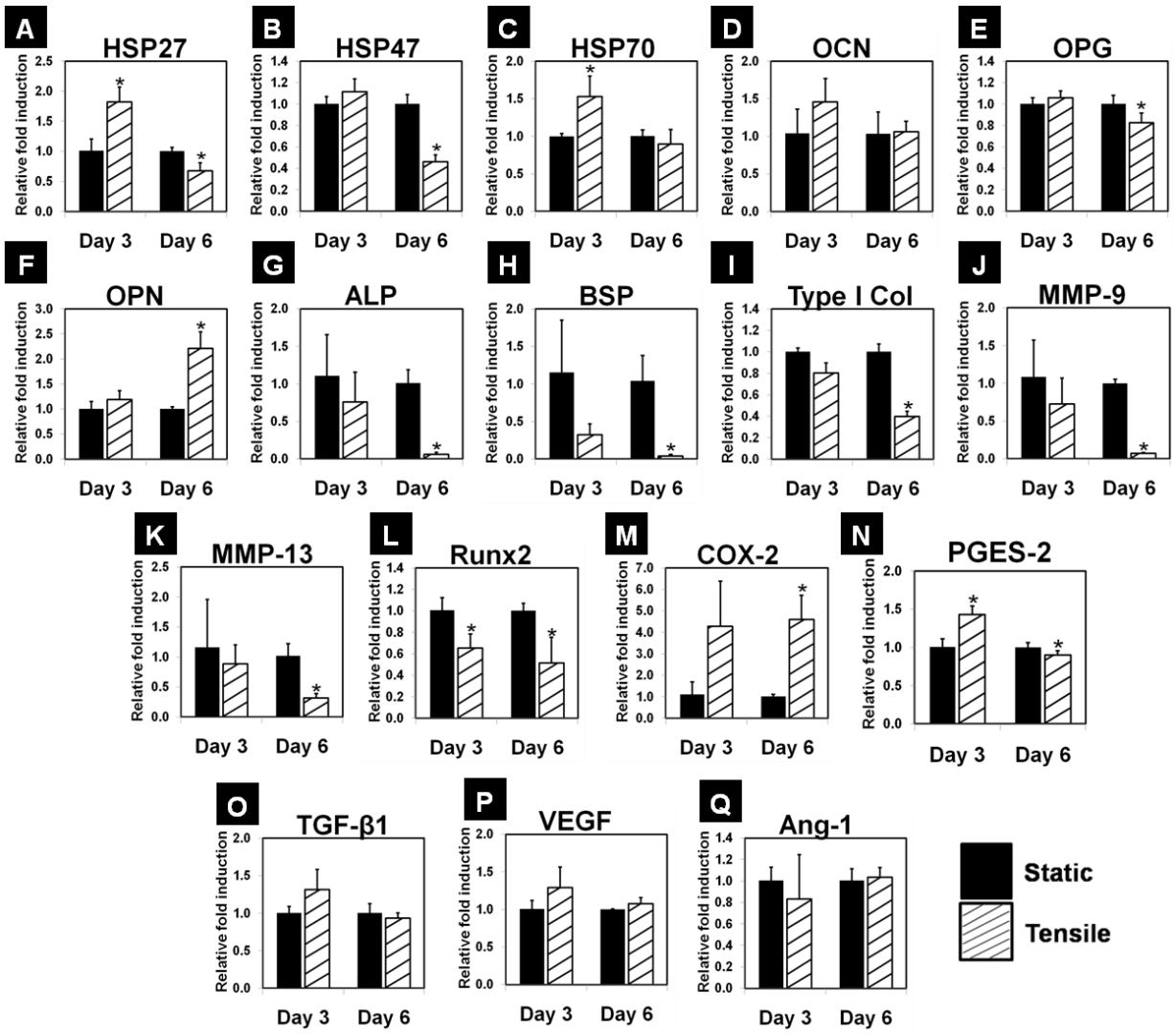

Figure 4-3. mRNA Expression of HSP and bone-related molecules on days 3 and 6 following cyclic tension (1% elongation). Expression of mRNA for HSP27 (A), HSP47 (B), HSP70 (C), OCN (D), OPG (E), OPN (F), ALP (G), BSP (H), type I collagen (I), MMP-9 (J), MMP-13 (K), Runx2 (L), COX-2 (M), PGES-2 (N), TGF-β1 (O), VEGF (P), and angiopoietin-1 (Ang-1) (Q). An asterisk (*) represents statistical significant difference between tension-treated groups and control ($p<0.05$).

4.4.2. Effect of long-term cyclic tensile stress conditioning (1% elongation, 0.2 Hz, 10 seconds tension on/10 seconds rest) without GF treatment on mRNA of HSPs and bone-related molecules

Messenger RNA from MC3T3-E1 cells was analyzed by real time RT-PCR following 3 and 6 days of tensile preconditioning. The mRNA levels of each HSP (HSP27, HSP47, and HSP70) increased following 3 days of tensile preconditioning, but decreased by days 6 (Figure 4-3A-C). Cyclic tension induced OPN, COX-2, and VEGF, mRNA by days 3 and 6 (Figure 4-3F, M, and P). OCN, prostaglandin E synthase 2 (PGES-2) (gene name: Ptges2), and TGF-β1 mRNA increased slightly after 3 days of stress conditioning (Figure 4-3D, N, and O). However, ALP, BSP, type I collagen, MMP-9, and MMP-13 mRNA were suppressed significantly and Runx2 mRNA decreased slightly at days 6 compared to days 3 (Figure 4-3G, H, I, J, K, and L). No modification of angiotensin I mRNA was detected on days 3 and 6 (Figure 4-3Q).

4.4.3. Combined conditioning effect of short-term (24 hours) cyclic tensile stress and GFs on mRNA expression of HSPs

The combinatorial effect of tensile stress and GFs was investigated by exposing confluent cells to different magnitudes of cyclic tension (1, 3, 5, and 10%, 0.2 Hz, 10 seconds tension on/10 seconds rest) and supplementing culture media with GFs (BMP-2 and TGF-β1) for 24 hours. Cells exposed to tension only expressed slightly higher or similar levels of HSP27 and HSP70 (Figure 4-4A, C). GF addition with tensile stress inhibited HSP27 and HSP70 induction (Figure 4-4A, C). Conversely, HSP47 mRNA showed significant increasing trends due to inclusion of GFs alone or in combination with tension; however, there was no significant difference in the induction effect by tension (Figure 4-4B).

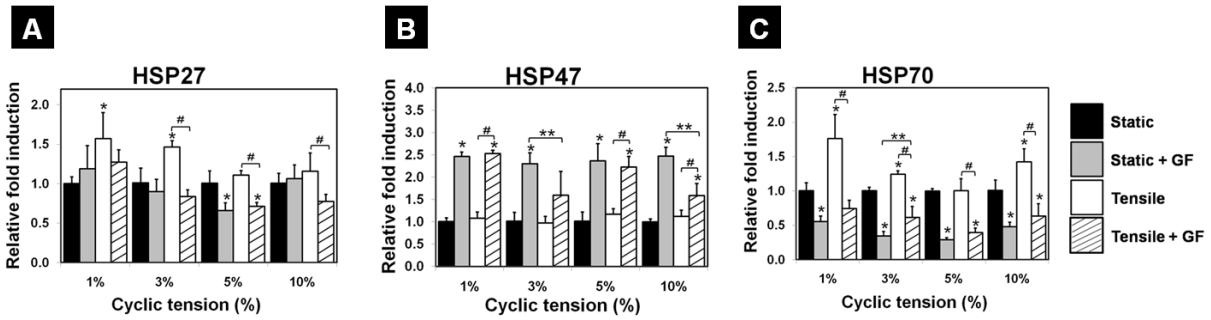

Figure 4-4. Expression of HSP mRNA following 24 hour cyclic tension (1, 3, 5, and 10% elongation) and GFs. Expression of mRNA for HSP27 (A), HSP47 (B), and HSP70 (C). * represents statistical significance between control group and treated groups (GF alone, tension alone for all protocols, GF and tension protocols in combination). ** denotes statistical significant difference between groups experiencing static with GFs and tension with GFs. # denotes statistical significant difference between groups exposed to tension alone and with GFs ($p<0.05$).

4.4.4. Combined conditioning effect of short-term (24 hours) cyclic tensile stress and GF treatment on mRNA expression of bone-related proteins

Identical methods as described in the previous section, were used to explore the effect of combined conditioning with tensile stress and GFs on bone-related protein mRNA. OPG and PGES-2 mRNA exhibited greater induction by cyclic tension (1, 3, and 5%) and GFs than individual conditioning of tension or GF treatment (Figure 4-5A and C). In contrast, OPN was suppressed by GFs in both static-cultured and tensile-loaded cells and demonstrated no significant difference following tension (except 1%) (Figure 4-5B). MMP-9 mRNA was inhibited by tension (3, 5, and 10%) and suppressed more by the combination of tensile stress (5 and 10%) and GFs (Figure 4-5H). GF addition induced much higher COX-2 mRNA expression in both static-cultured and tensile-loaded cells (Figure 4-5G). Type I collagen was induced by GFs, but tension inhibited the stimulating effect of GFs (Figure 4-5F) similar to BSP (3-10%) (Figure 4-5D). However, the BSP gene showed higher induction compared to type I collagen with GF addition (Figure 4-5D). There is no significant trend associated with induction of OCN

by tension or GF addition (Figure 4-5E). We also characterized endogenous production of TGF-β1 mRNA in combination with tensile stress. Addition of both GFs exogenously promoted endogenous significant induction of TGF-β1 mRNA in static-cultured and tensile-loaded cells (Figure 4-5I). Without GF treatment, endogenous inductions of TGF-β1 by tension were minimal.

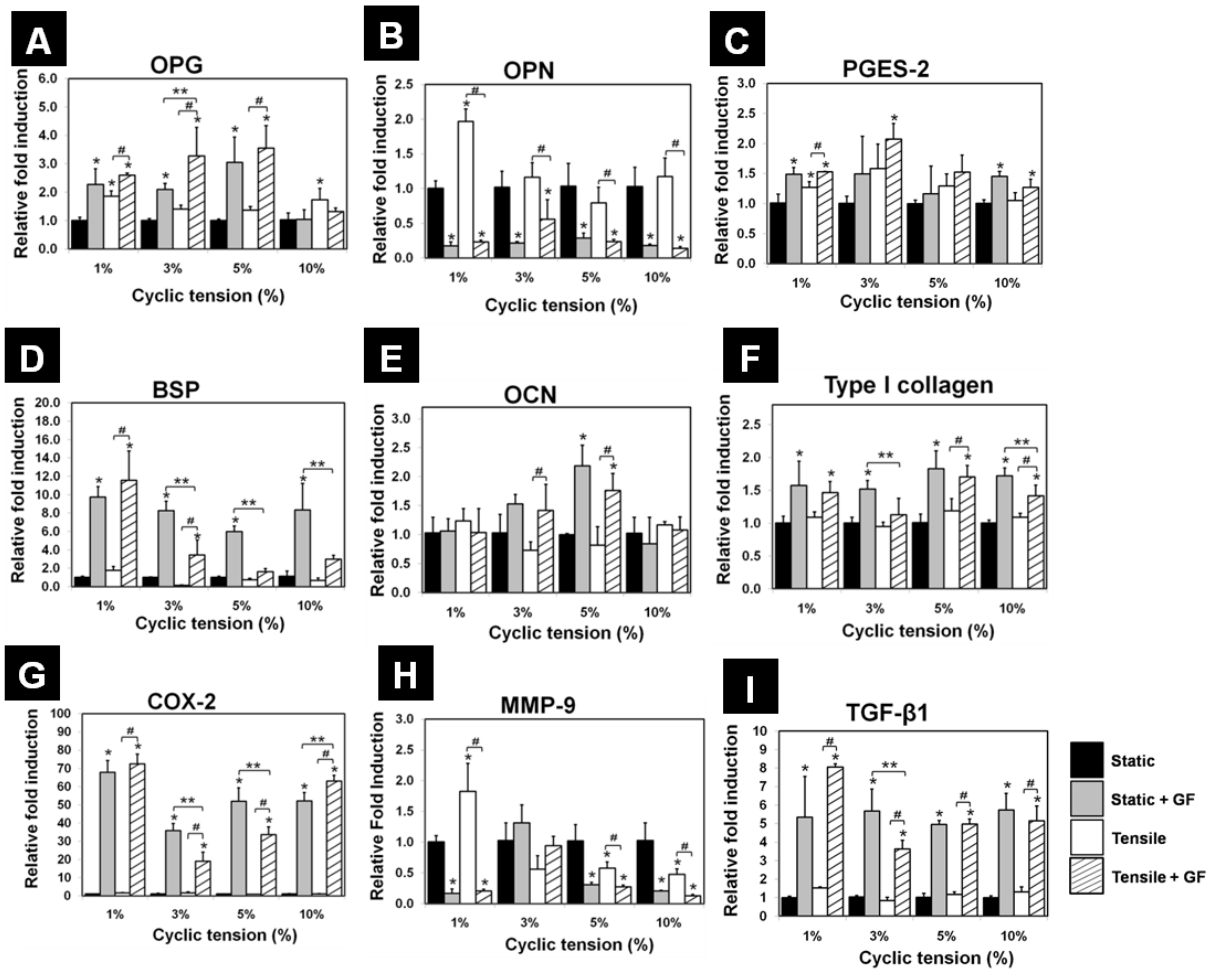

Figure 4-5. Expression of bone-related molecules mRNA following 24 hour conditioning with cyclic tension (1, 3, 5, and 10% elongation) and GFs. Expression of mRNA for OPG (A), OPN (B), PGES-2 (C), BSP (D), OCN (E), type I collagen (F), COX-2 (G), MMP-9 (H), and TGF-β1 (I). * represents statistical significance between control group and treated groups (GF alone, tension alone for all protocols, GF and tension protocols in combination). ** denotes statistical significant difference between groups experiencing static with GFs

and tension with GFs. # denotes statistical significant difference between groups exposed to tension alone and with GFs ($p<0.05$).

4.4.5. Induction of VEGF gene and protein by combined stress conditioning with cyclic tension and GFs.

All magnitudes (1, 3, 5, and 10%) of cyclic tensile stress in combination with GFs for 24 hours induced the greatest VEGF mRNA expression among test groups: tension and GFs alone, or in combination. However, GFs also increased VEGF gene expression significantly in static-cultured cells (Figure 4-6A). Similarly, VEGF protein secretion for both 0-3 and 3-6 days durations increased synergistically with tension (5%, 0.2 Hz, 10 seconds tension on/10 seconds rest) and GFs (significant interaction between tension and GFs for VEGF release, analyzed by two-way ANOVA, $p<0.05$) (Figure 4-6B). Without GFs, there was no detectable VEGF protein in the media.

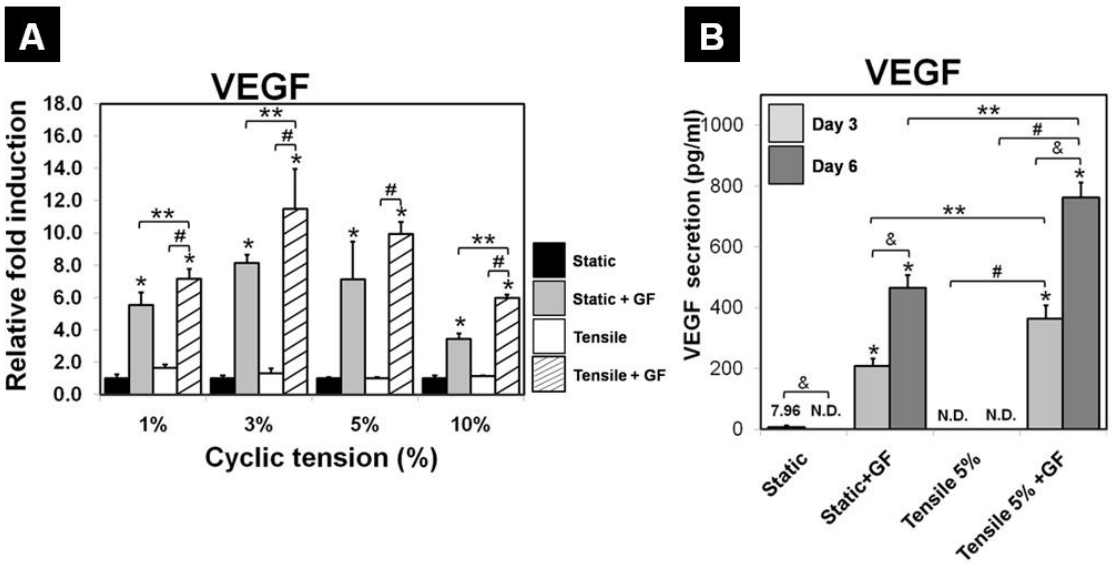

Figure 4-6. VEGF mRNA and protein secretion following conditioning with cyclic tension and GFs. PCR (A) was performed after 24 hour cyclic tension (1, 3, 5, and 10% elongation) with GFs and secreted concentration (B) was acquired from the culture media collected for two durations: days 0-3 and 3-6 of tensile stress (5% elongation) with GFs. In A, *

represents statistical significance between control group and treated groups (GF alone, tension alone for all protocols, GF and tension protocols in combination). ** denotes statistical significant difference between groups experiencing static conditions with GFs and tension with GFs. # denotes statistical significant difference between groups experiencing tension alone and tension with GFs ($p<0.05$). In B, * denotes statistical significant difference between each control group and treated groups (GF alone, tension alone for all protocols, GF and tension protocols in combination) measured on days 3 and 6 and & denotes statistical significant difference between tension for 3 and 6 days ($p<0.05$).

4.4.6. Protein release following combined conditioning with cyclic tension (5% elongation, 0.2 Hz, 10 seconds tension on/10 seconds rest) and GFs

Bone-related proteins (OPN, OCN, OPG, and MMP-9) in the media were measured by ELISA to determine the level of protein secretion for durations of days 0-3 and 3-6 of combinatorial tensile conditioning and GFs (Figure 4-7). OPN secretion was enhanced synergistically by tension and GFs on days 3 and 6, but OPN secretion diminished in the GF-treated cells for initial cultivation periods of days 0-3 (Figure 4-7A). GF-treated cells released more OPN with longer periods as compared to cells without GF treatment. However, cells without GFs secreted less OPN with increasing time. Tension without GFs stimulated higher secretion of OPG on days 3-6, but OPG expression decreased with addition of GFs (Figure 4-7B). There was no detection of OCN without the addition of GFs, however GF inclusion induced OCN protein release from cells (Figure 4-7C). With GFs, MMP-9 secretion was inhibited for both tension and non-tension treated groups. However, without GFs, secreted MMP-9 levels for longer cultivation time (days 3-6) increased more significantly under static conditions and were significantly inhibited under tensile conditioning (Figure 4-7D). Statistical analysis showed significant interaction between tension and GFs to cause secretion of the following proteins: OPN, OCN, and MMP-9 of days 0-3; OPN and MMP-9 of days 3-6 ($p<0.05$).

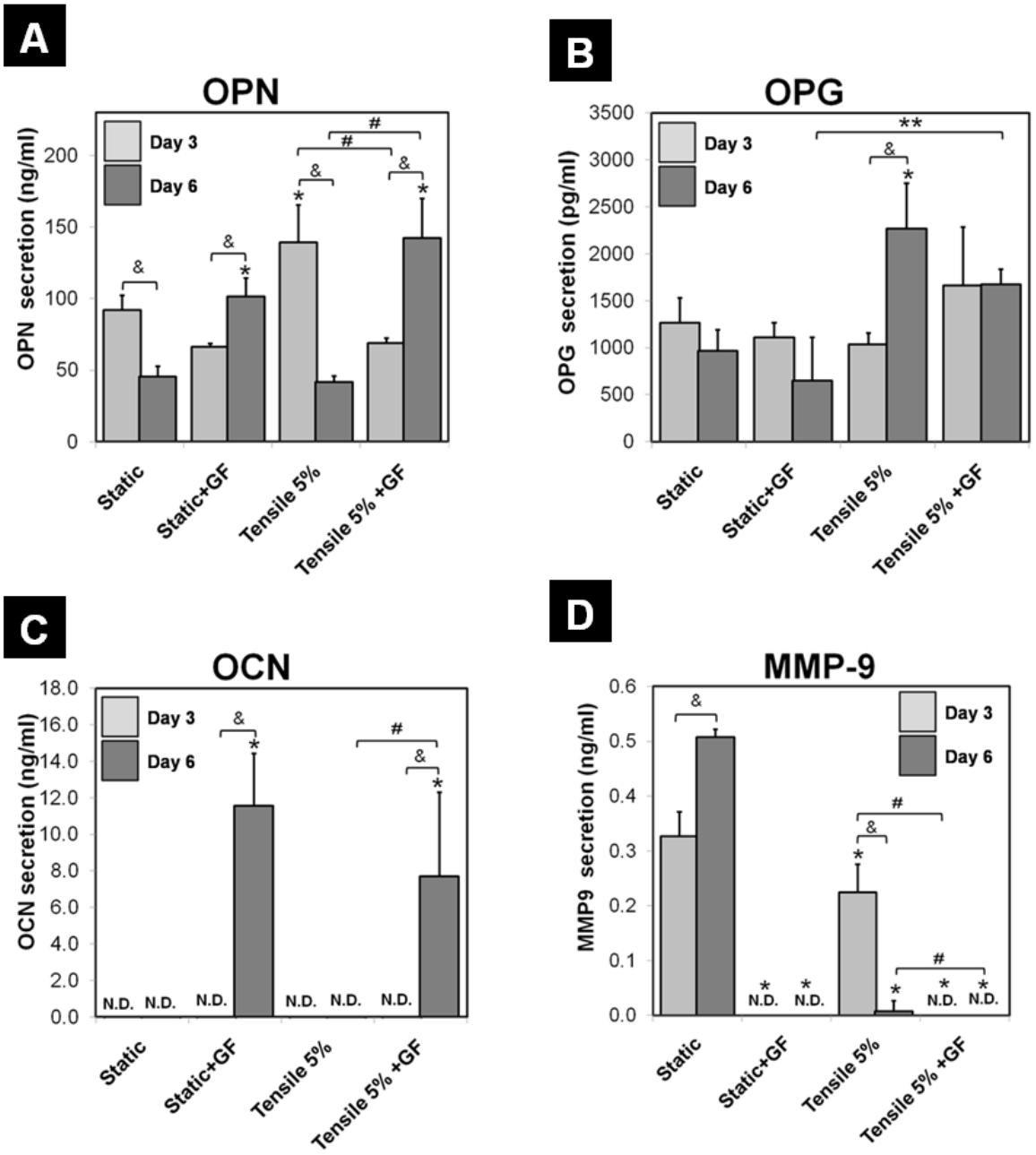

Figure 4-7. Secretion of bone proteins following cyclic tension (5% elongation) in combination with GFs for two cultivation durations: days 0-3 and 3-6. OPN (A), OPG (B), OCN (C), and MMP-9 (D) secretion. * denotes statistical significant difference between each control group and groups following tension measured on days 3 and 6 ($p<0.05$). ** denotes statistical significant difference between groups exposed to static conditions with GFs and those treated with tension and GFs. & denotes statistical significant difference between response for 3 and 6 days of tension ($p<0.05$).

