Graduate School ETD Form 9
(Revised 12/07)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Umut Atakan Gurkan

Entitled Engineering of Bone Marrow In Vitro for Investigating the Role of Growth Factors and Their Mechanoresponsiveness in Osteogenesis

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

Ozan Akkus	Eric A. Nauman
Chair	
Gert J. Breur	Gudrun Schmidt

To the best of my knowledge and as understood by the student in the *Research Integrity and Copyright Disclaimer (Graduate School Form 20)*, this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Approved by Major Professor(s): Ozan Akkus

Approved by: George Wodicka 21 April 2010
Head of the Graduate Program Date

Graduate School Form 20
(Revised 1/10)

PURDUE UNIVERSITY
GRADUATE SCHOOL

Research Integrity and Copyright Disclaimer

Title of Thesis/Dissertation:

Engineering of Bone Marrow In Vitro for Investigating the Role of Growth Factors and Their Mechanoresponsiveness in Osteogenesis

For the degree of Doctor of Philosophy

I certify that in the preparation of this thesis, I have observed the provisions of *Purdue University Teaching, Research, and Outreach Policy on Research Misconduct (VIII.3.1)*, October 1, 2008.*

Further, I certify that this work is free of plagiarism and all materials appearing in this thesis/dissertation have been properly quoted and attributed.

I certify that all copyrighted material incorporated into this thesis/dissertation is in compliance with the United States' copyright law and that I have received written permission from the copyright owners for my use of their work, which is beyond the scope of the law. I agree to indemnify and save harmless Purdue University from any and all claims that may be asserted or that may arise from any copyright violation.

Umut Atakan Gurkan

Printed Name and Signature of Candidate

20 April 2010

Date (month/day/year)

*Located at http://www.purdue.edu/policies/pages/teach_res_outreach/viii_3_1.html

ENGINEERING OF BONE MARROW *IN VITRO* FOR INVESTIGATING THE ROLE OF GROWTH FACTORS AND THEIR MECHANORESPONSIVENESS IN OSTEOGENESIS

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Umut Atakan Gurkan

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

May 2010

Purdue University

West Lafayette, Indiana

UMI Number: 3413893

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI 3413893
Copyright 2010 by ProQuest LLC.
All rights reserved. This edition of the work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I dedicate this dissertation to my wife, who has always been with me in this journey with her encouragement, support and love.

ACKNOWLEDGEMENTS

First and the foremost I would like to acknowledge my PhD advisor Dr. Ozan Akkus for being an inspiring mentor who helped me develop all the skills I will need as an independent researcher and scholar. Specifically, the following notable characteristics of Dr. Akkus have made my graduate school experience a very fruitful one: 1) his contagious affection for research which has been extremely stimulating for me and for everyone else, who are lucky enough to work with him, 2) his brilliant insight, unique perspective and multidisciplinary approach, which has always given me new ideas in seemingly hopeless situations, 3) his ability to see even the smallest details with a meticulous attention, 4) his memory and his amazing knowledge of the literature, 5) his amazing speed in reviewing/editing the full manuscripts (as short as 2 hours of response time), and 6) his willingness and efforts to be available for his students anytime, anywhere. I have learned a lot from him and I believe I will continue to do so.

I would like to acknowledge Dr. David Van Sickle for sharing his half-century long research experience through inspiring discussions and (life-)lessons not only about research but also about being a fine, responsible, focused scholar and individual.

I would like to acknowledge my PhD committee members: Dr. Eric A. Nauman, Dr. Gert J. Breur and Dr. Gudrun Schmidt for all the feedback that they have provided at every stage of my doctoral training.

I would like to specially thank Dr. Vipuil Kishore for all his help with the characterization studies and being available whenever I needed help with anything. It has always been comforting for me to know that there is someone like Vipuil around me, who I can always depend on.

I would like to thank Adam Krueger who has been a loyal undergraduate researcher in our lab and helped me with everything related to design and manufacturing of devices and prototypes. I also would like to acknowledge Josh Gargac and Ryan Golden for their all their help in various stages of this work.

I would like to acknowledge the help and colleagueship of all the previous and current lab members: Dr. Xingguo Cheng, Vikas Kaul, Jared Diegmueller, Nick Wasserman, Dr. Jihoon Jeon, Audrey Djibo, Serife Agcaoglu, Scott VanDyke, Jorge Alfredo Uquillas, Jason Toler and Xuanhao Sun.

I would like to thank Purdue University Histopathology service laboratory (David VanSickle Ph.D. DVM, Paul Snyder Ph.D. DVM, Carol Bain) for their support with histology and immunohistochemistry. I would like to acknowledge the supports of Pam Lachick with the μCT system and David Meyer with the microindenter. I also would like to thank Purdue Life Science Microscopy Facility director Debby Sherman for her support with EDS and BSEM systems.

Last but not the least; I would like to acknowledge the endless support and love (from all the way across the ocean) of my family. Without their life-long efforts and hard-work, I would not have been here.

Most of this study was funded by a grant from the Musculoskeletal Transplant Foundation.

TABLE OF CONTENTS

Page

Page

Page

Page

Page

Page

LIST OF FIGURES

Figure Page

Figure Page

LIST OF TABLES

ABSTRACT

Gurkan, Umut A. Ph.D., Purdue University, May 2010. Engineering of Bone Marrow *In Vitro* for Investigating the Role of Growth Factors and Their Mechanoresponsiveness in Osteogenesis. Major Professor: Ozan Akkus, Ph.D.

Bone regeneration is a complex process that involves the synergistic contribution of multiple cell types and numerous growth factors (GFs). It is widely accepted that effective and efficient reconstruction of critical size skeletal defects and non-unions can be achieved by tissue engineering approaches employing multi-factor and multi-phase GF delivery strategies. However, the studies investigating the involvement of multiple factors in osteogenesis are limited to simplified 2-dimensional *in vitro* studies with particular cell types or complex *in vivo* studies with associated experimental hurdles. There is a need for an *in vitro* model that embodies the multicellular and 3-dimensional (3D) nature of osteogenesis without the complexities of *in vivo* animal models. Bone marrow tissue consists of multiple cell types, houses the multipotent mesenchymal and hematopoietic stem cells, and plays a major role in bone regeneration. Marrow has a unique microenvironment and inherently ossifies *in vitro* under basal conditions (i.e. without addition of excipient osteoinductive factors). Therefore the main objective of this dissertation was to harness the inherent ossification potential of rat bone marrow tissue

and develop a representative 3D, multicellular, scaffold-free *in vitro* model of osteogenesis as a platform to study the temporal and interconnected involvement of multiple GFs. The specific aims of this work were: 1) optimizing and characterizing the *in vitro* ossification of marrow tissue, 2) tracing the sequential production profiles of key GFs in osteogenesis and their relation to ossified volume in marrow ossification model, and 3) assessing the mechanoresponsiveness of marrow ossification process and the effect of mechanical stimulation on the temporal production levels of GFs. Specifically, the osteogenic involvement of bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and transforming growth factor beta-1 (TGF-beta1) were studied. The key findings of this dissertation are: 1) *in vitro* ossification of bone marrow can be achieved under serum-free conditions resulting in a 3D tissue structure with characteristic morphological, compositional and cellular properties of newly forming bone, 2) BMP-2, VEGF, IGF-1 and TGF-beta1 are sequentially produced and secreted during *in vitro* ossification of marrow, 3) The levels of BMP-2, VEGF, IGF-1 and TGF-beta1 at specific time points correlate with the final ossified volume and they are highly interdependent to each other, 4) in vitro ossification model is mechanoresponsive and responds to mechanical stimulus by increased bone volume with enhanced or sustained release of VEGF, IGF-1 and TGF-beta1, but not BMP-2. These outcomes are essential for delineating the temporal and interconnected involvement of multiple growth factors in osteogenesis and the role of mechanical cues in this process.

INTRODUCTION

Bone marrow tissue is housed in the cavities of the bones housing various cell types including the essential multipotent cells including the mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) which are essential for the living organism [1-3]. It is the major source of HSCs which have the role of renewing the elements in the blood (monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets) [1]. Marrow also contains the mesenchymal stem cells which contribute to the regeneration of tissues such as bone, cartilage, muscle, adipose and tendon [3-9]. Bone marrow tissue has a unique microenvironment [10] and has the potential to undergo inherent ossification *in vivo* and *in vitro* [11-15]. Therefore, the major aim of this doctoral dissertation was to harness marrow's inherent ossification capacity to develop and characterize an *in vitro* 3-dimensional, scaffold-free, multicellular model of osteogenesis, which is conveniently located between the 2-dimensional single cell type culture models and complex animal models. This goal was achieved by 1) investigating the physiological environment of bone marrow tissue within the physiological and physical context (Chapter 1); developing and characterizing an *in vitro* 3-dimensional culture system that can support the marrow tissue and its ossification for at least 28 days (Chapter-2). Development of a 3-dimensional *in vitro* model of osteogenesis would be useful for studying osteogenesis in the following contexts: 1)

morphological, compositional evolution of ossifying tissue and cellular residency (Chapter-2), 2) production of the key osteoinductive factors during osteogenesis and their relation to each other (Chapter-3), and 3) mechanoresponsiveness of the marrow ossification model and the osteoinductive growth factor production (Chapter-4).

References

1. Cabrita, G.J.M., B.S. Ferreira, C.L. da Silva, R. Goncalves, G. Almeida-Porada, and J.M.S. Cabral, *Hematopoietic stem cells: from the bone to the bioreactor.* Trends in Biotechnology, 2003. **21**(5): p. 233-240.
2. Muschler, G.F., C. Boehm, and K. Easley, *Aspiration to obtain osteoblast progenitor cells from human bone marrow: The influence of aspiration volume.* Journal of Bone and Joint Surgery-American Volume, 1997. **79A**(11): p. 1699-1709.
3. Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak, *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-147.
4. Polak, J.M. and A.E. Bishop, *Stem cells and tissue engineering: Past, present, and future.* Ann N Y Acad Sci, 2006. **1068**: p. 352-366.
5. Braccini, A., D. Wendt, C. Jaquiery, M. Jakob, M. Heberer, L. Kenins, A. Wodnar-Filipowicz, R. Quarto, and I. Martin, *Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts.* Stem Cells, 2005. **23**(8): p. 1066-1072.
6. Miyanishi, K., M.C.D. Trindade, D.P. Lindsey, G.S. Beaupre, D.R. Carter, S.B. Goodman, D.J. Schurman, and R.L. Smith, *Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro.* Tissue Engineering, 2006. **12**(6): p. 1419-1428.

7. Schumann, D., R. Kujat, M. Nerlich, and P. Angele, *Mechanobiological conditioning of stem cells for cartilage tissue engineering.* Bio-Medical Materials and Engineering, 2006. **16**(4): p. S37-S52.

8. Dennis, J.E. and A.I. Caplan, *Bone Marrow Mesenchymal Stem Cells*, in *Stem Cells Handbook*, S. Sell, Editor. 2003, Humana Press Inc.: Totowa, NJ. p. 107-118.

9. Priller, J., *From Marrow to Brain*, in *Adult Stem Cells*, K. Turksen, Editor. 2004, Humana Press Inc.: Totowa, NJ. p. 215-233.

10. Gurkan, U.A. and O. Akkus, *The Mechanical Environment of Bone Marrow: A Review.* Annals of Biomedical Engineering, 2008. **36**(12): p. 1978-1991.

11. Friedenstein, A.J., K.V. Petrakov, A.I. Kuroleso, and G.P. Frolova, *Heterotopic Transplants of Bone Marrow - Analysis of Precursor Cells for Osteogenic and Hematopoietic Tissues.* Transplantation, 1968. **6**(2): p. 230-247.

12. Tavassoli, M. and W.H. Crosby, *Transplantation of Marrow to Extramedullary Sites.* Science, 1968. **161**(3836): p. 54-&.

13. Schoeters, G.E.R., L. Desaintgeorges, R. Vandenheuvel, and O. Vanderborght, *Mineralization of Adult-Mouse Bone-Marrow Invitro.* Cell and Tissue Kinetics, 1988. **21**(5): p. 363-374.

14. Luria, E.A., M.E. Owen, A.J. Friedenstein, J.F. Morris, and S.A. Kuznetsow, *Bone-Formation in Organ-Cultures of Bone-Marrow.* Cell and Tissue Research, 1987. **248**(2): p. 449-454.

15. Gurkan, U.A., J. Gargac, and O. Akkus, *The Sequential Production Profiles of Growth Factors and Their Relations to Bone Volume in Ossifying Bone Marrow Explants.* Tissue Eng Part A, 2010. **DOI: 10.1089/ten.TEA.2009.0565**.

1. THE MECHANICAL ENVIRONMENT OF BONE MARROW

(Annals of Biomedical Engineering, 2008, 36(12):1978-91)

1.1. Abstract

Bone marrow is a viscous tissue that resides in the confines of bones and houses the vitally important pluripotent stem cells. Due to its confinement by bones, the marrow has a unique mechanical environment which has been shown to be affected from external factors, such as physiological activity and disuse. The mechanical environment of bone marrow can be defined by determining hydrostatic pressure, fluid flow induced shear stress and viscosity. The hydrostatic pressure values of bone marrow reported in the literature vary in the range of 10.7 – 120 mmHg for mammals, which is generally accepted to be around one fourth of the systemic blood pressure. Viscosity values of bone marrow have been reported to be between 37.5 cP to 400 cP for mammals, which is dependent on the marrow composition and temperature. Marrow's mechanical and compositional properties have been implicated to be changing during common bone diseases, aging or disuse. *In vitro* experiments have demonstrated that the resident mesenchymal stem and progenitor cells in adult marrow are responsive to hydrostatic pressure, fluid shear or to local compositional factors such as medium viscosity.

Therefore, the changes in the mechanical and compositional microenvironment of marrow may affect the fate of resident stem cells *in vivo* as well, which in turn may alter the homeostasis of bone. The aim of this review is to highlight the marrow tissue within the context of its mechanical environment during normal physiology and underline perturbations during disease.

Key Terms: mesenchymal stem cells, marrow progenitor cells, physiological activity, osteoporosis, disuse, aging, pressure, fluid shear, rheology, viscosity

1.2. Introduction

Bone marrow is the soft tissue residing in the cavities of the bones housing the essential pluripotent precursor cells for the living organism. It is the major source of hematopoietic stem cells (HSCs) which have the role of renewing the elements (monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets) in the blood [1]. Adult bone marrow also contains the mesenchymal stem cells which contribute to the regeneration of tissues such as bone, cartilage, muscle, adipose, tendon, ligament and stroma by differentiating into osteoblasts, chondrocytes, myocytes, adipocytes, tenocytes and neuronal cells *in vivo* and *in vitro* [2-8]. Mesenchymal stem cells have been proven to be responsive to mechanical signals such as hydrostatic pressure [6, 9-11], fluid flow induced shear stress [12] and the rheological properties (viscosity) of their environment [13-15].

Bones are primarily responsible for countering physiological loads. The close proximity of marrow within medullary cavities of bones subject the marrow to physiological loads as well [16-20]. The key variables of the mechanical environment of marrow due to the external factors can be listed as the intramedullary pressure and the fluid flow generated by pressure gradients. These variables change during regular physical activities [19-22]. Intramedullary pressure elevations are reported in response to drugs and steroids [23-27]. Composition and rheological properties of marrow are also reported to change during aging and drug use [27, 28]. Studies also suggest these properties of marrow to change during aging, osteoporosis or disuse [24, 28, 29]. Since these anomalies have the common hallmark of bone loss, the possibility arises that the

mechanical environment of marrow may be a key player in homeostasis of bone by way of the mechanically responsive resident stem cells [30].

1.2.1. Structure and Function of Bone Marrow

In adults, marrow tissue located in the mid-diaphyseal portions of peripheral bones in the body mostly consists of adipose tissue which imparts a yellowish color (fatty marrow) [31]. In the axial skeleton however, the adipose tissue coexist with the hematopoietic tissue in a variable but roughly equal proportion. Bones provide a confined environment for marrow. Therefore, changes in the volume of active marrow (where hematopoiesis occurs) should be compensated by the expansion of a space-occupying component [32]. This component is commonly accepted to be the adipose tissue. When the marrow hematopoietic activity increases, adipose tissue undergoes resorption to provide more space, or vice versa.

Hematopoietic tissue imparts a reddish color to marrow (red marrow) due to the high content of heme chromogen [31]. Red marrow houses the red blood cells, platelets and white blood cells and resides in the flat bones such as skull, ribs, vertebrae, the proximal halves and the endosteal surfaces of the long bones. Red marrow tissue is rich in a variety of cellular components comprised mostly of hematopoietic cells. The hematopoietic cells give rise to monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets and osteoclasts [1, 8, 33-35]. Marrow's other cellular component is a highly organized stroma that supports the proliferation of the hematopoietic cells [33]. The organized stroma is composed of

reticular cells which form a spongy framework on which hematopoietic cells are arranged. Upon maturation, hematopoietic cells migrate into the blood stream. Therefore, hematopoietic cells are temporary residents of marrow. Conversely, the stroma remains as a scaffold for the differentiation and maturation of the hematopoietic cells [31]. Bone marrow also contains mesenchymal stem cells which have been shown to differentiate into osteoblasts, chondrocytes, myocytes, adipocytes and neuronal cells *in vivo* and *in vitro* [2-8].

The organization of the marrow can be best understood by following its vascular layout. In a tubular bone, the nutrient artery enters the marrow cavity, runs longitudinally in the center, then branches out toward the endosteum of the surrounding bone, leading to specialized vascular structures known as sinuses or sinusoids (Figure 1.1) [35, 36]. Several of these sinuses may then combine to form collecting sinuses which lead to the central sinus or vein. This vein runs longitudinally next to the nutrient artery. Blood in marrow flows from the center toward the bone and then returns back to the center [31, 35]. This structural configuration yields high numbers of vessels and sinuses in the periphery (resulting in a slower flow rate of blood and higher surface area) where most of the exchange occurs. Therefore, hematopoiesis is maximal in the closer proximities to the bone surface leaving the central parts with relatively little hematopoietic activity (Figure 1.1) [37]. Due to this fact, it is possible to observe a transition region between red marrow and fatty marrow radially (red marrow being closer to the endosteal surfaces, Figure 1.1) [31]. Similarly, a longitudinal macroscopic distribution is observed as red marrow in the proximal half and fatty marrow in the distal half of the bones [31]. Bone

and marrow are connected by vasculature. Bone nutrient vessels enter the marrow cavity to make connections with marrow vessels. Small arteries of marrow also enter the bone, make a loop and return back to where they originated from [31, 35]. A more detailed description of the structural organization and function of marrow can be found elsewhere [31, 35].

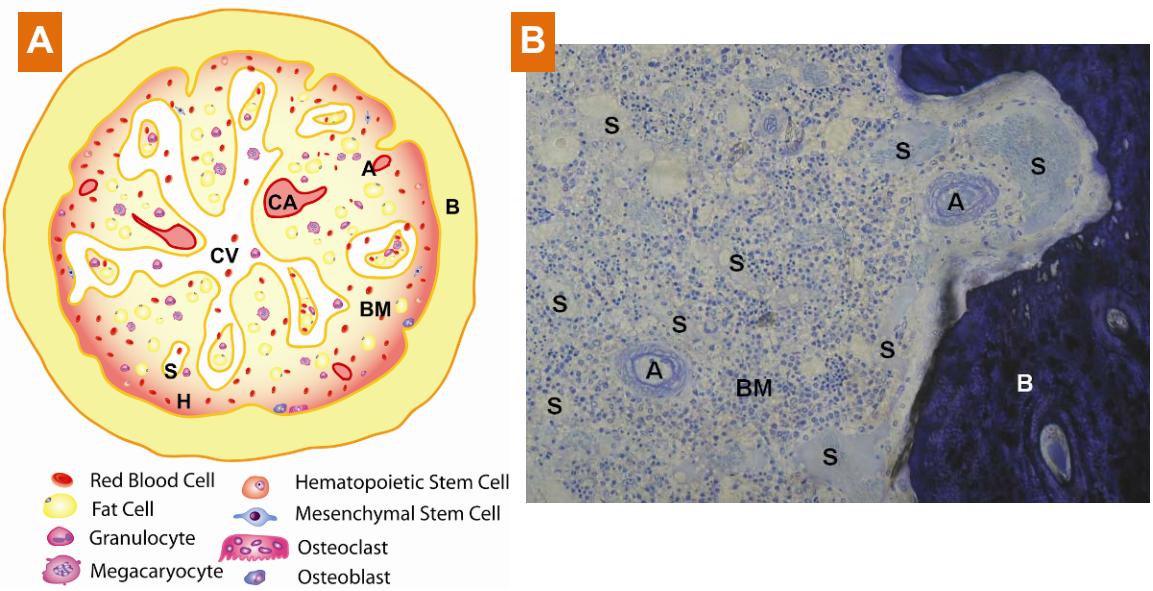


Figure 1.2: Overview of Bone Marrow Tissue. **A)** Layout of bone marrow in a cross-sectional view of a tubular bone. Bone (B) is surrounding the bone marrow (BM). Central artery (CA) and central vein (CV) are running parallel to each other and longitudinally along the long bone (perpendicular to the plane of the page). The central artery and central vein branch toward the periphery to form arterioles (A) and sinusoids (S) which then combine and join with the central vein. Hematopoietic space (H) is interspersed by the sinuses. Developing red blood cells and granulocytic cells appear in the hematopoietic space. Megakaryocytes develop subjacent to the endothelium of marrow sinuses. It is possible to observe the radial distribution of marrow as the yellow marrow in the central regions and the red marrow in the periphery. **B)** A toluidine-blue stained section taken transversely to the longer axis of a tubular bone. The micrograph displays the endosteal junction between bone and marrow (125×). The distribution of abundant number of red blood cells indicates that the bone marrow is hematopoietic (Courtesy of David C. Van Sickle, Purdue University).

1.3. The Mechanical Environment of Bone Marrow

1.3.1. Hydrostatic Pressure

One of the first studies on the bone medullary pressure reported extensive necrosis of the bone after intramedullary infusion of high pressure saline solution [38]. Numerous subsequent studies tried to elucidate the possible relationship between the medullary pressure and the hemodynamics of the bone [19, 23-26, 39-44] (Table 1.1). The systemic blood pressure in animals has been reported to be in the range of 110-140 mmHg, whereas the normal intramedullary pressure (IMP) was generally about 30 mmHg which is approximately one fourth of the systemic blood pressure (Table 1.1). This generalization is known as the one-forth rule [24, 29].

It was observed that intramedullary blood flow is directly related to the IMP and was suggested that the IMP is the resultant of the total blood flow entering the bone and the total blood flow leaving it [24, 45]. In addition, marrow pressure depends on the relative degree of resistance to flow between arteries and marrow blood vessels, and between marrow blood vessels and veins. Moreover, IMP and blood-pressure has variations with the phases of respiration such that IMP increases with inspiration and decreases with respiration [19]. Tondevold et al. investigated the changes in IMP in relation to mean arterial pressure. Interestingly, they observed that the medullary pressure remained essentially constant and independent of the mean arterial pressure as long as the latter remained above 81 mmHg [43]. Therefore, it was suggested that there has to be a regulatory system trying to keep the medullary pressure constant, which may be the

arterioles of the bone marrow that are supplied by sympathetic nerve fibers with a vasoconstrictor function [46].

Shim et al. observed the relationship between IMP and hemodynamics of bone by focusing on the blood supplies of bone [41]. This approach was in contrast to previous studies, which focused on systemic blood circulation. Experiments carried out by Shim et al. indicated that IMP rises if the arterial blood supply to the bone increases or venous congestion occurs in the limb (Table 1.2). It was further demonstrated that increased marrow cavity pressure due to venous congestion decreases the blood supply to the bone [41]. It was also suggested that the elevation of the IMP due to muscle contraction, which mimics physiological activity, may have significance in the maintenance of structural integrity of the bone. Although some researchers claimed to be able to define a relationship between the IMP and the blood circulation in bones, there is neither an agreement nor a consistency in the findings as it can be seen in Table 1.1. Nevertheless, a common finding in these studies is that the IMP has a pulsatile regime synchronous with the arterial blood pressure and respiration.

Table 1.1: Blood pressure and intramedullary pressure (IMP) values reported in the literature.

		Blood pressure		IMP	
	Animal	**Location**	**Value (mmHg)**	**Location**	**Value (mmHg)**
Stein *et al* [23]*	dog	femoral artery	110-140	tibial diaphysis	25-75
Shaw [24]*	cat	contralateral femoral or carotid artery	130	femoral diaphysis	37
Azuma [25]*	rabbit	carotid artery	100-110	femur or tibia	25 (4 - 70)
Michelsen [26]*	rabbit	carotid artery	73-118	tibia	18-36
Harrelson and Hills [40]*	mongrel dogs	femoral artery contralateral	110-140	mid-diaphysis of femur	35
		femoral vein contralateral	2		
Shim *et al* [41]*	rabbit	carotid artery	120	femur	20 - 60
	dog	carotid artery	130	femur	40-120
Wilkes and Visscher [44]†	dog	femoral artery	134.0 (± 13.2)	tibia	23 (± 5.3)
		nutrient vein	19.3 (± 6.3)		
Tondevold *et al* [43]*	mongrel dogs	left brachial artery	112.9 (±0.9)	femoral epiphysis	30.7 (± 2.6)
				femoral metaphysis	20.5 (± 1.5)
				femoral diaphysis	25.7 (± 1.7)
Thomas *et al* [42]*	rabbit	Not measured	-	lower femoral diaphysis	33 (7 - 81)
Bauer and Walker [39]*	dog	Not measured	-	femoral diaphysis	27.6 (± 15.4)
				femoral metaphysis	17.6 (± 10.5)
				tibial diaphysis	26.4 (± 13.0)
				tibial metaphysis	17.9 (± 11.8)
				humeral diaphysis	26.2 (± 15.8)
				humeral metaphysis	13.4 (± 7.7)
				radial diaphysis	15.4 (± 18.9)
Stevens *et al* [48]‡	mouse	Not measured	-	femur	10.7 (± 1.4)

* IMP was measured with a cannula inserted into the bone in anesthetized animals,

† IMP was measured with a tonometric pressure transducer in anesthetized dogs,

‡ IMP was measured by radiotelemetry in unanesthetized ambulatory mouse.

There was a significant variation in the intramedullary pressure measurements reported by various authors in the literature (Table 1.1). This could be due to the differences in the marrow tissue of different species [25]. Another explanation to these variations was that the blood channels in the bone marrow were damaged by the insertion of the cannula and it was quite possible that there was a blood pool forming at the tip of the cannula. Therefore, sizes of the damaged channels, the amount of the damage in blood channels and in the extravascular tissue could be causing the variation [25]. Similar concerns related to variations in pressure measurements due to lesions formed at the tip of the cannula were raised by various researchers [41, 47]. In order to eliminate the negative effects of hemorrhage or lesions forming, a tonometric pressure transducer was employed which was positioned on the intact endosteal membrane through a cortical hole of about 7.3 mm in diameter [44]. Although the enhancing characteristics of the measurement technique were not discussed in detail, less variation was observed in the IMP measurements (Table 1.1). However, the difficulties in the surgical techniques used in this study caused puncture of the endosteal membrane and gross trauma in half of the animals. Due to the advances in sensing electronics and telemetry, it has been recently possible to conduct *in vivo* IMP pressure measurements on unanesthetized ambulatory mice by implantation of a radiotelemetry pressure transducer [19, 48]. The transducer was composed of a 0.4 mm diameter catheter, which was inserted through a 0.5 mm hole in the femoral cortex and sealed with tissue cement. The catheter was connected to a transmitter, which was secured in the peritoneal cavity, onto the abdominal wall. The new technique was successfully employed for long term, *in vivo* measurements of IMP in venous ligation and hindlimb suspension mice models (Tables 1.1 and 1.2). This new

technique has proved to be more efficient in yielding *in vivo* pressure measurements with more accurate readings.

The response of the mechanical environment of the bone medullary cavity to various factors has revealed that the marrow environment is quite susceptible to external factors. In addition, this response has been shown to be quite robust in its recovery towards its normal state after its dynamics were disturbed. The studies in this regard aimed to elucidate the hemodynamic changes in bone marrow due to external effects such as occlusion of regional vessels, injection of epinephrine, norepinephrine, acetylcholine, pressor and depressor drugs, and skeletal muscle contraction (Table 1.2).

It is commonly anticipated that physiological loading (i.e. walking or running) perturbs the IMP[16-20]. This supposition was tested by externally stimulating skeletal muscles of anesthetized animals to mimic the physiological loading conditions. The results (Table 1.2) showed that IMP rises due to muscle contraction. In a more recent *in vivo* study, the effect of hindlimb suspension and venous ligation on the medullary pressure was successfully measured by telemetry in ambulatory mice[19]. In this study, surgical venous ligation increased the IMP by 25%. In addition, IMP decreased by 23% in normal mice and decreased by 33% in ligated mice upon hindlimb suspension (Table 1.2). In general, arterial occlusion yielded a decrease while venous occlusion yielded a rise in the medullary pressure, both of which verify the direct relationship between the blood flow into the bone and the IMP (Table 1.2). The effects of vasodilator, vasoconstrictor drugs and steroids on marrow pressure were investigated on anesthetized animals[23-27]. Vasodilator drugs tend to lower both the marrow and the systemic blood

pressure while vasoconstrictor drugs tend to increase both of them (Table 1.2). Miyanishi et al. attempted to relate steroid-induced osteonecrosis with intraosseous pressure rise in rabbits[27]. They observed that steroid treatment significantly increased marrow pressure, decreased bone blood flow and also caused fat cell enlargement.

1.3.2. Rheology

It has recently been indicated that marrow viscosity is a critical parameter modulating the shear stresses experienced by the trabecular surfaces in the vertebral bodies due to vibratory loads [49]. Since the potential rheological changes in the marrow due to bone diseases and aging is likely to affect the shear stresses experienced by the progenitor cells in marrow, it is essential to quantify and characterize these parameters. Bone marrow (bovine) has been shown to present Newtonian fluid characteristics (i.e. constant viscosity which is independent of shear rate) at near body temperatures [50]. It should be noted that marrow has a slightly lower temperature (1.6 – 4.8°C below) than normal body temperature in humans [51].

Table 1.2: The effects of external factors on intramedullary pressure (IMP) and systemic blood pressure.

	Occlusion				Drugs		Skeletal Muscle Contraction	
	Arterial	Venous	Epinephrine	Norepinephrine	Vasodilator	Vasoconstrictor	Abdominal	Lower limb
Stein *et al* [23]*			⇑⇓ ↑	⇑⇓ ↑	⇓ ↓	⇑ ↑		
Shaw [24]*	⇓ ↓	⇑ →	⇓ ↑	⇓ ↑	⇓ ↓	⇑ ↑		
Azuma [25]*	⇓ →	⇑ →	⇑⇓ ↑↓		⇓ ↓↑→		⇑⇓⇒	→
Michelsen [26]†	⇓ ↓	⇑ ↑		⇓ ↑	⇑⇓⇒ ↓↑→			
Shim *et al* [41]†	⇓ →	⇑ →	⇓ ↓	⇓ ↑			⇓ →	⇑ →
Stevens *et al* [48]‡		⇑						

⇑,⇓,⇒ : Increase, decrease or no change in IMP,
↑,↓,→: Increase, decrease or no change in systemic blood pressure,
⇑⇓ or ↑↓: First increase, then decrease or vice versa,
vasodilator: acetylcholine, benzyl-imidazoline,
vasoconstrictor: amphetamine, histamine,
***** IMP was measured with a cannula inserted into the bone in anesthetized animals,
† Blood pressure was measured at the femoral artery; vasodilator (acetylcholine) was injected into femoral artery,
‡ IMP was measured by radiotelemetry in unanesthetized ambulatory mouse.
All injections were made intravenously; systemic blood pressures were measured at the carotid artery.

Bryant et al. observed the dependence of bovine bone marrow's rheological properties on temperature and anatomical location [50]. The measured viscosity of the proximal marrow at 35 °C (≈400 cP, viscosity of water is 1 cP) was found to be about ten times that of the distal samples (≈40 cP) which can be associated with compositional variations of marrow along the bones (Table 1.3) [52]. Noting that the proximal ends of bones contain red marrow, whereas the distal ends contain yellow (fatty) marrow [31]; it is reasonable to infer that the increased fat content in marrow may reduce its viscosity. Interestingly, removal of cell debris, blood cells and other granular matter from the marrow by centrifugation decreased the dependence of its viscosity on temperature [50]. Considering the durations of viscosity measurement intervals of as long as 6 hours; the potential effect of coagulation of marrow samples was not discussed in this study [50]. In a more recent study on the measurement of femoral bovine marrow viscosity with an implantable wireless method, a viscosity value of 123 cP was reported [53]. The only study on the viscosity of human bone marrow (calcaneal marrow which was reported to be mainly yellow or fatty) reports values in the range of 37.5 cP (at 36°C) and Newtonian fluid characteristics [54]. However the authors report non-Newtonian behavior for the bone marrow specimens with red components (i.e. higher blood cell concentration) [54]. This finding is not surprising as it is known that human blood displays non-Newtonian characteristics [55]. In addition, the density of red bone marrow (1.06 g/cm^3) [56] has been reported to be comparable to that of blood (1.05 g/cm^3) [55]. It should be noted that there is limited information available in the literature on the density of fatty marrow (0.89 g/cm^3) [53] which is comparable to that of fat tissue (0.92 g/cm^3) [56]. The viscosity values reported in the literature for bone marrow and blood are summarized in Table 1.3.

Table 1.3: Reported viscosity values for bone marrow and blood

Bone Marrow	Subject	Location	Temp. (°C)	Value (cP)
Bryant *et al* [50]	bovine	proximal radius	35	400*
	bovine	distal radius	35	44*
Gurkan and Akkus [53]	bovine	femur	37	123†
Davis and Praveen [54]	human	calcaneus	36	37.5‡
Blood				
Eguchi and Karino [55]	human	-	37	66**

* Measurement was obtained with cone and plate viscometer.
† Measurement was obtained with parallel plate rheometer.
‡ Measurement was obtained with controlled oscillatory flow within a straight, cylindrical tube with circular cross section.
** Measurement was obtained with falling ball viscometer at a shear rate of $0.189 s^{-1}$ on a blood sample with a hemacrit value of 41%, and a density of 1.05 g/cm^3.

The variation of marrow composition along the bones and in the different parts of the body is still a question to be answered. This variation can be explained by the temperature dependence of marrow. Marrow composition is highly affected from variations in temperature. Huggins et al. evidenced that the fat content of bone marrow in the limb bones (femur, radius) are higher than the bones in the central parts of the body (ribs, vertebra) [57-59]. This was suggested to be associated with greater body temperatures in central bones. Similarly, Weiss et al. demonstrated that in summer times, the bony exoskeleton of the nine-banded armadillo (*Dasypus novemcinctus*) displayed a red or erythropoietic marrow; whereas in winter times, when the ambient temperature is low, the marrow was yellow or fatty [60].

The rheological properties of bone marrow in different parts of the body and in osteoporotic, disused or aged bones are not known. *In vivo* monitoring of marrow in bone disease and disuse models can provide more realistic values due to the elimination of

effects of extraction. The outcomes of future investigations in this area may prove to be valuable for researchers trying to simulate the natural environment of stem and progenitor cells in mechanically stimulated cell cultures in an effort to better understand bone loss.

While the intramedullary pressure and the rheology of marrow are relatively well investigated, the shear stress in the marrow during ambulation is largely unknown. Shear stress within the marrow as well as between the marrow and the endosteal bone may play a role in terms of modulating the biological response of marrow resident cells and the endosteal lining. The deformation and flow of marrow due to mechanical loading and associated effects on the stromal cells is an area open to investigation and it needs to be studied further [12, 61].

1.4. External Influences to the Mechanical Environment of Bone Marrow

1.4.1. Effects of Physiological Activity and Loading

When the mechanical loading on bones is removed due to extended periods of inactivity, such as bed rest or during space-flights, bone mineral density decreases [62-64]. On the other hand, elevated amounts of loading on bones due to exercise causes increased bone mass and bone turnover [65-70]. Even though there is *in vivo* evidence that exercise induced mechanical loading enhances bone mass, the cellular and molecular mechanisms underlying this fact are still being studied. Bone homeostasis is a balanced system of formation by osteoblasts and resorption by osteoclasts. Osteoblasts are the bone forming cells that originate from mesenchymal stem cells residing in marrow. Whereas osteoclasts are the bone resorbing cells that originate from the hematopoietic stem cells in marrow [71]. Bone marrow mechanical environment is known to be affected from loading of bones with variations in intramedullary pressure, intramedullary and interstitial fluid flow [16-19, 72]. Since marrow is housing the precursors of osteoblasts and osteoclasts, the changes in the mechanical environment of marrow due to physiological activity and loading may play a role in bone homeostasis.

The earlier studies on the effects of loading on the mechanical environment of bone marrow tried to elucidate the strengthening characteristics of marrow as a slightly compressed liquid in the trabecular regions. It has been suggested that intertrabecular fluid (marrow) pressurized by compressive deformation of the bone may provide load bearing capabilities [73-75]. The related *ex vivo* and *in vivo* studies proved that marrow

inside the trabecular regions of femoral head provides stiffness to subchondral trabecular bone [76, 77]. It was also suggested that bone marrow acts as an intermediate transferring the external loads by means of pressurization to trabecular bones, which are acting as trusses in subchondral regions. Therefore, the trabeculum bears only the modest pressure difference across it as a tensile load [78].

Researchers have used both *in viv*o and *in vitro* models to study the effects of physiological loading and activity on bone marrow. Kumar et al. loaded the fixed femurs of rats *in vivo* applying loads ranging from 0 to 12.25 kg for 1 minute. They observed pressure values to rise about two-fold (12 -14 mmHg increase) upon loading [79]. They also studied contraction of the quadriceps muscles with electrical stimulation, resulting in a pressure increase of 60 mmHg [79]. Another study loaded the tibiae of the sheep in impact *in vitro* with loads of 2000 N for durations of 0.015 seconds and observed elevations up to 300 mmHg [80]. Downey et al. studied the effects of *in vitro* compressive loading in the human femoral head [22]. The extracted human hips from cadavers were subjected to load by means of a mechanical testing device. They observed the IMP rise by 55 (±66) mmHg per 980 N of load applied over 0.1 seconds. The load values and the application durations were estimated to be comparable to physiologic conditions. They also presented an interesting finding about an osteoporotic bone with a much higher pressure rise of about 220 mmHg as a result of a relatively less load of 590 N. However, due to the lack of blood flow in the bone resting IMP of the bones was zero and the resemblance of these loading conditions and models to natural physiological loading conditions are disputable. These studies, regardless they are *in vivo* or *in vitro*,

apply artificial external loads with assumed similarity to physiological loads. Moreover, *in vitro* studies lack blood circulation in the bone and in the muscle tissue, both of which may have potent effects on the pressure of bone marrow during physiological loading. An ideal study investigating the effects of physiological loading on the mechanical environment of bone marrow should employ slightly invasive or noninvasive instrumentation which at the same time facilitates continuous measurements in unanesthetized ambulatory animal models. Furthermore it should be noted that the magnitudes of the loading induced IMP is dependent on factors such as loading rate, viscosity of marrow and porosity of the surrounding bone [18, 49]. Therefore these factors should be taken into consideration when evaluating the relative magnitudes of loading induced IMP.

An explanation for bone loss due to lack of exercise and physical activity is decreased medullary cavity pressurization [63]. It has been reported that bone loss is not equal throughout the skeleton in long-term bed rest. Bone loss is the greatest in lower extremities, where the medullary pressure is significantly decreased due to lack of activity. On the other hand, bone formation is observed in the head, where the intracranial pressure is increased due to body orientation and shift of body fluids [62]. This suggestion has recently been supported by a mouse hind limb suspension model with *in vivo* femoral IMP measurements for extended durations [19]. The study aimed to infer the interstitial fluid flow changes due to pressure gradients between the endosteum and periosteum. A decrease of 23% in the IMP was observed upon hindlimb suspension. Although the correlation between the IMP and the bone formation or bone loss was not

reported, it was shown that medullary pressure is sensitive to disuse with an *in vivo*, unanesthetized and ambulatory animal model. Correspondingly, a strengthening treatment technique for osteoporotic long bones has recently been put forward, which elicits cortical bone formation in the femoral neck region of mice by means of knee loading *in situ* [81, 82]. It has been verified by a following study that the intramedullary pressure is altered in synchrony with the knee loading [21]. It should be noted that 2/3 of the blood supply of cortical bone is provided by the endosteal surface [18, 83]. It has also been shown that the variations and oscillations in IMP play an important role on the loading induced fluid flow developed in the interstices of the cortical bones as well as of the trabecular bones [19, 81, 84]. Therefore it is likely that the variations in the IMP not only affect the resident pluripotent stem cells but also serve as one of the players which are driving the fluid flow within the intersticies of cortical bone by way of generating pressure gradients between the envelopes of bone. This flow is essential to provide nutrients and to remove the metabolic byproducts of osteocytes resident in mineralized cortex.

1.4.2. Effects of Osteoporosis and Aging

Osteoporosis is a disease of bone in which fracture susceptibility is compromised by decreased bone mineral density. It mostly involves the hip, the wrist and the lumbar vertebrae [85]. It is estimated that over 20% of women and 7% of men over the age of 50 have osteoporosis. Osteoporosis is responsible for over 1.5 million fractures in the United States annually [86]. It has been evidenced that there is a significant change in marrow

composition and mechanics due to osteoporosis and aging [24, 28, 29, 87]. The only study in this regard stated that the IMP in the tibial diaphysis of children with paralytic osteoporosis approximates the arterial blood pressure. Whereas the tibial IMP of the healthy children with the same age was only about one quarter of the arterial blood pressure [24, 29]. In addition, the adipose tissue fraction in marrow increases significantly with osteoporosis [28, 87], which may be yielding to obstruction of blood circulation [27]. Increased adipose fraction, thus decreased viscosity may be causing the overall shear stress in the medullary cavity to decrease in osteoporosis. Potential elevations in pressure or decrease in shear stress in osteoporotic bones may contribute to osteoporosis by way of altering the milieu of the bone marrow progenitor, precursor and stem cells.

Osteoblasts and adipocyte cells both originate from the mesenchymal stem cells [2]. It has been suggested that the commitment of stem cell fate is integral with mechanical cues experienced in developmental and adult contexts, embodied in cell shape, cytoskeletal tension and RhoA signaling [88]. RhoA is a small GTPase protein known to regulate the actin cytoskeleton in the formation of stress fibers. It has been shown that human mesenchymal stem cells subjected to different mechanical environments differentiate into either osteoblasts or adipocytes depending on the RhoA activity (RhoA active: osteogenesis, RhoA negative: adipogenesis) [88]. Likewise, adipocyte tissue volume in bone marrow has been shown to increase with aging and in patients with osteoporosis [28, 87]. Therefore, it can be hypothesized that osteoporosis may be due to a greater portion of mesenchymal stem cells differentiating into adipocytes

than osteoblasts due to the changes in the cell properties and/or the mechanical environment of the marrow.

1.5. Effects of the Mechanical Environment on Stem and Progenitor Cells

It should be noted that the responses of the marrow derived progenitor cells to physical factors such as hydrostatic pressure, fluid shear and the rheology of the environment have been extensively studied *in vitro*. The question about the natural physiological and altered mechanical conditions of these cells in the bone marrow remains to be answered. Therefore, the review focuses on the effects of mechanical loading to stem and progenitor cells *in vitro* to illustrate that stem cells are responsive to mechanical cues. However, it remains to be determined as to whether this mechanical responsiveness exists *in vivo*.

It is commonly accepted that stem cells' microenvironment, which includes biochemical and biomechanical factors, has an important role on their differentiation and phenotypic expression. Even though most studies in the literature place an emphasis on growth factors and cytokines, it has been evidenced that the differentiation of precursor, progenitor and stem cells are also significantly influenced by mechanical factors [9]. The main mechanical signals that are accepted to be effective on marrow precursor cell proliferation and function are: hydrostatic pressure and fluid-flow induced shear [9, 15]. The viscosity of marrow is also important since it directly relates to the fluid shear stress magnitudes experienced by the cells. The influences of these factors are considered to be significant in regulating the stem cell phenotype and may have synergistic and/or

supplemental effects in combination with the biochemical factors. The employment of such mechanical effects in specially designed "bioreactors" may enhance current standard biochemical signaling pathways for promoting engineered tissue growth [9, 89, 90].

Marrow-derived mesenchymal stem and/or progenitor cells offer novel treatment techniques in tissue engineering research. They are already cultured *ex vivo* in mechanically active environments for various purposes, such as tissue engineering of bone and cartilage [3, 4, 15, 34, 91-95]. The main goal of moving from conventional 2-D methods to 3-D mechanically active systems is to attain more physiological (or natural) culture environments for the cells. Although these studies prove improvements over conventional culturing techniques, they are far from *in vivo* conditions in efficiency.

1.5.1. Hydrostatic Pressure

There are two main reasons why hydrostatic pressure is considered as an important stimulating factor for bone marrow progenitor and stem cells. First of all, marrow derived mesenchymal progenitor cells can express chondrogenic phenotypes under appropriate hydrostatic pressure conditions. There are numerous *in vitro* studies subjecting marrow-derived mesenchymal stem cells to high hydrostatic pressures (750-75,000 mmHg) in an effort to induce chondrogenic phenotype [5, 6, 10, 96-98]. Even though the chondrogenic differentiation of the progenitor cells does not take place in marrow cavity, those studies are related in indicating the sensitivity of the progenitors to the magnitude and the mode (cyclic, intermittent or static) of loading. In general, higher pressure (~75,000 mmHg) over lower (~750 mmHg) and intermittent loading over static

have proved to be more effective in chondrogenesis [5, 96]. Second, hydrostatic pressures in the medullary cavities of long bones due to physiological loading (50-200 mmHg rise) [22, 30, 63, 79, 80] have osteogenic effects [11]. It has been shown that constant hydrostatic pressure applied *in-vitro* at physiologic levels (30-60 mmHg) decreases osteoclast formation [99]. Similarly, cyclic pressures of 75-300 mmHg in magnitude decreases formation of osteoclasts from their progenitors and decreases bone resorptive activity by osteoclasts *in vitro* [100]. Furthermore, this loading scheme enhances osteoblast functions pertinent to new bone formation by stimulating both synthesis and deposition of collagen accompanied by increased accumulation of calcium-containing crystals [100, 101]. A general conclusion that can be drawn from those *in vitro* studies is that the osteogenic response of the cells are not always linearly proportional to the value of the mechanical signals, rather there is an optimal amplitude interval for each loading pattern and frequency. Limited number of *in-vivo* studies in this regard report similar results. Oscillatory hydrostatic pressure (60 mmHg, 20 Hz) applied *in-vivo* for 10 minutes per day for four weeks to avian ulnae elicited new bone formation on the periosteal surface [20] possibly due to increased intracortical fluid flow as suggested by the authors. It was also suggested that since the applied loading regime has bone forming effects; it should be similar to the physiological loading conditions. Similarly, intramedullary hypertension due to venous occlusion (pressure increased to about 28.7mmHg from 15.5 mmHg) has periosteal (138%), endocortical (369%) and cancellous (889%) bone forming effects at the caprine tibial metaphysic [102]. One of the suggestions on how the marrow pressure affects bone formation is that the progenitors in the marrow may be directly reacting to marrow pressure or extracellular fluid flow

(developed by pressure gradients) by differentiating into osteoblasts and forming new bone [12, 30]. On the other hand, it is suggested that nitric oxide is imperative in signaling of mechanically induced bone formation [103-105]. Nitric oxide is a signaling molecule and it is synthesized by nitric oxide synthase which is produced by osteoprogenitor cells in bone marrow, by osteocytes in bone and bone lining cells [106]. Therefore, it is probable that the production of this signaling molecule by the osteoprogenitor cells in the marrow is mediated by bone marrow pressure or pressure gradients [30].

1.5.2. Fluid Shear

Mechanical loading and bending of bones cause strain gradients as well as local pressure gradients in the bone and in the medullary cavity that can drive the interstitial fluid flow [16-18, 72] and can result in shear stresses on the endosteal surface. Fluid flow induced fluid shear on bone marrow derived cells has been investigated extensively to assess its effects on cell differentiation, proliferation and function [4, 12, 34, 94, 100]. Besides, it has been evidenced by a number of researchers that interstitial fluid flow may play an important role in bone remodeling, formation and adaptation [16, 20, 107-110]. The mechanisms of flow-induced remodeling have been studied *in vitro* by subjecting osteoblasts [110-113], osteocytes [110, 114] and osteoclasts [16, 99] to fluid shear. The studies on osteoblasts and osteocytes showed that several osseoactive agents are stimulated by fluid flow such as nitric oxide [104, 115], prostaglandins E2 and I2 [109, 113, 116-118], cyclic adenosine monophosphate [117], intracellular free calcium [11,

101, 110, 111, 119, 120], inositol triphosphate [117, 119, 121], and transforming growth factor β [5, 9, 96, 113]. On the other hand, osteoblast-mediated mineralization is preceded by osteoclast-mediated resorption with osteoclast resorption rates being about 20 times higher than osteoblast deposition rates [122]. Therefore, osteoclast function may dominate the dynamic osteoclast-osteoblast balance that regulates bone turnover and degradation. Moreover, it is suggested that physiological load induced fluid flow in cortical bone is radially outward from endosteal surface to the periosteal surface [18, 123], which makes it difficult for osteoblast or osteocyte secreted mediators to diffuse against the current and reach the osteoclasts or osteoclast progenitors in the marrow cavity [16]. The osteoprogenitor cells are reported to be residing in bone marrow, one to three cell layers away from the endosteal surface [124]. Accordingly, it has been suggested that osteoclasts and their precursors, which are located in the close vicinity of endosteal surface, may be fluid flow induced shear sensitive and that osteoclast remodeling activities may be under the control of autocrine factors [16]. Bone marrow derived osteoclast-like cells are mechanosensitive to fluid flow induced shear and secrete autocrine factors, such as nitric oxide, prostaglandins E2 and I2, which can regulate local resorptive activity [16]. Therefore, fluid flow induced shear developing on the endosteal surface in the medullary cavity may be the significant stimulant for osteoprogenitors in the marrow that recruits them to bone formation sites. Human marrow stromal cells subjected to oscillatory fluid flow of 1 Hz are shown to have increased proliferation rates [12], which mean more osteoprogenitor cells to participate bone formation process. Similarly, the sensitivity of the cells in fluidic environment to flow frequency has been studied to find the potent optimal frequency values for cell phenotype determination

[125]. However, the flow mode that is naturally experienced by osteoprogenitors in marrow has not been revealed yet.

1.5.3. Rheology

Viscosity of the environment in which cells reside is important since it directly affects the shear stresses experienced by cells if the medium is flowing. The studies in this field investigate the viscosity of the environment in mechanically active cell cultures or perfusion bioreactors [13-15]. In one such study, the effect of medium viscosity on marrow stromal osteoblastic cells seeded on 3D fiber meshes were studied by adding dextran (a complex, branched polysaccharide) to flowing medium in a perfusion bioreactor [15]. Increasing medium viscosity with constant flow rates resulted in 2- to 3-fold increase in the shear stresses experienced by the cultured cells without changing chemotransport characteristics significantly. It was reported that increased medium viscosity not only enhanced mineralized matrix deposition but also provided a better matrix distribution in the porosity of the 3D scaffolds. In a similar study, the effect of fluid flow induced shear and chemotransport on bone cells was studied in oscillatory flow [13]. The medium viscosity was varied to obtain different shear stress values on the cultured cells under constant flow rates. It was shown that fluid flow induced chemotransport and shear stress acting on the cells play a synergistic role to elicit cell response to oscillatory fluid flow induced shear stress. In an effort to control the cell aggregate sizes in neural stem cell cultures, researchers tried to alter the kinematic viscosity of culture medium by adding dextran and carboxymethylcellulose [14]. The

results indicate that viscosity is an important parameter to consider for scale-up of stem cell bioreactors. The viscosity values of the media employed in these studies were in the range of 1 – 4 cP, whereas the reported viscosity values for human fatty marrow is about 37.5 cP [54]. Therefore there is a need to revisit these studies and assess stem cell response at these higher viscosity media.

1.6. Conclusion

Bone marrow mechanical environment is susceptible to external effects such as physiological activity and disuse. Moreover, there is a potential relation between the bone diseases such as osteoporosis, and aging or disuse-related bone loss and the marrow composition and mechanics. The changes in the bone marrow mechanical environment is likely to be effective on the occupant precursor and progenitor cells, which are accepted to be responsive to mechanical factors such as hydrostatic pressure, fluid shear and the viscosity of their environment. Although the effects of these mechanical factors and viscosity on mesenchymal stem and progenitor cells are being widely investigated in vitro, the naturally occurring and altered cues in the cells' natural environment (bone marrow) have not been well characterized yet.

Bone marrow mechanical environment can be completely defined by quantifying and characterizing the hydrostatic pressure, fluid flow induced shear and viscosity in natural and altered conditions. The results of these studies can be compiled to generate physiologically relevant in vitro mechanical environments for cell cultures, stem cell bioreactors and computational models to better understand the effects of mechanical

signals on stem, progenitor and precursor cells; and potentially the nature of associated bone diseases.

1.7. Acknowledgements

We would like to acknowledge Dr. David C. Van Sickle for inspiring discussions and providing the histological figure of bone marrow; Sharon Evander and Carol Bain for their help in preparing the histological figure for publication.

1.8. References

1. Cabrita, G.J.M., B.S. Ferreira, C.L. da Silva, R. Goncalves, G. Almeida-Porada, and J.M.S. Cabral, *Hematopoietic stem cells: from the bone to the bioreactor.* Trends in Biotechnology, 2003. **21**(5): p. 233-240.
2. Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak, *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-147.
3. Polak, J.M. and A.E. Bishop, *Stem cells and tissue engineering: Past, present, and future.* Skeletal Development and Remodeling in Health, Disease, and Aging, 2006. **1068**: p. 352-366.
4. Braccini, A., D. Wendt, C. Jaquiery, M. Jakob, M. Heberer, L. Kenins, A. Wodnar-Filipowicz, R. Quarto, and I. Martin, *Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts.* Stem Cells, 2005. **23**(8): p. 1066-1072.
5. Miyanishi, K., M.C.D. Trindade, D.P. Lindsey, G.S. Beaupre, D.R. Carter, S.B. Goodman, D.J. Schurman, and R.L. Smith, *Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro.* Tissue Engineering, 2006. **12**(6): p. 1419-1428.
6. Schumann, D., R. Kujat, M. Nerlich, and P. Angele, *Mechanobiological conditioning of stem cells for cartilage tissue engineering.* Bio-Medical Materials and Engineering, 2006. **16**(4): p. S37-S52.

7. Dennis, J.E. and A.I. Caplan, *Bone Marrow Mesenchymal Stem Cells*, in *Stem Cells Handbook*, S. Sell, Editor. 2003, Humana Press Inc.: Totowa, NJ. p. 107-118.

8. Priller, J., *From Marrow to Brain*, in *Adult Stem Cells*, K. Turksen, Editor. 2004, Humana Press Inc.: Totowa, NJ. p. 215-233.

9. Estes, B.T., J.M. Gimble, and F. Guilak, *Mechanical signals as regulators of stem cell fate.* Stem Cells in Development and Disease, 2004. **60**: p. 91-126.

10. Angele, P., J.U. Yoo, C. Smith, J. Mansour, K.J. Jepsen, M. Nerlich, and B. Johnstone, *Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro.* Journal of Orthopaedic Research, 2003. **21**(3): p. 451-457.

11. Rubin, J., C. Rubin, and C.R. Jacobs, *Molecular pathways mediating mechanical signaling in bone.* Gene, 2006. **367**: p. 1-16.

12. Li, Y.J., N.N. Batra, L.D. You, S.C. Meier, I.A. Coe, C.E. Yellowley, and C.R. Jacobs, *Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation.* Journal of Orthopaedic Research, 2004. **22**(6): p. 1283-1289.

13. Donahue, T.L.H., T.R. Haut, C.E. Yellowley, H.J. Donahue, and C.R. Jacobs, *Mechanosensitivity of bone cells to oscillating fluid flow induced shear stress may be modulated by chemotransport.* Journal of Biomechanics, 2003. **36**(9): p. 1363-1371.

14. Sen, A., M.S. Kallos, and L.A. Behie, *Expansion of mammalian neural stem cells in bioreactors: effect of power input and medium viscosity.* Developmental Brain Research, 2002. **134**(1-2): p. 103-113.

15. Sikavitsas, V.I., G.N. Bancroft, H.L. Holtorf, J.A. Jansen, and A.G. Mikos, *Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(25): p. 14683-14688.

16. McAllister, T.N., T. Du, and J.A. Frangos, *Fluid shear stress stimulates prostaglandin and nitric oxide release in bone marrow-derived preosteoclast-like cells.* Biochemical and Biophysical Research Communications, 2000. **270**(2): p. 643-648.

17. Weinbaum, S., S.C. Cowin, and Y. Zeng, *A Model for the Excitation of Osteocytes by Mechanical Loading-Induced Bone Fluid Shear Stresses.* Journal of Biomechanics, 1994. **27**(3): p. 339-360.

18. Qin, Y.X., W. Lin, and C. Rubin, *The pathway of bone fluid flow as defined by in vivo intramedullary pressure and streaming potential measurements.* Annals of Biomedical Engineering, 2002. **30**(5): p. 693-702.

19. Stevens, H.Y., D.R. Meays, and J.A. Frangos, *Pressure gradients and transport in the murine femur upon hindlimb suspension.* Bone, 2006. **39**(3): p. 565-572.

20. Qin, Y.X., T. Kaplan, A. Saldanha, and C. Rubin, *Fluid pressure gradients, arising from oscillations in intramedullary pressure, is correlated with the formation of bone and inhibition of intracortical porosity.* Journal of Biomechanics, 2003. **36**(10): p. 1427-1437.

21. Zhang, P., M. Su, Y.L. Liu, A. Hsu, and H. Yokota, *Knee loading dynamically alters intramedullary pressure in mouse femora.* Bone, 2007. **40**(2): p. 538-543.

22. Downey, D.J., P.A. Simkin, and R. Taggart, *The Effect of Compressive Loading on Intraosseous Pressure in the Femoral-Head Invitro.* Journal of Bone and Joint Surgery-American Volume, 1988. **70A**(6): p. 871-877.