4.4.7. Proliferation of MC3T3-E1 cell line by combined conditioning with cyclic tension (5% elongation, 0.2 Hz, 10 seconds tension on/10 seconds rest) and GFs (BMP-2 and TGF-β1)

In addition to protein secretion analysis, cell proliferation following tensile stress conditioning with the addition of GFs was measured using an MTS assay based on mitochondrial metabolic activity following 3 and 6 days of stress conditioning (Figure 4-8). The viability of all test groups on days 3 were not statistically different (tensile with GFs, 1.01 ± 0.07; tensile, 1.03 ± 0.07; static with GFs, 1.11 ± 0.04; static, 1.06 ± 0.03) even though static-cultured cells with GFs showed slightly higher proliferation. However, the viability level of cells treated with tensile stress and GFs on days 6 exhibited the greatest level of proliferation (1.79 ± 0.13) compared to other groups (tensile alone, 1.55 ± 0.12; static with GFs, 1.48 ± 0.03; and static, 1.46 ± 0.03). Tension without GFs also slightly increased cell proliferation compared to static-cultured cells.

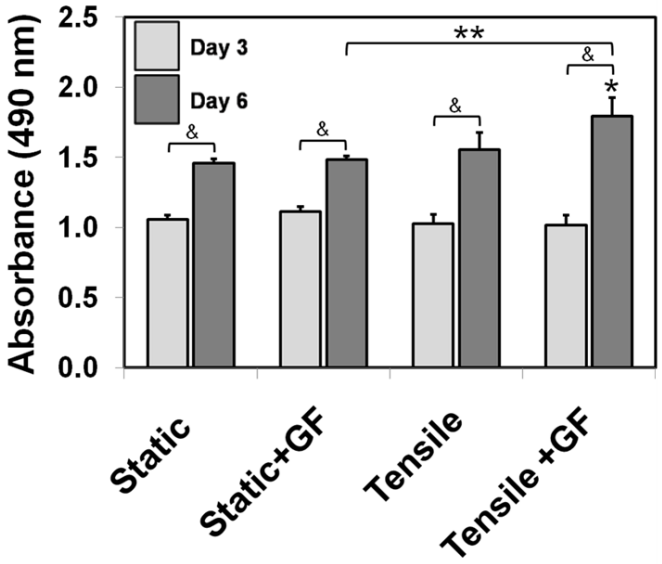

Figure 4-8. MC3T3-E1 cell proliferation following stress conditioning with cyclic tension (5% elongation) and GFs. MTS assay was performed following 3 and 6 days of tension. * statistical significant difference between control group and treated groups after 3 and 6 days of tension ($p<0.05$). & represents statistical significant difference between cellular response for 3 and 6 days of tension. ** represents statistical significant difference between groups treated with tension and GFs and groups exposed to static conditions with GFs ($p<0.05$).

4.5. Discussion

The ultimate goal of this study was to identify combinatorial stress conditioning protocols involving mechanical strain and osteoinductive GFs for bone tissue engineering applications. To demonstrate the hypothesis that the effective combination of tensile stress and GFs can synergistically enhance the stress responses and bone-forming capacity of preosteoblasts, a diverse magnitude (1-10%, 0.01-0.1 strain) of equibiaxial tensile stress conditioning protocols were applied to MC3T3-E1 cell monolayers using a Flexcell® tensile bioreactor cultivated in GF-containing media.

A wide array of tensile conditioning protocols and GFs has been demonstrated to be key modulators of myriad cellular responses. For example, *in vitro* tensile modulation of varying magnitude [60, 160], direction [161], continuity [130], duration [159], and frequency [59], have been investigated. Cyclic tension [59-61] has been reported as a mechanical stimulator capable of inducing several bone proteins with critical roles in controlling proliferation and the composition of bone matrix and relevant minerals. In addition, osteoinductive GFs of a wide range of concentrations [276], treatment duration [277], therapy method (*e.g.* direct [56] or carrier-mediated [270]), and diverse combinations of GFs [278] are considered critical determinants in bone-related studies. In spite of these endeavors, previous findings have shown large variation in the stress response of preosteoblasts and other bone cells *in vitro* depending on the strategy. Furthermore, each study has focused on measurement of specific proteins, lacking a broad investigation of the many target proteins involved in bone physiology. In addition, prior literature has explored strategies involving mechanical stress or GFs independently. However, recent advances in exogenous GF delivery techniques in bone tissue engineering require in-depth understanding of the collaborative healing effect between exogenously introduced biochemical cues and mechanical stress generated from the natural bone environment. Application of *in vitro* multi-stress strategies can provide a more representative system to the native bone environment and potentially enhance bone healing. However, few studies have explored a multi-stress protocol such as dual-mechanical stress [279, 280] or combinatorial treatment of mechanical stress and GFs [281, 282]. Therefore, our combinatorial strategies using tensile stress with GFs in combination can provide meaningful information regarding multi-stress conditioning effects on bone regeneration.

We confirmed morphological modifications of MC3T3-E1 cells could be induced under low level of 1% strain using a Flexcell® tensile bioreactor within 6 days as shown in Figure 4-2. Cells in the center of collagen-coated BioFlex® plates appeared equibiaxially stretched which is analogous to U937 cell morphology described in Matheson *et al.*'s study following tensile stress [161]. On the other hand, cells close to the edge of the circular loading post boundary were aligned and appeared uniaxially stretched. The variation in cell morphology across the culture plate may induce gene or protein expression by each preosteoblasts differently. Even though cell morphology exhibited location-dependent variation following equibiaxial strain, equibiaxial direction of stretching (10% elongation) is thought to be an attractive stimuli to promote VEGF, type I collagen, and TGF-β1 mRNA induction in osteoblasts within 24 hours [93].

We measured gene expression following tension for 6 days. HSP induction was observed to be transient, similar to heating-inductive trends observed for HSP70 following water bath and incubator heating [136, 243]. Initially, all HSPs increased slightly on days 3 in response to mechanical stress, but decreased by days 6 (HSP27 RFI=1.82 and 0.68 at days 3 and 6, respectively; HSP47=1.12 and 0.47; HSP70=1.53 and 0.90). COX-2 mRNA showed the greatest induction (4.28 and 4.60 relative fold induction at days 3 and 6, respectively) of all bone proteins by 1% cyclic tension. According to Narutomi *et al.*'s study [159], COX-2 induction can induce PGE_2 synthesis by *in vitro* cyclic tension (9% elongation, 3 hours, 0.1 Hz) in a magnitude-dependent manner. However, our PCR data measuring PGES-2 mRNA (1.43 and 0.90 relative fold induction at 3 and 6 days, respectively) following tension alone did not show significant induction and, possibly may have resulted from the use of lower stress magnitudes or different stress durations or frequencies (1%, 0.2 Hz, 10 seconds tension on/10 seconds rest, for 3 and 6 days). Since **PGE_2** has been reported to be induced under bone mechanical stress response such as fluid shear stress [119] and can enhance differentiation in osteoblast [120], the induction of these enzymes by mechanical strain suggests that cyclic tension may be a beneficial stressor to promote osteoblast differentiation.

We have shown for the first time, induction (Figure 4-5) of several HSPs by a preosteoblast cell line following short durations of tension alone or in combination with GFs. Based on measurement of gene induction, we determined short-term (24 hours) tensile conditioning with GFs was capable of inducing cell responses rapidly. Induction of HSP genes by tension is more significant relative to static cultured cells, however, induction of HSP genes

by the addition of GFs showed varying inductions during earlier periods of tensile stress (Figure 4-3 and 5). GF addition induced HSP47 mRNA in static or tensile-loaded cells. In contrast, GFs used with tension hindered HSP27 and HSP70 gene induction by tension. These differences may be caused by the fact that HSP27 and HSP70 are involved in apoptosis or differentiation in several intracellular or nuclear locations [212] and is distinct from the role of HSP47. In addition, it may indicate cells possess or lose initial anti-apoptotic capacity following cyclic tensile under exogenous TGF-β1/BMP-2 by suppressing HSP27 and HSP70. There may also be a conflict between cellular stress responses to mechanical stress and GFs.

Bone matrix proteins such as type I collagen, OPN, OCN, and BSP exhibited different responses to tensile conditioning and GFs (Figure 4-5and 7). Significant induction of BSP by GFs was reduced with the application of tension (3-10%) (Figure 4-5) indicating tensile stress may conflict with the cellular action of GFs for early durations (24 hours) of mechanical stress. Even though previous research on the effects of mechanical stress conditioning on BSP are few compared to other bone marker proteins, it can be presumed that BSP may be modulated by GFs [283] or mechanical stress in the bone regeneration process. One such prior work reported that cemetoblasts, similar to osteoblast-like cells, produced higher levels of BSP following tension [284]. In contrast, type I collagen gene induction by GFs was slightly inhibited when tension was used in combination with GFs (Figure 4-5). In previous literature type I collagen was shown to be induced by TGF-β1 in MC3T3-E1 cells [94] while cyclic tension (5-12.5%, 0.5 Hz, 24 hours) only slightly promoted type 1 collagen compared to static-cultivated SaOs-2 cells [160]. The contrasting effect of cyclic tension between our data and the literature may result from the use of different frequencies of cyclic tension or a unique cell type. Mechanical stress may not significantly affect the regulation of type I collagen or hamper the stimulatory effect of GFs in the early duration. On the other hand, the gene for OPN was significantly inhibited by GFs at 24 hours following stress treatment. Interestingly, protein secretion during the initial 72 hours (on days 0-3) was consistent with mRNA data derived from real time RT-PCR. However, for later cultivation times, GF addition induced synergistic promotion of OPN levels on days 3-6 (Figure 4-8), indicating a gradual promotion of OPN levels. OCN and MMP-9 exhibited opposite expression trends following treatment with GFs. GFs prevented MMP-9 production but enhanced OCN promotion significantly (Figure 4-8). The mRNA for COX-2, a synthase for PGE_2, was induced more significantly by GFs compared to PGES-2 (Figure 4-3), but PGES-2 showed

greater induction by 1-5% tension with GFs than tension or GF treatment alone. Significant COX-2 promotion is supported by previous literature documenting that COX-2 can be induced by BMP-2 in osteoblasts [285].

GFs in combination with cyclic tension (5%) induced MC3T3-E1 proliferation more on days 6 than individual preconditioning of tension or GF addition (Figure 4-4), but exhibited no difference on days 3. This result suggests that sufficient time is necessary to demonstrate beneficial effects on cell proliferation and positive mitogenic-stimulating effects. Even though metabolic activity level has often been used in the tissue engineering study to demonstrate the proliferative trends of cells, MTS assay may be not enough for proliferation investigation since this assay is used to measure metabolic activity. However, we saw the similar trends between MTS results and DNA contents in other experiments using MC3T3-E1 cells. Therefore, that can be used to investigate the proliferation of MC3T3-E1 cells. Individual doses of TGF-β1 [47, 270] and BMP-2 [47, 286] have been reported to promote proliferation of bone-related stem or progenitor cells in bone repair and mediate bone healing in an autocrine or paracrine manner [47]. However, the osteoinductive effect of exogenous TGF-β1 treatment is controversial compared to the bone regenerative capacity of BMP-2 [57, 287]. In spite of this controversy, we preferred to utilize TGF-β1 with BMP-2 because TGF-β1 is associated with regulation of several HSPs as well as bone physiology [94, 191, 194, 195, 288]. In addition, in previous literature, tension has been shown to have varying effects on bone cell proliferation including promotion [80, 99] or inhibition [59, 176] depending on each study strategy, possibly, due to tension conditioning factors, cell type, and culture system. Therefore, our results provide important evidence confirming the beneficial effect of dual GFs and tension on cell proliferation.

In addition to mitogenic effects, our novel combined strategy utilizing cyclic tension and GFs promoted angiogenic and anti-osteoclastic effects by inducing OPG and VEGF mRNA and suppressing MMP-9 (Figure 4-5 and Figure 4-6). Previous studies have demonstrated induction of VEGF by GFs [52] and cyclic tension [59] independently. Our results can expand prior understanding regarding the angiogenic activation of preosteoblasts by demonstrating greater VEGF induction by mechanical stimulation with exogenous GFs for all magnitudes of tensile stress than mechanical stress or GF addition alone. Furthermore, our PCR data showed exogenous delivery of BMP-2 and TGF-β1 can promote endogenous induction of TGF-β1 in preosteoblasts by promoting GF gene induction. However, the combination of tensile loading

with GFs did not cause an added production of TGF-β1 (Figure 4-6). In terms of OPG, greater induction of OPG gene and protein secretion at 3 days following tension with GFs than tension or GFs alone, strongly suggests rapid anti-osteoclastogenic effects of our combinatorial stress preconditioning. However, OPG secretion was the greatest on days 3-6 (later durations) when tension alone was applied (Figure 4-8). It is presumed that OPG regulation may be influenced more significantly by mechanical stress based on Tang, *et al.*'s study [60] in which OPG was induced in a magnitude-dependant manner following 6, 12, and 18% elongation by cyclic tension. Our data also showed that MMP-9 was suppressed by tension which is similar to prior work [184]. MMP-9 is a target molecule for bone diseases such as osteoporosis due to over-activated osteoclasts [31]. Inhibition of MMP-9 may be beneficial to preventing osteoclast functionality. From OPG and MMP-9 data, we presume that osteoclast activity can be hindered with tension alone or in combination with TGF-β1/BMP-2. Therefore, our conditioning protocols may have a positive effect in inhibiting osteoclastogenesis and inducing angiogenesis, which are critical processes to supplement bone growth.

In conclusion, we have demonstrated combined conditioning protocols using tensile stress and GFs can promote proliferation, diverse regulations of HSPs and bone-related molecules. Based on our data, we believe several complex down-stream signaling pathways may be activated by mechanical and biochemical cues and should be investigated in future studies. This study has demonstrated correlation between multiple stresses and *in vitro* response of preosteoblasts, which can provide a foundation for the development of an effective multi-stress conditioning protocol for bone tissue engineering.

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Chapter 5: Combined Effects of Heating and Cyclic Tension on the Induction of Heat Shock Proteins and Bone-related Proteins By MC3T3-E1 Cell Line

Eunna Chung[1] and Marissa Nichole Rylander[1, 2*]

[1]Virginia Tech –Wake Forest University, School of Biomedical Engineering and Sciences, Virginia Tech, Blacksburg, VA 24061

[2]Department of Mechanical Engineering, Virginia Tech, Blacksburg, VA 24061

5.1. Abstract

Conditioning with external stress (*e.g.* thermal, fluid shear, and tensile stress) has been known to induce bone-healing activity. However, prior studies have not investigated whether employing thermal and mechanical stress in combination could synergistically promote osteogenic capacity of bone cells. This study explored the impact of combined heating and cyclic strain on preosteoblast cell proliferation and expression of stress proteins in the form of heat shock proteins (HSPs) and bone-related proteins. A murine preosteoblast cell line (MC3T3-E1) was exposed to thermal stress using a water bath (44°C, 4 or 8 minutes) and post-heating incubation (37°C, 4 hours) followed by exposure to cyclic strain (equibiaxial 3%, 0.2 Hz, 10 seconds cyclic tensile stress/10 seconds rest) using a Flexcell® tension bioreactor. Thermal stress (for 8 minutes) and tensile stress (applied for 4 hours for HSP induction and 24 hours for osteopontin and matrix metalloproteinase 9 expression) utilized in combination induced HSP27 (1.41 relative fold induction (RFI) compared to sham-treated control), HSP70 (5.55 RFI), and osteopontin (1.44 RFI) mRNA more but, suppressed matrix metalloproteinase 9 (0.6 RFI) compared to thermal or tensile stress alone. Alkaline phosphate mRNA was suppressed similarly by thermal (0.47 RFI) and tensile (0.53 RFI) stress alone or in combination (0.50 RFI). Combinatorial thermal (for 8 minutes) and tensile stress (for 24 hours) induced the most significant level of secreted vascular endothelial growth factor into the culture supernatant (1.54 fold increase to control). However, cell proliferation and several bone proteins (*e.g.* osteocalcin, type I collagen, cyclooxygenase-2) were unaffected by combined stress. Therefore, combinatorial stress preconditioning, utilizing thermal and mechanical stimulation, can enhance the stress response by upregulating HSPs, and

can also increase angiogenic and anti-osteoclastic activity of preosteoblasts by regulating VEGF and MMP-9 providing a potentially promising stress protocol for bone tissue engineering applications.

5.2. Introduction

Bone is exposed to complex mechanical cues such as tensile and compressive loads during body motion as well as fluid shear stress throughout narrow channels (*i.e.* canaliculi) between osteocytes [289]. These mechanical signals modulate cell morphology, proliferation, migration, differentiation, and production of bone-related proteins such as matrix proteins, cytokines, and minerals in bone cells via mechanotransduction [185]. Mechanotransduction is initiated by activation of several membrane-deposited mechanoreceptors including integrin, connexin, stretch-activated cation channels, and G-protein-coupled receptors [185, 290]. In particular, complex environmental factors including mechanical stress, biochemical signals, and interactions with neighboring extracellular matrix control bone cell activity by modulating various downstream signaling cascades such as mitogen-activated protein kinase (MAPK) and calcium release [290, 291]. The MAPK signaling cascade is activated by phosphorylation of several downstream molecules such as extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinases (JNKs), and p38.

Despite the promise of tissue engineering for development of bone substitutes, numerous challenges exist including limited cell proliferation and in-growth within cell scaffolds, insufficient angiogenesis, and inadequate formation of bone extracellular matrix [36, 292, 293]. In order to better mimic the bone environment and promote bone regeneration, numerous conditioning protocols involving mechanical stress (*e.g.* mechanical strain [99], fluid shear stress [173], bending [180], heating (using an incubator and water bath [76], and laser irradiation [132]) have been applied *in vitro* to osteoblasts or osteogenic stem cells using a bioreactor system [160, 294]. Supraphysiological levels of mechanical conditioning can induce bone healing and the corresponding induction trends for bone proteins and mitogenic effects appear to be determined by mechanical stress parameters [59, 60, 130, 159, 160, 265, 280, 295]. Mechanical strain can be induced by vacuum pressure [159] and 4-point bending [280] and is dictated by parameters such as magnitude [60, 159], mode (*e.g.* continuous/intermittent [130], or uniaxial [130]/equibiaxial [160]), frequency [59], duration [265], and cyclic numbers [295].

Cyclic strain imposed by the Flexcell™ tension system, a commercial available tensile bioreactor, has been shown to upregulate bone-related proteins such as type I and III collagen [160], osteopontin (OPN) [61, 99], osteocalcin (OCN) [61, 99], vascular endothelial growth factor (VEGF) [99, 265], bone morphogenetic protein-2 (BMP-2) [99], transforming growth factor beta 1 (TGF-β1) [266], osteoprotegerin (OPG) [60], and cyclooxygenase-2 (COX-2) [159] in bone cells.

In addition to strain, thermal stress can activate varying intracellular mechanisms and cellular responses depending on the type of heating system (*e.g.* water bath and incubator), loading temperatures [136, 139, 213], and heating duration [136]. Cellular protective and regenerative capacity can be enhanced by mild stress via hormesis, which denotes the mechanism to induce beneficial cellular responses to low-level stress [296]. Numerous research groups agree that severe heating (over 45-50°C for shorter time such as 10 minutes and temperatures of 43-44°C for longer durations than 15 minutes) may induce cytotoxicity or decreased protein production [74, 136-138]. Endothelial cells showed enhanced angiogenic capacity at 24 hour after exposure to 41°C for 1 hour [296]. Application of thermal stress to bone [76] or dental [142] cells, and indirect thermal stress conditioning by adding conditioned media collected from heat-treated osteoblasts [131] can promote proliferation and upregulate alkaline phosphate (ALP) and OCN. From our previous study, water bath heating at 44°C for 8 minutes induced heat shock proteins (HSPs) and bone proteins such as OPN and OPG (Chapter 3, unpublished data). However, the hormetic responses of bone cells following thermal stress are not fully understood.

This stress-induced stimulation of cellular processes may be associated with induction of HSPs, which can be induced in response to stress such as elevated temperature [142], heavy metals [297], hypoxia [244], and mechanical loading [237]. Heating using an incubator and water bath at 42-50°C has been shown to upregulate HSP expression [76, 136, 141, 142]. Prior studies have shown HSP70 expression to increase in trabecular meshwork cells and tendon fibroblasts following cyclic tension [218, 219]. However, HSP70 is inhibited in response to tension in gastric mucosal cells during the process of wound healing [220]. As a molecular chaperone, HSPs are involved in mitosis [240], differentiation [212, 298], cytoskeleton stabilization [299], intracellular processing of matrix proteins (*e.g.* collagen) [199], immune system control [300], and the wound healing process [247, 301]. Therapeutic approaches using

beneficial aspects of HSPs have been investigated in sepsis, transplantation, skin damage, and ischemic diseases of bone, brain, and heart, as reviewed by Jäättelä [302]. Even though HSPs are named according to their molecular weight, *e.g.* HSP27 and HSP47, each HSP has distinct functions and induction trends depending on external stresses and cell type. HSP27 is induced by bone-related mediators such as prostaglandin and estrogen. HSP47, a collagen-specific chaperone operating in the endoplasmic reticulum, is associated with the collagen synthesis process by binding to procollagen. Both HSP27 and HSP70 rescue stressed cells from apoptotic cell death through varied mechanisms, corresponding to their location and relevant signaling pathways [212]. Furthermore, HSP27, HSP47, and HSP70 are highly expressed in bone-forming osteoblasts of rat bone determined by immunohistochemistry [208]. Our previous study demonstrated heating and tension alone induced gene expression for all previously mentioned HSPs (unpublished data, Chapter 3 and 4). Taken together, there may be a critical correlation between HSP modulations and protective/osteogenic responses of bone cells in response to stress.

This study investigated whether the combination of thermal and mechanical stress could facilitate synergistic enhancement in cell proliferation and induction of HSPs, and angiogenic/osteogenic proteins for potential use as a novel bone healing strategy. Gene/protein-based measurements of HSPs, bone and vessel representative markers, and proliferation analysis were performed following conditioning of preosteoblast with a single dose of water bath heating (44°C, 4 or 8 minutes) and cyclic strain (equibiaxial 3% elongation, 0.2 Hz, 10 seconds tension on/10 seconds rest).

5.3. Materials and Methods

5.3.1. Cell culture and preparation for stress treatment

A murine preosteoblastic cell line, MC3T3-E1, (subclone 4, American Type Culture Collection, Manassas, VA), was cultured as a monolayer with growth media composed of alpha Minimal Essential Medium (αMEM) (Mediatech, Manassas, VA) including 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in a CO_2 incubator at 37°C. Cells were plated

in a 6-well BioFlex® plate at 2×10^5 cells per well and cultured further for 16 hours to allow cell adhesion before stress preconditioning.

5.3.2. Combined stress conditioning of heating and cyclic tension

After cell adhesion, cells underwent the following stress conditioning protocols consisting of four different test groups: 1) sham-treated control, 2) thermal stress only, 3) tensile stress only, and 4) combined thermal and tensile stress. Sham-treated control groups were not exposed to any stress treatment but were cultivated with identical media as stress-treated groups and maintained in an incubator. Samples exposed to thermal stress were washed two times with PBS. Heating media which was composed of Eagle's MEM without L-glutamine (Sigma-Aldrich), was filled to the top of flasks. Thermal stress was applied by submerging the flask containing cell monolayer in a water bath (ISOTEMP 210, Fisher Scientific), which was set at 44°C for heating durations of 4 or 8 minutes, similar to prior work by Rylander *et al.*'s group [136, 249]. For post-heating recovery, cells were returned and incubated further for 4 hours in a CO_2 incubator at 37°C to permit induction of HSP and manifestation of cellular injury. For samples exposed to tension only, Flexcell® Tension Plus™ System (Flexcell International Corporation, Hillsborough, NC) was utilized to apply cyclic tensile stress conditioning protocols of equibiaxial 3% maximum elongation and 0.2 Hz (intermittent, 10 seconds tension on/10 seconds rest). A circular loading post (diameter=25 mm) was used to apply equibiaxial tension. Combinatorial stress treatment was conducted as depicted in Figure 5-1. using identical methods for thermal and tensile conditioning described previously. For these experiments, a single dose of water bath heating (44°C, 4 or 8 minutes) was applied followed by 4 hour post-heating incubation at 37°C. Subsequently, cells were exposed to cyclic tension (equibiaxial 3%, 0.2 Hz, 10 seconds tension on/10 seconds rest) using the Flexcell® Tension System for varying durations (1-72 hours post-tension (PT)) depending on test measurements. PT time denotes the duration of tensile stress conditioning and time for measurement. For each type of measurement within a single test group were performed at the same time regardless of whether either tension or heating were applied. During post-heating and tensile stress preconditioning, osteogenic media composed of αMEM including 50 μg/ml L-ascorbic acid, 10 mM β-glycerol phosphate, 10% FBS, and 1% PS was added to cells. Media formulation was based on the protocol for Chou, *et*

al.'s osteogenic media which was demonstrated to be conducive for differentiation of MC3T3-E1 cells [303].