23. Stein, A.H., H.C. Morgan, and R.F. Porras, *The Effect of Pressor and Depressor Drugs on Intramedullary Bone-Marrow Pressure.* Journal of Bone and Joint Surgery-American Volume, 1958. **40**(5): p. 1103-1110.

24. Shaw, N.E., *Observations on Intramedullary Blood-Flow and Marrow-Pressure in Bone.* Clinical Science, 1963. **24**(3): p. 311-&.

25. Azuma, H., *Intraosseous Pressure as Measure of Hemodynamic Changes in Bone Marrow.* Angiology, 1964. **15**(9): p. 396-&.

26. Michelsen, K., *Pressure Relationships in Bone Marrow Vascular Bed.* Acta Physiologica Scandinavica, 1967. **71**(1): p. 16-&.

27. Miyanishi, K., T. Yamamoto, T. Irisa, A. Yamashita, S. Jingushi, Y. Noguchi, and Y. Iwamoto, *Bone marrow fat cell enlargement and a rise in Intraosseous pressure in steroid-treated rabbits with osteonecrosis.* Bone, 2002. **30**(1): p. 185-190.

28. Justesen, J., K. Stenderup, E.N. Ebbesen, L. Mosekilde, T. Steiniche, and M. Kassem, *Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis.* Biogerontology, 2001. **2**(3): p. 165-171.

29. Shaw, N.E., *Studies on Intramedullary Pressure + Blood Flow in Bone.* American Heart Journal, 1964. **68**(1): p. 134-&.

30. Turner, C.H., V. Anne, and R.M.V. Pidaparti, *A uniform strain criterion for trabecular bone adaptation: Do continuum-level strain gradients drive adaptation?* Journal of Biomechanics, 1997. **30**(6): p. 555-563.

31. Tavassoli, M. and J.M. Yoffey, *Bone marrow, structure and function*. 1983, New York: A.R. Liss. xii, 300 p.

32. Gimble, J.M., C.E. Robinson, X. Wu, and K.A. Kelly, *The function of adipocytes in the bone marrow stroma: An update.* Bone, 1996. **19**(5): p. 421-428.

33. Weiss, L., *Hematopoietic Microenvironment of Bone-Marrow - Ultrastructural-Study of Stroma in Rats.* Anatomical Record, 1976. **186**(2): p. 161-184.

34. Chen, X., H. Xu, C. Wan, M. McCaigue, and G. Li, *Bioreactor expansion of human adult bone marrow-derived mesenchymal stem cells.* Stem Cells, 2006. **24**(9): p. 2052-2059.

35. Travlos, G.S., *Normal structure, function, and histology of the bone marrow.* Toxicologic Pathology, 2006. **34**(5): p. 548-565.

36. Branemark, P.I., *Vital microscopy of bone marrow in rabbit.* Scand J Clin Lab Invest, 1959. **11**(Supp 38): p. 1-82.

37. Tavassoli, M., *Ultrastructural development of bone marrow adipose cell.* Acta Anat (Basel), 1976. **94**(1): p. 65-77.

38. Larsen, R.M., *Intramedullary pressure with particular reference to massive diaphyseal bone necrosis experimental observations.* Annals of Surgery, 1938. **108**: p. 127-140.

39. Bauer, M.S. and T.L. Walker, *Intramedullary Pressure in Canine Long Bones.* American Journal of Veterinary Research, 1988. **49**(3): p. 425-427.

40. Harrelson, J.M. and B.A. Hills, *Changes in Bone Marrow Pressure in Response to Hyperbaric Exposure.* Aerospace Medicine, 1970. **41**(9): p. 1018-1021.

41. Shim, S.S., W.Y. Yu, and H.E. Hawk, *Relationship between Blood-Flow and Marrow Cavity Pressure of Bone.* Surgery Gynecology and Obstetrics with International Abstracts of Surgery, 1972. **135**(3): p. 353-&.

42. Thomas, I.H., P.J. Gregg, and D.N. Walder, *Intra-Osseous Phlebography and Intra-Medullary Pressure in the Rabbit Femur.* Journal of Bone and Joint Surgery-British Volume, 1982. **64**(2): p. 239-242.

43. Tondevold, E., J. Eriksen, and E. Jansen, *Observations on Long-Bone Medullary Pressure in Relation to Mean Arterial Blood-Pressure in the Anesthetized Dog.* Acta Orthopaedica Scandinavica, 1979. **50**(5): p. 527-531.

44. Wilkes, C.H. and M.B. Visscher, *Some Physiological Aspects of Bone-Marrow Pressure.* Journal of Bone and Joint Surgery-American Volume, 1975. **A 57**(1): p. 49-57.

45. Drinker, C.K., K.R. Drinker, and C.C. Lund, *The circulation in the mammalian bone-marrow - With especial reference to the factors concerned in the movement of red blood cells from the bone marrow into the circulating blood as disclosed by perfusion of the tibia of the dog and by injections of the bone-marrow in the rabbit and cat.* American Journal of Physiology, 1922. **62**(1): p. 1-92.

46. Drinker, C.K. and K.R. Drinker, *A Method for Maintaining an Artificial Circulation through the Tibia of the Dog, With a Demonstration of the Vasomotor Control of the Marrow Vessels.* American Journal of Physiology, 1916. **40**: p. 514-521.

47. Michelsen, K., *Hemodynamics of the bone marrow circulation.* Acta Physiol Scand, 1968. **73**(3): p. 264-80.

48. Stevens, H.Y., D.R. Meays, J. Yeh, L.M. Bjursten, and J.A. Frangos, *COX-2 is necessary for venous ligation-mediated bone adaptation in mice.* Bone, 2006. **38**(1): p. 93-104.

49. Dickerson, D.A., E.A. Sander, and E.A. Nauman, *Modeling the mechanical consequences of vibratory loading in the vertebral body: microscale effects.* Biomech Model Mechanobiol, 2007.

50. Bryant, J.D., T. David, P.H. Gaskell, S. King, and G. Lond, *Rheology of bovine bone marrow.* Proc Inst Mech Eng [H], 1989. **203**(2): p. 71-5.

51. Petrakis, N.L., *Temperature of Human Bone Marrow.* Journal of Applied Physiology, 1952. **4**(7): p. 549-553.

52. Dietz, A.A., *Composition of Normal Bone Marrow in Rabbits.* Journal of Biological Chemistry, 1946. **165**(2): p. 505-511.

53. Gurkan, U.A. and O. Akkus, *An implantable magnetoelastic sensor system for wireless physiological sensing of viscosity*, in *Proceeding of the Amse Summer Bioengineering Conference - 2007*. 2007, Amer Soc Mechanical Engineers: New York. p. 759-760.

54. Davis, L.B. and S.S. Praveen. *Nonlinear versus Linear Behavior of Calcaneal Bone Marrow at Different Shear Rates*. in *American Society of Biomechanics Annual Meeting*. 2006. Virginia Tech, Blacksburg, VA

55. Eguchi, Y. and T. Karino, *Measurement of rheologic property of blood by a falling-ball blood viscometer.* Annals of Biomedical Engineering, 2008. **36**(4): p. 545-553.

56. White, D.R., H.Q. Woodard, and S.M. Hammond, *Average Soft-Tissue and Bone Models for Use in Radiation-Dosimetry.* British Journal of Radiology, 1987. **60**(717): p. 907-913.

57. Huggins, C., B.H. Blocksom, and W.J. Noonan, *Temperature conditions in the bone marrow of rabbit, pigeon and albino rat.* American Journal of Physiology, 1936. **115**(2): p. 395-401.

58. Huggins, C. and W.J. Noonan, *An increase in reticulo-endothelial cells in outlying bone marrow consequent upon a local increase in temperature.* Journal of Experimental Medicine, 1936. **64**(2): p. 275-U7.

59. Huggins, C. and B.H. Blocksom, *Changes in outlying bone marrow accompanying a local increase of temperature within physiological limits.* Journal of Experimental Medicine, 1936. **64**(2): p. 253-U4.

60. Weiss, L.P. and G.B. Wislocki, *Seasonal Variations in Hematopoiesis in the Dermal Bones of the 9-Banded Armadillo.* Anatomical Record, 1956. **126**(2): p. 143-&.

61. Tate, M.L.K., *"Whither flows the fluid in bone?" An osteocyte's perspective.* Journal of Biomechanics, 2003. **36**(10): p. 1409-1424.

62. Leblanc, A.D., V.S. Schneider, H.J. Evans, D.A. Engelbretson, and J.M. Krebs, *Bone-Mineral Loss and Recovery after 17 Weeks of Bed Rest.* Journal of Bone and Mineral Research, 1990. **5**(8): p. 843-850.

63. Robling, A.G., A.B. Castillo, and C.H. Turner, *Biomechanical and molecular regulation of bone remodeling.* Annual Review of Biomedical Engineering, 2006. **8**: p. 455-498.

64. Zerwekh, J.E., L.A. Ruml, F. Gottschalk, and C.Y.C. Pak, *The effects of twelve weeks of bed rest on bone histology, biochemical markers of bone turnover, and calcium homeostasis in eleven normal subjects.* Journal of Bone and Mineral Research, 1998. **13**(10): p. 1594-1601.

65. Courteix, D., E. Lespessailles, S.L. Peres, P. Obert, P. Germain, and C.L. Benhamou, *Effect of physical training on bone mineral density in prepubertal girls: A comparative study between impact-loading and non-impact-loading sports.* Osteoporosis International, 1998. **8**(2): p. 152-158.

66. Krolner, B., B. Toft, S.P. Nielsen, and E. Tondevold, *Physical Exercise as Prophylaxis against Involutional Vertebral Bone Loss - a Controlled Trial.* Clinical Science, 1983. **64**(5): p. 541-546.

67. Mori, T., N. Okimoto, A. Sakai, Y. Okazaki, N. Nakura, T. Notomi, and T. Nakamura, *Climbing exercise increases bone mass and trabecular bone turnover through transient regulation of marrow osteogenic and osteoclastogenic potentials in mice.* Journal of Bone and Mineral Research, 2003. **18**(11): p. 2002-2009.

68. Mori, T., N. Okimoto, H. Tsurukami, A. Sakai, Y. Okazaki, S. Uchida, T. Notomi, and T. Nakamura, *Effects of tower climbing exercise on bone mass and turnover in growing mice.* Journal of Bone and Mineral Research, 2001. **16**: p. S362-S362.

69. Nakura, N., T. Mori, N. Okimoto, A. Sakai, and T. Nakamura, *The climbing exercise prevents trabecular bone loss with highly expressed mRNA of estrogen receptor alpha in bone cells after ovariectomy in mice.* Journal of Bone and Mineral Research, 2005. **20**(9): p. S73-S73.

70. Turner, C.H. and A.G. Robling, *Mechanisms by which exercise improves bone strength.* Journal of Bone and Mineral Metabolism, 2005. **23**: p. 16-22.

71. Canalis, E., *The fate of circulating osteoblasts.* New England Journal of Medicine, 2005. **352**(19): p. 2014-2016.

72. Piekarski, K. and M. Munro, *Transport Mechanism Operating between Blood-Supply and Osteocytes in Long Bones.* Nature, 1977. **269**(5623): p. 80-82.

73. Kafka, V., *On Hydraulic Strengthening of Bones.* Biorheology, 1983. **20**(6): p. 789-793.

74. Kafka, V., *On Hydraulic Strengthening of Bones.* Journal of Biomechanics, 1993. **26**(6): p. 761-762.

75. Bryant, J.D., *On Hydraulic Strengthening of Bones.* Journal of Biomechanics, 1995. **28**(3): p. 353-354.

76. Ochoa, J.A., A.P. Sanders, D.A. Heck, and B.M. Hillberry, *Stiffening of the Femoral-Head Due to Intertrabecular Fluid and Intraosseous Pressure.* Journal of Biomechanical Engineering-Transactions of the Asme, 1991. **113**(3): p. 259-262.

77. Ochoa, J.A., A.P. Sanders, T.W. Kiesler, D.A. Heck, J.P. Toombs, K.D. Brandt, and B.M. Hillberry, *In vivo observations of hydraulic stiffening in the canine femoral head.* Journal of Biomechanical Engineering-Transactions of the Asme, 1997. **119**(1): p. 103-108.

78. Simkin, P.A., *Hydraulically loaded the trabeculae may serve as springs within normal femoral head.* Arthritis and Rheumatism, 2004. **50**(10): p. 3068-3075.

79. Kumar, S., P.R. Davis, and B. Pickles, *Bone-Marrow Pressure and Bone Strength.* Acta Orthopaedica Scandinavica, 1979. **50**(5): p. 507-512.

80. Bryant, J.D., *The Effect of Impact on the Marrow Pressure of Long Bones Invitro.* Journal of Biomechanics, 1983. **16**(8): p. 659-&.

81. Zhang, P., M. Su, S.M. Tanaka, and H. Yokota, *Knee loading stimulates cortical bone formation in murine femurs.* Bmc Musculoskeletal Disorders, 2006. **7**: p. -.

82. Zhang, P., S.M. Tanaka, H. Jiang, M. Su, and H. Yokota, *Loading frequency-dependent enhancement of bone formation in mouse tibia with knee-loading modality.* Journal of Bone and Mineral Research, 2005. **20**(9): p. S134-S135.

83. Tate, M.L.K., P. Niederer, and U. Knothe, *In vivo tracer transport through the lacunocanalicular system of rat bone in an environment devoid of mechanical loading.* Bone, 1998. **22**(2): p. 107-117.

84. Tate, M.L.K. and U. Knothe, *An ex vivo model to study transport processes and fluid flow in loaded bone.* Journal of Biomechanics, 2000. **33**(2): p. 247-254.

85. Raisz, L.G., *Pathogenesis of osteoporosis: concepts, conflicts, and prospects.* Journal of Clinical Investigation, 2005. **115**(12): p. 3318-3325.

86. *Osteoporosis - Fast Facts*. 2006, National Osteoporosis Foundation, http://www.nof.org/osteoporosis/diseasefacts.htm.

87. Verma, S., J.H. Rajaratnam, J. Denton, J.A. Hoyland, and R.J. Byers, *Adipocytic proportion of bone marrow is inversely related to bone formation in osteoporosis.* J Clin Pathol, 2002. **55**(9): p. 693-8.

88. McBeath, R., D.M. Pirone, C.M. Nelson, K. Bhadriraju, and C.S. Chen, *Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment.* Developmental Cell, 2004. **6**(4): p. 483-495.

89. Semino, C.E., *Can We Build Artificial Stem Cell Compartments?* J Biomed Biotechnol, 2003. **2003**(3): p. 164-169.

90. Khademhosseini, A., R. Langer, J. Borenstein, and J.P. Vacanti, *Microscale technologies for tissue engineering and biology.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2480-7.

91. Nesic, D., R. Whiteside, M. Brittberg, D. Wendt, I. Martin, and P. Mainil-Varlet, *Cartilage tissue engineering for degenerative joint disease.* Advanced Drug Delivery Reviews, 2006. **58**(2): p. 300-322.

92. Kuo, C.K., W.J. Li, R.L. Mauck, and R.S. Tuan, *Cartilage tissue engineering: its potential and uses.* Current Opinion in Rheumatology, 2006. **18**(1): p. 64-73.

93. Stoltz, J.F., D. Bensoussan, V. Decot, P. Netter, A. Ciree, and P. Gillet, *Cell and tissue engineering and clinical applications: An overview.* Bio-Medical Materials and Engineering, 2006. **16**(4): p. S3-S18.

94. Sikavitsas, V.I., G.N. Bancroft, J.J. Lemoine, M.A.K. Liebschner, M. Dauner, and A.G. Mikos, *Flow perfusion enhances the calcified matrix deposition of marrow stromal cells in biodegradable nonwoven fiber mesh scaffolds.* Annals of Biomedical Engineering, 2005. **33**(1): p. 63-70.

95. van den Dolder, J., G.N. Bancroft, V.I. Sikavitsas, P.H.M. Spauwen, J.A. Jansen, and A.G. Mikos, *Flow perfusion culture of marrow stromal osteoblasts in titanium fiber mesh.* Journal of Biomedical Materials Research Part A, 2003. **64A**(2): p. 235-241.

96. Miyanishi, K., M.C.D. Trindade, D.P. Lindsey, G.S. Beaupre, D.R. Carter, S.B. Goodman, D.J. Schurman, and R.L. Smith, *Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta 3-induced chondrogenesis by adult human mesenchymal stem cells in vitro.* Tissue Engineering, 2006. **12**(8): p. 2253-2262.

97. Angele, P., D. Schumann, M. Angele, B. Kinner, C. Englert, R. Hente, B. Fuchtmeier, M. Nerlich, C. Neumann, and R. Kujat, *Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds.* Biorheology, 2004. **41**(3-4): p. 335-346.

98. Nerlich, M., D. Schumann, R. Kujat, and P. Angele, *Mechanobiological conditioning on mesenchymal stem cells during chondrogenesis.* Shock, 2004. **21**: p. 136-136.

99. Rubin, J., D. Biskobing, X.A. Fan, C. Rubin, K. McLeod, and W.R. Taylor, *Pressure regulates osteoclast formation and MCSF expression in marrow culture.* Journal of Cellular Physiology, 1997. **170**(1): p. 81-87.

100. Nagatomi, J., B.P. Arulanandam, D.W. Metzger, A. Meunier, and R. Bizios, *Effects of cyclic pressure on bone marrow cell cultures.* Journal of Biomechanical Engineering-Transactions of the Asme, 2002. **124**(3): p. 308-314.

101. Nagatomi, J., B.R. Arulanandam, D.W. Metzger, A. Meunier, and R. Bizios, *Cyclic pressure affects osteoblast functions pertinent to osteogenesis.* Annals of Biomedical Engineering, 2003. **31**(8): p. 917-923.

102. Welch, R.D., C.E. Johnston, M.J. Waldron, and B. Poteet, *Bone Changes Associated with Intraosseous Hypertension in the Caprine Tibia.* Journal of Bone and Joint Surgery-American Volume, 1993. **75A**(1): p. 53-60.

103. Fox, S.W., T.J. Chambers, and J.W.M. Chow, *Nitric oxide is an early mediator of the increase in bone formation by mechanical stimulation.* American Journal of Physiology-Endocrinology and Metabolism, 1996. **33**(6): p. E955-E960.

104. Pitsillides, A.A., S.C.F. Rawlinson, R.F.L. Suswillo, G. Zaman, P.I. Nijweide, and L.E. Lanyon, *Mechanical Strain-Induced Nitric-Oxide Production by Osteoblasts and Osteocytes.* Journal of Bone and Mineral Research, 1995. **10**: p. S217-S217.

105. Turner, C.H., Y. Takano, I. Owan, and G.A.C. Murrell, *Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats.* American Journal of Physiology-Endocrinology and Metabolism, 1996. **33**(4): p. E634-E639.

106. Fox, S.W. and J.W.M. Chow, *Nitric oxide synthase expression in bone cells.* Bone, 1998. **23**(1): p. 1-6.

107. Burr, D.B., A.G. Robling, and C.H. Turner, *Effects of biomechanical stress on bones in animals.* Bone, 2002. **30**(5): p. 781-786.

108. Turner, C.H., M.R. Forwood, and M.W. Otter, *Mechanotransduction in Bone - Do Bone-Cells Act as Sensors of Fluid-Flow.* Faseb Journal, 1994. **8**(11): p. 875-878.

109. Nauman, E.A., R.L. Satcher, T.M. Keaveny, B.P. Halloran, and D.D. Bikle, *Osteoblasts respond to pulsatile fluid flow with shortterm increases in PGE(2) but no change in mineralization.* Journal of Applied Physiology, 2001. **90**(5): p. 1849-1854.

110. Jacobs, C.R., C.E. Yellowley, B.R. Davis, Z. Zhou, J.M. Cimbala, and H.J. Donahue, *Differential effect of steady versus oscillating flow on bone cells.* Journal of Biomechanics, 1998. **31**(11): p. 969-976.

111. Donahue, S.W., C.R. Jacobs, and H.J. Donahue, *Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent.* American Journal of Physiology-Cell Physiology, 2001. **281**(5): p. C1635-C1641.

112. Kurokouchi, K., C.R. Jacobs, and H.J. Donahue, *Oscillating fluid flow inhibits TNF-alpha-induced NF-kappa B activation via an I kappa B kinase pathway in osteoblast-like UMR106 cells.* Journal of Biological Chemistry, 2001. **276**(16): p. 13499-13504.

113. KleinNulend, J., C.M. Semeins, and E.H. Burger, *Prostaglandin mediated modulation of transforming growth factor-beta metabolism in primary mouse osteoblastic cells in vitro.* Journal of Cellular Physiology, 1996. **168**(1): p. 1-7.

114. Kleinnulend, J., A. Vanderplas, C.M. Semeins, N.E. Ajubi, J.A. Frangos, P.J. Nijweide, and E.H. Burger, *Sensitivity of Osteocytes to Biomechanical Stress in-Vitro.* Faseb Journal, 1995. **9**(5): p. 441-445.

115. Johnson, D.L., T.N. McAllister, and J.A. Frangos, *Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts.* American Journal of Physiology-Endocrinology and Metabolism, 1996. **34**(1): p. E205-E208.

116. Nauman, E.A., R.L. Satcher, T.M. Keaveny, B.P. Halloran, and D.D. Bikle, *Osteoblasts in culture respond to in vitro pulsatile fluid flow with short term increases in PGE2 and long term increases in cell proliferation.* Journal of Bone and Mineral Research, 2000. **15**: p. S500-S500.

117. Reich, K.M. and J.A. Frangos, *Effect of Flow on Prostaglandin-E2 and Inositol Trisphosphate Levels in Osteoblasts.* American Journal of Physiology, 1991. **261**(3): p. C428-C432.

118. Li, J.L., D.W. Liu, H.Z. Ke, R.L. Duncan, and C.H. Turner, *The P2X(7) nucleotide receptor mediates skeletal mechanotransduction.* Journal of Biological Chemistry, 2005. **280**(52): p. 42952-42959.

119. Hung, C.T., S.R. Pollack, T.M. Reilly, and C.T. Brighton, *Real-Time Calcium Response of Cultured Bone-Cells to Fluid-Flow.* Clinical Orthopaedics and Related Research, 1995(313): p. 256-269.

120. Chen, N.X., K.D. Ryder, F.M. Pavalko, C.H. Turner, D.B. Burr, J.Y. Qiu, and R.L. Duncan, *Ca2+ regulates fluid shear-induced cytoskeletal reorganization and gene expression in osteoblasts.* American Journal of Physiology-Cell Physiology, 2000. **278**(5): p. C989-C997.

121. Reich, K.M., T.N. Mcallister, S. Gudi, and J.A. Frangos, *Activation of G proteins mediates flow-induced prostaglandin E(2) production in osteoblasts.* Endocrinology, 1997. **138**(3): p. 1014-1018.

122. Tettelbaum, S.L., M.M. Tondravi, and F.P. Ross, *Osteoclast Biology*, in *Osteoporosis*, S.E. Papapoulos, Editor. 1996, Academic Press: San Diego, CA. p. 61-71.

123. Montgomery, R.J., B.D. Sutker, J.T. Bronk, S.R. Smith, and P.J. Kelly, *Interstitial Fluid-Flow in Cortical Bone.* Microvascular Research, 1988. **35**(3): p. 295-307.

124. Tenenbaum, H.C., *Cellular Origins and Theories of Differentiation of Bone-Forming Cells*, in *In Bone: The Osteoblast and Osteocyte*, B.K. Hall, Editor. 1992, CRC Press: Boca Raton, FL. p. 41-69.

125. Balcells, M., M.F. Suarez, M. Vazquez, and E.R. Edelman, *Cells in fluidic environments are sensitive to flow frequency.* Journal of Cellular Physiology, 2005. **204**(1): p. 329-335.

2. CHARACTERIZATION OF INHERENTLY OSSIFYING BONE MARROW CULTURE AS A SCAFFOLD-FREE MULTICELLULAR THREE-DIMENSIONAL (3D) IN VITRO MODEL OF INTRAMEMBRANOUS OSTEOGENESIS

2.1. Abstract

Osteogenesis occurs in a complex three-dimensional (3D) extracellular environment under the regulation of chemical and physical cues. Furthermore, cells of both mesenchymal and hematopoietic origin are involved. *In vitro* models of osteogenesis range from two-dimensional (2D) monolayer cultures of single cell types to 3D systems with or without scaffolds. Such models are essential to understand the biology of osteogenesis as well as to assess the effects of biological, chemical and physical cues on bone formation. Previous studies have shown that bone marrow has an inherent ability to undergo osteogenesis without the addition of osteoinductive stimulants. Therefore the self-inductive ossification ability of bone marrow tissue can be harnessed *in vitro* to employ this natural phenomenon as a scaffold-free multicellular and 3D model of osteogenesis. However, little is known on the compositional, cellular and mechanical properties of bone-like structures emerging from this *in vitro* marrow ossification model. In the current study, rat bone marrow explants were cultured in an organ culture

environment for 28 days and the following analyses were performed: 1) structural characterization and matrix typification with micro-computed tomography (μCT), histochemical and immunohistochemical analyses, 2) cellular residency via flow cytometry, 3) compositional and micromechanical analyses of the extracellular matrix with Raman microspectroscopy, energy dispersive x-ray spectroscopy (EDS), backscattered electron microscopy (BSEM) and microindentation. The results indicated that marrow explants resulted in plate-like bone formations up to 5 mm in diameter and 160 μm (±55 μm) in thickness. Ossification of marrow occurred via an intramembranous mechanism without any evidence of intermediate endochondral phase. The cellular and compositional characteristics of the resulting ossified structures were comparable to newly forming bone tissue. Therefore, this scaffold-free multicellular *in vitro* 3D model has significant potential to study intramembranous osteogenesis in terms of morphological, compositional evolution and cellular residency.

Keywords: mesenchymal stem cells, marrow stromal cells, differentiation, osteogenesis, hematopoietic cells, bone tissue engineering, BSE, EDX, Raman, microhardness, CD44, CD45, STRO-1, collagen type-I, collagen type-III, Masson Trichrome, Safranin-O fast green, von Kossa, periosteal bone, intracortical bone.

2.2. Introduction

Bone formation takes place in a complex microenvironment under the regulation of chemical [1-3] and physical cues [4, 5] necessitating the involvement and interaction of multiple cells types [6-15]. There are two types of bone formation: endochondral ossification and intramembranous ossification. It is known that a fracture site that is not well stabilized (prone to motion) tend to heal through endochondral ossification [16, 17], in which ossification is preceded by the formation of cartilaginous matrix. On the other hand, a well stabilized fracture site undergoes healing through intramembranous ossification, in which mesenchymal stem cells directly differentiate into osteoblasts and form an ossified matrix [18, 19]. It was recently shown that healing through intramembranous ossification results in a stiffer and faster healed bone compared to endochondral ossification in mice [19]. Intramembranous ossification is critical in skeletal development and regeneration and takes place in the periosteum, in the regeneration of endosteum and in parts of cranium, scapula and clavicles [20-24]. Even though there is limited number of *in vivo* animal models of intramembranous ossification [18, 19], there seems to be a lack of *in vitro* models that effectively represents the complex natural environment of this process. Delineating the intramembranous bone formation process is essential to develop effective therapies towards regeneration and healing of bone tissue.

Various models have been developed and utilized to investigate bone formation. These models comprise of either *in vitro* monolayer culture systems that employ single cell types (i.e. calvarial osteoblastic cells, mesenchymal stem cells) [25-34], explant

cultures of bone tissue fragments (e.g. mouse metatarsals and calvaria) [35-37] or *in vivo* animal models (e.g. murine fracture healing models) [18, 19, 38-41]. It has been shown that the cells cultured in two-dimensional (2D) monolayer culture conditions display significant perturbations in gene expression compared to the cells in native tissue and the cells cultured in three-dimensional (3D) culture conditions [42]. These perturbations may be attributed to the elimination of the interactions between different types. Bone fragments/explants are formed readily; therefore, they are not particularly useful for studying the full-scale of bone formation. *In vivo* models are complex and the response observed is generally the result of multiple systems in the organism. In addition, with *in vivo* animal models it is harder to target specific proteins (i.e. transgenic mouse models) compared to *in vitro* systems which allow protein targeting methods such as silencing RNA (siRNA) [37]. Therefore, there is a need for novel 3D *in vitro* models which embody the multicellular and physiological characteristics of native tissues [14, 42-47] and hence complement the existing models in aiding to better understand osteogenesis. The 3D culture platforms are conveniently situated between the simplified 2D culture systems and *in vivo* models; and they can be an alternative to the currently used animal models [42, 45, 46]. Here, we propose and characterize a scaffold-free, multicellular 3D *in vitro* model based on the self-inductive ossification of bone marrow tissue, which can be used as a convenient model to study the process of intramembranous osteogenesis.

Earlier studies on the ossification potential of bone marrow tissue have shown that ectopic implantation of marrow induces ossification and forms a tissue that is composed of both bone and marrow components [48]. It was previously demonstrated that bone

marrow tissue inherently ossifies *in vitro* [49], and we have recently shown that explant cultures of rat marrow tissue results in 3D ossified structures [3]. However, the characteristics of the bone tissue formed by ossifying marrow explants are largely unknown. In the current study, rat bone marrow explants were cultured for 28 days and the compositional properties of ossified marrow were investigated and characterized by delineating: 1) the structural organization of the ossified matrix with micro-computed tomography (μCT), 2) the cellular and compositional organization of the ossified marrow by histology, immunohistochemistry and high magnification back-scattered electron microscopy (BSEM), 3) the temporal residency of STRO-1+ (MSCs) and CD45+ (hematopoietic) cells during ossification with flow cytometry, 4) the mineral to matrix ratio of the ossified marrow with Raman microspectroscopy, 5) the elemental analysis of the ossified matrix with energy dispersive x-ray spectroscopy (EDS), and 6) the mechanical properties of the ossified matrix with microindentation.

2.3. Methods

2.3.1. *In Vitro* Culture Conditions

Whole bone marrow tissue was isolated from the femurs and tibiae of 80-90 days old male Long-Evans rats (Purdue Animal Care and Use Committee approved). We have previously detailed a comprehensive description of the bone marrow extraction and culture procedure elsewhere [3] (see Appendix for detailed protocols). Briefly, bone marrow was extracted with a centrifugation based technique and directly pipetted onto

culture inserts with porous PET membranes (Transwell, Corning, 0.4 μm pore size) at a 7 μl (7 mm^3) volume (Fig.2.1A&B). The lower side of the porous membrane was in contact with 1ml of media (in a 6-well plate) (Fig.2.1A&B). The upper portion of the porous inserts was not filled with media (Fig.2.1B). Furthermore, the insert containing the explant was removed and placed on the side during media changes; therefore, the cellular composition of marrow tissue was fully maintained throughout this air-medium interface culture model. The marrow growth medium was osteogenic; however, it did not contain any osteoinductive factors (such as dexamethasone or BMP-2) and composed of (modified from Luria et al. [49]): α-MEM (Sigma), 10% MSC-qualified-FBS (Invitrogen), 60 U/ml Pen-Strep (Invitrogen), 2.5 μg/ml Fungizone (Sigma), 50 μg/ml ascorbic acid (Sigma), 5 mM Na-β-glycerophosphate (MP Biomedical) and 3.5 mg/ml glucose (Sigma). The culture medium was changed three times a week and marrow culture was maintained at 37°C and 5% CO_2 throughout the experiment (28 days).

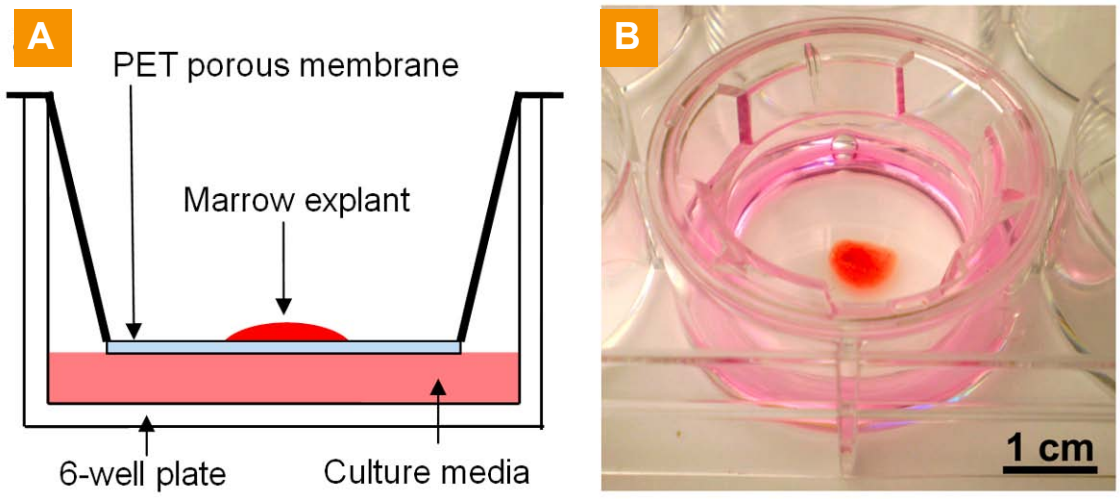


Figure 2.1: The culture system used for culturing bone marrow explants. **A)** Schematic drawing of the marrow culture system that is composed of a culture insert with a PET porous membrane (0.4 μm pore size, Transwell, Corning) placed in a culture plate (6-well). **B)** Overview of a typical bone marrow explant (at day-0) placed in a culture plate with a a sufficient volume of media added underneath the insert membrane. The inside of the insert (i.e. above the porous membrane) was not filled with media to prevent the immersion and dispersion of marrow explant and to maintain the compositional integrity during media changes.

2.3.2. Micro-Computed Tomography (μCT) Analysis

μCT scans were performed on the ossified marrow explants on day 28. The ossified samples were fixed in 10% formalin and kept in the fixative before and during the scans (SCANCO Medical AG, μCT 40, Brüttisellen, Switzerland), which were performed with a 16 μm voxel resolution (I = 145 μA, E = 55 kVp, integration time = 200 ms). The scanned images were reconstructed and analyzed with a commercial software (SCANCO evaluation software) by using the standard segmentation parameters for bone tissue [50-52]. An ossified marrow explant was stained with 2% silver nitrate

(vonKossa staining protocol[28]) to visualize the mineralized matrix and compare with the detected ossified volume by μCT.

2.3.3. Histology of Ossified Marrow Explants

At the end of the culture period (28 days), 10% formalin was used to fix the ossified marrow explants, followed by decalcification with formic acid solution (1:1 solution of 50% aqueous formic acid and 20% sodium citrate) for 12 hours. The decalcified explants were then washed in tap water for 30 to 45 minutes, embedded in paraffin, sectioned and dried overnight in a 37°C oven. The sections were deparaffinized and hydrated in a graded series of alcohol solutions (100%, 95%, 70% and water). The slides were then stained with Masson's trichrome for visualizing the collagen-rich ossifying regions. In addition, Safranin-O fast green staining was performed to check for cartilaginous formation via the presence of proteoglycans. The light microscope images were taken with Olympus Vanox microscope equipped with Qimaging Micropublisher 5.0 RTV 5 megapixel CCD camera.

2.3.4. Immunohistochemistry on Ossified Marrow Explants

The protocol followed for immunohistochemistry of the ossified marrow explants was modified from previously published literature [53, 54]. The sections of decalcified ossified marrow explants were deparaffinized and treated with 10 mM sodium citrate solution (pH 6.0) for 10 minutes at 95 ºC for epitope recovery. The sections were then

cooled for 15 minutes, washed in tris buffered saline (TBS) and treated with 3% H_2O_2 for 10 minutes to quench endogenous peroxidase activity. Following this step, the sections were rinsed with TBS and immersed in a protein block solution (Dako, Carpinteria, CA) for 30 minutes to prevent non-specific binding. The blocking solution was then drained and the sections were incubated with the primary antibody for 1 hour at room temperature. The anti-rat primary antibodies included anti-CD44 (OX-49) mouse monoclonal IgG antibody to detect the presence of osteocytes [54] (1:100 dilution, Biolegend, San Diego, CA), anti-collagen type I rabbit polyclonal IgG antibody (1:10 dilution, AbD Serotec, Raleigh, NC) and anti-collagen type III rabbit polyclonal IgG antibody (1:300 dilution, AbD Serotec, Raleigh, NC) to characterize matrix composition. After incubation with the primary antibody, the sections were washed with TBS and sequentially incubated with a prediluted biotinylated secondary antibody (30 minutes), a peroxidase-labelled streptavidin (30 minutes) and a 3,3'-diaminobenzidine chromogen solution (5 minutes) using the Universal Dako LSAB + kit (Dako, Carpinteria, CA) following the manufacturer's instructions. The sections were then washed with DI water, counter-stained with hematoxylin and coverslipped. Section stained in a similar manner but without the primary antibody served as the negative control. The light microscope images of the sections were taken as described in the previous section.

2.3.5. Back-Scattered Electron Microscopy (BSEM)

Ossified marrow explants were fixed in 70% ethanol followed by serial dehydration (ethanol 80%, 90%, 100%×2). The dehydrated samples were embedded in

(poly) methylmethacrylate (PMMA) for undemineralized assessment of the ossified matrix. PMMA embedding process involved infiltration of the samples with 80% methyl methacrylate (Sigma) and 20% dibutyl phthalate (Sigma) (M1) for 1 day followed by infiltration with M1 supplemented with 1 g/L benzoyl peroxide (Sigma) for 1 day. After infiltration the samples were embedded with M1 supplemented with 3.5 g/L benzoyl peroxide for 2 days followed by 2 day curing at 45°C oven. The PMMA embedded samples were sectioned transversely with a diamond low-speed saw exposing the cross-section of the ossified marrow explants. The exposed surfaces were polished with increasingly fine sandpaper up to 2000 grit. The polished samples were visually checked with a light microscope. BSEM imaging was performed on the polished surface with a gaseous analytical detector (GAD) at an accelerator voltage of 10kV, with a 4.4mm working distance and at 10,000X magnification (FEI NanoSEM).

2.3.6. Assessment of Cellular Residency by Flow Cytometry

The cells were harvested by pooling at least ten marrow explants per time point at periodic intervals (days 0, 14 and 28) by incubating the explants in 1 mg/ml collagenase (Sigma) in 0.25% trypsin-EDTA solution (Invitrogen) for 15 minutes at 37 °C with gentle mixing at intervals. After enzymatic treatment, the cell suspension harvested was filtered through a 70 micron cell strainer and centrifuged. Following centrifugation, the red blood cells (RBCs) were lysed using 1X RBC lysis buffer (150 mM Ammonium Chloride, 10 mM Sodium Bicarbonate and 1 mM EDTA) for 5 minutes at room temperature. Prior to incubation with the specific antibodies, the cells were suspended in

5% mouse serum for 20 minutes at 4 °C to prevent non-specific binding of the antibodies. The cells were then stained with FITC labeled CD45 antibody (Biolegend, San Diego, CA) to identify hematopoietic cells [55], PE labeled STRO-1 antibody (Santa Cruz Biotech, Santa Cruz, CA) to identify MSCs [11, 56-60] and analyzed by a flow cytometer (Beckman Coulter Cell Lab Quanta MPL). The data obtained was analyzed by performing a dot plot analysis using the WinList 6.0 software (Fig.2.6A).

2.3.7. Raman Microspectroscopy

After 28 days in culture, formalin fixed ossified marrow explants were transferred onto glass slides and the Raman spectra were obtained with 660 nm laser (LabRam HR800, Horiba Jobin Yvon, Edison, NJ). A wavenumber range of 250-1800 cm^{-1} was selected to visualize the characteristic peaks of bone tissue. As a control, diaphyseal portion of the tibia of a rat used in this study was fixed and analyzed the same way. The ratio of the intensities of 959 cm^{-1} peak (apatitic mineral, symmetric stretch for PO_4^{-3}) and 1450 cm^{-1} peak (N-H bending, methyl deformation; CH_2 wagging) was quantified [61] in ossified marrow explants (n=10) and rat tibiae (n=3) to compare the mineral/matrix ratio.

2.3.8. Energy Dispersive X-Ray Spectroscopy (EDS)

The elemental analysis of ossified marrow explants was conducted with EDS by using FEI Quanta 3D FEG Dual-beam SEM equipped with OXFORD INCAPentaFET-x3

large area crystal Energy Dispersive X-ray (EDX) detector in low vacuum mode. The diaphyseal sections of the rat tibiae were included as positive controls and similarly analyzed with EDS. Elemental analysis on rat tibia was conducted on the periosteal surface (newly forming bone) and on the intracortical regions (older bone) separately, due to different maturity levels of bone tissue at these locations [62, 63]. Ca/P weight ratio was measured for ossified marrow (OM) explants (n=21), periosteal rat tibiae (PRT) (n=5), intracortical rat tibiae (IRT) (n=6) and used to compare the crystalline level of the samples [64]. Ca/P ratio for human bone (HB) (ages: 15-55, n=80) was obtained from a previous study in the literature [65] for comparison.

2.3.9. Microindentation of Ossified Marrow Explants

Native rat tibiae were fixed with 70% ethanol, dehydrated, PMMA embedded, sectioned and polished as described above. The embedded tibiae were sectioned transversely to expose the cross-section of the mid-diaphyses. Microindentation on rat tibial diaphysis was performed on the periosteal (PRT) and intracortical (IRT) regions separately due to the differences in the micromechanical properties, which is associated with the different maturity of the bone tissue at these locations [62]. Microindentation was performed on the polished PMMA embedded samples using a Vickers diamond micro-indenter with a microhardness tester (Leco LM247AT). The indentation load was selected to be 200 gf with a dwell time of 15 seconds [66, 67]. Vickers Hardness (HV) was calculated by using the manufacturer's software (Leco Amh43) by averaging the measurements obtained from 10-26 individual indentations for each sample group.

2.3.10. Statistical Analysis

The Ca/P ratio and the Vickers microhardness values of the samples were analyzed statistically with Kruskal–Wallis one-way analysis of variance, followed by Mann-Whitney U-test and a *post hoc* test with Bonferroni correction for multiple comparisons. The measured ratio of the Raman peak intensities at 959 cm^{-1} (apatitic mineral, symmetric stretch for PO_4^{-3}) and 1450 cm^{-1} peak (N-H bending, methyl deformation; CH_2 wagging) in the samples was statistically analyzed by Mann-Whitney U-test. Statistical significance threshold was set at $p < 0.05$ and the p-value obtained for each test was adjusted based on the number of comparisons according to Bonferroni correction (p-value obtained from the test multiplied by number of comparisons). Error bars in the figures were displayed as standard deviation.

2.4. Results

2.4.1. Appearance and Structural Organization of Ossified Marrow

Rat bone marrow explants underwent ossification without addition of any osteoinductive factors forming a plate-shaped bone-like tissue by day-28 with a diameter up to 5 mm (Fig.2.2A) and a thickness up to 0.16 mm (160µm ±55µm) (Fig.2.3). Ossified marrow explants were visible to the naked eye (Fig.2.2A). The presence of calcified tissue was verified by von Kossa staining (Fig.2.2A) and µCT scans further confirmed the presence of mineralized tissue (Fig.2.2B). The mineralized volume

quantified by µCT was observed to be about 10% (0.73 mm^3 ±0.41mm^3) of the initial bone marrow volume (7µl which is equivalent to 7 mm^3).

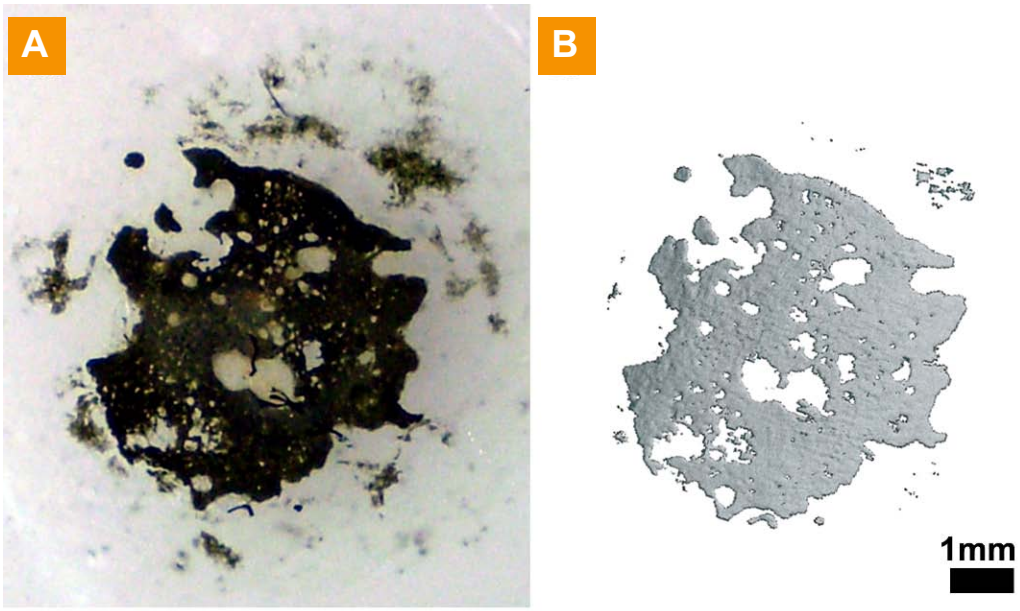


Figure 2.2: General overview of ossifying marrow explants. **A)** von Kossa stained marrow explant at day 28 confirms the presence of calcification. **B)** 3-dimensional µCT reconstructed appearance of an ossified explant that displays a plate-like ossified structure at day-28.

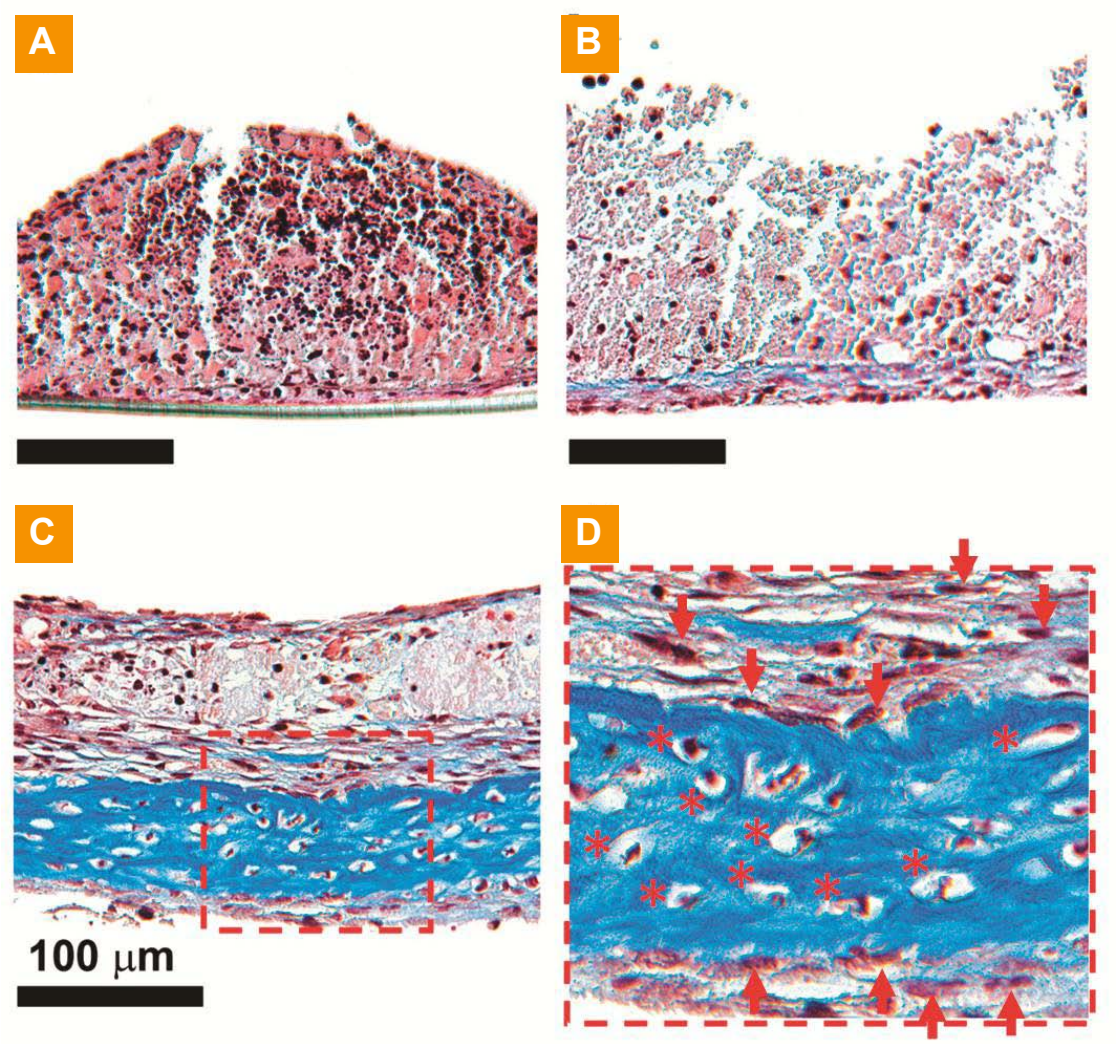


Figure 2.3: Masson's trichrome histology of ossifying marrow over time. **A)** Day-7, **B)** Day-14, **C)** Day-28, **D)** A close-up view of the day-28 section. Blue color represents the collagen-rich sections of the explants. At day-7 it was possible to see a multi-cellular composition of bone marrow tissue without any traces of ossification **(A)**. At day-14 the collagen-rich matrix began to originate at the bottom of the explant adjacent to the porous membrane **(B)**. At day-28, the collagen-rich matrix deposition increased compared to day-14 **(C)**. Osteocyte-like cells were observed to be embedded in the collagen-rich matrix **(D)**.

2.4.2. Cellular and Compositional Organization

Histological assessment at day-7 (Fig.2.3A) showed the presence of a multi-cellular composition within the marrow explants. Masson's trichrome staining revealed high density collagen regions (blue color) originating around day-14 (Fig.2.3B) at the sections of the explants interfacing with the porous membrane. The collagen dense zone increased in thickness and covered a large portion of the sample by day-28 (Fig.2.3C and Fig.2.4A). Viable osteoblast-like cells (arrowheads in Fig.2.3E) were observed around the collagen-rich regions (Fig.2.3D). In addition, viable osteocyte-like cells (* in Fig.2.3D and Fig.2.5) were embedded in the collagen-rich sections.

Safranin-O fast green staining indicated the absence of proteoglycans (orange color absent in Fig.2.4B) and hence no trace of cartilaginous tissue was found. The collagen matrix synthesized during the ossification of marrow explants was predominantly type I collagen (Fig.2.4C) and was weakly positive for type III collagen (Fig.2.4D). CD44 staining was observed on the surface of some of the osteocyte-like cells present within the collagen matrix (Fig.2.4E). Negative control without the primary antibody was included to negate any non-specific/background staining (Fig.2.4F). High magnification BSEM imaging revealed the ossified regions of the marrow explants embedded in PMMA resin (light-grey areas in Fig.2.5). The osteocyte lacunae were present in the ossified matrix (* in Fig.2.5) with the connecting canalicular extensions (arrowheads in Fig.2.5), which are the typical characteristics of bone tissue observed with BSEM.

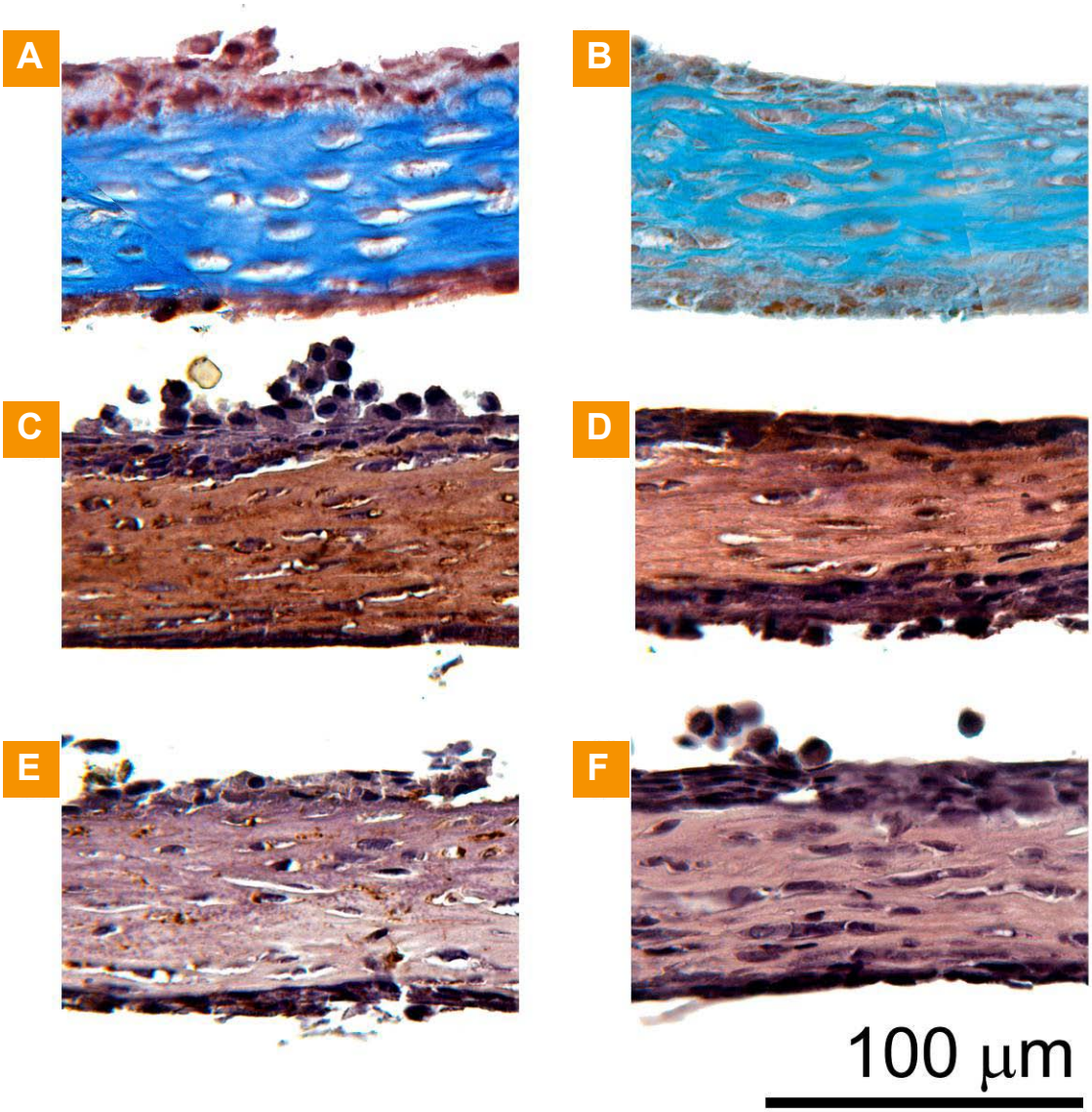


Figure 2.4: Histology and immunohistology images of ossifying marrow explants. **A)** Masson trichrome staining showed the presence of a collagen rich matrix. **B)** Safranin-o fast green staining indicated absence of proteoglycans (no orange color). **C-F)** Immunohistochemistry images for collagen type I, collagen type III, CD44 and negative control. The collagen-rich matrix was predominantly collagen type I **(C)** and weakly positive for collagen type III **(D)**. CD44 staining **(E)** was observed on the surface of osteocyte-like cells embedded within the collagen-rich matrix. Section stained without the primary antibody served as the negative control for immunohistochemical analysis **(F)**.

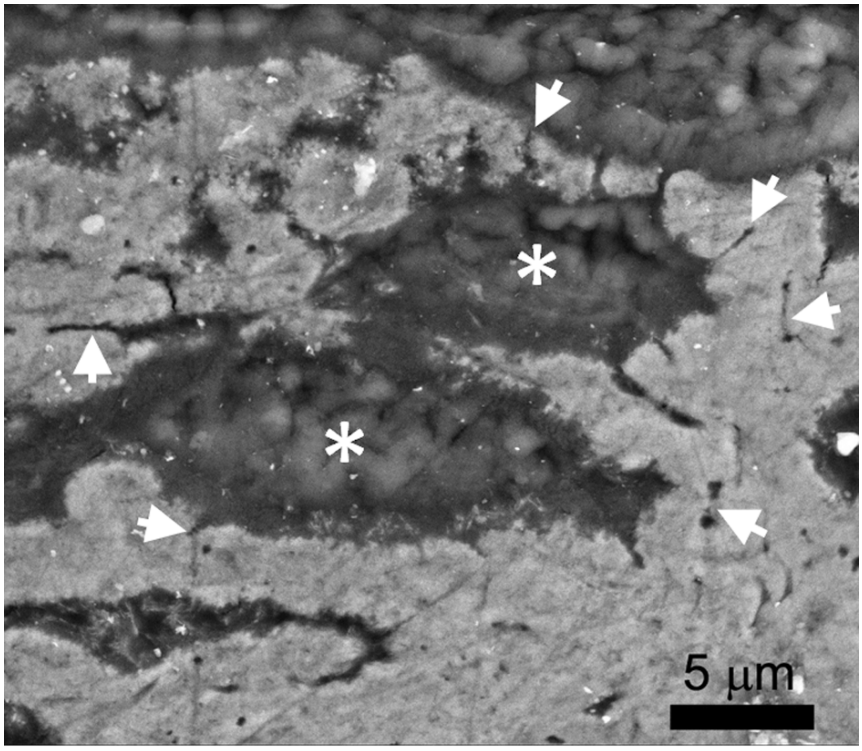


Figure 2.5: High magnification BSEM image of an ossified marrow explant embedded and polished in a PMMA resin. The gray regions in the figure correspond to the mineralized/ossified sections of the explants with * indicating the location of a lacunae where the osteocytes reside. The arrowheads indicate the sections of canalicular extensions which is a typical characteristic of osteocyte cell network.

2.4.3. Temporal Residency of STRO-1+ And CD45+ Cells

The CD45(+) hematopoietic cell fraction within the marrow explants declined with the duration of the culture from >95% at day-0 to 22% by day-28. The STRO-1(+) stromal cell fraction was 3% on day-0, increased to 6% at day-14 and declined to below 1% by day-28. The CD45(-) STRO-1(-) fraction was observed to progressively increase with the duration of the culture from below 2% at day-0 to > 75% by day-28 (Fig.2.6A&B).