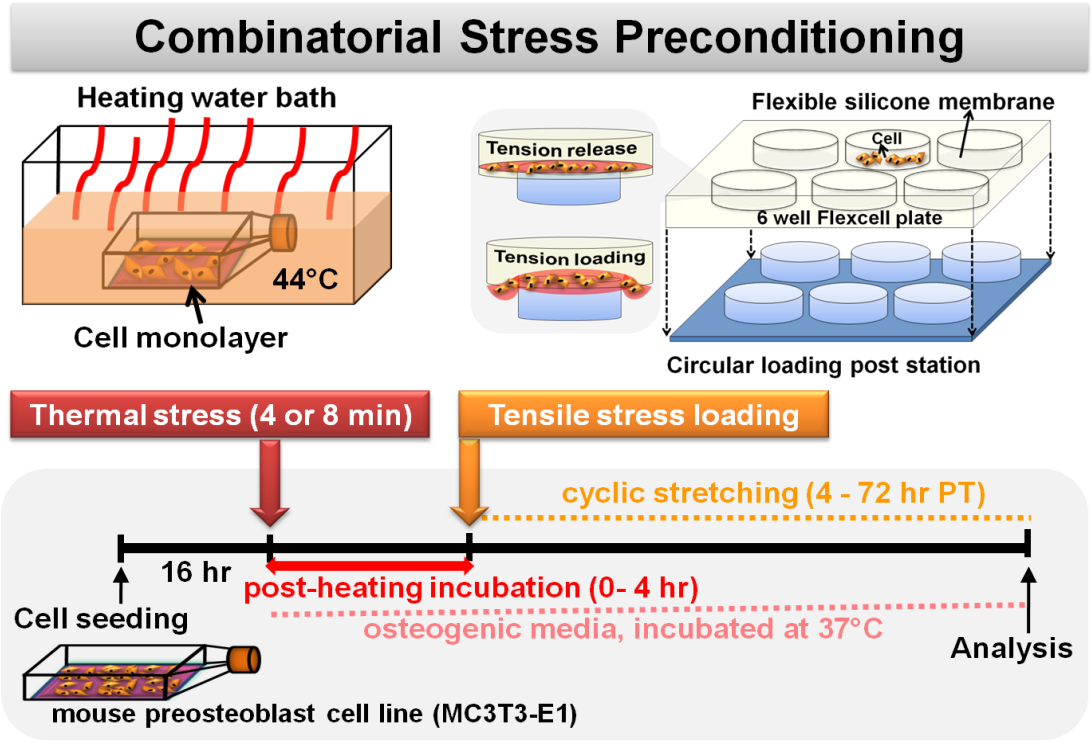

Figure 5-1. Combinatorial conditioning protocol utilizing thermal and tensile stress for *in vitro* MC3T3-E1 monolayer. Cells were seeded on 6-well BioFlex® plates with flexible culture substrate 16 hours before tensile stress preconditioning. Thermal stress was applied by heating in a water bath at 44°C for 4 or 8 minutes followed by 4 hour post-heating incubation at 37°C and cyclic tension using Flexcell® Tension™ system. PT denotes the period of tensile stress treatment before measurement.

5.3.3. Quantitative real time RT-PCR

The gene expression of HSPs, several bone-related proteins was measured following individual or combinatorial treatment of thermal and tensile stress. RNA was isolated by spin protocol using an RNeasy Mini kit (Qiagen) and a QIAshredder (Qiagen), according to the manufacturer's protocol, immediately after 4-72 hour cyclic tension following heating at 44°C

and 4 hour post-heating incubation at 37°C. Isolated RNA was converted to cDNA using Reverse Transcription System (Promega). RNA sample was reacted at 25°C for 10 minutes and 42°C for 45 minutes followed by heating at 99°C for 5 minutes. After reverse transcription, cDNA samples were mixed with Taqman PCR Master Mix (Applied Biosystems) and each specific primer, and polymerized in a 7300 Real-Time PCR System (Applied Biosystems). The PCR reaction was performed at 50°C for 2 minutes followed by 95°C for 10 minutes. For each polymerization (total PCR reaction=45 cycles), temperature was set at 95°C for 15 seconds and 60°C for 1 minutes. Taqman® Gene Expression Assay (Applied Biosystems) for specific gene detection was used as a primer and probe as follows: GAPDH (Mm99999915_g1), HSP27 (Mm00517908_m1), HSP47 (Mm00438056_m1), HSP70 (Mm03038954_s1), OPN (Mm01611440_mH), OPG (Mm01205928_m1), MMP-9 (Mm00600164_g1), ALP (liver/bone/kidney) (Mm01187113_g1), OCN (Mm00649782_gH), type I collagen (alpha 1) (Mm00801666_g1), and VEGF (Mm00437308_m1). Relative fold induction (RFI) of each mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method used in Lee *et al.*'s study [251]. Threshold cycle (C_T), derived using SDS v1.2× system software of 7300 Real-Time PCR System, denotes the fractional cycle number at threshold polymerized gene and $-\Delta\Delta C_T$ was derived from (C_T of target gene-C_T of GAPDH)$_{\text{treated group}}$-(C_T of target gene-C_T of GAPDH)$_{\text{control group}}$ [251]. Treated groups denote heated, tensile-treated alone or in combinated treated groups and control groups indicated sham-treated cells without heating and tension.

5.3.4. Enzyme-linked immunosorbent assay analysis (ELISA)

Protein secretion by MC3T3-E1 cells following thermal and tensile stress independently or in combination were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. In brief, immediately after 24 hour (for VEGF) and 72 hour (for OPN, OPG, and MMP-9) cyclic tension loading, conditioned osteogenic culture supernatant was collected. For untreated and heated samples supernatant was isolated at identical timepoints although no tension was applied. The concentrations of OPG, VEGF, OPN, and MMP-9 in the conditioned cell culture medium were determined using Quantikine® ELISA (R&D Systems). The culture supernatant was added into a 96-well microplate coated with antibodies for the desired proteins and incubated for 2 hours at room temperature. Further 2 hours and 30 minutes incubations in the conjugate and peroxidase substrate solutions, respectively, were followed to

initiate the enzyme reaction between TMB (3,3',5,5'-tetra methyl benzidine) and antibodies to specific proteins for visualization of protein expression. The optical absorbance of the colored solution was measured at 450 nm by a microplate reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA) and converted into the concentration level using a standard absorbance curve.

5.3.5. Proliferation assay

Cell proliferation was measured at 24 and 72 hour following thermal (44°C, 4 and 8 minutes) and tensile stress applied independently or in combination. We implemented two different assays:3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI), and DNA assay using Quan-Ti™ PicoGreen ds DNA reagent kit (Invitrogen), according to the manufacturer's protocol. MTS stock solution was mixed with basal αMEM without FBS and PS (the volume ratio of MTS stock to media was 1:5). Diluted MTS working solution was added to cultured cells. After 4 hour incubation at 37°C, the solution was transferred to a 96-well plate and optical density was measured at 490 nm by a microplate reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA). DNA was isolated at identical timepoints as the MTS assay. In brief, cells were lysed using Tris-EDTA buffer (10 mM Tris, 1mM EDTA, pH 8.0, Fisher Scientific) including 0.1% Triton® X-100 (Sigma) and 0.1 mg/ml proteinase K (Fisher Scientific). Cell lysate was incubated at 56°C at least overnight and transferred into a 96 well-plate with standard solutions. Quan-Ti™ PicoGreen ds DNA reagent was added into each sample at a volume ratio of 1:1 and incubated at room temperature in the dark for 3 minutes. Fluorescence of each sample was measured by a microplate reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA) set at the 480/520 nm (excitation/emission).

5.3.6. Statistical analysis

All data and graphs are presented as mean ± standard deviation. Experimental groups with a minimum of three repetitions (n=3-8) were tested and analyzed independently. To demonstrate statistical significance between groups, one-way ANOVA and a Tukey multiple comparison test were performed to compare the means between each group. The significance of

each treatment in the study was defined by a p-value lower than 0.05. This statistical analysis was performed using JMP® 8.0 statistical software.

5.4. Results

5.4.1. Combined stress conditioning effect of a single dose of heating and cyclic tension on HSP expression

Messenger RNA (mRNA) expression for each HSP (HSP27, HSP47, and HSP70) and the protein level of HSP70 following individual and combined heating and cyclic mechanical strain conditioning are shown in Figure 5-2. There was no significant induction of the mRNA of any of the HSPs (HSP27, HSP47, and HSP70) following heating at 44^{o}C for 4 minutes and 24 hours of cyclic tension alone or in combination at 24 hour PT. However, the combination of heating and tension induced HSP27 and HSP70 mRNA slightly (Figure 5-2A-C). Similarly, HSP mRNA expression following 8 minute heating with identical tensile stress conditioning protocols did not induce HSPs significantly (data were not shown here). However, for shorter durations of tensile conditioning (4 hours) following 8 minute heating and 4 hour post-heating incubation, combined stress conditioning induced significantly greater induction of HSP70 (5.55 RFI) and slightly greater induction of HSP27 (1.41 RFI) compared to tensile or thermal stress alone (Figure 5-2E-G). Heating (8 minutes) alone induced 1.87 RFI of HSP70 and tension (4 hours) suppressed HSP70 (0.73 RFI). Protein induction of HSP70 following heating (at 44°C for 4 minutes) and 24 hour tension (Figure 5-2D) was significant promoted. In contrast, HSP47 mRNA was suppressed by tension alone (0.76 RFI) or in combination (0.72 RFI) (Figure 5-2F).

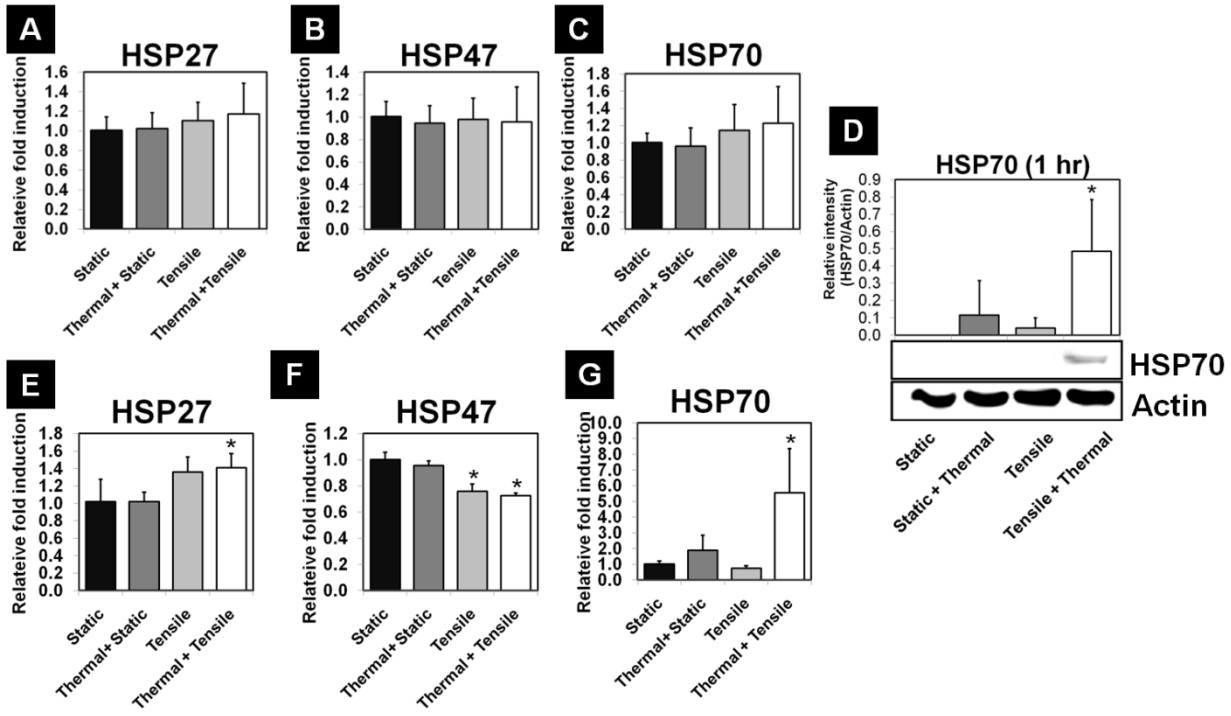

Figure 5-2. HSP (HSP27, HSP47, and HSP70) expression following a single dose of heating (44°C, 4 or 8 minutes, post-heating incubation=4 hours) and cyclic tension (equibiaxial 3%, 0.2 Hz, 10 seconds tension on/10 seconds rest) individually or in combination. Gene expression of HSP27 (A), HSP47 (B), and HSP70 (C) following 4 minute heating and 24 hour cyclic strain. Gene expression of HSP27 (E), HSP47 (F), and HSP70 (G), and protein expression of HSP70 (D) following 8 minute heating and 1 hour cyclic tension. * denotes statistical significance between stress-treated and sham-treated control groups ($p<0.05$).

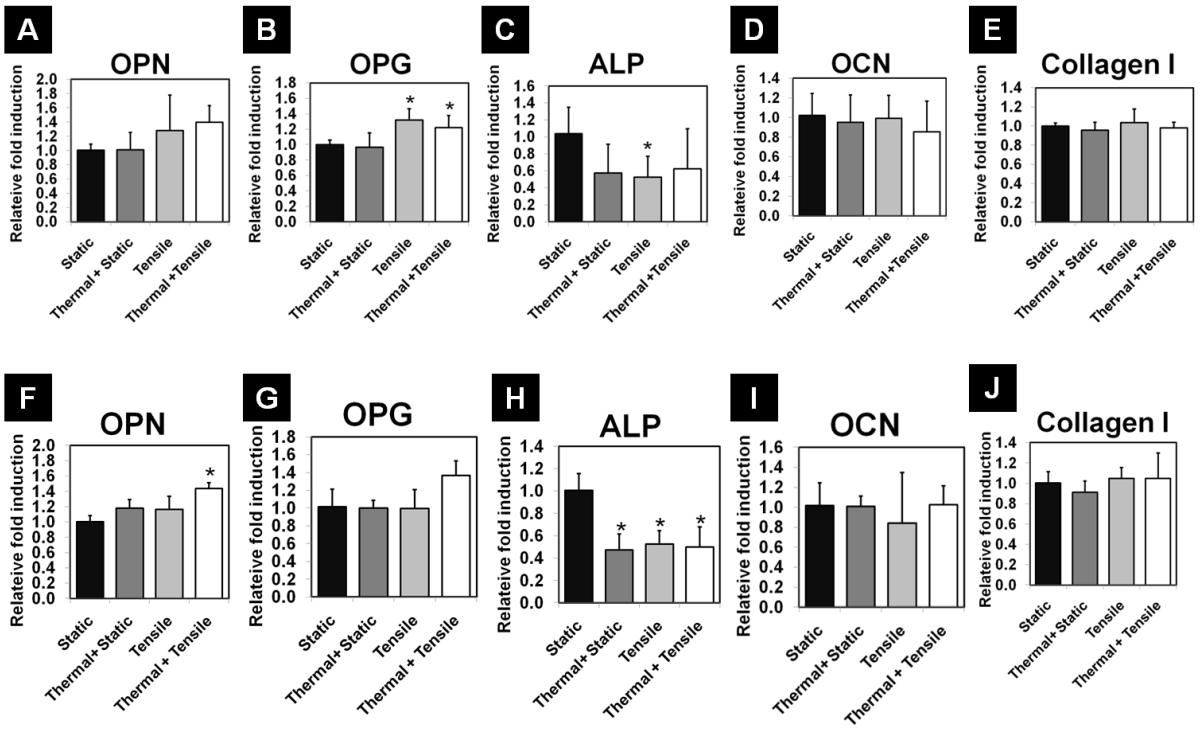

Figure 5-3. Gene expression of bone-related proteins by MC3T3-E1 cells following a single dose of heating (44°C, 4 or 8 minutes, post-heating incubation=4 hours) and cyclic tension (equibiaxial 3%, 0.2 Hz, 10 seconds tension on/10 seconds rest, 24 hours) individually or in combination. Expression of OPN (A and F), OPG (B and G), ALP (C and H), OCN (D and I), and type I collagen (E and J) mRNA was measured with varying heating durations of 4 (A, B, C, D, and E) and 8 minutes (F, G, H, I, and J). * denotes statistical significance between stress-treated and sham-treated control groups ($p<0.05$).

5.4.2. Combined stress conditioning effect of a single dose of heating and cyclic tension on bone-related proteins

Gene expressions of bone-related proteins are shown in Figure 5-3. OPN was slightly induced more by combined heating (44°C, 4 and 8 minutes) and 24 hour tension than individual stress. OPG mRNA increased slightly following 24 hour tension alone or with 4 minute heating. ALP mRNA was inhibited significantly by all stress conditioning protocols (tension and heating alone, or in combination). However, type I collagen and OCN expression showed no apparent modification of genes following all stress conditioning protocols. Secreted OPN and OPG into

the culture media were analyzed by ELISA kits following heating and tension for 24 and 72 hour (Figure 5-4). There was a statistical difference between OPG induction following tension for 72 hours compared to the control. However, the secretion of OPN did not show significant different between test groups at 24 and 72 hour PT (Figure 5-4).

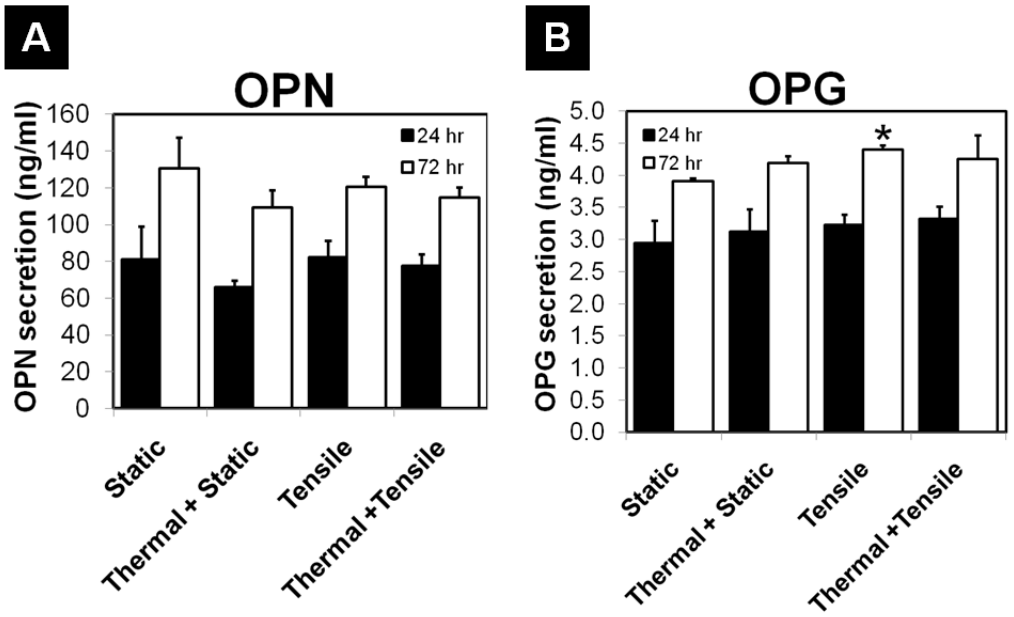

Figure 5-4. OPN and OCN secretion following a single dose of heating (44°C, 4 minutes, post-heating incubation=4 hours) and cyclic tension (equibiaxial 3%, 0.2 Hz, 10 seconds tension on/10 seconds rest, 24 and 72 hours) individually or in combination. ELISA was applied to analyze the concentrations of OPN (A) and OPG (B) secreted in the culture supernatant. * denotes statistical significance between stress-treated and sham-treated control groups ($p<0.05$).

5.4.3. Combined stress conditioning effect of a single dose of heating and cyclic tension on MMP-9 expression

MMP-9 mRNA was suppressed in response to thermal (4 or 8 minutes) and tensile stress (24 hours) alone or in combination. MMP-9 mRNA levels following 24 hour tension in combination with heating at 44°C for 4 (Figure 5-5A) and 8 minutes (Figure 5-5B) was lower

than either stress applied independently. MMP-9 mRNA was suppressed slightly by heating (4 and 8 minutes) alone at 24 hour PT, as shown in Figure 5-5A and B. Tensile stress alone suppressed MMP-9 mRNA more significantly compared to thermal stress (8 minutes) (Figure 5-5B): control, 1.02 RFI ; thermal, 0.83; tensile, 0.64; combined, 0.60. Similarly, MMP-9 secretion decreased at 72 hour PT following tension alone or in combination with heating (Figure 5-5D-E). MMP-9 secretion at 72 hour PT slightly decreased following 4 minutes of heating, but increased with 8 minute heating (Figure 5-5D-E). Figure 5-5C showed MMP-9 mRNA modification when cells were exposed to 24 hour tensile stress continuously after heating, without 4 hour post-heating incubation (Figure 5-5C). There were significant suppressions of MMP-9 mRNA by tension alone, or in combination with heating. However, there no change in MMP-9 expression was observed in response to heating alone.