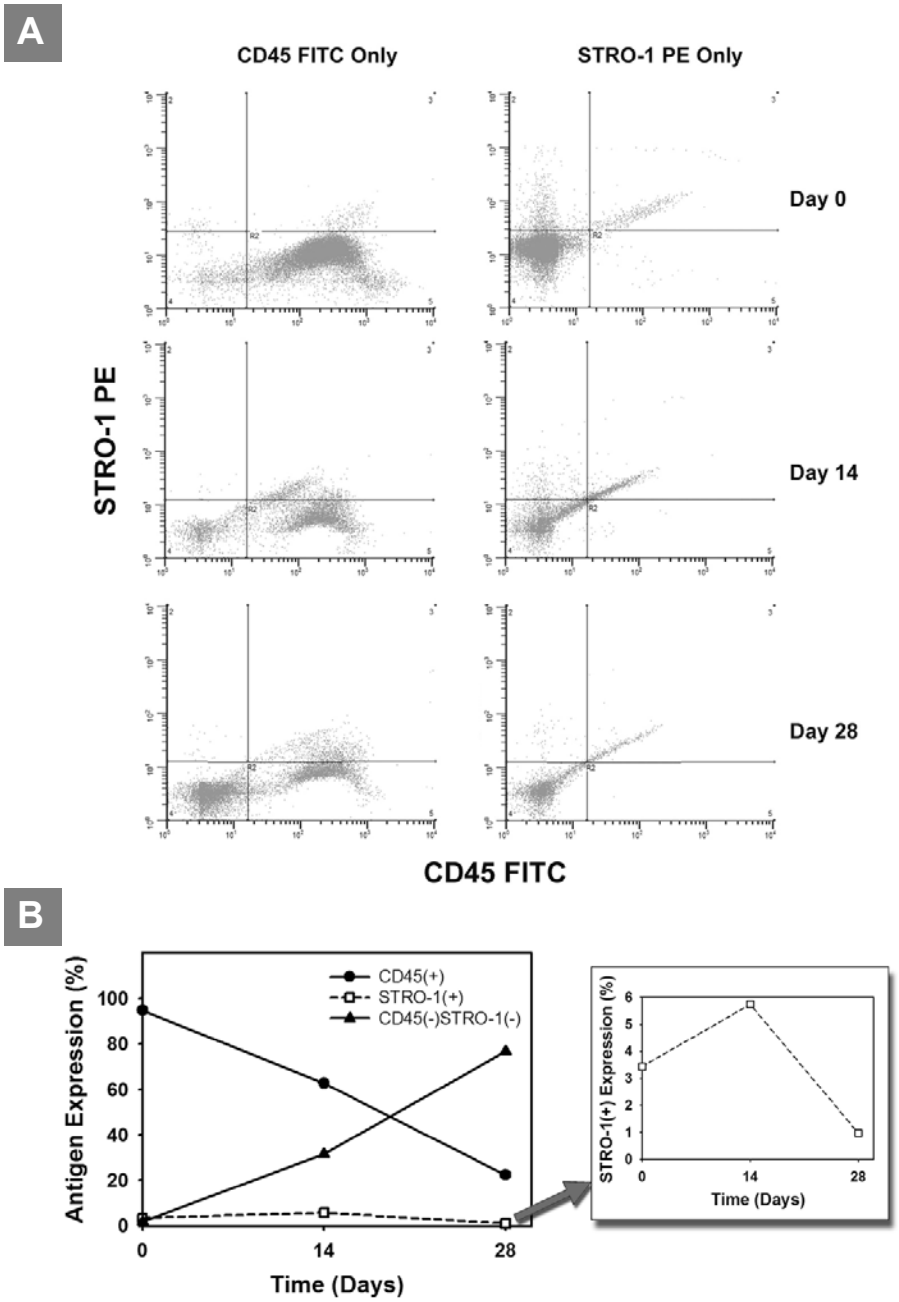


Figure 2.6: Cellular residency of ossifying bone marrow explants. **A)** Flow cytometry data analysis for the expression of CD45(+) hematopoietic cells and STRO-1(+) mesenchymal stem cell progenitors. Events in the lower right quadrant were analyzed as CD45(+) and the events in the upper left quadrant were analyzed as STRO-1(+). **B)** Change is the percentage of CD45(+), STRO-1(+) and CD45(-)STRO-1(-) cells with time within the ossifying marrow explants. The CD45(+) hematopoietic fraction decreased with time. The CD45(-)STRO-1(-) fraction progressively increased with the duration of the culture. The STRO-1(+) mesenchymal stem cell progenitor fraction was maintained throughout the culture period with a peak at day 14 (inset).

2.4.4. Mineral to Matrix Ratio of Ossified Marrow

Raman microspectroscopy analysis indicated that ossified marrow explants displayed all the key vibrational modes characteristic to native bone tissue (Fig.2.7A) [61]. Specifically, the phosphate vibrations (symmetric bending, asymmetric bending and symmetric stretch at about 450 cm^{-1}, 650 cm^{-1} and 959 cm^{-1} respectively) and type-B carbonate substitution vibrations (at about 1070 cm^{-1}) were present. It was also observed that apatitic mineral (symmetric stretch for PO_4^{-3}; 959 cm^{-1}) to N-H bend (methyl deformation; CH_2 wagging; 1450 cm^{-1}) ratio [61] was significantly lower in ossified marrow explants relative to native bone tissue (Fig.2.7B).

2.4.5. The CA/P Ratio Analysis of Ossified Marrow

Ca/P (weight ratio) analysis was conducted by EDS on the ossified marrow (OM), native periosteal rat tibia (PRT) and native intracortical rat tibia (IRT) (Fig.2.7C). OM displayed both Ca and P peaks and had a Ca/P value of 1.32 (st. dev: 0.20, n=21), which was significantly less than IRT (1.79, st. dev:0.30, n=6) and comparable to PRT (1.28, st. dev:0.10, n=5) (Fig.2.7C). Human bone has been reported to have a Ca/P ratio of 2.21 (st. dev: 0.29, n=80, ages: 15-55)[65], which was significantly greater than that of native rat tibia and OM.

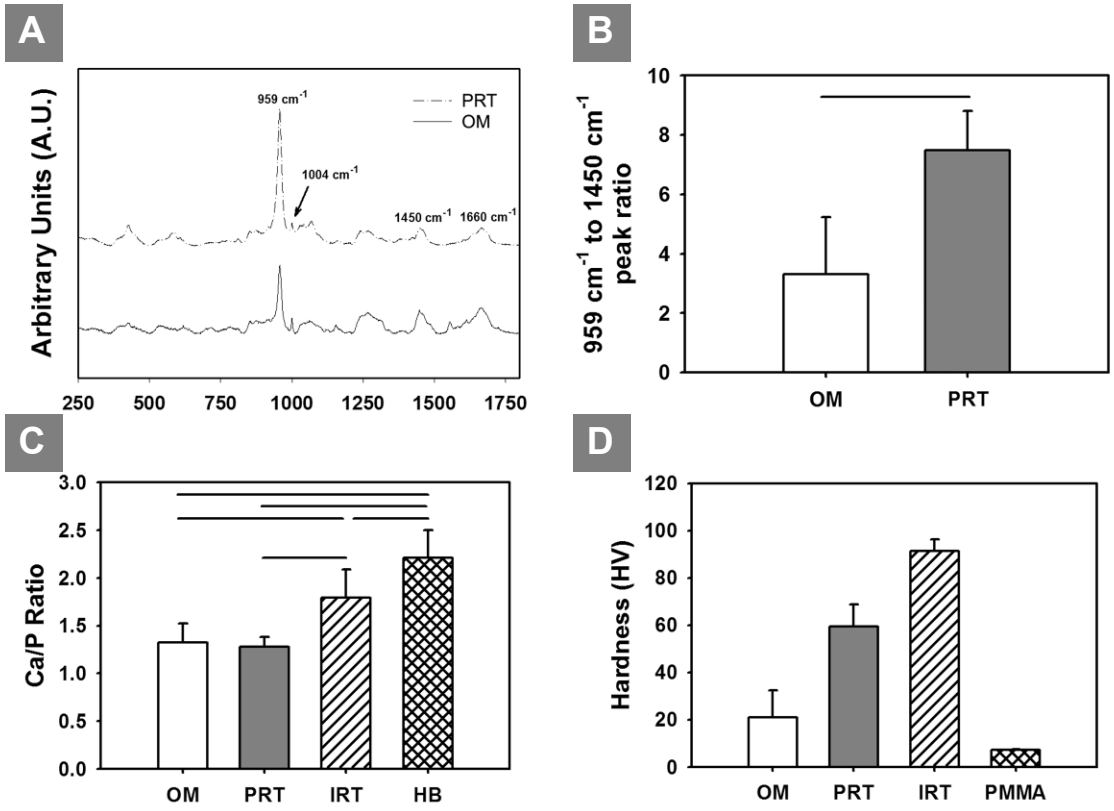


Figure 2.7: Compositional and micromechanical characterization of ossified marrow (OM) in comparison to intracortical rat tibia (IRT) and periosteal rat tibia (PRT). **A)** Raman microspectroscopic analysis of OM in comparison to PRT indicated that OM display all the characteristic peaks of PRT (450 cm^{-1}, 650 cm^{-1}, 959 cm^{-1}, 1070 cm^{-1}, 1450 cm^{-1} and 1660 cm^{-1}) with a lower intensity apatitic mineral-peak (959 cm^{-1}) which is an indicator of newly mineralizing bone matrix. **B)** Apatitic mineral (959 cm^{-1}) to N-H bend (1450 cm^{-1}) ratio indicated the crystallinity of OM (n=10) to be lower ($p<0.05$) than PRT (n=3). **C)** Comparison of Ca/P ratio of OM with PRT, IRT and human bone (HB). Ca/P ratio of HB (n=80) was significantly greater than IRT (n=6), PRT (n=5) and OM (n=21). IRT displayed a greater Ca/P ratio than OM, whereas Ca/P ratio of PRT was not statistically different than that of ossified marrow. Lines connecting individual groups indicate statistical significance ($p<0.05$). (Ca/P ratio of HB was based on: [65]). **D)** Vickers Hardness (HV) values for OM in comparison to PRT and IRT measured by Microindentation tests. IRT displayed a significantly higher HV compared to PRT as well as OM. The HV value of the embedding resin (PMMA) was included for comparison purposes. All the groups displayed in the figure are significantly different from each other ($p<0.05$).

2.4.6. Microhardness of Ossified Marrow Explants

Microindentation test indicated that intracortical rat tibia (IRT, n=10) had a significantly greater Vickers Hardness (HV) value than periosteal rat tibia (PRT, n=10) and ossified marrow (OM, n=26) (Fig.2.7D). The hardness of the embedding resin (PMMA, n=10) was also measured for comparison purposes, which was observed to be significantly less than all the groups (Fig.2.7D).

2.5. Discussion and Conclusion

The results of this study showed that: 1) rat marrow tissue ossified inherently *in vitro* without addition of excipient osteoinductive factors, 2) ossified marrow formed a visible 3D plate-like bone tissue with viable osteoblast-like cells laying the mineralized matrix and viable osteocytes embedded within the ossified matrix, 3) marrow ossification process supported the residency of STRO-1+ MSCs and CD45+ hematopoietic cells throughout the culture period, 4) hematopoietic cell fraction decreased over time whereas MSC fraction was maintained throughout the culture period, 5) Ca/P ratio was comparable between ossified marrow tissue and newly forming native bone tissue (periosteal tibia), 6) ossified marrow tissue displayed all the characteristic Raman peaks of bone tissue with a lower mineral to matrix ratio, 7) microhardness of ossified marrow tissue was less than native bone tissue.

Histological assessment of the Masson's trichrome stained ossifying explant sections revealed collagen-rich regions (blue color) starting day-14 (Fig.2.3B), which was

observed to be corresponding to the ossified volume of the explants as detected by μCT conducted at day-28 (Fig.2.2B & Fig.2.3C). These collagen-rich sections (Fig.2.3C & Fig.2.4A) were verified to be mostly type-I collagen (Fig.2.4C) which is the most abundant type in bone tissue. In addition to type-I collagen, type-III collagen was observed to be present at a lower extent in the ossified marrow tissue (Fig.2.4D). Collagen type-III is known to be expressed by early-stage osteoblasts during the synthesis of woven bone tissue, which is then replaced by the osteoid (rich in collagen type-I) during the remodeling phase reducing the collagen type-III presence dramatically [68]. Therefore the low-level presence of type-III collagen in ossified marrow explant is an indicator that the type of bone present is woven bone.

The ossified matrix was observed to be surrounded by or in contact with an unmineralized tissue (Fig.2.3C&D). Viable osteoblast-like cells were apparent surrounding the ossification site, which were in the process of laying the mineralized matrix and getting trapped as osteocytes (Fig.2.3D). In addition, viable CD44 positive osteocyte-like cells were observed to be present in the collagen-rich ossified matrix (Fig.2.4E) with typical lacunae and primitive canalicular extensions (Fig.2.5). These results indicate that marrow explants form a 3-dimensional plate-like ossified matrix (Fig.2.2), which houses the basic cellular elements (osteoblasts and osteocytes) of bone tissue.

The histological analysis of the *in vitro* ossified marrow explants with Safranin-O/fast green staining displayed (Fig.2.4B) the absence of proteoglycans at all time points which can be found abundantly in cartilaginous matrix. This finding is expected

considering the fact that the *in vitro* ossifying marrow tissue in this study did not experience any mechanical stimulation and as described above, intramembranous ossification is generally observed in well-stabilized fracture sites with minimal deformation of the fracture callus [18, 19]. Therefore it would be reasonable to expect endochondral ossification if mechanical deformations at the level that is observed at nonstabilized fracture sites are applied during *in vitro* ossification of marrow tissue. It should be noted that intramembranous ossification commonly takes place in various locations in the skeletal system [20-24] and produces a stronger and accelerated healing of the fracture than that healed by endochondral ossification [19]. Therefore, understanding the biology of intramembranous healing via such *in vitro* models would allow developing biological therapies for improving fracture healing.

Earlier *in vivo* studies of ectopically implanted explants demonstrated that the cells of hematopoietic origin left the marrow explants [69, 70]. Our study supports this observation which is evident by the decrease in the CD45+ hematopoietic cell fraction throughout the culture period (Fig.2.6). The STRO-1+ MSC fraction was maintained throughout the culture period indicating the perennial presence of a stem cell progenitor population in our culture system that gave rise to the differentiated cells. By the end of the culture, a large fraction (>75%) of the cells were negative for both CD45 and STRO-1 suggesting the presence of committed/differentiated cells of mesenchymal origin, including osteoblasts and osteocytes (Fig.2.6).

In this study, the Ca/P ratio of ossified marrow was compared with human bone tissue (value obtained from literature: [65]) and rat bone tissue in terms of crystalline

level [64]. It was previously shown that Ca/P ratio increases in osteoblast cultures (30 days long) with time suggesting maturation of crystal growth, which was supported by X-ray and infrared spectroscopic analyses [64]. Our results indicated that the Ca/P ratio and hence the maturity of the ossified marrow tissue was comparable to that of the newly forming bone tissue (i.e. rat tibial diaphysis periosteal surface [63]) (Fig.2.7C). In attestation, Raman microspectroscopy showed that the crystals making up the mineralized component of the marrow explants were carbonated apatite displaying all the key vibrational modes typical of those observed in natural bone tissue (rat tibial periosteal diaphysis) (Fig.2.7A). The phosphate symmetric stretch vibrations from marrow explants' crystals appeared at the same wavelength with that of periosteal bone (959 cm^{-1}) indicating that the maturity of crystals in explants was comparable to that of early stage bone. However, it was also observed (Raman microspectroscopy) that 959 cm^{-1} (apatitic mineral, symmetric stretch for PO_4^{-3}) to 1450 cm^{-1} (N-H bending, methyl deformation; CH_2 wagging) ratio was lower in ossified marrow explants relative to native bone tissue (Fig.2.7B), which is an indicator of lower mineralization and hence newly ossifying bone tissue[61]. In addition, since the spectra were collected under similar data integration times and the phosphate band intensities were lower in the case of ossified marrow explants at x50 high magnification objective, the amount of mineral marrow explants was less than that of bone.

Microscopic hardness of normal bone tissue has been suggested to be a direct indicator of its degree of mineralization and an increase in microhardness has been associated with maturation of the ossified tissue [66]. Therefore Vickers hardness of the

ossified marrow explants was used to estimate the maturity and compare with PRT (newly formed periosteal bone) and IRT (relatively more mature intracortical bone). The results based on the hardness measurements indicated that IRT and PRT were stiffer (hence more mineralized) than ossified marrow (Fig.2.7D), which is expected considering the culture duration of the marrow explants (28 days) which is significantly less than the age of the donor rats (80-90 days) of the tested native bone samples [62]. It should also be noted that the ossified marrow explants display a woven bone structure whereas the IRT and PRT are lamellar bone. Therefore, the maturity and hence the microhardness of the ossified marrow explants are expected to increase with longer culture durations, which warrants further investigation.

Bone formation involves the differentiation of MSCs or osteoblast progenitors into osteoblasts, which are responsible for producing the mineralized bone matrix [71, 72]. The *in vitro* monolayer models of bone formation that are aimed to study the differentiation into osteoblastic phenotype generally employ various purification steps to isolate the progenitors from sources such as bone marrow [73]. These purification steps include the isolation of the adherent multipotent MSCs by eliminating the non-adherent hematopoietic stem cells (HSCs) and the osteopoietic accessory cells (OACs) [11]. In order for these purified population of cells to display osteogenic potential, a variety of osteoinductive factors (e.g. dexamethasone and BMPs) are added in a range of concentrations [27, 28]. It has been suggested that the presence of other cell types (i.e. HSCs, OACs) play a role in differentiation of MSCs to osteoprogenitors and osteoblasts and these cell types regulate each other's functions [6-15]. These cell types are housed

cumulatively in bone marrow tissue making it a critical component in bone regeneration [74-76]. Previous studies have demonstrated that bone marrow explants inherently undergo ossification *in vivo* [48, 70] or *in vitro* without the addition of excipient osteoinductive factors [3, 49, 77]. In fact, we have recently shown that when the cellular integrity of intact bone marrow tissue is disrupted and the cells are cultured according to the traditional methods (i.e. eliminating the non-adherent cells with media change), the inherent ossification potential of bone marrow tissue is lost [3]. Therefore, *in vitro* marrow culture system presented here demonstrates the inherent ossification potential of marrow tissue allowing it to be used as a model to study osteogenesis to develop therapeutic strategies for skeletal reconstruction.

The outcomes of this study showed that rat bone marrow tissue inherently ossified *in vitro* with the typical characteristics of newly forming bone tissue, such as viable embedded osteocyte-like cells, viable osteoblast-like cells and compositional properties including specific Raman peaks and a typical Ca/P ratio. Marrow ossification process displayed the typical characteristics of intramembranous ossification and therefore, the inherent *in vitro* ossification of bone marrow tissue can be a useful as a scaffold-free, multicellular and 3D model to study the intramembranous osteogenesis process in terms of morphological differentiation, cellular composition, mechanoresponsiveness and gene and protein expression. This model may also be useful for developing high-throughput drug screening models of osteogenesis for drug discovery programs [46, 78].

2.6. Acknowledgements

This study was funded by a grant from the Musculoskeletal Transplant Foundation. We would like to thank Purdue University Histopathology service laboratory (David VanSickle Ph.D. DVM, Paul Snyder Ph.D. DVM, Carol Bain) for their support with histology and immunohistochemistry. We would like to acknowledge the supports of Pam Lachick with the μCT system and David Meyer with the microindenter. We also would like to thank Purdue Life Science Microscopy Facility director Debby Sherman for her support with EDS and BSEM systems.

2.7. References

1. Gerstenfeld, L.C., D.M. Cullinane, G.L. Barnes, D.T. Graves, and T.A. Einhorn, *Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation.* Journal of Cellular Biochemistry, 2003. **88**(5): p. 873-884.
2. Barnes, G.L., P.J. Kostenuik, L.C. Gerstenfeld, and T.A. Einhorn, *Growth factor regulation of fracture repair.* Journal of Bone and Mineral Research, 1999. **14**(11): p. 1805-1815.
3. Gurkan, U.A., J. Gargac, and O. Akkus, *The Sequential Production Profiles of Growth Factors and Their Relations to Bone Volume in Ossifying Bone Marrow Explants.* Tissue Eng Part A, 2010: p. DOI: 10.1089/ten.TEA.2009.0565.
4. Palomares, K.T.S., R.E. Gleason, Z.D. Mason, D.M. Cullinane, T.A. Einhorn, L.C. Gerstenfeld, and E.F. Morgan, *Mechanical Stimulation Alters Tissue Differentiation and Molecular Expression during Bone Healing.* Journal of Orthopaedic Research, 2009. **27**(9): p. 1123-1132.
5. Gurkan, U.A. and O. Akkus, *The Mechanical Environment of Bone Marrow: A Review.* Annals of Biomedical Engineering, 2008. **36**(12): p. 1978-1991.
6. Jung, Y.G., J.H. Song, Y. Shiozawa, J.C. Wang, Z. Wang, B. Williams, A. Havens, A. Schneider, C.X. Ge, R.T. Franceschi, L.K. McCauley, P.H. Krebsbach, and R.S. Taichman, *Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche.* Stem Cells, 2008. **26**(8): p. 2042-2051.

7. Moioli, E.K., P.A. Clark, M. Chen, J.E. Dennis, H.P. Erickson, S.L. Gerson, and J.J. Mao, *Synergistic Actions of Hematopoietic and Mesenchymal Stem/Progenitor Cells in Vascularizing Bioengineered Tissues.* Plos One, 2008. **3**(12): p. e3922.

8. Wu, J.Y., D.T. Scadden, and H.M. Kronenberg, *Role of the Osteoblast Lineage in the Bone Marrow Hematopoietic Niches.* Journal of Bone and Mineral Research, 2009. **24**(5): p. 759-764.

9. Porter, R.L. and L.M. Calvi, *Communications between bone cells and hematopoietic stem cells.* Archives of Biochemistry and Biophysics, 2008. **473**(2): p. 193-200.

10. Eipers, P.G., S. Kale, R.S. Taichman, G.G. Pipia, N.A. Swords, K.G. Mann, and M.W. Long, *Bone marrow accessory cells regulate human bone precursor cell development.* Experimental Hematology, 2000. **28**(7): p. 815-825.

11. van den Dolder, J. and J.A. Jansen, *Enrichment of osteogenic cell populations from rat bone marrow stroma.* Biomaterials, 2007. **28**(2): p. 249-255.

12. Taichman, R.S., M.J. Reilly, and S.G. Emerson, *Human osteoblasts support human hematopoietic progenitor cells in in vitro bone marrow cultures.* Blood, 1996. **87**(2): p. 518-524.

13. Taichman, R.S., M.J. Reilly, R.S. Verma, K. Ehrenman, and S.G. Emerson, *Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors.* British Journal of Haematology, 2001. **112**(2): p. 438-448.

14. de Barros, A.P.D.N., C.M. Takiya, L.R. Garzoni, M.L. Leal-Ferreira, H.S. Dutra, L.B. Chiarini, M.N. Meirelles, R. Borojevic, and M.I.D. Rossi, *Osteoblasts and Bone Marrow Mesenchymal Stromal Cells Control Hematopoietic Stem Cell Migration and Proliferation in 3D In Vitro Model.* Plos One, 2010. **5**(2): p. -.

15. Chitteti, B.R., Y.-H. Cheng, B. Poteat, S. Rodriguez-Rodriguez, W.S. Goebel, N. Carlesso, M.A. Kacena, and E.F. Srour, *Impact of interactions of cellular components of the bone marrow microenvironment on hematopoietic stem and progenitor cell function.* Blood, 2010. **(Blood-2009-09-246173)**.

16. Probst, A. and H.U. Spiegel, *Cellular mechanisms of bone repair.* Journal of Investigative Surgery, 1997. **10**(3): p. 77-86.

17. Ferguson, C., E. Alpern, T. Miclau, and J.A. Helms, *Does adult fracture repair recapitulate embryonic skeletal formation?* Mechanisms of Development, 1999. **87**(1-2): p. 57-66.

18. Thompson, Z., T. Miclau, D. Hu, and J.A. Helms, *A model for intramembranous ossification during fracture healing.* Journal of Orthopaedic Research, 2002. **20**(5): p. 1091-1098.

19. Histing, T., P. Garcia, R. Matthys, M. Leidinger, J.H. Holstein, A. Kristen, T. Pohlemann, and M.D. Menger, *An Internal Locking Plate to Study Intramembranous Bone Healing in a Mouse Femur Fracture Model.* Journal of Orthopaedic Research, 2010. **28**(3): p. 397-402.

20. Opperman, L.A., *Cranial sutures as intramembranous bone growth sites*. Developmental Dynamics, 2000. **219**(4): p. 472-485.

21. Shorey, S., J.N.M. Heersche, and M.F. Manolson, *The relative contribution of cysteine proteinases and matrix metalloproteinases to the resorption process in osteoclasts derived from long bone and scapula.* Bone, 2004. **35**(4): p. 909-917.

22. Hall, B.K., *Development of the clavicles in birds and mammals.* Journal of Experimental Zoology, 2001. **289**(3): p. 153-161.

23. Fujii, T., T. Ueno, T. Kagawa, Y. Sakata, and T. Sugahara, *Comparison of bone formation ingrafted periosteum harvested from tibia and calvaria.* Microscopy Research and Technique, 2006. **69**(7): p. 580-584.

24. Colnot, C., *Skeletal Cell Fate Decisions Within Periosteum and Bone Marrow During Bone Regeneration.* Journal of Bone and Mineral Research, 2009. **24**(2): p. 274-282.

25. Aronow, M.A., L.C. Gerstenfeld, T.A. Owen, M.S. Tassinari, G.S. Stein, and J.B. Lian, *Factors That Promote Progressive Development of the Osteoblast Phenotype in Cultured Fetal-Rat Calvaria Cells.* Journal of Cellular Physiology, 1990. **143**(2): p. 213-221.

26. Yamamoto, N., K. Furuya, and K. Hanada, *Progressive development of the osteoblast phenotype during differentiation of osteoprogenitor cells derived from fetal rat calvaria: Model for in vitro bone formation.* Biological & Pharmaceutical Bulletin, 2002. **25**(4): p. 509-515.

27. Satomura, K. and M. Nagayama, *Ultrastructure of Mineralized Nodules Formed in Rat Bone-Marrow Stromal Cell-Culture Invitro.* Acta Anatomica, 1991. **142**(2): p. 97-104.

28. Huang, Z.N., E.R. Nelson, R.L. Smith, and S.B. Goodman, *The sequential expression profiles of growth factors from osteroprogenitors to osteoblasts In vitro.* Tissue Engineering, 2007. **13**(9): p. 2311-2320.

29. Beresford, J.N., S.E. Graves, and C.A. Smoothy, *Formation of Mineralized Nodules by Bone Derived Cells-Invitro - a Model of Bone-Formation.* American Journal of Medical Genetics, 1993. **45**(2): p. 163-178.

30. Beresford, J.N., C.J. Joyner, C. Devlin, and J.T. Triffitt, *The Effects of Dexamethasone and 1,25-Dihydroxyvitamin D-3 on Osteogenic Differentiation of Human Marrow Stromal Cells in-Vitro.* Archives of Oral Biology, 1994. **39**(11): p. 941-947.

31. Jaiswal, N., S.E. Haynesworth, A.I. Caplan, and S.P. Bruder, *Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro.* Journal of Cellular Biochemistry, 1997. **64**(2): p. 295-312.

32. Cheng, S.L., J.W. Yang, L. Rifas, S.F. Zhang, and L.V. Avioli, *Differentiation of Human Bone-Marrow Osteogenic Stromal Cells in Vitro - Induction of the Osteoblast Phenotype by Dexamethasone.* Endocrinology, 1994. **134**(1): p. 277-286.

33. Malaval, L., D. Modrowski, A.K. Gupta, and J.E. Aubin, *Cellular Expression of Bone-Related Proteins during in-Vitro Osteogenesis in Rat Bone-Marrow Stromal Cell-Cultures.* Journal of Cellular Physiology, 1994. **158**(3): p. 555-572.

34. Bodine, P.V.N. and B.S. Komm, *Tissue culture models for studies of hormone and vitamin action in bone cells.* Vitamins and Hormones - Advances in Research and Applications, Vol 64, 2002. **64**: p. 101-151.

35. Minkin, C., S. Stjames, H.H. Tao, X.H. Yu, S. Pockwinse, C. Mackay, and S.C. Marks, *Skeletal Development and Formation of Osteoclast-Like Cells from Insitu Progenitors in Fetal Mouse Metatarsals Cultured in Chemically Defined Medium.* Bone and Mineral, 1991. **12**(3): p. 141-155.

36. Tao, H. and C. Minkin, *The Effects of 1,25-Dihydroxyvitamin D-3 on Osteoclast Formation in Fetal Mouse Metatarsal Organ-Cultures.* Bone, 1994. **15**(2): p. 217-223.

37. Gupta, A.K., Y. Eshraghi, C. Gliniak, and A.K. Gosain, *Nonviral Transfection of Mouse Calvarial Organ In Vitro Using Accell-Modified siRNA.* Plastic and Reconstructive Surgery, 2010. **125**(2): p. 494-501.

38. Cho, T.J., L.C. Gerstenfeld, and T.A. Einhorn, *Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing.* Journal of Bone and Mineral Research, 2002. **17**(3): p. 513-520.

39. Le, A.X., T. Miclau, D. Hu, and J.A. Helms, *Molecular aspects of healing in stabilized and non-stabilized fractures.* Journal of Orthopaedic Research, 2001. **19**(1): p. 78-84.

40. Tatsuyama, K., Y. Maezawa, H. Baba, Y. Imamura, and M. Fukuda, *Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone.* European Journal of Histochemistry, 2000. **44**(3): p. 269-278.

41. Muraglia, A., I. Martin, R. Cancedda, and R. Quarto, *A nude mouse model for human bone formation in unloaded conditions.* Bone, 1998. **22**(5): p. 131s-134s.

42. Birgersdotter, A., R. Sandberg, and I. Ernberg, *Gene expression perturbation in vitro - A growing case for three-dimensional (3D) culture systems.* Seminars in Cancer Biology, 2005. **15**(5): p. 405-412.

43. Yamada, K.M. and E. Cukierman, *Modeling tissue morphogenesis and cancer in 3D.* Cell, 2007. **130**(4): p. 601-610.

44. Tibbitt, M.W. and K.S. Anseth, *Hydrogels as Extracellular Matrix Mimics for 3D Cell Culture.* Biotechnology and Bioengineering, 2009. **103**(4): p. 655-663.

45. Mazzoleni, G., D. Di Lorenzo, and N. Steimberg, *Modelling tissues in 3D: the next future of pharmaco-toxicology and food research?* Genes and Nutrition, 2009. **4**(1): p. 13-22.

46. Kunz-Schughart, L.A., J.P. Freyer, F. Hofstaedter, and R. Ebner, *The use of 3-D cultures for high-throughput screening: The multicellular spheroid model.* Journal of Biomolecular Screening, 2004. **9**(4): p. 273-285.

47. Petersen, M.C., J. Lazar, H.J. Jacob, and T. Wakatsuki, *Tissue engineering: a new frontier in physiological genomics.* Physiological Genomics, 2007. **32**(1): p. 28-32.

48. Tavassoli, M. and W.H. Crosby, *Transplantation of Marrow to Extramedullary Sites.* Science, 1968. **161**(3836): p. 54-&.

49. Luria, E.A., M.E. Owen, A.J. Friedenstein, J.F. Morris, and S.A. Kuznetsow, *Bone-Formation in Organ-Cultures of Bone-Marrow.* Cell and Tissue Research, 1987. **248**(2): p. 449-454.

50. Morgan, E.F., Z.D. Mason, K.B. Chien, A.J. Pfeiffer, G.L. Barnes, T.A. Einhorn, and L.C. Gerstenfeld, *Micro-computed tomography assessment of fracture healing: Relationships among callus structure, composition, and mechanical function.* Bone, 2009. **44**(2): p. 335-344.

51. Muller, R., H. Van Campenhout, B. Van Damme, G. Van Der Perre, J. Dequeker, T. Hildebrand, and P. Ruegsegger, *Morphometric analysis of human bone biopsies: A quantitative structural comparison of histological sections and micro-computed tomography.* Bone, 1998. **23**(1): p. 59-66.

52. Oest, M.E., K.M. Dupont, H.J. Kong, D.J. Mooney, and R.E. Guldberg, *Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects.* Journal of Orthopaedic Research, 2007. **25**(7): p. 941-950.

53. Majewski, M., P.E. Ochsner, F. Liu, R. Fluckiger, and C.H. Evans, *Accelerated healing of the rat Achilles tendon in response to autologous conditioned serum.* Am J Sports Med, 2009. **37**(11): p. 2117-25.

54. Noonan, K.J., J.W. Stevens, R. Tammi, M. Tammi, J.A. Hernandez, and R.J. Midura, *Spatial distribution of CD44 and hyaluronan in the proximal tibia of the growing rat.* Journal of Orthopaedic Research, 1996. **14**(4): p. 573-581.

55. Ralph, S.J., M.L. Thomas, C.C. Morton, and I.S. Trowbridge, *Structural variants of human T200 glycoprotein (leukocyte-common antigen).* EMBO J, 1987. **6**(5): p. 1251-7.

56. De Ugarte, D.A., Z. Alfonso, P.A. Zuk, A. Elbarbary, M. Zhu, P. Ashjian, P. Benhaim, M.H. Hedrick, and J.K. Fraser, *Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow.* Immunology Letters, 2003. **89**(2-3): p. 267-270.

57. Dennis, J.E., J.P. Carbillet, A.I. Caplan, and P. Charbord, *The STRO-1+marrow cell population is multipotential.* Cells Tissues Organs, 2002. **170**(2-3): p. 73-82.

58. Gronthos, S., S.E. Graves, S. Ohta, and P.J. Simmons, *The Stro-1(+) Fraction of Adult Human Bone-Marrow Contains the Osteogenic Precursors.* Blood, 1994. **84**(12): p. 4164-4173.

59. Simmons, P.J. and B. Torokstorb, *Identification of Stromal Cell Precursors in Human Bone-Marrow by a Novel Monoclonal-Antibody, Stro-1.* Blood, 1991. **78**(1): p. 55-62.

60. Byers, R.J., J. Brown, C. Brandwood, P. Wood, W. Staley, L. Hainey, A.J. Freemont, and J.A. Hoyland, *Osteoblastic differentiation and mRNA analysis of STRO-1-positive human bone marrow stromal cells using primary in vitro culture and poly (A) PCR.* Journal of Pathology, 1999. **187**(3): p. 374-381.

61. Wang, C.Y., Y. Wang, N.T. Huffman, C.Y. Cui, X.M. Yao, S. Midura, R.J. Midura, and J.P. Gorski, *Confocal Laser Raman Microspectroscopy of Biomineralization Foci in UMR 106 Osteoblastic Cultures Reveals Temporally Synchronized Protein Changes Preceding and Accompanying Mineral Crystal Deposition.* Journal of Biological Chemistry, 2009. **284**(11): p. 7100-7113.

62. Donnelly, E., A.L. Boskey, S.P. Baker, and M.C.H. van der Meulen, *Effects of tissue age on bone tissue material composition and nanomechanical properties in the rat cortex.* Journal of Biomedical Materials Research Part A, 2010. **92A**(3): p. 1048-1056.

63. Frost, H.M. and W.S.S. Jee, *On the Rat Model of Human Osteopenias and Osteoporosis.* Bone and Mineral, 1992. **18**(3): p. 227-236.

64. Rohde, M. and H. Mayer, *Exocytotic process as a novel model for mineralization by osteoblasts In vitro and In vivo determined by electron microscopic analysis.* Calcified Tissue International, 2007. **80**(5): p. 323-336.

65. Tzaphlidou, M. and V. Zaichick, *Sex and age related Ca/P ratio in cortical bone of iliac crest of healthy humans.* Journal of Radioanalytical and Nuclear Chemistry, 2004. **259**(2): p. 347-349.

66. Weaver, J.K., *Microscopic Hardness of Bone.* Journal of Bone and Joint Surgery-American Volume, 1966. **A 48**(2): p. 273-&.

67. Dall'Ara, E., C. Ohman, M. Baleani, and M. Viceconti, *The effect of tissue condition and applied load on Vickers hardness of human trabecular bone.* Journal of Biomechanics, 2007. **40**(14): p. 3267-3270.

68. Carter, D.H., P. Sloan, and J.E. Aaron, *Immunolocalization of Collagen Type-I and Type-Iii, Tenascin, and Fibronectin in Intramembranous Bone.* Journal of Histochemistry & Cytochemistry, 1991. **39**(5): p. 599-606.

69. Friedenstein, A.J., A.A. Ivanovsmolenski, R.K. Chajlakjan, U.F. Gorskaya, A.I. Kuralesova, N.W. Latzinik, and U.W. Gerasimow, *Origin of Bone-Marrow Stromal Mechanocytes in Radiochimeras and Heterotopic Transplants.* Experimental Hematology, 1978. **6**(5): p. 440-444.

70. Friedenstein, A.J., K.V. Petrakov, A.I. Kuroleso, and G.P. Frolova, *Heterotopic Transplants of Bone Marrow - Analysis of Precursor Cells for Osteogenic and Hematopoietic Tissues.* Transplantation, 1968. **6**(2): p. 230-247.

71. Bruder, S.P., D.J. Fink, and A.I. Caplan, *Mesenchymal Stem-Cells in in Bone-Development, Bone Repair, and Skeletal Regeneration Therapy.* Journal of Cellular Biochemistry, 1994. **56**(3): p. 283-294.

72. Caplan, A.I., *Mesenchymal Stem-Cells.* Journal of Orthopaedic Research, 1991. **9**(5): p. 641-650.

73. Ohishi, M. and E. Schipani, *Bone Marrow Mesenchymal Stem Cells.* Journal of Cellular Biochemistry, 2010. **109**(2): p. 277-282.

74. Devine, M.J., C.M. Mierisch, E. Jang, P.C. Anderson, and G. Balian, *Transplanted bone marrow cells localize to fracture callus in a mouse model.* Journal of Orthopaedic Research, 2002. **20**(6): p. 1232-1239.

75. Granero-Molto, F., J.A. Weis, M.I. Miga, B. Landis, T.J. Myers, L. O'Rear, L. Longobardi, E.D. Jansen, D.P. Mortlock, and A. Spagnoli, *Regenerative Effects of Transplanted Mesenchymal Stem Cells in Fracture Healing.* Stem Cells, 2009. **27**(8): p. 1887-1898.

76. Muschler, G.F., H. Nitto, C.A. Boehm, and K.A. Easley, *Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors.* Journal of Orthopaedic Research, 2001. **19**(1): p. 117-125.

77. Schoeters, G.E.R., L. Desaintgeorges, R. Vandenheuvel, and O. Vanderborght, *Mineralization of Adult-Mouse Bone-Marrow Invitro.* Cell and Tissue Kinetics, 1988. **21**(5): p. 363-374.

78. Giese, K., J. Kaufmann, G.J. Pronk, and A. Klippel, *Unravelling novel intracellular pathways in cell-based assays.* Drug Discovery Today, 2002. **7**(3): p. 179-186.

3. THE SEQUENTIAL PRODUCTION PROFILES OF GROWTH FACTORS AND THEIR RELATIONS TO BONE VOLUME IN OSSIFYING BONE MARROW EXPLANTS

(Tissue Engineering Part A, 2010, vol.16, DOI: 10.1089/ten.TEA.2009.0565)

3.1. Abstract

Osteogenesis is a complex process that involves the synergistic contribution of multiple cell types and numerous growth factors (GFs). In order to develop effective bone tissue engineering strategies employing GFs, it is essential to delineate the complex and interconnected role of GFs in osteogenesis. The studies investigating the temporal involvement of GFs in osteogenesis are limited to *in vitro* studies with single cell types or complex *in vivo* studies. There is a need for platforms that embody the physiological characteristics and the multicellular environment of natural osteogenesis. Marrow tissue houses various cell types that are known to be involved in osteogenesis and *in vitro* cultures of marrow inherently undergo osteogenesis process. Self-inductive ossification of marrow explants *in vitro* can be employed as a representative multicellular and 3-dimensional model of osteogenesis. Therefore, the aims of this study were to employ the rat bone marrow explant ossification model to determine: 1) the temporal production

profiles of key GFs involved in osteogenesis, 2) the relation between GF production and ossification, and, 3) the relations between the GF levels throughout ossification. Temporal production profiles of transforming growth factor beta-1 (TGF-β1), bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1); and the bone-related proteins alkaline phosphatase (AP), osteocalcin (OC) were obtained by enzyme-linked immunosorbent assay (ELISA) conducted at days 2, 7, 12, 14, 19 and 21. The final amount of ossification (ossified volume, OV) was measured by micro computed tomography (μCT) at day 21. TGF-β1, BMP-2, VEGF, IGF-1, AP and OC were produced by the ossifying marrow explants differentially over time. The early production of IGF-1 (day 2) correlated positively ($r=0.868$) with OV; however, latent production of IGF-1 correlated negatively (day14: $r=-0.813$; and day-19: $r=-0.865$) with OV. OV also correlated with VEGF levels at day-12 ($r=0.988$) and at day-14 ($r=0.970$). Production of GFs also correlated to each other across time points which indicate the complex and interconnected contribution of various GFs in osteogenesis. Therefore tissue engineering strategies towards bone regeneration should consider the richness of GFs involved in osteogenesis as well as their dynamically varying participation over time.

Keywords: marrow stromal cells, mesenchymal stem cells, organ culture, mineralization.

3.2. Introduction

Osteogenesis is a complex process that involves the contribution of multiple cell types and numerous cytokines or growth factors (GFs). GFs are known to influence cell division, differentiation, matrix synthesis, and to play an important role in bone regeneration, fracture healing and repair of other musculoskeletal tissues [1-4]. It was suggested that there is a crosstalk between the GF signaling pathways in osteogenesis and the overall osteogenic outcome may be resulting from the synergistic contribution of numerous GFs [1-3, 5-15]. In order to develop effective bone tissue engineering strategies that can control and modulate bone formation, it is essential to investigate the temporal and interconnected involvement of GFs in osteogenesis [2, 3, 6, 8, 11, 12, 14, 15]. However, the studies investigating the osteogenesis-related GF expression, production and secretion are limited to 2-dimensional *in vitro* studies with single cell types (i.e. osteoblasts, marrow stromal cells) [14, 16-18] or complex *in vivo* studies with associated experimental hurdles [2, 3, 19-21]. In order to study the complex and interconnected involvement of multiple GFs, there is a need for a multicellular and 3-dimensional *in vitro* platform that embodies the intricate physiology of natural osteogenesis. Bone marrow tissue houses multiple cell types (i.e. hematopoietic stem cells, mesenchymal stem cells, accessory cells) that are known to be collaboratively involved in osteogenesis [22-27]. Therefore, bone marrow plays a critical role in bone regeneration [28] and has been shown to have osteogenic potential [29]. Marrow explants inherently ossify i*n vitro* [30] without the addition of excipient osteoinductive factors (under basal conditions) and throughout the ossification process, osteoinductive factors are produced by the ossifying marrow tissue [31]. Therefore, *in vitro* bone marrow

explant cultures reflect the physiological diversity of bone formation and hold the potential to be used as a platform to study osteogenesis in a more realistic and natural context. This model is conveniently situated between the 2-dimensional *in vitro* culture systems employing single cell types and the complex *in vivo* animal models.

The most potent GFs known to be involved in osteogenesis are: TGF-β1, BMPs (2, 4 and 7), FGF-2, VEGF, IGF-1 and PDGF [1-4, 14, 32, 33]. BMP-2 and -7 have been introduced clinically for treatment of open tibial fractures [13, 34, 35]. While there is some appreciation of the sequential expression of these potent GFs in fracture healing [2, 3, 19, 20] and in single type cell culture models [14, 16-18]; little is known about their associations with the final amount of bone formation during osteogenesis. Moreover, the knowledge on the relationships between the production levels of growth factors during osteogenesis is limited as well. We hypothesized that *in vitro* bone marrow self-inductive ossification model can be used as a platform to delineate the temporal involvement of multiple GFs, their relations with the ossification level and with each other in osteogenesis. Therefore, the aims of this study were: a) to analyze the temporal production patterns of the key GFs in osteogenesis: TGF-β1, BMP-2, VEGF and IGF-1 using the inherently ossifying bone marrow explant model, b) to investigate the relations between the temporal concentrations of GFs and the final ossified volume (OV) of marrow explants, and c) to investigate relations between the production of GFs, bone-related proteins and between the GFs themselves throughout the ossification process. These aims were accomplished by: a) measuring the concentrations of GFs in the conditioned media via quantitative ELISA at days 2, 7, 12, 14, 19 and 21, b) quantifying

the end point of ossification (day-21, OV) of marrow explants and correlating OV with temporal concentrations of the GFs, and c) correlating the concentration levels of the GFs and bone-related proteins to each other at all time points and across time points.

3.3. Methods

3.3.1. Extraction And Culture Of Bone Marrow Explants

Bone marrow was isolated from the tibiae of 80-90 days old male Long-Evans rats (Harlan, Indianapolis, IN) under IACUC (Institutional Animal Care and Use Committee) approval with a centrifugation based extraction technique. Briefly, one of the diaphyseal end of the bones was cut with a high-speed circular saw, the medullary components (marrow) was exuded with a brief centrifugation of the cut bone, and the centrifugate was gently pipetted onto the culture inserts (Transwell, Corning) at a 7 µl volume with a low protein binding pipette tip. Bone marrow isolation procedure did not involve dispersion of the marrow contents in a solution, and therefore entailed minimal manipulation and processing of the tissue in order to preserve the cellular integrity (both adherent and non-adherent) and structural composition of the extracted marrow explant. The growth medium was added underneath the culture insert (below the membrane with 0.4 µm pore size) which resulted in an air-medium interface culture system (Fig.3.1). The membrane allowed the attachment and the growth of cells. Explants were cultured under serum free conditions to identify the baseline ability of marrow explants to ossify. The serum-free growth medium was modified from Lennon et al. [36] and composed of 60%

DMEM, 40% MCDB-201 supplemented with 1% ITS+1 (Sigma), 50 µg/ml ascorbic acid, 5 mM Na-β-glycerophosphate, 3.5 mg/ml glucose, 40 U/ml Penicillin and 40 µg/ml Streptomycin, 1.5 µg/ml Fungizone. No osteoinductive factors (e.g. dexamethasone, BMP-2) were added into the culture media at any point in time. The explants were cultured for 21 days and media was changed on days: 2, 5, 7, 10, 12, 14, 17, 19 and 21. Since the inserts carrying the explants were set aside during culture medium change, the cellular and compositional integrity of the marrow explants were maintained, which kept the adherent and non-adherent cells together throughout the culture period (Fig.3.1). Due to the small pore size (0.4 µm) of the membrane above which the marrow explants were cultured, the cells were not able to migrate through the holes and hence were contained within the insert. This feature of the culture system kept the bone marrow cells above the membrane and allowed the secreted products to be released into the culture medium. The medium conditioned by bone marrow explants was collected from each ossifying explant before each fresh media addition and stored in sterile low protein binding tubes (LoBind, Eppendorf) separately. The samples were stored at -80°C freezer for ELISA measurements which were performed at the end of the experiment collectively. Repeated freezing and thawing of the collected conditioned media was eliminated with appropriate aliquoting.

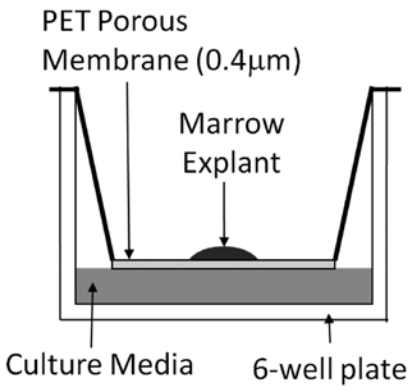


Figure 3.1: Cross-sectional view of air-medium interface culture system designed to preserve the adherent and non-adherent cellular composition of marrow tissue throughout the culture period. Marrow explants were placed on PET porous membrane (0.4μm pore size) of culture inserts and supplemented with sufficient amount of culture media underneath the membrane. Culture medium was not added above the membrane and therefore marrow explants were not in direct contact nor were they dispersed in the medium, which prevented the non-adherent marrow cells from being washed away during media changes.

In order to test the effect of dispersion or disruption of the cellular integrity of marrow explants on their self-inductive ossification potential, an additional experimental group was included in which marrow explants above the porous membrane were dispersed with the addition of culture media inside the culture insert (Fig.3.1). In this group, same volume of marrow tissue (7 μl) was utilized in the same culture setting with the culture media added above the membrane (Fig.3.1) submerging and disrupting the marrow explants. This group was similar to the regular marrow culture studies in the literature, in which nonadherent marrow cells are gradually washed away with each media change, purifying the adherent marrow stromal cells. In this experimental group, the same culture conditions and the same culture media was used for the same duration as the regular intact marrow explant culture described above. The mineralization of

submerged (dispersed) marrow culture samples was evaluated with Raman Microspectroscopy and micro Computed Tomography. Raman microspectroscopy analysis (LabRam, Horiba Jobin-Yvon, Edison, NJ, USA) was performed on the randomly selected nodule-like structures (8 samples, 6 nodule-like structure per sample) observed in the dispersed marrow culture samples. The presence of apatitic mineral peak (located at 959 cm^{-1}) was evaluated by performing a scan in the wavenumber range of 250–1800 cm^{-1}[37].

3.3.2. Micro Computed Tomography (μCT) of Ossifying Marrow Explants

The ossified marrow samples and the dispersed marrow cultures were fixed with 10% formalin and kept in the fixative before and throughout the scans. The ossified volume of the marrow explants was measured by micro computed tomography (SCANCO Medical AG, μCT 40, Brüttisellen, Switzerland) with a 16 μm voxel resolution (I = 145 μA, E = 55 kVp, integration time = 200 ms). The porous membranes supporting the dispersed marrow cultures were cut out of the culture inserts and scanned with μCT with the same settings. The scanned images were reconstructed and analyzed with a commercial software (SCANCO evaluation software) and the segmentation parameters of 0.8 (sigma), 1 (support) and 100 (threshold) were used [38-40]. The total bone volume (BV, mm^3) calculated by the software was used and reported as the final ossified volume (OV) of the marrow explants.

3.3.3. Baseline Levels of Factors in Freshly Isolated Bone Marrow

Two male Long-Evans rats, 80-90 days old, were euthanized under IACUC approval. Bone marrow was removed from the tibiae using a centrifugation based extraction technique explained in the previous section. Extracted marrow tissue (7 µl) was diluted in tubes which allow minima protein binding (protein LoBind, Eppendorf) with the same serum- free growth medium described above. Marrow extracts were incubated for 30 min at 37°C to allow the soluble factors to solubilize in the media. The suspension was then centrifuged for 10 min at 300g to precipitate the cells as a pellet at the bottom of the tube. The supernatant was aspirated and filtered through a 0.2 µm filter using a syringe to remove the remaining cells. The solubilized bone marrow deficient of cells was then aliquoted and stored at -80°C for the quantitative ELISA assays described below. Appropriate conversion of the quantified concentrations of the factors was performed based on the dilution ratios employed.

3.3.4. Histology of Ossified Marrow Explants

At the end of the culture period, the ossified explants were fixed in 10% formalin. Samples were decalcified in formic acid solution (1:1 solution of 50% aqueous formic acid and 20% sodium citrate) for 8 to 12 hours, washed in tap water for 30 to 45 minutes, embedded in paraffin, sectioned and dried overnight in 37°C oven. For all staining procedures, the sections were deparaffinized and hydrated in gradually decreasing percentages of alcohol solutions (100%, 95%, 70% and water). The sections were stained with safranine-O/fast green for assessing the presence of proteoglycans and hematoxylin

& eosin (H&E) according to standard procedures. Safranine-O/fast green staining is a common method used for staining cartilage-bone interface. AP activity in the histological sections was stained with naphthol AS-MX based commercial AP staining kit (Sigma 85L1). After staining, the sections were dehydrated, cleared in xylene and cover-slipped.

3.3.5. Quantification of AP, OC, BMP-2, IGF-1, VEGF and TGF-β1

The quantification of these factors was performed on the media conditioned by ossifying explants (days 2, 7, 12, 14, 19 and 21) and the fresh marrow tissue (day 0). AP level was measured with the colorimetric p-Nitrophenyl phosphate (pNPP) substrate AP assay kit (SensoLyte^{TM}, Anaspec Corp., San Jose, CA). 50 µL of samples and standards were added to each well of a 96-well plate. Fifty µL of pNPP reaction mixture was added to each well and incubated 2-3 hrs until color developed. Optical density was determined using a microplate reader (Molecular Devices, Spectramax M5) set to 405 nm. Absorbance values were converted to AP concentration with the utilization of the calibration curve. OC levels were measured using a Rat Osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA). One hundred µl of samples and standards were added to a 96-well plate pre-coated with OC capture antibody, incubated for 20 hr at 4°C, washed three times with phosphate-saline wash buffer, 100 µL of OC antiserum was added to each well, and incubated at 37°C for 1 hr. Following another set of washes, 100 µL of diluted donkey anti-goat IgG peroxidase was added to each well, incubated for 1 hour at room temperature, rerinsed, 100 µL of substrate mix (1:1 of hydrogen peroxide solution and tetramethyl benzidine) was added and incubated at room temperature for 30 minutes,

avoiding direct light. One hundred μL of stop solution was added to each well, and the absorbance was measured using a microplate reader set at 450 nm with a wavelength correction set at 540 nm. The concentrations of BMP-2, VEGF, TGF-β1 and IGF-1 in the conditioned media were measured by quantitative ELISA development kits (BMP-2: PeproTech; VEGF, IGF-1 and TGF-β1: R&D Systems). Briefly, 96-well microplates (MaxiSorp, Nalge) were coated with capture antibody, the wells were blocked for at least 1 hour, 100 μl of samples or standards were added to wells followed by incubation for 2-3 hours at room temperature. After thorough washing, detection antibody was added at the specified concentration for each kit and incubated for 2 hours at the room temperature. The peroxidase substrate solution was added (protected from direct light) and incubated at room temperature for 20 minutes, the enzyme reaction was stopped with 2N hydrochloric acid solution. The color product was detected by a microplate reader set at 450 nm with wavelength correction set at 540 nm. Quantification was also carried out on the non-conditioned serum-free medium to determine baseline levels of the GFs, OC and AP. TGF-β1 in the samples was first activated to its immunoreactive form using 1 N HCl followed by addition of 1.2 N NaOH/0.5 M HEPES before being used in ELISA assays.

3.3.6. Statistical Analysis

The measured concentration profiles were analyzed statistically with Kruskal–Wallis one-way analysis of variance followed by a *post hoc* Mann-Whitney U-test with Bonferroni correction for multiple comparisons. Statistical significance threshold was set

at $p < 0.05$ and the p-value obtained for each test was adjusted based on the number of comparisons according to Bonferroni correction (p-value obtained from the test multiplied by number of comparisons). Error bars in the figures were displayed as standard error. Relations between the concentrations and the final ossified volume; and between the measured concentrations themselves were analyzed by calculating the Pearson product moment correlation coefficient. The statistical significance between the day-0 baseline concentrations (n=10-12) of the bone-related proteins and GFs and their day-2 levels (n=6) produced by the ossifying explants was tested with a Mann-Whitney U-test with the significance threshold set at $p<0.05$.

3.4. Results

3.4.1. Bone Marrow Explants Inherently Ossified

Bone marrow explants cultured under serum-free conditions without any osteoinductive factors (e.g. dexamethasone, BMP-2, etc.) inherently ossified to form a matrix which was visible through low magnification light microscopy (Fig.3.2A). μCT scans of ossified explants revealed a plate-like ossified structure (Fig.3.2B). The absence of orange-red stain in safranine-o/fast green stained sections was an indication of proteoglycan deficiency, indicating the absence of cartilaginous matrix (Fig.3.2C). H&E staining displayed viable cells embedded in the matrix (Fig.3.2D). Naphthol AS-MX based AP staining indicated that AP activity (dark purple-red regions, Fig.3.2E) was concentrated in the lower and the upper surfaces of the ossified matrix, indicating the

locations of the actively ossifying regions in the marrow explant. However, when the marrow explants were dispersed and submerged in the culture media, which gradually eliminated the non-adherent cell population with each media change, no indication of mineralization was observed as per Raman Microspectroscopy (absence of apatitic mineral peak) and μCT (no detectable mineralized volume).

3.4.2. AP and OC were Produced by Ossifying Marrow

The baseline levels of AP and OC in day 0 marrow tissue were quantified as 14.8 ng/ml (st. dev: 3.8 ng/ml) and 514 pg/ml (st.dev: 212 pg/ml) respectively (Fig.3.3). Both of these baseline concentrations were significantly lower than the production of these bone-related proteins on day 2 by *in vitro* ossifying marrow explants as seen in Fig.3.3. AP concentration measured in conditioned media displayed a high level at the beginning of the culture period and decreased significantly at day-7 and further decreased after day-12 (Fig.3.3A). The decrease in AP concentration was significant by day 21 relative to day-12. OC concentration profile displayed a similar pattern at the beginning of the culture period, which was significantly greater on day-2 than all the following time points (Fig.3.3B). A significant decrease in OC concentration was observed on day-7. There was no statistically significant decrease in OC concentration between day-12 and day-21.