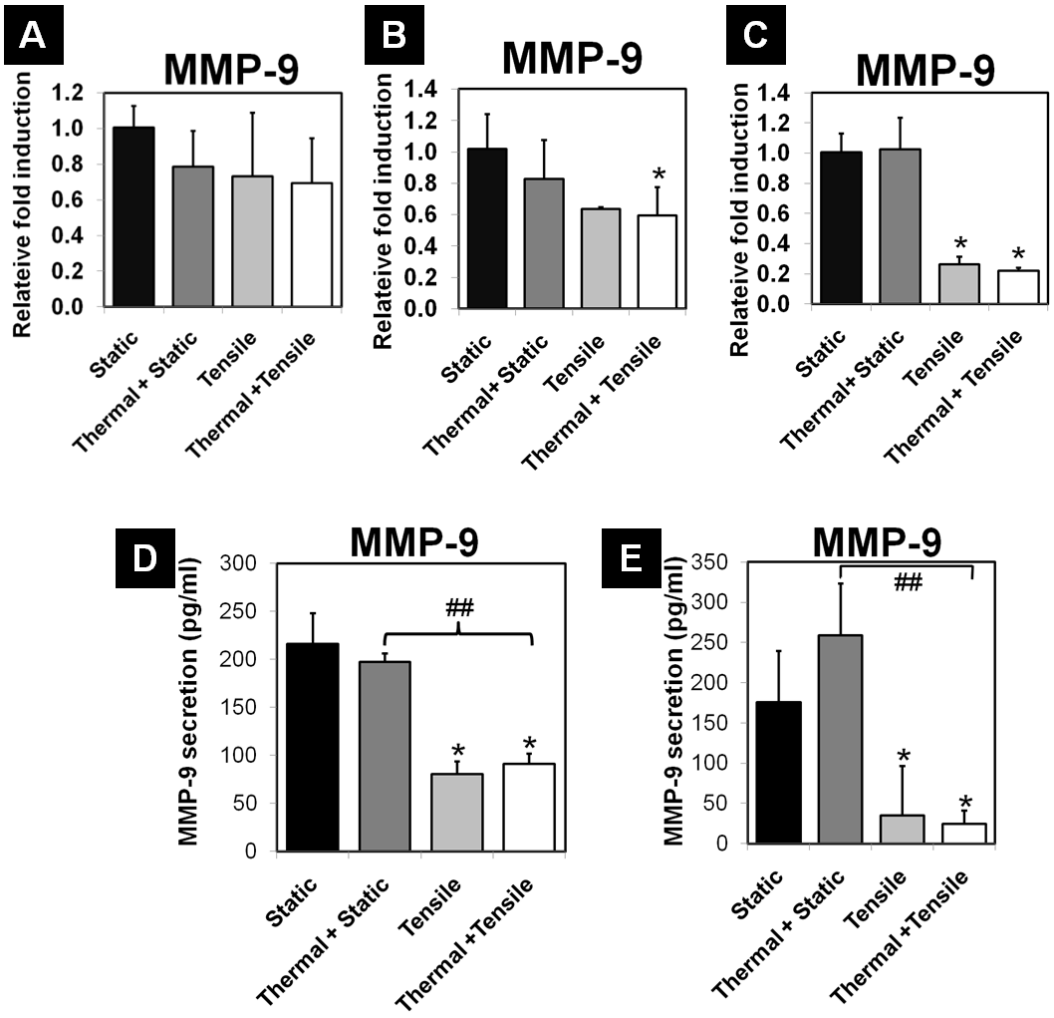

Figure 5-5. Expression of MMP-9 gene and protein by MC3T3-E1 cells following a single dose of heating (44°C, 4 or 8 minutes, post-heating incubation=4 hours) and cyclic tension (equibiaxial 3%, 0.2 Hz, 10 seconds tension on/10 seconds rest) individually or in combination. MMP-9 mRNA expression (24 hour tension) (A-C) and protein secretion (72 hour tension) (D-E) were examined by RT-PCR and ELISA, respectively with varying heating durations 4 (A and D) and 8 minutes (B and E). MMP-9 mRNA (C) was also measured following heating (4 minutes) and tension (72 hours) without post-heating incubation. * denotes statistical significance between stress-treated and sham-treated control groups ($p<0.05$).

5.4.4. Combined stress conditioning effect of a single dose of heating and cyclic tension on VEGF expression

VEGF mRNA did not show apparent modification trends following stress conditioning (Figure 5-6A-C). However, RFI (1.3) for VEGF mRNA following 8 minute heating alone was higher than other groups: control, tension alone, heating alone, and tension and heating in combination (Figure 5-6B).

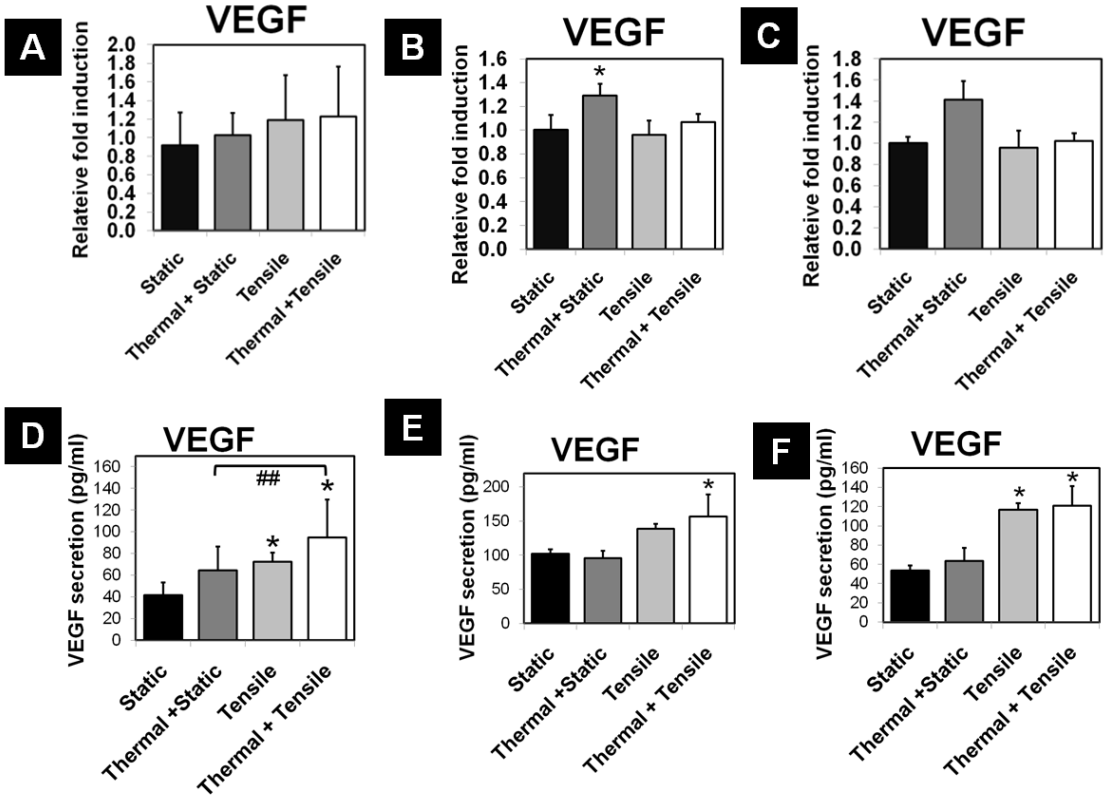

Figure 5-6. Expression of VEGF gene and protein by MC3T3-E1 cells following a single dose of heating (44°C, 4 or 8 minutes, post-heating incubation=4 hours) and cyclic tension (equibiaxial 3%, 0.2 Hz, 10 seconds tension on/10 seconds rest) individually or in combination. VEGF mRNA expression (8 and 24 hour tension) (A-C) and protein secretion (24 hour tension) (D-F) were examined by RT-PCR and ELISA, respectively, with varying heating durations 4 (A and D) and 8 minutes (B and E). VEGF mRNA (C) and protein (F) were also measured following heating (4 minutes) and tension (24 hours) without post-

heating incubation. * denotes statistical significance between stress-treated and sham-treated control groups ($p<0.05$).

VEGF secretion showed greater induction by combined heating and tensile stress than individual treatment, as demonstrated by ELISA data (Figure 5-6D-F): 4 minute heating and tension (Figure 5-6D) (static 41.5 pg/ml; thermal 64.2; tensile 72.5; combined 94.9); 8 minute and tension (Figure 5-6E) (102.0; 95.5; 138.9; 156.6); 4 minute heating with tension Figure 5-6F) (53.3; 63.6; 116.5; 120.9). Likewise, VEGF secretion (Figure 5-6F) following heating (4 minute) and tension (24 hours) without PH incubation showed similar trends to conditioning with 4 hour PH incubation (Figure 5-6D and E).

5.4.5. MC3T3-E1 proliferation by combined stress preconditioning

We investigated MC3T3-E1 proliferation through examination of metabolic activity using MTS assay and level of DNA using PicoGreen assay as shown in Figure 5-7. Cells did not show any associated cytotoxicity following heating for individual and combinatorial conditioning of 4 (Figure 5-7A and B) or 8 minutes (Figure 5-7C and D) heating and tension (days 1and 3). In addition, cell proliferation increased for longer cultivation (3 day PT) periods as evidenced by increased metabolic activity and DNA amount.

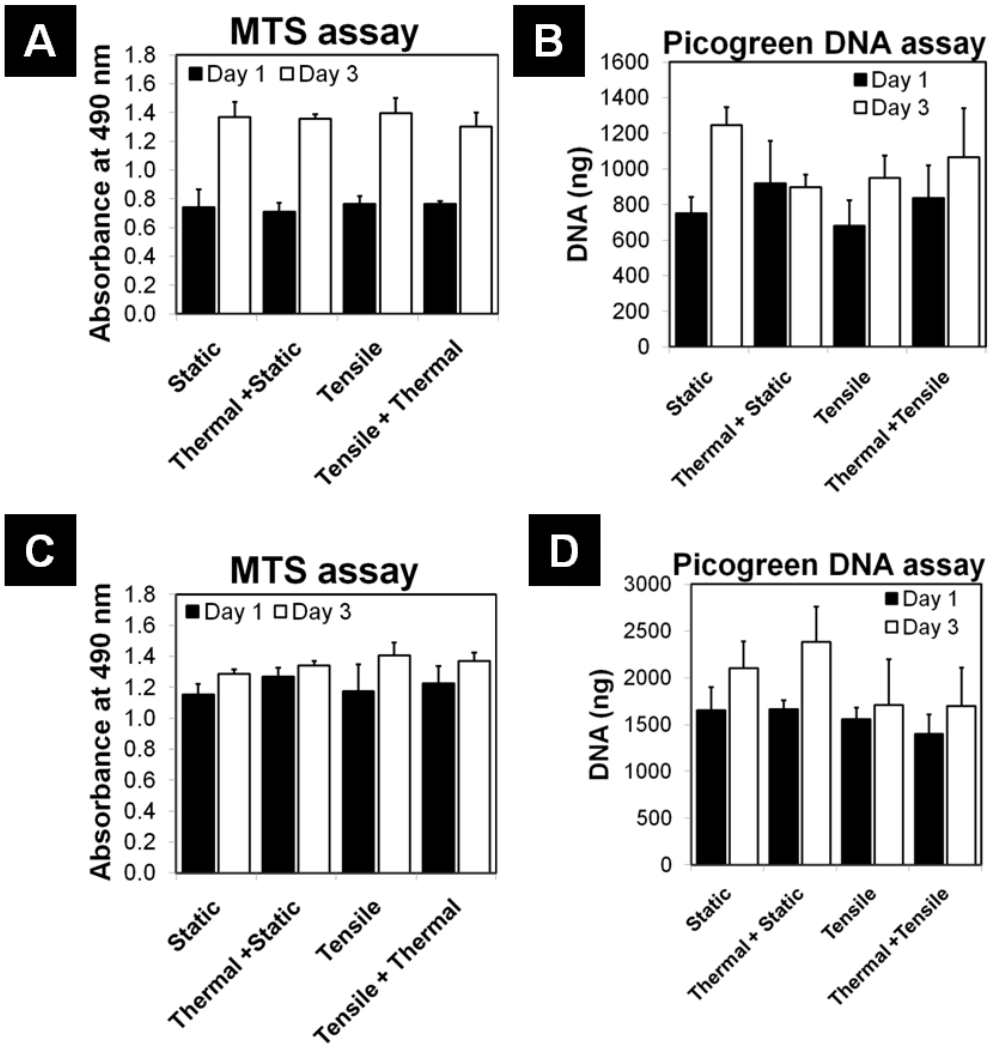

Figure 5-7. MC3T3-E1 proliferation following a single dose of heating (44°C, 4 or 8 minutes, post-heating incubation=4 hours) and cyclic tension (equibiaxial 3%, 0.2 Hz, 10 seconds tension on/10 seconds rest, 24 and 72 hours) individually or in combination. Cell proliferation was measured using MTS (A and C) and PicoGreen DNA assay (B and D) with varying heating durations of 4 (A and B) and 8 minutes (C and D). * denotes statistical significance between stress-treated and sham-treated control groups ($p<0.05$).

5.5. Discussion

Prior studies utilizing mechanical [60, 61, 99, 159, 160, 173, 180, 265, 266, 294] and thermal [76, 304] stress conditioning have suggested that these stresses can be a beneficial stimulator for bone cell activity. Our study explored for the first time the effects of combined thermal and mechanical stress on preosteoblasts. To investigate overall responses of bone cells to *in vitro* stress, we evaluated cell proliferation, inductions of HSPs and diverse bone-related proteins from osteogenic matrix proteins (*e.g.* collagens, OPN, and OCN), and enzymes including ALP and MMP-9. An angiogenic growth factor, VEGF, and an anti-osteoclastic cytokine such as OPG served as additional metrics for the evaluating the contribution of stress conditioning to osteogenesis or bone regenerative process in bone microenvironment.

Thermal stress preconditioning: Our study can expand the understanding of preosteoblasts' combinated responses following low-level heating (44°C for 2 and 8 minutes) and cyclic mechanical strain (3% magnitude) during short periods (4 hours- 76 hours). According to Rylander *et al.*'s research [136], cells experienced greater injury when they were exposed to higher temperatures or longer heating durations. Our heating protocols were chosen because heating at 44°C for periods less than 10 minutes induced HSPs rapidly without any cytotoxicity, in our previous study (unpublished data, Chapter 3). Our heating protocols are comparable to those used by other groups at temperatures of 40-43°C (for 30 minutes-1 hour) [75, 131, 142]. Although the water bath was set to *ca.* 44°C, the flask required time to equilibrate to the surrounding water bath temperature (data was not shown here) causing the cells to experience temperatures in the range of 40-43°C for short periods.

Tensile stress preconditioning: Prior literature employing tensile stress conditioning typically lower than 18 % strain documented positive osteogenic effects inducing collagens, VEGF, and COX-2 [93, 159, 160, 305]. Several research groups employed low level strain at 0.2%, since low-level tension can more closely mimic the mechanical situation in natural bone environment or bone healing processes such as distraction osteogenesis [130, 180]. 0.1-1 Hz frequency and 6 cycle numbers per minute also have been investigated commonly in bone-related studies [60, 79, 160, 305]. In Winter *et al.*'s study, intermittent stretching (uniaxial, 0.1%, 15 minutes rest inserted, 1 Hz) induced higher levels of DNA and calcium in osteoblasts compared to continuous tensile stress [130]. Equibiaxial strain has been demonstrated to induce collagens in osteoblast-like SaOs-2 cells [160], cell proliferation, expression of TGF-β1, FGF-2, collagen, and VEGF in primary osteoblast cells [93] and production of COX-2 and prostaglandin

E_2 in MC3T3-E1 cells [159]. In addition, the adherent matrix proteins (*e.g.* type I collagen, vitronectin, laminin, and fibronectin) caused variation in tensile stress-modulated (3%, 0.1 Hz, 1, 3, and 5 days) differentiation from mesenchymal cells to osteoblasts, emphasizing the importance of a matrix protein [79]. Selection of type I collagen-coated substrate in this study can best mimic bone matrix, given that it resembles natural bone matrix. Our tensile stress protocols employed 3% cyclic equibiaxial stretching with 0.2 Hz frequency and 6 cycle numbers per minute in an intermittent manner (10 seconds tension/10 seconds rest) for MC3T3-E1 preosteoblasts cultured on type I collagen. Taken together, this can be a promising tensile protocol to mimic natural bone tissue and modify bone activity.

Heat shock proteins: To the best of our knowledge, we have demonstrated for the first time the expression of HSP27, HSP47, and HSP70 in preosteoblasts in response to combined conditioning with mechanical strain and heating. We determined that HSP27 and HSP70 mRNA were induced more following heating at 44^{o}C for 8 minutes and tension (4 hours) than individual stress of heating or tension, suggesting this may be a more effective method for inducing HSPs. HSP70 showed the greatest induction in response to combined stress treatment compared to HSP27 and HSP47. Our data for induction of HSP70 expression by heating is consistent with prior studies showing elevated HSP70 in chondrocytes [223] and endothelial cells [136] in response to heating. Shui *et al*. demonstrated that thermal stress (*ca.* 41-45°C for 1 hour or 39°C for 96 hours) conditioning of bone cells induced beneficial osteogenic effects by significantly enhancing calcium production, ALP activity, and HSP70 following heating at 39°C for 96 hours [76]. Our prior work (unpublished data, Chapter 3) showed significant induction of HSP27 (2.4 RFI), HSP47 (4.4), HSP70 (63.0) mRNA at 8 hour post-heating following 8 minute heating, however but mRNA modification of HSPs (HSP70, 1.87 RFI;) by heating in this study were much lower level or did not show induction (HSP27, 1.02; HSP47, 0.95) by heating. This may be caused by differences in cell density and culture plates used (general plastic T-flask at previous study and Flexcell™ BioFlex® plate coated with type I collagen). Our previous studies showed 1% tension induced transient induction of HSP27 (1.82 RFI) and HSP70 (1.53 RFI) mRNA at days 3 but this induction level was relatively lower than induction by heating. However, as far as we know, there is no previous study except our study measuring HSP70 induction in preosteoblasts by cyclic tension.

Bone-related proteins: Gene expression of several bone-related proteins (*e.g.* type I collagen, OCN, ALP, OPN, and OPG) were unaffected or slightly modified following thermal or tensile stress alone or in combination. Given that HSP47 is a procollagen-binding chaperone and was not induced by our stress strategy as aforementioned, no effect on type I collagen by heating or tensile stress was expected. Previous studies (unpublished data, in Chapter 3 and 4) both heating (for 4 or 8 minutes) and tension (for 24 hours) did not modify type I collagen mRNA for short periods of post-heating incubation and tension loading. Likewise, mechanical strain of 10-12% magnitude (0.1 Hz, cycle number 6 per minute for 24 hours) did not affect collagen production by rat primary osteoblasts [305]. According to Liu *et al.*, 5% stretching (equibiaxial, 0.5 Hz, 24 hours) slightly increased gene expression of both collagen type I and III in human osteoblast-like SaOs-2 cells [160]. Similar to type I collagen, OCN was unaffected by individual and combinatorial heating and tensile stress. Suppression of ALP mRNA by our stress conditioning protocols (heating or tension) was similar to our previous studies (Chapter 3 and 4, unpublished). However, suppression of ALP by heating was different to induced trends of ALP activity, shown in another group's study [76]. The possible reasons for this are our stress treatments may have involved 1) insufficient heating duration or 2) insufficient post-stress recovery incubation durations to allow preosteoblasts to induce bone proteins *in vitro*. OPN, a popular osteogenic marker protein, was induced slightly more by 8 minute heating and 24 hour tensile stress than heating or tension alone. However, OPN was suppressed by 4 hour tension alone, or in combination with 8 minute heating (data was not shown here). OPN secretion was unaffected by stress at 24 and 72 PH. OPG did not show induction by combinatorial stress but tensile stress for 72 hours increased OPG secretion. Anti-osteoclastogenic effects by osteoblasts can be accomplished by mediating the balance between OPG and RANK, which are essential in controlling the interaction between osteoblasts and osteoclasts. In Tang *et al.*'s study, OPG mRNA in MC3T3-E1 cells was increased in a magnitude-dependent manner following 6-18% (6 cycles per minute) for 24 hours but the relative fold induction of 6% stretching to sham-treated cells was lower than 2 [60]. Similar to my previous study in Chapter 4 (unpublished data), OCN, OPN, and OPG genes appear not to be modified by 24 hour tension alone. However, heating (for 8 minutes) alone induced mRNA of OCN (3.8RFI), OPN (1.8 RFI), and OPG (2.1 RFI) genes at 8 hour post-heating in our previous study (Chapter 3, unpublished data). The discrepancies might be caused from type I collagen-coated flexible substrate and different FBS concentration in the

osteogenic culture media used in the current study. It should be investigated further to clarify this issue.

MMP-9: We demonstrated the suppression of MMP-9 following tension alone, or combinatorial tensile and thermal stress preconditioning. Similar, to our previous study, MMP-9 secretion was inhibited by heating as shown in Chapter 3 and 3% mechanical strain described in Chapter 4 (unpublished data). Therefore, MMP-9 mRNA in MC3T3-E1 cells appear to be suppressed by cyclic tensile stress following short periods (24 hours-6 days) of tension. Our results compare well with a prior study using a Flexcell® bioreactor applying 10% tension (0.5 Hz) which caused diminished MMP-9 gene expression of RAW264.7 osteoclastic cells [184]. Although MC3T3-E1 cells express several types of MMPs including MMP-2, MMP-9, and MMP-13 [111], MMP-9 was chosen to allow investigation of the effects of stress on osteoclast-like activity of bone resorption. MMP-9 is known to be an osteoclast marker protein for enzymes involved in fracture healing and bone remodeling/development [184, 306, 307]. However, MMP-9 overexpression in bone microenvironment could be an osteoclastic activator for bone resorption. Recently, MMP-9 has become a promising therapeutic target for bone diseases with high osteoclast activity such as osteoporosis. Therefore, MMP-9 suppression by stress condition could provide a beneficial impact for bone regeneration. Despite the suggested MMP-9 suppression by tension alone or in combination with heating, this phenomena should be investigated further and protocols optimized to determine whether suppressed MMP-9 can diminish bone remodeling. In addition, since MMP-9 from osteoclasts is a stimulating factor for angiogenesis [308], suppression of MMP-9 may be an inhibitory factor for angiogenesis.

VEGF: In our previous studies in Chapter 3 (unpublished data), VEGF gene was induced at 8 post-heating by heating (44°C, 8 minutes) and more significantly with growth factors (GFs) (*i.e*. BMP-2 and TGFβ-1). For tensile stress (Chapter 4, unpublished data), tension alone did not show VEGF gene induction but, the combination of tension and growth factors increased VEGF gene and protein more compared to growth factor addition or tension alone, suggesting tension may be a positive stimulator for VEGF. Even though GFs are thought as the most powerful angiogenic stress compared to heating and tension, VEGF secretion increased following combined conditioning with heating at 44°C for 4 or 8 minutes and 24 hour tension compared to individual stresses and sham-treated control. The effect of combined thermal and mechanical stress on HSP70 and VEGF has not yet been explored in prior studies, although prior studies

have suggested thermal or mechanical stress may stimulate VEGF induction [93, 309, 310]. Heating at 42°C for 15 minutes using a heating blanket has been shown to induce VEGF with rapid induction of HSP70 in rat cardiac tissue at 4-72 hour post-heating [309]. VEGF was not only induced after 90 minute heat stress at 43°C by a heating pad but also at 42°C by infrared radiation both of which also induced HSP70 [310]. On the other hand, tensile stress rapidly promoted VEGF gene expression in osteoblasts in response to 3 hour equibiaxial tension (10% magnitude) [93]. Therefore, given that angiogenesis and VEGF are critical in bone healing process [52], our stress conditioning protocols utilizing heating and cyclic tension may enhance VEGF-mediated communication between osteoblasts and endothelial cells. These protocols could potentially be used to stimulate blood vessel formation in a bone microenvironment or within bone scaffolds.

Proliferation: Depending on stress conditioning parameters, investigations of proliferation or cytotoxicity in response to stress showed contradicting outcomes [74]. Low-level heat treatment at 42°C for 1 hour using an incubator did not significantly influence the proliferation rate of human myoblasts [75]. Another study described that 39-41°C water bath heating for 1 hour induced a slight increase in cell proliferation, but heating at 42.5 and 45°C induced cytotoxic responses in bone marrow-derived cells and osteoblast-like MG-63 cells [76]. Similarly, the effect of tensile stress on proliferation of bone-related mature cells or stem cells appear controversial. Furthermore, it is difficult to directly compare the response to stress conditioning in these prior studies due to differences in the tensile stress protocols. For example, Song *et al.*, suggested tensile stress (cyclic equibiaxial 2-8 %, 1 Hz, 15-60 minutes) increased mesenchymal stem cell (MSCs) proliferation at 6 hour post-tension [77]. However, Simmons et al. reported that 3% equibiaxial mechanical strain with 0.25 Hz decreased growth of MSCs around days 10 [78]. Huang *et al.*, showed tension (3%, 0.1 Hz) slightly induced the metabolic activity of MSCs on day 1 but exhibited similar levels as static-cultured cells on days 3 and 5 [79]. In addition, osteoblastic cells showed decreased cell numbers following 24 and 48 hours of 12% tension and demonstrated 0.2 Hz stretching inhibited cell growth more than 0.1 Hz [59]. However, 0.17% biaxial tensile stress (1Hz, 2 hours) promoted osteoblast growth at 24 hour post-stretching [80]. Taken together, tensile stress with higher magnitudes and frequency or longer durations causes toxicity but low-level mechanical strain may increase cell proliferation. From our MTS and DNA data, there were no significant differences following our stress

treatment on the proliferation of MC3T3-E1 cells. However, due to limitations of our current heating system, cell proliferation trends had variations depending on the experiments.