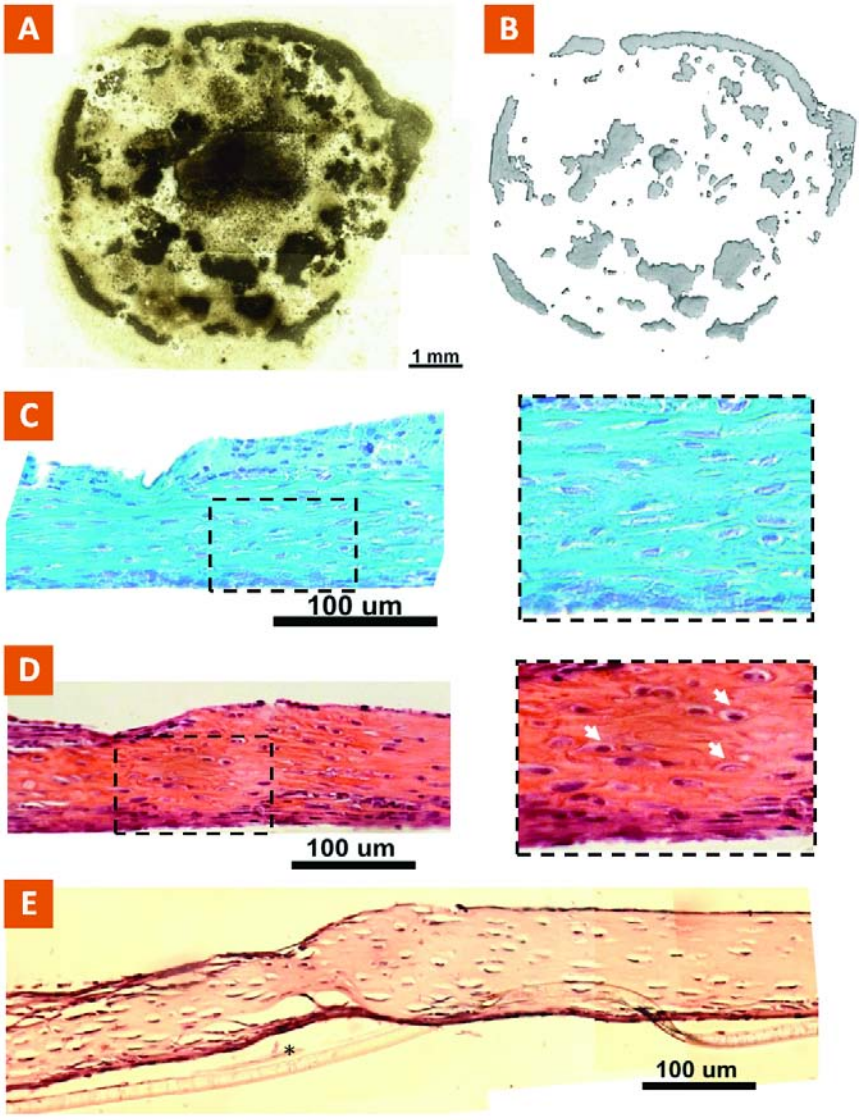


Figure 3.2: The appearance of a marrow explant cultured for 21 days, **A)** Light microscope image of the ossified explant, **B)** the reconstructed 3-D view of the ossified regions of the explant from micro-CT scan indicate a plate-like ossified structure, **C)** safranine-O/fast green stained histological section image of an ossified region in the marrow explant and the magnified inset. The absence of orange-red stain indicates that the extracellular matrix does not contain glycosaminoglycans and thus it is not a cartilaginous matrix. **D)** H&E stained histological section image and the magnified inset, displaying viable osteocyte-like cells (arrowheads). **E)** Alkaline phosphatase stained (dark purple-red regions, counter stain: hematoxylin) histological section image. Alkaline phosphatase activity is observed in the upper and lower sections of the ossified plate like structures. The membrane (*) is visible in the image which lines the bottom of the ossified matrix.

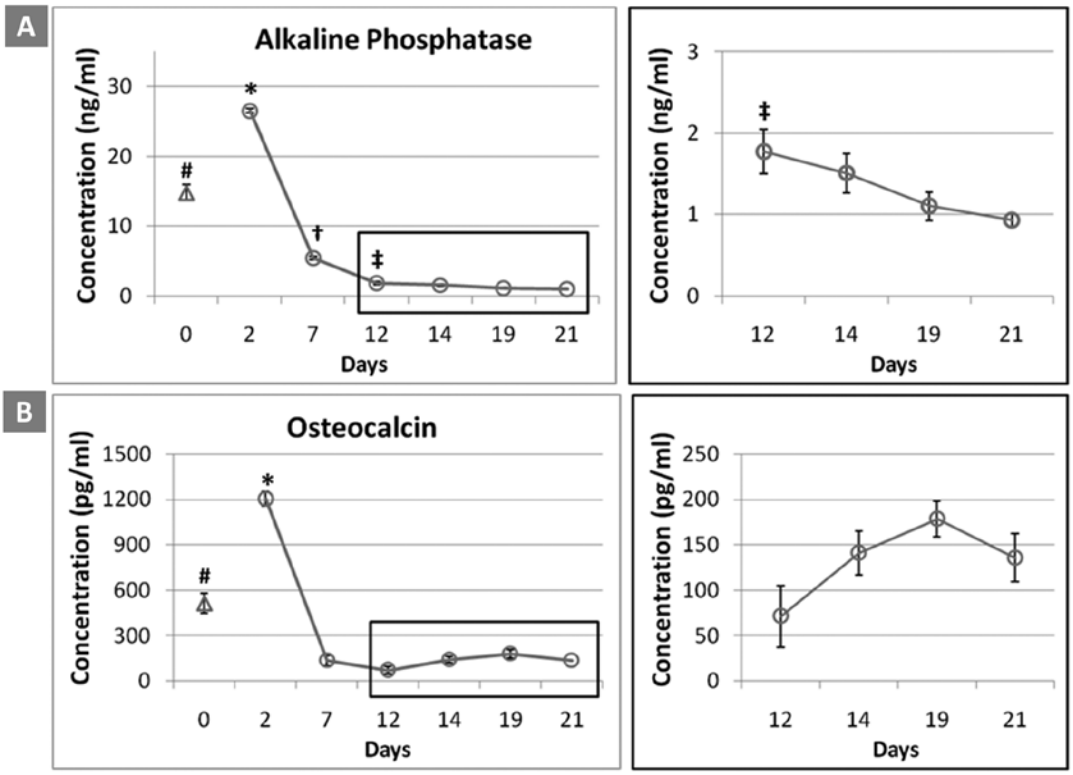


Figure 3.3: Concentration profiles of alkaline phosphatase and osteocalcin measured in conditioned media (n=6 per time-point), a close-up view of days 12-21 highlighted by insets is displayed on the right. The triangular marker at day-0 indicates the baseline concentration in bone marrow tissue at day-0 (#: $p<0.05$ between the baseline concentration in bone marrow tissue at day-0 and the expression of that factor on day-2.) **A)** Alkaline phosphatase concentration profile in conditioned media, *$p<0.05$ for day 2 vs. days 7, 12, 14, 19 and 21; †$p<0.05$ for day 7 vs. day 12, 14, 19 and 21, ‡$p<0.05$ for day 12 vs. day 21. **B)** Osteocalcin concentration profile in conditioned media, *$p<0.05$ day 2 vs. day 7, 12, 14, 19 and 21.

3.4.3. Osteoinductive GFs were Produced by Ossifying Marrow

The baseline levels of BMP-2, IGF-1, TGF-β1 and VEGF in day-0 marrow tissue were significantly lower than the production of these factors by *in vitro* ossifying marrow explants on day-2 (Fig.3.4). BMP-2 concentration in conditioned media was at a high level early on at days 2 and 7 (Fig.3.4A). BMP-2 concentration decreased significantly by day-12 and displayed further significant decrease on days 19 and 21 (Fig.3.4A). IGF-1

concentration was significantly higher on day-2 than the later time points, which decreased significantly and stayed around 50 pg/ml between days 7 and 14 (Fig.3.4B). A significant increase in IGF-1 concentration was observed in the later stage starting day 19 and beyond. TGF-β1 concentration displayed a significantly high level on days-2, -7, and day-12 than all of the subsequent time points (Fig.3.4C). TGF-β1 concentration decreased significantly by day-14 and stayed constant around 50 pg/ml till the end of the culture period. Similarly, VEGF concentration was significantly higher at the early and mid phase of the culture period (days 2, 7, 12 and 14) than all the following time points (Fig.3.4D). VEGF concentration displayed a significant steady decrease during the entire culture period.

3.4.4. Correlations Between Levels of Factors and Ossified Volume

The rightmost column of Table 3.1 displays the correlation between the levels of measured GFs and the bone markers (OC and AP) at different time points and the final ossified volume measured at day-21. VEGF concentration in conditioned media displayed a high correlation (Table 3.1) with the final ossified volume (OV) on day 12 (0.988) and day 14 (0.970). IGF-1 concentration in conditioned media early on in the culture (day 2) correlated positively with OV (0.868, Table 3.1), whereas latent IGF-1 concentration correlated negatively on day 14 (-0.813) and 19 (-0.865) with OV. OC level displayed a high negative correlation with OV on day 19 (-0.931, Table 3.1).

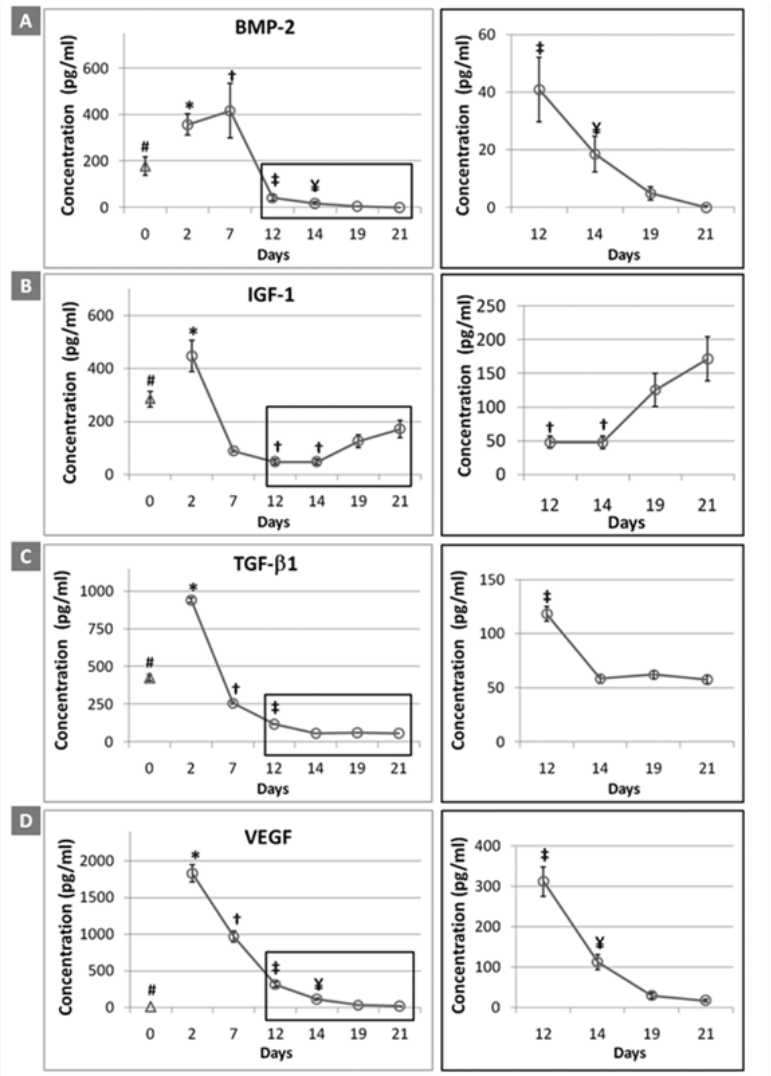


Figure 3.4: Concentration profiles BMP-2, IGF-1, TGF-β1 and VEGF measured in conditioned media (n=6 per time-point), a close-up view of days 12-21 highlighted by insets is displayed on the right. The triangular marker at day-0 indicates the baseline concentration in bone marrow tissue at day-0 (#: $p<0.05$ between the baseline concentration in bone marrow tissue at day-0 and the expression of that factor on day-2.) **A)** BMP-2 concentration profile in conditioned media, *$p<0.05$ for day 2 vs. day 12, 14, 19 and 21; †$p<0.05$ for day 7 vs. day 12, 14, 19 and 21, ‡$p<0.05$ for day 12 vs. day 19 and 21; ¥$p<0.05$ for day 14 vs. day 21, **B)** IGF-1 concentration profile in conditioned media, *$p<0.05$ for day 2 vs. day 7, 12, 14, 19 and 21; ‡$p<0.05$ for day 12 vs. day 19 and 21, and for day 14 vs. day 19 and 21, **C)** TGF-β1 concentration profile in conditioned media, *$p<0.05$ for day 2 vs. day 7, 12, 14, 19 and 21; †$p<0.05$ for day 7 vs. day 12, 14, 19 and 21, ‡$p<0.05$ for day 12 vs. day 14, 19 and 21, **D)** VEGF concentration profile in conditioned media, *$p<0.05$ for day 2 vs. day 7, 12, 14, 19 and 21; †$p<0.05$ for day 7 vs. day 12, 14, 19 and 21, ‡$p<0.05$ for day 12 vs. day 14, 19 and 21, ¥$p<0.05$ for day 14 vs. day 19 and 21.

3.4.5. Correlations of Factors Across the Timeline of Production

Table 3.1 displays the significant correlations among the quantified GFs as well as between the GFs and the bone related proteins (AP and OC). The correlations in the table can be categorized into two groups; correlation between the factors at the same time point (e.g. VEGF at day-2 correlating positively with BMP-2 at day-2, 0.895) and the correlation between the factors across time points (e.g. VEGF at day-2 correlating positively with BMP-2 at day-7, 0.877). The second type of correlation can only be possible between an earlier time point and a later time point (e.g. day-2 level of a factor and a day-7 level of another factor). Since correlation of a later time point with an earlier time point lacks physical meaning, such correlations were not reported.

3.4.6. Correlation Between the Factors at the Same Time Point

BMP-2 level correlated with IGF-1 level (Table 3.1) on day-14, and also correlated with VEGF level on day-2. OC level correlated with BMP-2 and VEGF on day-2; and with BMP-2 on day-7 and day-14. AP level displayed a negative correlation with BMP-2 level on day-2 (Table 3.1).

Table 3.1: Correlations between the levels of factors across time points and the final ossified volume (OV) of the marrow explants.

Days		2		7				12			14			19			21				OV
	Factors	BMP-2	VEGF	BMP-2	VEGF	TGF-β	OC	BMP-2	VEGF	IGF-1	BMP-2	IGF-1	OC	VEGF	IGF-1	OC	BMP-2	VEGF	IGF-1	OC	(day 21)
2	BMP-2		0.893				0.986					0.864	0.896			0.872					
	VEGF	0.893		0.877		0.923	0.866	0.888			0.817										
	IGF-1			-0.833					0.918							-0.914					0.868
	OC	0.845	0.977	0.847		0.961												0.848			
	AP	-0.817					-0.861	-0.814		-0.948	-0.841	-0.931	-0.895		-0.859	-0.910					
7	BMP-2						0.957									0.830					
	OC			0.957				0.945			0.926	0.856									
	AP																			-0.814	
12	BMP-2											0.897	0.944			0.831					
	VEGF											-0.829			-0.874	-0.942					0.988
	IGF-1												0.896			0.837					
	OC																				
	AP																			-0.922	
14	BMP-2											0.946	0.977		0.847	0.870					
	VEGF														-0.845	-0.890					0.970
	IGF-1										0.946					0.906					-0.813
	OC										0.977			-0.890							
	AP																			-0.828	
19	BMP-2																	0.812			
	VEGF																0.842				
	IGF-1																				-0.865
	OC																				-0.931
	AP																			-0.957	

Pearson product moment correlation coefficient is presented in the cells (all the presented correlations are significant with $p<0.05$). The cells corresponding to the correlations between the same factor at the same time points and reverse correlations in time were shaded due to the lack of physical significance. BMP-2, bone morphogenetic protein-2; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; TGF-β1, transforming growth factor beta 1; AP, alkaline phosphatase; OC, osteocalcin.

3.4.7. Correlation Between the Factors Across Time Points

There were a great number of correlations between the quantified factors across time points (Table 3.1). Most correlations were observed between the day-2 levels of BMP-2, VEGF, IGF-1, OC, AP and later time point levels of BMP-2, VEGF, TGF-β1, OC and IGF-1 (14 positive and 9 negative correlations). The number of significant correlations across time points decreased for days 7, 12, 14 and 19.

3.5. Discussion and Conclusion

The importance of the GFs included in this study (BMP-2, VEGF, TGF-β1, OC and IGF-1) and their synergistic combinatorial role on bone regeneration is widely accepted [1-3, 14, 16, 17, 19-21, 32, 34, 41, 42]. On the other hand, data on sequential expression of GFs in osteogenesis are either limited to 2-dimensional single type cell culture studies [14, 16, 17] or complex animal models of fracture healing [2, 3, 19-21]. The current model of marrow explant cultures is situated in between single type cell culture studies and animal models and present several unique advantages. First, it encompasses multiple cell types (adherent and non-adherent, hematopoietic and mesenchymal stem cells). Second it displays a natural osteogenic potential (under serum free conditions without any excipient osteoinductive factors), which results in a significant volume of bone formation. Third, it presents a platform in which the protein production can be quantified via the conditioned media. In the future, this model would also allow interrogation with silencing-RNA or gene transfection to control GF production to assess their ultimate effect on the amount of bone formation. Therefore, *in*

vitro bone marrow explant culture model presented here is a useful model for studying the temporal production profiles of the osteogenic factors and their synergistic combinatorial roles on bone generation/regeneration process.

The importance of preserving the cellular integrity of marrow tissue in terms of osteogenic potential was verified by including an experimental group in which marrow explants were dispersed in the culture medium. This condition allowed the gradual elimination of non-adherent cells (i.e. hematopoietic cells) with each media change and left the adherent cells (i.e. marrow stromal cells) in the culture. The absence of mineralization (as per Raman microspectroscopy and μCT) in this group indicated that the multicellular nature of marrow tissue needs to be preserved to achieve self-inductive ossification. On the other hand, bone marrow explants whose integrity was maintained ossified to varying extents. Therefore, the final ossified volume of the marrow explants was used as an indicator of the osteogenic capacity of individual samples, which may be directly related to levels of osteoinductive factors produced. The varying ossification levels of the samples allowed us to evaluate the correlation between the final ossified volume (OV) and the concentration levels of individual factors at different time points (Table 3.1). To the best of our knowledge, the correlation between the levels of osteoinductive factors at different time points, across time points and the final ossified volume in an *in vitro* ossification model is being reported for the first time in this study. This information can be used to determine the most critical osteoinductive factors, as well as the most critical time of application of these factors to obtain a more efficient and natural ossification mechanism. In addition, the correlation between the levels of factors

at different time points and across time points can be used to delineate the synergistic involvement of multiple osteoinductive factors in the ossification process.

GFs present in the serum that is commonly used as a supplement in *in vitro* cell culture studies confound the picture and makes it hard to study the involvement of growth factors in various processes such as osteogenesis. Therefore we confirmed the ability of marrow explants to ossify in serum-free culture conditions and without application of any excipient osteoinductive factors. This property facilitates carrying out more comprehensive *in vitro* analysis of various proteins involved in osteogenesis without the inhibiting and/or interfering effects of serum supplements.

The histochemical analysis on the *in vitro* ossified marrow explants with safranine-o/fast green staining displayed (Fig.3.2C) the absence of proteoglycans, which can be found abundantly in cartilaginous matrix. In addition, the presence of AP activity on the lower and upper surfaces of the ossifying explants indicated the presence of differentiated osteoblast-like cells forming a mineralized matrix. Therefore the absence of a cartilaginous matrix and the presence of AP activity indicate that the ossification mechanism in this *in vitro* bone marrow culture model to resemble the intramembranous ossification mechanism.

The baseline levels of AP, OC, BMP-2, IGF-1, TGF-β1 and VEGF in day-0 marrow tissue have been quantified and all of them have been observed to be significantly lower than the day-2 production levels of these proteins by ossifying marrow explants (Fig.3.3 and Fig.3.4, triangular markers at day-0). Therefore the

presented concentration profiles of these quantified proteins could not be related merely to the baseline concentrations in fresh marrow tissue.

AP and OC are commonly accepted bone markers [18]. AP is expressed by many cell types to some extent. AP is also associated with osteoblast differentiation and its production is high in preosteoblasts and osteoblasts [43]. Using AP as an ossification marker with a heterogeneous population of marrow cells is complicated since only a small population of the cells in marrow stroma are AP positive osteoblast precursors and many other cell types in marrow express AP, such as adipocytic cells [44]. It should be noted that the marrow explants employed in this study were handled minimally, which preserved the natural components together, including all the resident cell types, the extracellular matrix, as well as the soluble proteins and factors. Therefore it may be reasonable to suggest that the early high production of AP in this study (Fig.3.3A) may be attributed to the other cell types in the marrow tissue which are in high concentration, such as adipocytes. Due to the difficulties with quantifying ossification related AP activity, a secondary ossification marker (OC) was used to assess ossification. OC is a specific marker of mature osteoblast phenotype [18]. Previously, OC level was observed to be increasing after about 10 days with *in vitro* mineralization models that employed marrow cells [14, 18]. A peak in OC level around day-14 was also reported when the marrow stromal cells were stimulated with dexamethasone and $1,25(OH)_2D_3$ [18]. However the high OC level observed early on in this study (Fig.3.3B) is not in agreement with the previous findings. At the earlier stage of the marrow explant culture, the high level of OC at day-2 decreased dramatically by day-7 and did not increase significantly

between day 12 and day 21 (Fig.3.3B). This observation may be attributed to the complex nature of the current model due to the presence of multiple cell types.

In vivo studies indicate that BMP-2 expression in the fracture site displays an early increase during the phase in which mesenchymal stem cells are recruited to the injury site [2, 4, 45]. The high level of BMP-2 production in the early phase of fracture healing is followed by a decrease as it was also observed in this study (Fig.3.4A). A similar trend, in which an upregulation of BMP-2 around day 4 followed by a down-regulation around day 12 was previously observed during mineralization of osteoprogenitors *in vitro* [14]. BMP-2 level measured at day-14 highly correlated with the IGF-1 level on day-14 (Table 3.1 and Fig.3.4C), which may be an indication of interaction or a similarity in terms of source cells for these two factors at this phase. Similarly, it was previously shown that early application of BMP-2 (day-1) followed by later application of combination of BMP-2 and IGF-1 (after day-5) resulted in the highest amount of cell number and AP activity in pluripotent C3H10T1/2 cells [15]. The high positive correlation between the OC level and the BMP-2 concentration at day-14 and day-19 may be an indication on the role of BMP-2 in mature osteoblast function in mineralization. On the other hand, BMP-2 production on day-2 was observed to correlate negatively with AP level on day-2 (Table 3.1). This could be due to the early high levels of AP, which suppressed the production of BMP-2 which later peaked at day-7 when AP level decreased significantly. It is also important to note that the early level of BMP-2 (days 2-7) also correlated with later levels of OC (at days 7, 14 and 19) and IGF-1 (at day 14). This observation suggests that an early involvement of BMP-2 has repercussions on

the latent stages of osteogenesis. The early involvement of BMP-2 in osteogenesis has recently been shown in sequential growth-factor delivery studies with BMP-2 and BMP-7 [46, 47]. In these studies, early release of BMP-2 and latent release of BMP-7 was achieved with nanocapsules in an *in vitro* study with MSCs. Superior osteoinductive effects of the sequential application of BMP-2 and BMP-7 was demonstrated over individual and simultaneous applications based on elevated alkaline phosphatase activity on day 14 and day 21.

IGF-1 production by *in vitro* ossifying marrow explants in this study was bimodal. It displayed a high level at the early stage, followed by a decrease during days 7-14 and then a latent increase during days 19-21 (Fig.3.4B). A similar pattern of IGF-1 production in the later stage was observed in fracture healing in an *in vivo* model by Wildemann et al [33]. As the earliest time point included in the study by Wildemann et al. was day-5, it is not possible to compare the early level of IGF-1 to that was observed in our study. However in an *in vitro* mineralization model utilizing osteoprogenitor cells, a high level of IGF-1 was observed early on followed by a decrease between days 5-12 and then followed by an increase starting day-13 [14]. Therefore the results presented in this study in terms of IGF-1 concentration profile agree with the previous findings in the literature. The high IGF-1 level at day-2 was observed to correlate positively with OV (Table 3.1), which may be an indication that early application of IGF-1 is critical in bone regeneration. On the other hand, we observed that IGF-1 levels at later stages (days 14-19) correlated negatively with OV (Table 3.1), which may be an indication that IGF-1 application in the later stages may have a deterring role in ossification. IGF-1

concentration was also observed to correlate with OC level positively on days 14 and 19 (Table 3.1). When this information is combined with the findings presented above (IGF-1 correlated negatively with OV on days 14-19) and the fact that OC correlated negatively with OV on day-19 (Table 3.1); it is reasonable to suggest a connection between IGF-1 and OC in curbing further ossification of the marrow explants starting day-14 and allowing the mineralization to reach a steady state. In attestation, OC is known to curb mineralization [48] and current results imply that IGF-1 may be associated in this pathway.

Early involvement of TGF-β1 during the proliferation phase in fracture healing process has been shown previously [2-4, 33]. Similarly, TGF-β1 was observed at a high level early on in the marrow explant culture model and decreased continually to reach a steady-state level by day-14 (Fig.3.4C). Therefore TGF-β1 production profile presented here agrees with the previous findings. However TGF-β1 levels at various time points displayed a limited number of correlations with other factors. The only TGF-β1 level that has displayed a significant correlation was day-7 level which correlated positively with day-2 levels of VEGF and OC. Therefore a link between VEGF and TGF-β1 involvement could be present between days 2 and 7 during ossification.

VEGF is considered to play its most important role in fracture healing in the earlier stages [49]. Therefore the high concentration of VEGF observed in this study during day-2, which gradually decreased starting day-7 up to day-21 (Fig.3.4D) agrees with the previous findings. A similar trend of VEGF expression during mineralization by osteoprogenitors was also observed in an *in vitro* study [14]. Even though VEGF

concentration was high at the earlier stage of ossification and decreased gradually till day 21 (Fig.3.4D), VEGF concentration and OV was observed to highly correlate only during days 12-14 (Table 3.1). This high correlation between VEGF concentration and OV indicates that the presence of VEGF during days 12-14 was associated with final amount of ossification. Importance of VEGF at the mid-phase of fracture repair was also shown and emphasized before [4].

It should be noted that the correlations that were reported between the GF levels and the final ossified volume are useful for identifying the temporal involvement of the GFs in osteogenesis. However, these correlations do not necessarily imply causations. The correlations observed in this study require further analysis with studies employing targeted inhibition of growth factors at a specific time points to assess the overall effect in the ossification of marrow tissue.

There are many GFs involved in osteogenesis and they are not limited to the four factors (BMP-2, VEGF, IGF-1 and TGF-β1) studied here. Other factors that are known to be actively involved in osteogenesis are BMPs (4, 6, 7 and 13), FGF-2, Wnt, GDF-5 and PDGF [14, 21, 50-54]. A detailed characterization of the involvement many GFs and signaling molecules is necessary and proteomic analysis could be used for high-throughput screening of all the proteins. Therefore, we are currently conducting experiments to analyze a myriad of proteins and their temporal expression profiles during inherent *in vitro* ossification of bone marrow explants.

In conclusion, it was shown that BMP-2, IGF-1, TGF-β1 and VEGF are expressed differentially over time by the ossifying marrow explants and the concentration

of IGF-1 and VEGF correlate at different time points with the final ossified volume. IGF-1 has dichotomous effect on the final ossified volume which is indicated by positive correlation on day-2 and negative correlations on days 14 and 19. The GF levels and the production of bone markers (AP and OC) have been shown to be highly interdependent due to correlations to each other at same time points as well as across various time points. The results presented in this study provide a more robust understanding of the osteogenesis process in terms of the involvement of BMP-2, IGF-1, TGF-β1, VEGF, AP and OC in marrow explants and the secretion sequence and the amounts of key osteoinductive factors involved in this osteogenesis model. The information obtained from the marrow ossification model can be used to develop multi-factor and multi-phase GF delivery strategies for fracture healing and bone tissue engineering applications.

3.6. Acknowledgements

This study was supported by a grant from the Musculoskeletal Transplant Foundation (MTF). We would like to thank Purdue University Histopathology service laboratory (David VanSickle Ph.D. DVM, Paul Snyder Ph.D. DVM, Sharon Evander, Carol Bain, Tracy Wiegandt) for their support in histological work and Pam Lachick for her support with the μCT system. In addition, we would like to acknowledge the Summer Undergraduate Research Fellowships (SURF) program at Purdue University for supporting this study.

3.7. References

1. Lieberman, J.R., A. Daluiski, and T.A. Einhorn, *The role of growth factors in the repair of bone - Biology and clinical applications.* Journal of Bone and Joint Surgery-American Volume, 2002. **84A**(6): p. 1032-1044.
2. Cho, T.J., L.C. Gerstenfeld, and T.A. Einhorn, *Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing.* Journal of Bone and Mineral Research, 2002. **17**(3): p. 513-520.
3. Tatsuyama, K., et al., *Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone.* European Journal of Histochemistry, 2000. **44**(3): p. 269-278.
4. Gerstenfeld, L.C., et al., *Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation.* Journal of Cellular Biochemistry, 2003. **88**(5): p. 873-884.
5. Wozney, J.M. and V. Rosen, *Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair.* Clinical Orthopaedics and Related Research, 1998(346): p. 26-37.
6. Wozney, J.M., et al., *Novel Regulators of Bone-Formation - Molecular Clones and Activities.* Science, 1988. **242**(4885): p. 1528-1534.
7. Bessho, K., et al., *Comparison of recombinant and purified human bone morphogenetic protein.* British Journal of Oral & Maxillofacial Surgery, 1999. **37**(1): p. 2-5.

8. Balemans, W. and W. Van Hul, *Extracellular regulation of BMP signaling in vertebrates: A cocktail of modulators.* Developmental Biology, 2002. **250**(2): p. 231-250.

9. Maegawa, N., et al., *Enhancement of osteoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2).* J Tissue Eng Regen Med, 2007. **1**(4): p. 306-13.

10. Farhadi, J., et al., *Differentiation-dependent up-regulation of BMP-2, TGF-beta1, and VEGF expression by FGF-2 in human bone marrow stromal cells.* Plast Reconstr Surg, 2005. **116**(5): p. 1379-86.

11. Peng, H.R., et al., *Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4.* Journal of Clinical Investigation, 2002. **110**(6): p. 751-759.

12. Schmidmaier, G., et al., *Synergistic effect of IGF-I and TGF-beta 1 on fracture healing in rats - Single versus combined application of IGF-I and TGF-beta 1.* Acta Orthopaedica Scandinavica, 2003. **74**(5): p. 604-610.

13. Gautschi, O.P., S.P. Frey, and R. Zellweger, *Bone morphogenetic proteins in clinical applications.* Anz Journal of Surgery, 2007. **77**(8): p. 626-631.

14. Huang, Z.N., et al., *The sequential expression profiles of growth factors from osteroprogenitors to osteoblasts In vitro.* Tissue Engineering, 2007. **13**(9): p. 2311-2320.

15. Raiche, A.T. and D.A. Puleo, *In vitro effects of combined and sequential delivery of two bone growth factors.* Biomaterials, 2004. **25**(4): p. 677-685.

16. Robey, P.G., et al., *Osteoblasts Synthesize and Respond to Transforming Growth Factor-Type-Beta (Tgf-Beta) Invitro.* Journal of Cell Biology, 1987. **105**(1): p. 457-463.

17. Kassem, M., M. Kveiborg, and E.F. Eriksen, *Production and action of transforming growth factor-beta in human osteoblast cultures: dependence on cell differentiation and modulation by calcitriol.* European Journal of Clinical Investigation, 2000. **30**(5): p. 429-437.

18. Malaval, L., et al., *Cellular Expression of Bone-Related Proteins during in-Vitro Osteogenesis in Rat Bone-Marrow Stromal Cell-Cultures.* Journal of Cellular Physiology, 1994. **158**(3): p. 555-572.

19. Le, A.X., et al., *Molecular aspects of healing in stabilized and non-stabilized fractures.* Journal of Orthopaedic Research, 2001. **19**(1): p. 78-84.

20. Andrew, J.G., et al., *Insulin-Like Growth-Factor Gene-Expression in Human Fracture Callus.* Calcified Tissue International, 1993. **53**(2): p. 97-102.

21. Kugimiya, F., et al., *Involvement of endogenous bone morphogenetic protein (BMP)2 and BMP6 in bone formation.* Journal of Biological Chemistry, 2005. **280**(42): p. 35704-35712.

22. Cabrita, G.J.M., et al., *Hematopoietic stem cells: from the bone to the bioreactor.* Trends in Biotechnology, 2003. **21**(5): p. 233-240.

23. Dennis, J.E. and A.I. Caplan, *Bone Marrow Mesenchymal Stem Cells*, in *Stem Cells Handbook*, S. Sell, Editor. 2003, Humana Press Inc.: Totowa, NJ. p. 107-118.

24. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-147.

25. Muschler, G.F., C. Boehm, and K. Easley, *Aspiration to obtain osteoblast progenitor cells from human bone marrow: The influence of aspiration volume.* Journal of Bone and Joint Surgery-American Volume, 1997. **79A**(11): p. 1699-1709.

26. Eipers, P.G., et al., *Bone marrow accessory cells regulate human bone precursor cell development.* Experimental Hematology, 2000. **28**(7): p. 815-825.

27. Jung, Y.G., et al., *Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche.* Stem Cells, 2008. **26**(8): p. 2042-2051.

28. Devine, M.J., et al., *Transplanted bone marrow cells localize to fracture callus in a mouse model.* Journal of Orthopaedic Research, 2002. **20**(6): p. 1232-1239.

29. Muschler, G.F., et al., *Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors.* Journal of Orthopaedic Research, 2001. **19**(1): p. 117-125.

30. Luria, E.A., et al., *Bone-Formation in Organ-Cultures of Bone-Marrow.* Cell and Tissue Research, 1987. **248**(2): p. 449-454.

31. Gurkan, U.A. and A. O. *The Osteoinductive Potential of Bone Marrow Conditioned Media is Superior to Dexamethasone and rhBMP-2*. in *Orthopedic Research Society 55th Annual Meeting*. 2009. Las Vegas, NV.

32. Phillips, A.M., *Overview of the fracture healing cascade.* Injury, 2005. **36 Suppl 3**: p. S5-7.

33. Wildemann, B., et al., *Quantification, localization, and expression of IGF-I and TGF-beta 1 during growth factor-stimulated fracture healing.* Calcified Tissue International, 2004. **74**(4): p. 388-397.

34. Ludwig, S.C., J.M. Kowalski, and S.D. Boden, *Osteoinductive bone graft substitutes.* European Spine Journal, 2000. **9**: p. S119-S125.

35. Termaat, M.F., et al., *Bone morphogenetic proteins - Development and clinical efficacy in the treatment of fractures and bone defects.* Journal of Bone and Joint Surgery-American Volume, 2005. **87A**(6): p. 1367-1378.

36. Lennon, D.P., et al., *A Chemically-Defined Medium Supports in-Vitro Proliferation and Maintains the Osteochondral Potential of Rat Marrow-Derived Mesenchymal Stem-Cells.* Experimental Cell Research, 1995. **219**(1): p. 211-222.

37. Dziak, K.L. and O. Akkus, *Effects of polyelectrolytic peptides on the quality of mineral crystals grown in vitro.* Journal of Bone and Mineral Metabolism, 2008. **26**(6): p. 569-575.

38. Morgan, E.F., et al., *Micro-computed tomography assessment of fracture healing: Relationships among callus structure, composition, and mechanical function.* Bone, 2009. **44**(2): p. 335-344.

39. Muller, R., et al., *Morphometric analysis of human bone biopsies: A quantitative structural comparison of histological sections and micro-computed tomography.* Bone, 1998. **23**(1): p. 59-66.

40. Oest, M.E., et al., *Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects.* Journal of Orthopaedic Research, 2007. **25**(7): p. 941-950.

41. Reddi, A.H., *Role of morphogenetic proteins in skeletal tissue engineering and regeneration.* Nature Biotechnology, 1998. **16**(3): p. 247-252.

42. Hing, K.A., *Bone repair in the twenty-first century: biology, chemistry or engineering?* Philosophical Transactions of the Royal Society of London Series a-Mathematical Physical and Engineering Sciences, 2004. **362**(1825): p. 2821-2850.

43. Turksen, K. and J.E. Aubin, *Positive and Negative Immunoselection for Enrichment of 2 Classes of Osteoprogenitor Cells.* Journal of Cell Biology, 1991. **114**(2): p. 373-384.

44. Udagawa, N., et al., *The Bone Marrow-Derived Stromal Cell-Lines Mc3t3-G2/Pa6 and St2 Support Osteoclast-Like Cell-Differentiation in Cocultures with Mouse Spleen-Cells.* Endocrinology, 1989. **125**(4): p. 1805-1813.

45. Barnes, G.L., et al., *Growth factor regulation of fracture repair.* Journal of Bone and Mineral Research, 1999. **14**(11): p. 1805-1815.

46. Yilgor, P., N. Hasirci, and V. Hasirci, *Sequential BMP-2/BMP-7 delivery from polyester nanocapsules.* J Biomed Mater Res A, 2009.

47. Yilgor, P., et al., *Incorporation of a sequential BMP-2/BMP-7 delivery system into chitosan-based scaffolds for bone tissue engineering.* Biomaterials, 2009. **30**(21): p. 3551-9.

48. Boskey, A.L., et al., *Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin.* Bone, 1998. **23**(3): p. 187-196.

49. Pufe, T., et al., *Quantitative measurement of the splice variants 120 and 164 of the angiogenic peptide vascular endothelial growth factor in the time flow of fracture healing: a study in the rat.* Cell Tissue Res, 2002. **309**(3): p. 387-92.

50. Globus, R.K., J. Plouet, and D. Gospodarowicz, *Cultured Bovine Bone-Cells Synthesize Basic Fibroblast Growth-Factor and Store It in Their Extracellular-Matrix.* Endocrinology, 1989. **124**(3): p. 1539-1547.

51. Ross, R., E.W. Raines, and D.F. Bowenpope, *The Biology of Platelet-Derived Growth-Factor.* Cell, 1986. **46**(2): p. 155-169.

52. Buxton, P., et al., *Growth/differentiation factor-5 (GDF-5) and skeletal development.* Journal of Bone and Joint Surgery-American Volume, 2001. **83A**: p. S23-S30.

53. Jane, J.A., et al., *Ectopic osteogenesis using adenoviral bone morphogenetic protein (BMP)-4 and BMP-6 gene transfer.* Molecular Therapy, 2002. **6**(4): p. 464-470.

54. De Boer, J., H.J. Wang, and C. Van Blitterswijk, *Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells.* Tissue Engineering, 2004. **10**(3-4): p. 393-401.

4. OSSIFYING BONE MARROW EXPLANT CULTURE AS A THREE-DIMENSIONAL (3D) MECHANORESPONSIVE *IN VITRO* MODEL OF OSTEOGENESIS

(Tissue Engineering Part A, under review)

4.1. Abstract

Mechanical cues play an important role in bone regeneration and affect production and secretion dynamics of growth factors (GFs) involved in osteogenesis. The *in vitro* models for investigating the mechanoresponsiveness of the involvement of GFs in osteogenesis are limited to two-dimensional monolayer cell culture studies, which do not effectively embody the physiological interactions with the neighboring cells of different types and the interactions with a natural extracellular matrix. Natural bone formation is a complex process that necessitates the contribution of multiple cell types, physical and chemical cues in a three-dimensional (3D) setting. There is a need for *in vitro* models that represent the physiological diversity and characteristics of bone formation to realistically study the effects of mechanical cues on this process. *In vitro* cultures of marrow explants inherently ossify and they embody the multicellular and 3D nature of osteogenesis. Therefore, the aim of this study was to assess the

mechanoresponsiveness of the scaffold-free, multicellular and 3D model of osteogenesis based on inherent marrow ossification and to investigate the effects of mechanical loading on the osteoinductive GF production dynamics of this model. These aims were achieved by: a) culturing rat bone marrow explants for 28 days under basal conditions which facilitate inherent ossification, b) employing mechanical stimulation (compressive loading) between days 12 and 26; c) quantifying the final ossified volume and the production levels of BMP-2, VEGF, IGF-1 and TGF-β1. The results showed that the final ossified volume of the marrow explants increased by about 4-fold in mechanically stimulated samples. Furthermore, mechanical stimulation sustained the production level of VEGF (starting day-21) which otherwise declined temporally under static conditions. The production levels of IGF-1 and TGF-β1 were enhanced under the effect of loading after day-21. In addition, significant correlations were observed between the final ossified volume and the levels of GFs analyzed. In conclusion, this study demonstrates that the scaffold-free, multicellular and 3D model of bone formation based on inherent ossification of marrow tissue is mechanoresponsive and mechanical loading improves *in vitro* osteogenesis in this model with sustaining or enhancing osteoinductive GF production levels which otherwise would decline with increasing time.

Keywords: bone tissue engineering, cytokines, regeneration, mechanotransduction, bone marrow stromal cells, mechanical loading, mechanical stimulation.

4.2. Introduction

Bone regeneration is a complex process which involves the direct contribution of multiple cell types, physical, chemical and mechanical cues [1-8]. Numerous growth factors (GFs) are involved in osteogenesis in a sequential and interrelated manner [1, 9-12]. However, the studies investigating the involvement of mechanical cues in osteogenesis and osteogenesis-related GF expression, production and secretion are limited to 2-dimensional (2D) *in vitro* studies with particular cell types [13-24] or complex *in vivo* studies with associated experimental hurdles [25-28]. It was shown that gene expression of the cells in 2D monolayer cultures display significant differences compared to the cells of the native tissue origin and the cells cultured in 3D platforms [29]. These differences are possibly due to the limited presence of physiologically relevant interactions with the neighboring cells of different types and the absence of a natural extracellular matrix in 2D monolayer culture models [29]. In order to delineate the effects of mechanical cues on bone formation and regeneration processes, extensive *in vitro* studies have been carried out on purified populations of cells with 2D monolayer culture models. The effect of mechanical cues on mesenchymal stem cells (MSCs) [13-18, 30] and osteoblasts [19-24] have been investigated previously. These studies, almost without any exceptions, involve a purified (e.g. Ficoll purification, cell sorting) population of cells (e.g. excluding the non-adherent cells of marrow tissue), which do not fully represent the complex cellular and compositional characteristics of bone formation milieu and do not include all the cells that are normally present in bone regeneration process: hematopoietic stem cells (HSC) and the osteopoietic accessory cells (OACs) [31, 32]. Differentiation of MSCs into osteoblasts (among many other connective tissue cell

types) is essential for bone regeneration [4, 6, 33, 34]. There is increasing evidence suggesting that in addition to the extracellular microenvironment of MSC niche, the presence of other cell types (i.e. HSCs, OACs) play a role in differentiation of MSCs to osteoprogenitors and osteoblasts. It has been suggested that a close interaction exists between the HCSs, OACs, MSCs, osteoprogenitors, osteoblasts and they regulate each other's functions [31, 32, 35-38]. Therefore, when the mechanoresponsiveness of MSCs during bone regeneration process is investigated, possible contribution of other cell types should also be considered and there is a need for *in vitro* osteogenesis models which reflect the physiological diversity of cell populations.

Bone marrow tissue houses OACs, HSCs and MSCs [31, 32, 39-42] and hence partially reflects the physiological diversity of osteogenic milieu. Bone marrow is known to play a role in bone regeneration [3] and has been shown to have osteogenic potential [43]. Bone marrow explants inherently ossify i*n vitro* without the addition of excipient osteoinductive factors [12, 44]. Therefore, *in vitro* bone marrow explant cultures hold the potential to study bone regeneration in a more natural context. The scaffold-free, multicellular and 3D model of osteogenesis based on self-inductive bone marrow ossification bridges the gap between the *in vitro* 2D monolayer culture systems employing single cell types and the complex *in vivo* animal models.

We have recently showed that throughout the ossification process of bone marrow tissue (under basal conditions) osteoinductive GFs are produced with a temporal pattern with highly correlating to the ossification level [12]. Therefore studying the mechanoresponsiveness of this natural *in vitro* ossification model, the effect of

mechanical cues on the production dynamics of key osteoinductive GFs can be elucidated. There are multiple GFs involved in bone regeneration, some of the most potent ones being BMP-2, VEGF, IGF-1 and TGF-β1 [1, 9-11]. The current study tested the hypotheses that *in vitro* ossifying bone marrow tissue is mechanoresponsive as reflected by greater amount of bone formation in mechanically loaded marrow explants, and, the mechanical stimulation will enhance the production levels of BMP-2, VEGF, IGF-1 and TGF-β1 by the ossifying marrow explants. To validate these hypotheses, rat bone marrow explants undergoing ossification were stimulated with compressive load in culture (starting day-12 up to day-26). The levels of BMP-2, VEGF, IGF-1 and TGF-β1 by the ossifying explants was measured with quantitative ELISA throughout the culture period (at days 7, 14, 21 and 28) and compared to unloaded controls. The results of this study show that mechanical stimulation sustains and/or enhances the production levels of VEGF, IGF-1 and TGF-β1, but not of BMP-2 by inherently ossifying marrow explants *in vitro*.

4.3. Methods

4.3.1. *In Vitro* Culture Conditions

Bone marrow was isolated from the femurs and tibiae of 80-90 days old male Long-Evans rats (Purdue Animal Care and Use Committee approved) with a centrifugation based technique and transferred onto PET culture inserts (Transwell, Corning, 0.4 μm pore size) at a 7 μl volume (Fig.4.1A). The complete details of the

extraction and culture procedures were explained elsewhere [12]. The growth medium was composed of (modified from [44]): α-MEM (Sigma), 10% MSC-qualified-FBS (Invitrogen), 60 U/ml Pen-Strep (Invitrogen), 2.5 μg/ml Fungizone (Sigma), 50 μg/ml ascorbic acid (Sigma), 5 mM Na-β-glycerophosphate (MP Biomedical) and 3.5 mg/ml glucose (Sigma). The cultures were kept at 37°C and 5% CO_2 throughout the experiment (28 days). The unused culture media was aliquoted in appropriate volumes and kept frozen till needed. The insert including the marrow explants were set aside and the culture media in the well was changed three times a week and the spent (or conditioned) media was collected and stored at -80°C.

4.3.2. In Vitro Mechanical Loading System

The mechanical stimulation was applied to the ossifying bone marrow nodules by means of a custom made device developed in our laboratory (Fig.4.1B&C, see Appendix for the drawings of the system). The actuation is provided by Flexinol actuator wires (arrowheads in Fig.4.1C) and the frequency can be adjusted with a current control circuit driven by a 555 timer circuit. The loading chamber (sterile inside) is sealed from the outside with the elastic sealing membrane and a filtered (0.2 μm pore size) air vent. The adjustable height loading rod engages with the inside-chamber PTFE loading tip with a custom-made load cell by means of magnetic coupling. The isolated chamber houses a PET membrane insert with the ossifying bone marrow nodule at its center. The elastic PET membrane is supported by a porous polyethylene polymeric block (100 μm pore size) which at the same time allows the flow of media (Fig.4.1B).

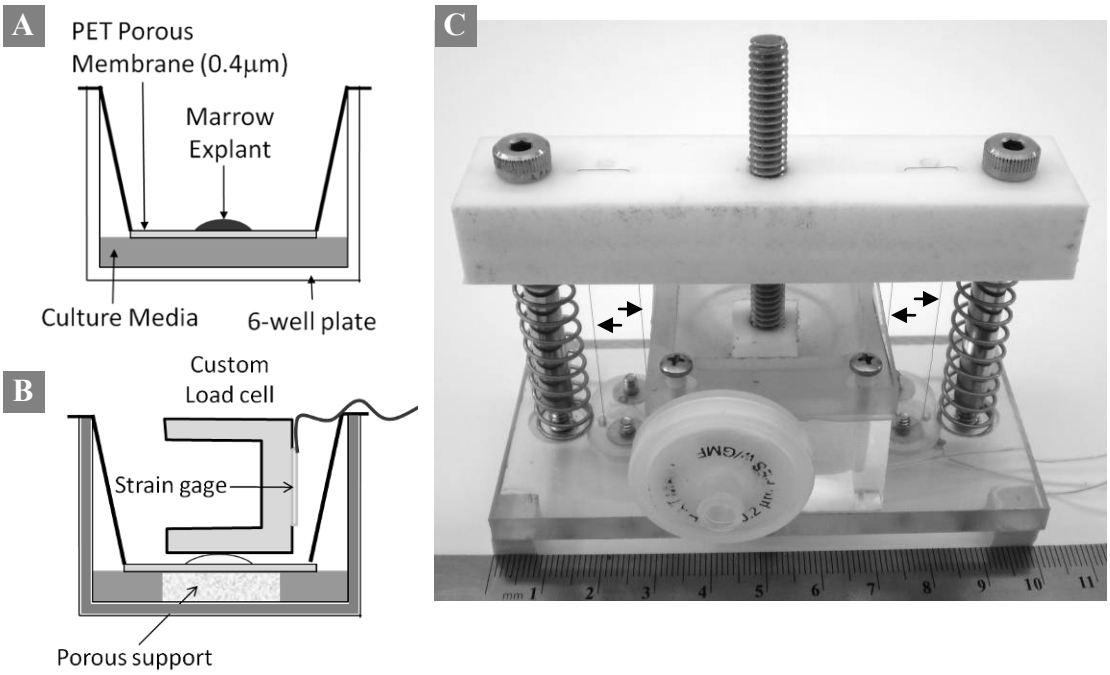


Figure 4.1: In vitro culture and loading system. **A)** Cross-sectional view of air-medium interface culture system designed to preserve the adherent and non-adherent cellular composition of marrow tissue throughout the culture period. Marrow explants were placed on PET porous membrane (0.4μm pore size) of culture inserts and supplemented with sufficient amount of culture media underneath the membrane. Culture medium was not added above the membrane and therefore marrow explants were not in direct contact nor were they immersed in the medium, which prevented the non-adherent marrow cells from being washed away during media changes. **B)** The cross-sectional view of the custom-made *in vitro* loading chamber with an ossifying explant positioned inside. The culture insert was located inside the loading chamber and a custom-made load cell (left facing C structure) was engaged, which is shown to be in contact with the partially ossified marrow explant. In this illustration, marrow explant was sandwiched between the load cell and the membrane supported with a porous polymeric block underneath. Starting day-12, ossifying marrow explants were placed in the custom-made loading chamber every two days up to day-26. Mechanical stimulation was applied as a compressive load for 900 cycles per day at 0.5 Hz. **C)** A photograph of the fully functional custom loading setup. The loading system is composed of a polycarbonate base with springs under compression and guide rods which are attached to the upper loading bar with the adjustable height loading rod. The loading chamber (sterile inside) is sealed from the outside with the elastic sealing membrane and a filtered (0.2 μm pore size) air vent. The adjustable height loading rod engages with the inside-chamber PTFE loading tip (custom load cell) by means of a magnet coupling. The force is generated by four Flexinol actuator wires running in parallel between the lower base and the upper loading bar (arrowheads).

The loading system operates under displacement control such that displacement occurs proportionally to the applied current. The displacement-current relation is linear and calibrated by a displacement gage before use. The displacement (Δ, μm) of the loading system in response to the applied current (I, mA) displayed a linear calibration curve ($\Delta = 3.9*I - 499$, $R^2=0.997$). The error between the set displacement and the actual displacement was measured to be varying between ±3.1% and ±5.6% for the minimum and maximum displacement set values respectively. Prolonged tests of the system resulted in no detectable drift in the set displacement values.

4.3.3. Mechanical Stimulation of Ossifying Marrow Explants

The inserts were removed from the culture wells, transferred to the loading setup inside a laminar flow hood and placed back in the incubator for mechanical loading under compression. Loading began at day 12 and it was applied once in every 2 days for 900 cycles per day at 0.5 Hz up to day 26 (Fig.4.2). Upon completion of loading, the inserts were placed back in culture wells and incubated until the next bout of mechanical stimulation. Five marrow explants were loaded as such longitudinally over time. There were also nine control explants which were also transferred to the loading set up, the actuator tip was engaged; however, the loading was not performed. The total peak load was measured by the custom-made load cell (Fig.4.1B) and the maximum stress experienced by the explants was adjusted to 0.0313 N/mm^2 (31.3 kPa), which was estimated to induce a maximum apparent strain of about 5% in ossifying explants. The maximum stress and strain values were approximately determined by assuming an elastic

modulus of woven bone tissue (4 GPa, [45]) and estimating the ossifying area of the marrow explants by using the projected light microscope images. The conditioned media was collected 2 days after the application of each loading bout (Fig.4.2) and stored at -80°C before being used in ELISA assays.

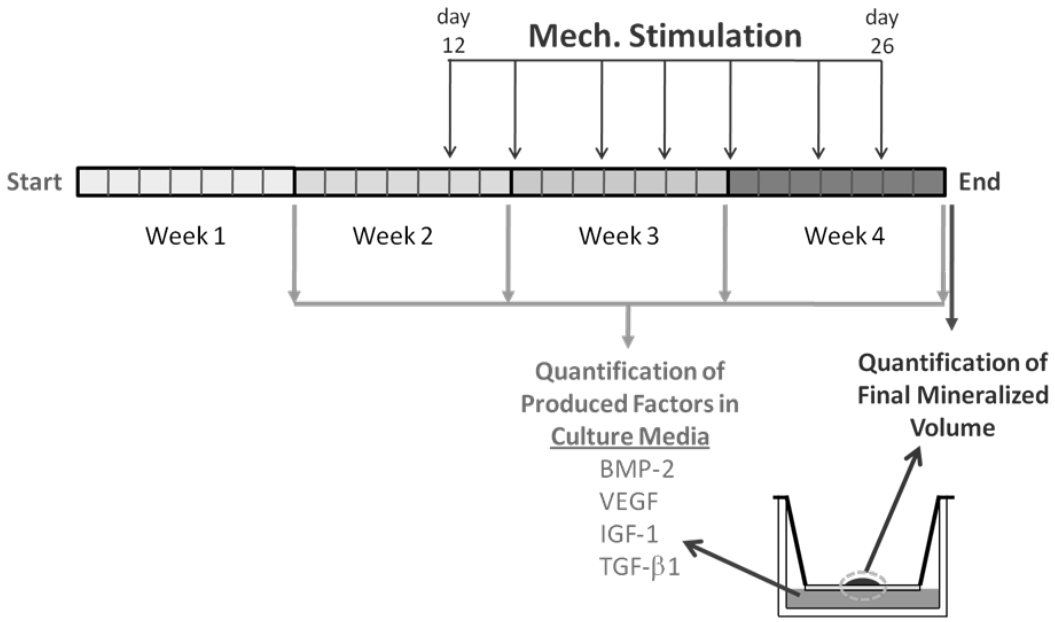


Figure 4.2: Timeline of the loading experiment of ossifying marrow. Mechanical stimulation was applied to the ossifying marrow explants starting day-12 up to day-26. The concentration levels of BMP-2, VEGF and IGF-1 and TGF-β1 in conditioned culture media were measured every 7 days starting day-7. The final ossified volume of the marrow explants was quantified at the end of the experiment with micro computed tomography (day-28).

4.3.4. Quantification of BMP-2, VEGF, IGF-1 and TGF-β1 Levels

The quantification of GFs was performed on the media conditioned by loaded and control groups of ossifying explants at four time points: at day 7 (5 days before the loading cycles started) and at days 14, 21 and 28 (after the loading cycles started, Fig.4.2). The GF concentrations in the conditioned media were measured by quantitative

enzyme-linked immunosorbent assay (ELISA) development kits (BMP-2: PeproTech; VEGF, IGF-1 and TGF-β1: R&D Systems). TGF-β1 in the conditioned media was acid-activated to make it immunoreactive and render it detectable by the immunoassay. Acid-activation was carried out by incubating aliquots of the conditioned media with 1N HCl followed by neutralization with 1.2N NaOH in 0.5M HEPES buffer. Then the standard ELISA protocols provided by the manufacturers of the kits were followed. Briefly, 96-well microplates (MaxiSorp, Nalge) were coated with capture antibody, and the wells were blocked for 1 hour. Samples and standards were added to wells followed by incubation for 2-3 hours at room temperature. After thorough washing, detection antibody was added at the specified concentration for each kit and the plates were incubated for 2 hours at the room temperature. The peroxidase substrate solution was added (protected from direct light) and incubated at room temperature for 20 minutes. The enzyme reaction was stopped with 2N hydrochloric acid solution. The color product was detected by a microplate reader set at 450 nm with wavelength correction set at 540 nm. The unknown concentrations of GFs in the samples were calculated based on the standard curves obtained. The levels of the GFs in the non-conditioned growth medium were also measured to determine the baseline levels.

4.3.5. Micro Computed Tomography (μCT)

At the end of the 28 day long experiment, the ossified marrow samples were fixed in 10% formalin and kept in the fixative before and throughout the μCT scans (SCANCO Medical AG, μCT 40, Brüttisellen, Switzerland). μCT scans were performed with a 16

μm voxel resolution (I = 145 μA, E = 55 kVp, integration time = 200 ms). The scanned images were reconstructed and analyzed with a commercial software (SCANCO evaluation software) and the standard segmentation parameters were used [46-48]. The total bone volume (BV, μm^3) calculated by the software was used and reported as the final ossified volume (OV) of the marrow explants.

4.3.6. Histology of Ossified Marrow Explants

At the end of the 28-day culture period (Fig.4.2), the ossified marrow explants were fixed in 10% formalin. Decalcification of the samples was performed in formic acid solution (1:1 solution of 50% aqueous formic acid and 20% sodium citrate) for 12 hours. Samples were then washed in tap water for 30 to 45 minutes, embedded in paraffin, sectioned and dried overnight in 37°C oven. The sections were deparaffinized and hydrated in gradually decreasing percentages of alcohol solutions (100%, 95%, 70% and water). The slides were then stained with Masson's trichrome method for visualizing the collagen-rich ossifying regions. The light microscope images were taken with Olympus Vanox microscope equipped with Qimaging Micropublisher 5.0 RTV 5 megapixel CCD camera.

4.3.7. Statistical Analysis

The ossified volume in the loaded samples (n=5) and the controls (n=9) was compared statistically with Mann-Whitney U-test with a significance threshold was set at

0.05 ($p < 0.05$). The levels of GFs produced by control (n=4) and loaded (n=4) samples at various time points (days 7, 14, 21 and 28) were statistically analyzed by using General Linear Model with Tukey's posthoc test with statistical significance threshold set at 0.05. Relations between the GF concentrations and the final ossified volume were analyzed by calculating the Pearson product moment correlation coefficient (PCC) with a significance threshold of 0.01 ($p<0.01$). Error bars in the figures were displayed as standard error.

4.4. Results

4.4.1. The Effect of Mechanical Stimulation on Marrow Explants

The ossified volume in the loaded samples was significantly greater (about 4 times) than the control samples (Fig.4.3). At the end of the 28 day culture period, the ossification of the bone marrow explants was visible through light microscopy (Fig.4.4A and 4B). The ossified center of the loaded samples (Fig.4.4B) was observed to appear darker under light microscope compared to control samples (Fig.4.4A). The ossified volume was further visualized and quantified by μCT. Three dimensional reconstructed images obtained from μCT scans revealed a smaller ossified area and volume in the control samples (Fig.4.4C, a well ossified sample in the control group is shown) compared to loaded samples (Fig.4.4D, a well-ossified sample in the loaded group is shown).