In conclusion, our study revealed that combinatorial stress conditioning for short periods induced HSPs and VEGF more with suppression of MMP-9 than individual stress of heating or tension. Therefore, our conditioning protocol for tensile stress (*i.e.* equibiaxial 3%, 0.2 Hz, intermittent mode of 10 seconds tension and 10 seconds rest) and thermal stress at 44°C for shorter duration than 10 minutes can be a candidate stress conditioning protocol to stimulate angiogenesis and diminish bone resorptive activity via HSP expression. Future research based on this study should focus on developing conditioning for application of simultaneous thermal and mechanical stress. Through this system, intermittent or repeated treatment of thermal or tensile stress for long-term conditioning should also be explored to confirm the effect of combinatorial strategy on mineralization process by preosteoblasts.

Chapter 6: Effect of HSP70 Small Interfering RNA on Proliferation and Regulation of Bone-related Proteins Under Thermal Stress Preconditioning

Eunna Chung[1] and Marissa Nichole Rylander[1, 2*]

[1]Virginia Tech –Wake Forest University, School of Biomedical Engineering and Sciences, Virginia Tech, Blacksburg, VA 24061

[2]Department of Mechanical Engineering, Virginia Tech, Blacksburg, VA 24061

6.1. Abstract

Molecular chaperones known as heat shock proteins (HSPs) regulate cellular processes under both normal conditions and in response to stress. In particular, thermal stress conditioning has been shown to cause induction of HSPs and stimulate bone regeneration. However, the role of HSPs in the thermal stress-mediated bone regeneration process has not been investigated. This study explored whether HSP70 directly are related to expressions of bone-related proteins and other HSPs by bone cells under normal conditions and in response to thermal stress. MC3T3-E1 murine preosteoblasts were subjected to thermal stress conditioning (44°C, 8 minutes in a heating water bath) alone or in combination with suppression of HSP70 with small interfering RNA (siRNA). According to western blot analysis, HSP27 was induced more significantly in cells with HSP70 silencing following heating compared to only heat-treated cells while HSP47 levels remained unchanged. HSP70 suppression hampered cell proliferation in unstressed and heated cells. HSP70 silencing in combination with heating induced significant increases in secretion of vascular endothelial growth factors (VEGF). Osteopontin (OPN) and osteoprotegerin (OPG) secretion were induced by heating, but secretion levels remained unchanged by HSP70 silencing. Therefore, HSP70 expression in preosteoblasts following mild thermal stress may be involved in HSP27 regulation, mitogenic activity, and the production of angiogenic factor (VEGF).

6.2. Introduction

Heat shock proteins (HSP): HSPs, known as stress proteins, are a multifarious protein superfamily including HSP27, HSP47, HSP70, and HSP90, named according to their molecular weights [207, 311]. HSPs are constitutively expressed [244] and can be induced in response to physiological [312], pathological (*e.g.* ischemia [244]), and environmental cues such as heat [136] and mechanical stress [207, 237]. HSPs are involved in normal cellular processes such as proliferation [240] and differentiation [212] and are instrumental in rescuing dying cells exposed to severe stress. HSP27, one of the small HSPs, can inhibit stress-induced cell death [209], regulate actin kinetics [313], and undergo induction by bone-related molecules such as estrogen and prostaglandins [191, 193]. HSP27 is expressed in bone and cartilage, primarily in osteoblasts and hypertrophic chondrocytes [216]. HSP47 resides in the endoplasmic reticulum and functions in collagen processing [207]. HSP70 includes a highly thermally inducible form occurring in many cells (*e.g.* endothelial cells and chondrocytes) [136, 223, 243] and a constitutive (cognate) type, HSC70 [314]. The molecular structure of HSP70 contains an ATPase domain (44 kDa), peptide binding domain (18 kDa), and 542-640 amino acid resides with 10 kDa size from N- to C-terminal [207]. HSP70 can be also induced by mechanical stress [315] and hypoxia [244]. Furthermore, HSP70 is expressed highly in the actively healing wound site [316]. HSP-specific staining of bone cells have demonstrated expression of several HSPs including HSP27, HSP47, and HSP70 during regeneration [208].

HSPs and Therapy: Even though many of the diverse cytoprotective and physiological functions of HSPs have been demonstrated, the purposeful upregulation of HSPs by exogenous protein or gene delivery [231, 317] and the use of stress conditioning remain expanding areas of research. Direct gene or protein introduction of HSPs into target cells have been investigated by utilizing fusion protein transduction with polymer microsphere carriers [231] and membrane-permeable carriers such as virus vector systems [231, 317]. For example, the exogenous treatment of HSP27 protein combined with a virus-originated transcriptional activator (TAT) encapsulated within PLGA microspheres improved cardiomyoblast survival and growth rate under hypoxia suggesting HSP27 as a novel therapeutic molecule for curing ischemic disease [231]. HSP70 overexpression by introducing human HSP70 cDNA with pBK-CMV phagemid vector into rat gastric mucosal epithelial cell line following mechanical stretching enhanced the

wound healing capacity of these cells [220]. Exogenous HSP70 gene or protein delivery can enhance cell protection and repair of abnormal proteins in chondrocytes [230], myocardium [318], and nerve tissue [319]. In addition, HSP70 delivery has previously showed inhibition of amyloid-β for Alzheimer's disease [320].

Since bone experiences numerous mechanical stresses (*e.g.* tensile, shear, and compressive stress) due to physiological loading conditions which influence bone mass, strength, and cellular morphology, [321, 322], it is only reasonable that application of stress conditioning using physiological and supraphysiological parameters could enhance bone formation and remodeling. Supra-physiological levels of stress conditioning have been shown to promote cell protective capacity, induction of HSPs [59, 76, 141, 221, 323], and bone tissue regeneration. Mechanical strain and fluid shear stress can accelerate osteogenesis by inducing bone-related proteins (*e.g.* osteopontin (OPN), and osteocalcin (OCN)) or growth factors such as bone morphogenic protein-2 (BMP-2) and transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF) [59, 173]. Microgravity conditions (e.g. space flight) have been shown to down-regulate HSP70 and HSP47 mRNA with associated decreases in secretion of TGF-β1 [217] suggesting stress may affect the secretion of bone-related proteins. Thermal stress conditioning applied through methods such as incubator (at 39°C for 96 hours) and water bath (at 39-41°C for 1 hour) can enhance proliferation and activity of bone marker protein such as alkaline phosphate (ALP) [76]. Heating induced HSP70 expression in bone marrow stromal cells, which were cultured on denatured collagen substrate, and was attributed to hindering the *in vitro* aging process [324]. Thermal stress conditioning using a water bath for the temperature range of 44-50°C (for 1-30 minutes) has been previously shown to induce endogenous HSP70 expression in endothelial cells [136]. HSP70 gene-deleted C57/BL6 mice fetuses showed shorter upper limb and lower fibroblast growth factor-8 immunolocalization, and insufficient collagen fiber structure compared to non-heated wild type mice fetuses following water bath heating at 43°C (body central temperature) for 5 minutes [325]. Taken together, HSPs and bone physiology may be closely correlated with each other that can be modulated through stress conditioning. However, to the best of our knowledge, the role of HSP70 expression in osteogenesis physiologically and in response to stress conditioning is unknown, preventing assessment of its potential therapeutic benefit for enhancing bone regeneration and healing.

Artificial manipulation of HSP expression can elucidate the role of HSP expression in osteogenesis alone or in combination with stress conditioning by revealing changes in cellular activity due to absence of this target protein. **Small inferring RNA** (siRNA) is a useful tool to investigate the physiological impact of specific proteins via gene silencing by impairing normal mRNA transition into a specific protein. In the tissue engineering field, siRNA strategies have been applied to silence inhibitors for healing-related molecules such as hyaluronic acid, which contributes to the wound healing process [66]. For example, using siRNA techniques, inhibition of G protein alpha stimulating activity polypeptide 1 (GNAS1) stimulated core-binding factor α-1 (cbfa1), which is an osteogenesis-mediated transcription factor [66]. This result demonstrated GNAS1 as a novel target in the bone tissue engineering field [66]. In addition, this technique is suggested as a therapeutic strategy to inhibit the role of harmful proteins which are involved in disease pathology and to assist with novel drug development. In particular, HSP27 silencing using siRNA has confirmed HSP27's antiapoptotic role to treat chronic lung failure [65]. Downregulation of HSP47-homolog protein, gp46, can be applied to inhibit liver fibrosis [232].

In this study, we investigated whether downregulation of constitutive and heat stress-inducible HSP70 could alter induction of other HSPs (*e.g.* HSP27 and HSP47), bone-related proteins (*e.g.* OPN, OPG, and ALP), and growth factors such as TGF-β and VEGF and cell proliferation to determine whether HSP modulation could be a novel therapeutic target for bone regeneration. Preosteoblasts (MC3T3-E1) were heated at 44°C for 8 minutes with a water bath alone or in combination with treatment with HSP70-specific siRNA. Subsequently, gene and protein expression of HSPs and bone-related and growth factors were measured with real time RT-PCR, western blot, and enzyme-linked immunosorbent assay (ELISA).

6.3. Materials and Methods

6.3.1. Cell preparation

MC3T3-E1, a murine preosteoblastic cell line (subclone 4), (American Type Culture Collection, Manassas, VA), was cultured with growth media composed of alpha Minimum Essential Medium (αMEM) (Mediatech, Manassas, VA), 10% fetal bovine serum (FBS) (Sigma), and 1% penicillin-streptomycin (PS) (Invitrogen) in a 5% CO_2 incubator at 37°C for cell

expansion. For PCR, western blot, and ELISA, cells were seeded in 25 cm^2 cell culture flasks at a concentration of 2×10^5 cells per flask. For cell proliferation assays, cells were seeded in 12 multi-well plates at 1×10^4 cells per well. Cells were cultured for 48 hours with growth media without PS before gene silencing to provide sufficient time for cell adhesion. Cells experienced the following conditions: 1) no heating without HSP70 siRNA transfection (control); 2) no heating with HSP70 siRNA silencing; 3) heating without HSP70 siRNA silencing; 4) heating with HSP70 siRNA silencing; 5) (optionally) heating with negative control.

6.3.2. Gene silencing using HSP70 SiRNA

HSP70 gene silencing was accomplished using Silencer® Select Pre-designed & Validated siRNA (ID number=S201486, Applied Biosystems) and targeting heat shock protein 1B gene. 100 pmol HSP70 siRNA, 12.5 ul Lipofectamine™ 2000 (Invitrogen), and Opti-MEM® I Reduced Serum-Medium (Invitrogen) were combined. This solution was combined with growth media without PS and then added to cells 48 hours after cell seeding. After 6 hour incubation in a 5% CO_2 incubator at 37°C, previous media including siRNA-Lipofectamine™ complexes was removed and fresh growth media without PS was added. Then cells underwent 42 hour incubation before thermal stress conditioning to give sufficient time for HSP70 gene silencing.

6.3.3. Thermal stress treatment

Thermal stress was applied at 48 hours after transfection by submerging the flask containing the cell monolayer in a water bath (ISOTEMP 210, Fisher Scientific) at 44°C for 8 minutes. This thermal conditioning protocol was selected because this condition may allow the cells to induce HSP induction without cytotoxicity, according to Rylander *et al.*'s study [136] and our previous studies (unpublished data, Chapter 3). Following thermal stress, osteogenic media was added to promote osteogenesis during the post-treatment incubation (PTI) period. Osteogenic media was composed of αMEM including 50 μg/ml L-ascorbic acid, 10 mM β-glycerol phosphate, and 10% (proliferation) or 1% (other tests) FBS with PS. To permit induction of HSPs and bone proteins and manifestation of cellular injury, cells were returned to a 5% CO_2 incubator at 37°C for varying post-treatment incubation (PTI) periods depending on measurement type: cell proliferation (1, 2, 4, and 6 day PTI), PCR (8 hours), western blot (24

hours), and ELISA (2 and 6 days). PTI denotes the cultivation periods after heating until measurement, as depicted in Figure 6-1. Measurements in other unheated test groups (*i.e.* sham-treated and HSP70 silencing alone) were also performed at identical PTI timepoints as heated groups (heating alone and heating with HSP70 silencing).

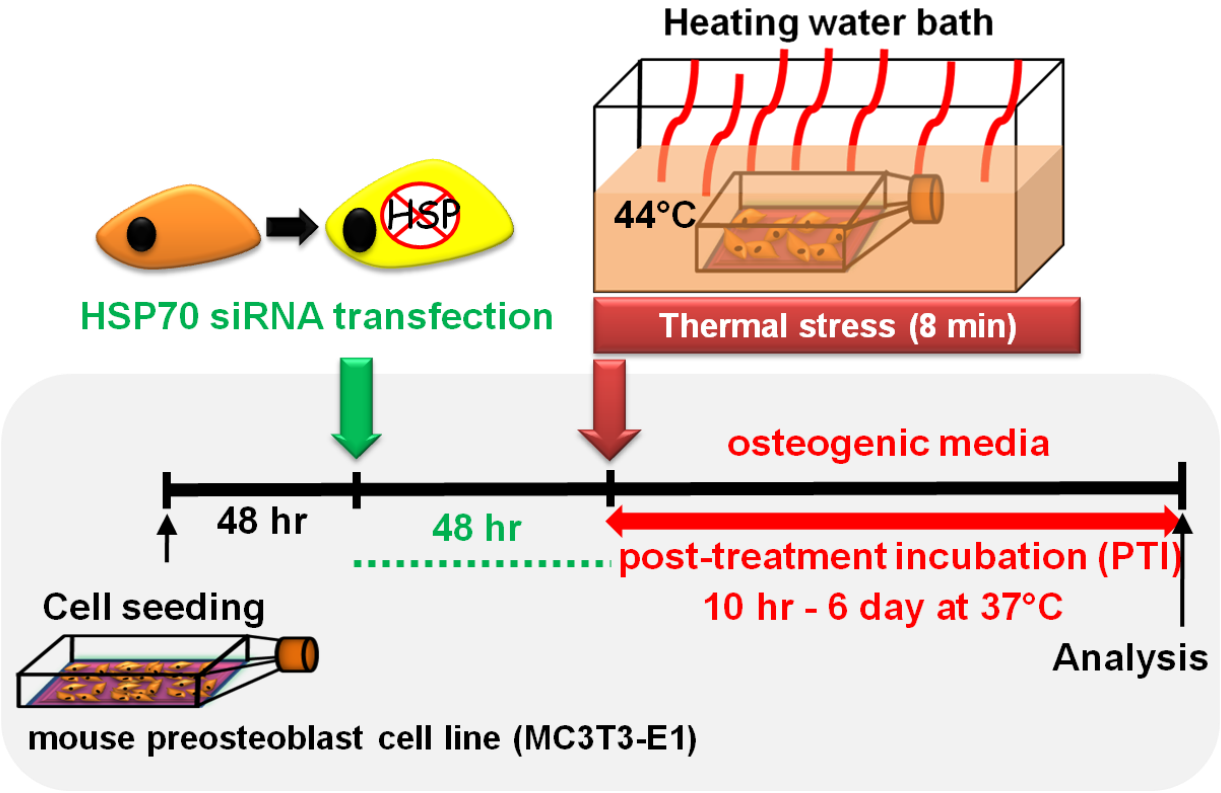

Figure 6-1. Illustration of combinatorial treatment process of siRNA treatment and thermal stress, utilized in this study. For the test groups for heating with HSP70 silencing, cells were transfected using HSP70-spefici siRNA, incubated for 48 hours, and heating with a water bath at 44°C for 8 minutes.

6.3.4. Western blot

HSP70 expression was measured with western blotting following water bath heating at 44°C for 8 minutes at 24 hour PTI. Cells were lysed with RIPA buffer (Santa Cruz

Biotechnology). Isolated protein was quantified using BCA Protein Assay Kit (Pierce) to permit loading of identical amounts of protein sample in each well of the electrophoresis gel. Proteins were diluted using Laemmli sample buffer (Bio-Rad Laboratories) followed by heating at 95°C for 5 minutes. For electrophoresis, protein sample was loaded in 10% Criterion Tris-HCl Gel (Bio-Rad Laboratories). Proteins transferred onto the membranes were immunoblotted using specific primary and secondary antibodies in 5% non-fat dry milk solution in Tween-20/Tris buffer saline for blocking. Protein bands detected using SuperSignal West Dura Extended Duration Chemiluminescent Substrate (Pierce) were visualized by LAS3000 Image Analyzer (Fujifilm). After imaging, the membranes were stripped for actin normalization and actin was detected using goat/rabbit polyclonal anti-actin as a primary antibody (SC-1616, dilution ratio=1:1000) and a secondary antibody (SC-2020, 1:2000). Quantification of each protein band on western blot images was derived by calculating intensity difference between a band of interest and background per unit using Multi Gauge V3.0 program (Fujifilm) and normalized with actin band level (n=3). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) except HSP70 primary antibody (Stressgen). HSP27 (SC-51956, 1:200), HSP47 (SC-13150, 1:500), HSP70 (SPA-810, 1:1000) were used as mouse monoclonal primary antibodies and HRP-conjugated secondary antibodies (SC-2005 for HSP27 and HSP47 and SC-2969 for HSP70, all diluted at 1:2000) matching specific species of each primary antibody were used.

6.3.5. Double immunofluorescence (IF) staining (HSP70 and COX-2)

The expression of HSP70 and cyclooxygenase 2 (COX-2) following heating at 44°C for 8 minutes and 16 and 48 hour PTI were measured with IF staining. IF was used to investigate HSP70 expressions at specific timepoints (16 and 48 hours). Since COX-2 functions in intracellular locations, IF was performed instead of ELISA to compare with RT-PCR data. Cells were fixed with Histochoice MB (Electron Microscopy Sciences, Hatfield, PA) for 20 minutes following permeablization with 0.5% Triton X-100 in deionized water for 15 minutes. Then, cells were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hour. Blocked samples were incubated with primary antibodies for HSP70 (mouse anti-HSP70, SPA-810, Stressgen, 1:50) and COX-2 (SC-1747, Santa Cruz Biotechnology, 1:50) diluted in 1%

BSA/PBS solution overnight at 4°C. On the next day, cells were washed with PBS and incubated with secondary antibodies, donkey anti-mouse IgG (Molecular Probes, Carlsbad, California, A21203), and anti-goat IgG (Molecular Probes, A11055) diluted in the ratio of 1:200 for 1 hour. After washing with the antibody solution, cells were counterstained and mounted with DAPI-including mounting medium, VECTASHIELD Mounting Medium with DAPI (DAPI: 4',6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA). Immunofluorescence stained images were obtained using a fluorescent inverted microscope (CTR6500, Leica Microsystems) with a magnification of 400X.

6.3.6. Real-time RT-PCR

RNA was isolated by spin protocol using RNeasy Mini kit (Qiagen) and QIAshredder (Qiagen) at 10 hour PTI according to the manufacturer's protocol. Isolated RNA was converted to cDNA using Reverse Transcription System (Promega) by serial reactions at 25°C for 10 minutes and 42°C for 45 minutes followed by heating at 99°C for 5 minutes. After reverse transcription, cDNA samples were mixed with Taqman PCR Master Mix (Applied Biosystems) and each specific primer and further polymerized in a 7300 Real-Time PCR System (Applied Biosystems). For each polymerization (total 45 cycles), temperature was set at 95°C for 15 seconds and 60°C for 1 minute. The PCR reaction was performed at 50°C for 2 minutes followed by 95°C for 10 minutes. Taqman® Gene Expression Assay (Applied Biosystems) for specific gene detection was used as a primer and probe as follows: GAPDH (Mm99999915_g1), HSP70 (Mm03038954_s1), HSP27 (Mm00517908_m1), HSP47 (Mm00438056_m1), osteopontin (OPN) (Mm01611440_mH), osteoprotegerin (OPG) (Mm01205928_m1), osteocalcin (OCN) (Mm00649782_gH), matrix metalloproteinase 9 (MMP-9) (Mm00600164_g1), ALP (liver/bone/kidney) (Mm01187113_g1), cyclooxygenase-2 (COX-2) (Mm01307330_g1), BSP (Mm00492555_m1), type I collagen (alpha 1) (Mm00801666_g1), transforming growth factor beat 1 (TGF-β1) (Mm00441724_m1), and vascular endothelial growth factor (VEGF) (Mm00437308_m1). Threshold cycle (C_T), derived using SDS v1.2× system software of 7300 Real-Time PCR System, denotes the fractional cycle number at threshold polymerized gene and -$\Delta\Delta C_T$ was derived from (C_T of target gene-C_T of GAPDH)$_{\text{treated group}}$-(C_T of target gene-C_T of GAPDH)$_{\text{control group}}$ [251]. Treated groups denote heated, HSP70 siRNA-treated alone or in

combined treated groups and control groups indicated sham-treated cells without heating and HSP70 siRNA treatment.

6.3.7. Protein secretion measurement

Protein release into the culture media was analyzed using enzyme-linked immunosorbent assay (ELISA). Conditioned media from monolayer cells was collected at 72 hour PTI after thermal stress (44°C, 8 minutes). OPG, TGF-β1, VEGF, OPN, and MMP-9 were measured using Quantikine® ELISA (R&D Systems) according to the manufacturer's protocol. Culture supernatant was added to a 96 well microplate coated with specific antibodies for each protein. The absorbance of the colorized solution was acquired at 450 nm using a microplate reader and used for concentration determination.

6.3.8. Measurement of cell proliferation

For investigation of cell growth, we utilized two assays including PicoGreen DNA and MTS assay. DNA was isolated using the TE lysis buffer (10 mM Tris, 1mM EDTA, 0.1% Triton X-100, and 0.1 mg/ml proteinase K) at 1, 2, 4, and 6 day PTI. Cell lysate was heated at 56°C at least overnight and DNA concentration was measured using Quan-Ti™ PicoGreen ds DNA reagent kit (Invitrogen) according to the manufacturer's protocol. DNA samples and standards were transferred into a 96 well-plate and Quan-Ti™ PicoGreen ds DNA reagent was added to each sample at the volume ratio of 1:1 following 3 minute incubation at room temperature in the dark. Fluorescence of each sample was measured by a microplate reader set at excitation and emission wavelengths of 480 and 520 nm, respectively. In addition, cell proliferation was also measured with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI) to determine cell proliferation rate. At specific time points (1, 2, 4, and 6 day PTI), MTS stock solution at 20% of the culture media volume used during cultivation was mixed with a basal culture media which did not contain FBS and PS. Diluted MTS working solution was added to cultured cells. After 4 hour incubation at 37°C, the solution was transferred into a 96 well plate and optical density was measured at 490 nm by a microplate reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA).

6.3.9. Statistical analysis

All data are presented as mean ± standard deviation (SD). Experimental groups with a minimum of three repetitions (3 or 4) were tested and analyzed independently. One-way ANOVA supplemented by a Tukey multiple comparison test was applied to determine statistical significances and the difference of means between each group using JMP-8. The statistical significance was defined as p value lower than 0.05.