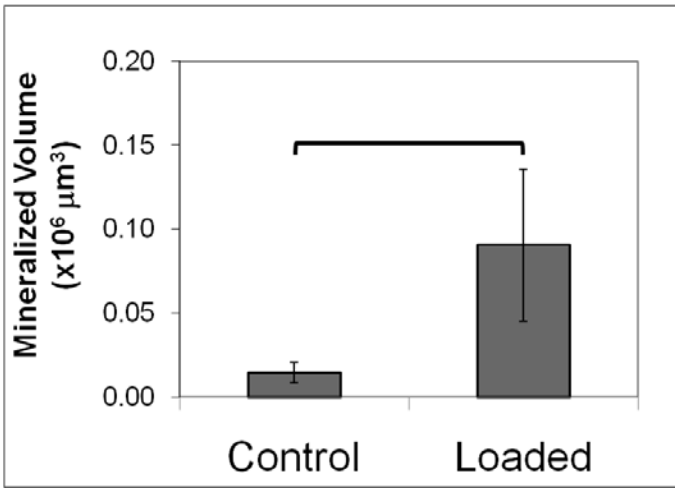


Figure 4.3: Effect of mechanical loading on ossified volume. The final mineralized (ossified) volume of the loaded samples (n=5) was significantly more compared to controls (n=9). The bracket connecting the control and loaded groups indicates statistical significance ($p<0.05$, Mann-Whitney U test).

4.4.2. Morphological Characterization of Ossified Marrow

Histological assessment of marrow explants at day-28 (Fig.4.4E) displayed a multi-cellular composition. Masson's trichrome stain revealed high density collagen regions (blue color) starting day-14 at the bottom of the explants (the figure is not shown), which is the surface in contact with the porous membrane. The collagen density increased and covered a large portion of the sample by day-28 (Fig.4.4E). Viable cells with osteoblast-like morphology (arrows in Fig.4.4E) above and below the collagen-rich regions were observed. In addition, viable cells with osteocyte-like morphology (* in Fig.4.4E) could be seen embedded in the collagen-rich sections.

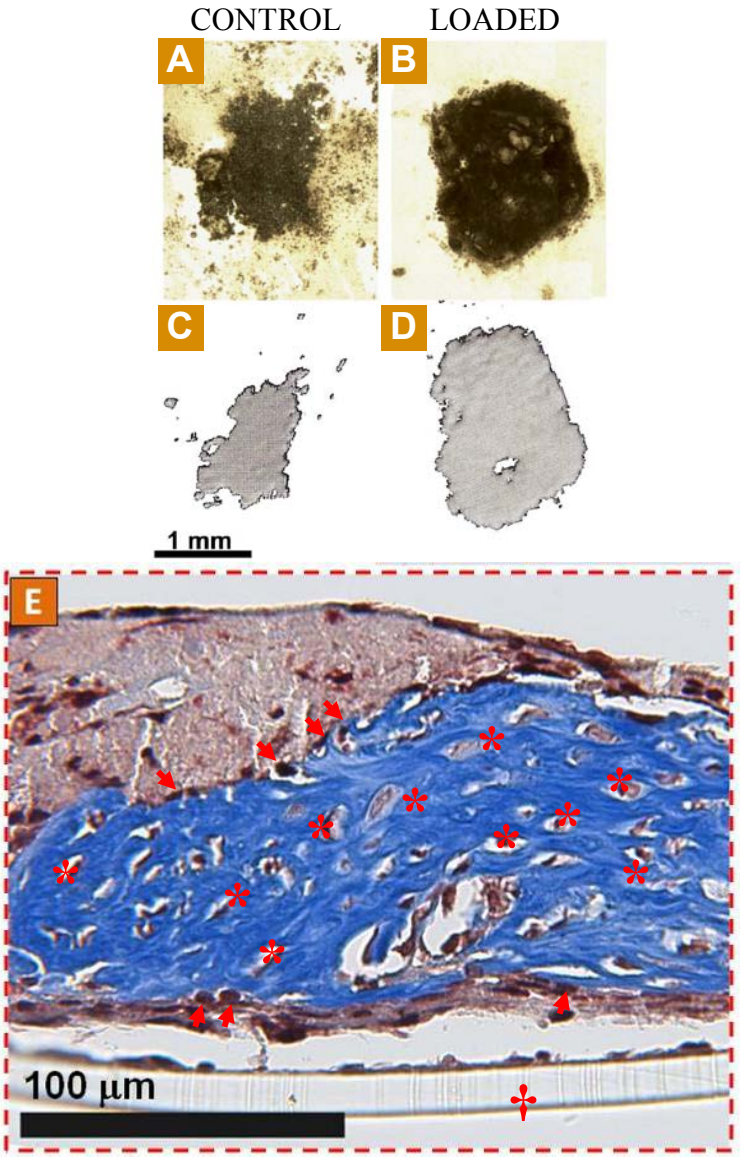


Figure 4.4: Typical appearance of ossified marrow under light microscope (at day-28) which were cultured in the absence (**A**) and in the presence (**B**) of mechanical stimulation. Corresponding three dimensional reconstructed images (from µCT scans) of the ossified explants for a control sample (**C**) and a loaded sample (**D**). **E)** Masson's Trichrome stained section of a bone marrow explant that underwent mechanical stimulation. Blue color indicates the collagen-rich regions where ossification took place. Arrows indicate viable cells with osteoblast-like morphology which are in the process of laying the ossified matrix and getting ready to be engulfed by the ossified matrix. * indicates viable cells with osteocyte-like morphology trapped in the ossified matrix. † indicates the 0.4 µm pore sized supporting membrane located below the explants.

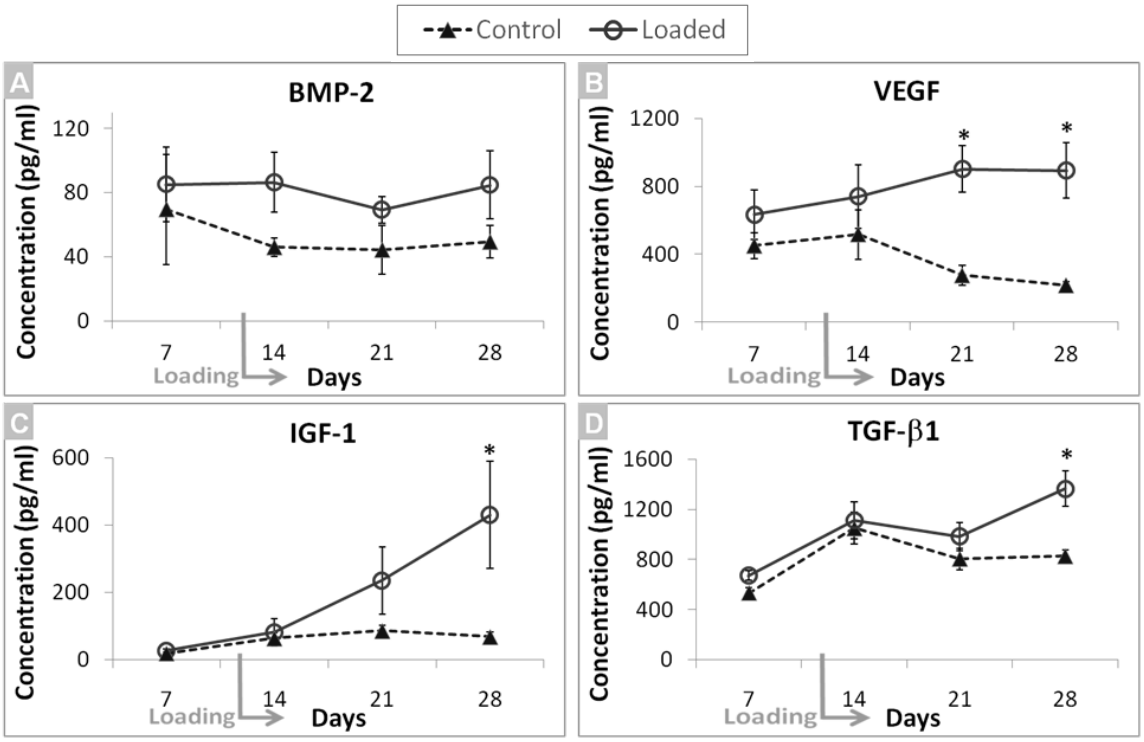


Figure 4.5: Effect of mechanical stimulation on the production of BMP-2, VEGF, IGF-1 and TGF-β1 by ossifying marrow explants. **A)** BMP-2 production was not affected significantly from mechanical stimulation. **B)** VEGF production in the loaded samples was significantly higher at days 21 and 28. **C)** IGF-1 production at day-28 was significantly greater in the loaded samples compared to controls. **D)** TGF-β1 production was significantly higher in the loaded samples than the controls at day-28. * indicates statistical significance ($p<0.05$) between the loaded samples and the controls at marked time points (n=4 for each sample at each time point, General Linear Model with Tukey post-hoc comparisons).

4.4.3. The Effect of Mechanical Stimulation on the Production of Factors

Even though BMP-2 was observed to be produced by both groups of samples, mechanical loading of the ossifying marrow explants did not have a significant effect on the production of BMP-2 at any time point (Fig.4.5A). VEGF production by the non-loaded ossifying marrow explants declined after day-14. However, loaded marrow explants sustained VEGF production after day-14 which was significantly greater (Fig.4.5B) on day-21 (about 3 times) and on day-28 (about 4 times) in the loaded samples

compared to controls. IGF-1 production was increased about 6 times on day-28 by mechanical stimulation (Fig.4.5C) in the loaded samples. Similarly, TGF-β1 production was also increased about 1.5 times on day-28 by mechanical loading (Fig.4.5D).

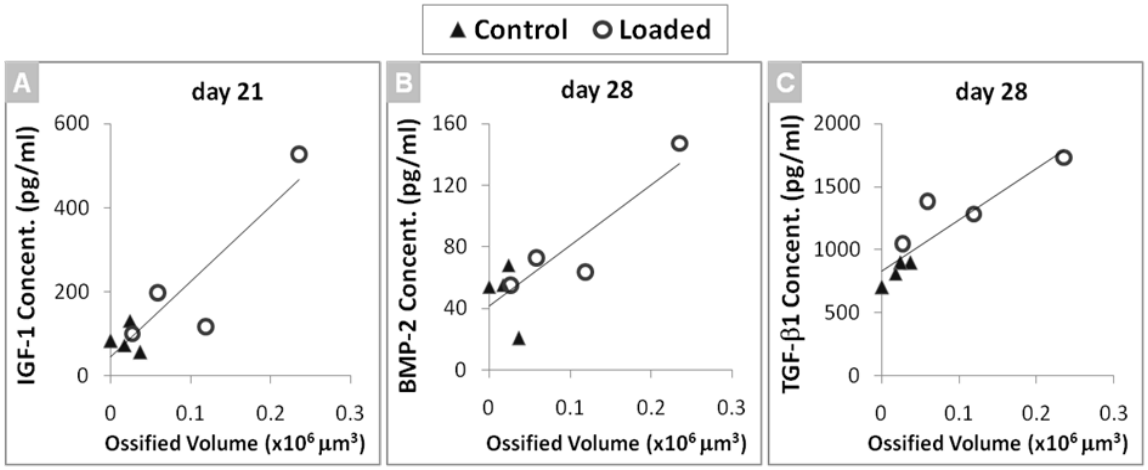


Figure 4.6: Correlation between the growth factor levels and the ossified volume (OV) of marrow explants. **A)** IGF-1 level at day 21 correlated with OV (Pearson product moment correlation coefficient, PCC: 0.899, $p<0.01$). **B)** BMP-2 level at day 28 correlated with OV (PCC: 0.850, $p<0.01$). **C)** TGF-β1 level at day 28 correlated with OV (PCC: 0.907, $p<0.01$). Triangles indicate control (non-loaded) samples, and hollow circles indicate loaded samples.

4.4.4. Correlation between the Levels of Factors and the Ossified Volume

IGF-1 level at day-21 was observed to correlate significantly (PCC: 0.899, $p<0.01$) with the final ossified volume (Fig.4.6A). Similarly, final ossified volume correlated significantly with the day-28 levels of BMP-2 (Fig.4.6B, PCC: 0.850, $p<0.01$) and TGF-β1 (Fig.4.6C, PCC: 0.907, $p<0.01$).

4.5. Discussion and Conclusion

It was shown that *in vitro* ossifying marrow explants were mechanoresponsive since compressive mechanical stimulation induced significantly more bone formation in the loaded samples. In addition, mechanical loading sustained the production level of VEGF between days 21 and 28 and enhanced production levels of IGF-1 and TGF-β1 after day-21 by in vitro ossifying bone marrow explants compared to non-loaded controls. However mechanical stimulation did not induce a statistically significant effect on BMP-2 production level at any time point.

Histological assessment of the Masson's Trichrome stained ossified marrow sections revealed collagen-rich regions in the central regions of the explants (Fig.4.4E) which corresponded to the ossified volume of the explants as detected by μCT. Viable cells with osteoblast-like morphology were observed surrounding the lower and upper surfaces of the ossification site, which were in the process of laying the mineralized matrix and getting trapped within the ossified matrix as cells with osteocyte-like morphology. Therefore it can be suggested that the applied compressive mechanical load is experienced by the resident cells in different forms. Mechanical stimulation information is acquired by bone and bone marrow cells in different forms: compression, tension and fluid shear. These different types of stimulation and their effects on bone marrow cells has been discussed before in a detailed review [49]. Even though the *in vitro* loading model presented in this study is predominantly compression based, it would be reasonable to suggest that there may be other forms of stimulation experienced by the resident cells. A potential secondary stimulation mechanism induced by this loading

system may be through fluid flow induced shear due to compressive deformation of the ossifying matrix [30, 50]. It was previously shown that unconfined compression of fibrous tissues resulted in loading-induced convection inside the tissue [51]. Therefore, the *in vitro* loading system combined with 3D marrow ossification presented in this study may possibly result in a more complex stress-strain field (compression and fluid shear) for the resident cells. It can be suggested that the cells with osteoblast-like morphology surrounding the ossifying site may be experiencing predominantly compressive stress as they are either positioned at the interface of the ossifying site with the actuator tip; or, between the ossifying site and the bottom support. On the other hand, the cells with osteocyte-like morphology encapsulated in the ossified matrix may be undergoing compression induced fluid flow based shear stress. This stress-strain field may be similar to the stress-strain field experienced by bone and bone marrow cells under natural conditions [49, 52]. Therefore the results of this study should be interpreted considering the possibly complex stress-strain field experienced by the resident cells.

The results indicated that the VEGF production was sustained over time due to mechanical loading. VEGF plays a critical role in BMP-induced osteogenesis [53]. In addition, the effectiveness of low level sustained VEGF release over burst-release for effective blood vessel formation in ischemic tissues was shown before [54]. Therefore, sustained production of VEGF in response to mechanical stimulation may be valuable for enhanced vascularization of newly forming bone tissue. In attestation, it was previously shown with an *in vivo* animal model (employing VEGF inhibitory antibody in the experimental animals) that VEGF signaling is essential for bone formation induced by

mechanical strain [55]. In addition, VEGF gene expression was shown to be upregulated by pulsatile fluid shear stress in osteoblasts [56] and in bone marrow stromal cells [57]. Therefore it can be suggested that the sustained VEGF production in the loaded ossifying explants was maintained by the resident marrow stromal cells and osteoblasts experiencing a complex stress-train field of compression and fluid shear. This proposition requires further verification via immunohistochemical methods and computational simulations.

There is evidence suggesting that marrow stromal cells regulate osteoblast proliferation with the involvement of IGF-1 and IGF-2 [58]. It was shown that compressive loading enhanced IGF-1 gene expression in MSCs [59]. Similarly, tensile stretch increased the mRNA expression of IGF-1 in human osteoblastic cell cultures [19]. The synergistic involvement of IGF-1 and mechanical loading was studied with an *in vivo* transgenic mouse study with osteoblasts selectively overexpressing IGF-1 [26]. It was shown that bone formation in the transgenic mouse was elevated in response to mechanical loading in comparison to wild-type animals. The marrow platform presented here allowed quantification of IGF-1 production and indicated that IGF-1 was one of the most responsive GFs to mechanical loading. IGF-1 level increased substantially after day-21, during the second week of loading. Furthermore, there was a correlation between the amount of IGF-1 production level at day-21 and the final ossified matrix volume. Therefore, mechanical anabolism in this model seems to occur through mediation of IGF-1; however, this assertion needs to be proven by targeted inhibition of IGF in this culture model.

TGF-β is considered to enhance proliferation of osteoprogenitor cells at all stages of bone regeneration [1]. It was also shown that cyclic strain induced TGF-β1 production in human osteoblasts [60]. Similarly, fluid flow induced shear increased the gene expression of TGF-β1 in rat calvarial osteoblastic cultures [61] and in bone marrow stromal cells [57]. Furthermore, equibiaxial strain enhanced the expression of both TGF-β1 and VEGF (short term: 3 hours) in calvarial osteoblasts [62]. The current study was able to investigate longer term response of GFs. Unlike VEGF which readily responded to mechanical loading without delay, it took about one more week for TGF-β1 production to respond to mechanical stimulation. The level of TGF-β1 at day-28 was observed to be highly correlated with the final ossified volume of the explants which supports the importance of TGF-β1 in osteogenesis.

BMP-2 has been shown to play an important role in bone regeneration by means of its capacity to promote the differentiation of MSCs to osteochondroblastic phenotype [1, 63]. It was previously observed that compressive stimulation of osteoblasts in 3-dimensional electrospun poly(e-caprolactone) (PCL) scaffolds resulted in up-regulation of BMP-2 mRNA at both 10% and 20% strain compression levels [20]. However, in this study, BMP-2 production by in vitro ossifying marrow explants was not observed to be affected significantly by an estimated 5% compressive strain level. This may be due to the presence of other cell types in the presented ossification model or the differences in loading regime and intensity employed. Even though BMP-2 production level was not significantly affected from mechanical stimulation, BMP-2 level at day-28 was observed to be highly correlating with the final ossified volume of the explants.

The majority of the in vitro studies investigating the bone cell mechano-responsiveness have used 2D monolayer cultures. There have been attempts to develop 3-dimensional *in vitro* models to better mimic the natural microenvironment of bone formation site by seeding cells in collagen-based matrices or scaffolds [20, 30, 50, 64]. Even though these studies offer improvements over the traditional 2D models, they still neglect the coexistent multi-cellular composition of the bone formation environment (i.e. HSCs, MSCs and OACs). It has long been considered that the microenvironment of the MSCs is the most critical parameter affecting the lineage decisions. However it has recently been shown that HSCs are able to induce osteoblastic differentiation of MSCs under basal conditions [31]. Therefore the inherent ossification of bone marrow explants under basal conditions presented in this study can be attributed to the coexistence of MSCs and HSCs in addition to the other resident cells of bone marrow.

It is known that mechanical stimulation has osteogenic effects on fracture repair [5, 65-67] and has anabolic effect on healthy bone *in vivo* [26, 27, 68]. Strains employed in fracture healing models (5% range) are greater than those employed in anabolic stimulation of healthy bone (0.5%-2% range). However, commonly, these studies employ loading bouts with rest periods in between and frequency levels at about 0.5-2 Hz are applied. A particular loading model for enhancing fracture healing demonstrated that the mechanical stimulation applied after about 10 days following fracture has an accelerating effect on bone healing, whereas the mechanical stimulation applied after 3 days of fracture has a deleterious effect on bone healing [65]. Therefore, in the light of the existing *in vivo* loading models, we have adopted a 5% strain and 0.5 Hz loading regime

applied 900 cycles every 2 days beginning from the 12th day after the initiation of culturing, which provided sufficient time for the early formation of a partially ossified matrix. In attestation, histological assessment (Masson's trichrome) of earlier time-points (data not reported) indicated that high collagen production was evident (emergence of blue color) by day-14, which can be considered as the indicator of earlier stages of ossification.

It should be noted that, in this study, the response of the GF production levels to mechanical stimulation is an accumulated response spread over a 2 day period (i.e. GF levels were quantified 2 days after mechanical stimulation, Fig.4.2). Therefore, the results presented here should be considered accordingly and should not be confused with the short term response of these factors. A detailed analysis of short term responses of BMP-2, VEGF, IGF-1 and TGF-β1 to mechanical stimulation with this ossification model warrants further investigation. The correlations reported between the GF levels and the final ossified volume can be used to study the temporal involvement of the GFs in bone formation. However, it should be noted that the correlations presented here do not necessarily imply causations, which require further targeted inhibition studies of the specific factors.

Mechanical stimulation was previously shown to enhance cellular proliferation in osteogenesis with in vitro studies [15, 16, 18, 21, 60]. Therefore, the increase in the final ossified volume of marrow explants observed in this study in response to mechanical stimulation (Fig.4.3 and Fig.4.4) can be attributed to two factors: 1) enhanced cellular proliferation, and 2) enhanced total collagenous matrix production by the increased cell

population. Therefore, the effect of mechanical stimulation on cellular proliferation and non-mineralized matrix production (that was not detected by μCT scans in this study) dynamics in marrow ossification model warrants further investigation.

GFs control cell division, differentiation and extracellular matrix synthesis. They are also known to play an important role in bone formation, and regeneration [1, 8, 10, 69]. It has been suggested that there is a crosstalk between the GF signaling pathways and the overall osteogenic outcome may be a synergistic contribution [1, 9, 10, 53, 69-74]. Therefore it is critical to investigate the effect of mechanical cues on the expression of multiple factors to better understand their individual and cooperative involvement in bone regeneration. GFs involved in osteogenesis are many and they are not limited to the ones studied here. A comprehensive analysis of other potent factors that are affected from mechanical stimulation can be investigated using the inherently ossifying marrow explant platform.

In conclusion, it was demonstrated that the *in vitro* ossifying marrow explants are mechanoresponsive and can be used to study the effect of mechanical stimulation on the production of various GFs. It was shown that the final ossified volume increased in the mechanically stimulated marrow samples. The production levels of VEGF, IGF-1 and TGF-β1 were enhanced or sustained in response to compressive mechanical stimulation. The outcomes of this study are essential for understanding the nature's way of regenerating bone tissue in terms of the complex involvement of multiple GFs in a multicellular 3D environment and the effect of mechanical stimulation on this process.

4.6. Acknowledgements

This study was funded a grant from Musculoskeletal Transplant Foundation.

4.7. References

1. Lieberman, J.R., A. Daluiski, and T.A. Einhorn, *The role of growth factors in the repair of bone - Biology and clinical applications.* Journal of Bone and Joint Surgery-American Volume, 2002. **84A**(6): p. 1032-1044.
2. Caplan, A.I., *Bone-Development and Repair.* Bioessays, 1987. **6**(4): p. 171-175.
3. Devine, M.J., C.M. Mierisch, E. Jang, P.C. Anderson, and G. Balian, *Transplanted bone marrow cells localize to fracture callus in a mouse model.* Journal of Orthopaedic Research, 2002. **20**(6): p. 1232-1239.
4. Bruder, S.P., A.A. Kurth, M. Shea, W.C. Hayes, N. Jaiswal, and S. Kadiyala, *Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells.* J Orthop Res, 1998. **16**(2): p. 155-62.
5. Hannouche, D., H. Petite, and L. Sedel, *Current trends in the enhancement of fracture healing.* Journal of Bone and Joint Surgery-British Volume, 2001. **83B**(2): p. 157-164.
6. Petite, H., V. Viateau, W. Bensaid, A. Meunier, C. de Pollak, M. Bourguignon, K. Oudina, L. Sedel, and G. Guillemin, *Tissue-engineered bone regeneration.* Nature Biotechnology, 2000. **18**(9): p. 959-63.
7. Braccini, A., D. Wendt, C. Jaquiery, M. Jakob, M. Heberer, L. Kenins, A. Wodnar-Filipowicz, R. Quarto, and I. Martin, *Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts.* Stem Cells, 2005. **23**(8): p. 1066-1072.

8. Gerstenfeld, L.C., D.M. Cullinane, G.L. Barnes, D.T. Graves, and T.A. Einhorn, *Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation.* Journal of Cellular Biochemistry, 2003. **88**(5): p. 873-884.

9. Huang, Z.N., E.R. Nelson, R.L. Smith, and S.B. Goodman, *The sequential expression profiles of growth factors from osteroprogenitors to osteoblasts In vitro.* Tissue Engineering, 2007. **13**(9): p. 2311-2320.

10. Tatsuyama, K., Y. Maezawa, H. Baba, Y. Imamura, and M. Fukuda, *Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone.* European Journal of Histochemistry, 2000. **44**(3): p. 269-278.

11. Reddi, A.H., *Role of morphogenetic proteins in skeletal tissue engineering and regeneration.* Nature Biotechnology, 1998. **16**(3): p. 247-252.

12. Gurkan, U.A., J. Gargac, and O. Akkus, *The Sequential Production Profiles of Growth Factors and Their Relations to Bone Volume in Ossifying Bone Marrow Explants.* Tissue Eng Part A, 2010. **16**(TBD).

13. Simmons, C.A., S. Matlis, A.J. Thornton, S.Q. Chen, C.Y. Wang, and D.J. Mooney, *Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway.* Journal of Biomechanics, 2003. **36**(8): p. 1087-1096.

14. Sumanasinghe, R.D., S.H. Bernacki, and E.G. Loboa, *Osteogenic differentiation of human mesenchymal stem cells in collagen matrices: Effect of uniaxial cyclic tensile strain on bone morphogenetic protein (BMP-2) mRNA expression.* Tissue Engineering, 2006. **12**(12): p. 3459-3465.

15. Choi, K.M., Y.K. Seo, H.H. Yoon, K.Y. Song, S.Y. Kwon, H.S. Lee, and J.K. Park, *Effects of mechanical stimulation on the proliferation of bone marrow-derived human mesenchymal stem cells.* Biotechnology and Bioprocess Engineering, 2007. **12**(6): p. 601-609.

16. Koike, M., H. Shimokawa, Z. Kanno, K. Ohya, and K. Soma, *Effects of mechanical strain on proliferation and differentiation of bone marrow stromal cell line ST2.* Journal of Bone and Mineral Metabolism, 2005. **23**(3): p. 219-225.

17. Qi, M.C., J. Hu, S.J. Zou, H.Q. Chen, H.X. Zhou, and L.C. Han, *Mechanical strain induces osteogenic differentiation: Cbfa1 and Ets-1 expression in stretched rat mesenchymal stem cells.* International Journal of Oral and Maxillofacial Surgery, 2008. **37**(5): p. 453-458.

18. Song, G.B., Y. Ju, X.D. Shen, Q. Luo, Y.S. Shi, and J. Qin, *Mechanical stretch promotes proliferation of rat bone marrow mesenchymal stem cells.* Colloids and Surfaces B-Biointerfaces, 2007. **58**(2): p. 271-277.

19. Cillo, J.E., R. Gassner, R.R. Koepsel, and M.J. Buckley, *Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: Implications for distraction osteogenesis.* Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics, 2000. **90**(2): p. 147-154.

20. Rath, B., J. Nam, T.J. Knobloch, J.J. Lannutti, and S. Agarwal, *Compressive forces induce osteogenic gene expression in calvarial osteoblasts.* Journal of Biomechanics, 2008. **41**(5): p. 1095-1103.

21. Kaspar, D., W. Seidl, C. Neidlinger-Wilke, A. Ignatius, and L. Claes, *Dynamic cell stretching increases human osteoblast proliferation and CICP synthesis but decreases osteocalcin synthesis and alkaline phosphatase activity.* Journal of Biomechanics, 2000. **33**(1): p. 45-51.

22. Kadow-Romacker, A., J.E. Hoffmann, G. Duda, B. Wildemann, and G. Schmidmaier, *Effect of Mechanical Stimulation on Osteoblast- and Osteoclast-Like Cells in vitro.* Cells Tissues Organs, 2009. **190**(2): p. 61-68.

23. Ziros, P.G., A.P.R. Gil, T. Georgakopoulos, I. Habeos, D. Kletsas, E.K. Basdra, and A.G. Papavassiliou, *The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells.* Journal of Biological Chemistry, 2002. **277**(26): p. 23934-23941.

24. Harter, L.V., K.A. Hruska, and R.L. Duncan, *Human Osteoblast-Like Cells Respond to Mechanical Strain with Increased Bone-Matrix Protein-Production Independent of Hormonal-Regulation.* Endocrinology, 1995. **136**(2): p. 528-535.

25. Duty, A.O., M.E. Oest, and R.E. Guldberg, *Cyclic mechanical compression increases mineralization of cell-seeded polymer scaffolds in vivo.* Journal of Biomechanical Engineering-Transactions of the Asme, 2007. **129**(4): p. 531-539.

26. Gross, T.S., S. Srinivasan, C.C. Liu, T.L. Clemens, and S.D. Bain, *Noninvasive loading of the murine tibia: An in vivo model for the study of mechanotransduction.* Journal of Bone and Mineral Research, 2002. **17**(3): p. 493-501.

27. Akhter, M.P., D.M. Cullen, E.A. Pedersen, D.B. Kimmel, and R.R. Reeker, *Bone response to in vivo mechanical loading in two breeds of mice.* Calcified Tissue International, 1998. **63**(5): p. 442-449.

28. Palomares, K.T.S., R.E. Gleason, Z.D. Mason, D.M. Cullinane, T.A. Einhorn, L.C. Gerstenfeld, and E.F. Morgan, *Mechanical Stimulation Alters Tissue Differentiation and Molecular Expression during Bone Healing.* Journal of Orthopaedic Research, 2009. **27**(9): p. 1123-1132.

29. Birgersdotter, A., R. Sandberg, and I. Ernberg, *Gene expression perturbation in vitro - A growing case for three-dimensional (3D) culture systems.* Seminars in Cancer Biology, 2005. **15**(5): p. 405-412.

30. Mauney, J.R., S. Sjostorm, J. Blumberg, R. Horan, J.P. O'Leary, G. Vunjak-Novakovic, V. Volloch, and D.L. Kaplan, *Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro.* Calcified Tissue International, 2004. **74**(5): p. 458-468.

31. Jung, Y.G., J.H. Song, Y. Shiozawa, J.C. Wang, Z. Wang, B. Williams, A. Havens, A. Schneider, C.X. Ge, R.T. Franceschi, L.K. McCauley, P.H. Krebsbach, and R.S. Taichman, *Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche.* Stem Cells, 2008. **26**(8): p. 2042-2051.

32. Eipers, P.G., S. Kale, R.S. Taichman, G.G. Pipia, N.A. Swords, K.G. Mann, and M.W. Long, *Bone marrow accessory cells regulate human bone precursor cell development.* Experimental Hematology, 2000. **28**(7): p. 815-825.

33. Bruder, S.P., D.J. Fink, and A.I. Caplan, *Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy.* J Cell Biochem, 1994. **56**(3): p. 283-94.

34. Caplan, A.I., *Mesenchymal Stem-Cells.* Journal of Orthopaedic Research, 1991. **9**(5): p. 641-650.

35. van den Dolder, J. and J.A. Jansen, *Enrichment of osteogenic cell populations from rat bone marrow stroma.* Biomaterials, 2007. **28**(2): p. 249-255.

36. Wu, J.Y., D.T. Scadden, and H.M. Kronenberg, *Role of the Osteoblast Lineage in the Bone Marrow Hematopoietic Niches.* Journal of Bone and Mineral Research, 2009. **24**(5): p. 759-764.

37. Taichman, R.S., M.J. Reilly, and S.G. Emerson, *Human osteoblasts support human hematopoietic progenitor cells in in vitro bone marrow cultures.* Blood, 1996. **87**(2): p. 518-524.

38. Taichman, R.S., M.J. Reilly, R.S. Verma, K. Ehrenman, and S.G. Emerson, *Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors.* British Journal of Haematology, 2001. **112**(2): p. 438-448.

39. Cabrita, G.J.M., B.S. Ferreira, C.L. da Silva, R. Goncalves, G. Almeida-Porada, and J.M.S. Cabral, *Hematopoietic stem cells: from the bone to the bioreactor.* Trends in Biotechnology, 2003. **21**(5): p. 233-240.

40. Dennis, J.E. and A.I. Caplan, *Bone Marrow Mesenchymal Stem Cells*, in *Stem Cells Handbook*, S. Sell, Editor. 2003, Humana Press Inc.: Totowa, NJ. p. 107-118.

41. Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak, *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-147.

42. Muschler, G.F., C. Boehm, and K. Easley, *Aspiration to obtain osteoblast progenitor cells from human bone marrow: The influence of aspiration volume.* Journal of Bone and Joint Surgery-American Volume, 1997. **79A**(11): p. 1699-1709.

43. Muschler, G.F., H. Nitto, C.A. Boehm, and K.A. Easley, *Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors.* Journal of Orthopaedic Research, 2001. **19**(1): p. 117-125.

44. Luria, E.A., M.E. Owen, A.J. Friedenstein, J.F. Morris, and S.A. Kuznetsow, *Bone-Formation in Organ-Cultures of Bone-Marrow.* Cell and Tissue Research, 1987. **248**(2): p. 449-454.

45. Cowin, S.C., *Bone mechanics handbook*. 2nd ed. 2001, Boca Raton, Fla.: CRC Press. 1 v. (various pagings).

46. Morgan, E.F., Z.D. Mason, K.B. Chien, A.J. Pfeiffer, G.L. Barnes, T.A. Einhorn, and L.C. Gerstenfeld, *Micro-computed tomography assessment of fracture healing: Relationships among callus structure, composition, and mechanical function.* Bone, 2009. **44**(2): p. 335-344.

47. Muller, R., H. Van Campenhout, B. Van Damme, G. Van Der Perre, J. Dequeker, T. Hildebrand, and P. Ruegsegger, *Morphometric analysis of human bone biopsies: A quantitative structural comparison of histological sections and micro-computed tomography.* Bone, 1998. **23**(1): p. 59-66.

48. Oest, M.E., K.M. Dupont, H.J. Kong, D.J. Mooney, and R.E. Guldberg, *Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects.* Journal of Orthopaedic Research, 2007. **25**(7): p. 941-950.

49. Gurkan, U.A. and O. Akkus, *The Mechanical Environment of Bone Marrow: A Review.* Annals of Biomedical Engineering, 2008. **36**(12): p. 1978-1991.

50. Tanaka, S.M., H.B. Sun, R.K. Roeder, D.B. Burr, C.H. Turner, and H. Yokota, *Osteoblast responses one hour after load-induced fluid flow in a three-dimensional porous matrix.* Calcified Tissue International, 2005. **76**(4): p. 261-271.

51. Huang, C.Y. and W.Y. Gu, *Effects of tension-compression nonlinearity on solute transport in charged hydrated fibrous tissues under dynamic unconfined compression.* Journal of Biomechanical Engineering-Transactions of the Asme, 2007. **129**(3): p. 423-429.

52. Cowin, S.C., *Bone poroelasticity.* Journal of Biomechanics, 1999. **32**(3): p. 217-238.

53. Peng, H.R., V. Wright, A. Usas, B. Gearhart, H.C. Shen, J. Cummins, and J. Huard, *Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4.* Journal of Clinical Investigation, 2002. **110**(6): p. 751-759.

54. von Degenfeld, G., A. Banfi, M.L. Springer, R.A. Wagner, J. Jacobi, C.R. Ozawa, M.J. Merchant, J.P. Cooke, and H.M. Blau, *Microenvironmental VEGF distribution is critical for stable and functional vessel growth in ischemia.* Faseb Journal, 2006. **20**(14): p. 2657-+.

55. Yao, Z.Q., M.H. Lafage-Proust, J. Plouet, S. Bloomfield, C. Alexandre, and L. Vico, *Increase of both angiogenesis and bone mass in response to exercise depends on VEGF.* Journal of Bone and Mineral Research, 2004. **19**(9): p. 1471-1480.

56. Thi, M.M., D.A. Iacobas, S. Iacobas, and D.C. Spray, *Fluid shear stress upregulates vascular endothelial growth factor gene expression in osteoblasts.* Skeletal Biology and Medicine, Pt B, 2007. **1117**: p. 73-81.

57. Sharp, L.A., Y.W. Lee, and A.S. Goldstein, *Effect of Low-Frequency Pulsatile Flow on Expression of Osteoblastic Genes by Bone Marrow Stromal Cells*. Annals of Biomedical Engineering, 2009. **37**(3): p. 445-453.

58. Zhang, R.W., D.J. Simmons, R.S. Crowther, S. Mohan, and D.J. Baylink, *Contribution of Marrow Stromal Cells to the Regulation of Osteoblast Proliferation in Rats - Evidence for the Involvement of Insulin-Like Growth-Factors.* Bone and Mineral, 1991. **13**(3): p. 201-215.

59. Hamrick, M.W., X. Shi, W. Zhang, C. Pennington, H. Thakore, M. Haque, B. Kang, C.M. Isales, S. Fulzele, and K.H. Wenger, *Loss of myostatin (GDF8) function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading*. Bone, 2007. **40**(6): p. 1544-1553.

60. NeidlingerWilke, C., I. Stalla, L. Claes, R. Brand, I. Hoellen, S. Rubenacker, M. Arand, and L. Kinzl, *Human osteoblasts from younger normal and osteoporotic donors show differences in proliferation and T-GF beta-release in response to cyclic strain*. Journal of Biomechanics, 1995. **28**(12): p. 1411-1418.

61. Gonzalez, O., K.D. Fong, M.C.D. Trindade, S.M. Warren, M.T. Longaker, and R.L. Smith, *Fluid shear stress magnitude, duration, and total applied load regulate gene expression and nitric oxide production in primary calvarial osteoblast cultures.* Plastic and Reconstructive Surgery, 2008. **122**(2): p. 419-428.

62. Fong, K.D., R.P. Nacamuli, E.G. Loboa, J.H. Henderson, T.D. Fang, H.M. Song, C.M. Cowan, S.M. Warren, D.R. Carter, and M.T. Longaker, *Scientific foundations - Equibiaxial tensile strain affects calvarial osteoblast biology.* Journal of Craniofacial Surgery, 2003. **14**(3): p. 348-355.

63. Gautschi, O.P., S.P. Frey, and R. Zellweger, *Bone morphogenetic proteins in clinical applications.* Anz Journal of Surgery, 2007. **77**(8): p. 626-631.

64. Gabbay, J.S., P.A. Zuk, A. Tahernia, M. Askari, C.M. O'Hara, T. Karthikeyan, K. Azari, J.O. Hollinger, and J.P. Bradley, *In vitro microdistraction of preosteoblasts: Distraction promotes proliferation and oscillation promotes differentiation.* Tissue Engineering, 2006. **12**(11): p. 3055-3066.

65. Weaver, A.S., Y.P. Su, D.L. Begun, R.T. Zade, A.I. Alford, K.D. Hankenson, J.M. Kreider, S.A. Ablowitz, M.R. Kilbourn, and S.A. Goldstein, *Exogenous Mesenchymal Stem Cells and Controlled Mechanical Stimulation Accelerate Fracture Healing*, in *The Seventh Annual Meeting of the Midwest Tissue Engineering Consortium (M-TEC)*. 2008: University of Cincinnati.

66. Guldberg, R.E., N.J. Caldwell, X.E. Guo, R.W. Goulet, S.J. Hollister, and S.A. Goldstein, *Mechanical stimulation of tissue repair in the hydraulic bone chamber.* Journal of Bone and Mineral Research, 1997. **12**(8): p. 1295-1302.

67. Claes, L.E., C.A. Heigele, C. Neidlinger-Wilke, D. Kaspar, W. Seidl, K.J. Margevicius, and P. Augat, *Effects of mechanical factors on the fracture healing process.* Clinical Orthopaedics and Related Research, 1998(355): p. S132-S147.

68. Turner, C.H., M.R. Forwood, J.Y. Rho, and T. Yoshikawa, *Mechanical Loading Thresholds for Lamellar and Woven Bone-Formation.* Journal of Bone and Mineral Research, 1994. **9**(1): p. 87-97.

69. Cho, T.J., L.C. Gerstenfeld, and T.A. Einhorn, *Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing.* Journal of Bone and Mineral Research, 2002. **17**(3): p. 513-520.

70. Wozney, J.M. and V. Rosen, *Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair.* Clinical Orthopaedics and Related Research, 1998(346): p. 26-37.

71. Wozney, J.M., V. Rosen, A.J. Celeste, L.M. Mitsock, M.J. Whitters, R.W. Kriz, R.M. Hewick, and E.A. Wang, *Novel Regulators of Bone-Formation - Molecular Clones and Activities.* Science, 1988. **242**(4885): p. 1528-1534.

72. Balemans, W. and W. Van Hul, *Extracellular regulation of BMP signaling in vertebrates: A cocktail of modulators.* Developmental Biology, 2002. **250**(2): p. 231-250.

73. Schmidmaier, G., B. Wildemann, T. Gabelein, J. Heeger, F. Kandziora, N.P. Haas, and M. Raschke, *Synergistic effect of IGF-I and TGF-beta 1 on fracture healing in rats - Single versus combined application of IGF-I and TGF-beta 1.* Acta Orthopaedica Scandinavica, 2003. **74**(5): p. 604-610.

74. Raiche, A.T. and D.A. Puleo, *In vitro effects of combined and sequential delivery of two bone growth factors.* Biomaterials, 2004. **25**(4): p. 677-685.

CONCLUSIONS

The major findings of this doctoral dissertation can be listed as:

1. The changes in the bone marrow mechanical environment is likely to be effective on the occupant precursor and progenitor cells, which are accepted to be responsive to mechanical factors such as hydrostatic pressure, fluid shear and the viscosity of their environment.
2. Rat marrow tissue inherently ossifies *in vitro* without addition of excipient osteoinductive factors and forms a plate-like bone tissue with viable osteocyte-like cells embedded in the matrix and osteoblast-like cells laying the mineralized matrix.
3. Marrow ossification supports the residency of STRO-1+ MSCs and CD45+ hematopoietic cells throughout the culture period.
4. In *in vitro* marrow ossification model, hematopoietic cell residency decreases over time whereas MSC cell residency peaks around day-14.
5. Ossified marrow tissue displays all the characteristic Raman peaks of bone tissue with a lower mineral to matrix ratio.
6. Ca/P ratio of ossified marrow tissue is comparable to newly forming bone tissue (periosteal rat tibia) and significantly less than mature bone tissue (i.e. intracortical rat tibia and human bone).

7. Marrow ossification process displayed the typical characteristics of intramembranous ossification and therefore, the inherent *in vitro* ossification of bone marrow tissue can be a useful as a scaffold-free, multicellular and 3D model to study the intramembranous osteogenesis process in terms of morphological differentiation, cellular composition, mechanoresponsiveness and gene and protein expression.
8. It was demonstrated for the first time that inherent ossification of marrow tissue can be achieved under serum free culture conditions without any excipient osteoinductive factors.
9. It was shown that BMP-2, IGF-1, TGF-β1 and VEGF are expressed differentially over time by the ossifying marrow explants and the concentration of IGF-1 and VEGF correlate at different time points with the final ossified volume.
10. The growth factor levels and the production of bone markers (alkaline phosphatase and osteocalcin) are highly interdependent due to correlations to each other at same time points as well as across various time points.
11. The results presented in this study provide a more robust understanding of the osteogenesis process in terms of the involvement of BMP-2, IGF-1, TGF-β1, VEGF, AP and OC in marrow explants and the secretion sequence and the amounts of key osteoinductive factors involved in this osteogenesis model.
12. It was shown that *in vitro* ossifying marrow explants are mechanoresponsive since compressive mechanical stimulation induced significantly more bone formation in the loaded samples.

13. The information obtained from the marrow ossification model can be used to develop multi-factor and multi-phase GF delivery strategies for fracture healing and bone tissue engineering applications.
14. Mechanical stimulation sustained the production level of VEGF between days 21 and 28 and enhanced production levels of IGF-1 and TGF-β1 after day-21 by in vitro ossifying bone marrow explants compared to non-loaded controls.
15. Mechanical stimulation did not induce a significant effect on BMP-2 production level at any time point in ossifying marrow tissue.
16. GFs involved in osteogenesis are many and they are not limited to the ones studied here. A comprehensive analysis of other potent factors that are affected from mechanical stimulation can be investigated using the inherently ossifying marrow explant platform.

APPENDIX

APPENDIX

Rat Bone Marrow Culture Protocols

Bone Marrow Organ Culture Materials

Name	**Order Info**	**Package**	**Addn. Info**	**Storage**
α-MEM	Sigma, M4526	500 ml	For FBS growth media	R
DMEM low glucose	Sigma, D5546	500 ml	For serum-free growth media (60%)	R
MCDB 201	Sigma, M6770	500 ml	For serum-free growth media (40%)	R
ITS+1	Sigma, I2521	5ml	For serum-free growth media	R
Fetal Bovine Serum (FBS)	Invitrogen 12662-011	100 ml	MSC qualified	F
Ascorbic Acid (Vitamin C)	Sigma, A4544	25 g	MW=176.12 Solubility: 10 mg/ml 5.0mg/ml stock	RT
L-glutamine	Sigma, G8540	25 g	MW=146.5	F
Glucose (45% in water)	Sigma, G8769	100 ml	MW=180.16	RT
Penicillin-Streptomycin	Lilly (Invitrogen, 15140-122)	100 ml	10,000U/ml stock	F
Fungizone	Sigma A9528	50 mg	5,000 µm/ml stock	F

DMSO, Hybrimax	Sigma, D2650	5 ml	Glass vial	RT
β-Glycerophosphate disodium salt pentahydrate (β-GP)	MP Biomedicals, 195206	10 g	MW=216.04 Solubility: 50 mg/ml	R

R: Refrigerator (4°C), F: Freezer (-20°C), RT: room temperature

Storage Notes for Culture Materials

- Media without L-glutamine lasts 6-9 months at 4°C.
- Once glutamine, antibiotics and serum are added the storage time of medium is reduced to 2-3 weeks, or it can be stored at -20°C. The half-life of glutamine at 37°C is 1 week.
- Ascorbic acid can be stored at -20°C in 1mg ascorbic acid / 1ml α-MEM form.

1. Glycerol 2-phosphate disodium salt hydrate can be stored at -20°C in 1 M solution form.

Standard Procedures for Using Laminar Flow Hood

1) Remove the unnecessary things off the hood and minimize the number of items in the hood to allow better air circulation.
2) Turn on the hood UV light and keep it on for at least 30 minutes for sterilization before each use.
3) While waiting for the UV sterilization of the hood, thaw the frozen components by placing them in the 37°C water bath.
4) Turn off the UV light; turn on the blower and the normal light in the hood.
5) Swab the work surface and all other inside surfaces of the laminar-flow hood with 70% ethanol and a lint-free swab or tissue, from **back** to **front**.
6) When extracting solutions from the bottles, tilt the bottles towards the pipette so that your hand will not be over the opening of the bottle.
7) When a bottle is uncapped, place the cap in the back of the work place open side down.
8) Do not let your hand, your arm or any other items to be hovering above open cell culture containers.

Preparation of Bone Marrow Extraction Medium

Lab-ware to be autoclaved beforehand:

1 × 100 ml medium bottle

1 ml pipettes

Sterile:

Chemicals

α-MEM

Penicillin-streptomycin stock

Lab ware

10 ml pipettes, 1 ml pipettes

50 ml centrifuge tubes

Non-sterile:

Pipettor, Pipette aid

70% Ethanol in spray bottle

Lint-free swabs

Marker pen, Lab notebook, pen, protocols

Protocol:

1) Follow the regular cleaning/sterilization procedures before starting to work in the hood.
2) Take the chemicals to the hood with the other supplements and additions.
3) Place the pipettes at the side of the work place in an accessible position.
4) Unwrap the bottles if polythene wrapped and swap with 70% ethanol.
5) Slacken, but do not remove the caps of all bottles about to be used.
6) Remove the cap of the **α-MEM** bottle and place the cap open side down behind the bottle so that your hand will not be passing over it.
7) Uncap a 100 ml sterile medium bottle and place the cap open side down behind the bottle so that your hand will not be passing over it.
8) Tilt the **α-MEM** bottle towards the pipette or the syringe so that your hand will not be over the opening of the bottle.
9) Mix the following in the laminar-flow hood in sterile conditions into a 100 ml medium bottle:
    - α-MEM **100 ml (**Sigma, M4526**)**
    - 50 U/ml Penicillin-streptomycin (Invitrogen, 15140-122)
        - Use **0.5 ml** of 10000 U/ml of Penicillin-streptomycin
10) Cap the 100 ml medium bottle and shake it to mix the ingredients.
11) Fill sterile 50ml centrifuge tubes with **25 ml** of the rinsing media.

Preparation of Regular Growth Medium

This medium is used for culturing bone marrow tissue. Media is changed 3 times a week (based on Luria, 1987 [1]).

Lab ware to be autoclaved beforehand:

1 × 500 ml medium bottles

Sterile:

Chemicals:

α-MEM

MSC qualified FBS

Ascorbic Acid stock solution (5 mg/ml)

L-glutamine

Glucose solution (45%)

Penicillin-streptomycin stock solution (10,000 U/ml)

Fungizone stock solution (5 mg/ml)

β-Glycerophosphate disodium salt pentahydrate (β-GP)

Lab ware:

Pipettes, 10 ml, 30 ml syringes and needles

50 ml centrifuge tubes

500 ml bottle top filter

Non-sterile:

Pipettor, pipetting aid

70% Ethanol in spray bottle

Lint-free swabs, Absorbent paper tissues

Marker pen, Lab notebook, pen, protocols, labeling tapes

Protocol:

1. Follow the regular cleaning/sterilization procedures before starting to work in the hood.
2. Take the chemicals into the hood with the other supplements and additions.
3. Unwrap the bottles if polythene wrapped and swap with 70% ethanol.
4. Slacken, but do not remove the caps of all bottles about to be used.
5. Mix the following in a 500 ml bottom-top filter fitted on a 500 ml medium bottle in sterile conditions (Per **500 ml** of medium):
    - α-MEM **450 ml**
    - MSC qualified FBS (10%) **50 ml**
    - 50 μg/ml Vitamin-C
        - Use **5 ml** of 5.0mg/ml stock solution
    - 200 mM L-glutamine

    - Use **0.146 g** L-glutamine for 500 ml of medium
- 3.5 mg/ml Glucose
    - Use **3.9 ml** of Glucose stock solution (45%)
- 60 IU/ml penicillin-streptomycin
    - Use **3 ml** of 10000 U/ml stock solution of Penicillin-streptomycin
- 2.5 μg/ml Fungizone
    - Use 250 μl of 5.0 mg/ml stock Fungizone solution
- 5 mM β-Glycerophosphate
    - Use 0.765 g of powder β-GP

6. Filter the mixed solution by applying vacuum.
7. After filtration, aliquot the media into 50ml centrifuge tubes in appropriate volumes, seal with parafilm and label the tubes with labeling tape.
8. Freeze the 50 ml centrifuge tubes at -20°C freezer for future use.

Preparation of Serum-Free Growth Medium

This medium is used for culturing bone marrow tissue under serum-free culture conditions (based on [1] and [2]). Media is changed 3 times a week.

Lab ware to be autoclaved beforehand:

1 × 500 ml medium bottles

Sterile:

Chemicals:

DMEM low glucose

MCDB-201

ITS+1

Ascorbic Acid stock solution (5 mg/ml)

Glucose solution (45%)

Penicillin-streptomycin stock solution (10,000 U/ml)

Fungizone stock solution (5 mg/ml)

β-Glycerophosphate disodium salt pentahydrate (β-GP)

Lab ware:

Pipettes, 10 ml, 30 ml syringes and needles

50 ml centrifuge tubes

500 ml bottle top filter

Non-sterile:

Pipettor, pipetting aid

70% Ethanol in spray bottle

Lint-free swabs, Absorbent paper tissues

Marker pen, Lab notebook, pen, protocols, labeling tapes

Protocol:

9. Follow the regular cleaning/sterilization procedures before starting to work in the hood.
10. Take the chemicals into the hood with the other supplements and additions.
11. Unwrap the bottles if polythene wrapped and swap with 70% ethanol.
12. Slacken, but do not remove the caps of all bottles about to be used.
13. Mix the following in a 500 ml bottom-top filter fitted on a 500 ml medium bottle in sterile conditions (Per **500 ml** of medium):
    - DMEM (60%) **300 ml**
    - MCDB-201 (40%) **200 ml**
    - ITS+1 (1%) **5 ml**
    - 50 μg/ml Vitamin-C
        - Use **5 ml** of 5.0mg/ml stock solution

- 3.5 mg/ml Glucose
    - Use **3.9 ml** of Glucose stock solution (45%)
- 40 IU/ml penicillin-streptomycin
    - Use **2 ml** of 10000 U/ml stock solution of Penicillin-streptomycin
- 1.5 μg/ml Fungizone
    - Use **150 μl** of 5.0 mg/ml stock Fungizone solution
- 5 mM β-Glycerophosphate
    - Use **0.765 g** of powder β-GP

14. Filter the mixed solution by applying vacuum.
15. After filtration, aliquot the media into 50ml centrifuge tubes in appropriate volumes, seal with parafilm and label the tubes with labeling tape.
16. Freeze the 50 ml centrifuge tubes at -20°C freezer for future use.

Extraction of Bone Marrow from Rat

Lab-ware to be autoclaved beforehand:

20mm radius diamond bit for Dremel rotary tool

2 x 100 ml containers with white lids

Multiple forceps in sterilization pouches (2 forceps in 1 pouch, multiple pouches needed)

1.5 ml micro centrifuge tubes with silicon stoppers for bones

200 µl, wide orifice, low binding pipette tips.

Sterile:

Chemicals

Bone marrow extraction rinsing medium (25 ml) in a 50 ml centrifuge tube

Lab ware

Petri dishes (at least 3)

Non-sterile:

Dremel 7700 high speed rotary cutting tool

70% Ethanol in spray bottle

Lint-free swabs, Absorbent paper tissues

Marker pen, Lab notebook, pen, protocols

Ice in a foam container (rinsing media in 50 ml tube is placed on ice)

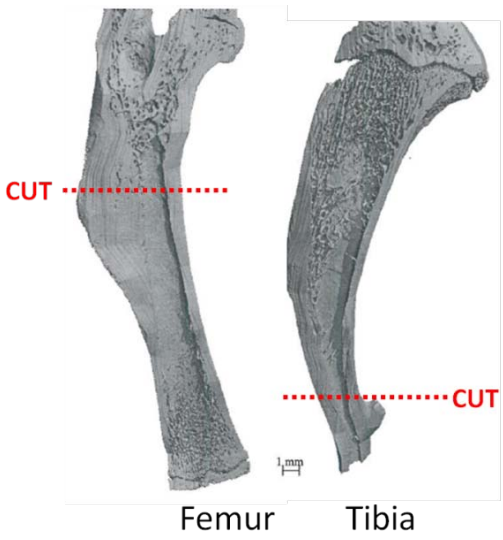
Note: Large laminar flow hood should be used for this procedure due to space requirements.

Bone marrow extraction Protocol:

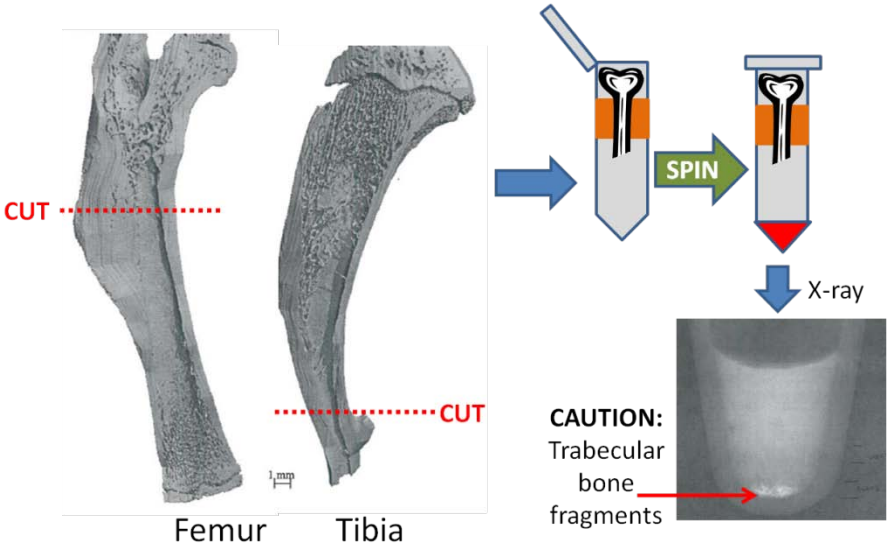
1. THE CO_2 SACRIFICE OF RATS SHOULD BE CARRIED OUT BY AUTHORIZED PERSONNEL WITH APPROPRIATE TRAINING. THE PRCEDURE SHOULD BE CARRIED OUT ACCORDING TO THE APPROVED PACUC PROTOCOL. THE AUTHORIZED PERSONNEL SHOULD BE LISTED IN THE APPROVED PACUC PROTOCOL. IF YOU DO NOT HAVE THE APPROPRIATE TRAINING, CONTACT THE DEPARTMENT'S ANIMAL HANDLING PERSONNEL.
2. The rat is taken from the animal husbandry room in the basement and transported to the operation room of BME on a cart. DURING TRANSPORTATION, THE ANIMAL CAGE SHOULD BE COVERED WITH A CLOTH OR TOWEL.
3. The rat is placed in the induction chamber, which is connected to the CO_2 line and activated carbon air filter.
4. CO_2 gas is turned on quarter-turn and the hissing sound of the gas is checked.
5. The animal is observed and once it becomes motionless, CO_2 gas is kept on for 5 more minutes.

6. The CO_2 line is turned off, the line is disconnected and the CO2 rich air in the chamber is allowed to equilibrate through the activated carbon filter for 5 minutes.
7. The induction chamber is opened and the heartbeat of the animal is checked inside the induction chamber.
8. The euthanized animal is transferred onto a lab bench which is covered with a layer of absorbing pad.
9. The hind limbs and the chest of the animal are shaved with clippers.
10. Thoracic puncture of the chest cavity is performed with scalpels and surgical scissors. THIS PROCEDURE SHOULD BE PERFORMED BY TRAINED PERSONNEL.
11. The animal is wrapped in a towel or cloth and transferred to sample preparation room for dissection.
12. The animal is placed in a clean dissection tray, face down and the feet are fixed with dissection pins.
13. The skin is cleaned with 70% ethanol and swabs.
14. Using tweezers lift the skin above the femur on the hind leg.
15. Cut lifted skin using scalpels to expose the underlying muscles and bones.
16. Using fresh instruments cut the overlying muscle to expose the femur and tibia
17. Remove muscles surrounding the femur and tibia using scissors and/or scalpels. Dissect out tibia first followed by femur.
18. Once the tibia and the femur are dissected out, place them in the rinsing media in 50 ml tube which was previously placed on ice.