6.4. Results

6.4.1. Confirmation of HSP70 silencing and other HSP expression

To demonstrate whether HSP70 siRNA effectively suppressed HSP70 expression in both sham-treated and heated (44°C, heating duration=8 minutes) MC3T3-E1 cells, isolated HSP70 gene and protein were measured using samples isolated at 10 and 24 hour PTI, respectively, by real time RT-PCR (Figure 6-2A) and western blot (Figure 6-2B). HSP70 siRNA treatment successfully inhibited HSP70 messenger RNA (mRNA) induction compared to cells without siRNA treatment before and following heating; 0.15 RFI in unheated cells with HSP70 siRNA, 9.07 in heated cells without HSP70 siRNA, and 3.05 in cells heated with HSP70 siRNA. HSP70 genes in cells exposed to both heating alone (9.07 RFI) and heating with HSP70 siRNA (3.05 RFI) groups showed much higher induction levels than non-heated cells. However, there was no significant HSP27 and HSP47 mRNA difference between test groups at 10 hour PTI (Figure 6-2C and D). Similar to PCR data, western blot analysis demonstrated that HSP70 protein increased significantly due to heating and HSP70 siRNA treatment diminished HSP70 induction levels following heating at 24 hour PTI. HSP27 protein expression in HSP70-silenced cells exposed to heating increased compared to cells exposed to heating without siRNA treatment. HSP47 protein expression was similar in all groups (Figure 6-2B). Nondetectable levels of HSP27 and HSP70 were observed for unheated cells; however, HSP47 expression was high for all groups (Figure 6-2B).

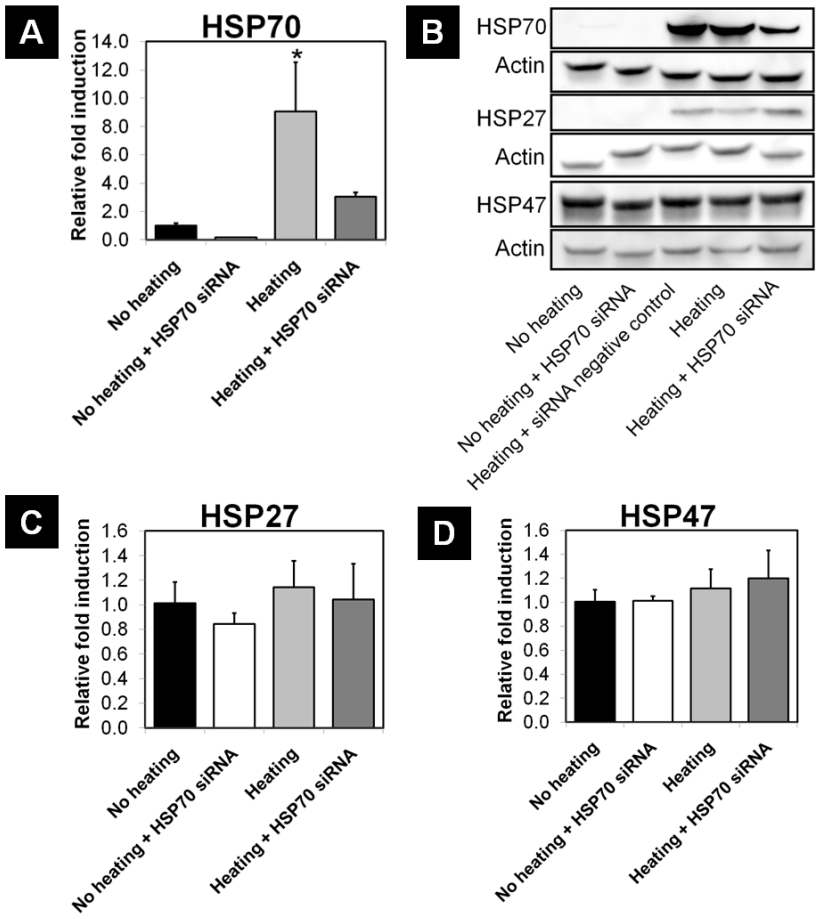

Figure 6-2. HSP mRNA and protein expressions of MC3T3-E1 cells experiencing, no heating, HSP70 silencing, heating (44°C, heating duration=8 minutes), and heating with HSP70 silencing. Relative fold induction (RFI) of HSP70 (A), HSP27 (C), and HSP47 (D) mRNA expression at 10 hour PTI measured with RT-PCR. Protein levels of all HSPs at 24 hour PTI measured with western blot (B). * denotes statistical significant difference between control and treated groups (HSP70 siRNA silencing alone, heating alone, and heating with HSP70 siRNA silencing). # represents statistical significance among heated groups-including heating alone, heating with HSP70 siRNA, and heating with negative control (in B) ($p<0.05$).

6.4.2. Cell Proliferation

Cell proliferation was measured for cells exposed to normal culturing conditions, heating alone (44°C, heating duration=8 minutes), HSP70 siRNA gene silencing alone (without heating), or HSP70 silencing in combination with heating (Figure 6-3). Two different methods were utilized to measure cell viability for varying PTI durations (measured at 24, 48, 97, and 145 hours): MTS assay for characterization of mitochondria metabolic activity and PicoGreen fluorescence assay for DNA quantification.

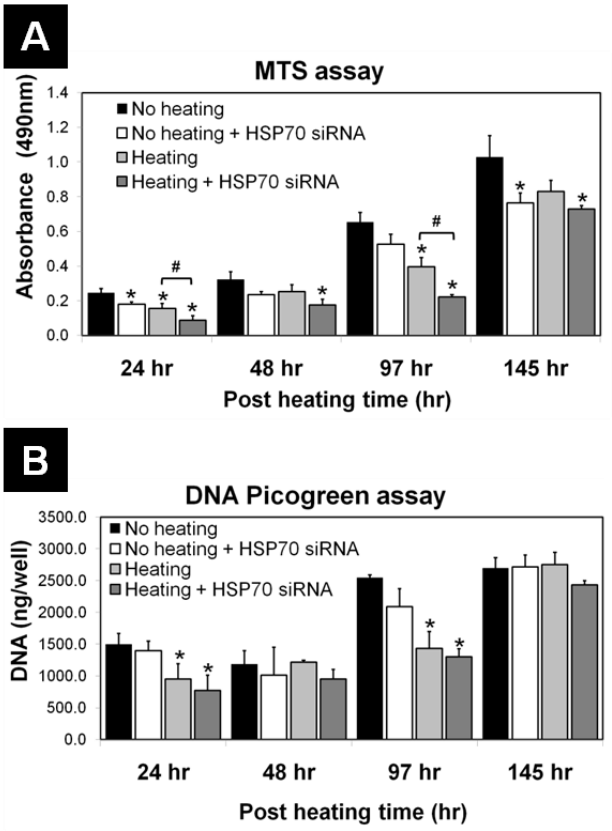

Figure 6-3. MC3T3-E1 proliferation for cells experiencing, no heating, HSP70 silencing, heating (44°C, heating duration=8 minutes), and heating with HSP70 silencing measured at 24, 48, 97, and 145 hour PTI. Bar graph (A) was analyzed by MTS assay and DNA concentration was determined by PicoGreen assay (B). * denotes statistical significant difference between control groups and samples exposed to HSP70 siRNA silencing alone, heating alone, and heating with HSP70 siRNA silencing. # represents statistical significant difference between heated groups: heating and heating with HSP70 siRNA silencing ($p<0.05$).

As shown in Figs. 2A and B, MTS viability assay revealed that HSP70 silencing inhibited MC3T3-E1 cell proliferation in both normal and heated cells. Cells heated with HSP70 siRNA silencing showed the lowest metabolic level of all test groups. Heated cells (heating alone and heating with HSP70 siRNA) exhibited lower cell metabolic activity indicating slow cell growth. However, the MTS absorbance level of all test groups increased with longer cell cultivation period.

6.4.3. Modification of bone-related protein mRNA

The induction of bone-related protein mRNA for cells exposed to normal culturing conditions, heating alone (44°C, heating duration=8 minutes), HSP70 siRNA gene silencing alone (without heating), or HSP70 silencing in combination with heating was quantitatively analyzed at 10 hour PTI using real time RT-PCR as shown in Figure 6-4. Several bone-related proteins including OPN, OPG, OCN, MMP-9, ALP, COX-2, BSP, and type I collagen were chosen as bone phenotypic markers to determine whether heating and HSP70 silencing could influence *in vitro* osteogenic activity of preosteoblasts. Without heating, HSP70 silencing did not significantly modify expression of OPN, OPG, OCN, COX-2, and type I collagen mRNA (Figure 6-4A, B, C, F, and H). However, MMP-9, ALP ($p<0.05$), and BSP mRNA were suppressed in unheated cells by HSP70 siRNA silencing (Figure 6-4D, E, and G) at 10 hour PTI. Heating caused suppression of MMP-9 (Figure 6-4D) and ALP (Figure 6-4E) mRNA in cells both with and without HSP70 silencing. In contrast, OCN mRNA was enhanced in heat-treated cells. HSP70 siRNA silenced cells exhibited suppressed BSP mRNA for both unheated and heated samples (Figure 6-4G). However, gene expressions of all markers at 10 hour PTI showed only slight modification by heating and HSP70 siRNA silencing alone or in combination.

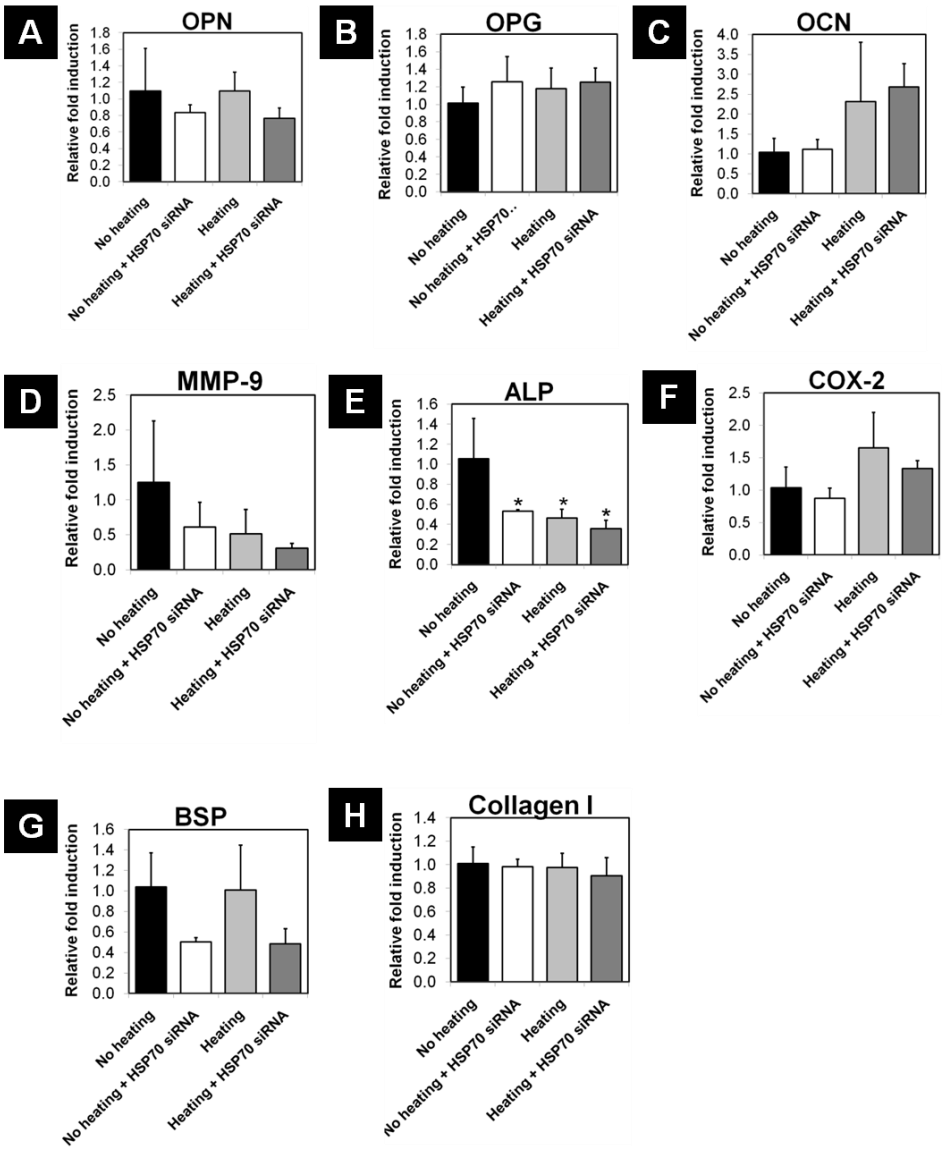

Figure 6-4. Bone-related protein mRNA expressions of MC3T3-E1 cells experiencing, no heating, HSP70 silencing, heating (44°C, heating duration=8 minutes), and heating with HSP70 silencing measured at 10 hour PTI. Gene expression of OPN (A), OPG (B), OCN (C), MMP-9 (D), ALP (E), COX-2 (F), BSP (G), and type I collagen (H). * denotes statistical significant difference between control and groups experiencing HSP70 siRNA silencing alone, heating alone, and heating with HSP70 siRNA silencing. # represents statistical significant difference between heated groups: heating alone and heating with HSP70 siRNA silencing ($p<0.05$).

6.4.4. Protein expression and localization of HSP70 and bone-related proteins

IF staining was utilized to measure the expression and localization of intracellular protein at 16 hour and 2 day PTI for cells exposed to normal culturing conditions, heating alone (44°C, heating duration=8 minutes), HSP70 siRNA gene silencing alone (without heating), or HSP70 silencing in combination with heating. Co-expression of COX-2 and HSP70 was visualized in the merged fluorescence images (Figure 6-5) acquired by double IF staining. At 16 hour PTI (Figure 6-5A-D), HSP70, similar to gene analysis, was significantly induced following heating but induction of HSP70 was diminished with HSP70 siRNA silencing. There was no detectable level of HSP70 expression in unheated cells. Higher HSP70 expression in the cytosol was detected compared to that observed in the nucleus whereas COX-2 expression was greater in nucleus. On the other hand, at 2 day PTI (Figure 6-5E-H), HSP70 expression following heating was decreased and was at comparable levels to HSP70 siRNA-silenced cells following heating.

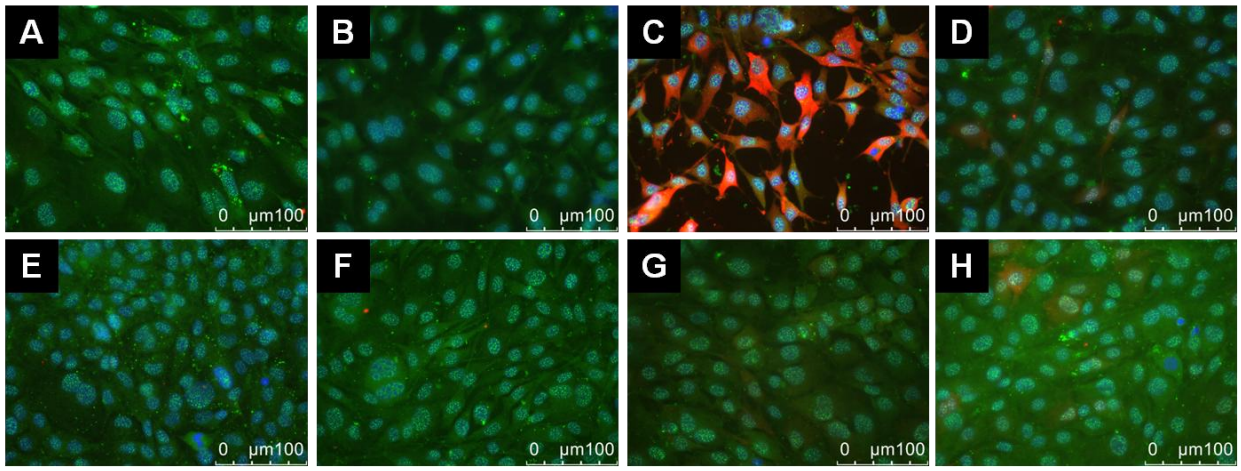

Figure 6-5. Immunofluorescence of HSP70 and COX-2. It was acquired at 16 hour PTI (A-D) and 48 hour PTI (E-H) for samples experiencing no heating (A and E), no heating with HSP70 siRNA silencing (B and F), heating alone (44°C, heating duration=8 minutes) (C and G), and heating with HSP70 siRNA (D and H). HSP70 and COX-2 expression are stained as red and green fluorescence, respectively. Scale bar=100 µm.

6.4.5. ELISA analysis of secreted bone proteins

ELISA analysis was used to measure secreted levels of bone-related proteins in the culture supernatant collected at 2 and 6 day PTI for cells exposed to normal culturing conditions, heating alone (44°C, heating duration= 8 minutes), HSP70 siRNA gene silencing alone (without heating), or in combination with heating. OPN, OPG, and MMP-9 secretion was measured with ELISA. OPN secretion was measured to investigate the combined heating and HSP70 silencing effect on OPN matrix protein production by MC3T3-E1 cells (Figure 6-6A and B), and OPG and MMP-9 release were chosen to investigate whether heating or HSP70 silencing could result in anti-osteoclastic effects of bone resorption (Figure 6-6C-F). OPN and OPG concentrations at 2 and 6 day PTI showed significant elevation following heating for all heated groups: heating, heating with HSP70 silencing, and heating with siRNA negative control. However, there was no effect on OPG and OPN at 2 and 6 day PTI by HSP70 silencing. All heated groups showed significantly lower levels of MMP-9 secretion. HSP70 silencing without heating caused suppression of MMP-9 secretion compared to the control group at days 2 (Figure 6-6E and F) but this result should be confirmed further with negative control-included test. However, at 6 day PTI (Figure 6-6F), all groups showed higher MMP-9 concentration than that measured at 2 day PTI (Figure 6-6E) suggesting MC3T3-E1 may secrete increased MMP-9 for longer PTI times.

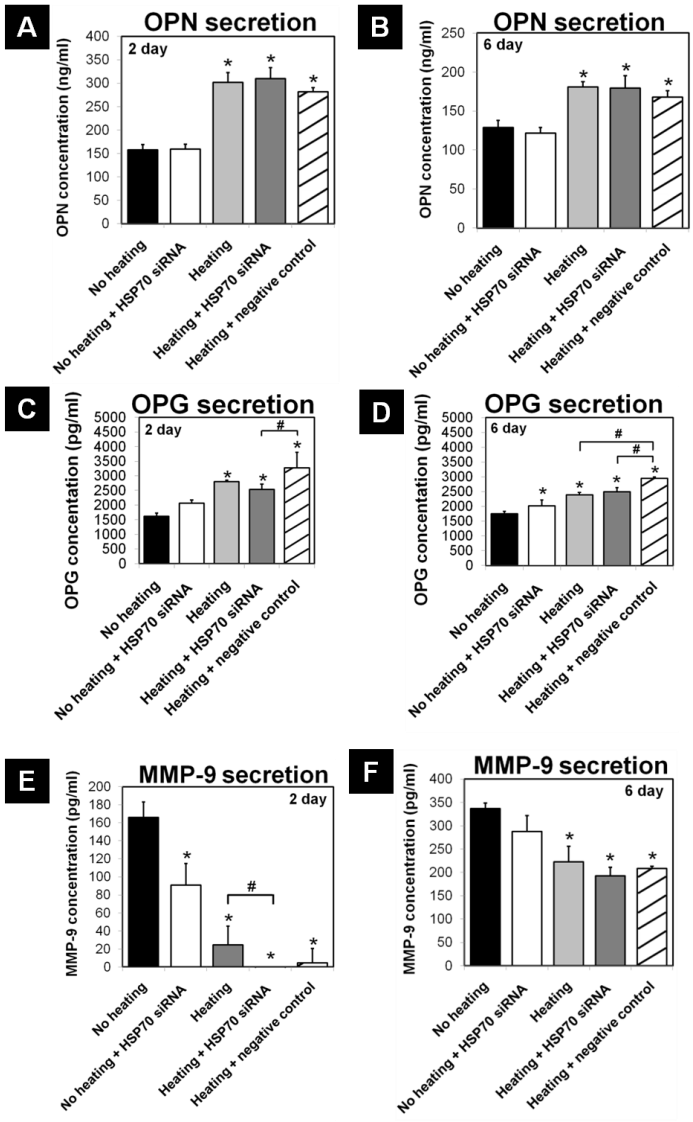

Figure 6-6. Protein secretions into the culture media at 2 and 6 day PTI following no heating, HSP70 silencing, heating (44°C, heating duration=8 minutes), and heating with HSP70 silencing. The culture supernatant measured at day 2 and 6 after heating was collected and analyzed by ELISA. Secretion of OPN at 2 (A) and 6 (B) days; OPG at 2 (C) and 6 (D) days; MMP-9 at 2 (E) and 6 (F) days. * denotes statistical significant difference between control and stressed groups: HSP70 siRNA silencing alone, heating alone, and heating with HSP70 siRNA silencing. # represents statistical significant differences between heated groups: heating alone, heating with HSP70 siRNA, and heating with negative control ($p<0.05$).

6.4.6. Growth factor mRNA expression and release

Using identical methods of real time RT-PCR (10 hours) and ELISA (2 days) as described in previous sections, the induction of bone-related growth factors including VEGF and TGF-β1 by cells exposed to normal culturing conditions, heating alone (44°C, heating duration=8 minutes), HSP70 siRNA gene silencing alone (without heating), or HSP70 silencing in combination with heating, was investigated (Figure 6-7). VEGF mRNA at 10 hour PTI was not affected by HSP70 silencing in spite of a slight increase in heated groups (Figure 6-7A). At 2 day PTI, secreted VEGF following heating in HSP70-silenced cells significantly increased compared to cells exposed to only heating (Figure 6-7B). TGF-β1 mRNA showed the lowest level of RFI (Figure 6-7C). TGF-β1 secretion showed decreased trends by heating but this result should be confirmed further by negative control-included tests.

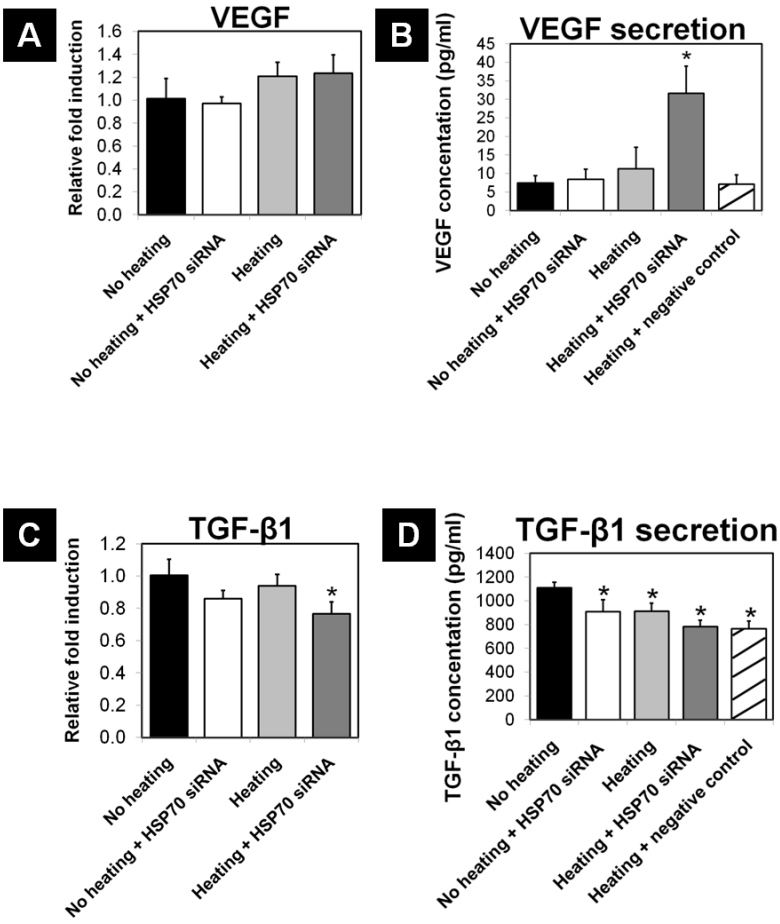

Figure 6-7. Gene and protein secretions of VEGF and TGF-β1 from MC3T3-E1 cells experiencing, no heating, HSP70 silencing, heating (44°C, heating duration=8 minutes), and heating with HSP70 silencing, measured by ELISA. Expression of VEGF mRNA (A) and protein secretion (B); TGF-β1 mRNA (C) and protein secretion (D). PTI=2 day. * denotes statistical significant difference between control and treated groups (cells exposed toHSP70 siRNA silencing alone, heating alone, and heating with HSP70 siRNA silencing). # represents statistical significant differences between heated groups: heating alone, heating with HSP70 siRNA silencing, and heating with negative control (in B and D) ($p<0.05$).