19. When the dissection is complete, take the ice box to cell culture room.
20. Carry out the standard cleaning and preparation procedures for the large laminar flow hood.
21. Spend 2 minutes to plan your actions strategically in the hood and place the following at strategic locations based on your plan:
    a. Two Sterile 100 ml containers with white lids. One of them is filled with 50 ml of 70% ethanol.
    b. Three petri dishes. Three Gauze pads are placed in one of them for drying the extracted bones.
    c. The sterile forceps in pouches.
    d. The container of the sterile 1.5 ml tubes.
    e. Dremel tool with the sterile diamond bit attached.
    f. Corning culture wells with inserts (6 well inserts).
22. Pour the rinsing media with the bones into the sterile container.
23. Pick the bones with sterile forceps and drop them into the 70% ethanol container. Count to 15.
24. After counting to 15, remove the bones from 70% ethanol container and place them onto the sterile gauze pads in the Petri dish. Let them dry for 2 minutes.
25. Hold the bones with sterile forceps and cut the ends of the femur and tibia at the locations shown below (the cut locations were chosen strategically to minimize the amount of trabecular bone pieces in the extracted bone marrow):



26. After cutting the ends of the bones, place them immediately in the sterile, labeled (F or T) 1.5 ml tubes with the silicon stoppers as shown below and spin them at 2000RPM for 2 minutes (as seen below).



27. After centrifugation, the bone marrow tissue will be accumulated at the tip of the centrifuge tube. If there is very little (approximately less than 50 µl) marrow tissue in the tube, it means that all the marrow tissue was not extracted efficiently.

Increase the centrifuge speed to 3000 RPM and spin one more time for 1 minute. Caution should be use as some bone fragments may also be present in the marrow tissue as seen in the above figure.

28. The bones and the silicon stoppers are removed from the tubes.
29. By using sterile 200 µl low binding wide orifice pipette tips, combine femur and tibia marrow in individually labeled (F or T) 1.5 ml tubes each and mix them by gently pipetting and mixing.
30. Spin the combined and mixed tube at 1000RPM for 1 minute to settle the bone fragments.
31. The marrow tissue in the tubes is ready for seeding on the culture inserts. Start seeding immediately.

Seeding and Culturing Bone Marrow Explants

Note: 6 well plates with inserts require 1ml of media underneath to provide a medium-air interface. This volume of medium provides a reasonable liquid level in the wells without flooding the upper surface of the insert.

Sterile:

Chemicals

Growth media

Lab ware

Corning Transwell Cell Inserts, 6 well, 24 mm, Polyester, Clear, 0.4µm pore size, #3450.

1 ml pipette tips

Non-sterile:

Pipettor, pipetting aid

70% Ethanol in spray bottle

Lint-free swabs, Absorbent paper tissues

Marker pen, Lab notebook, pen, protocols

Protocol:

1. Follow the regular cleaning/sterilization procedures before starting to work in the hood.
2. Thaw and take the mediums to the hood.
3. Follow the bone marrow extraction protocol to obtain the marrow tissue.
4. Transfer 7 μl of marrow tissue onto the culture inserts.
5. When aspirating marrow from the 1.5ml centrifuge tube, pay attention to the bone fragments and do not aspirate them. Aspiration should be done from the top of the marrow layer.
6. Spread the marrow tissue with the tip of the pipette to obtain a 5 mm diameter uniform layer.
7. Add 1m ml of regular growth medium underneath the culture insert, label the wells accordingly (pay attention to samples that are coming from tibia and femur)
8. Close the lid of the 6-well plate and transfer it into the incubator immediately.
9. After all the seeding is complete, inspect the samples and note down the ones which contain bone fragments.

Cryopreservation Protocol

Note: Bone marrow in a mice femur contains about 1.5 to 2.0 $\times 10^7$ [3], whereas rat femur contains 6 $\times 10^7$ nucleated cells [4]. For cryopreservation it is recommended to have 1×10^6 - 1×10^7 cells/ml of freezing medium [3].

Sterile:

Chemicals

FBS (Sigma, F6178)

DMSO

Lab ware

15 ml centrifuge tubes

1 × 100 ml medium bottle

Cryo vials

Non-sterile:

Extracted rat femurs

70% Ethanol in spray bottle

Lint-free swabs, Absorbent paper tissues,

Marker pen, Lab notebook, pen, protocols

Freezing container with isopropyl alcohol

Protocol:

1. Follow the standard sterilization procedures in the laminar-flow hood.
2. Thaw the FBS for 30 minutes at 37°C water bath.
3. Add **13.5 ml** of **FBS** to a 15 ml centrifuge tube.
4. Add **1.5 ml** of **DMSO** to the centrifuge tube and mix by repeated pipetting.
5. If the cells are in suspension tentrifuge at **1200 RPM** for **5 minutes**.
6. Discard the supernatant.
7. Add the freezing medium onto the pellet.
8. Disperse the mixture to a suspension by pipetting through a 10 ml pipette.
9. Aliquot **1.8 ml** of the mixture to the cryo vials.
10. Put the cryo vials into freeze container and place it at -80°C freezer overnight.
11. The next day, take out the cryo vials and immediately transfer to the liquid nitrogen tank.
12. Do not forget to take note in the frozen cell inventory.

Protocol for Thawing Frozen Cells

Note: Bone marrow in a mice femur contains about 1.5 to 2.0 $\times 10^7$ [3], whereas rat femur contains 6 $\times 10^7$ nucleated cells [4]. For cryopreservation it is recommended to have 1×10^6 - 1×10^7 cells/ml of freezing medium [3].

Sterile:

Chemicals

Regular growth medium that contains 10% FBS

Lab ware

15 ml centrifuge tubes

1 × 100 ml medium bottle

Culture flask

Non-sterile:

70% Ethanol in spray bottle

Lint-free swabs, Absorbent paper tissues

Marker pen, Lab notebook, pen, protocols

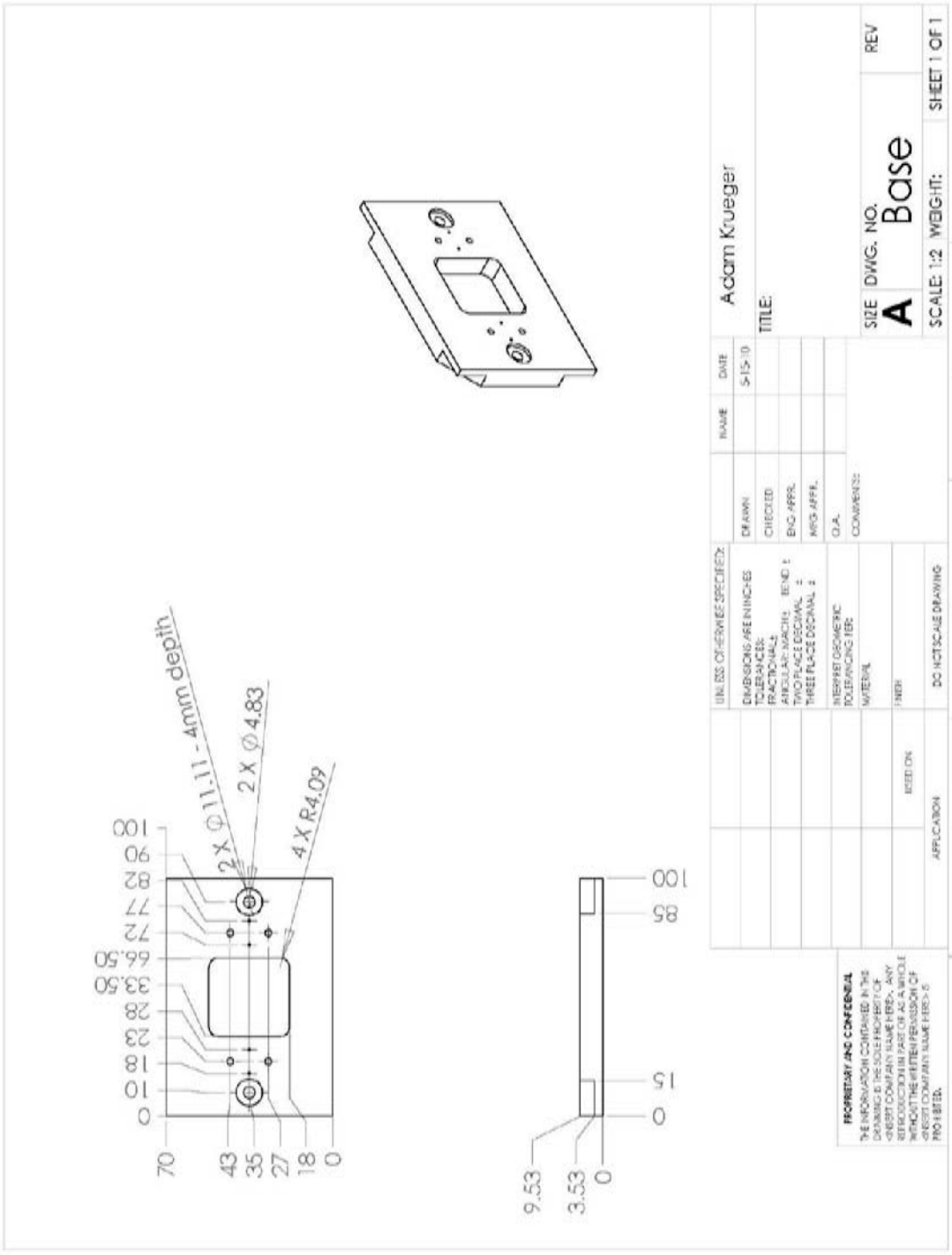
Protocol:

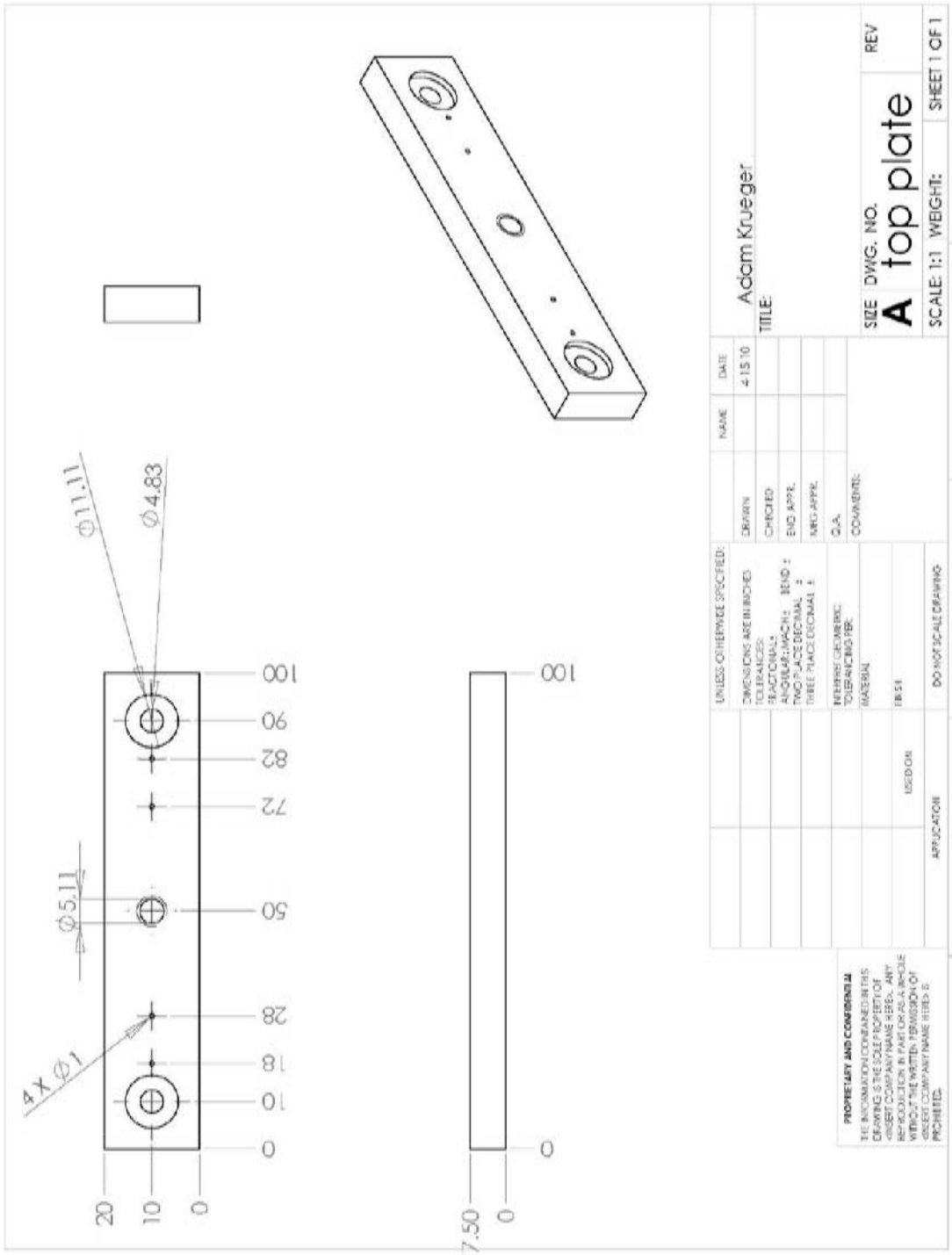
1. Retrieve the vial from the freezer and check that it is the correct one, carry it with glove.
2. Take note in the frozen cell inventory.
3. Place the vial immediately in water bath at 37°C and close the lid.
4. When the vial is completely thawed, wipe down outside of the vial with 70% ethanol.
5. Measure 9 ml of regular plating media into a 15ml centrifuge tube.
6. Draw up 1.5 ml of media from 15ml tube then draw up cells from vial into this 1.5 ml slowly. This gradual process is particularly important with DMSO, with which sudden dilution can cause severe osmotic damage to the cells.
7. Pipette up and down a few times into cell vial to ensure all cells are taken. Be careful not to overflow the contents.
8. Put the contents of the cell vial into the 15ml tube with media slowly.
9. Spin the centrifuge tube at 1200rpm for 5min.
10. Decant media and resuspend the pellet in a volume of regular growth medium appropriate for a flask or a macrowell.
11. Transfer the cells to a flask or macrowell and incubate at 37°C and 5% CO_2.
12. Change the media the next day to remove residual DMSO. No need to tyrpsinize, just pour off media and replace with 5ml of fresh media. Pass cells as normal after that.

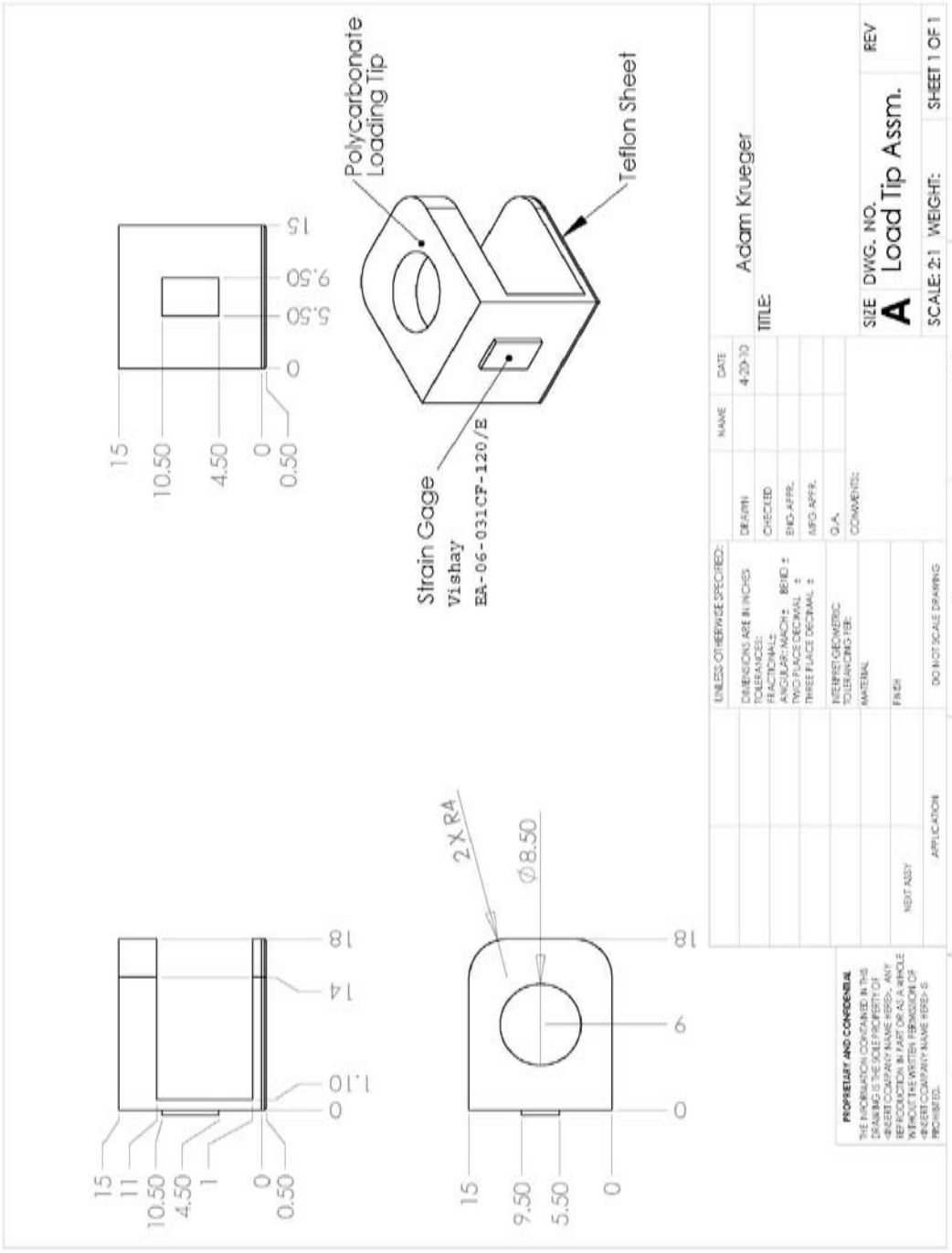
References

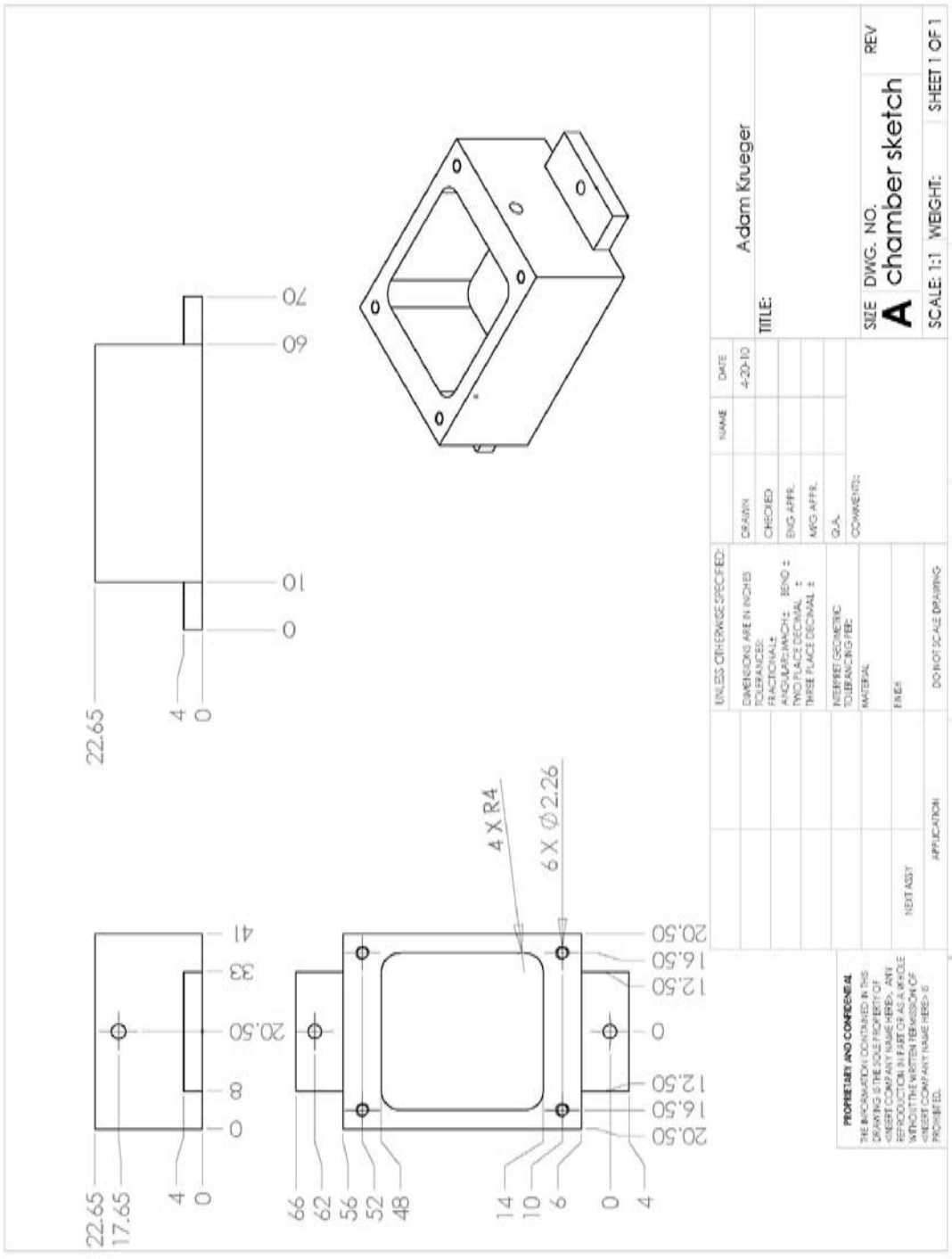
1. Luria, E.A., M.E. Owen, A.J. Friedenstein, J.F. Morris, and S.A. Kuznetsow, *Bone-Formation in Organ-Cultures of Bone-Marrow.* Cell and Tissue Research, 1987. **248**(2): p. 449-454.
2. Lennon, D.P., S.E. Haynesworth, R.G. Young, J.E. Dennis, and A.I. Caplan, *A Chemically-Defined Medium Supports in-Vitro Proliferation and Maintains the Osteochondral Potential of Rat Marrow-Derived Mesenchymal Stem-Cells.* Experimental Cell Research, 1995. **219**(1): p. 211-222.
3. Freshney, R.I., *Culture of animal cells : a manual of basic technique*. 4th ed. 2000, New York: Wiley-Liss. xxvi, 577 p., [12] p. of plates.
4. Ben-Ishay, Z., F. Reichert, and S. Sharon, *Migration of lymphoid cells to the bone marrow of rat following eradication of cells in DNA synthesis and in mitosis.* Experientia, 1978. **34**(10): p. 1369-70.

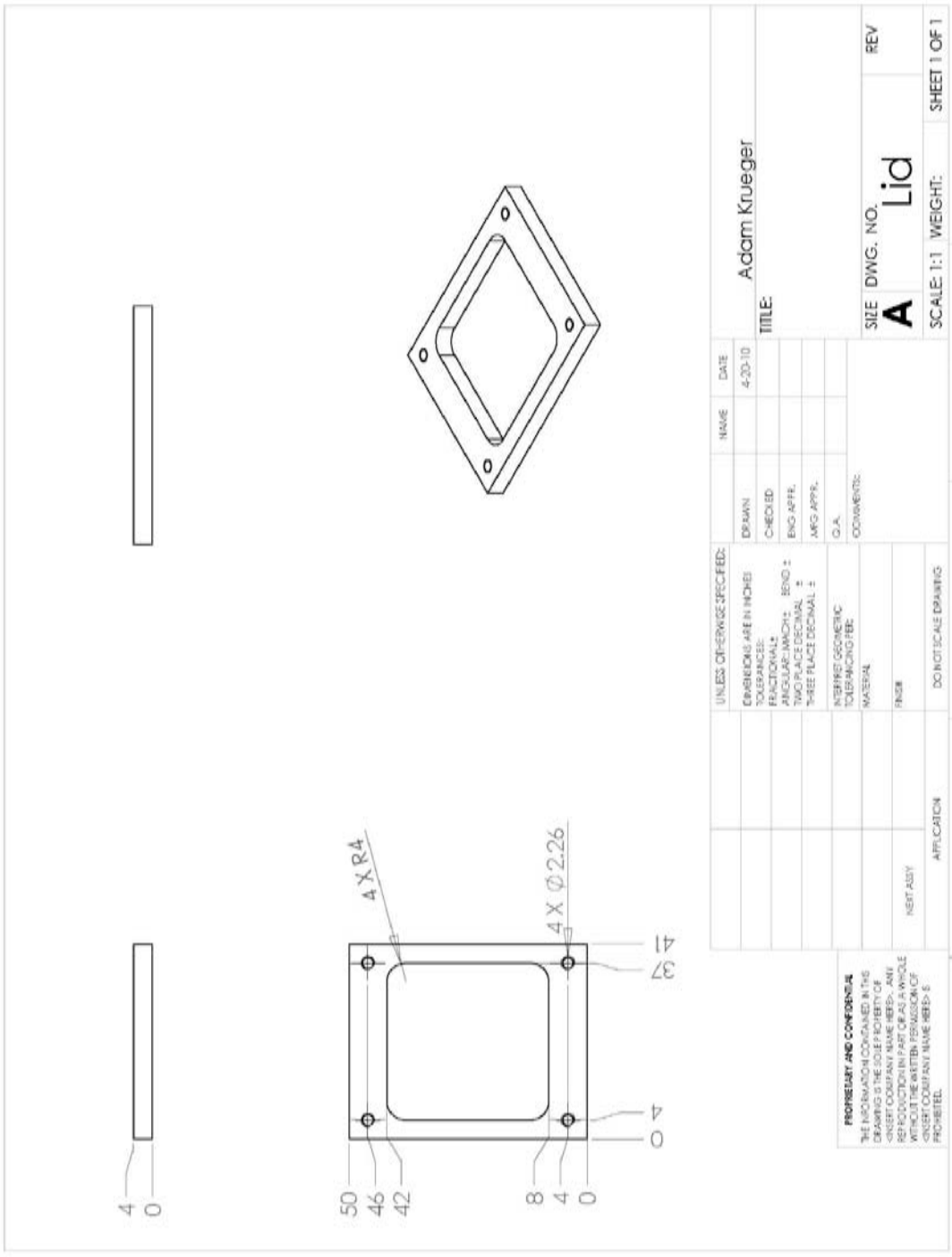
CAD Drawings of the Mechanical Loading System




Adam Krueger
A top plate
SIZE DWG. NO. REV
SCALE: 1:1 WEIGHT: SHEET 1 OF 1
TITLE:
DRAWN
CHECKED
ENG APPR.
MFG APPR.
Q.A.
COMMENTS:
MATERIAL
FINISH
DO NOT SCALE DRAWING
APPLICATION
USED ON
PROPRIETARY AND CONFIDENTIAL
4 X Ø1
Ø5.11
Ø11.11
Ø4.83
100
90
82
72
50
28
18
10
0
20
10
0
7.50


Polycarbonate
Loading Tip
Teflon Sheet
Strain Gage
Vishay
EA-06-031CF-120/E
2 X R4
Ø8.50
Adam Krueger
TITLE:
A Load Tip Assm.
SIZE DWG. NO.
REV
SCALE: 2:1 WEIGHT:
SHEET 1 OF 1
DRAWN
CHECKED
ENG APPR.
MFG APPR.
Q.A.
COMMENTS:
DATE
4-20-10
PROPRIETARY AND CONFIDENTIAL


Adam Krueger
TITLE:
SIZE DWG. NO. REV
A chamber sketch
SCALE: 1:1 WEIGHT: SHEET 1 OF 1
UNLESS OTHERWISE SPECIFIED:
DIMENSIONS ARE IN INCHES
TOLERANCES:
FRACTIONAL ±
ANGULAR: MACH ± BEND ±
TWO PLACE DECIMAL ±
THREE PLACE DECIMAL ±
INTERPRET GEOMETRIC TOLERANCING PER:
MATERIAL
FINISH
DO NOT SCALE DRAWING
NAME DATE
DRAWN 4-20-10
CHECKED
ENG APPR.
MFG APPR.
Q.A.
COMMENTS:
NEXT ASSY
APPLICATION
PROPRIETARY AND CONFIDENTIAL
THE INFORMATION CONTAINED IN THIS DRAWING IS THE SOLE PROPERTY OF <INSERT COMPANY NAME HERE>. ANY REPRODUCTION IN PART OR AS A WHOLE WITHOUT THE WRITTEN PERMISSION OF <INSERT COMPANY NAME HERE> IS PROHIBITED.
4 X R4
6 X Ø2.26
22.65
17.65
4
0
8
33
41
20.50
16.50
12.50
0
66
62
56
52
48
14
10
6
Ø
70
60
10
22.65


4 X R4
4 X Ø2.26
50
46
42
8
4
0
41
37
4
0
4
0
UNLESS OTHERWISE SPECIFIED:
DRAWN
CHECKED
ENG APPR.
MFG APPR.
Q.A.
COMMENTS:
NAME
DATE
4-20-10
Adam Krueger
TITLE:
Lid
SIZE
A
DWG. NO.
REV
SCALE: 1:1
WEIGHT:
SHEET 1 OF 1
MATERIAL
FINISH
NEXT ASSY
APPLICATION
DO NOT SCALE DRAWING
PROPRIETARY AND CONFIDENTIAL

VITA

VITA

Umut Atakan Gurkan, Ph.D.
Research Assistant
Weldon School of Biomedical Engineering, Purdue University
206 S. Martin Jischke Drive, West Lafayette, IN 47907-2032
Ph: (765) 413-8936; E-mail: ugurkan@purdue.edu

EDUCATION

2010	Ph.D. in Biomedical Engineering Purdue University, Weldon School of Biomedical Engineering ▪ *Dissertation title:* Engineering of Bone Marrow *In Vitro* for Investigating the Role of Growth Factors and Their Mechanoresponsiveness in Osteogenesis ▪ *Advisor:* Ozan Akkus, Ph.D.
2005	B.S. in Mechanical Engineering, *High Honor* Middle East Technical University, METU
2004	B.S. in Chemical Engineering, *High Honor* Middle East Technical University, METU

AWARDS AND SCHOLARSHIPS

2009	Geddes-Laufman-Greatbatch Outstanding Graduate Student Award, Purdue University
2009	Top Dissertation Proposal Award, Weldon School of Biomedical Engineering, Purdue University
2009	Graduate Teacher Certificate (GTC), Purdue University Center for Instructional Excellence
2009	A. H. Ismail Interdisciplinary Program Doctoral Research Travel Award, Purdue University
2009	Joe Bourland Graduate Student Travel Award, Purdue University
2008	Purdue Graduate Student Government Travel Grant
1999 – 2004	Sabanci Foundation Scholarship

PEER REVIEWED PUBLICATIONS

1. **Gurkan U.A.**, V. Kishore and O. Akkus, *Characterization of Inherently Ossifying Bone Marrow Culture as a Scaffold-free Multicellular Three-dimensional (3D) In Vitro Model of Intramembranous Osteogenesis,* BONE*, under review.*
2. **Gurkan U.A.**, A. Krueger and O. Akkus, *Ossifying Bone Marrow Explant Culture as a Three-dimensional (3D) Mechanoresponsive In Vitro Model of Osteogenesis,* Tissue Engineering Journal Part A*, under review.*
3. **Gurkan U.A.,** J. Gargac and O. Akkus, *The Sequential Production Profiles of Growth Factors and Their Relations to Bone Volume in Ossifying Bone Marrow Explants*, Tissue Engineering Journal Part A, 2010, *in press (DOI: 10.1089/ten.tea.2009.0565).*
4. **Gurkan U.A.**, X. Cheng, V. Kishore, J.A. Uquillas and O. Akkus, *Comparison of Morphology, Orientation, and Migration of Tendon Derived Fibroblasts and Bone Marrow Stromal Cells on Electrochemically Aligned Collagen Constructs,* Journal of Biomedical Materials Research Part A, 2010*, in press (DOI: 10.1002/jbm.a.32783).*
5. Meldrum R. D., **U.A. Gurkan**, S. A. Kattaya, O. Akkus, *Osteogenic Effects of Preparations of Rat Pulmonary Alveolar Macrophages Challenged with Staphylococcus Aureus*, Indiana Orthopaedic Journal, Volume 3, 2009, Pages: 28-29.
6. **Gurkan U.A.** and O. Akkus, *The mechanical environment of bone marrow: a review*, Annals of Biomedical Engineering, Volume 36, Issue 12, 2008, Pages: 1978-1991.
7. Cheng X., **U.A. Gurkan**, C. J. Dehen, M. P. Tate, H. W. Hillhouse, G. J. Simpson and O. Akkus, *An electrochemical fabrication process for the assembly of anisotropically oriented collagen bundles*, Biomaterials, Volume 29, Issue 22, 2008, Pages: 3278-3288.
8. Yildiz, U., **U.A. Gurkan**, C. Ozgen, and K. Leblebicioglu, *State estimator design for multicomponent batch distillation columns.* Chemical Engineering Research & Design, 2005. 83(A5): p. 433-444.

CONFERENCE PROCEEDINGS

1. **Gurkan U. A.,** A. Krueger and O. Akkus, *The Effect of Mechanical Stimulation on the Production of BMP-2, VEGF, IGF-1 and TGF-b1 by In Vitro Ossifying Rat Bone Marrow Explants*, The 17th Congress of the European Society of Biomechanics, Edinburgh, Scotland, July 2010
2. **Gurkan U. A.** and O. Akkus, *Temporal Productions of BMP-2, IGF-1, VEGF and TGF-21 Correlate Highly with Each Other in Ossifying Marrow Explants*, Orthopedic Research Society 56th Annual Meeting, New Orleans, Louisiana, March 2010
3. **Gurkan U. A.** and O. Akkus, *Temporal Production Levels of VEGF and IGF-1 Correlate with the Final Ossified Volume in Inherently Ossifying Marrow Explants In Vitro*, Orthopedic Research Society 56th Annual Meeting, New Orleans, Louisiana, March 2010
4. Kishore V., **U.A. Gurkan,** J. A. Uquillas and O. Akkus, *Effects of Cell Type and Fabric Orientation on the Population Rates of Collagen Constructs*, Orthopedic Research Society 56th Annual Meeting, New Orleans, Louisiana, March 2010

5. Kishore V., **U.A. Gurkan,** J. A. Uquillas and O. Akkus, *Comparison between the Migration Rates of Bone Marrow Stromal Cells and Tendon Derived Fibroblasts on Random and Electrochemically Aligned Collagen Constructs*, Society for Biomaterials, Biomaterials Day, Lexington, Kentucky, September 2009
6. **Gurkan U. A.**, X. Cheng, O. Akkus, *Migration of Tendon Derived Fibroblasts and Bone Marrow Stromal Cells on Electrochemically Aligned Collagen Constructs***,** The 15th International Biomedical Science and Technology Symposium, Middle East Technical University Northern Cyprus Campus, Turkish Republic of Northern Cyprus, August 2009
7. **Gurkan U. A.**, O. Akkus, *In Vitro Ossifying Bone Marrow Explants Have Osteoinductive Potential via the Media Conditioned By Them***,** The 15th International Biomedical Science and Technology Symposium, Middle East Technical University Northern Cyprus Campus, Turkish Republic of Northern Cyprus, August 2009
8. **Gurkan U. A.**, A. Krueger and O. Akkus, *Mechanical Stimulation Enhances the Production of Bmp-2 in Ossifying Rat Bone Marrow Organ Cultures***,** ASME Summer Bioengineering Conference, Lake Tahoe , CA, June 2009
9. **Gurkan U. A.** and O. Akkus, *Facilitating the Exodus of Adherent Cells Improves In Vitro Bone Formation in Bone Marrow Explants*, Orthopedic Research Society 55th Annual Meeting, Las Vegas, Nevada, February 2009
10. **Gurkan U. A.** and O. Akkus, *The Osteoinductive Potential of Bone Marrow Conditioned Media is Superior to Dexamethasone and rhBMP-2*, Orthopedic Research Society 55th Annual Meeting, Las Vegas, Nevada, February 2009
11. Meldrum R. D., **U.A. Gurkan**, S. A. Kattaya, O. Akkus, *Staphylococcus Aureus Inhibits the Osteogenesis Induced by Rat Pulmonary Alveolar Macrophages*, Orthopedic Research Society 55th Annual Meeting, Las Vegas, Nevada, February 2009
12. Santoso A., **U. A. Gurkan**, J. Uquillas, O. Akkus, A. Ivanisevic, *Collagen Fiber Orientation and Proteoglycan Influence on Retinal Pigment Epithelial (RPE) Cell Attachment and Morphology,* Biomedical Engineering Society, 2008 Annual Fall Meeting, St. Louis, Missouri
13. **Gurkan U. A.**, X. Cheng, O. Akkus, *Structural Organization and cellular response of electrochemically aligned collagen bundles*, The Seventh Annual Meeting of the Midwest Tissue Engineering Consortium (M-TEC 2008), University of Cincinnati, Cincinnati OH, April 2008
14. Cheng X., **U. A. Gurkan**, O. Akkus, *Feasibility of electrochemically aligned collagen bundles for ligament/tendon tissue engineering*, International Symposium on Ligaments & Tendons - VIII, Stanford University, March 2008
15. Cheng X., **U. A. Gurkan**, O. Akkus, *Biomechanical evaluation of a novel electrochemically synthesized collagen constructs for tendon/ligament tissue engineering*, 54th Annual Meeting of the Orthopaedic Research Society, San Francisco, CA, March 2008
16. **Gurkan U. A.**, O. Akkus, *An implantable magnetoelastic sensor system for wireless physiological sensing of viscosity*, ASME Summer Bioengineering Conference, Keystone CO, June 2007

INVITED SEMINARS

1. University of Notre Dame Bioresearch Seminar (November 19, 2009), Seminar title: *Engineering of Bone Marrow In Vitro for Investigating the Role of Growth Factors and Their Mechanoresponsiveness in Osteogenesis.*
2. Bilkent University Mechanical Engineering Department Seminar (January 28, 2009), Seminar title: *Engineering of Bone Marrow In Vitro for Investigating the Role of Growth Factors and Their Mechanoresponsiveness in Osteogenesis.*

PROPOSAL WRITING EXPERIENCE (SUPERVISED BY DR. OZAN AKKUS)

Principal Investigator (PI):

1. National Institutes of Health Small Business Innovation Research (SBIR) Phase I Award, *Mechanically Actuatable Patch For Stimulation of Wound Healing*, *pending, $215,075* **(**FlexTissue Biomedical LLC).

Co-author:

1. Musculoskeletal Transplant Foundation Peer-reviewed Research Grant for 2009, *Osteoinductive Growth Factor Harvest from Allogeneic Human Marrow*, *funded: $125,000*
2. National Institutes of Health R21, 2008, *Engineering of Bone Marrow Organ Cultures for Production of Osteoinductive Cocktails***,** not funded
3. Purdue University Ralph W. and Grace M. Showalter Research Trust Fund 2007, *Implantable Sensors for Remote Physiological Sensing in Orthopaedics,* not funded
4. National Institutes of Health R21, 2006, for the Program Announcement (PA) Number: PAS-06-208 (Interactions between Stem and Progenitor Cells and the Microenvironment), *In-Vivo Characterization of Mechanical Environment of Bone Marrow by Immersed Magnetoelastic Sensor Array,* not funded

PATENTS

1. Akkus O., **U. A. Gurkan**, A. Aref and R. Meldrum, *Prevention of Hypertrophic Scars by Actuatable Patch*, filed to USPTO: June 2009, S/N:61/220661. (Co-founder, shareholder and CTO of FlexTissue Biomedical LLC, http://www.flextissue.com)

AD HOC REVIEWER

- Journal of Orthopedic Research
- Tissue Engineering Journal
- Journal of Medical Devices
- Purdue University Society for Biomaterials
- Purdue Graduate Student Government Travel Grant Committee (invited reviewer)

PROFESSIONAL MEMBERSHIPS

- Materials Research Society (MRS)
- Tissue Engineering And Regenerative Medicine International Society (TERMIS)

RESEARCH EXPERIENCE

Purdue University (2006-present)

- Developed a mechanically actuatable wound patch (MATCH) for stimulating large open wounds, diabetic ulcers, pressure sores.
- Performed in vivo animal experiments (porcine) with the portable MATCH system to stimulate large open wounds.
- Developed a multicellular 3D *in vitro* organ culture platform for studying the osteogenic and osteoinductive properties of bone marrow tissue.
- Developed and performed a SILAC based proteomic analysis method for studying the expression of signaling proteins in osteogenesis with self inductive ossification of bone marrow.
- Investigated the production and sequential expression of osteoinductive factors (BMP-2, TGF-β1, VEGF and IGF-1) by ossifying bone marrow explants in an effort to develop more effective multi-factor and multi-phase delivery methods for bone tissue engineering.
- Developed a sterilizable low-cost *in vitro* loading system with shape memory alloy actuator (Flexinol) for loading ossifying marrow explants.
- Investigated the effects of mechanical stimulation on the osteogenic potential and the osteoinductive factor production by ossifying marrow explants.
- Carried out preliminary investigation on the effect of early administration of rhBMP-2 on *in vitro* ossifying bone marrow explants.
- Carried out preliminary investigation on the metastasis of breast cancer cells to bone tissue by developing a 3D primary organ culture model.
- Investigated the osteogenesis induced by rat pulmonary alveolar macrophages challenged by an infectious agent (*staphylococcus aureus*) to understand the ossification mechanism observed in wounds that involve bone fracture.
- Carried out mechanical tests and real-time high magnification imaging on cortical bone wafers with a mechanical testing system located in an environmental SEM in an effort to detect the emergence of micro cracks upon loading.
- Developed and carried out migration and population assays for bone marrow stromal cells and tendon derived fibroblasts on electrochemically aligned collagen bundles for tissue engineering of tendon/ligament.
- Developed a wireless magnetoelastic sensor for measuring the viscosity of bone marrow tissue *in vivo*.

Middle East Technical University (2001-2005)

- Designed a magnetocaloric refrigeration cycle that can be used in household refrigerators.
- Designed and produced a scientific calculator for the blind that employed Braille alphabet.
- Developed a neural-network based state estimator for control systems used for batch distillation columns.

TEACHING EXPERIENCE

2008-2009 **Graduate Teacher Certificate (GTC)** awarded by Purdue University Center for Instructional Excellence
- Completed a two-semester program designed to enhance teaching skills
- Participated in video-taped critiques of teaching
- Received mid-semester end-semester and student feedback and evaluations
- Attended professional development seminars/workshops

Graduate Student Mentorship

2008, 2009 Purdue Summer Undergraduate Research Fellowship Program

Teaching

Fall 2007, 2008 Scanning Electron Microscopy Laboratory course (HORT 595B), Graduate Level, Purdue University Life Sciences Microscopy Facility

Teaching Assistantship

Spring 2006, 2007 Biomechanics of Hard and Soft Tissues course (BME204), Sophomore Level, Purdue University

Fall 2005 Biomaterials course (BIOE2200), Sophomore Level, The University of Toledo

2000 – 2004 Student Assistant for Computer Education on University Campus, Middle East Technical University Computer Center

STUDENTS/RESEARCHERS MENTORED

Fall 2009 **Adam Krueger**, *Manufacturing and characterization of mechanically actuatable wound patch.*

Ryan Golden, *Data processing for SILAC-based proteomic analysis on in vitro ossifying bone marrow explants.*

Summer 2009 **Joshua Gargac**, *Quantifying the baseline levels of osteoinductive growth factors in rat bone marrow tissue.*

Spring 2009	**Adam Krueger,** *Incorporating a load measurement component into the In vitro loading system for mechanical stimulation of bone marrow organ cultures*
	Abdulrahman Aref, *Developing a mechanically active wound patch*
Fall 2008	**Xiaomei Liu,** *Characterization and immunohistology studies on in vitro cultured bone marrow explants*
Summer 2008	**Arden Santoso,** *Collagen fiber orientation and proteoglycan influence on retinal pigment epithelial cell architecture and morphology*
	Adam Krueger, *Developing a low cost in vitro loading system for mechanical stimulation of bone marrow organ cultures*
	Yena Chokshi, *Osteogenesis induced by rat pulmonary alveolar macrophages challenged by staphylococcus aureus*
Spring 2008	**Seema A. Kattaya,** *Osteogenesis induced by rat pulmonary alveolar macrophages challenged by staphylococcus aureus*
Spring 2007	**Benjamin McQuiston,** *Printed square spiral inductors for use in biomedical magnetoelastic sensors*
Fall 2006	**Arun Mohan,** *Effect of radioprotectants and radiosensitizers on the sterility of gamma irradiated bone*

LEADERSHIP

2007 – 2008	President of the Purdue University Turkish Students Association – elected to represent 100+ Turkish students on Purdue Campus
2006 – 2007	Secretary of the Purdue University Turkish Students Association Administrative Board

PROFESSIONAL TRAININGS AND WORKSHOPS

- Learning Styles: Empowering College Teaching and Learning (by Anthony F. Gregorc, Ph.D., organized by Purdue Teaching Academy)
- College Teaching Workshop Series: What Should We Be Teaching Beyond Content?
- Basics of Teaching: Presentation Techniques to Enhance Learning
- Creating Teaching Philosophies and Portfolios
- Grant Proposal Writing Workshop
- Rodent handling/surgery hands-on training
- Rabbit handling training
- Laser Safety Training (Purdue Radiological and Environmental Management)
- Radiation Safety Training (Purdue Radiological and Environmental Management)
- Bloodborn Pathogen Training (Purdue Radiological and Environmental Management)
- COMSOL Multiphysics Hands-on Workshop

TECHNICAL EXPERIENCE

- Involvement in the establishment of a cell/tissue culture facility at Weldon School of Biomedical Engineering under the supervision of Dr. Ozan Akkus
- Cell and tissue culture (rat bone marrow stromal cells, tendon derived fibroblasts, bone marrow organotypic culture, human mesenchymal stem cells, alveolar macrophages, rat breast cancer cells, retinal pigment epithelial cells, cytotoxicity, cell proliferation and cell migration assays)
- *In vitro* three-dimensional (3D) culture and mechanical stimulation
- Scanning Electron Microscopy (SEM)
  - Low vacuum and environmental mode
  - Dual beam mode
  - Real-time imaging during mechanical testing with in-chamber tensile/compressive loading stage
  - Quantitative backscattered electron imaging
  - Energy-dispersive X-ray spectroscopy (EDS)
  - Biological sample preparation techniques
- Transmission Electron Microscopy (TEM) and biological sample preparation techniques
- Confocal microscopy
- Fluorescent microscopy
- Raman microspectroscopy
- Micro Computed Tomography (μCT)
- Mineralization assays in cell and organ cultures (alkaline phosphatase activity, alizarin red, von Kossa)
- Enzyme-Linked Immunosorbent Assay (ELISA) for protein detection and quantification
- Flow Cytometry (marrow mesenchymal stem cells, hematopoietic cells)
- RNA isolation, purification and amplification
- Histology and immunohistology (plastic and paraffin embedding)
- Mechanical testing, microindentation
- Sterilization techniques (γ-radiation, autoclave)
- Printed Circuit Board design and patterning, electronic circuitry, programmable Intelligent Computer (PIC) programming

NON-ACADEMIC WORK EXPERIENCE

2009 - present **FlexTissue Biomedical LLC** (http://www.flextissue.com)
Chief Technical Officer (CTO)

2004 – 2005 **TR.NET Internet Service Provider**
Technical support and customer service.

Summer 2003 **SASA-Dupont SABANCI Polyester Inc.**
Chemical Engineering internship at Dimethyl Terephthalate (DMT) petro-chemical plant.

Summer 2002 **SAKOSA Industrial Yarn and Tire Cord Manuf. and Trading Inc.**
Mechanical engineering internship at the maintenance service of the plant and chemical engineering internship at the process control unit.

HOBBIES AND INTERESTS

Running, swimming, basketball and indoor rock climbing.

PUBLICATIONS

Annals of Biomedical Engineering, Vol. 36, No. 12, December 2008 (© 2008) pp. 1978–1991
DOI: 10.1007/s10439-008-9577-x

The Mechanical Environment of Bone Marrow: A Review

UMUT ATAKAN GURKAN and OZAN AKKUS

Weldon School of Biomedical Engineering, Purdue University, 206 S. Martin Jischke Drive, West Lafayette, IN 47907-2032, USA

(Received 29 December 2007; accepted 29 September 2008; published online 15 October 2008)

Abstract—Bone marrow is a viscous tissue that resides in the confines of bones and houses the vitally important pluripotent stem cells. Due to its confinement by bones, the marrow has a unique mechanical environment which has been shown to be affected from external factors, such as physiological activity and disuse. The mechanical environment of bone marrow can be defined by determining hydrostatic pressure, fluid flow induced shear stress, and viscosity. The hydrostatic pressure values of bone marrow reported in the literature vary in the range of 10.7–120 mmHg for mammals, which is generally accepted to be around one fourth of the systemic blood pressure. Viscosity values of bone marrow have been reported to be between 37.5 and 400 cP for mammals, which is dependent on the marrow composition and temperature. Marrow's mechanical and compositional properties have been implicated to be changing during common bone diseases, aging or disuse. *In vitro* experiments have demonstrated that the resident mesenchymal stem and progenitor cells in adult marrow are responsive to hydrostatic pressure, fluid shear or to local compositional factors such as medium viscosity. Therefore, the changes in the mechanical and compositional microenvironment of marrow may affect the fate of resident stem cells *in vivo* as well, which in turn may alter the homeostasis of bone. The aim of this review is to highlight the marrow tissue within the context of its mechanical environment during normal physiology and underline perturbations during disease.

Keywords—Mesenchymal stem cells, Marrow progenitor cells, Physiological activity, Osteoporosis, Disuse, Aging, Pressure, Fluid shear, Rheology, Viscosity.

INTRODUCTION

Bone marrow is the soft tissue residing in the cavities of the bones housing the essential pluripotent precursor cells for the living organism. It is the major source of hematopoietic stem cells (HSCs) which have the role of renewing the elements (monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets) in the blood.[12] Adult bone marrow also contains the mesenchymal stem cells which contribute to the regeneration of tissues such as bone, cartilage, muscle, adipose, tendon, ligament and stroma by differentiating into osteoblasts, chondrocytes, myocytes, adipocytes, tenocytes and neuronal cells *in vivo* and *in vitro*.[6,18,57,76–78,87] Mesenchymal stem cells have been proven to be responsive to mechanical signals such as hydrostatic pressure,[2,27,86,87] fluid flow induced shear stress[51] and the rheological properties (viscosity) of their environment.[21,89,93]

Bones are primarily responsible for countering physiological loads. The close proximity of marrow within medullary cavities of bones subject the marrow to physiological loads as well.[53,79,80,97,116] The key variables of the mechanical environment of marrow due to the external factors can be listed as the intramedullary pressure and the fluid flow generated by pressure gradients. These variables change during regular physical activities.[23,79,97,123] Intramedullary pressure elevations are reported in response to drugs and steroids.[3,55,59,90,96] Composition and rheological properties of marrow are also reported to change during aging and drug use.[39,59] Studies also suggest these properties of marrow to change during aging, osteoporosis or disuse.[39,90,91] Since these anomalies have the common hallmark of bone loss, the possibility arises that the mechanical environment of marrow may be a key player in homeostasis of bone by way of the mechanically responsive resident stem cells.[110]

Structure and Function of Bone Marrow

In adults, marrow tissue located in the mid-diaphyseal portions of peripheral bones in the body mostly consists of adipose tissue which imparts a yellowish color (fatty marrow).[104] In the axial skeleton however, the adipose tissue coexist with the hematopoietic tissue in a variable but roughly equal proportion. Bones provide a confined environment for marrow.

Address correspondence to Ozan Akkus, Weldon School of Biomedical Engineering, Purdue University, 206 S. Martin Jischke Drive, West Lafayette, IN 47907-2032, USA. Electronic mail: ozan@purdue.edu

0090-6964/08/1200-1978/0 © 2008 Biomedical Engineering Society

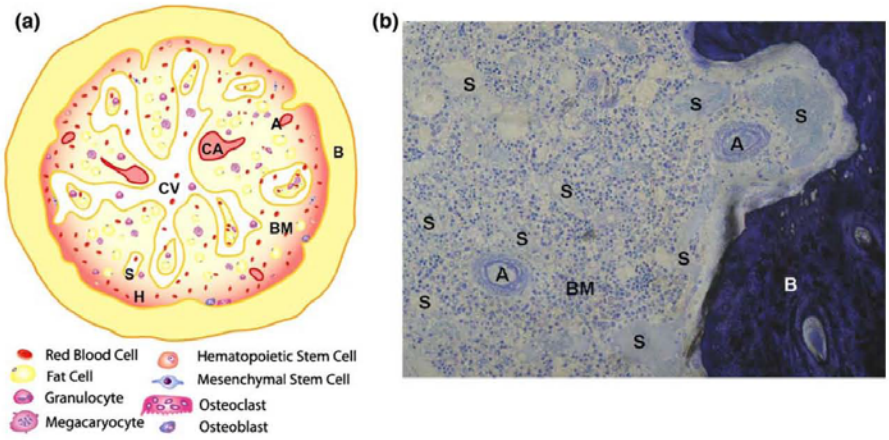


FIGURE 1. (a) Layout of bone marrow in a cross-sectional view of a tubular bone. Bone (B) is surrounding the bone marrow (BM). Central artery (CA) and central vein (CV) are running parallel to each other and longitudinally along the long bone (perpendicular to the plane of the page). The central artery and central vein branch toward the periphery to form arterioles (A) and sinusoids (S) which then combine and join with the central vein. Hematopoietic space (H) is interspersed by the sinuses. Developing red blood cells and granulocytic cells appear in the hematopoietic space. Megakaryocytes develop subjacent to the endothelium of marrow sinuses. It is possible to observe the radial distribution of marrow as the yellow marrow in the central regions and the red marrow in the periphery (Adapted from Tavassoli and Yoffey[104] and reprinted with permission of John Wiley & Sons, Inc.). (b) A toluidine-blue stained section taken transversely to the longer axis of a tubular bone. The micrograph displays the endosteal junction between bone and marrow (125×). The distribution of abundant number of red blood cells indicates that the bone marrow is hematopoietic (Courtesy of David C. Van Sickle, Purdue University).

Therefore, changes in the volume of active marrow (where hematopoiesis occurs) should be compensated by the expansion of a space-occupying component.[30] This component is commonly accepted to be the adipose tissue. When the marrow hematopoietic activity increases, adipose tissue undergoes resorption to provide more space, or vice versa.

Hematopoietic tissue imparts a reddish color to marrow (red marrow) due to the high content of heme chromogen.[104] Red marrow houses the red blood cells, platelets and white blood cells and resides in the flat bones such as skull, ribs, vertebrae, the proximal halves and the endosteal surfaces of the long bones. Red marrow tissue is rich in a variety of cellular components comprised mostly of hematopoietic cells. The hematopoietic cells give rise to monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets, and osteoclasts.[12,15,78,109,117] Marrow's other cellular component is a highly organized stroma that supports the proliferation of the hematopoietic cells.[117] The organized stroma is composed of reticular cells which form a spongy framework on which hematopoietic cells are arranged. Upon maturation, hematopoietic cells migrate into the blood stream. Therefore, hematopoietic cells are temporary residents of marrow. Conversely, the stroma remains as a scaffold for the differentiation and maturation of the hematopoietic cells.[104] Bone marrow also contains mesenchymal stem cells which have been shown to differentiate into osteoblasts, chondrocytes, myocytes, adipocytes and neuronal cells *in vivo* and *in vitro*.[6,18,57,76–78,87]

The organization of the marrow can be best understood by following its vascular layout. In a tubular bone, the nutrient artery enters the marrow cavity, runs longitudinally in the center, then branches out toward the endosteum of the surrounding bone, leading to specialized vascular structures known as sinuses or sinusoids (Fig. 1).[7,109] Several of these sinuses may then combine to form collecting sinuses which lead to the central sinus or vein. This vein runs longitudinally next to the nutrient artery. Blood in marrow flows from the center toward the bone and then returns back to the center.[104,109] This structural configuration yields high numbers of vessels and sinuses in the periphery (resulting in a slower flow rate of blood and higher surface area) where most of the exchange occurs. Therefore, hematopoiesis is maximal in the closer proximities to the bone surface leaving the central parts with relatively little hematopoietic activity (Fig. 1).[103] Due to this fact, it is possible to observe a transition region between red marrow and fatty marrow radially (red marrow being closer to the endosteal surfaces, Fig. 1).[104] Similarly, a longitudinal macroscopic

distribution is observed as red marrow in the proximal half and fatty marrow in the distal half of the bones.[104] Bone and marrow are connected by vasculature. Bone nutrient vessels enter the marrow cavity to make connections with marrow vessels. Small arteries of marrow also enter the bone, make a loop and return back to where they originated from.[104,109] A more detailed description of the structural organization and function of marrow can be found elsewhere.[104,109]

THE MECHANICAL ENVIRONMENT OF BONE MARROW

Hydrostatic Pressure

One of the first studies on the bone medullary pressure reported extensive necrosis of the bone after intramedullary infusion of high pressure saline solution.[49] Numerous subsequent studies tried to elucidate the possible relationship between the medullary pressure and the hemodynamics of the bone (Table 1). The systemic blood pressure in animals has been reported to be in the range of 110 140 mmHg, whereas the normal intramedullary pressure (IMP) was generally about 30 mmHg which is approximately one fourth of the systemic blood pressure (Table 1). This generalization is known as the one-forth rule.[90,91]

It was observed that intramedullary blood flow is directly related to the IMP and was suggested that the IMP is the resultant of the total blood flow entering the bone and the total blood flow leaving it.[25,90] In addition, marrow pressure depends on the relative degree of resistance to flow between arteries and marrow blood vessels, and between marrow blood vessels and veins. Moreover, IMP and blood-pressure has variations with the phases of respiration such that IMP increases with inspiration and decreases with respiration.[97] Tondevold *et al.*[108] investigated the changes in IMP in relation to mean arterial pressure. Interestingly, they observed that the medullary pressure remained essentially constant and independent of the mean arterial pressure as long as the latter remained above 81 mmHg. Therefore, it was suggested that there has to be a regulatory system trying to keep the medullary pressure constant, which may be the arterioles of the bone marrow that are supplied by sympathetic nerve fibers with a vasoconstrictor function.[24] Shim *et al.*[92] observed the relationship between IMP and hemodynamics of bone by focusing on the blood supplies of bone. This approach was in contrast to previous studies, which focused on systemic blood circulation. Experiments carried out by Shim *et al.* indicated that IMP rises if the arterial blood supply to the bone increases or venous congestion occurs in the

TABLE 1. Blood pressure and intramedullary pressure (IMP) values reported in the literature.