6.5. Discussion

Successful adaptation of bone to a stressful environment is essential to maintaining normal bone mass via dynamic bone remodeling processes consisting of apoptosis, proliferation, and differentiation of bone cells [5, 326]. Given the importance of stress-mediation in bone health, heat shock (or stress) proteins, which not only increase in response to external cues but also are responsible for cell survival and protein folding, can be key modulators in controlling bone physiology [207].

In this study, we explored the influence of HSP70 in cell proliferation and induction of other stress proteins (*i.e.* HSP27 and HSP47), bone-related proteins (*e.g.* OPN, OPG, and ALP), and growth factors such as TGF-β and VEGF under normal conditions and in response to thermal stress. MC3T3-E1 cells were exposed to HSP70 gene silencing through siRNA transfection or untreated and subsequently subjected to a single dose of mild thermal stress at 44°C for 8 minutes using a heating water bath. The use of HSP70 gene silencing allows the effect of HSP70 expression on proliferation, expression of HSPs, and bone-related proteins to be specifically investigated by eliminating its involvement in these processes. However, deciphering the exact relationship between thermal stress, HSP70 expression, and its ultimate effect on bone regeneration is difficult because of the opposing effects thermal stress and HSP70 exert on the heat shock factor (HSF). HSFs, which upregulate HSPs by binding to specific DNA region (*i.e.* heat shock element) as an activated trimer form, are induced by heating. However, HSP70

induction causes deactivation of HSF [214]. In order to better elucidate the impact of thermal stress on HSP70 expression and ultimate bone response, we applied thermal stress protocols previously documented to cause significant HSP70 induction [136] and subsequently suppressed HSP70 expression with gene silencing to better understand the correlation between HSP70 expression and *in vitro* responses of preosteoblasts following thermal stress. Since bone healing activity can be evaluated by demonstrating enhanced level of proliferation or regulations of gene/protein in osteoblasts, examinations of cell growth rate and inductions of HSP and bone proteins using biochemical and biomolecular assays such as real time RT-PCR and ELISA were utilized.

Mild heating (also known as low-level or sub-lethal heating), for periods of several minutes to hours at the supraphysiological temperature range of 37-45°C, can improve cellular protective or regenerative activity in various cells/tissues related to dentin, cartilage, bone, and skin [74-76, 131, 142, 323]. Selection of our heating protocol of 44°C for 8 minutes was chosen based on prior work by Rylander et al. which demonstrated water bath heating at 44°C for heating durations to cause significant elevations of HSP70 expression in bovine aortic endothelial (1-24 minutes) and PC3 prostate cancer cells (1-12 minutes) [136, 249]. We have also demonstrated that water bath heating at 44°C for 8 minutes to upregulate genes of osteogenic (*e.g.* OCN, OPN, OPG, and COX-2) and angiogenic marker (VEGF) by preosteoblast cells at 8 hour post-heating timepoints in our previous research (unpublished data).

We confirmed suppressed HSP70 gene or protein levels by HSP70 siRNA silencing using real time RT-PCR (10 hour PTI), IF stain (16 and 48 hours), and western blot (24 hours) for all samples (Figure 6-2 and 5). In spite of nondetectable difference of HSP70 expression in normal cells due to weak signals from western blot and IF stain, RT-PCR analysis revealed the suppressed mRNA level of HSP70 by HSP70 siRNA silencing in normal cells. This suggests that constitutive HSP70 can be also silenced by our siRNA transfection technique. Heating at 44°C for 8 minutes using a water bath significantly induced HSP70 expression rapidly in several hours, similar to previous studies by our group (unpublished data) and other literature [136, 243]. Therefore, this mode of thermal stress provided a mechanism to study the effect of siRNA silencing in combination with thermal stress conducive to HSP70 induction. Thermal induction of HSP27 and HSP70 were evident based on western blot analysis, however, HSP47 showed no noticeable change in response to heat. High universal inductions of HSP47 in all groups (*i.e.*

sham-treated, HSP70-silenced, heat-treated, and combined groups of heating and HSP silencing) indicated that HSP47 function may not be related to either HSP70-mediated or a heat-stimulated mechanism in preosteoblasts. However, the induction level or duration of HSP47 expression may be distinct from other HSPs. This can be presumed from current knowledge that HSP47 is a molecular chaperone capable of being activated thermally [327] and its obligation is to transfer procollagen stably to Golgi by binding with procollagen in the endoplasmic reticulum undergoing collagen process [199]. In addition, HSP70 may interact with HSP27 more closely than HSP47 because they both operate as molecular chaperones related to mitogenic and apoptotic processes [209, 212]. Higher HSP27 expression was measured in the HSP70-silenced cells under thermal stress compared to cells exposed to only heating. A possible hypothesis for this is that HSP27 may have a compensating operation system for hampered HSP70-mediated protective processes, which are caused by HSP70 silencing, when exposed to thermal stress.

Our cell proliferation results (Figure 6-3A) provide evidence that HSP70 may be involved in the proliferative process of unstressed cells as well as heat-stressed cells. Thermal stress conditioning diminished cell proliferation rate but allowed cells to continue growing at 3 day PTI compared to day 1. This demonstrates that the thermal conditioning protocols can be considered to be sublethal. In addition, HSP70-silenced cells under thermal stress showed lower cell viability than cells exposed to heating alone. Therefore, thermal induction of HSP70 may play a role in the beneficial auto-protective capacity of stress-treated cells. Silencing of HSP70 production impaired mitogenic responses of MC3T3-E1 cell line under normal and thermal stress environment suggesting HSP70 has a critical role in preosteoblast proliferation. The relationship between HSP70 and cell growth and death has been documented in prior studies [209, 212, 328, 329]. According to Rylander *et al.*'s study using endothelial cells, it appears to have thermally induced kinetic relationship between HSP70 expression levels and cytotoxicity depending on thermal stress conditioning protocols (44, 46, 48, and 50°C) [136]. Higher HSP70 induction level (3.6 fold) following heating at 48-50°C for 1-2 minutes was examined with *ca.* 94 % viability compared to 2.3-2.6 fold induction of HSP70 due to heating at 46 and 44°C for 10 and 22 minutes, respectively, with comparable viability [136]. HSP70 interrupts apoptosis by retarding the release of cytochrome C from mitochondria, modifying procaspase, and inhibiting apoptosis-inducing factor (AIF) location-switch into nucleus, where AIF modifies chromatin causing apoptosis [328, 329]. From Garrido *et al.*'s [209] and Lanneau *et al.*'s [212] review,

HSP70, similar to HSP27 and HSP90, modulates a variety of mediators and pathways associated with apoptosis. For example, HSP70 hampers normal operation of several apoptotic mediators such as caspase 3 and 8, Bax, and c-Jun N-terminal kinases (JNK) in diverse locations such as proteasomes, mitochondria, and nucleus [209, 212].

Evaluation of *in vitro* osteogenic and anti-osteoclastic responses of preosteoblasts under thermal stress in combination with HSP70 silencing was accomplished by investigating gene or protein inductions of bone-related proteins (Figure 6-4, 5, 6, and 7). Bone-related proteins can be defined as secreted or produced proteins from preosteoblasts and include collagens (*i.e.* type I collagen) and non-collagenous matrix proteins (*e.g.* OPN, OCN, and BSP), growth factors (*e.g.* VEGF and TGF-beta 1), cytokines (OPG), and relevant enzymes (*e.g.* ALP, COX-2, and MMP-9). These marker proteins have been investigated as critical indicators of MC3T3-E1 cells to evaluate preosteoblast activity or differentiation by numerous prior studies [41, 59, 60, 89, 105, 330]. At an early timepoint (10 hour PTI), HSP70 silencing diminished BSP, MMP-9, and ALP mRNA expression suggesting HSP70 is associated with the regulation of these bone proteins. In addition, heating suppressed MMP-9 and ALP mRNA significantly and induced OCN mRNA suggesting heat stress can induce varied responses for each protein. Similar to HSP47, there was no change in type I collagen mRNA as a result of HSP70 siRNA silencing or heating. Therefore, HSP47 and type I collagen may not be related to HSP70 modulation in both rest and thermal stressed cells. OPN mRNA at 10 hour PTI showed no significant modifications by our stresses (heating and HSP70 silencing alone or in combination) but OPN secreted in the culture supernatant showed more significant elevated levels following thermal stress at day 2 and 6 PTI than unheated groups. OPG secretion at 2 day PTI was induced slightly following heating. However, there were no significant modifications in OPN and OPG secretion due to HSP70 silencing, suggesting HSP70 may not be involved in osteogenesis in preosteoblasts.

MMPs are enzymes that can degrade extracellular matrix (ECM) by causing various ECM-mediated cell/tissue-level modifications such as cell migration, development, and wound healing [109]. MMP-9 is one of more than 20 types of MMPs, known as 92 kDa gelatinase or type IV collagenase and is an osteoclast phenotyping protein [109]. From Blavier *et al.*'s study, down-regulation of MMPs resulting from treatment with MMP inhibitors (*i.e.* CI-1 and RP59794) caused the hindrance of osteoclast-mediated calcium release and migration [112]. Bone healing is abnormally hampered by MMP-9 knockout mice [307]. Even though the

regulation of MMP-9 is a very important factor in bone physiology, MMP-9 expression in osteoporosis is controversial. For example, MMP-9 is highly expressed in rat osteoporotic bone tissue identified through dot blot hybridization and in situ hybridization [113]. In contrast, bone of osteoporotic women exhibited low levels of MMP-9 gene [114]. However, given that overexpression of MMP-9 can accelerate bone resorption, blocking MMP-9 activity, with cathepsin K, is a promising target in bone diseases [31, 306]. In this study, MMP-9 mRNA (10 hour PTI) and protein secretion showed suppressed trends by HSP70 silencing without heating (Figure 6-4D and 7E). Even though little is known about the correlation between HSP70 and MMP-9, it can be presumed from Lee *et al.*'s study that HSP70 regulation may be related to MMP-9 [116]. Lee *et al.*'s group demonstrated HSP70 transfection diminished MMP-9 elevation in astrocytes exposed to neuro-ischemic-like stimuli (*i.e.* oxygen glucose deprivation) and normal culturing conditions [116]. Taken together, suppression of MMP-9 by silencing HSP70 may be effective to block abnormal activity of osteoclasts in bone resorption, but the level of MMP-9 should be optimized further for controlling bone remodeling and regeneration.

The communicative mechanism between HSP70 and GF release of biomolecules such as VEGF or TGF-β1 has not yet been explored. VEGF not only induces neo-blood vessel formation in fracture healing but also is an important communicator between osteoblasts and endothelial cells [19]. Therefore, in engineering artificial bone tissue, neovascularization via VEGF delivery, microsurgery, construction of microvessel-like structures, and EC seeding alone or in combination with osteoblasts or mesenchymal stem cells is a critical factor to promote bone healing capacity [35]. In our previous studies (unpublished data), thermal stress conditioning at 44°C for 8 minutes induced VEGF mRNA at 8 hour PTI. Similarly, rats exposed to hyperthermia (42°C for 15 minutes) using a heating blanket induced VEGF and HSP70 rapidly in cardiac tissue at 4-24 hour PTI [309]. These studies suggest HSP70 may be involved in the process of VEGF secretion in response to thermal stress. Interestingly, VEGF secretion following heating and HSP70 silencing at day 2 PTI was significantly increased even though VEGF mRNA was slightly induced by heat treatment and was not affected by HSP70 silencing at 10 hour PTI. As demonstrated from ELISA analysis, VEGF showed significantly elevated levels (5-fold induction compared to control groups) following heating with HSP70 silencing. This result suggests that HSP70 gene silencing with heating could induce VEGF. However, detailed interactions between HSP70 and VEGF are not fully understood.

On the other hand, the suppressed trends of TGF-β1 mRNA by HSP70 silencing and heating were shown in this study. TGF-β1 is known to be a pivotal osteoinductive growth factor and frequently applied as a bone regenerative chemical cue [47, 270]. TGF-β can be produced by osteoblasts [331]. This growth factor exerts a variety of effects in bone physiology by recruiting and controlling both osteoblasts and osteoclasts via receptor-mediated downstream signaling pathways such as smad and MAPK [23]. According to recent work by Cao *et al.*, both TGF-β1 and HSP70 expression in human skin fibroblasts were elevated by ultraviolet B, and HSP70 induction by UVB and heating also was diminished by TGF-β1 receptor inhibition [332]. One possible explanation is that heating [333] induced downstream signaling such as MAPK signaling, which is also related to TGF-β [23]. Further works should be required to determine if MAPK signaling might be involved in between VEGF or TGF-beta and HSP70.

In conclusion, *in vitro* effects of HSP70 gene silencing on preosteoblasts revealed that HSP70 is involved in preosteoblast proliferation under both normal culturing conditions and thermal stress. Furthermore, HSP70 down-regulation modified HSP27 protein level and secretion of bone-related proteins such as VEGF. Our study can provide new insight regarding HSP70 function in osteoblast proliferation and differentiation. However, proliferation, PCR and ELISA results should be confirmed further with negative control tests to demonstrate HSP silencing effects more exactly. For this test, non-heated or heated with negative control-treated groups will be perfomed with the groups with heating and HSP silencing alone or in combination. In addition, there are still several questions to be answered. Detailed intracellular mechanisms between HSP and GF expression/activation are still unclear. In addition, the correlation between osteogenesis and other HSPs such as HSP27 and HSP47 in response to stress should be investigated by gene silencing and HSP delivery techniques.

Chapter 7: Concluding Remark and Perspectives

7.1. Conclusion

The current study is focused on demonstrating whether applying individual or combinatorial stress conditioning (thermal, tensile, and biochemical) and effective HSP modulation could induce *in vitro* responses in preosteoblasts indicating mitogenic/osteogenic/angiogenic/anti-osteoclastic effects. This hypothesis was approached by measuring the various responses of preosteoblasts following individual or combined treatment of heating, cyclic tensile stress, and growth factors. This knowledge is necessary to advance development of stress conditioning protocols to enhance bone regeneration and treat bone diseases. Since induction of HSPs by stress can potentially influence cell proliferation, angiogenesis, and extracellular matrix production, it is critical to determine the relationship between stress, HSP expression, and bone formation to enable development of HSP-based therapies for bone healing. Therefore, we determined the correlation between HSP induction and *in vitro* osteogenic activity by silencing HSP70 gene expression in normal and heat-treated preosteoblasts using HSP70-specific siRNA.

In **Chapter 3**, a preosteoblast cell line (MC3T3-E1) was exposed to **elevated temperature (44°C)** with varying heating durations (0-8 minutes) alone or in combination with two **osteoinductive GFs (BMP-2 and TGF-β1)**. This study revealed that heating (44°C) showed the induced trends of mRNA levels of HSPs (*e.g.* HSP27, HSP47, and HSP70), bone-related proteins (*e.g.* OPN and OCN), anti-osteoclastogenic cytokines (*e.g.* OPG), and angiogenic growth factor (*e.g.* VEGF) and inhibited a bone ECM-degrading enzyme (*e.g.* MMP-9). GF addition induced significant increases in COX-2 mRNA and secretion of OCN, OPG, and OPN proteins. The expressions of VEGF and OPG mRNA were induced more by tension with GF addition than tension or GF alone. However, combined treatment with heating and GFs caused varying responses by bone proteins and HSPs depending on heating temperature and duration, and post-heating time.

In **Chapter 4**, **mechanical cyclic strain** (equibiaxial 1, 3, 5, and 10% magnitude, 0.2 Hz, 10 seconds tension on/10 seconds rest) using Flexcell® tensile bioreactor alone or with **two osteoinductive GFs** (BMP-2 and TGF-β1) was applied to MC3T3-E1 monolayers cultured on

type I collagen-coated BioFlex® plates. Long-term mechanical strain alone (at days 3 and 6, under confluent in 10 % FBS osteogenic media) induced transient HSP27 and HSP70 mRNA for early durations (at days 3) of tension loading and also promoted PGES-2 (at days 3), and COX-2 and OPN gene (at days 6) expression in preosteoblasts. In contrast, alkaline phosphate (ALP) and bone sialoprotein (BSP), type I collagen, MMP-9, MMP13, OPG, and Runx2 decreased in response to tension at days 6. Short-term (for 24 hours, confluent monolayer in 1% FBS osteogenic media) tension (1-5%, 0.2 Hz, 10 seconds tension on/10 seconds rest) in combination with GFs induced PGES-2, OPG, and VEGF genes more than tension or GF addition alone. However, GF addition in combination with tension diminished HSP70 and OPN expression in contrast to the inductions of COX-2 and BSP mRNA by GFs. Combinatorial conditioning using tensile stress and GFs induced greater levels of cell proliferation (for 5% strain), and genes for OPG, PGES-2 (both for 1, 3, 5% strain), and VEGF (for all stress magnitudes), and secretion of VEGF and OPN (for 5% strain) than individual stress of tension or GF treatment. GF addition in combination with tension diminished HSP27 and HSP70 gene expression compared to tension alone. In addition, tension alone or with GFs decreased MMP-9. GFs induced genes for HSP47, COX-2, BSP, and type I collagen, but suppressed HSP70 and OPN genes.

In **Chapter 5**, the effect of combined **thermal and mechanical stress** on preosteoblast response was measured. A single dose of heating using a water bath (44°C, 4 or 8 minutes) followed by cyclic stretching (3%, 0.2 Hz, 10 seconds tension on/10 seconds rest) using a Flexcell® tensile bioreactor was applied to MC3T3-E1 monolayers cultured on the type I collagen-coated BioFlex® plates. HSP27 and HSP70 mRNA expression increased more following heating (8 minutes) and tension (4 hours) than heating or tension alone. There was the greatest increase in VEGF release and decrease in MMP-9 mRNA expression following heating and tension (24 hours). ALP decreased in response to all stress treatment protocols. On the other hand, combined stress did not affect mRNA expression of OCN, type I collagen, and COX-2.

In **Chapter 6**, the role of HSP70 in the expression of bone-related molecules or other HSPs under normal culturing conditions and in response to stress was investigated by silencing HSP70 with small interfering RNA (siRNA). HSP70 was silenced in MC3T3-E1 cells by transfecting HSP70-specific siRNA followed by mild heating (44°C, 8 minutes in a heating water bath). Real time RT-PCR and western blot analysis revealed that HSP70-silenced cells did not show significant modification of HSP47 gene and protein expression. The protein expression

of HSP27 was promoted more by heating and HSP70 silencing than heating alone. Thermal stress impaired cell proliferation with more significant reductions by HSP70-silenced cells, indicating HSP70 may mediate the mitogenic activity of preosteoblasts. VEGF increased significantly by heating and HSP70 silencing. On the other hand, OPN and OPG secretions into culture media increased in response to heating, was not affected by HSP70 manipulation. Therefore, HSP70 may be associated closely with HSP27 and be involved in the cell proliferation and production of pivotal bone-related proteins by preosteoblasts in response to mild thermal stress.

Although we have conducted a comprehensive study to measure the response of preosteoblasts to stress conditioning, further work is needed to optimize these protocols to achieve maximum benefit for bone tissue engineering applications or bone disease therapy. Since only a single dose of water bath heating was applied in this study, this stimulus may be insufficient to induce long-term osteogenesis. In addition, the length of post-stress cultivation or tensile stress treatment periods of several hours to days used in this study were chosen to induce HSP induction and may not be sufficient to cause sufficient osteogenic benefit. Future work should focus on repeated heating and the use of longer post-heating incubation periods. The use of 3D hydrogels or polymeric scaffold will allow long-term combined stress conditioning and permit measurement of the anabolic response of preosteoblasts for several weeks or months which is the ideal time period for measuring bone markers. Furthermore, the use of a bioreactor system which will allow combined thermal, tensile, and shear stress to be applied to bone cells should be implemented to enable investigation of the impact of all three stresses alone or in combination on *in vitro* bone formation. This system would allow thermal and tensile stress to be applied simultaneously rather than using the Flexcell system to apply tensile stress followed by water bath heating. In addition, overexpression effects of HSP70 via exogenous HSP70 delivery system should be investigated to better understand HSP70 roles in bone physiology. Similar to HSP70, the role of other HSPs (*e.g.* HSP27 and HSP47) on bone formation should also be investigated to determine whether these HSPs could be better targets for achieving therapeutic advantage.

7.2. Future Research

7.2.1. Application of other types of stress preconditioning: laser and fluid shear stress

7.2.1.1. Laser irradiation

Thermal stress preconditioning can be applied by exposing cells to the elevated temperature by treating laser. To choose the superior thermal stressor between laser treatment and water bath heating for proliferation or diffentiation of preosteoblastic cells, the impacts of laser irradiation should be compared to the altered cell responses by water-bath heating. Low-level laser treatment (LLLT) has been shown to induce bone and teeth growth, as described in Chapter 1. However, protocols for effective laser conditioning have not yet been confirmed and the cellular responses to laser heating have not been thoroughly characterized. Initial studies have been conducted to investigate the effect of *in vitro* laser treatment (single or multiple doses) on cell proliferation, regulations of bone-related molecules, and HSP expression in MC3T3-E1 monolayers, as shown in Figure 7-1 and 7-2. A continuous fiber Nd:YAG laser (wavelength= 1064 nm) (Model YLR-10-1064-LP, IPG Photonics Corporation, Oxford, MA) with a concave lens was utilized for thermal preconditioning. Multiple laser treatments with a power range of 0.9-1.2 W) increased cell proliferation (Figure 7-1B).

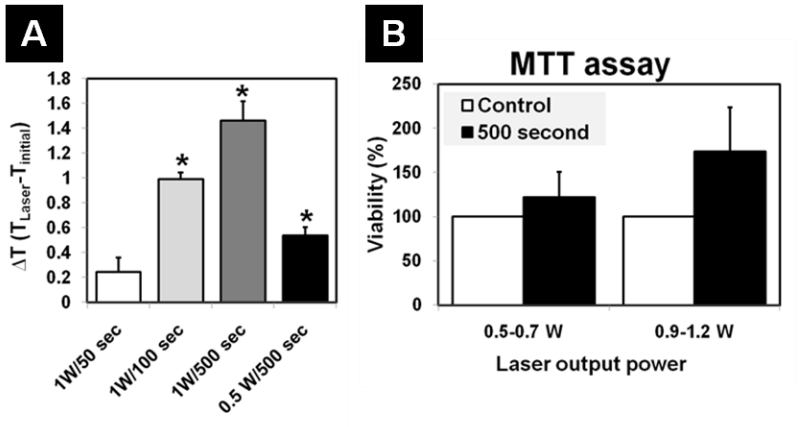

Figure 7-1. Temperature change and cell proliferation effect of in response to 1064 nm continuous laser treatment. Temperature changes (A) with a concave lens depending on different laser irradiation conditions and the viability (B) at 24 hour PH after multiple

doses (4 times, every 2 days) for two different laser output powers (0.5-0.7 W and 0.9-1.2 W).