		Blood pressure		IMP	
	Animal	Location	Value (mmHg)	Location	Value (mmHg)
Stein *et al.*[96,a]	Dog	Femoral artery	110–140	Tibial diaphysis	25–75
Shaw[90,a]	Cat	Contralateral femoral or carotid artery	130	Femoral diaphysis	37
Azuma[3,a]	Rabbit	Carotid artery	100–110	Femur or tibia	25 (4–70)
Michelsen[55,a]	Rabbit	Carotid artery	73–118	Tibia	18–36
Harrelson and Hill[32,a]	Mongrel dogs	Femoral artery contralateral	110–140	Mid-diaphysis of femur	35
		Femoral vein contralateral	2		
Shim *et al.*[92,a]	Rabbit	Carotid artery	120	Femur	20–60
	Dog	Carotid artery	130	Femur	40–120
Wilkes and Visscher[121,b]	Dog	Femoral artery	134.0 (±13.2)	Tibia	23 (±5.3)
		Nutrient vein	19.3 (±6.3)		
Tondevold *et al.*[108,a]	Mongrel dogs	Left brachial artery	112.9 (±0.9)	Femoral epiphysis	30.7 (±2.6)
				Femoral metaphysis	20.5 (±1.5)
				Femoral diaphysis	25.7 (±1.7)
Thomas *et al.*[107,a]	Rabbit	Not measured	–	Lower femoral diaphysis	33 (7–81)
Bauer and Walker[5,a]	Dog	Not measured	–	Femoral diaphysis	27.6 (±15.4)
				Femoral metaphysis	17.6 (±10.5)
				Tibial diaphysis	26.4 (±13.0)
				Tibial metaphysis	17.9 (±11.8)
				Humeral diaphysis	26.2 (±15.8)
				Humeral metaphysis	13.4 (±7.7)
				Radial diaphysis	15.4 (±18.9)
Stevens *et al.*[97,c]	Mouse	Not measured	–	Femur	10.7 (±1.4)

[a]IMP was measured with a cannula inserted into the bone in anesthetized animals.
[b]IMP was measured with a tonometric pressure transducer in anesthetized dogs.
[c]IMP was measured by radiotelemetry in unanesthetized ambulatory mouse.

TABLE 2. The effects of occlusion, epinephrine, norepinephrine, vasodilators, vasoconstrictors and skeletal muscle contraction on intramedullary pressure (IMP) and systemic blood pressure.

	Occlusion				Drugs		Skeletal muscle contraction	
	Arterial	Venous	Epinephrine	Norepinephrine	Vasodilator	Vasoconstrictor	Abdominal	Lower limb
Stein *et al.*[96,a]			⇑⇓↑	⇑⇓↑	⇓↓	⇑↑		
Shaw[90,a]	⇓↓	⇑ →	⇓↑	⇓↑	⇓↓	⇑↑		
Azuma[3,a]	⇓ →	⇑ →	⇑⇓↑↓		⇓↓↑ →		⇑⇓⇒	→
Michelsen[55,a,b]	⇓↓	⇑↑		⇓↑	⇑⇓⇒ ↓↑ →			
Shim *et al.*[92,b]	⇓ →	⇑ →	⇓↓	⇓↑			⇓ →	⇑ →
Stevens *et al.*[97,c]		⇑						

⇑,⇓,⇒: Increase, decrease or no change in IMP; ↑,↓, → : Increase, decrease or no change in systemic blood pressure; ⇑⇓ or ↑↓: First increase, then decrease or vice versa; vasodilator: acetylcholine, benzyl-imidazoline; vasoconstrictor: amphetamine, histamine.
[a]IMP was measured with a cannula inserted into the bone in anesthetized animals.
[b]Blood pressure was measured at the femoral artery; vasodilator (acetylcholine) was injected into femoral artery.
[c]IMP was measured by radiotelemetry in unanesthetized ambulatory mouse.
All injections were made intravenously; systemic blood pressures were measured at the carotid artery.

limb (Table 2). It was further demonstrated that increased marrow cavity pressure due to venous congestion decreases the blood supply to the bone.[92] It was also suggested that the elevation of the IMP due to muscle contraction, which mimics physiological activity, may have significance in the maintenance of structural integrity of the bone. Although some researchers claimed to be able to define a relationship between the IMP and the blood circulation in bones, there is neither an agreement nor a consistency in the findings as it can be seen in Table 1. Nevertheless, a common finding in these studies is that the IMP has a pulsatile regime synchronous with the arterial blood pressure and respiration.

There was a significant variation in the intramedullary pressure measurements reported by various authors in the literature (Table 1). This could be due to the differences in the marrow tissue of different species.[3] Another explanation to these variations was that the blood channels in the bone marrow were damaged by the insertion of the cannula and it was quite possible that there was a blood pool forming at the tip of the cannula. Therefore, sizes of the damaged channels, the amount of the damage in blood channels and in the extravascular tissue could be causing the variation.[3] Similar concerns related to variations in pressure measurements due to lesions formed at the tip of the cannula were raised by various researchers.[56,92] In order to eliminate the negative effects of hemorrhage or lesions forming, a tonometric pressure transducer was employed which was positioned on the intact endosteal membrane through a cortical hole of about 7.3 mm in diameter.[121] Although the enhancing characteristics of the measurement technique were not discussed in detail, less variation was observed in the IMP measurements (Table 1). However, the difficulties in the surgical techniques used in this study caused puncture of the endosteal membrane and gross trauma in half of the animals. Due to the advances in sensing electronics and telemetry, it has been recently possible to conduct *in vivo* IMP pressure measurements on unanesthetized ambulatory mice by implantation of a radiotelemetry pressure transducer.[97,98] The transducer was composed of a 0.4 mm diameter catheter, which was inserted through a 0.5 mm hole in the femoral cortex and sealed with tissue cement. The catheter was connected to a transmitter, which was secured in the peritoneal cavity, onto the abdominal wall. The new technique was successfully employed for long term, *in vivo* measurements of IMP in venous ligation and hindlimb suspension mice models (Tables 1 and 2). This new technique has proved to be more efficient in yielding *in vivo* pressure measurements with more accurate readings.

The response of the mechanical environment of the bone medullary cavity to various factors has revealed that the marrow environment is quite susceptible to external factors. In addition, this response has been shown to be quite robust in its recovery towards its normal state after its dynamics were disturbed. The studies in this regard aimed to elucidate the hemodynamic changes in bone marrow due to external effects such as occlusion of regional vessels, injection of epinephrine, norepinephrine, acetylcholine, pressor and depressor drugs, and skeletal muscle contraction (Table 2).

It is commonly anticipated that physiological loading (i.e. walking or running) perturbs the IMP.[53,79,80,97,116] This supposition was tested by externally stimulating skeletal muscles of anesthetized animals to mimic the physiological loading conditions. The results (Table 2) showed that IMP rises due to muscle contraction. In a more recent *in vivo* study, the effect of hindlimb suspension and venous ligation on

the medullary pressure was successfully measured by telemetry in ambulatory mice.[97] In this study, surgical venous ligation increased the IMP by 25%. In addition, IMP decreased by 23% in normal mice and decreased by 33% in ligated mice upon hindlimb suspension (Table 2). In general, arterial occlusion yielded a decrease while venous occlusion yielded a rise in the medullary pressure, both of which verify the direct relationship between the blood flow into the bone and the IMP (Table 2). The effects of vasodilator, vasoconstrictor drugs and steroids on marrow pressure were investigated on anesthetized animals.[3,55,59,90,96] Vasodilator drugs tend to lower both the marrow and the systemic blood pressure while vasoconstrictor drugs tend to increase both of them (Table 2). Miyanishi *et al.*[59] attempted to relate steroid-induced osteonecrosis with intraosseous pressure rise in rabbits. They observed that steroid treatment significantly increased marrow pressure, decreased bone blood flow and also caused fat cell enlargement.

Rheology

It has recently been indicated that marrow viscosity is a critical parameter modulating the shear stresses experienced by the trabecular surfaces in the vertebral bodies due to vibratory loads.[19] Since the potential rheological changes in the marrow due to bone diseases and aging is likely to affect the shear stresses experienced by the progenitor cells in marrow, it is essential to quantify and characterize these parameters. Bone marrow (bovine) has been shown to present Newtonian fluid characteristics (i.e. constant viscosity which is independent of shear rate) at near body temperatures.[10] It should be noted that marrow has a slightly lower temperature (1.6 4.8 °C below) than normal body temperature in humans.[73] Bryant *et al.*[10] observed the dependence of bovine bone marrow's rheological properties on temperature and anatomical location. The measured viscosity of the proximal marrow at 35 °C (≈400 cP, viscosity of water is 1 cP) was found to be about ten times that of the distal samples (≈40 cP) which can be associated with compositional variations of marrow along the bones (Table 3).[20] Noting that the proximal ends of bones contain red marrow, whereas the distal ends contain yellow (fatty) marrow[104]; it is reasonable to infer that the increased fat content in marrow may reduce its viscosity. Interestingly, removal of cell debris, blood cells and other granular matter from the marrow by centrifugation decreased the dependence of its viscosity on temperature.[10] Considering the durations of viscosity measurement intervals of as long as 6 h; the potential effect of coagulation of marrow samples was not discussed in this study.[10] In a more recent study on the measurement of femoral bovine marrow viscosity with an implantable wireless method, a viscosity value of 123 cP was reported.[31] The only study on the viscosity of human bone marrow (calcaneal marrow which was reported to be mainly yellow or fatty) reports values in the range of 37.5 cP (at 36 °C) and Newtonian fluid characteristics (i.e. viscosity is not dependent on the shear rate).[17] However the authors report non-Newtonian behavior for the bone marrow specimens with red components (i.e. higher blood cell concentration).[17] This finding is not surprising as it is known that human blood displays non-Newtonian characteristics.[26] In addition, the density of red bone marrow (1.06 g/cm^3)[120] has been reported to be comparable to that of blood (1.05 g/cm^3).[26] It should be noted that there is limited information available in the literature on the density of fatty marrow (0.89 g/cm^3)[31] which is comparable to that of fat tissue (0.92 g/cm^3).[120] The viscosity values reported in the literature for bone marrow and blood are summarized in Table 3.

The variation of marrow composition along the bones and in the different parts of the body is still a

TABLE 3. Viscosity measurement values reported in the literature for bone marrow and blood.

	Subject	Location	Temperature (°C)	Value (cP)
Bone marrow				
Bryant *et al.*[10]	Bovine	Proximal radius	35	400[a]
	Bovine	Distal radius	35	44[a]
Gurkan and Akkus[31]	Bovine	Femur	37	123[b]
Davis and Praveen[17]	Human	Calcaneus	36	37.5[c]
Blood				
Eguchi and Karino[26]	Human	–	37	66[d]

[a]Measurement was obtained with cone and plate viscometer.
[b]Measurement was obtained with parallel plate rheometer.
[c]Measurement was obtained with controlled oscillatory flow within a straight, cylindrical tube with circular cross section.
[d]Measurement was obtained with falling ball viscometer at a shear rate of 0.189 s^{-1} on a blood sample with a hematrit value of 41%, and a density of 1.05 g/cm^3.

question to be answered. This variation can be explained by the temperature dependence of marrow. Marrow composition is highly affected from variations in temperature. Huggins *et al.*[33–35] evidenced that the fat content of bone marrow in the limb bones (femur, radius) are higher than the bones in the central parts of the body (ribs, vertebra). This was suggested to be associated with greater body temperatures in central bones. Similarly, Weiss *et al.* demonstrated that in summer times, the bony exoskeleton of the nine-banded armadillo (*Dasypus novemcinctus*) displayed a red or erythropoietic marrow; whereas in winter times, when the ambient temperature is low, the marrow was yellow or fatty.[118]

The rheological properties of bone marrow in different parts of the body and in osteoporotic, disused or aged bones are not known. *In vivo* monitoring of marrow in bone disease and disuse models can provide more realistic values due to the elimination of effects of extraction. The outcomes of future investigations in this area may prove to be valuable for researchers trying to simulate the natural environment of stem and progenitor cells in mechanically stimulated cell cultures in an effort to better understand bone loss.

While the intramedullary pressure and the rheology of marrow are relatively well investigated, the shear stress in the marrow during ambulation is largely unknown. Shear stress within the marrow as well as between the marrow and the endosteal bone may play a role in terms of modulating the biological response of marrow resident cells and the endosteal lining. The deformation and flow of marrow due to mechanical loading and associated effects on the stromal cells is an area open to investigation and it needs to be studied further.[51,100]

EXTERNAL INFLUENCES TO THE MECHANICAL ENVIRONMENT OF BONE MARROW

Effects of Physiological Activity and Loading

When the mechanical loading on bones is removed due to extended periods of inactivity, such as bed rest or during space-flights, bone mineral density decreases.[50,84,122] On the other hand, elevated amounts of loading on bones due to exercise causes increased bone mass and bone turnover.[16,45,61,62,65,112] Even though there is *in vivo* evidence that exercise induced mechanical loading enhances bone mass, the cellular and molecular mechanisms underlying this fact are still being studied. Bone homeostasis is a balanced system of formation by osteoblasts and resorption by osteoclasts. Osteoblasts are the bone forming cells that originate from mesenchymal stem cells residing in marrow. Whereas osteoclasts are the bone resorbing cells that originate from the hematopoietic stem cells in marrow.[13] Bone marrow mechanical environment is known to be affected from loading of bones with variations in intramedullary pressure, intramedullary and interstitial fluid flow.[53,74,80,97,116] Since marrow is housing the precursors of osteoblasts and osteoclasts, the changes in the mechanical environment of marrow due to physiological activity and loading may play a role in bone homeostasis.

The earlier studies on the effects of loading on the mechanical environment of bone marrow tried to elucidate the strengthening characteristics of marrow as a slightly compressed liquid in the trabecular regions. It has been suggested that intertrabecular fluid (marrow) pressurized by compressive deformation of the bone may provide load bearing capabilities.[9,40,41] The related *ex vivo* and *in vivo* studies proved that marrow inside the trabecular regions of femoral head provides stiffness to subchondral trabecular bone.[70,71] It was also suggested that bone marrow acts as an intermediate transferring the external loads by means of pressurization to trabecular bones, which are acting as trusses in subchondral regions. Therefore, the trabeculum bears only the modest pressure difference across it as a tensile load.[95]

Researchers have used both *in vivo* and *in vitro* models to study the effects of physiological loading and activity on bone marrow. Kumar *et al.*[46] loaded the fixed femurs of rats *in vivo* applying loads ranging from 0 to 12.25 kg for 1 min. They observed pressure values to rise about two-fold (12 14 mmHg increase) upon loading. They also studied contraction of the quadriceps muscles with electrical stimulation, resulting in a pressure increase of 60 mmHg.[46] Another study loaded the tibiae of the sheep in impact *in vitro* with loads of 2000 N for durations of 0.015 s and observed elevations up to 300 mmHg.[7] Downey *et al.*[23] studied the effects of *in vitro* compressive loading in the human femoral head. The extracted human hips from cadavers were subjected to load by means of a mechanical testing device. They observed the IMP rise by 55 (±66) mmHg per 980 N of load applied over 0.1 s. The load values and the application durations were estimated to be comparable to physiologic conditions. They also presented an interesting finding about an osteoporotic bone with a much higher pressure rise of about 220 mmHg as a result of a relatively less load of 590 N. However, due to the lack of blood flow in the bone resting IMP of the bones was zero and the resemblance of these loading conditions and models to natural physiological loading conditions are disputable. These studies, regardless they are *in vivo* or *in vitro*, apply artificial external loads with assumed similarity to physiological loads. Moreover, *in vitro*

studies lack blood circulation in the bone and in the muscle tissue, both of which may have potent effects on the pressure of bone marrow during physiological loading. An ideal study investigating the effects of physiological loading on the mechanical environment of bone marrow should employ slightly invasive or noninvasive instrumentation which at the same time facilitates continuous measurements in unanesthetized ambulatory animal models. Furthermore it should be noted that the magnitudes of the loading induced IMP is dependent on factors such as loading rate, viscosity of marrow and porosity of the surrounding bone.[19,80] Therefore these factors should be taken into consideration when evaluating the relative magnitudes of loading induced IMP.

An explanation for bone loss due to lack of exercise and physical activity is decreased medullary cavity pressurization.[84] It has been reported that bone loss is not equal throughout the skeleton in long-term bed rest. Bone loss is the greatest in lower extremities, where the medullary pressure is significantly decreased due to lack of activity. On the other hand, bone formation is observed in the head, where the intracranial pressure is increased due to body orientation and shift of body fluids.[50] This suggestion has recently been supported by a mouse hind limb suspension model with *in vivo* femoral IMP measurements for extended durations.[97] The study aimed to infer the interstitial fluid flow changes due to pressure gradients between the endosteum and periosteum. A decrease of 23% in the IMP was observed upon hindlimb suspension. Although the correlation between the IMP and the bone formation or bone loss was not reported, it was shown that medullary pressure is sensitive to disuse with an *in vivo*, unanesthetized and ambulatory animal model. Correspondingly, a strengthening treatment technique for osteoporotic long bones has recently been put forward, which elicits cortical bone formation in the femoral neck region of mice by means of knee loading *in situ*.[124,125] It has been verified by a following study that the intramedullary pressure is altered in synchrony with the knee loading.[123] It should be noted that 2/3 of the blood supply of cortical bone is provided by the endosteal surface.[80,102] It has also been shown that the variations and oscillations in IMP play an important role on the loading induced fluid flow developed in the interstices of the cortical bones as well as of the trabecular bones.[97,101,124] Therefore it is likely that the variations in the IMP not only affect the resident pluripotent stem cells but also serve as one of the players which are driving the fluid flow within the intersticies of cortical bone by way of generating pressure gradients between the envelopes of bone. This flow is essential to provide nutrients and to remove the metabolic byproducts of osteocytes resident in mineralized cortex.

Effects of Osteoporosis and Aging

Osteoporosis is a disease of bone in which fracture susceptibility is compromised by decreased bone mineral density. It mostly involves the hip, the wrist and the lumbar vertebrae.[81] It is estimated that over 20% of women and 7% of men over the age of 50 have osteoporosis. Osteoporosis is responsible for over 1.5 million fractures in the United States annually.[72] It has been evidenced that there is a significant change in marrow composition and mechanics due to osteoporosis and aging.[39,90,91,115] The only study in this regard stated that the IMP in the tibial diaphysis of children with paralytic osteoporosis approximates the arterial blood pressure. Whereas the tibial IMP of the healthy children with the same age was only about one quarter of the arterial blood pressure.[90,91] In addition, the adipose tissue fraction in marrow increases significantly with osteoporosis,[39,115] which may be yielding to obstruction of blood circulation.[59] Increased adipose fraction, thus decreased viscosity may be causing the overall shear stress in the medullary cavity to decrease in osteoporosis. Potential elevations in pressure or decrease in shear stress in osteoporotic bones may contribute to osteoporosis by way of altering the milieu of the bone marrow progenitor, precursor and stem cells.

Osteoblasts and adipocyte cells both originate from the mesenchymal stem cells.[76] It has been suggested that the commitment of stem cell fate is integral with mechanical cues experienced in developmental and adult contexts, embodied in cell shape, cytoskeletal tension and RhoA signaling.[54] RhoA is a small GTPase protein known to regulate the actin cytoskeleton in the formation of stress fibers. It has been shown that human mesenchymal stem cells subjected to different mechanical environments differentiate into either osteoblasts or adipocytes depending on the RhoA activity (RhoA active: osteogenesis, RhoA negative: adipogenesis).[54] Likewise, adipocyte tissue volume in bone marrow has been shown to increase with aging and in patients with osteoporosis.[39,115] Therefore, it can be hypothesized that osteoporosis may be due to a greater portion of mesenchymal stem cells differentiating into adipocytes than osteoblasts due to the changes in the cell properties and/or the mechanical environment of the marrow.

EFFECTS OF THE MECHANICAL ENVIRONMENT ON STEM AND PROGENITOR CELLS

It should be noted that the responses of the marrow derived progenitor cells to physical factors such as hydrostatic pressure, fluid shear and the rheology of

the environment have been extensively studied *in vitro*. The question about the natural physiological and altered mechanical conditions of these cells in the bone marrow remains to be answered. Therefore, the review focuses on the effects of mechanical loading to stem and progenitor cells *in vitro* to illustrate that stem cells are responsive to mechanical cues. However, it remains to be determined as to whether this mechanical responsiveness exists *in vivo*.

It is commonly accepted that stem cells' microenvironment, which includes biochemical and biomechanical factors, has an important role on their differentiation and phenotypic expression. Even though most studies in the literature place an emphasis on growth factors and cytokines, it has been evidenced that the differentiation of precursor, progenitor and stem cells are also significantly influenced by mechanical factors.[27] The main mechanical signals that are accepted to be effective on marrow precursor cell proliferation and function are: hydrostatic pressure and fluid-flow induced shear.[27,93] The viscosity of marrow is also important since it directly relates to the fluid shear stress magnitudes experienced by the cells. The influences of these factors are considered to be significant in regulating the stem cell phenotype and may have synergistic and/or supplemental effects in combination with the biochemical factors. The employment of such mechanical effects in specially designed "bioreactors" may enhance current standard biochemical signaling pathways for promoting engineered tissue growth.[27,42,88]

Marrow-derived mesenchymal stem and/or progenitor cells offer novel treatment techniques in tissue engineering research. They are already cultured *ex vivo* in mechanically active environments for various purposes, such as tissue engineering of bone and cartilage.[6,15,47,69,77,93,94,99,114] The main goal of moving from conventional 2-D methods to 3-D mechanically active systems is to attain more physiological (or natural) culture environments for the cells. Although these studies prove improvements over conventional culturing techniques, they are far from *in vivo* conditions in efficiency.

Hydrostatic Pressure

There are two main reasons why hydrostatic pressure is considered as an important stimulating factor for bone marrow progenitor and stem cells. First of all, marrow derived mesenchymal progenitor cells can express chondrogenic phenotypes under appropriate hydrostatic pressure conditions. There are numerous *in vitro* studies subjecting marrow-derived mesenchymal stem cells to high hydrostatic pressures (750 75,000 mmHg) in an effort to induce chondrogenic phenotype.[1,2,57,58,68,87] Even though the chondrogenic differentiation of the progenitor cells does not take place in marrow cavity, those studies are related in indicating the sensitivity of the progenitors to the magnitude and the mode (cyclic, intermittent or static) of loading. In general, higher pressure (~75,000 mmHg) over lower (~750 mmHg) and intermittent loading over static have proved to be more effective in chondrogenesis.[57,58] Second, hydrostatic pressures in the medullary cavities of long bones due to physiological loading (50 200 mmHg rise)[8,23,46,84,110] have osteogenic effects.[86] It has been shown that constant hydrostatic pressure applied *in-vitro* at physiologic levels (30 60 mmHg) decreases osteoclast formation.[85] Similarly, cyclic pressures of 75 300 mmHg in magnitude decreases formation of osteoclasts from their progenitors and decreases bone resorptive activity by osteoclasts *in vitro*.[63] Furthermore, this loading scheme enhances osteoblast functions pertinent to new bone formation by stimulating both synthesis and deposition of collagen accompanied by increased accumulation of calcium-containing crystals.[63,64] A general conclusion that can be drawn from those *in vitro* studies is that the osteogenic response of the cells are not always linearly proportional to the value of the mechanical signals, rather there is an optimal amplitude interval for each loading pattern and frequency. Limited number of *in-vivo* studies in this regard report similar results. Oscillatory hydrostatic pressure (60 mmHg, 20 Hz) applied *in-vivo* for 10 min per day for 4 weeks to avian ulnae elicited new bone formation on the periosteal surface[79] possibly due to increased intracortical fluid flow as suggested by the authors. It was also suggested that since the applied loading regime has bone forming effects; it should be similar to the physiological loading conditions. Similarly, intramedullary hypertension due to venous occlusion (pressure increased to about 28.7 mmHg from 15.5 mmHg) has periosteal (138%), endocortical (369%) and cancellous (889%) bone forming effects at the caprine tibial metaphysis.[119] One of the suggestions on how the marrow pressure affects bone formation is that the progenitors in the marrow may be directly reacting to marrow pressure or extracellular fluid flow (developed by pressure gradients) by differentiating into osteoblasts and forming new bone.[51,110] On the other hand, it is suggested that nitric oxide is imperative in signaling of mechanically induced bone formation.[28,75,113] Nitric oxide is a signaling molecule and it is synthesized by nitric oxide synthase which is produced by osteoprogenitor cells in bone marrow, by osteocytes in bone and bone lining cells.[29] Therefore, it is probable that the production of this signaling molecule by the osteoprogenitor cells in the marrow is mediated by bone marrow pressure or pressure gradients.[110]

Fluid Shear

Mechanical loading and bending of bones cause strain gradients as well as local pressure gradients in the bone and in the medullary cavity that can drive the interstitial fluid flow[53,74,80,116] and can result in shear stresses on the endosteal surface. Fluid flow induced fluid shear on bone marrow derived cells has been investigated extensively to assess its effects on cell differentiation, proliferation and function.[6,15,51,63,94] Besides, it has been evidenced by a number of researchers that interstitial fluid flow may play an important role in bone remodeling, formation and adaptation.[11,37,53,67,79,111] The mechanisms of flow-induced remodeling have been studied *in vitro* by subjecting osteoblasts,[22,37,43,48] osteocytes[37,44] and osteoclasts[53,85] to fluid shear. The studies on osteoblasts and osteocytes showed that several osseoactive agents are stimulated by fluid flow such as nitric oxide,[38,75] prostaglandins E2 and I2,[43,52,66,67,82] cyclic adenosine monophosphate,[82] intracellular free calcium,[14,22,36,37,64,86] inositol triphosphate,[36,82,83] and transforming growth factor β.[27,43,57,58] On the other hand, osteoblast-mediated mineralization is preceded by osteoclast-mediated resorption with osteoclast resorption rates being about 20 times higher than osteoblast deposition rates.[106] Therefore, osteoclast function may dominate the dynamic osteoclast-osteoblast balance that regulates bone turnover and degradation. Moreover, it is suggested that physiological load induced fluid flow in cortical bone is radially outward from endosteal surface to the periosteal surface,[60,80] which makes it difficult for osteoblast or osteocyte secreted mediators to diffuse against the current and reach the osteoclasts or osteoclast progenitors in the marrow cavity.[53] The osteoprogenitor cells are reported to be residing in bone marrow, one to three cell layers away from the endosteal surface.[105] Accordingly, it has been suggested that osteoclasts and their precursors, which are located in the close vicinity of endosteal surface, may be fluid flow induced shear sensitive and that osteoclast remodeling activities may be under the control of autocrine factors.[53] Bone marrow derived osteoclast-like cells are mechanosensitive to fluid flow induced shear and secrete autocrine factors, such as nitric oxide, prostaglandins E2 and I2, which can regulate local resorptive activity.[53] Therefore, fluid flow induced shear developing on the endosteal surface in the medullary cavity may be the significant stimulant for osteoprogenitors in the marrow that recruits them to bone formation sites. Human marrow stromal cells subjected to oscillatory fluid flow of 1 Hz are shown to have increased proliferation rates,[51] which means more osteoprogenitor cells to participate bone formation process. Similarly, the sensitivity of the cells in fluidic environment to flow frequency has been studied to find the potent optimal frequency values for cell phenotype determination.[4] However, the flow mode that is naturally experienced by osteoprogenitors in marrow has not been revealed yet.

Rheology

Viscosity of the environment in which cells reside is important since it directly affects the shear stresses experienced by cells if the medium is flowing. The studies in this field investigate the viscosity of the environment in mechanically active cell cultures or perfusion bioreactors.[21,89,93] In one such study, the effect of medium viscosity on marrow stromal osteoblastic cells seeded on 3D fiber meshes was studied by adding dextran (a complex, branched polysaccharide) to flowing medium in a perfusion bioreactor.[93] Increasing medium viscosity with constant flow rates resulted in 2- to 3-fold increases in the shear stresses experienced by the cultured cells without changing chemotransport characteristics significantly. It was reported that increased medium viscosity not only enhances mineralized matrix deposition but also provided a better matrix distribution in the porosity of the 3D scaffolds. In a similar study, the effect of fluid flow induced shear and chemotransport on bone cells was studied in oscillatory flow.[21] The medium viscosity was varied to obtain different shear stress values on the cultured cells under constant flow rates. It was shown that fluid flow induced chemotransport and shear stress acting on the cells play a synergistic role to elicit cell response to oscillatory fluid flow induced shear stress. In an effort to control the cell aggregate sizes in neural stem cell cultures, researchers tried to alter the kinematic viscosity of culture medium by adding dextran and carboxymethylcellulose.[89] The results indicate that viscosity is an important parameter to consider for scale-up of stem cell bioreactors. The viscosity values of the media employed in these studies were in the range of 1 4 cP, whereas the reported viscosity values for bovine marrow was in the range of 44 400 cP.[10] Therefore there is a need to revisit these studies and assess stem cell response at these higher viscosity media.

CONCLUSION

Bone marrow mechanical environment is susceptible to external effects such as physiological activity and disuse. Moreover, there is a potential relation between the bone diseases such as osteoporosis, and aging or disuse-related bone loss and the marrow composition and mechanics. The changes in the bone marrow mechanical environment is likely to be effective on the

occupant precursor and progenitor cells, which are accepted to be responsive to mechanical factors such as hydrostatic pressure, fluid shear and the viscosity of their environment. Although the effects of these mechanical factors and viscosity on mesenchymal stem and progenitor cells are being widely investigated *in vitro*, the naturally occurring and altered cues in the cells' natural environment (bone marrow) have not been well characterized yet.

Bone marrow mechanical environment can be completely defined by quantifying and characterizing the hydrostatic pressure, fluid flow induced shear and viscosity in natural and altered conditions. The results of these studies can be compiled to generate physiologically relevant *in vitro* mechanical environments for cell cultures, stem cell bioreactors and computational models to better understand the effects of mechanical signals on stem, progenitor and precursor cells; and potentially the nature of associated bone diseases.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. David C. Van Sickle for inspiring discussions and providing the histological figure of bone marrow; Sharon Evander and Carol Bain for their help in preparing the histological figure for publication.

REFERENCES

[1]Angele, P., D. Schumann, M. Angele, B. Kinner, C. Englert, R. Hente, B. Fuchtmeier, M. Nerlich, C. Neumann, and R. Kujat. Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds. *Biorheology* 41(3–4):335–346, 2004.

[2]Angele, P., J. U. Yoo, C. Smith, J. Mansour, K. J. Jepsen, M. Nerlich, and B. Johnstone. Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro. *J. Orthop. Res.* 21(3):451–457, 2003. doi:10.1016/S0736-0266(02)00230-9.

[3]Azuma, H. Intraosseous pressure as measure of hemodynamic changes in bone marrow. *Angiology* 15(9):396, 1964. doi:10.1177/000331976401500903.

[4]Balcells, M., M. F. Suarez, M. Vazquez, and E. R. Edelman. Cells in fluidic environments are sensitive to flow frequency. *J. Cell. Physiol.* 204(1):329–335, 2005. doi:10.1002/jcp.20281.

[5]Bauer, M. S., and T. L. Walker. Intramedullary pressure in canine long bones. *Am. J. Vet. Res.* 49(3):425–427, 1988.

[6]Braccini, A., D. Wendt, C. Jaquiery, M. Jakob, M. Heberer, L. Kenins, A. Wodnar-Filipowicz, R. Quarto, and I. Martin. Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts. *Stem Cells* 23(8):1066–1072, 2005. doi:10.1634/stemcells.2005-0002.

[7]Branemark, P. I. Vital microscopy of bone marrow in rabbit. *Scand. J. Clin. Lab. Invest.* 11(Suppl 38):1–82, 1959. doi:10.3109/00365515909060400.

[8]Bryant, J. D. The effect of impact on the marrow pressure of long bones in vitro. *J. Biomech.* 16(8):659, 1983. doi:10.1016/0021-9290(83)90117-3.

[9]Bryant, J. D. On hydraulic strengthening of bones. *J. Biomech.* 28(3):353–354, 1995. doi:10.1016/0021-9290(95)90563-L.

[10]Bryant, J. D., T. David, P. H. Gaskell, S. King, and G. Lond. Rheology of bovine bone marrow. *Proc. Inst. Mech. Eng. [H]* 203(2):71–75, 1989. doi:10.1243/PIME_PROC_1989_203_013_01.

[11]Burr, D. B., A. G. Robling, and C. H. Turner. Effects of biomechanical stress on bones in animals. *Bone* 30(5):781–786, 2002. doi:10.1016/S8756-3282(02)00707-X.

[12]Cabrita, G. J. M., B. S. Ferreira, C. L. da Silva, R. Goncalves, G. Almeida-Porada, and J. M. S. Cabral. Hematopoietic stem cells: from the bone to the bioreactor. *Trends Biotechnol.* 21(5):233–240, 2003. doi:10.1016/S0167-7799(03)00076-3.

[13]Canalis, E. The fate of circulating osteoblasts. *N. Engl. J. Med.* 352(19):2014–2016, 2005. doi:10.1056/NEJMe058080.

[14]Chen, N. X., K. D. Ryder, F. M. Pavalko, C. H. Turner, D. B. Burr, J. Y. Qiu, and R. L. Duncan. Ca2+ regulates fluid shear-induced cytoskeletal reorganization and gene expression in osteoblasts. *Am. J. Physiol. Cell Physiol.* 278(5):C989–C997, 2000.

[15]Chen, X., H. Xu, C. Wan, M. McCaigue, and G. Li. Bioreactor expansion of human adult bone marrow-derived mesenchymal stem cells. *Stem Cells* 24(9):2052–2059, 2006. doi:10.1634/stemcells.2005-0591.

[16]Courteix, D., E. Lespessailles, S. L. Peres, P. Obert, P. Germain, and C. L. Benhamou. Effect of physical training on bone mineral density in prepubertal girls: a comparative study between impact-loading and non-impact-loading sports. *Osteoporos. Int.* 8(2):152–158, 1998. doi:10.1007/BF02672512.

[17]Davis, L. B., and S. S. Praveen. Nonlinear versus linear behavior of calcaneal bone marrow at different shear rates. In: American Society of Biomechanics Annual Meeting, Blacksburg, VA, 2006. Available from: http://www.asbweb.org/conferences/2006/pdfs/304.pdf.

[18]Dennis, J. E., and A. I. Caplan. Bone marrow mesenchymal stem cells. In: Stem Cells Handbook, edited by S. Sell. Totowa, NJ: Humana Press Inc., 2003, pp. 107–118.

[19]Dickerson, D. A., E. A. Sander, and E. A. Nauman. Modeling the mechanical consequences of vibratory loading in the vertebral body: microscale effects. *Biomech. Model. Mechanobiol.*, 2007.

[20]Dietz, A. A. Composition of normal bone marrow in rabbits. *J. Biol. Chem.* 165(2):505–511, 1946.

[21]Donahue, T. L. H., T. R. Haut, C. E. Yellowley, H. J. Donahue, and C. R. Jacobs. Mechanosensitivity of bone cells to oscillating fluid flow induced shear stress may be modulated by chemotransport. *J. Biomech.* 36(9):1363–1371, 2003. doi:10.1016/S0021-9290(03)00118-0.

[22]Donahue, S. W., C. R. Jacobs, and H. J. Donahue. Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent. *Am. J. Physiol. Cell Physiol.* 281(5):C1635–C1641, 2001.

[23]Downey, D. J., P. A. Simkin, and R. Taggart. The effect of compressive loading on intraosseous pressure in the femoral-head in vitro. *J. Bone Joint Surg. Am.* 70A(6): 871–877, 1988.

[24]Drinker, C. K., and K. R. Drinker. A method for maintaining an artificial circulation through the tibia of the dog, with a demonstration of the vasomotor control of the marrow vessels. *Am. J. Physiol.* 40:514–521, 1916.

[25]Drinker, C. K., K. R. Drinker, and C. C. Lund. The circulation in the mammalian bone-marrow—with especial reference to the factors concerned in the movement of red blood cells from the bone marrow into the circulating blood as disclosed by perfusion of the tibia of the dog and by injections of the bone-marrow in the rabbit and cat. *Am. J. Physiol.* 62(1):1–92, 1922.

[26]Eguchi, Y., and T. Karino. Measurement of rheologic property of blood by a falling-ball blood viscometer. *Ann. Biomed. Eng.* 36(4):545–553, 2008. doi:10.1007/s10439-008-9454-7.

[27]Estes, B. T., J. M. Gimble, and F. Guilak. Mechanical signals as regulators of stem cell fate. *Stem Cells Dev. Dis.* 60:91–126, 2004. doi:10.1016/S0070-2153(04)60004-4.

[28]Fox, S. W., T. J. Chambers, and J. W. M. Chow. Nitric oxide is an early mediator of the increase in bone formation by mechanical stimulation. *Am. J. Physiol. Endocrinol. Metab.* 33(6):E955–E960, 1996.

[29]Fox, S. W., and J. W. M. Chow. Nitric oxide synthase expression in bone cells. *Bone* 23(1):1–6, 1998. doi:10.1016/S8756-3282(98)00070-2.

[30]Gimble, J. M., C. E. Robinson, X. Wu, and K. A. Kelly. The function of adipocytes in the bone marrow stroma: an update. *Bone* 19(5):421–428, 1996. doi:10.1016/S8756-3282(96)00258-X.

[31]Gurkan, U. A., and O. Akkus. An implantable magnetoelastic sensor system for wireless physiological sensing of viscosity. In: *Proceeding of the ASME Summer Bioengineering Conference - 2007*. New York: Amer. Soc. Mechanical Engineers, 2007, pp. 759–760.

[32]Harrelson, J. M., and B. A. Hills. Changes in bone marrow pressure in response to hyperbaric exposure. *Aerospace Med.* 41(9):1018–1021, 1970.

[33]Huggins, C., and B. H. Blocksom. Changes in outlying bone marrow accompanying a local increase of temperature within physiological limits. *J. Exp. Med.* 64(2):253–274, 1936. doi:10.1084/jem.64.2.253.

[34]Huggins, C., B. H. Blocksom, and W. J. Noonan. Temperature conditions in the bone marrow of rabbit, pigeon and albino rat. *Am. J. Physiol.* 115(2):395–401, 1936.

[35]Huggins, C., and W. J. Noonan. An increase in reticuloendothelial cells in outlying bone marrow consequent upon a local increase in temperature. *J. Exp. Med.* 64(2):275–280, 1936. doi:10.1084/jem.64.2.275.

[36]Hung, C. T., S. R. Pollack, T. M. Reilly, and C. T. Brighton. Real-time calcium response of cultured bone-cells to fluid-flow. *Clin. Orthop. Relat. Res.* 313:256–269, 1995.

[37]Jacobs, C. R., C. E. Yellowley, B. R. Davis, Z. Zhou, J. M. Cimbala, and H. J. Donahue. Differential effect of steady versus oscillating flow on bone cells. *J. Biomech.* 31(11):969–976, 1998. doi:10.1016/S0021-9290(98)00114-6.

[38]Johnson, D. L., T. N. McAllister, and J. A. Frangos. Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts. *Am. J. Physiol. Endocrinol. Metab.* 34(1):E205–E208, 1996.

[39]Justesen, J., K. Stenderup, E. N. Ebbesen, L. Mosekilde, T. Steiniche, and M. Kassem. Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. *Biogerontology* 2(3):165–171, 2001. doi:10.1023/A:1011513223894.

[40]Kafka, V. On hydraulic strengthening of bones. *Biorheology* 20(6):789–793, 1983.

[41]Kafka, V. On hydraulic strengthening of bones. *J. Biomech.* 26(6):761–762, 1993. doi:10.1016/0021-9290(93)90038-G.

[42]Khademhosseini, A., R. Langer, J. Borenstein, and J. P. Vacanti. Microscale technologies for tissue engineering and biology. *Proc. Natl. Acad. Sci. USA* 103(8):2480–2487, 2006. doi:10.1073/pnas.0507681102.

[43]Kleinnulend, J., C. M. Semeins, and E. H. Burger. Prostaglandin mediated modulation of transforming growth factor-beta metabolism in primary mouse osteoblastic cells in vitro. *J. Cell. Physiol.* 168(1):1–7, 1996. doi:10.1002/(SICI)1097-4652(199607)168:1<1::AID-JCP1>3.0.CO;2-T.

[44]Kleinnulend, J., A. Vanderplas, C. M. Semeins, N. E. Ajubi, J. A. Frangos, P. J. Nijweide, and E. H. Burger. Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB J.* 9(5):441–445, 1995.

[45]Krolner, B., B. Toft, S. P. Nielsen, and E. Tondevold. Physical exercise as prophylaxis against involutional vertebral bone loss – a controlled trial. *Clin. Sci.* 64(5):541–546, 1983.

[46]Kumar, S., P. R. Davis, and B. Pickles. Bone-marrow pressure and bone strength. *Acta Orthop. Scand.* 50(5): 507–512, 1979.

[47]Kuo, C. K., W. J. Li, R. L. Mauck, and R. S. Tuan. Cartilage tissue engineering: its potential and uses. *Curr. Opin. Rheumatol.* 18(1):64–73, 2006. doi:10.1097/01.bor.0000198005.88568.df.

[48]Kurokouchi, K., C. R. Jacobs, and H. J. Donahue. Oscillating fluid flow inhibits TNF-alpha-induced NF-kappa B activation via an I kappa B kinase pathway in osteoblast-like UMR106 cells. *J. Biol. Chem.* 276(16): 13499–13504, 2001. doi:10.1074/jbc.M003795200.

[49]Larsen, R. M. Intramedullary pressure with particular reference to massive diaphyseal bone necrosis experimental observations. *Ann. Surg.* 108:127–140, 1938. doi:10.1097/00000658-193807000-00009.

[50]Leblanc, A. D., V. S. Schneider, H. J. Evans, D. A. Engelbretson, and J. M. Krebs. Bone-mineral loss and recovery after 17 weeks of bed rest. *J. Bone Miner. Res.* 5(8):843–850, 1990.

[51]Li, Y. J., N. N. Batra, L. D. You, S. C. Meier, I. A. Coe, C. E. Yellowley, and C. R. Jacobs. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. *J. Orthop. Res.* 22(6):1283–1289, 2004. doi:10.1016/j.orthres.2004.04.002.

[52]Li, J. L., D. W. Liu, H. Z. Ke, R. L. Duncan, and C. H. Turner. The P2X(7) nucleotide receptor mediates skeletal mechanotransduction. *J. Biol. Chem.* 280(52):42952–42959, 2005. doi:10.1074/jbc.M506415200.

[53]McAllister, T. N., T. Du, and J. A. Frangos. Fluid shear stress stimulates prostaglandin and nitric oxide release in bone marrow-derived preosteoclast-like cells. *Biochem. Biophys. Res. Commun.* 270(2):643–648, 2000. doi:10.1006/bbrc.2000.2467.

[54]McBeath, R., D. M. Pirone, C. M. Nelson, K. Bhadriraju, and C. S. Chen. Cell shape, cytoskeletal tension, and

RhoA regulate stem cell lineage commitment. *Dev. Cell* 6(4):483–495, 2004. doi:10.1016/S1534-5807(04)00075-9.
[55]Michelsen, K. Pressure relationships in bone marrow vascular bed. *Acta Physiol. Scand.* 71(1):16, 1967.
[56]Michelsen, K. Hemodynamics of the bone marrow circulation. *Acta Physiol. Scand.* 73(3):264–280, 1968.
[57]Miyanishi, K., M. C. D. Trindade, D. P. Lindsey, G. S. Beaupre, D. R. Carter, S. B. Goodman, D. J. Schurman, and R. L. Smith. Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro. *Tissue Eng.* 12(6):1419–1428, 2006. doi:10.1089/ten.2006.12.1419.
[58]Miyanishi, K., M. C. D. Trindade, D. P. Lindsey, G. S. Beaupre, D. R. Carter, S. B. Goodman, D. J. Schurman, and R. L. Smith. Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta 3-induced chondrogenesis by adult human mesenchymal stem cells in vitro. *Tissue Eng.* 12(8):2253–2262, 2006. doi:10.1089/ten.2006.12.2253.
[59]Miyanishi, K., T. Yamamoto, T. Irisa, A. Yamashita, S. Jingushi, Y. Noguchi, and Y. Iwamoto. Bone marrow fat cell enlargement and a rise in intraosseous pressure in steroid-treated rabbits with osteonecrosis. *Bone* 30(1):185–190, 2002. doi:10.1016/S8756-3282(01)00663-9.
[60]Montgomery, R. J., B. D. Sutker, J. T. Bronk, S. R. Smith, and P. J. Kelly. Interstitial fluid-flow in cortical bone. *Microvasc. Res.* 35(3):295–307, 1988. doi:10.1016/0026-2862(88)90084-2.
[61]Mori, T., N. Okimoto, A. Sakai, Y. Okazaki, N. Nakura, T. Notomi, and T. Nakamura. Climbing exercise increases bone mass and trabecular bone turnover through transient regulation of marrow osteogenic and osteoclastogenic potentials in mice. *J. Bone Miner. Res.* 18(11):2002–2009, 2003. doi:10.1359/jbmr.2003.18.11.2002.
[62]Mori, T., N. Okimoto, H. Tsurukami, A. Sakai, Y. Okazaki, S. Uchida, T. Notomi, and T. Nakamura. Effects of tower climbing exercise on bone mass and turnover in growing mice. *J. Bone Miner. Res.* 16(Suppl 1):S362, 2001.
[63]Nagatomi, J., B. P. Arulanandam, D. W. Metzger, A. Meunier, and R. Bizios. Effects of cyclic pressure on bone marrow cell cultures. *J. Biomech. Eng. Trans. ASME* 124(3):308–314, 2002. doi:10.1115/1.1468867.
[64]Nagatomi, J., B. R. Arulanandam, D. W. Metzger, A. Meunier, and R. Bizios. Cyclic pressure affects osteoblast functions pertinent to osteogenesis. *Ann. Biomed. Eng.* 31(8):917–923, 2003. doi:10.1114/1.1590663.
[65]Nakura, N., T. Mori, N. Okimoto, A. Sakai, and T. Nakamura. The climbing exercise prevents trabecular bone loss with highly expressed mRNA of estrogen receptor alpha in bone cells after ovariectomy in mice. *J. Bone Miner. Res.* 20(9):S73, 2005.
[66]Nauman, E. A., R. L. Satcher, T. M. Keaveny, B. P. Halloran, and D. D. Bikle. Osteoblasts in culture respond to in vitro pulsatile fluid flow with short term increases in PGE2 and long term increases in cell proliferation. *J. Bone Miner. Res.* 15(Suppl 1):S500, 2000.
[67]Nauman, E. A., R. L. Satcher, T. M. Keaveny, B. P. Halloran, and D. D. Bikle. Osteoblasts respond to pulsatile fluid flow with shortterm increases in PGE(2) but no change in mineralization. *J. Appl. Physiol.* 90(5):1849–1854, 2001.
[68]Nerlich, M., D. Schumann, R. Kujat, and P. Angele. Mechanobiological conditioning on mesenchymal stem cells during chondrogenesis. *Shock* 21:136, 2004.
[69]Nesic, D., R. Whiteside, M. Brittberg, D. Wendt, I. Martin, and P. Mainil-Varlet. Cartilage tissue engineering for degenerative joint disease. *Adv. Drug Deliv. Rev.* 58(2):300–322, 2006. doi:10.1016/j.addr.2006.01.012.
[70]Ochoa, J. A., A. P. Sanders, D. A. Heck, and B. M. Hillberry. Stiffening of the femoral-head due to intertrabecular fluid and intraosseous pressure. *J. Biomech. Eng. Trans. ASME* 113(3):259–262, 1991. doi:10.1115/1.2894882.
[71]Ochoa, J. A., A. P. Sanders, T. W. Kiesler, D. A. Heck, J. P. Toombs, K. D. Brandt, and B. M. Hillberry. In vivo observations of hydraulic stiffening in the canine femoral head. *J. Biomech. Eng. Trans. ASME* 119(1):103–108, 1997. doi:10.1115/1.2796051.
[72]*Osteoporosis - Fast Facts*. 2006, National Osteoporosis Foundation, http://www.nof.org/osteoporosis/diseasefacts.htm.
[73]Petrakis, N. L. Temperature of human bone marrow. *J. Appl. Physiol.* 4(7):549–553, 1952.
[74]Piekarski, K., and M. Munro. Transport mechanism operating between blood-supply and osteocytes in long bones. *Nature* 269(5623):80–82, 1977. doi:10.1038/269080a0.
[75]Pitsillides, A. A., S. C. F. Rawlinson, R. F. L. Suswillo, G. Zaman, P. I. Nijweide, and L. E. Lanyon. Mechanical strain-induced nitric-oxide production by osteoblasts and osteocytes. *J. Bone Miner. Res.* 10(Suppl 1):S217, 1995.
[76]Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak. Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411):143–147, 1999. doi:10.1126/science.284.5411.143.
[77]Polak, J. M., and A. E. Bishop. Stem cells and tissue engineering: past, present, and future. *Ann. NY Acad. Sci.* 1068:352–366, 2006.
[78]Priller, J. From marrow to brain. In: Adult Stem Cells, edited by K. Turksen. Totowa, NJ: Humana Press Inc., 2004, pp. 215–233.
[79]Qin, Y. X., T. Kaplan, A. Saldanha, and C. Rubin. Fluid pressure gradients, arising from oscillations in intramedullary pressure, is correlated with the formation of bone and inhibition of intracortical porosity. *J. Biomech.* 36(10):1427–1437, 2003. doi:10.1016/S0021-9290(03)00127-1.
[80]Qin, Y. X., W. Lin, and C. Rubin. The pathway of bone fluid flow as defined by in vivo intramedullary pressure and streaming potential measurements. *Ann. Biomed. Eng.* 30(5):693–702, 2002. doi:10.1114/1.1483863.
[81]Raisz, L. G. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J. Clin. Invest.* 115(12):3318–3325, 2005. doi:10.1172/JCI27071.
[82]Reich, K. M., and J. A. Frangos. Effect of flow on prostaglandin-E2 and inositol trisphosphate levels in osteoblasts. *Am. J. Physiol.* 261(3):C428–C432, 1991.
[83]Reich, K. M., T. N. Mcallister, S. Gudi, and J. A. Frangos. Activation of G proteins mediates flow-induced prostaglandin E(2) production in osteoblasts. *Endocrinology* 138(3):1014–1018, 1997. doi:10.1210/en.138.3.1014.
[84]Robling, A. G., A. B. Castillo, and C. H. Turner. Biomechanical and molecular regulation of bone remodeling. *Annu. Rev. Biomed. Eng.* 8:455–498, 2006. doi:10.1146/annurev.bioeng.8.061505.095721.
[85]Rubin, J., D. Biskobing, X. A. Fan, C. Rubin, K. McLeod, and W. R. Taylor. Pressure regulates osteoclast

formation and MCSF expression in marrow culture. *J. Cell. Physiol.* 170(1):81–87, 1997. doi:10.1002/(SICI)1097-4652(199701)170:1<81::AID-JCP9>3.0.CO;2-H.

[86]Rubin, J., C. Rubin, and C. R. Jacobs. Molecular pathways mediating mechanical signaling in bone. *Gene* 367:1–16, 2006. doi:10.1016/j.gene.2005.10.028.

[87]Schumann, D., R. Kujat, M. Nerlich, and P. Angele. Mechanobiological conditioning of stem cells for cartilage tissue engineering. *Biomed. Mater. Eng.* 16(4):S37–S52, 2006.

[88]Semino, C. E. Can we build artificial stem cell compartments? *J. Biomed. Biotechnol.* 2003(3):164–169, 2003. doi:10.1155/S1110724303208019.

[89]Sen, A., M. S. Kallos, and L. A. Behie. Expansion of mammalian neural stem cells in bioreactors: effect of power input and medium viscosity. *Dev. Brain Res.* 134(1–2):103–113, 2002. doi:10.1016/S0165-3806(01)00328-5.

[90]Shaw, N. E. Observations on intramedullary blood-flow and marrow-pressure in bone. *Clin. Sci.* 24(3):311, 1963.

[91]Shaw, N. E. Studies on intramedullary pressure + blood flow in bone. *Am. Heart J.* 68(1):134, 1964. doi:10.1016/0002-8703(64)90250-9.

[92]Shim, S. S., W. Y. Yu, and H. E. Hawk. Relationship between blood-flow and marrow cavity pressure of bone. *Surgery Gynecology and Obstetrics with International Abstracts of Surgery* 135(3):353, 1972.

[93]Sikavitsas, V. I., G. N. Bancroft, H. L. Holtorf, J. A. Jansen, and A. G. Mikos. Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. *Proc. Natl. Acad. Sci. USA* 100(25):14683–14688, 2003. doi:10.1073/pnas.2434367100.

[94]Sikavitsas, V. I., G. N. Bancroft, J. J. Lemoine, M. A. K. Liebschner, M. Dauner, and A. G. Mikos. Flow perfusion enhances the calcified matrix deposition of marrow stromal cells in biodegradable nonwoven fiber mesh scaffolds. *Ann. Biomed. Eng.* 33(1):63–70, 2005. doi:10.1007/s10439-005-8963-x.

[95]Simkin, P. A. Hydraulically loaded the trabeculae may serve as springs within normal femoral head. *Arthritis Rheum.* 50(10):3068–3075, 2004. doi:10.1002/art.20563.

[96]Stein, A. H., H. C. Morgan, and R. F. Porras. The effect of pressor and depressor drugs on intramedullary bone-marrow pressure. *J. Bone Joint Surg. Am.* 40(5):1103–1110, 1958.

[97]Stevens, H. Y., D. R. Meays, and J. A. Frangos. Pressure gradients and transport in the murine femur upon hindlimb suspension. *Bone* 39(3):565–572, 2006. doi:10.1016/j.bone.2006.03.007.

[98]Stevens, H. Y., D. R. Meays, J. Yeh, L. M. Bjursten, and J. A. Frangos. COX-2 is necessary for venous ligation-mediated bone adaptation in mice. *Bone* 38(1):93–104, 2006. doi:10.1016/j.bone.2005.07.006.

[99]Stoltz, J. F., D. Bensoussan, V. Decot, P. Netter, A. Ciree, and P. Gillet. Cell and tissue engineering and clinical applications: an overview. *Biomed. Mater. Eng.* 16(4):S3–S18, 2006.

[100]Tate, M. L. K. "Whither flows the fluid in bone?" An osteocyte's perspective *J. Biomech.* 36(10):1409–1424, 2003. doi:10.1016/S0021-9290(03)00123-4.

[101]Tate, M. L. K., and U. Knothe. An ex vivo model to study transport processes and fluid flow in loaded bone. *J. Biomech.* 33(2):247–254, 2000. doi:10.1016/S0021-9290(99)00143-8.

[102]Tate, M. L. K., P. Niederer, and U. Knothe. In vivo tracer transport through the lacunocanalicular system of rat bone in an environment devoid of mechanical loading. *Bone* 22(2):107–117, 1998. doi:10.1016/S8756-3282(97)00234-2.

[103]Tavassoli, M. Ultrastructural development of bone marrow adipose cell. *Acta Anat. (Basel)* 94(1):65–77, 1976.

[104]Tavassoli, M., and J. M. Yoffey. Bone Marrow, Structure and Function. New York: A.R. Liss, pp. xii, 300, 1983.

[105]Tenenbaum, H. C. Cellular origins and theories of differentiation of bone-forming cells. In: In Bone: The Osteoblast and Osteocyte, edited by B. K. Hall. Boca Raton, FL: CRC Press, 1992, pp. 41–69.

[106]Tettelbaum, S. L., M. M. Tondravi, and F. P. Ross. Osteoclast biology. In: Osteoporosise, edited by S. E. Papapoulos. San Diego, CA: Academic Press, 1996, pp. 61–71.

[107]Thomas, I. H., P. J. Gregg, and D.N. Walder. Intraosseous phlebography and intra-medullary pressure in the rabbit femur. *J. Bone Joint Surg. Br.* 64(2):239–242, 1982.

[108]Tondevold, E., J. Eriksen, and E. Jansen. Observations on long-bone medullary pressure in relation to mean arterial blood-pressure in the anesthetized dog. *Acta Orthop. Scand.* 50(5):527–531, 1979.

[109]Travlos, G. S. Normal structure, function, and histology of the bone marrow. *Toxicol. Pathol.* 34(5):548–565, 2006. doi:10.1080/01926230600939856.

[110]Turner, C. H., V. Anne, and R. M. V. Pidaparti. A uniform strain criterion for trabecular bone adaptation: do continuum-level strain gradients drive adaptation? *J. Biomech.* 30(6):555–563, 1997. doi:10.1016/S0021-9290(97)84505-8.

[111]Turner, C. H., M. R. Forwood, and M. W. Otter. Mechanotransduction in bone – do bone-cells act as sensors of fluid-flow. *FASEB J.* 8(11):875–878, 1994.

[112]Turner, C. H., and A. G. Robling. Mechanisms by which exercise improves bone strength. *J. Bone Miner. Metab.* 23:16–22, 2005.

[113]Turner, C. H., Y. Takano, I. Owan, and G. A. C. Murrell. Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats. *Am. J. Physiol. Endocrinol. Metab.* 33(4):E634–E639, 1996.

[114]van den Dolder, J., G. N. Bancroft, V. I. Sikavitsas, P. H. M. Spauwen, J. A. Jansen, and A. G. Mikos. Flow perfusion culture of marrow stromal osteoblasts in titanium fiber mesh. *J. Biomed. Mater. Res. A* 64(2):235–241, 2003. doi:10.1002/jbm.a.10365.

[115]Verma, S., J. H. Rajaratnam, J. Denton, J. A. Hoyland, and R. J. Byers. Adipocytic proportion of bone marrow is inversely related to bone formation in osteoporosis. *J. Clin. Pathol.* 55(9):693–698, 2002. doi:10.1136/jcp.55.9.693.

[116]Weinbaum, S., S. C. Cowin, and Y. Zeng. A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. *J. Biomech.* 27(3):339–360, 1994. doi:10.1016/0021-9290(94)90010-8.