Multiple laser treatment (4 times, every 2 days, output power=1.8-2 W) showed higher expression of HSP70 and ALP (Figure 7-2). Future research should be focused on optimizing laser protocols to further enhance proliferation and bone-related protein induction. In addition, long-term cultivation using a 2D sheet or 3D scaffold system should be performed to allow measurement of the long-term response. Finally, laser-induced stimulating effects should be compared to of the response to water bath heating.

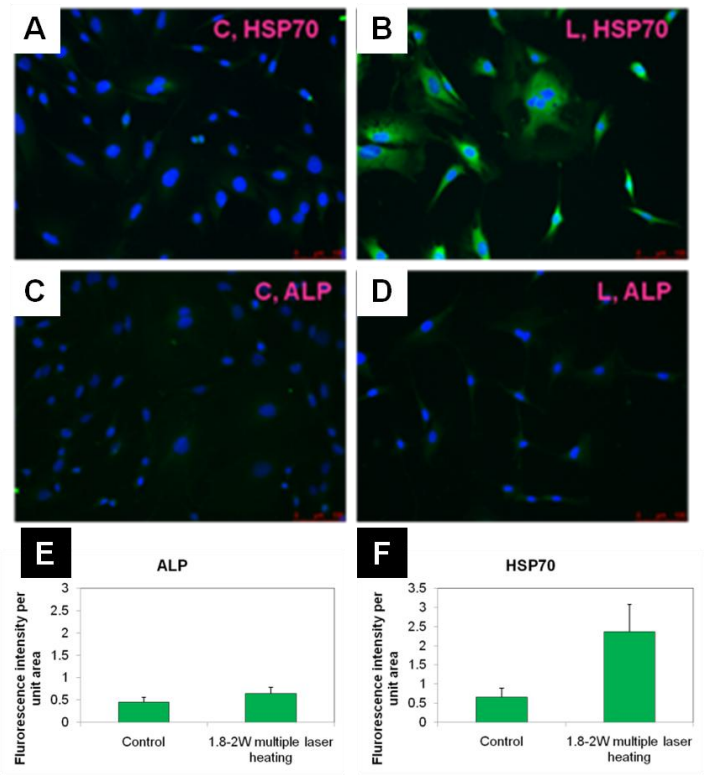

Figure 7-2. Immunofluorescence staining for HSP70 and ALP on 24 hours after multiple laser treatment (4 times, every 2 days) (output power=1.8-2 W, with an expanding lens). Fluorescence images of HSP70 (A and B) and ALP (C and D). Sham-treated (A and C) and laser-irradiated (B and D) cells. C and L denote sham-treated control and laser-treated

group, respectively. Semi-quantification (E and F) of HSP70 (A and B) and ALP (C and D) expression.

7.2.1.2. Fluid shear stress

In the current study described in prior Chapters (4 and 5), mechanical stress was in the form of cyclic tension using a Flexcell® bioreactor. However, fluid shear stress can be another critical stress for enhancing bone formation. HSPs such as HSP60 and HSP27 has been induced or phosphorylated in endothelial cells in response to fluid shear stresses of 30 and 16 dyne/cm^2, respectively [236, 334]. Similar to endothelial cells that experience blood flow, bone cells experience low level of shear stress through canaliculi between osteocytes. Using parallel plate flow chambers connected with a peristaltic pump, the correlation between HSPs and shear stress should be investigated.

7.2.2. Long-term stress conditioning and controlled HSP delivery using a polymeric system.

3D polymeric scaffolds/carriers enable bone cells time for mineralization by preosteoblasts, and to continuously provide growth factors or exogenous HSP. Utilization of exogenous growth factors requires high cost *in vitro* due to the frequent exchange necessary to achieve desired response. On the other hand, long-term gene or protein delivery of HSPs requires a polymeric shuttle, which can penetrate through the cell membrane. Future work could focus on long-term stress conditioning using a 3D scaffold as a platform for growth factor or HSP delivery. This system will be exposed to optimal stress conditioning protocols determined in the prior chapters. As a preliminary study, we have fabricated two different polymeric scaffolds with fibrous and spherical structure, as shown in Figure 7-3 and 4. Detailed fabrication methods are described in Appendix 2.

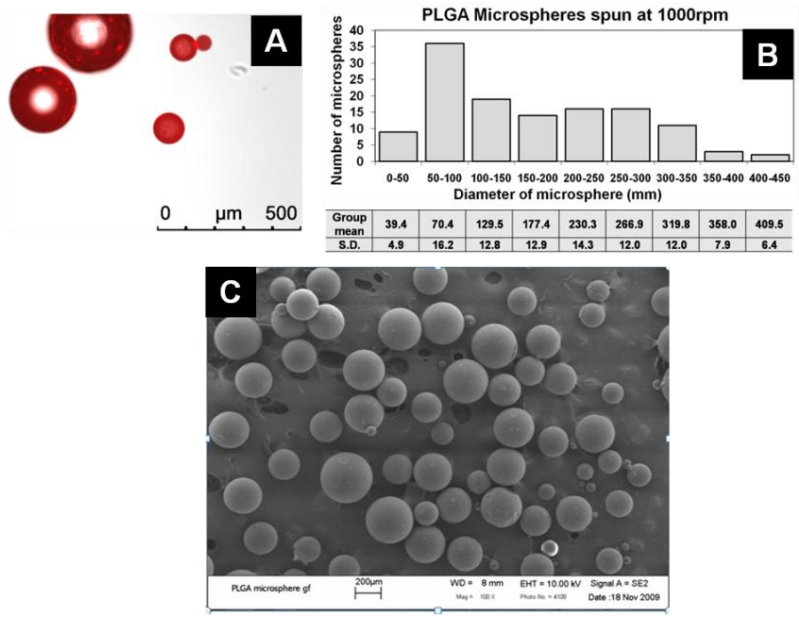

Figure 7-3.The merged fluorescence and bright field- image of DiI-labeled-PLGA microspheres (A). Red color visualization was due to DiI fluorescence dye. Size distribution of PLGA microspheres fabricated by poly(vinyl alcohol)-mediated emulsion method (rotating velocity=1000 rpm) (B). SEM of PLGA microsphere (C). Works related to PLGA microsphere were performed by Robert D. Olsen, an undergraduate mechanical engineering student.

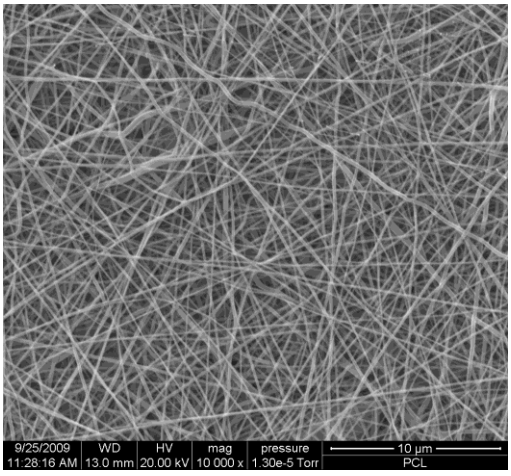

Figure 7-4. PCL:collagen (1:1 w/w) electrospun scaffold. The scale bar in the right bottom of the image denotes 10 µm length.

HSP gene or protein delivery will be accomplished following two candidate methods and it should be determined which methodologies between gene or protein delivery can upregulate HSPs more effectively for longer durations. **Candidate method 1: TAT-HSP protein transduction using PLGA microsphere:** This study will be conducted according to methods described in works by Tan *et al.* [335], Lee *et al.* [231], and Kwon *et al.* [228]. From this prior research, HSP27 protein was successfully delivered using transcription activator (TAT) from human immunodeficiency virus (HIV virus). TAT oligonucleotide and a full length HSP27 were fused in the bacterial expression vector pRSET-A with 6-His tag. TAT-HSP27 will be combined with PLGA solution before emulsion. **Candidate method 2: Gene livery of PLGA nanospheres:** This study will be conducted based on the previous reports of Kang *et al.* [336] and He *et al.* [337]. Nanospheres will be fabricated by a double-emulsion method with a probe sonicator or a homogenizer. For first emulsion, 1 mg HSP70 cDNA (1 ml in Tris-EDTA) in expression vector combined with PLGA (85:15) polymer. We will develop HSP47 and HSP70 gene or protein delivery system using the same processes as described above.

7.2.3. Construction of a novel multi-stress bioreactor

The current design of a Flexcell® Tension™ bioreactor only permits stress conditioning using tension. To explore the combined effects of stress conditioning with thermal, tensile, and shear stress in union, a flow device has been designed which can be integrated into the current Flexcell® system to allow fluid flow for shear conditioning and heating fluid flow for thermal conditioning. A prototype device composed of flow-tubing and a novel flow compartment was designed and fabricated by Jonathan Hopkins (an undergraduate mechanical engineering student). As shown in Figure 7-5, the flow compartment can be easily integrated with the 6 well plate of the BioFlex® culture plate. This shear flow compartment has an inlet and an outlet to allow connection to the entire flow loop for circulating media. The inlet and outlet are on the top side of the device and these holes are connected to the slit on the bottom surface. Slits on the bottom of the device generate one-directional shear stress to cells growing on the well of a culture plate. This device will be incorporated in the Flexcell® FX-5000™ Tension System. Based on the flow compartment prototype, we will fabricate a device composed of plastic material such as polycarbonate or Teflon in the future. By connecting the Flexcell® Tension System, a peristaltic pump, flow compartment, and a heating water bath as illustrated in Figure

7-5, a multi-stress bioreactor will be constructed. The heated media will be circulated through this tubing from a reservoir via a peristaltic pump to a Flexcell® Tension culture plate. Cell-based analysis will be performed following multi-stress preconditioning, according to identical methods to the current study.

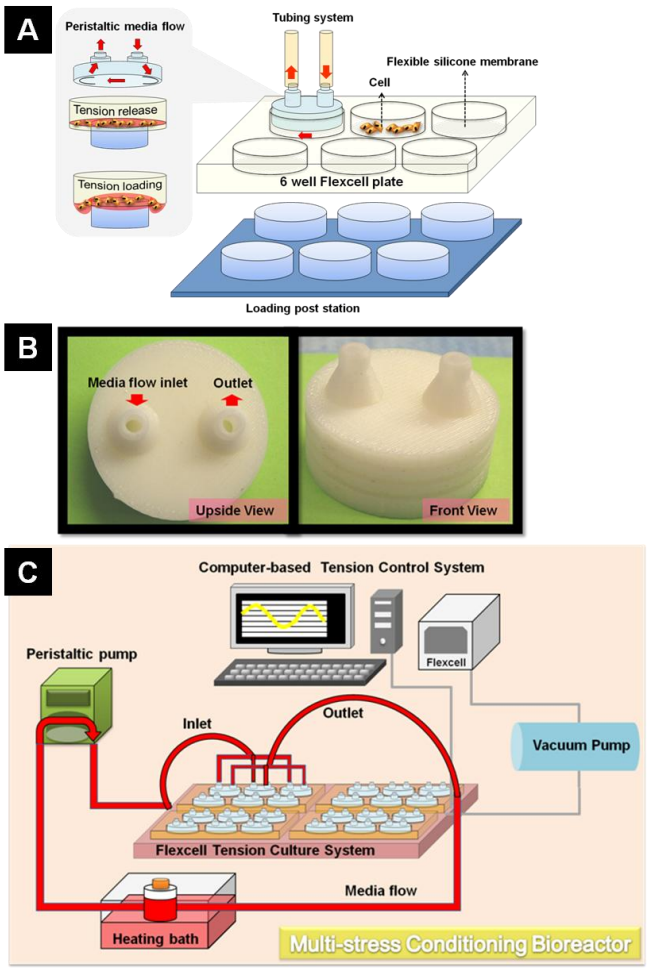

Figure 7-5. A novel multi-stress bioreactor which incorporates a Flexcell® Tension bioreactor system with a novel shear stress device (A). Image of a tubing lid (prototype) for shear stress treatment (B). Inner diameter of inlet and outlet is 3 mm and the diameter of the bottom surface is 3.5 mm. The surface area of the BioFlex® silicone membrane=9.62 cm^2. The gap between fluid-tubing device and BioFlex® membrane is 1 mm. Designed and fabricated by Jonathan Hopkins. Schematic illustration of a novel multi-stress bioreactor (C).

7.2.4. Gene silencing of other HSPs (HSP27 and HSP47)

HSP27 and HSP47 gene silencing will be completed with identical method to HSP70 siRNA transfection described in Chapter 6. Silencer® Select Pre-Designed & Validated siRNA (S2486 for HSP27 and S87618 for HSP47, Applied Biosystems) will be used to silence HSP27 and HSP47 gene expression specifically. Cellular responses of HSP silencing in the osteogenic media will be measured in both sham-treated (normal) and stress-treated cells. Stress conditioning using thermal and tensile stress conditioning in combination with gene silencing.

7.2.5 Confirmation of angiogenic effects of combined stress conditioning using mouse brain microvascular endothelial cell (mBMEC)

Given that our stress conditioning induced VEGF expression, the effects of elevated VEGF expression on endothelial cells should be demonstrated further according to two following methods: ***Method 1. Co-culture of endothelial cells and stress-preconditioned preosteoblasts:*** An insert membrane will be used to culture mBMECs (dEnd.3 cell line, ATCC, catalog no. CRL-2299) and preosteoblasts together in a 6-well plate. After exposure to stress such as heating, or tension or in combination, preosteoblasts will be co-cultured with mBMECs for several days before the measurement of angiogenic responses of HUVES. ***Method 2. Utilization of conditioned medium from stress-preconditioned preosteoblasts:*** Conditioned media from stressed preosteoblasts will be added to the growth medium for mBMEC culture. Angiogenic activity will be characterized by measuring blood vessel formation and endothelial cell activity as described subsequently. This experiment will confirm the results of our previous ELISA data showing conditioned media collected after combined stress induced higher VEGF level compared to unstressed cells.

Analysis 1. ***In vitro*** **blood vessel formation** can be studied by measuring endothelial cell spreading on the specific gel-typed matrix using Geltrex™ Reduced Growth Factor Basement Membrane Matrix (Invitrogen) according to the manufacturer's protocol.

Analysis 2. ***Analysis of Endothelial Cell Activity*** Gene/protein-based assay using ELISA, immunofluorescence (IF) staining, and real-time RT-PCR will be performed to identify endothelial cell activity, according to similar protocols described in prior sections. Endothelial cell-specific marker proteins such as PECAM (CD31), von Willebrand factor, and ve-Cadherin

will be detected by using specific primers for PCR, and antibodies or antibody-included kits for IF stain and ELISA, respectively.

Appendix 1. Strain Energy Function [157]

Geest *et al.* demonstrated strain level depending on the positions of the loading post using strain energy function and finite element analyses.

Assumption about the Flexcell® membrane : 1) nonlinear, 2) hyperelastic, 3) isotropic, and 4) incompressible

$$W = C_1(I_1 - 3) \text{ ----- (1)}$$

$$\boldsymbol{T} = -p\boldsymbol{I} + 2\frac{dW}{dI_1}\boldsymbol{B} + 2\frac{dW}{dI_2}\boldsymbol{B}^{-1} \text{----- (2)}$$

$$\boldsymbol{T_1} = 2C_1(\lambda_1^2 - \lambda_1^{-1}) \text{----- (3)}$$

, where

W: non-Hookean strain energy function

I_1: the first strain invariant of the left Cauchy-Green tensor B

I_2: the second strain invariant of the left Cauchy-Green tensor B

C_1: material parameter

T: Cauchy stress tensor

λ: stretch ratio

Appendix 2. 3D scaffold/carrier fabrication

Fabrication of a microsphere-hydrogel scaffold Poly(lactide-co-glycolide) (PLGA) microspheres were fabricated by oil-water emulsion method. DiI fluorescence dye (Molecular Probes, Carlsbad, CA, catalog no. D282) was combined with PLGA for fluorescence tagging. On the other hand, the gelatin hydrogel was prepared by cross-linking method. Gelatin isolated from porcine skin (Type A, Sigma, St. Louis, MO G1890) was dissolved in DI water with a hot plate heated at 40°C and cooled down until room temperature. 200 mg of PLGA microspheres were added to 2 ml gelatin solution. For cross-linking, 3% gelatin solution in DI water was mixed with glutaraldehyde (0.16%) followed by incubation at 4°C for 12 hours in a 6 well plate. After casting, the hydrogel was incubated further in 100 mM glycine (Fisher Scientific, Pittsburg, PA, catalog no. BP381-1) solution at 37°C for 1 hour for the inactivation of residual aldehyde functional groups of glutaraldehyde. These **PLGA microspheres** will be incorporated into the gelatin hydrogel to allow delivery of growth factors and HSPs for continuous release over 2-4 week duration. We will test a protein release assay using BSA-coated PLGA microspheres embedded in gelatin hydrogel, by surface–coating different concentrations of protein solution. We will also fabricate **PLGA nanospheres** by double emulsion method using a homogenizer.

On other hand, successful fabrication of a nanofibrous electrospun scaffold will enable elongated cultivation with MC3T3-E1 cells under thermal and mechanical stress conditioning similar to the methodology described in Chapter 3 and 4. Cells cultured in a scaffold will be harvested at several time points until 1 month for morphological and molecular biological analysis. For fabrication of a **nanofibrous** sheet scaffold, poly ε-caprolactone (PCL) (LACTEL Absorbable Polymers, Pelham, AL, B-6003-23P) was mixed with Type I collagen (Elastin Products Co., INC, Owensville, MO, C857). SEM image in Figure 7-4 showed a random nanofibrous 3D sheet made of co-polymers: PCL and collagen. To investigate the effect of long-term stress treatment on preosteoblast differentiation and mineralization, ALP activity will be quantified using SensoLyte® pNPP Alkaline Phosphatase Assay kit (AnaSpec, San Jose, CA) following the vendor's protocol. Mineralization staining will be performed using Osteogenesis Assay Kit (Millipore, Billerica, MA) based on Alizarin Red staining following the manufacturer's protocol.

Appendix 3. Specification of assay materials for evaluation of bone-related proteins: primers, siRNAs, and antibodies, ELISA kits (Applied Biosystems)

- Primer for PCR analysis

Target protein	Gene symbol	Gene name	Assay ID	Sequence	Size
OPN	Spp1	secreted phosphoprotein 1	Mm01611440_mH	GAACAGTATCCTGATGCCACAGATG	102
ON	Spock2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	Mm01168758_m1	TGTTTCTGGAGGGAGAAGCCCCCCT	81
OCN	Bglap-rs1	bone gamma-carboxyglutamate protein, related sequence 1	Mm00649782_gH	CCTTGGAGCTTCAGTCCCCAGCCCA	89
OPG	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Mm01205928_m1	AGTGTGGAATAGATGTCACCCTGTG	75
Bone morphogenic protein receptor, type II	Bmpr2	bone morphogenic protein receptor, type II (serine/threonine kinase)	Mm03023976_m1	TGGAGTATTATCCCAATGGATCTCT	94
MMP13	Mmp13	matrix metallopeptidase 13	Mm01168713_m1	CTTTAGAGGGAGAAAATTCTGGGCT	124
HSP27	Hspb2	heat shock protein 2	Mm00517908_m1	TCGGAGAAGGCCTCCTGCCAGAAGA	115
HSP47	Serpinh1	serine (or cysteine) peptidase inhibitor, clade H, member 1	Mm00438056_m1	TGGTAAACCCTCACAGGTCCTCTGT	76
HSP70	Hspa1b	heat shock protein 1B	Mm03038954_s1	GTTAAGGTTTTGTGGTATAACCAGT	141
MMP9	Mmp9	matrix metallopeptidase 9	Mm00600164_g1	TCTTCAAGGACGGTTGGTACTGGAA	72
Cox-2	Ptgs2	prostaglandin-endoperoxide synthase 2	Mm01307330_g1	TGTACTACACCTGAATTTCTGACAA	73
ALP	Alpl	alkaline phosphatase,	Mm01187113_g1	TCCTGGGAGATGGTATGGGCGTCTC	71

		liver/bone/kidney			
Col1(Col1a1)	Col1a1	collagen, type I, alpha 1	Mm00801666_g1	CGATGGATTCCCGTTCGAGTACGGA	89
GAPDH	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1	GTGAACGGATTTGGCCGTATTGGGC	107
Runx2	Runx2	runt related transcription factor 2	Mm00501580_m1	GACGAGGCAAGAGTTTCACCTTGAC	129
VEGF	Vegfa	vascular endothelial growth factor A	Mm00437308_m1	CAAAGCCAGAAAATCACTGTGAGCC	66
TGFb1	Tgfb1	transforming growth factor, beta 1	Mm00441724_m1	TGGTGGACCGCAACAACGCCATCTA	99
BSP	Ibsp	integrin binding sialoprotein	Mm00492555_m1	GGTTTCCAGTCCAGGGAGGCAGTGA	98
Prostaglandin E synthase 2	Ptges2	prostaglandin E synthase 2	Mm00460181_m1	CAGGAAGGAGACAGCTTGCAACAGC	73
Ang-2	Angpt2	angiopoietin 2	Mm00545822_m1	TCACCCAACTCCAAGAGCTCGGTTG	71
Ang-1	Angpt1	angiopoietin 1	Mm00456498_m1	AAAAAACAGTTTACTAGAGCACAAA	118

- ELISA kits for protein secretion analysis

Protein	ELISA kit	Catalog #
OPN	R&D Quantikine	MOST00
OCN	Biomedical T	BT-470
OPG	R&D Quantikine	MOP00
MMP9	R&D Quantikine	MMPT90
VEGF	R&D Quantikine	MMV00
PGE_2	Assay Designs	900-001
TGF-b1	R&D Quantikine	MB100B

- SiRNA

Product name	Silencing target	Catalog no.	Vendor
Silencer® Negative Control #1 siRNA (50 µM)		AM4611	Applied Biosystems
Silencer® Select Pre-Designed & Validated siRNA	heat shock protein 1B (HSPa1b)	S201486	Applied Biosystems

- Antibodies for western blot analysis

1' antibody									2' antibody		
Protein	Genetic locus	Size	Catalog no.	Dilution	Host	m/p	Isotype	Vendor	Catalog no.	Vendor	Dilution
HSP27	Hspb1	27	sc-51956	200	mouse	mono	IgG2a	santa-cruz	sc-2005	santa-cruz	2000
HSP47	Serpinh1	47	sc-13150	500	mouse	mono	IgG1	santa-cruz	sc-2005	santa-cruz	2000
HSP70	Amino acid residues 436-503 of human Hsp70. No reactivity with the constitutive Hsc70 (Hsp73)	70	spa-810	1000	mouse	mono	IgG1	stressgen	sc-2969	santa-cruz	2000
Actin-I		43	sc-1616	1000	goat/rabbit	poly	IgG	santa-cruz	sc-2020	santa-cruz	2000

Appendix 4. BioFlex 25 mm loading station (post) conversion chart

Press (-kPa)	% Elongation	Strain
6.13	1	0.010
16.74	3	0.030
25.52	5	0.050
42.50	10	0.100

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