[117]Weiss, L. Hematopoietic microenvironment of bone-marrow – ultrastructural-study of stroma in rats. *Anat. Rec.* 186(2):161–184, 1976. doi:10.1002/ar.1091860204.

[118]Weiss, L. P., and G. B. Wislocki. Seasonal variations in hematopoiesis in the dermal bones of the 9-banded armadillo. *Anat. Rec.* 126(2):143, 1956. doi:10.1002/ar.1091260203.

[119]Welch, R. D., C. E. Johnston, M. J. Waldron, and B. Poteet. Bone changes associated with intraosseous

hypertension in the caprine tibia. *J. Bone Joint Surg. Am. A* 75(1):53–60, 1993.

[120]White, D. R., H. Q. Woodard, and S. M. Hammond. Average soft-tissue and bone models for use in radiation-dosimetry. *Br. J. Radiol.* 60(717):907–913, 1987.

[121]Wilkes, C. H., and M. B. Visscher. Some physiological aspects of bone-marrow pressure. *J. Bone Joint Surg. Am. A* 57(1):49–57, 1975.

[122]Zerwekh, J. E., L. A. Ruml, F. Gottschalk, and C. Y. C. Pak. The effects of twelve weeks of bed rest on bone histology, biochemical markers of bone turnover, and calcium homeostasis in eleven normal subjects. *J. Bone Miner. Res.* 13(10):1594–1601, 1998. doi:10.1359/jbmr.1998.13.10.1594.

[123]Zhang, P., M. Su, Y. L. Liu, A. Hsu, and H. Yokota. Knee loading dynamically alters intramedullary pressure in mouse femora. *Bone* 40(2):538–543, 2007. doi:10.1016/j.bone.2006.09.018.

[124]Zhang, P., M. Su, S. M. Tanaka, and H. Yokota. Knee loading stimulates cortical bone formation in murine femurs. *BMC Musculoskelet. Disord.* 7, 2006.

[125]Zhang, P., S. M. Tanaka, H. Jiang, M. Su, and H. Yokota. Loading frequency-dependent enhancement of bone formation in mouse tibia with knee-loading modality. *J. Bone Miner. Res.* 20(9):S134–S135, 2005.

TISSUE ENGINEERING: Part A
Volume 00, Number 00, 2010
© Mary Ann Liebert, Inc.
DOI: 10.1089/ten.tea.2009.0565

Original Article

The Sequential Production Profiles of Growth Factors and their Relations to Bone Volume in Ossifying Bone Marrow Explants

Umut Atakan Gurkan, Ph.D.,[1] Joshua Gargac,[2] and Ozan Akkus, Ph.D.[1]

Osteogenesis is a complex process that involves the synergistic contribution of multiple cell types and numerous growth factors (GFs). To develop effective bone tissue engineering strategies employing GFs, it is essential to delineate the complex and interconnected role of GFs in osteogenesis. The studies investigating the temporal involvement of GFs in osteogenesis are limited to *in vitro* studies with single cell types or complex *in vivo* studies. There is a need for platforms that embody the physiological characteristics and the multicellular environment of natural osteogenesis. Marrow tissue houses various cell types that are known to be involved in osteogenesis, and *in vitro* cultures of marrow inherently undergo osteogenesis process. Self-inductive ossification of marrow explants *in vitro* can be employed as a representative multicellular and three-dimensional model of osteogenesis. Therefore, the aims of this study were to employ the rat bone marrow explant ossification model to determine (1) the temporal production profiles of key GFs involved in osteogenesis, (2) the relation between GF production and ossification, and (3) the relations between the GF levels throughout ossification. Temporal production profiles of transforming GF β-1 (TGF-β1), bone morphogenetic protein-2 (BMP-2), vascular endothelial GF (VEGF), and insulin-like GF-1 (IGF-1) and the bone-related proteins alkaline phosphatase and osteocalcin were obtained by enzyme-linked immunosorbent assays conducted at days 2, 7, 12, 14, 19, and 21. The final amount of ossification (ossified volume [OV]) was measured by microcomputed tomography at day 21. TGF-β1, BMP-2, VEGF, IGF-1, alkaline phosphatase, and osteocalcin were produced by the ossifying marrow explants differentially over time. The early production of IGF-1 (day 2) correlated positively ($r = 0.868$) with OV; however, latent production of IGF-1 correlated negatively (day 14: $r = -0.813$; day 19: $r = -0.865$) with OV. OV also correlated with VEGF levels at day 12 ($r = 0.988$) and at day 14 ($r = 0.970$). Production of GFs also correlated to each other across time points, which indicates the complex and interconnected contribution of various GFs in osteogenesis. Therefore, tissue engineering strategies toward bone regeneration should consider the richness of GFs involved in osteogenesis and their dynamically varying participation over time.

Introduction

Osteogenesis is a complex process that involves the contribution of multiple cell types and numerous cytokines or growth factors (GFs). GFs are known to influence cell division, differentiation, and matrix synthesis and to play an important role in bone regeneration, fracture healing, and repair of other musculoskeletal tissues.[1–4] It was suggested that there is a crosstalk between the GF signaling pathways in osteogenesis, and the overall osteogenic outcome may be resulting from the synergistic contribution of numerous GFs.[1–3,5–15] To develop effective bone tissue engineering strategies that can control and modulate bone formation, it is essential to investigate the temporal and interconnected involvement of GFs in osteogenesis.[2,3,6,8,11,12,14,15] However, the studies investigating osteogenesis-related GF expression, production, and secretion are limited to two-dimensional *in vitro* studies with single cell types (i.e., osteoblasts and marrow stromal cells)[14,16–18] or complex *in vivo* studies with associated experimental hurdles.[2,3,19–21] To study the complex and interconnected involvement of multiple GFs, there is a need for a multicellular and three-dimensional *in vitro* platform that embodies the intricate physiology of natural osteogenesis. Bone marrow tissue houses multiple cell types

[1]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, Indiana.
[2]Mechanical Engineering Department, Ohio Northern University, Ada, Ohio.

(i.e., hematopoietic stem cells, mesenchymal stem cells, and accessory cells) that are known to be collaboratively involved in osteogenesis.[22–27] Therefore, bone marrow plays a critical role in bone regeneration[28] and has been shown to have osteogenic potential.[29] Marrow explants inherently ossify *in vitro*[30] without the addition of excipient osteoinductive factors (under basal conditions) and throughout the ossification process, osteoinductive factors are produced by the ossifying marrow tissue.[31] Therefore, *in vitro* bone marrow explant cultures reflect the physiological diversity of bone formation and hold the potential to be used as a platform to study osteogenesis in a more realistic and natural context. This model is conveniently situated between the two-dimensional *in vitro* culture systems employing single cell types and the complex *in vivo* animal models.

The most potent GFs known to be involved in osteogenesis are transforming GF β-1 (TGF-β1), bone morphogenetic proteins (BMPs; 2, 4, and 7), fibroblast growth factor-2, vascular endothelial GF (VEGF), insulin-like GF-1 (IGF-1), and platelet-derived growth factor.[1–4,14,32,33] BMP-2 and −7 have been introduced clinically for treatment of open tibial fractures.[13,34,35] Although there is some appreciation of sequential expression of these potent GFs in fracture healing[2,3,19,20] and in single-type cell culture models,[14,16–18] little is known about their associations with the final amount of bone formation during osteogenesis. Moreover, the knowledge on the relationships between the production levels of GFs during osteogenesis is limited as well. We hypothesized that *in vitro* bone marrow self-inductive ossification model can be used as a platform to delineate the temporal involvement of multiple GFs, and their relations with the ossification level and with each other in osteogenesis. Therefore, the aims of this study were (1) to analyze the temporal production patterns of the key GFs in osteogenesis, TGF-β1, BMP-2, VEGF, and IGF-1, using the inherently ossifying bone marrow explant model, (2) to investigate the relations between the temporal concentrations of GFs and the final ossified volume (OV) of marrow explants, and (3) to investigate relations between the production of GFs, bone-related proteins, and between the GFs themselves throughout the ossification process. These aims were accomplished by (1) measuring the concentrations of GFs in the conditioned medium via quantitative enzyme-linked immunosorbent assay (ELISA) at days 2, 7, 12, 14, 19, and 21, (2) quantifying the end point of ossification (day 21, OV) of marrow explants and correlating OV with temporal concentrations of the GFs, and (3) correlating the concentration levels of the GFs and bone-related proteins to each other at all time points and across time points.

Materials and Methods

Extraction and culture of bone marrow explants

Bone marrow was isolated from the tibiae of 80–90-day-old male Long-Evans rats (Harlan) under Purdue Animal Care and Use Committee approval with a centrifugation-based extraction technique. Briefly, one of the diaphyseal end of the bones was cut with a high-speed circular saw, the medullary components (marrow) were exuded with a brief centrifugation of the cut bone, and the centrifugate was gently pipetted onto the culture inserts (Transwell, Corning) at a 7 μL volume with a low protein binding pipette tip. Bone marrow isolation procedure did not involve dispersion of the marrow contents in a solution, and therefore entailed minimal manipulation and processing of the tissue to preserve the cellular integrity (both adherent and nonadherent) and structural composition of the extracted marrow explant. The growth medium was added underneath the culture insert (below the membrane with 0.4 μm pore size), which resulted in an air–medium interface culture system (Fig. 1). The membrane allowed the attachment and the growth of cells. Explants were cultured under serum-free conditions to identify the baseline ability of marrow explants to ossify. The serum-free growth medium was modified from Lennon *et al.*[36] and composed of 60% Dulbecco's modified Eagle's medium, 40% MCDB-201 supplemented with 1% ITS+1 (Sigma), 50 μg/mL ascorbic acid, 5 mM Na-β-glycerophosphate, 3.5 mg/mL glucose, 40 U/mL penicillin, 40 μg/mL streptomycin, and 1.5 μg/mL Fungizone. No osteoinductive factors (e.g., dexamethasone and BMP-2) were added into the culture medium at any point in time. The explants were cultured for 21 days and the medium was changed on days 2, 5, 7, 10, 12, 14, 17, 19, and 21. Since the inserts carrying the explants were set aside during culture medium change, the cellular and compositional integrity of the marrow explants were maintained, which kept the adherent and nonadherent cells together throughout the culture period (Fig. 1). Due to the small pore size (0.4 μm) of the membrane above which the marrow explants were cultured, the cells were not able to migrate through the pores and hence were contained within the insert. This feature of the culture system kept the bone marrow cells above the membrane and allowed the secreted products to be released

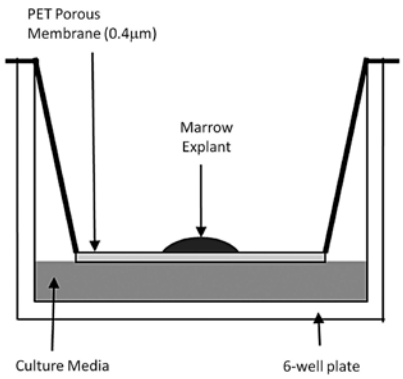


FIG. 1. Cross-sectional view of air–medium interface culture system designed to preserve the adherent and nonadherent cellular composition of marrow tissue throughout the culture period. Marrow explants were placed on PET porous membrane (0.4 μm pore size) of culture inserts and supplemented with a sufficient amount of culture medium underneath the membrane. Culture medium was not added above the membrane, therefore marrow explants were not in direct contact nor were they dispersed in the medium, which prevented the nonadherent marrow cells from being washed away during medium changes.

into the culture medium. The medium conditioned by bone marrow explants was collected from each ossifying explant before each fresh medium addition and stored in sterile low protein binding tubes (LoBind; Eppendorf) separately. The samples were stored at −80°C freezer for ELISA measurements, which were performed at the end of the experiment collectively. Repeated freezing and thawing of the collected conditioned medium was eliminated with appropriate aliquoting.

To test the effect of dispersion or disruption of the cellular integrity of marrow explants on their self-inductive ossification potential, an additional experimental group was included in which marrow explants above the porous membrane were dispersed with the addition of the culture medium inside the culture insert (Fig. 1). In this group, same volume of marrow tissue (7 μL) was utilized in the same culture setting with the culture medium added above the membrane (Fig. 1) submerging and disrupting the marrow explants. This group was similar to the ones used in regular marrow culture studies, in which nonadherent marrow cells are gradually washed away with each medium change, purifying the adherent marrow stromal cells. In this experimental group, the same culture conditions and the same culture medium were used for the same duration as the regular intact marrow explant culture described above. The mineralization of submerged (dispersed) marrow culture samples was evaluated with Raman microspectroscopy and microcomputed tomography. Raman microspectroscopy analysis (LabRam, Horiba Jobin-Yvon) was performed on the randomly selected nodule-like structures (eight samples, six nodule-like structure per sample) observed in the dispersed marrow culture samples. The presence of apatitic mineral peak (located at 959 cm^{-1}) was evaluated by performing a scan in the wavenumber range of 250–1800 cm^{-1}.[37]

Microcomputed tomography of ossifying marrow explants

The ossified marrow samples and the dispersed marrow cultures were fixed with 10% formalin and kept in the fixative before and throughout the scans. The OV of the marrow explants was measured by microcomputed tomography (μCT 40; SCANCO Medical AG) with a 16 μm voxel resolution ($I = 145$ μA, $E = 55$ kVp, integration time = 200 ms). The porous membranes supporting the dispersed marrow cultures were cut out of the culture inserts and scanned with μCT with the same settings. The scanned images were reconstructed and analyzed with commercial software (SCANCO evaluation software), and the segmentation parameters of 0.8 (sigma), 1 (support), and 100 (threshold) were used.[38–40] The total bone volume (mm^3) calculated by software was used and reported as the final OV of the marrow explants.

Quantification of baseline levels of alkaline phosphatase, osteocalcin, BMP-2, IGF-1, VEGF, and TGF-β1 in freshly isolated bone marrow tissue at day 0

Two male Long-Evans rats, 80–90 days old, were euthanized under Purdue Animal Care and Use Committee approval. Bone marrow was removed from the tibiae using a centrifugation-based extraction technique explained in the previous section. Extracted marrow tissue (7 μL) was diluted in tubes that allow minimal protein binding (protein LoBind; Eppendorf) with the same serum-free growth medium described above. Marrow extracts were incubated for 30 min at 37°C to allow the soluble factors to solubilize in the medium. The suspension was then centrifuged for 10 min at 300 *g* to precipitate the cells as a pellet at the bottom of the tube. The supernatant was aspirated and filtered through a 0.2 μm filter using a syringe to remove the remaining cells. The solubilized bone marrow deficient of cells was then aliquoted and stored at −80°C for the quantitative ELISA assays described below. Appropriate conversion of the quantified concentrations of the factors was performed based on the dilution ratios employed.

Histology of ossified marrow explants for matrix typification

At the end of the culture period, the ossified explants were fixed in 10% formalin. Samples were decalcified in formic acid solution (1:1 solution of 50% aqueous formic acid and 20% sodium citrate) for 8–12 h, washed in tap water for 30–45 min, embedded in paraffin, sectioned, and dried overnight in 37°C oven. For all staining procedures, the sections were deparaffinized and hydrated in gradually decreasing percentages of alcohol solutions (100%, 95%, 70%, and water). The sections were stained with safranin-O/fast green for assessing the presence of proteoglycans and hematoxylin and eosin according to standard procedures. Safranin-O/fast green staining is a common method used for staining cartilage–bone interface. Alkaline phosphatase (AP) activity in the histological sections was stained with naphthol AS-MX–based commercial AP staining kit (85L1; Sigma). After staining, the sections were dehydrated, cleared in xylene, and cover-slipped.

Quantification of AP, osteocalcin, BMP-2, IGF-1, VEGF, and TGF-β1

The quantification of these factors was performed on the medium conditioned by ossifying explants (days 2, 7, 12, 14, 19, and 21) and the fresh marrow tissue (day 0). AP level was measured with the colorimetric *p*-nitrophenyl phosphate substrate AP assay kit (SensoLyte™; Anaspec Corp.). Fifty microliters of samples and standards was added to each well of a 96-well plate. Fifty microliters of *p*-nitrophenyl phosphate reaction mixture was added to each well and incubated 2–3 h until color developed. Optical density was determined using a microplate reader (Spectramax M5; Molecular Devices) set to 405 nm. Absorbance values were converted to AP concentration with the utilization of the calibration curve. Osteocalcin (OC) levels were measured using a Rat Osteocalcin EIA kit (Biomedical Technologies). One hundred microliters of samples and standards were added to a 96-well plate precoated with OC capture antibody, incubated for 20 h at 4°C, and washed three times with phosphate saline wash buffer, and 100 μL of OC antiserum was added to each well and incubated at 37°C for 1 h. After another set of washes, 100 μL of diluted donkey anti-goat IgG peroxidase was added to each well, incubated for 1 h at room temperature, and rerinsed, and then 100 μL of substrate mix (1:1 of hydrogen peroxide solution and tetramethyl benzidine) was added and incubated at room temperature for 30 min, avoiding direct light. One hundred microliters of

stop solution was added to each well, and the absorbance was measured using a microplate reader set at 450 nm with a wavelength correction set at 540 nm. The concentrations of BMP-2, VEGF, TGF-β1, and IGF-1 in the conditioned medium were measured by quantitative ELISA development kits (BMP-2: PeproTech; VEGF, IGF-1, and TGF-β1: R&D Systems). Briefly, 96-well microplates (MaxiSorp; Nalge) were coated with capture antibody, the wells were blocked for at least 1 h, and 100 μL of samples or standards was added to wells followed by incubation for 2–3 h at room temperature. After thorough washing, detection antibody was added at the specified concentration for each kit and incubated for 2 h at room temperature. The peroxidase substrate solution was added (protected from direct light) and incubated at room temperature for 20 min, and the enzyme reaction was stopped with 2 N HCl solution. The color product was detected by a microplate reader set at 450 nm with wavelength correction set at 540 nm. Quantification was also carried out on the nonconditioned serum-free medium to determine baseline levels of the GFs, OC, and AP. TGF-β1 in the samples was activated to its immunoreactive form using 1 N HCl followed by the addition of 1.2 N NaOH/0.5 M HEPES before being used in ELISA.

Statistical analysis

The measured concentration profiles were analyzed statistically with Kruskal–Wallis one-way analysis of variance followed by a *post hoc* Mann–Whitney *U*-test with Bonferroni correction for multiple comparisons. Statistical significance threshold was set at $p < 0.05$, and the p-value obtained for each test was adjusted based on the number of comparisons according to Bonferroni correction (p-value obtained from the test multiplied by number of comparisons). Error bars in the figures were displayed as standard error. Relations between the concentrations and the final OV and between the measured concentrations themselves were analyzed by calculating the Pearson product moment correlation coefficient. The statistical significance between the day 0 baseline concentrations ($n = 10$–12) of the bone-related proteins and GFs and their day 2 levels ($n = 6$) produced by the ossifying explants was tested with a Mann–Whitney *U*-test with the significance threshold set at $p < 0.05$.

Results

Bone marrow explants inherently ossified without the addition of any osteoinductive factors

Bone marrow explants cultured under serum-free conditions without any osteoinductive factors (dexamethasone, BMP-2, etc.) inherently ossified to form a matrix that was visible through low magnification light microscopy (Fig. 2A). μCT scans of ossified explants revealed a plate-like ossified structure (Fig. 2B). The absence of orange-red stain in safranin-o/fast green–stained sections was an indication of proteoglycan deficiency, indicating the absence of cartilaginous matrix (Fig. 2C). Hematoxylin and eosin staining displayed viable cells embedded in the matrix (Fig. 2D). Naphthol AS-MX–based AP staining indicated that AP activity (dark purple-red regions, Fig. 2E) was concentrated in the lower and the upper surfaces of the ossified matrix, indicating the locations of the actively ossifying regions in the marrow explant. However, when the marrow explants were dispersed and submerged in the culture medium, which gradually eliminated the nonadherent cell population with each medium change, no indication of mineralization was observed as per Raman microspectroscopy (absence of apatitic mineral peak) and μCT (no detectable mineralized volume).

AP and OC were produced by in vitro ossifying marrow explants

The baseline levels of AP and OC in day 0 marrow tissue were quantified as 14.8 ng/mL (SD: 3.8 ng/mL) and 514 pg/mL (SD: 212 pg/mL), respectively (Fig. 3). Both of these baseline concentrations were significantly lower than the day 2 production levels of these bone-related proteins by *in vitro* ossifying marrow explants as seen in Figure 3. AP concentration measured in the conditioned medium displayed a high level at the beginning of the culture period and decreased significantly at day 7 and further decreased after day 12 (Fig. 3A). The decrease in AP concentration was significant by day 21 relative to day 12. OC concentration profile displayed a similar pattern at the beginning of the culture period, which was significantly greater on day 2 than all the following time points (Fig. 3B). A significant decrease in OC concentration was observed on day 7. There was no statistically significant decrease in OC concentration between day 12 and day 21.

Osteoinductive GFs (BMP-2, IGF-1, TGF-β1, and VEGF) were produced by ossifying marrow explants

The baseline levels of BMP-2, IGF-1, TGF-β1, and VEGF in day 0 marrow tissue were significantly lower than the day 2 production levels of these factors by *in vitro* ossifying marrow explants (Fig. 4). BMP-2 concentration in the conditioned medium was at a high level early on at days 2 and 7 (Fig. 4A). BMP-2 concentration decreased significantly by day 12 and displayed further significant decrease on days 19 and 21 (Fig. 4A). IGF-1 concentration was significantly higher on day 2 than the later time points, which decreased significantly and stayed around 50 pg/mL between days 7 and 14 (Fig. 4B). A significant increase in IGF-1 concentration was observed in the later stage, starting day 19 and beyond. TGF-β1 concentration displayed a significantly high level on days 2, 7, and 12 than all of the subsequent time points (Fig. 4C). TGF-β1 concentration decreased significantly by day 14 and stayed constant around 50 pg/mL till the end of the culture period. Similarly, VEGF concentration was significantly higher at the early and mid phase of the culture period (days 2, 7, 12, and 14) than all the following time points (Fig. 4D). VEGF concentration displayed a significant steady decrease during the entire culture period.

Levels of OC, AP, BMP-2, IGF-1, TGF-β1, VEGF, and final OV display significant correlations at specific time points

The rightmost column of Table 1 displays the correlation between the levels of measured GFs and the bone markers (OC and AP) at different time points and the final OV measured at day 21. VEGF concentration in the conditioned medium displayed a high correlation (Table 1) with the final OV on days 12 (0.988) and 14 (0.970). IGF-1 concentration

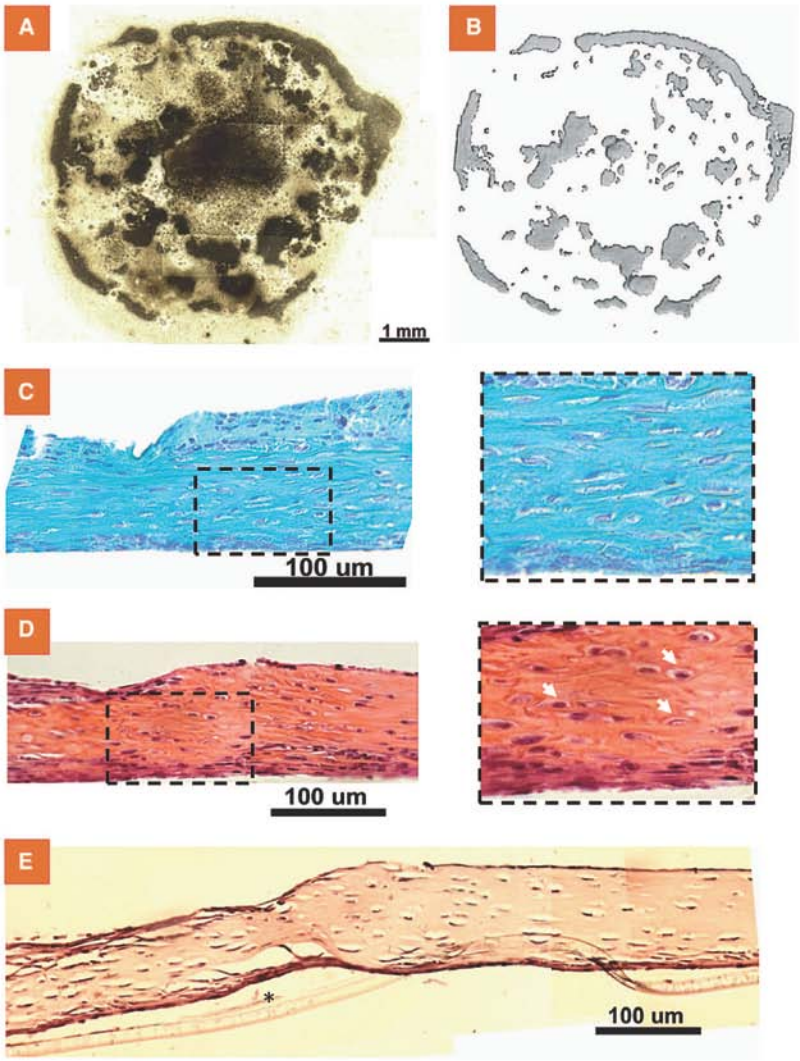


FIG. 2. The appearance of a marrow explant cultured for 21 days. (**A**) Light microscope image of the ossified explant. (**B**) The reconstructed three-dimensional view of the ossified regions of the explant from microcomputed tomography scan indicates a plate-like ossified structure. (**C**) Safranin-O/fast green–stained histological section image of an ossified region in the marrow explant and the magnified inset. The absence of orange-red stain indicates that the extracellular matrix does not contain proteoglycans, and thus it is not a cartilaginous matrix. (**D**) Hematoxylin and eosin–stained histological section image and the magnified inset, displaying viable osteocyte-like cells (arrows). (**E**) Alkaline phosphatase (AP)–stained (dark purple-red regions; counter stain, hematoxylin) histological section image. AP activity is observed in the upper and lower sections of the ossified plate-like structures. The membrane (*) is visible in the image, which lines the bottom of the ossified matrix. Color images available online at www.liebertonline.com/ten.

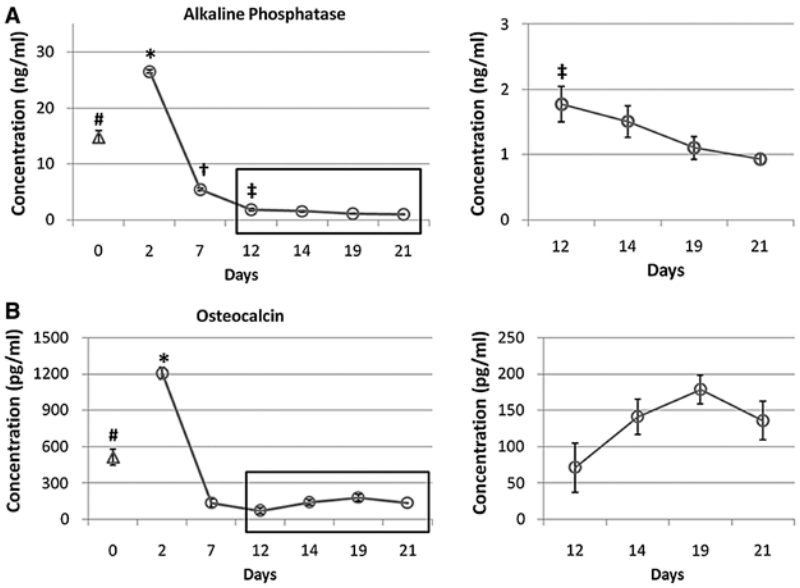


FIG. 3. Concentration profiles of AP and osteocalcin measured in the conditioned medium ($n = 6$ per time-point). A close-up view of days 12–21 highlighted by insets is displayed on the right. The triangular marker at day 0 indicates the baseline concentration in bone marrow tissue at day 0 ($^{\#}p < 0.05$ between the baseline concentration in bone marrow tissue at day 0 and expression of that factor on day 2). (**A**) AP concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus day 21. (**B**) Osteocalcin concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21.

in the conditioned medium early on in the culture (day 2) correlated positively with OV (0.868; Table 1), whereas latent IGF-1 concentration correlated negatively on days 14 (−0.813) and 19 (−0.865) with OV. OC level displayed a high negative correlation with OV on day 19 (−0.931; Table 1).

Discussion

The importance of the GFs included in this study (BMP-2, VEGF, TGF-β1, OC, and IGF-1) and their synergistic combinatorial role on bone regeneration is widely accepted.[1–3,14,16,17,19–21,32,34,41,42] On the other hand, data on sequential expression of GFs in osteogenesis are either limited to two-dimensional single-type cell culture studies[14,16,17] or complex animal models of fracture healing.[2,3,19–21] The current model of marrow explant cultures is situated in between single-type cell culture studies and animal models and present several unique advantages. First, it encompasses multiple cell types (adherent and nonadherent, hematopoietic, and mesenchymal stem cells). Second, it displays a natural osteogenic potential (under serum-free conditions without any excipient osteoinductive factors), which results in a significant volume of bone formation. Third, it presents a platform in which the protein production can be quantified

FIG. 4. Concentration profiles of potent osteoinductive factors (bone morphogenetic protein-2 [BMP-2], insulin-like growth factor-1 (IGF-1), transforming growth factor β-1 [TGF-β1], and vascular endothelial growth factor [VEGF]) measured in the conditioned medium ($n = 6$ per time-point). A close-up view of days 12–21 highlighted by insets is displayed on the right. The triangular marker at day 0 indicates the baseline concentration in bone marrow tissue at day 0 ($^{\#}p < 0.05$ between the baseline concentration in bone marrow tissue at day 0 and expression of that factor on day 2). (**A**) Bone morphogenetic protein-2 concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus days 19 and 21; $^{¥}p < 0.05$ for day 14 versus day 21. (**B**) Insulin-like growth factor-1 concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 12 versus days 19 and 21, and for day 14 versus days 19 and 21. (**C**) Transforming growth factor β-1 concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus days 14, 19, and 21. (**D**) Vascular endothelial growth factor concentration profile in the conditioned medium; $^{*}p < 0.05$ for day 2 versus days 7, 12, 14, 19, and 21; $^{\dagger}p < 0.05$ for day 7 versus days 12, 14, 19, and 21; $^{\ddagger}p < 0.05$ for day 12 versus days 14, 19, and 21; $^{¥}p < 0.05$ for day 14 versus days 19 and 21.

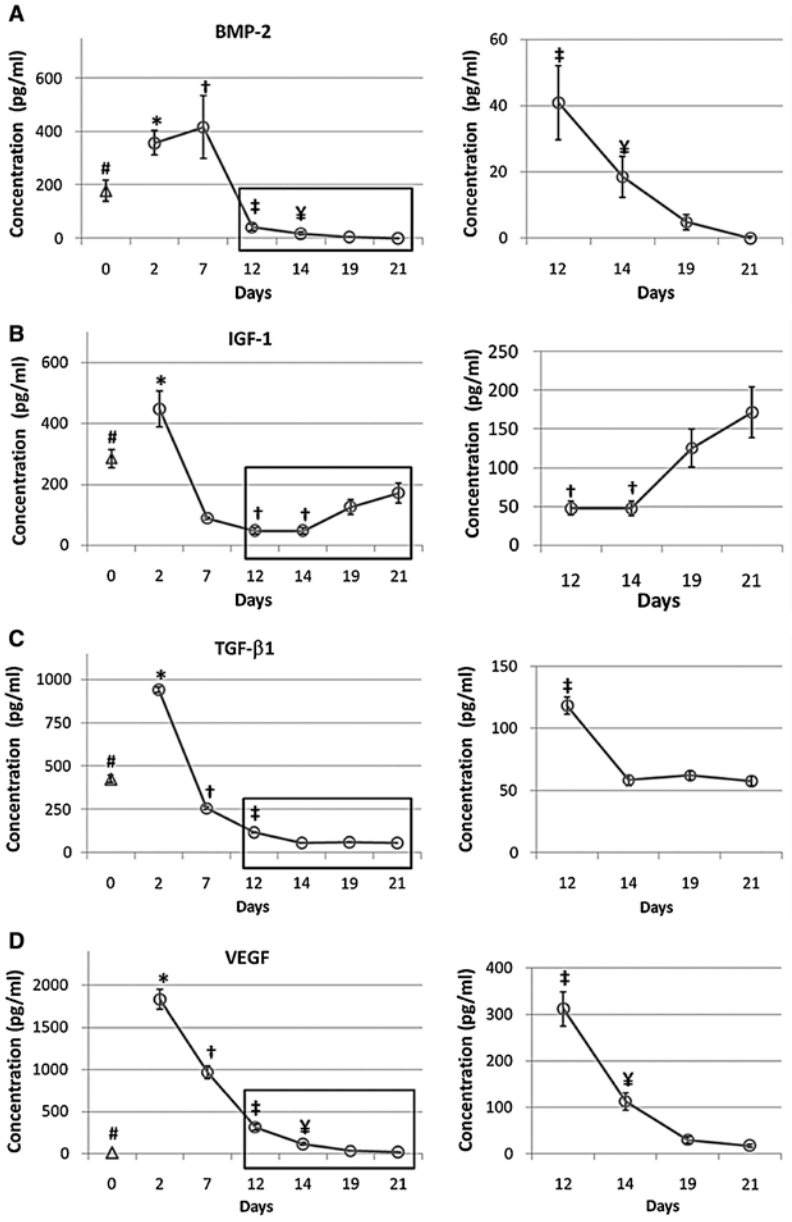

A
BMP-2
Concentration (pg/ml)
Days
B
IGF-1
C
TGF-β1
D
VEGF

TABLE 1. CORRELATION BETWEEN THE MEASURED CONCENTRATIONS ACROSS TIME POINTS AND THE FINAL OSSIFIED VOLUME OF THE MARROW EXPLANTS

Days		*2*		*7*				*12*			*14*			*19*			*21*				*Ossified volume*
	Factors	BMP-2	VEGF	BMP-2	VEGF	TGF-β1	OC	BMP-2	VEGF	IGF-1	BMP-2	IGF-1	OC	VEGF	IGF-1	OC	BMP-2	VEGF	IGF-1	OC	*(day 21)*
2	BMP-2		0.893				0.986					0.864	0.896			0.872					
	VEGF	0.893		0.877		0.923	0.866	0.888			0.817										
	IGF-1			-0.833					0.918							-0.914					0.868
	OC	0.845	0.977	0.847		0.961												0.848			
	AP	-0.817					-0.861	-0.814		-0.948	-0.841	-0.931	-0.895		-0.859	-0.910					
7	BMP-2						0.957									0.830					
	OC			0.957				0.945			0.926	0.856									
	AP																			-0.814	
12	BMP-2											0.897	0.944			0.831					
	VEGF											-0.829			-0.874	-0.942					0.988
	IGF-1												0.896			0.837					
	OC																				
	AP																			-0.922	
14	BMP-2											0.946	0.977		0.847	0.870					
	VEGF														-0.845	-0.890					0.970
	IGF-1										0.946					0.906					-0.813
	OC										0.977			-0.890							
	AP																			-0.828	
19	BMP-2																	0.812			
	VEGF																0.842				
	IGF-1																				-0.865
	OC																				-0.931
	AP																			-0.957	

Pearson product moment correlation coefficient is presented in the cells (all the presented correlations are significant with $p < 0.05$). The cells corresponding to the correlations between the same factor at the same time points and reverse correlations in time were shaded due to the lack of physical significance. BMP-2, bone morphogenetic protein-2; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; TGF-β1, transforming growth factor beta 1; AP, alkaline phosphatase; OC, osteocalcin.

via the conditioned medium. In the future, this model would also allow interrogation with silencing-RNA or gene transfection to control GF production to assess their ultimate effect on the amount of bone formation. Therefore, *in vitro* bone marrow explant culture model presented here is a useful model for studying the temporal production profiles of the osteogenic factors and their synergistic combinatorial roles on bone generation/regeneration process.

The importance of preserving the cellular integrity of marrow tissue in terms of osteogenic potential was verified by including an experimental group in which marrow explants were dispersed in the culture medium. This condition allowed the gradual elimination of nonadherent cells (i.e., hematopoietic cells) with each medium change and left the adherent cells (i.e., marrow stromal cells) in the culture. The absence of mineralization (as per Raman microspectroscopy and μCT) in this group indicated that the multicellular nature of marrow tissue needs to be preserved to achieve self-inductive ossification. On the other hand, bone marrow explants whose integrity was maintained ossified to varying extents. Therefore, the final OV of the marrow explants was used as an indicator of the osteogenic capacity of individual samples, which may be directly related to levels of osteoinductive factors produced. The varying ossification levels of the samples allowed us to evaluate the correlation between the final OV and the concentration levels of individual factors at different time points (Table 1). To the best of our knowledge, the correlation between the levels of osteoinductive factors at different time points, across time points, and the final OV in an *in vitro* ossification model is being reported for the first time in this study. This information can be used to determine the most critical osteoinductive factors, as well as the most critical time of application of these factors to obtain a more efficient and natural ossification mechanism. In addition, the correlation between the levels of factors at different time points and across time points can be used to delineate the synergistic involvement of multiple osteoinductive factors in the ossification process.

GFs present in the serum that is commonly used as a supplement in *in vitro* cell culture studies confound the picture and make it hard to study the involvement of GFs in various processes such as osteogenesis. Therefore, we confirmed the ability of marrow explants to ossify in serum-free culture conditions and without application of any excipient osteoinductive factors. This property facilitates carrying out more comprehensive *in vitro* analysis of various proteins involved in osteogenesis without the inhibiting and/or interfering effects of serum supplements.

The histochemical analysis on the *in vitro* ossified marrow explants with safranin-o/fast green staining displayed (Fig. 2C) the absence of proteoglycans, which can be found abundantly in cartilaginous matrix. In addition, the presence of AP activity on the lower and upper surfaces of the ossifying explants indicated the presence of differentiated osteoblast-like cells forming a mineralized matrix. Therefore, the absence of a cartilaginous matrix and the presence of AP activity indicate that the ossification mechanism in this *in vitro* bone marrow culture model resembles the intramembranous ossification mechanism.

The baseline levels of AP, OC, BMP-2, IGF-1, TGF-β1, and VEGF in day 0 marrow tissue have been quantified, and all of them have been observed to be significantly lower than the day 2 production levels of these proteins by ossifying marrow explants (Figs. 3 and 4, triangular markers at day 0). Therefore, the presented concentration profiles of these quantified proteins could not be related merely to the baseline concentrations in fresh marrow tissue.

AP and OC are commonly accepted bone markers.[18] AP is expressed by many cell types to some extent. AP is also associated with osteoblast differentiation and its production is high in preosteoblasts and osteoblasts.[43] Using AP as an ossification marker with a heterogeneous population of marrow cells is complicated since only a small population of the cells in marrow stroma are AP-positive osteoblast precursors and many other cell types in marrow express AP, such as adipocytic cells.[44] The marrow explants employed in this study were handled minimally, which preserved the natural components together, including all the resident cell types, the extracellular matrix, as well as the soluble proteins and factors. Therefore, the early high production of AP in this study (Fig. 3A) may be attributed to the other cell types in the marrow tissue that are in high concentration, such as adipocytes. Due to the difficulties with quantifying ossification-related AP activity, a secondary ossification marker (OC) was used to assess ossification. OC is a specific marker of mature osteoblast phenotype.[18] Previously, OC level was observed to be increasing after about 10 days with *in vitro* mineralization models that employed marrow cells.[14,18] A peak in OC level around day 14 was also reported when the marrow stromal cells were stimulated with dexamethasone and $1,25(OH)_2D_3$.[18] However, the high OC level observed early on in this study (Fig. 3B) is not in agreement with the previous findings. At the earlier stage of the marrow explant culture, the high level of OC at day 2 decreased dramatically by day 7 and did not increase significantly between day 12 and day 21 (Fig. 3B). This observation may be attributed to the complex nature of the current model due to the presence of multiple cell types.

In vivo studies indicate that BMP-2 expression in the fracture site displays an early increase during the phase in which mesenchymal stem cells are recruited to the injury site.[2,4,45] The high level of BMP-2 production in the early phase of fracture healing is followed by a decrease as it was also observed in this study (Fig. 4A). A similar trend, in which upregulation of BMP-2 around day 4 followed by downregulation around day 12 was previously observed during mineralization of osteoprogenitors *in vitro*.[14] BMP-2 level measured at day 14 highly correlated with the IGF-1 level on day 14 (Table 1; Fig. 4C), which may be an indication of interaction or a similarity in terms of source cells for these two factors at this phase. Similarly, it was previously shown that early application of BMP-2 (day 1) followed by later application of combination of BMP-2 and IGF-1 (after day 5) resulted in the highest amount of cell number and AP activity in pluripotent C3H10T1/2 cells.[15] The high positive correlation between the OC level and the BMP-2 concentration at days 14 and 19 may be an indication on the role of BMP-2 in mature osteoblast function in mineralization. On the other hand, BMP-2 production on day 2 was observed to correlate negatively with AP level on day 2 (Table 1). This could be due to the early high levels of AP, which suppressed the production of BMP-2, which later peaked at day 7 when AP level decreased significantly. The

early level of BMP-2 (days 2–7) also correlated with later levels of OC (at days 7, 14, and 19) and IGF-1 (at day 14). This observation suggests that an early involvement of BMP-2 has repercussions on the latent stages of osteogenesis. The early involvement of BMP-2 in osteogenesis has recently been shown in sequential growth factor delivery studies with BMP-2 and BMP-7.[46,47] In these studies, early release of BMP-2 and latent release of BMP-7 were achieved with nanocapsules in an *in vitro* study with MSCs. Superior osteoinductive effects of the sequential application of BMP-2 and BMP-7 were demonstrated over individual and simultaneous applications based on elevated AP activity on days 14 and 21.

IGF-1 production by *in vitro* ossifying marrow explants in this study was bimodal. It displayed a high level at the early stage, followed by a decrease during days 7–14, and then a latent increase during days 19–21 (Fig. 4B). A similar pattern of IGF-1 production in the later stage was observed in fracture healing in an *in vivo* model by Wildemann *et al.*[33] As the earliest time point included in the study by Wildemann *et al.* was day 5, it is not possible to compare the early level of IGF-1 to that was observed in our study. However, in an *in vitro* mineralization model utilizing osteoprogenitor cells, a high level of IGF-1 was observed early on, followed by a decrease between days 5–12, and then followed by an increase starting day 13.[14] Therefore, the results presented in this study in terms of IGF-1 concentration profile agree with the previous findings in the literature. The high IGF-1 level at day 2 was observed to correlate positively with OV (Table 1), which may be an indication that early application of IGF-1 is critical in bone regeneration. On the other hand, we observed that IGF-1 levels at later stages (days 14–19) correlated negatively with OV (Table 1), which may be an indication that IGF-1 application in the later stages may have a deterring role in ossification. IGF-1 concentration was also observed to correlate with OC level positively on days 14 and 19 (Table 1). When this information is combined with the findings presented above (IGF-1 correlated negatively with OV on days 14–19) and the fact that OC correlated negatively with OV on day 19 (Table 1), it is reasonable to suggest a connection between IGF-1 and OC in curbing further ossification of the marrow explants starting day 14 and allowing the mineralization to reach a steady state. In attestation, OC is known to curb mineralization[48] and current results imply that IGF-1 may be associated in this pathway.

Early involvement of TGF-β1 during the proliferation phase in fracture-healing process has been shown previously.[2–4,33] Similarly, TGF-β1 was observed at a high level early on in the marrow explant culture model and decreased continually to reach a steady-state level by day 14 (Fig. 4C). Therefore, TGF-β1 production profile presented here agrees with the previous findings. However, TGF-β1 levels at various time points displayed a limited number of correlations with other factors. The only TGF-β1 level that has displayed a significant correlation was day 7 level, which correlated positively with day 2 levels of VEGF and OC. Therefore, a link between VEGF and TGF-β1 involvement could be present between days 2 and 7 during ossification.

VEGF is considered to play its most important role in fracture healing in the earlier stages.[49] Therefore, the high concentration of VEGF observed in this study during day 2, which gradually decreased starting day 7 up to day 21 (Fig. 4D), agrees with the previous findings. A similar trend of VEGF expression during mineralization by osteoprogenitors was also observed in an *in vitro* study.[14] Even though VEGF concentration was high at the earlier stage of ossification and decreased gradually till day 21 (Fig. 4D), VEGF concentration and OV was observed to highly correlate only during days 12–14 (Table 1). This high correlation between VEGF concentration and OV indicates that the presence of VEGF during days 12–14 was associated with final amount of ossification. Importance of VEGF at the mid-phase of fracture repair was also shown and emphasized before.[4]

The correlations that were reported between the GF levels and the final OV are useful for identifying the temporal involvement of the GFs in osteogenesis. However, these correlations do not necessarily imply causations. The correlations observed in this study require further analysis with studies employing targeted inhibition of GFs at specific time points to assess the overall effect in the ossification of marrow tissue.

There are many GFs involved in osteogenesis, and they are not limited to the four factors (BMP-2, VEGF, IGF-1, and TGF-β1) studied here. Other factors that are known to be actively involved in osteogenesis are BMPs (4, 6, 7, and 13), fibroblast growth factor-2, Wnt, growth/differentiation factor-5, and platelet-derived growth factor.[14,21,50–54] A detailed characterization of the involvement of many GFs and signaling molecules is necessary, and proteomic analysis could be used for high-throughput screening of all the proteins. Therefore, we are currently conducting experiments to analyze a myriad of proteins and their temporal expression profiles during inherent *in vitro* ossification of bone marrow explants.

Conclusions

It was shown that BMP-2, IGF-1, TGF-β1, and VEGF are expressed differentially over time by the ossifying marrow explants and the concentration of IGF-1 and VEGF correlate at different time points with the final OV. IGF-1 has dichotomous effect on the final OV, which is indicated by a positive correlation on day 2 and negative correlations on days 14 and 19. The GF levels and the production of bone markers (AP and OC) have been shown to be highly interdependent due to correlations to each other at same time points as well as across various time points. The results presented in this study provide a more robust understanding of the osteogenesis process in terms of the involvement of BMP-2, IGF-1, TGF-β1, VEGF, AP, and OC in marrow explants and the secretion sequence and the amounts of key osteoinductive factors involved in this osteogenesis model. The information obtained from the marrow ossification model can be used to develop multifactor and multiphase GF delivery strategies for fracture healing and bone tissue engineering applications.

Acknowledgments

This study was supported by a grant from the Musculoskeletal Transplant Foundation. We would like to thank Purdue University Histopathology service laboratory (David VanSickle, Ph.D., D.V.M.; Paul Snyder, Ph.D., D.V.M.; Sharon Evander; Carol Bain; Tracy Wiegandt) for their support in histological work and Pam Lachick for her

support with the μCT system. We would also like to acknowledge the Summer Undergraduate Research Fellowships program at Purdue University for supporting this study.

Disclosure Statement

No competing financial interests exist.

References

1. Lieberman, J.R., Daluiski, A., and Einhorn, T.A. The role of growth factors in the repair of bone—Biology and clinical applications. J Bone Joint Surg Am **84A,** 1032, 2002.
2. Cho, T.J., Gerstenfeld, L.C., and Einhorn, T.A. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res **17,** 513, 2002.
3. Tatsuyama, K., Maezawa, Y., Baba, H., Imamura, Y., and Fukuda, M. Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone. Eur J Histochem **44,** 269, 2000.
4. Gerstenfeld, L.C., Cullinane, D.M., Barnes, G.L., Graves, D.T., and Einhorn, T.A. Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation. J Cell Biochem **88,** 873, 2003.
5. Wozney, J.M., and Rosen, V. Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin Orthop Relat Res **242,** 1528, 1988.
6. Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M., and Wang, E.A. Novel regulators of bone-formation—molecular clones and activities. Science **242,** 1528, 1988.
7. Bessho, K., Kusumoto, K., Fujimura, K., Konishi, Y., Ogawa, Y., Tani, Y., and Iizuka, T. Comparison of recombinant and purified human bone morphogenetic protein. Br J Oral Maxillofac Surg **37,** 2, 1999.
8. Balemans, W., and Van Hul, W. Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Dev Biol **250,** 231, 2002.
9. Maegawa, N., Kawamura, K., Hirose, M., Yajima, H., Takakura, Y., and Ohgushi, H. Enhancement of osteoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2). J Tissue Eng Regen Med **1,** 306, 2007.
10. Farhadi, J., Jaquiery, C., Barbero, A., Jakob, M., Schaeren, S., Pierer, G., Heberer, M., and Martin, I. Differentiation-dependent up-regulation of BMP-2, TGF-beta1, and VEGF expression by FGF-2 in human bone marrow stromal cells. Plast Reconstr Surg **116,** 1379, 2005.
11. Peng, H.R., Wright, V., Usas, A., Gearhart, B., Shen, H.C., Cummins, J., and Huard, J. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. J Clin Invest **110,** 751, 2002.
12. Schmidmaier, G., Wildemann, B., Gabelein, T., Heeger, J., Kandziora, F., Haas, N.P., and Raschke, M. Synergistic effect of IGF-I and TGF-beta 1 on fracture healing in rats—single versus combined application of IGF-I and TGF-beta 1. Acta Orthop Scand **74,** 604, 2003.
13. Gautschi, O.P., Frey, S.P., and Zellweger, R. Bone morphogenetic proteins in clinical applications. Anz J Surg **77,** 626, 2007.
14. Huang, Z.N., Nelson, E.R., Smith, R.L., and Goodman, S.B. The sequential expression profiles of growth factors from osteroprogenitors to osteoblasts *In vitro.* Tissue Eng **13,** 2311, 2007.
15. Raiche, A.T., and Puleo, D.A. *In vitro* effects of combined and sequential delivery of two bone growth factors. Biomaterials **25,** 677, 2004.
16. Robey, P.G., Young, M.F., Flanders, K.C., Roche, N.S., Kondaiah, P., Reddi, A.H., Termine, J.D., Sporn, M.B., and Roberts, A.B. Osteoblasts synthesize and respond to transforming growth factor-type-beta (TGF-beta) *in vitro.* J Cell Biol **105,** 457, 1987.
17. Kassem, M., Kveiborg, M., and Eriksen, E.F. Production and action of transforming growth factor-beta in human osteoblast cultures: dependence on cell differentiation and modulation by calcitriol. Eur J Clin Invest **30,** 429, 2000.
18. Malaval, L., Modrowski, D., Gupta, A.K., and Aubin, J.E. Cellular expression of bone-related proteins during *in-vitro* osteogenesis in rat bone-marrow stromal cell-cultures. J Cell Physiol **158,** 555, 1994.
19. Le, A.X., Miclau, T., Hu, D., and Helms, J.A. Molecular aspects of healing in stabilized and non-stabilized fractures. J Orthop Res **19,** 78, 2001.
20. Andrew, J.G., Hoyland, J., Freemont, A.J., and Marsh, D. Insulin-Like Growth-Factor Gene-Expression in Human Fracture Callus. Calcified Tissue International **53,** 97, 1993.
21. Kugimiya, F., Kawaguchi, H., Kamekura, S., Chikuda, H., Ohba, S., Yano, F., Ogata, N., Katagiri, T., Harada, Y., Azuma, Y., Nakamura, K., and Chung, U.G. Involvement of endogenous bone morphogenetic protein (BMP)2 and BMP6 in bone formation. J Biol Chem **280,** 35704, 2005.
22. Cabrita, G.J.M., Ferreira, B.S., da Silva, C.L., Goncalves, R., Almeida-Porada, G., and Cabral, J.M.S. Hematopoietic stem cells: from the bone to the bioreactor. Trends Biotechnol **21,** 233, 2003.
23. Dennis, J.E., and Caplan, A.I. Bone marrow mesenchymal stem cells. In: Sell, S., ed. Stem Cells Handbook. Totowa, NJ: Humana Press, Inc., 2003, pp. 107–118.
24. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143, 1999.
25. Muschler, G.F., Boehm, C., and Easley, K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. J Bone Joint Surg Am **79A,** 1699, 1997.
26. Eipers, P.G., Kale, S., Taichman, R.S., Pipia, G.G., Swords, N.A., Mann, K.G., and Long, M.W. Bone marrow accessory cells regulate human bone precursor cell development. Exp Hematol **28,** 815, 2000.
27. Jung, Y.G., Song, J.H., Shiozawa, Y., Wang, J.C., Wang, Z., Williams, B., Havens, A., Schneider, A., Ge, C.X., Franceschi, R.T., McCauley, L.K., Krebsbach, P.H., and Taichman, R.S. Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche. Stem Cells **26,** 2042, 2008.
28. Devine, M.J., Mierisch, C.M., Jang, E., Anderson, P.C., and Balian, G. Transplanted bone marrow cells localize to fracture callus in a mouse model. J Orthop Res **20,** 1232, 2002.
29. Muschler, G.F., Nitto, H., Boehm, C.A., and Easley, K.A. Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. J Orthop Res **19,** 117, 2001.

30. Luria, E.A., Owen, M.E., Friedenstein, A.J., Morris, J.F., and Kuznetsow, S.A. Bone-formation in organ-cultures of bone-marrow. Cell Tissue Res **248,** 449, 1987.
31. Gurkan, U.A., and Akkus, O. The osteoinductive potential of bone marrow conditioned media is superior to dexamethasone and rhBMP-2. Orthopedic Research Society 55th Annual Meeting, Las Vegas, NV, 2009.
32. Phillips, A.M. Overview of the fracture healing cascade. Injury **36 Suppl 3,** S5, 2005.
33. Wildemann, B., Schmidmaier, G., Brenner, N., Huning, M., Stange, R., Haas, N.P., and Raschke, M. Quantification, localization, and expression of IGF-I and TGF-beta 1 during growth factor-stimulated fracture healing. Calcif Tissue Int **74,** 388, 2004.
34. Ludwig, S.C., Kowalski, J.M., and Boden, S.D. Osteoinductive bone graft substitutes. Eur Spine J **9,** S119, 2000.
35. Termaat, M.F., Den Boer, F.C., Bakker, F.C., Patka, P., and Haarman, H.J.T.M. Bone morphogenetic proteins—development and clinical efficacy in the treatment of fractures and bone defects. J Bone Joint Surg Am **87A,** 1367, 2005.
36. Lennon, D.P., Haynesworth, S.E., Young, R.G., Dennis, J.E., and Caplan, A.I. A chemically-defined medium supports *in-vitro* proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem-cells. Exp Cell Res **219,** 211, 1995.
37. Dziak, K.L., and Akkus, O. Effects of polyelectrolytic peptides on the quality of mineral crystals grown *in vitro*. J Bone Miner Metab **26,** 569, 2008.
38. Morgan, E.F., Mason, Z.D., Chien, K.B., Pfeiffer, A.J., Barnes, G.L., Einhorn, T.A., and Gerstenfeld, L.C. Micro-computed tomography assessment of fracture healing: relationships among callus structure, composition, and mechanical function. Bone **44,** 335, 2009.
39. Muller, R., Van Campenhout, H., Van Damme, B., Van Der Perre, G., Dequeker, J., Hildebrand, T., and Ruegsegger, P. Morphometric analysis of human bone biopsies: a quantitative structural comparison of histological sections and micro-computed tomography. Bone **23,** 59, 1998.
40. Oest, M.E., Dupont, K.M., Kong, H.J., Mooney, D.J., and Guldberg, R.E. Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects. Journal of Orthopaedic Research **25,** 941, 2007.
41. Reddi, A.H. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nature Biotechnology **16,** 247, 1998.
42. Hing, K.A. Bone repair in the twenty-first century: biology, chemistry or engineering? Philos Trans R Soc Lond Ser A Math Phys Eng Sci **362,** 2821, 2004.
43. Turksen, K., and Aubin, J.E. Positive and negative immunoselection for enrichment of 2 classes of osteoprogenitor cells. J Cell Biol **114,** 373, 1991.
44. Udagawa, N., Takahashi, N., Akatsu, T., Sasaki, T., Yamaguchi, A., Kodama, H., Martin, T.J., and Suda, T. The bone marrow-derived stromal cell-lines Mc3t3-G2/Pa6 and St2 support osteoclast-like cell-differentiation in cocultures with mouse spleen-cells. Endocrinology **125,** 1805, 1989.
45. Barnes, G.L., Kostenuik, P.J., Gerstenfeld, L.C., and Einhorn, T.A. Growth factor regulation of fracture repair. J Bone Miner Res **14,** 1805, 1999.
46. Yilgor, P., Hasirci, N., and Hasirci, V. Sequential BMP-2/BMP-7 delivery from polyester nanocapsules. J Biomed Mater Res Part A **93,** 528, 2009.
47. Yilgor, P., Tuzlakoglu, K., Reis, R.L., Hasirci, N., and Hasirci, V. Incorporation of a sequential BMP-2/BMP-7 delivery system into chitosan-based scaffolds for bone tissue engineering. Biomaterials **30,** 3551, 2009.
48. Boskey, A.L., Gadaleta, S., Gundberg, C., Doty, S.B., Ducy, P., and Karsenty, G. Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin. Bone **23,** 187, 1998.
49. Pufe, T., Wildemann, B., Petersen, W., Mentlein, R., Raschke, M., and Schmidmaier, G. Quantitative measurement of the splice variants 120 and 164 of the angiogenic peptide vascular endothelial growth factor in the time flow of fracture healing: a study in the rat. Cell Tissue Res **309,** 387, 2002.
50. Globus, R.K., Plouet, J., and Gospodarowicz, D. Cultured bovine bone-cells synthesize basic fibroblast growth-factor and store it in their extracellular-matrix. Endocrinology **124,** 1539, 1989.
51. Ross, R., Raines, E.W., and Bowenpope, D.F. The biology of platelet-derived growth-factor. Cell **46,** 155, 1986.
52. Buxton, P., Edwards, C., Archer, C.W., and Francis-West, P. Growth/differentiation factor-5 (GDF-5) and skeletal development. J Bone Joint Surg Am **83A,** S23, 2001.
53. Jane, J.A., Dunford, B.A., Kron, A., Pittman, D.D., Sasaki, T., Li, J.Z., Li, H.W., Alden, T.D., Dayoub, H., Hankins, G.R., Kallmes, D.F., and Helm, G.A. Ectopic osteogenesis using adenoviral bone morphogenetic protein (BMP)-4 and BMP-6 gene transfer. Mol Ther **6,** 464, 2002.
54. De Boer, J., Wang, H.J., and Van Blitterswijk, C. Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. Tissue Eng **10,** 393, 2004.

Address correspondence to:
Ozan Akkus, Ph.D.
Weldon School of Biomedical Engineering
Purdue University
206 S. Martin Jischke Drive
West Lafayette, IN 47907-2032

E-mail: ozan@purdue.edu

Received: August 19, 2009
Accepted: February 25, 2010
Online Publication Date: March 26, 2010